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A STUDY OF THE EFFECTS OF ADENOSINE ON THE GROWTH AND METABOLISM OF *MICROCOCCUS SODONENSIS*

by

© CHARLES R. SHOBE

A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES

IN PARTIAL FULFILMENT OF THE REQUIREMENTS

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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies for acceptance, a thesis entitled "A study of the Effects of Adenosine on the Growth and Metabolism of Micrococcus sodonensis" submitted by Charles R. Shobe in partial fulfilment of the requirements for the degree of Doctor of Philosophy.

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ABSTRACT

The purine nucleoside, adenosine, exerts a characteristic bacteriostatic effect on the growth of Micrococcus sodonensis, ATCC 11880. The typical growth response of this organism to the inclusion of adenosine in a synthetic medium involves an initial period wherein no inhibition is evident, an inhibited phase which varies in duration depending upon the initial adenosine concentration and a recovery phase, during which the generation time again approaches that of normal exponentially growing cells. Except for inosine and perhaps hypoxanthine, which produce a slight inhibition, no other purines or purine derivatives can induce this abnormal response. Differences in the transport of purines and their derivatives does not explain the observed selective inhibition, since many of the non-inhibitory compounds can be transported at rates comparable with those of the inhibitors. However, the lessened inhibitory effects of inosine and hypoxanthine appear to correlate with their lower transport rates as compared with that of adenosine.

In order to eliminate the possibility that M. sodonensis was defective in some aspect of purine metabolism, extracts of the organism were shown to contain most of the enzymes known to be involved in the synthesis of nucleotides from purine bases and nucleosides, with the exception of adenosine phosphorylase, as well as those enzymes involved in purine nucleotide interconversions. The incorporation of nucleotides into nucleic acids was eliminated as a possible site of inhibition since DNA and RNA synthesis continued, although at an apparently reduced rate, in the presence of inhibitory concentrations of adenosine.

Adenosine-induced inhibition can be reversed by including either thiamine or the pyrimidine precursor of thiamine, but not the thiazole precursor of thiamine, in the growth medium. It was shown directly that in the presence of exogenous adenosine, de novo thiamine biosynthesis is inhibited. When adenosine, which is metabolized by the inhibited cells, is exhausted from the medium, thiamine synthesis is re-initiated.

In the presence of exogenous adenosine, at an initial concentration of 0.1 mM or greater, de novo purine biosynthesis is inhibited in excess of 97%. In vivo studies implicate phosphoribosyl-pyrophosphate amidotransferase, a shared enzyme in the pathways for de novo purine biosynthesis and for the synthesis of the pyrimidine precursor of thiamine, as the apparent site of adenosine-induced inhibition. A study of the changes in the intracellular levels of purine nucleotides in response to exogenous purine derivatives indicates that the rate of de novo purine biosynthesis is closely controlled by the adenine nucleotide concentrations.

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LIST OF ABBREVIATIONS

3-AcPyNAD$^+$	- 3-acetyl pyridine nicotinamide adenine dinucleotide
3-AcPyNADH	- Reduced 3-acetyl pyridine nicotinamide adenine dinucleotide
AICA	- 5-aminoimidazole carboxamide
AICAR	- 5-aminoimidazole carboxamide ribotide
AIR	- 5-aminoimidazole ribotide
AMP-S	- Succinyladenosine 5'-monophosphate
AMP, GMP, IMP, XMP, UMP, CMP	- 5'-monophosphates of adenosine, guanosine, inosine, xanthosine, uridine and cytidine, respectively
ADP, GDP, etc.	- 5'-diphosphates of the corresponding nucleosides
ATP, GTP, etc.	- 5'-triphosphates of the corresponding nucleosides
BN medium	- Synthetic medium for culturing M. sodonensis
B_1-pyrimidine	- 2-methyl-4-amino-5-hydroxymethyl pyrimidine
B_1-thiazole	- 4-methyl-5-(2-hydroxymethyl)thiazole
BSA	- Bovine serum albumin
DNA	- Deoxyribonucleic acid
EDTA	- Ethylenediaminetetraacetic acid
GAR	- Glycinamide ribotide
NAD$^+$	- Nicotinamide adenine dinucleotide

NADH	- Reduced nicotinamide adenine dinucleotide
PEI	- Polyethyleneimine
P_i	- Inorganic phosphate
$P\text{-}P_i$	- Inorganic pyrophosphate
PRA	- 5-phosphoribosyl-1-amine
PRPP	- 5-phosphoribosyl-1-pyrophosphate
RNA	- Ribonucleic acid
TCA	- Trichloroacetic acid
TCS	- Trypticase soy
TLC	- Thin-layer chromatography
TRIS	- TRIS (hydroxymethyl) aminomethane
U.V.	- Ultraviolet

INTRODUCTION

The phenomenon of purine-induced bacteriostasis was first described by Brooke and Magasanik (1954) during their investigations of purineless mutants of Aerobacter aerogenes. One of their mutants, Strain P-14, required guanine, guanosine, guanylic acid or 2,6-diamino-purine for growth, but was inhibited when adenine or hypoxanthine was also included in the medium. This inhibition was characteristically variable, depending upon the initial inoculum size, since increasing the inoculum reduced the degree of inhibition. Increasing the concentrations of the required guanine derivatives did not reverse the inhibition. A second strain of A. aerogenes, strain PD-1, which was blocked before 4-amino-5-imidazole carboxamide ribotide (AICAR) in the pathway for de novo purine biosynthesis, was shown to have an absolute requirement for thiamine as well as purine nucleotide precursors, but these two phenomena were not, at that time, considered to be related. Subsequently, Yara (1956) was able to show, by transduction experiments, that all mutants of Salmonella typhimurium blocked before 5-aminoimidazole ribotide (AIR) on the de novo purine pathway could be classified as Ath mutants (adenine and thiamine-requiring mutants), while almost all mutants blocked after AIR required only adenine (Ad mutants). These results suggested the possibility of a shared pathway for thiamine and purine biosynthesis. However, the occurrence of the Ath phenotype in certain mutants blocked after AIR obscured these results.

Neidhardt (1963) described more completely the bacteriostatic effects of adenine and adenosine on A. aerogenes 5-P14 and Escherichia coli K12W-6.

Guanosine also had a slight inhibitory effect, but only at very high concentrations. Both adenine and adenosine produced an inhibition of logarithmic growth which resulted in sustained linear growth at a rate approximately equal to the differential growth rate at the moment of the addition of the inhibitor. The inhibition also varied as the inoculum size was changed. In all cases, the addition of thiamine to the culture medium either prevented or reversed the adenine or adenosine-induced growth inhibition.

Moyed (1964) was the first author to report that both thiamine and its pyrimidine precursor (B_1-pyrimidine) were able to reverse purine-induced bacteriostasis. The thiazole precursor of thiamine (B_1-thiazole) had no protective properties even at very high concentrations. Moyed demonstrated that B_1-pyrimidine was twice as effective as thiamine, on a weight basis, in reversing adenine, adenosine and inosine-induced bacteriostasis in A. aerogenes. However, since the molecular weight of thiamine is approximately twice that of B_1-pyrimidine, these results can best be interpreted as indicating that B_1-pyrimidine and thiamine are equally effective in preventing the inhibition. Moyed was also able to show that, in fact, thiamine, B_1-pyrimidine and B_1-thiazole biosynthesis were completely inhibited when cells were grown in the presence of the growth inhibitors, whereas he, as well as Neidhardt (1963), Yara (1956), and Brooke and Magasanik (1954), had shown that other cellular processes (e.g. nucleic acid and protein biosynthesis) were only partially inhibited. Moyed, therefore, postulated that one of the intracellular derivatives of adenosine was specifically inhibiting B_1-pyrimidine biosynthesis, either by some pseudo-

feedback mechanism, or by competing with one of the intermediates of B_1-pyrimidine biosynthesis for the active site of an enzyme.

More recently, de Repentigny, et al. (1968 and personal communication), and Mathieu, et al. (1969) have demonstrated adenine and adenosine inhibition of the growth of certain strains of Staphylococcus aureus. Their findings are basically different from those of earlier investigators since the inhibition in S. aureus is readily reversed by other purine nucleosides and bases (guanine, guanosine, inosine and hypoxanthine), but not by thiamine. The mechanism for growth inhibition in this case appears to involve, at least in part, inhibition of de novo purine biosynthesis concomitant with an inability to convert either adenine or adenosine to guanine nucleotides for nucleic acid biosynthesis. Although the mechanism postulated below for adenosine-induced, thiamine-reversible growth inhibition also involves the inhibition of de novo purine biosynthesis, this would appear, at present, to be the only feature common to both systems.

Newell and Tucker (1966a and 1966b) have also shown that adenosine inhibits de novo synthesis of the B_1-pyrimidine moiety in Salmonella typhimurium. Furthermore, they (Newell and Tucker 1967 and 1968b) have conclusively demonstrated that B_1-pyrimidine is synthesized by a pathway which branches off the pathway for de novo purine biosynthesis at the level of 5-aminoimidazol ribotide. This finding explains the results of Brooke and Magasanik (1954) and of Yara (1956) concerning the thiamine requirements of mutants blocked at early reactions in the de novo purine

pathway. It also explains the isotope incorporation results of investigators who have looked for thiamine and B_1-pyrimidine precursors (Pine and Guthrie, 1959; Goldstein and Brown, 1963, Johnson, et al., 1966; Tomlinson, et al., 1967; David, et al., 1967) and supports the conclusion reached by Goldstein and Brown (1963), by Newell and Tucker (1968a), and by Linnett and Walker (1968), that the biosynthetic pathway for nucleic acid pyrimidines is not involved in B_1-pyrimidine biosynthesis.

Knowing that B_1-pyrimidine is synthesized in a pathway branching off the pathway for de novo purine synthesis, one can postulate three mechanisms for purine inhibition of B_1-pyrimidine synthesis. Either the B_1-pyrimidine pathway can be directly inhibited by adenosine or its derivatives, or the purine pathway can be so completely inhibited by adenosine deerivatives that no intermediates (i.e. AIR) are available for B_1-pyrimidine synthesis. The third possible mechanism involves competition between adenosine or its derivatives and some precursor of B_1-pyrimidine for an enzyme-active site, as was previously suggested by Moyed (1964).

The second hypothetical mechanism suggested above would seem to be the most probable since feedback inhibition of the de novo purine pathway by purine nucleotides has been demonstrated in a number of systems (Wyngaarden and Ashton, 1959; Caskey, et al., 1964; Nierlich and Magasanik, 1965; Brockman and Chumley, 1965; Hill and Bennett, 1969; Reem, 1968). Even if this is true, however, the question still arises as to why only certain purines can induce the inhibition whereas others cannot. The answer to this question may lie in the area of permeability differences

among various purine derivatives, or it could be attributed to variations in the ability to synthesize nucleotides from the different purine bases and nucleosides, to variations in the control of purine nucleotide interconversions, to variations in the effects of different purine nucleotides on the control of de novo purine biosynthesis, or to a complex combination of these factors.

The following investigation originated as an extension of earlier work in which it was reported (Campbell, Evans, Perry and Niven, 1961) that the addition to a culture of Micrococcus sodonensis of yeast extract, or high levels of purine derivatives, resulted in the elaboration by the cells of large quantities of extracellular materials consisting primarily of DNA, but also containing lesser amounts of RNA and protein. The report below describes the investigation into the effects of adenosine on the growth and metabolism of M. sodonensis, and postulates a mechanism whereby the observed adenosine-induced growth inhibition is effected.

MATERIALS AND METHODS

I. Reagents

All reagents used were of reagent grade and all reagents and materials were obtained from commercial suppliers.

^{14}C-labelled compounds were purchased from Calbiochem. Five-phosphoribosyl-1-pyrophosphate was purchased from Mann Research Laboratories.

Takadiastase powder was the generous gift of Parke, Davis and Company. Two-Methyl-4-amino-5-hydroxymethyl pyrimidine and 4-methyl-5-(2-hydroxy-methyl)thiazole, the pyrimidine and thiazole precursors, respectively, of thiamine were kindly provided by the Research Laboratories Division of Merck and Co., Inc.

II. Organisms and Culturing Conditions

1. Micrococcus sodonensis, ATCC 11880.

Stock cultures were maintained at -4°C, with weekly transfers, on Trypticase Soy (TCS) agar plates. Fluid cultures were grown in flasks, with vigorous aeration, on a Brunswick Gyrotary Incubator Shaker, Model G-25. All culturing procedures, as well as all experimental procedures in which whole cells were used, were carried out at 30°C.

2. Lactobacillus viridescens, ATCC 12706.

Stock cultures of the thiamine assay organism were maintained at

30°C, with transfers every three days, as stab cultures in tubes of APT agar (Difco) supplemented with thiamine at 1 mg/ml. Static fluid cultures were grown at 30°C in tubes of APT broth (Difco) supplemented with thiamine at 1 mg/ml.

III. Culture Media

1. TCS broth (Baltimore Biological Laboratories), at pH 7.3.

To prepare a solid medium, 1.5% Difco agar was added to the medium prior to autoclaving.

2. APT broth (Difco), at pH 6.3.

3. APT agar (Difco), at pH 6.3.

4. Bacto-thiamine assay medium LV (Difco), at pH 6.0.

5. Synthetic medium (BN medium)

The synthetic medium described by Campbell, Evans, Perry and Niven (1961) for the growth of M. sodonensis was employed with the minor modification that 0.02 mg/100 ml of sodium molybdate was substituted for the 0.08 mg/100 ml of ammonium molybdate previously used. The pH was adjusted to 8.1, giving a final pH, after autoclaving, of 7.2.

6. Supplemented BN medium

When supplements were heat stable, they were included in the media prior to autoclaving (e.g. purine and pyrimidine bases and nucleosides). Heat labile compounds were sterilized by Millipore filtration and added aseptically to pre-sterilized BN medium.

IV. Preparation of Standard Inocula

1. Standard inoculum of M. sodonensis.

The inoculum was prepared from 18-hour cultures grown in BN medium. Cells were harvested by centrifugation, washed twice with sterile 1% NaCl and re-suspended in BN medium to an optical density of 5.0, measured at 600 mμ. The standard inoculum was 4.0 ml of this suspension per 100 ml of culture medium.

2. Standard inoculum of L. viridescens.

The inoculum was prepared from 24-hour cells grown in APT broth supplemented with thiamine at 1 mg/ml. Cells were harvested by centrifugation, washed four times with sterile 1% NaCl and re-suspended in sterile 1% NaCl to an optical density of 0.1, measured at 600 mμ. The standard inoculum consisted of one drop per assay tube (4 ml) of a 1/100 dilution of this suspension.

V. Determination of Cell Density

Cell densities were determined by measuring the absorbance of cultures at 600 mμ with a Beckman Model DB-G spectrophotometer and, in the case of M. sodonensis, by dry weight and total cell protein determinations.

1. Dry weight determinations.

Measurements were carried out on washed 18-hour cells. Cell suspensions with known absorbances at 600 mμ were prepared and 10 ml samples of each were evaporated to dryness at 90°C in pre-weighed petri dishes.

The samples were transferred to an oven at 110°C and dried to constant weight. A standard curve relating absorbance at 600 mμ to dry weight of cells/ml was prepared.

2. Total cell protein determinations.

Measurements were carried out on washed 18-hour cells using a modification of the Lowry technique for insoluble proteins (Lowry, Rosenbrough, Farr and Randall, 1951). Crystalline bovine serum albumin (BSA) was used as the reference protein. Washed 18-hour cells re-suspended in distilled water were diluted to known absorbances at 600 mμ, and 10 ml samples treated as described below. Ten ml aliquots of known concentrations of the BSA standard were subjected to the same treatment.

Samples were extracted with 5% TCA twice, for 30 minutes each time, in an ice bath. The residue was collected by centrifugation, the tubes and pellets blotted dry, and the pellets re-suspended in 10 ml of 1.0N NaOH. The resulting mixture was maintained at 37°C for 12-15 hours, then heated in a boiling water bath for 10 minutes to ensure complete solubilization of the residue. The volume was adjuted to 10 ml with distilled water. To 1.0 ml of this solution was added 10 ml of neutral carbonate-copper reagent (50 vol. of 2.0% Na_2CO_3 in H_2O to 1 vol. of 0.5% $CuSO_4 \cdot 5H_2O$ in 1.0% potassium tartarate). After 10 minutes at room temperature, 1.0 ml of diluted (1:1 with H_2O) Folin-Ciocalteau Phenol Reagent solution (Fisher Scientific) was added rapidly with immediate mixing. After a further 30 minutes at room temperature, the absorbance of the solution was measured at 660 mμ. A standard curve relating absorbance at 660 mμ to BSA

concentration was prepared, and from this curve total cell protein concentrations were determined. A second standard curve relating absorbance of the cell suspension at 600 mμ to total cell protein/ml was prepared from this data.

VI. Estimation of Growth Responses

The effects of various additions to BN medium on the growth of M. sodonensis was determined as follows: cultures, generally consisting of 100 ml of medium in a 250 ml Erlenmeyer flask, were inoculated as described above and incubated at 30°C on a New Brunswick Gyrotary incubator shaker operating at 250 rpm. At various intervals, samples were withdrawn from the flasks and the absorbances of the cultures measured at 600 mμ. Absorbances were then related either to dry weight of cells or total cell protein by means of the standard curves, and the growth compared with growth in BN medium.

VII. Transport of Purine Bases and Nucleosides

For the purposes of these experiments, purine transport was defined as the disappearance of ^{14}C-labelled compounds from the supernatant of the cell suspension.

Transport experiments were carried out with washed 24-hour cells re-suspended in cold BN medium to a concentration of 4.0 mg dry weight/ml. Cells were held at 0°C until required. Ten ml portions of the cell suspension were incubated in a water-bath shaker at 30°C for 20 minutes prior to the addition of the purine.

Eight-^{14}C-labelled purine bases and nucleosides of high specific activity (usually 15-25 μc/mmole) were individually diluted in BN medium with the corresponding unlabelled compound to a final specific activity of 25 μc/mmole and a final concentration of 0.4 mM. These solutions were also warmed to 30°C before being used.

Transport experiments were initiated by the addition of 10 ml of the purine-containing solution to 10 ml of the cell suspension in a 50 ml flask. The mixtures were incubated at 30°C, with vigorous aeration, in a Brunswick water-bath shaker. Transport was terminated at 10 minutes by rapid chilling, followed immediately by centrifugation at 43,000 x g for 5 minutes at 0°C. Tests showed that if all glassware was cooled to -20°C before being used in the termination manipulations, the temperature of a 5 ml sample could be reduced from 30°C to 0°C, or less, in less than 6 seconds.

Samples of the supernatant were mixed with 10 volumes of Bray's scintillation fluid (see below) and counted in a Nuclear Chicago Mark I Liquid Scintillation counter. Counts obtained were corrected for background and for quenching, by means of a previously prepared quench correction curve, and apparent final purine concentrations were calculated. The differences between initial and final concentrations were taken as a measure of purine transport. Bray's scintillation fluid consists of:

Naphthalene	60.0 gm
2, 5-diphenyloxazole (PPO)	4.0 gm
1,4-bis-(2-5-Phenyloxazolyl) benzene (POPOP)	0.2 gm
Ethylene glycol	20.0 ml
Methanol (absolute)	100.0 ml
1,4-dioxane	to 1000.0 ml

VIII. Distribution of Adenosine Derivatives in a culture of M. sodonensis

Washed 18-hour cells were re-suspended in BN medium to a density of 4.8 mg cell protein/ml. ^{14}C-labelled adenosine (specific activity, 26 μc/mmole) was added to a final concentration of 0.5 mM and the culture incubated at 30°C, with vigorous aeration, for 3 hours. At various intervals, 4.0 ml samples of the culture were treated as follows: cells were harvested by centrifugation, washed twice with 4 ml of cold BN medium and the supernatants pooled (supernatant fraction). The cells were extracted twice (for 30 minutes each time) combined and saved. The residue (nucleic acid fraction) was re-suspended in 4 ml of 1N NaOH and left 12 hours at 37°C.

The 60-minute nucleic acid fraction was further treated as follows: 2 ml of the solution were neutralized with concentrated HCl and an equal volume of 10% TCA was added. The precipitate, after 30 minutes at 0°C, was collected by centrifugation, re-extracted with 4 ml of 5% TCA and the supernatants, containing nucleotides derived from RNA, were pooled. The pellet (DNA-containing fraction) was re-dissolved in 2 ml of 1N NaOH.

The various fractions were diluted with 10 volumes of Bray's scintillation fluid and counted in the liquid scintillation counter. Concentrations of adenosine derivatives present in the culture fractions were calculated for all samples, and the distribution of adenosine-derived compounds within the 60-minute nucleic acid-containing fraction was determined.

In a parallel experiment, the distribution of derivatives of ^{14}C-labelled guanosine within the nucleic acid fraction was also determined after 60 minutes incubation.

IX. Quantitation of Intracellular Purine Nucleotides

1. Extraction of the nucleotide pool.

Ten ml of ice-cold 60% $HClO_4$ were added to 100 ml of culture and the mixture cooled rapidly in a 3M NaCl ice bath (-7°C). After 30 minutes in the cold bath, the mixture was centrifuged at 25,000 x g for 10 minutes and the supernatant immediately neutralized with ice-cold 5 N KOH. The pellet was re-extracted four times with 30 ml of cold 6% $HClO_4$ and the supernatants neutralized and pooled with the original supernatant after each extraction. No additional U.V.-absorbing material could be extracted after the fourth extraction. The neutralized supernatants were filtered through Whatman No. 1 filter paper to remove the precipitated $KClO_4$ and the precipitate washed with 50 ml of ice-cold 0.01 mM phosphate buffer pH 7.0 containing 0.1 mM EDTA. Less than 1% of the nucleotides were retained on the filter paper when EDTA was included in the washing buffer. The filtrate was frozen at -60°C until required.

2. Desalting with and elution from octanol-treated charcoal.

Frozen extracts were thawed and filtered through a bed of octanol-treated charcoal (preparation described below) and the filtrates checked for U.V. absorbance. Using octanol-deactivated charcoal it was generally necessary to pass the extract through the charcoal bed at least three times to obtain complete adsorption of the nucleic acid precursors. More than 99% of these compounds, as determined by absorbance at 260 mμ, were adsorbed in this way.

The charcoal disc was washed with five 50 ml portions of distilled water to remove all salts, and the U.V. absorbing compounds eluted from the charcoal with four 50 ml portions of 28% NH_4OH: distilled water: 95% ethanol (5: 45:50). The eluting solvent was pre-cooled to -20°C and the filtrate collected in a vacuum flask seated in a dry ice acetone bath (-70°C). The frozen filtrate was thawed, filtered through a Millipore filter (0.45 μ) to remove the small amounts of charcoal fines which were present, and evaporated to dryness in a Buchii Rotavapor-R.

3. Quantitation of purine nucleotides.

The desalted mixture of bases, nucleosides and nucleotides was fractionated (see below) with Dowex 50 (H^+) and the nucleotide fraction concentrated to 0.1 ml. Twenty-five μl of the nucleotide extract were chromatographed on PEI-cellulose TLC layers as described below, and nucleotides located under U.V. light. The spots were cut out and scraped into small test tubes, and the nucleotides eluted with 1.5 ml of 2.0M LiCl in 0.1N HCl. Compounds were identified from their absorbance spectra and from their positions on the chromatogram and were quantitated using their known extinction coefficients in acid solutions. Absorbance values were corrected for each compound by subtracting the absorbance contribution of the corresponding region of a blank control chromatogram which was activated and developed in the same manner.

X. Quantitation of Intracellular Thiamine

1. Extraction and Takadiastase treatment.

Thiamine and its phosphorylated derivatives were extracted from M. sodonensis as follows: 10 ml of culture were centrifuged, and the cells washed with 1% NaCl and re-suspended in 5 ml of 0.12 N HCl. The samples were hydrolyzed in an autoclave at 121°C (15 psi) for 20 minutes, evaporated to dryness in a Rotary Evapomix and the residue re-suspended in 2.0 ml of 0.1 M acetate buffer, pH 4.9, containing 1 mg/ml Takadiastase powder (Parke, Davis and Co.). The mixture was incubated at 45°C for 4 hours, centrifuged to clarify and the supernatant assayed for thiamine as described below. Samples of the culture supernatant were also assayed for thiamine content.

2. Microbiological assay for thiamine.

Double strength Bacto-thiamine assay medium LV (Difco), pH 6.0, specially formulated for use with L. viridescens, ATCC 12706, was prepared according to directions and dispensed at 2.0 ml per assay tube. Two ml of thiamine solution or cell extract containing between 0.5 and 10 μg of thiamine were added and the tubes capped and sterilized by autoclaving for 5 minutes. Tubes were inoculated as described above. Both known and unknown thiamine samples were run in quadruplicate and a new standard curve was prepared for each experiment. Inoculated assay tubes were incubated for 18 hours at 30°C and then placed in an ice water bath. The absorbances of the cell suspensions at 600 mμ were measured and a standard curve prepared from the absorbance values obtained for known thiamine concentrations.

XI. Enzyme Assays

1. Preparation of cell-free extracts.

Intracellular enzyme activities were determined in extracts of 18-hour cultures grown in BN medium. Washed cells were re-suspended to an OD, measured at 600 mμ, of 20.0 in 0.01 M phosphate buffer, pH 6.7, containing 0.01 M $MgCl_2$. Crystalline lysozyme was added to a final concentration of 100 μg/ml and the cell suspension incubated, with gentle stirring, at 37°C for 30 minutes. The lysate was centrifuged at 45,000 x g for 20 minutes at 4°C and the pellet re-extracted at 4°C with 0.01 M Tris-HCl buffer, pH 7.5. The combined supernatants contained 68% of the total cell protein. Repeated extraction of the pellet would release only an addition 3 - 5% of the cell protein. Sonication of the lysate prior to centrifugation gave higher protein yields but reduced the activity of the enzymes.

2. Adenosine deaminase.

The standard reaction mixture contained: 0.1 ml of 1.0 M Tris-HCl buffer, pH 8.0; 2.9 ml of cell-free extract containing 0.5 mg protein/ml; 10 μl of 10 mM adenosine. The reaction was started by the addition of adenosine and followed by measuring the decrease in absorbance at 265 m according to the method of Kalcar (1947b).

3. Purine nucleosidases.

(a) Hydrolytic nucleosidase: The standard reaction mixture contained: 5 μl of 1.0 M Tris-HCl buffer, pH 8.0; 10 μl of 10 mM

8-^{14}C-labelled purine nucleoside (specific activity, 20 μc/mmole); 10 μl of distilled water; 10 μl of cell-free extract containing 5 mg protein/ml. The reaction was initiated by the addition of the cell-free extract. After 30 minutes at 37°C, the reaction was stopped by the addition of 5 μl of cold 60% perchloric acid and the reaction vessels placed in an ice bath for 30 minutes. Thirty μl of 2.0 M KOH were then added and the reaction mixture held in the ice bath an additional 20 minutes. The precipitate was removed by centrifugation, and 40 μl of the supernatant were chromatographed on 4 x 20 cm strips of Whatman No. 1 filter paper using the butanol-20% NH_4OH solvent system described below.

The developed chromatograms were dried in an oven at 110°C and scanned with a Nuclear Chicago Actigraph III strip scanner. Radioactive compounds were eluted from the paper with 1.0 ml of 1.0N HCl, diluted with 10 volumes of Bray's scintillation fluid, and counted in a Nuclear Chicago Mark 1 liquid scintillation counter.

(b) Purine nucleoside phosphorylase: Five μl of 1.0 M phosphate buffer, pH 7.5 were substituted for the Tris-HCl buffer employed in the assay for hydrolytic nucleosidase activity. Otherwise, reaction conditions and post-reaction manipulations were the same as described in the hydrolytic nucleosidase assay.

4. Guanosine deaminase activity.

The standard reaction mixture contained: 0.1 ml of 1.0 M Tris-HCl buffer, pH 8.0; 0.5 ml of cell-free extract containing 10 mg protein/ml; 10 µl of 10 mM guanosine; and water to a final volume of 3.0 ml. The reaction was initiated by the addition of the guanosine and was terminated after 30 minutes at 37°C by adding 0.3 ml of cold 70% $HClO_4$. After 30 minutes at 0°C, the precipitate was removed by centrifugation. Control values were obtained by adding $HClO_4$ prior to the addition of guanosine. Guanosine deaminase activity was estimated from the decrease in absorbance at 260 mµ.

5. Adenase.

The standard reaction mixture contained: 0.1 ml of 1.0 M Tris-HCl buffer, pH 7.53; 0.2 ml of cell-free extract containing 10 mg protein/ml; 0.1 ml of 1.0 mM adenine; 0.2 units of xanthine oxidase (Mann Research Laboratories); and water to a final volume of 1.0 ml. The reaction was initiated by the addition of adenine and followed by measuring the increase in absorbance at 292 mµ as a result of uric acid production in the reaction mixture according to Kalcar (1947 a and b).

6. Guanase.

Guanase activity was estimated in the same manner as adenase activity (above) with the addition of 0.1 ml of 1.0 mM guanine in place of adenine in the reaction mixture.

7. Purine Nucleoside Phosphokinase.

a) Purine nucleoside phosphokinase activity was measured by a modification of the technique of Kornberg (1955). The reaction mixture contained 100 µl of 0.5 M succinate buffer, pH 6.0; 50 µl of 10 mM ^{14}C-labelled purine nucleoside (specific activity, 25 µc/mmole); 10 µl of 0.1 M $MgCl_2$; 10 µl of 0.02 M $MnCl_2$; 20 µl of 0.16 glutathione; 10 µl of 5 mM ATP; 200 µl of cell-free extract containing 8.6 mg protein/ml; and distilled water to a final volume of 0.5 ml. The reaction was initiated by the simultaneous addition of purine nucleoside and ATP and was terminated after 30 minutes at 37°C by the addition of 50 µl of cold 60% $HClO_4$. The mixture was held at 0°C for 30 minutes and the resulting precipitate removed by centrifugation. One hundred µl of the supernatant were spotted on 4 x 20 cm Whatman No. 1 filter paper strips, and the chromatograms developed with the n-butanol: 20% NH_4OH (83:17) solvent system. The strips were scanned with a Nuclear Chicago Actigraph III strip scanner. Twenty µl samples were spotted on silica gel TLC sheets (Eastman Kodak) and the chromatograms developed with the same solvent system. In this solvent system, nucleotides do not migrate from the origin, whereas bases and nucleosides do migrate. The origins of the developed chromatograms were cut out, eluted with 0.1 N HCl (37°C for 1 hour), diluted with 10 volumes of Bray's solution and counted in the liquid scintillation counter.

b) Cell-free extracts were assayed for adenosine phosphokinase activity in the laboratory of Dr. J. F. Henderson (J. S. McEachern Laboratory, University of Alberta) using 6-(Methylmercapto) purine ribonucleoside (methyl-^{14}C) as the substrate, as described by Caldwell, Henderson and Paterson (1966).

8. Purine nucleoside-5'-monophosphate pyrophosphorylase activity.

AMP-, GMP-, and IMP- pyrophosphorylase activities were assayed by the method of Kornberg, Lieberman and Simms (1955). The standard reaction mixture contained: 10 µl of 10 mM 5-phosphoribosyl-1-pyrophosphate (PRPP); 10 µl of 100 mM $MgCl_2$; 10 µl of 1.0 M Tris-HCl buffer, pH 7.6; 50 µl of 10 mM ^{14}C-labelled purine base (30 µc/mmole); and 20 µl of cell-free extract containing 10 mg protein/ml. After 20 minutes at 37°C, the reaction was stopped by the addition of 100 µl of cold 10% TCA, and the reaction vessels placed in an ice bath. After 15 minutes, the mixture was clarified by centrifugation. To the supernatant were added: 0.25 ml of a 100 mM solution of the appropriate carrier purine nucleoside-5'-monophosphate; 1 drop of 0.04% bromothymol blue; 2 drops of 2N KOH; 0.1 ml of saturated $BaCl_2$ solution; and 3.0 ml of absolute ethanol. After 15 minutes at 0°C, the precipitate was collected by centrifugation, washed twice with 3.0 ml of cold absolute ethanol and dissolved in 0.5 ml of 0.1N HCl. Ten volumes of Bray's scintillation fluid were added and the samples counted in the liquid sctintillation counter.

9. Adenylosuccinate synthetase-lyase activities.

Combined activity was estimated by a modification of the technique described by Lieberman (1956). The standard reaction mixture contained 0.1 ml of 1.0 M glycine buffer, pH 8.0; 0.01 ml of 4M $MgCl_2$; 0.04 ml of 1.0 mM ATP; 0.04 ml of 10 mM phosphoenol pyruvate; 0.01 ml of pyruvate kinase (30 units); 0.1 ml of 1.0 mM GTP; 0.01 ml of 0.03 IMP; 0.05 ml of 10 mM

L-aspartate; 0.14 ml of water and 0.2 ml of cell-free extract containing 10 mg protein/ml. The reaction was initiated by the addition of the L-aspartic acid and was carried out for 30 minutes at 37°C, at which time 0.3 ml of 5% $HClO_4$ was added. The mixture was held at 0°C for 30 minutes, centrifuged to remove the precipitate, and the increase in absorbance at 260 mμ determined. The overall reaction measures the synthesis of adenylo-succinate (AMP-S) from IMP and aspartate, and the subsequent hydrolysis of AMP-S to AMP.

10. Adenylic acid deaminase.

The standard reaction mixture contained: 25 μl of 2.5mM AMP; 5 μl of 1.0 Tris-HCl buffer, pH 8.0; 50 μl of cell-free extract containing 12 mg protein/ml. After 30 minutes at 37°C, the mixture was diluted with 3.0 ml of distilled water and the decrease in absorbance at 265 mμ determined.

11. Inosine monophosphate dehydrogenase.

The standard reaction mixture contained: 0.1 ml of 1.0 M Tris-HCl buffer, pH 7.5; 0.05 ml of 0.1 M glutathione; 0.1 ml of 1.0 M KCl; 0.1 ml of 0.03 M IMP; 0.2 ml of 0.0125 M NAD; 0.5 ml cell-free extract containing 12 μg cell protein/ml; and water to give a final volume of 3.0 ml. The reaction was initated by the addition of NAD and followed at room temperature by measuring the increase in absorbance at 340 mμ according to the method of Magasanik (1963).

12. Xanthine monophosphate aminase.

XMP aminase activity was determined by a modification of the

technique of Magasanik (1963). The standard reaction mixture contained: 40 µl of 1.0 M Tris-HCl buffer, pH 8.5; 20 µl of 0.05 M ATP (pH 7.0); 10 µl of 0.4 M $MgCl_2$; 60 µl of 0.1 M XMP; 50 µl cell-free extract containing 10 mg protein/ml; and water to give a final volume of 210 µl. The reaction was initiated by the addition of 40 µl of 2.0 M $(NH_4)_2SO_4$, incubated at room temperature for 30 minutes and then terminated by the addition of 2.75 ml of 3.5% H ClO_4. Control values were obtained by adding $HClO_4$ prior to the addition of $(NH_4)_2SO_4$. Acidified samples were held at 0°C for 30 minutes and the precipitate removed by centrifugation. The increase in absorbance at 290 mµ was used as a measure of XMP aminase activity.

13. Guanosine monophosphate reductase.

GMP reductase activity was estimated according to the method of Magasanik (1963). The standard reaction mixture contained: 0.2 ml of 0.2 M Tris-HCl buffer, pH 7.5; 0.05 ml of 0.05 M cysteine (freshly dissolved and neutralized); 0.2 ml of 0.01 M GMP: 0.1 ml of 1.0 mM NaOH; 0.25 ml of cell-free extract containing 10 mg protein/ml; and water to a final volume of 1.0 ml. The cell-free extract was, in some experiments, pre-treated with 0.2 ml of 0.1 M KCN per ml of extract for 5 minutes at 0°C. The reaction was initiated by the addition of NADH and followed at room termperature by measuring the decrease in absorbance at 340 mµ.

14. 5-Phosphoribosyl-1-pyrophosphate amidotransferase.

Attempts were made to estimate PRPP amidotransferase activity after

the methods of Hartman (1965), by measuring pyrophosphate production according to Kornberg (1950); of Wyngaarden and Ashton (1959), by measuring NADH production in a coupled reaction; and by a modification of the procedure of Hartman and Buchanan (1958) which couples PRPP amidotransferase-dependent formation of phosphoribosylamine with subsequent reactions in the de novo purine pathway.

(a) Pyrophosphate production: The standard reaction mixture contained: 3 µl of 100 mM PRPP; 3 µl of 200 mM $MgCl_2$; 3 µl of 1.0 M Tris-HCl buffer, pH 8.6; 1 µl of 500 mM NaF; 25 µl of enzyme extract containing 15 mg protein/ml; 20 µl of water; and 5 µl of 200 mM glutamine. PRPP, $MgCl_2$, Tris-HCl, NaF and enzyme were pre-incubated for 15 minutes at 37°C prior to the addition of glutamine. Controls omitted either glutamine or enzyme extract. After 30 minutes at 37°C, the reaction was terminated by the addition of 100 µl of 0.5 M acetate buffer, pH 5.0 containing 0.10 M $MnCl_2$. The manganese pyrophosphate precipitate formed after 30 minutes at 0°C was collected by centrifugation, washed at 0°C with 50 µl of 10% acetone containing 0.01 M $MnCl_2$, and hydrolyzed in a boiling water bath for 15 minutes in 100 µl of 1 N H_2SO_4. Inorganic phosphate was determined by the Ames-Dubin (1960) technique.

(b) NADH production: The standard reaction mixture contained: 10 µl of 1.0 M Tris-HCl buffer, pH 8.6; 10 µl of 200 mM $MgCl_2$; 10 µl of 100 mM PRPP; 50 µl of 200 mM glutamine;

25 µl of 1.0 mM 3-acetyl-pyridine NAD (3-AcPyNAD); 25 µl of bovine liver glutamic dehydrogenase (Sigma Chemical Co.) diluted to 2 mg/ml with 0.1 Tris-HCl buffer, pH 7.4; 50 µl of enzyme extract containing 15 mg protein/ml; and water to a volume of 250 µl. The control omitted PRPP. The reaction mixture, without glutamine, was pre-incubated at room temperature for 15 minutes, then glutamine was added and the formation of glutamate followed by determining the increase in absorbance at 363 mµ due to PRPP-dependent reduction of 3-AcPyNAD. Three-AcPyNAD is used instead of NAD because in the normal glutamic dehydrogenase reaction the quilibrium is far in the direction of glutamate synthesis and NAD production. However, 3-AcPyNADH is not re-oxidizable and the reaction to form α-ketoglutarate can go to completion (Wyngaarden and Ashton, 1959).

(c) Phosphoribosylamine (PRA)synthesis: An attempt was made to estimate PRPP amidotransferase activity by measuring the utilization of glycine in the reaction mixture, assuming that under the influence of glycinamide ribotide kinosynthase, 1 mole of glycine would react per mole of PRA formed by PRPP amidotransferase. The standard reaction mixture contained: 10 µl of 1.0 M Tris-HCl buffer pH 8.0; 10 µl of 100 mM PRPP; 10 µl of 200 mM $MgCl_2$; 20 µl of 200 mM glutamine; 10 µl of 0.25 M 2-^{14}C-glycine (specific activity 12 µc/m mole); 10 µl of 50 mM ATP; and 30 µl of enzyme containing 15 mg protein/ml.

Controls included reaction mixtures omitting PRPP and reaction mixtures in which $HClO_4$ was added prior to the addition of the enzyme extract. The reaction was initiated by the addition of glutamine, was incubated for 30 minutes at 30°C and was terminated by the addition of 10 µl of ice cold 70% $HClO_4$. The mixture was held at 0°C for 30 minutes and centrifuged.

Portions of the supernatant (25 µl) were co-chromatographed with an equal volume of unlabelled 0.1 M glycine in three solvent systems: butanol:glacial acetic acid:H_2O; 85% ethanol; and butanol: 20% ($^v/_v$) NH_4OH (see chromatographic procedures). The glycine spots were detected using the ninhydrin reagent (0.2% ninhydrin in 5% glacial acetic acid, 90% acetone) and collected into scintillation vials containing 1 ml of 0.1 N HCl. The vials were left at room temperature overnight. 15 ml Bray's solution was added to each vial, and the samples counted in the liquid scintillation counter.

In addition, duplicate strips of the chromatograms run on paper were scanned in the Actigraph III strip scanner (Nuclear Chicago). The origins of the chromatograms developed with the butanol NH_4OH solvent were also eluted and counted as described above.

XII. Isolation and Purification of 5-Phosphoribosyl-1-Pyrophosphate Amidotransferase (PRPP Amidotransferase)

Attempts were made to isolate and purify PRPP amidotransferase, the first enzyme in the pathway for de novo purine biosynthesis. The best purification sequence developed was one similar to that described by Hartman (1963) for the purification of the enzyme from pigeon liver. Eighteen-hour cells, grown in BN medium, were harvested by centrifugation, washed with 0.01 M phosphate buffer pH 7.2 and re-suspended to a density of 2.5 mg cell protein per ml in 0.01 M phosphate buffer pH 7.2, containing 0.01 M Mg Cl_2 and lysozyme at 100 µg/ml. After 30 minutes at 37°C, the mixture was centrifuged for 20 minutes at 43,000 x g and the pellet re-extracted with 0.1 M Tris HCl,pH 7.2. The supernatants were combined and cooled to 0°C, the pH adjusted to 5.3 with 10% ($^{V}/v$) acetic acid and the mixture stirred slowly at 0°C for 30 minutes. The resulting precipitate was collected by centrifugation and discarded. The pH of the supernatant was adjusted to 7.2 with 0.5 M Na_3PO_4, and the solution heated to 60°C, held for 15 minutes, then rapidly cooled and held at 0°C for 30 minutes. The solution was centrifuged and the pellet discarded. The pH of the supernatant was adjusted to 5.3 with cold glacial acetic acid and 27.8 gm of solid $(NH_4)_2SO_4$ was slowly added per 100 ml of supernatant (0.45 saturation). The mixture was stirred gently in an ice bath for 30 minutes and the precipitate collected by centrifugation. The precipitate was re-dissolved in a minimal amount of distilled water and dialyzed for 16

hours in the cold against three changes of 4.0 l of distilled water. The pH of the solution in the dialysis sac was adjusted to 5.3 with 1% acetic acid and the precipitate, after 30 minutes at 0°C, collected by centrifugation. The precipitate was re-suspended in 0.05 M K_2HPO_4. After one hour at 0°C the precipitate was collected by centrifugation and the supernatant saved. The pellet was re-extracted with an equal volume of distilled water for 1 hour, and the supernatant pooled with the initial supernatant. The solution was evaporated under vacuum to a small volume (avoiding precipitation) and then diluted in 0.05 M Tris HCl, pH 7.5, to a final protein concentration of 15 mg/ml.

XIII. Estimation of de novo Purine Biosynthesis

De novo purine biosynthesis was estimated by following the incorporation of 2- ^{14}C-glycine into cell purines as described below.

1. Determination of optimum glycine concentration for glycine transport.

The optimum concentration for glycine transport was determined by measuring the incorporation of ^{14}C-glycine into M. sodonensis over a 15-minute interval. Washed 24-hour cells were incubated with ^{14}C-labelled glycine (specific activity 5.0 μc/mM), at various concentrations for 15 minutes, chilled, centrifuged, washed twice with cold BN medium, and re-suspended in 2.0 ml 1N HCl. Cells were hydrolyzed in an autoclave at 121°C (15 psi) and the hydrolysate neutralized with 2N NaOH, diluted with 10 volumes of Bray's scintillation fluid, and counted in the liquid scintillation counter.

2. Incorporation of glycine into cell purines.

Twenty-four-hour cells grown in BN medium were washed twice and re-suspended in BN medium to a density of 1 mg cell protein/ml. Three flasks, each containing 200 ml of the cell suspension, were incubated at 30°C, with vigorous aeration, for 15 minutes. ^{14}C-glycine, diluted with unlabelled glycine to give a specific activity of 10 μc/m mole at a concentration of 60 mM was added to the cell suspension to give a final concentration of 6 mM glycine. At 5 minutes, and thereafter at 8-minute intervals, 5.0 ml samples of the cell suspension were removed, frozen in a dry ice-acetone bath and stored at -60°C until sampling was completed. At 10 minutes, adenosine was added to one flask to a final concentration of 1 mM, and guanosine was added to another flask to the same final concentration. Sampling was continued as before until 40 minutes.

The frozen samples were treated as follows: 3.0 ml of 1.0 N HCl was added to 0.5 ml portions of the thawed samples and the mixtures hydrolyzed in an autoclave at 121°C (15 psi) for one hour. One ml of a 20% solution of activated charcoal was added to the cooled hydrolysates to adsorb the solubilized nucleic acid components. The mixture was centrifuged and the charcoal washed three times with 10 ml of distilled water. The adsorbed compounds were eluted from the charcoal with four 5 ml portions of 10% pyridine in 50% ethanol, evaporated to dryness in a Rotary Evapomix and the residue re-suspended in 0.5 ml of 0.1 N HCl. These solutions were diluted in 10 ml of Bray's solution and counted in the liquid scintillation counter.

XIV. Analytical Techniques

1. Protein

Soluble protein was determined by the method of Lowry et al. (1951). Crystalline bovine serum albumin was employed as the standard reference protein.

2. Inorganic phosphate.

(a) Microassay: The technique of Ames and Dubin (1960) was employed for the assay of inorganic phosphate in the range of 5 - 200 mμ moles.

(b) Macroassay: The technique of Fiske and Subba Row (1925) was employed for the assay of inorganic phosphate in the range of 0.1 to 2.0 μmoles.

3. Nucleic acid precursors.

(a) General techniques: Free bases, nucleosides and nucleotides were generally identified by their mobilities in a variety of chromatographic systems (see below), by their U.V. absorption spectra and by their 250/260 and 280/260 absorbance ratios. Purines were routinely eluted from paper chromatograms with 0.1 N HCl with an efficiency of about 96%. Eluents could be scanned spectrophotometrically or counted in the liquid scintillation counter without further treatment.

(b) Specific purine assays.

(i) Adenosine: An enzymatic assay modified from the procedure of Kalcar (1947b) was used for adenosine determinations. The standard reaction mixture contained: 0.05 to 0.4 mM adenosine; 0.1 M Tris buffer, pH 7.53; 10 µg adenosine deaminase (Calbiochem, specific activity 255 IU/mg); and water to a volume of 1.0 ml. The decrease in absorbance at 265 mµ and/or the increase in absorbance at 240 mµ were used as a measure of adenosine concentration. Since the deamination reaction to form inosine goes to completion, the absorbance change accurately reflects the adenosine concentrations. Experimental results with known adenosine concentrations indicated an accuracy of ± 1.6% for the technique. The co-efficients for the absorbance changes, at pH 7.53, at 265 mµ and 240 mµ are -7.84/mM and + 4.29/mM respectively.

(ii) Hypoxanthine: An enzymatic assay modified from the method of Kalcar (1947a) was used in the determination of hypoxanthine. The reaction mixture contained 0.05 - 0.4 mM hypoxanthine; 0.1 M Tris buffer, pH 7.53; 0.2 units of xanthine oxidase (Mann Research Laboratories); and water to a final volume of 1.0 ml. The decrease in

absorbance at 255 mμ and/or the increase in absorbance at 292 mμ were used as a measure of hypoxanthine concentration. This reaction also goes to completion, and experimental results with known hypoxanthine concentrations indicated an accuracy of ± 2.2% for this method. The co-efficients for the absorbance changes, at pH 7.53, at 255 mμ and 292 mμ are - 6.42/mM and + 11.77/mM respectively.

(iii) Inosine: When cell-free extracts were used, inosine could be determined by a differential method. In mixtures of adenosine and inosine, adenosine was determined as described above. Using the experimentally determined extinction co-efficient for inosine, at pH 7.53, at 265 mμ, of 5.94/mM, the absorbance of the inosine produced by deamination of adenosine could be calculated. If this value is subtracted from the final absorbance at 265 mμ, the difference is a measure of the inosine that was initially present. This procedure is accurate to within ± 1.9%.

In mixtures of hypoxanthine and inosine, a similar determination could be made by first determining hypoxanthine as described above and then calculating the contribution of the uric acid produced to the total absorbance at 255 mμ using the experimentally determined

extinction co-efficient for uric acid, at pH 7.53, at 255 mμ, of 3.22/mM. The difference between the final absorbance at 255 mμ and the absorbance at 255 mμ due to uric acid was a measure of the initial inosine concentration, which could be calculated using the absorption co-efficient for inosine, at pH 7.53, at 255 mμ of 10.61/mM. The error in this procedure was shown to be ± 1.4%.

(iv) Mixture of adenosine, inosine and hypoxanthine: Adenosine was determined first, and then hypoxanthine and total inosine determined as for a normal hypoxanthine-inosine mixture. Initial inosine was determined as the difference between total inosine and inosine produced by the deamination of adenosine. The method is accurate to within ± 3.5%.

Difference techniques for inosine determinations cannot be performed on culture supernatants because of the variable contributions of unknown compounds which are produced by the cells during growth, to the total U.V absorbance of the supernatant.

XV. Preparation of Activated Charcoal

Ten gm of acid washed charcoal (Norite A) was treated as follows: the charcoal was washed on a filter with 600 ml of 1N HCl, then washed with water to neutrality; the charcoal was further treated with 600 ml of 1 N NH_4OH and washed with water until the filtrate was again neutral. The charcoal was re-suspended in 500 ml of distilled water, washed five times by decanting to remove the very fine charcoal particles present, and re-suspended to a total volume of 100 ml in distilled water. The 10% charcoal suspension was thereafter handled with extreme care to minimize the formation of new fines.

As required, 5 ml (500 mg) of the charcoal suspension were layered onto Whatman No. 1 filter paper supported on a Millipore filtration apparatus (sealed at the joint with parafilm), and washed under suction with 50 ml of distilled water. Twenty-five ml of 8% 2-octanol in 50% ethanol were passed through the bed of charcoal in order to partially deactivate it (Carroll and Graham, 1965; Threlfall, 1957) and the charcoal washed with a further 25 ml of distilled water prior to its use.

XVI. Preparation of PEI Cellulose

The anion exchanger was prepared according to the method of Dr. E. Townsend (Department of Biochemistry, University of Alberta; personal communication), which is a modification of the technique described by Randerath (1965). Fifty percent PEI (Chemirad Corporation) was neutralized with concentrated HCl, diluted to 10% with distilled water, and

stored in the dark at 0°C. As required, portions of this solution were dialyzed for 24 hours at 0°C against three changes of 100 volumes of distilled water, and the dialysate diluted to give a final concentration of 1% PEI in distilled water. Thirty gm of MN-300 cellulose powder (Macherey, Nagel and Co.) were added per 100 ml of 1% PEI solution and a uniform slurry formed by vigorous mixing.

XVII. Chromatographic Techniques

1. Paper and thin layer chromatography.

(a) 85% Ethanol with $(NH_4)_2SO_4$ saturated paper: Whatman No. 1 paper was saturated with 10% $(NH_4)_2SO_4$ and dried for 24 hours. Eighty-five percent ethanol was used as a solvent for the separation of bases, nucleosides and nucleotides by descending chromatography as described by Singh and Lane (1965).

(b) n-Butanol: 20% ($^v/v$) NH_4OH (83:17). (Modified from Markham and Smith, 1949): This solvent system was used in the separation of purine bases and nucleosides by descending paper chromatography or by silica gel thin layer chromatography, and for the separation of glycine and glycinamide ribotide by paper chromatography. The system is particularly useful in the separation of adenine and hypoxanthine and their ribonucleosides, but less effective in the separation of guanine and xanthine derivatives. Nucleotides do not migrate from the origin in this system.

(c) Butanol; glacial acetic acid:H_2O (120:30:70): This solvent system was used in the separation of glycine from glycinamide ribotide by silica gel thin layer chromatography.

(d) i-Propanol: formic acid: 20% urea (64:24:12): This solvent system was employed for the separation of thiamine pyrophosphate, thiamine phosphate and free thiamine by ascending chromatography on 5" x 5" Schleicher and Schuell No. 589 analytical filter paper and by thin-layer chromatography on Eastman Kodak prepared silica gel TLC sheets with fluorescent indicator.

(e) PEI-cellulose thin layer chromatography.

(i) Preparation: TLC plates were prepared by spreading 100 ml of a PEI-cellulose slurry on an 8" x 48" sheet of Polyester TLC film (Kensington Scientific Corporation) with an adjustable Desaga spreader set to permit a layer thickness of 0.35 mm. The layer was dried overnight and cut to the desired size (usually 8" x 8"). A 1.0 cm margin was removed from the edges of the plates and lines 2.0 cm long and 0.75 cm apart were scribed inward from one edge of the layer. A start line was drawn across the layer 1 cm above the ends of the scribe marks. The dried layers were mechanically very stable and could be treated as paper (i.e. they could be written on and marked with pencil in the same way as paper chromatograms).

Plates were stored at 0°C in the dark until required, at which time they were activated by developing with 1.7 M LiCl to 5 cm above the start line and then to the top of the sheet with distilled water. After drying for 2 hours, developing again with distilled water and re-drying overnight, the layers were ready for use. Commercially available PEI-cellulose TLC sheets (J. T. Baker Chemical Co.) were also employed during the later stages of the investigation with good results.

(ii) Development: The gradient elution techniques previously described by Randerath (1965) were used for the rapid separation of mixtures of nucleotides by PEI-cellulose TLC.

The following two-dimensional system proved very useful in separating complex nucleotide mixtures. First dimension employed 0.75 M LiCl for 2.5 cm above the origin; 0.95 M LiCl for 6.5 cm; 1.4 M LiCl for 7.0 cm. Second dimension employed 0.5 M $HCOONH_4$, pH 3.4, for 2 cm above the origin; 0.85 M $HCOONH_4$, pH 3.4, for 3 cm; 2.0 M $HCOONH_4$, pH 3.4, for 4 cm; 4.0 M $HCOONH_4$, pH 3.4, for 7 cm. After development in the first dimension, the LiCl was extracted from the chromatogram by submerging the layer in a tank

of anhydrous methanol for 30-45 minutes. Nucleotides were not eluted from the layer by this procedure. By substituting 1.7 M LiCl for the 10% NaCl recommended by Randerath (1965) for the activation of the chromatogram (see above) it was possible to completely remove salts from the PEI-cellulose layer and thus obtain a more uniform development of the solvent front in the second dimension.

2. Column chromatography.

(a) PEI cellulose column chromatography.

(i) Preparation of resin (Miller and Kirkpatrick 1968): A PEI cellulose slurry was prepared as described above, spread in layers 0.25 cm thick and allowed to dry in the dark at room temperature for 96 hours, then transferred to 37°C for an additional 48 hours to ensure complete dryness. The dried layers were ground to a fine powder in a mortar, washed on a filter with absolute methanol until all of the excess PEI was removed, dried under vacuum and reground in a mortar. The powder was suspended in distilled water and washed by decanting to remove fines.

Whatman CC 31 microgranular cellulose (mean wet particle size 15-40 mμ)was washed by decanting with distilled water to remove fines, and equal weights of

washed PEI cellulose and microgranular cellulose were mixed in distilled water to form a homogeneous slurry. The microgranular cellulose is included to reduce column back pressure and thus increase the flow rate. Increasing the proportion of microgranular cellulose will permit faster flow rates, but larger columns are required to maintain the same exchanging capacity.

(ii) Packing and conditioning of the resin: Fifty gm wet weight of the slurry were diluted with 3 volumes of distilled water and poured into a column 1.75 x 30 cm. The settled resin was packed under a hydrostatic pressure head of 35 cm for one hour and then further packed under a head of 150 cm for 12 hours. After 12 hours the resin bed had packed down to 20 cm in height and the column flow rate was 65 ml/hr.

The column resin was conditioned (and regenerated after use) with: a) 10 ml of 4.0 M NaCl, 0.05 M NaH_2PO_4, pH 3.6; b) 20 ml of 0.05 M NaH_2PO_4, pH 4.0; c) 25 ml distilled water. Although it was not necessary to repack the column after each run, it was found that after a number of runs the flow rate began to decrease.

In order to maintain a good flow rate it was necessary to remove the resin, wash, by decanting, to remove newly formed fines and repack the column. Normally, it was possible to make 10 - 12 runs before repacking was necessary.

(iii) Elution of nucleotides: A mixture of nucleotides dissolved in 0.001 M phosphate buffer, pH 6.95, was applied to the column, which was then washed with 0.001 M phosphate buffer, pH 6.95, until no further U.V. absorbing materials could be detected in the effluent. Any bases and nucleosides in the mixture were washed from the column by this procedure. Nucleotides were eluted with two successive gradients: first, a pH gradient consisting of 100ml 0.001 M phosphate buffer, pH 6.95, in the mixing chamber, and 100ml of 0.001 M NaH_2PO_4, pH 3.0, in the reservoir; and second, a NaCl gradient consisting of 150ml of 0.001 M NaH_2PO_4, pH 3.0, in the first mixing chamber, 150ml of 0.1 M NaCl, 0.05 M NaH_2PO_4, pH 3.0, in the second mixing chamber, and 150ml of 3.0 M NaCl, 0.05 M NaH_2PO_4, pH 3.0, in the reservoir. Column effluent was collected in 3 ml fractions with an LKB automatic fraction collector. The pH gradient resolved TMP, CMP, AMP, UMP and IMP, and the salt gradient resolved GMP, XMP and the nucleoside di- and tri-phosphates.

The column was calibrated with simple and complex mixtures of known nucleotides and the elution profiles of these nucleotides were reproducible almost tube for tube in several different determinations.

(b) Dowex 50 column chromatography. Dowex AG 50W - X4 (200 - 400 mesh) in the hydrogen form was employed in the separation of nucleotides from bases and nucleosides in desalted mixtures of these compounds. The hydrogen form of the resin was prepared and regenerated by washing the resin alternately with 5 volumes of water and 2N NaOH, ethanol, acetone, 3N HCl, and then water until the effluent was neutral. A column of resin 2.5 by 10 cm, supported by a sintered glass disc, was poured and washed with 200 ml of distilled water. A thin layer of acid-cleaned glass beads was introduced to maintain an intact resin surface.

Desalted mixtures of bases, nucleosides and nucleotides were introduced into the column and forced through the resin bed with compressed air. Generally, the solution was passed through the column of resin several times until the adsorbance at 260 mμ of the effluent could no longer be reduced by subsequent passages. If very high concentrations of bases and nucleosides were being handled, larger columns were required, but in these experiments a 2.5 by 10 cm column was large enough to completely absorb the bases and nucleosides present.

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Nucleotides were washed out of the column with distilled water until the absorbance of the effluent measured at 260 mμ was less than 0.001. Bases and nucleosides could then be eluted from the resin with 1.0N NH_4OH. Under these conditions, nucleotides were completely separated from bases and nucleosides, and both fractions were quantitatively recovered from the column.

Because of the large diameter of the column, the resin may be regenerated in situ, even in columns of 25 - 30 cm in height, without causing significant packing of the resin bed.

(c) Dowex 1 column chromatography: Dowex AG1 - X 2 (200 - 400 mesh) was used for the separation of purine and pyrimidine nucleotides by column chromatography. The formate form of the resin was prepared and regenerated by washing the chloride form of the resin on a filter alternately with 10 volumes of water and 5.0 M naCl, ethanol, acetone, and then with 3.5 $HCOONH_4$ until Cl^- was not detectable in the effluent, and water until the excess $HCOONH_4$ was eluted. The resin was packed in a column 1 cm in diameter to a height of 60 cm and washed with 100 ml of distilled water. A salt-free nucleotide mixture was introduced into the column and washed on with an additional 10 ml of distilled water.

Nucleotides were eluted from the column under a hydrostatic pressure head of 2.5 meters with a phosphate/pH gradient consisting of: 200 ml of 0.225M K_2HPO_4, pH 5.5; 200 ml of 0.3375M KH_2PO_4, pH 4.5; 0.4425M K_2HPO_4, pH 4.5; and 0.482M K_2HPO_4, pH 4.5. Solutions of K_2HPO_4 were adjusted to the designated pH with 85% H_3PO_4.

After UDP was eluted from the column, the gradient flow was halted, and the buffer covering the resin bed permitted to drain to a level 4 cm above the surface of the resin. Just as UTP was eluted from the column, a further chamber containing 150 ml of 5.0 M NaCl was added in series with the first four chambers. These latter two steps are required in order to facilitate the elution and increase the resolution of the nucleotides which come off the column during the later stages of the gradient. The order of elution with this buffer system is CMP, UMP, CDP, UDP, CTP, AMP, UTP, GMP, ADP, GDP, ATP, and GTP. Compounds eluted from the column were identified by their adsorbance spectra and by their positions in the elution profile relative to those of previously chromatographed standards.

RESULTS AND DISCUSSION

I. The Effects of Purines and Purine Derivatives on the growth of M. sodonensis

In BN medium, M. sodonensis grows exponentially with a mean generation time of 2.8 hours until the culture reaches a cell density of approximately 0.8 to 0.9 mg cell protein/ml, at which time the growth rate decreases and the cells enter a stationary phase with a final cell yield of 1.1 to 1.4 mg cell protein/ml (Fig. 1, Fig. 2 and Fig. 3). When purine bases or nucleosides are individually added to BN medium at concentrations of 1.0 mM they are slightly stimulatory to the growth of the organism (Fig. 1), with the exception of adenosine, which is inhibitory at concentrations of 0.1 mM or greater (Fig. 1, Fig. 2, Fig. 3) and, to a lesser extent, inosine and hypoxanthine, which are inhibitory at concentrations of 1.0 mM (Fig. 2).

The growth curve for M. sodonensis growing in the presence of adenosine is characteristically tetraphasic (Fig. 1, Fig. 2, Fig. 3). The first phase involves a lag of approximately two generation times (5 - 6 hours) before inhibition is observed, and the length of the lag phase was noted to be constant at the adenosine concentrations tested. This observation suggests either that two generations are required for an inhibitor to be synthesized, or for it to exert its effect, or that an inhibitor is blocking the synthesis of an essential metabolite, the intracellular pool of which must be depleted before the inhibition can be

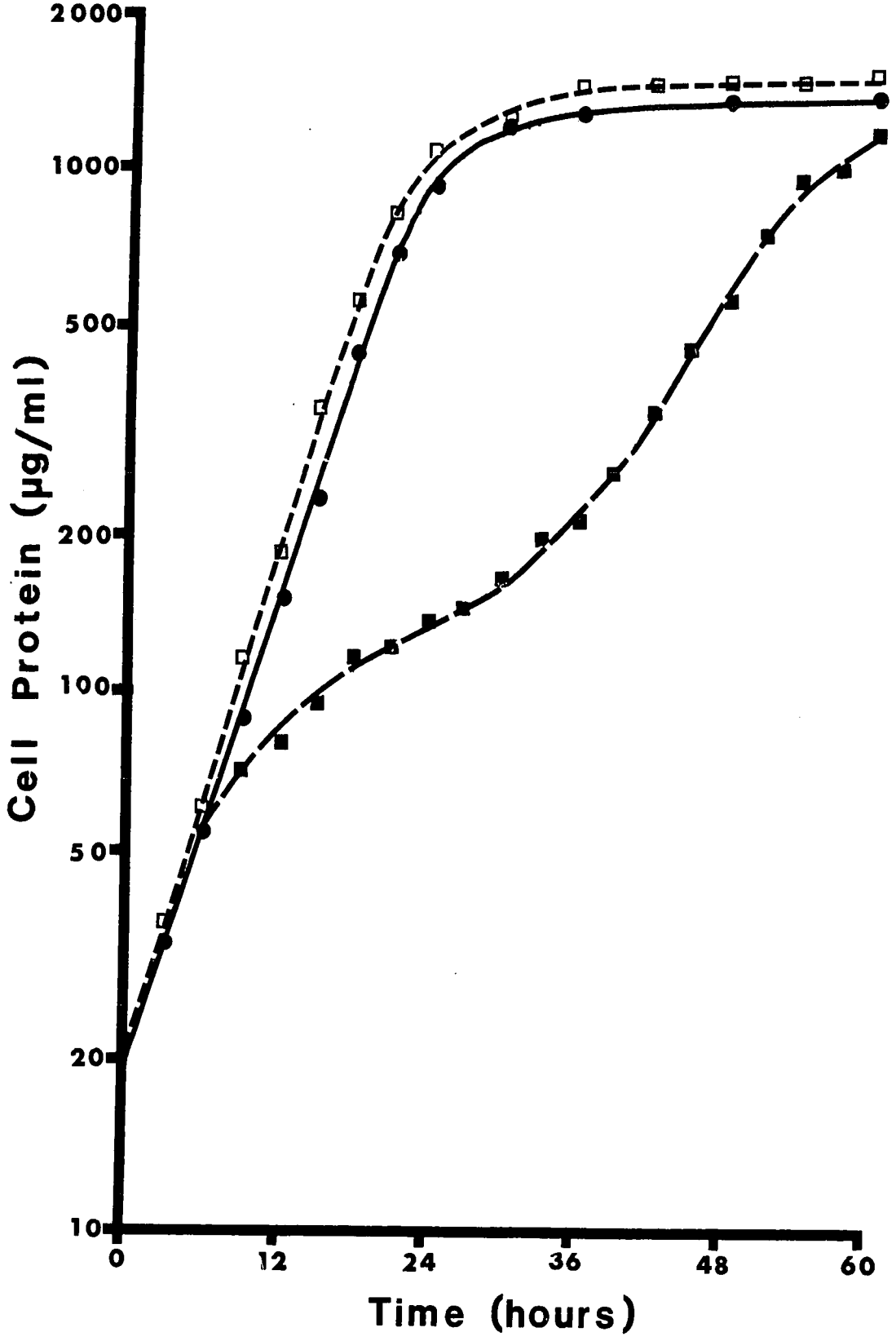
2000
1000
500
200
100
50
20
10
Cell Protein (µg/ml)
0
12
24
36
48
60
Time (hours)

FIGURE 1

EFFECTS OF PURINE BASES AND NUCLEOSIDES ON THE GROWTH OF M. SODONENSIS

Cell protein was determined by a modification of the procedure of Lowry et al., (1951) for insoluble protein.

BN-medium ●——●

BN-medium + 1.0 mM guanine, guanosine, adenine, xanthine or xanthosine □-----□

BN-medium + 1.0 mM adenosine ■— — —■

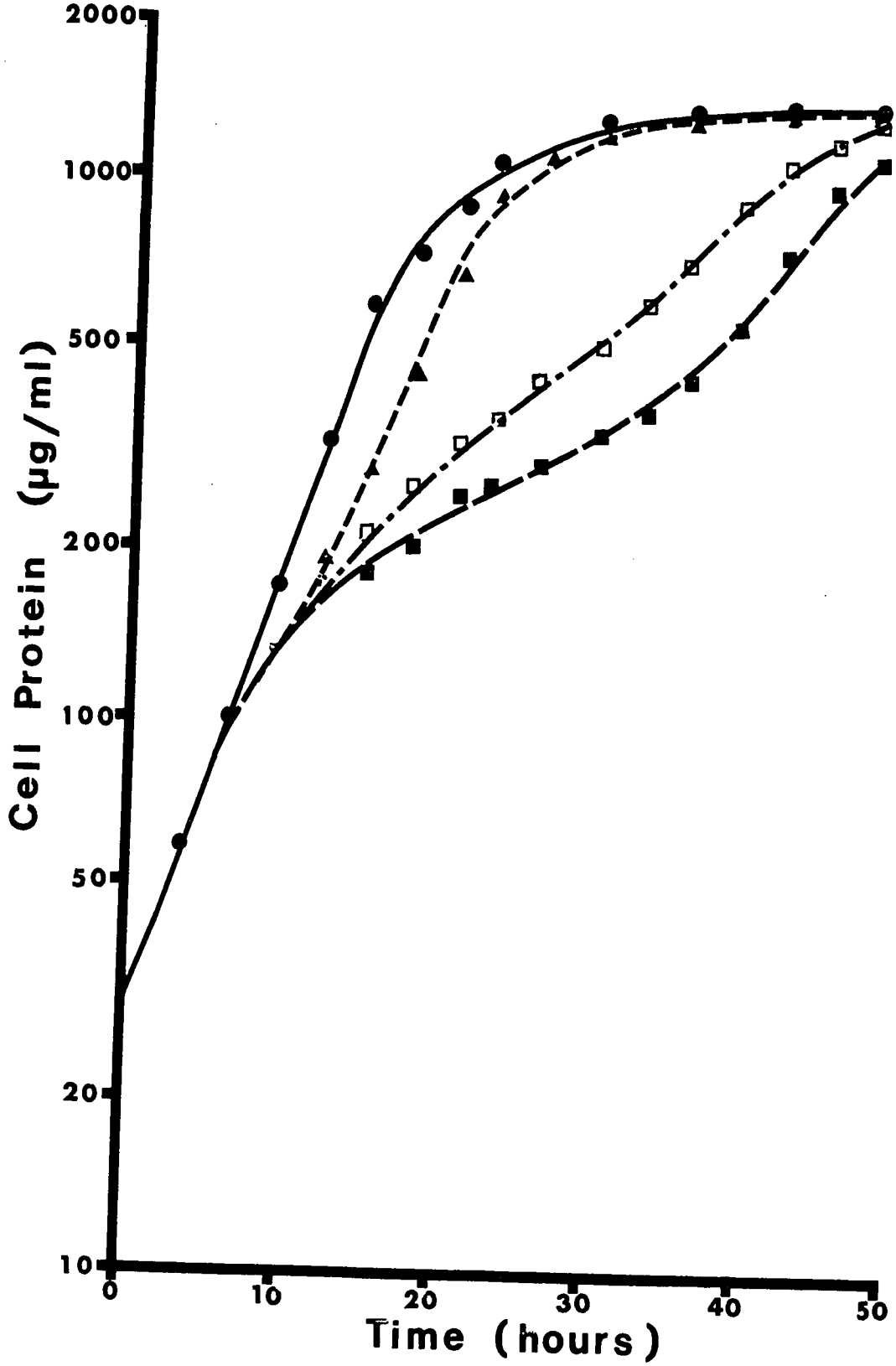
2000
1000
500
200
100
50
20
10
Cell Protein (µg/ml)
0
10
20
30
40
50
Time (hours)

FIGURE 2

COMPARISON OF THE EFFECTS OF ADENOSINE, INOSINE AND HYPOXANTHINE AT 1.0 mM ON THE GROWTH OF M. SODONENSIS

Cell protein was determined by a modification of the procedure of Lowry et al., (1951) for insoluble protein.

BN-medium

BN-medium + 1.0 mM adenosine

BN-medium + 1.0 mM inosine

BN-medium + 1.0 mM hypoxanthine

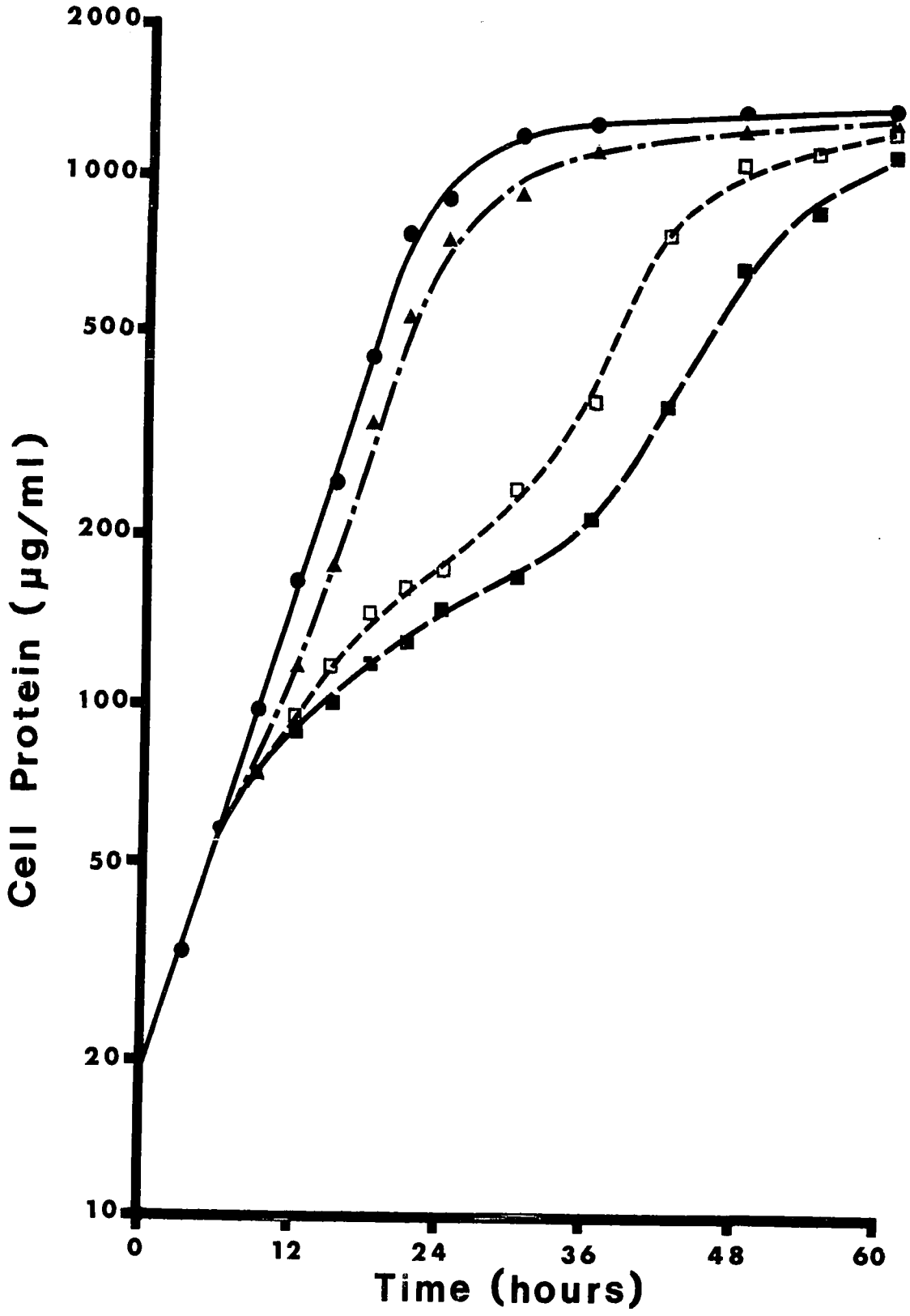
2000
1000
500
200
100
50
20
10
Cell Protein (µg/ml)
0
12
24
36
48
60
Time (hours)

FIGURE 3

EFFECT OF INITIAL ADENOSINE CONCENTRATION ON THE INHIBITION OF M. SODONENSIS BY ADENOSINE

Cell protein was determined by a modification of the procedure of Lowry, et al., (1951) for insoluble protein.

BN-medium ●——●

BN-medium + 1.0 mM adenosine ■— — —■

BN-medium + 0.5 mM adenosine □-----□

BN-medium + 0.1 mM adenosine ▲— – —▲

observed. As will be seen, the experimental results favour the latter possibility.

The second phase (or inhibited phase) of growth in the presence of adenosine varies in duration, depending upon the initial adenosine concentration (Fig. 3). This observation suggests that adenosine or an inhibitory metabolite is eliminated from the culture. At lower adenosine concentrations, less inhibitor would be present and, if it were metabolizable, it would be eliminated more rapidly by a given mass of cells. This would permit an earlier initiation of the recovery phase (third phase) in cultures with lower initial adenosine concentrations. The fourth phase appears to be a stationary phase corresponding to that in BN medium.

The growth rate during the recovery phase more closely approximates the growth rate in BN medium, in those cultures with lower initial adenosine concentrations (Fig. 3). This could be a reflection either of damage to the cells as a result of prolonged inhibition, or of residual traces of inhibitor in cultures with higher initial adenosine concentrations. The latter possibility is most likely, since inocula prepared at 48 hours from cultures supplemented with 1.0 mM adenosine grow normally when inoculated into BN medium.

Contrary to the report of Brooke and Magasanik (1954) that the recovery of A. aerogenes from adenine-induced growth inhibition was the result of a mutation to adenine-resistance, it could easily be demonstrated that this was not true for M. sodonensis. If cells harvested from 1.0 mM adenosine-supplemented medium after 48 hours growth

were washed and re-inoculated into fresh 1.0 mM adenosine-supplemented BN medium, the characteristic tetraphasic, inhibited growth response was observed. This finding suggests that the cells are capable of resuming their normal growth function in the absence of a genetic alteration of the cells, and supports the previous suggestion that recovery is a result of the elimination from the culture of the inhibitory substance.

Figure 4, (a re-plot of the data for Figure 3), illustrates that during the lag and inhibited phases, growth in adenosine-supplemented medium is non-exponential. A similar observation has been reported by Neidhardt (1963) on adenosine-inhibited cultures of A. aerogenes. This observation suggests either that the synthesis of an essential metabolite is partially inhibited, or that the synthesis of a stable re-cycling catalyst is completely inhibited so that only a small, but constant amount is available for reaction. In both instances, if there was initially a pool of the substance inside the cell which would allow normal growth to occur until the pool size became limiting, the growth responses that have been observed could occur. It is not possible to differentiate between these two possible mechanisms on the basis of these observations alone.

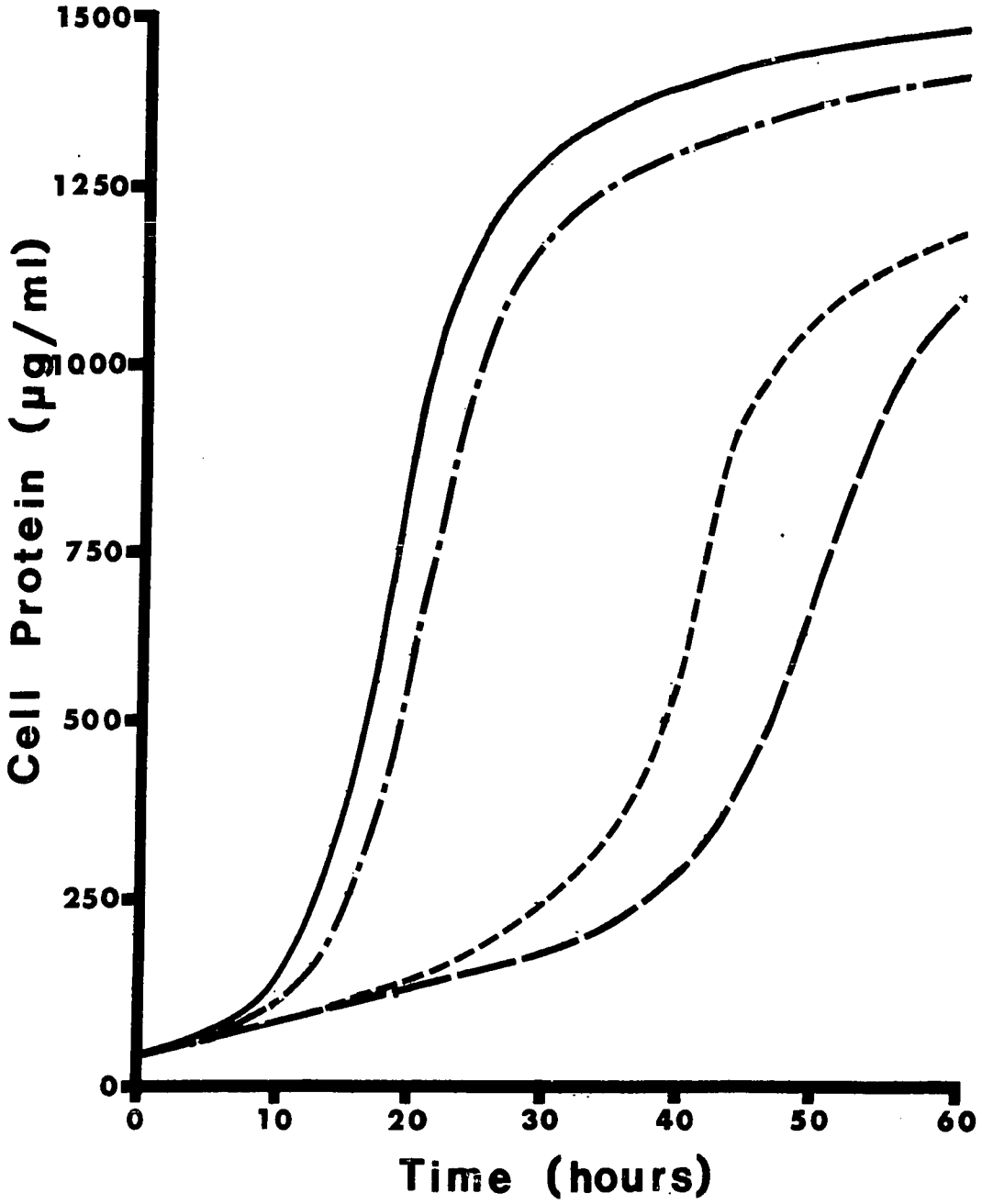
1500
1250
1000
750
500
250
0
Cell Protein (µg/ml)
0
10
20
30
40
50
60
Time (hours)

FIGURE 4

NON-EXPONENTIAL GROWTH DURING THE INHIBITED PHASE
IN ADENOSINE-SUPPLEMENTED CULTURES
OF M. SODONENSIS

Cell protein was determined by a modification of the procedure of Lowry, et al., (1951), for insoluble protein.

BN medium ————————

BN medium + 1.0 mM adenosine — — —

BN medium + 0.5 mM adenosine ----------

BN medium + 0.1 mM adenosine —— – ——

FIGURE 4

NON-EXPONENTIAL GROWTH DURING THE INHIBITED PHASE
IN ADENOSINE-SUPPLEMENTED CULTURES
OF M. SODONENSIS

Cell protein was determined by a modification of the procedure of Lowry, et al., (1951), for insoluble protein.

BN medium ————————

BN medium + 1.0 mM adenosine — — —

BN medium + 0.5 mM adenosine ----------

BN medium + 0.1 mM adenosine —— – ——

II. The Relationship Between the Transport of Purines and Purine Derivatives and their Effects on Cell Growth

The question was asked as to why only certain purine bases and nucleosides are bacteriostatic, while most others are not. In attempting to answer this, the first consideration was that differences in the transport rates of the purine bases and nucleosides could be of importance in determining their effectiveness as inhibitors.

The incorporation by M. sodonensis of a number of purine bases and nucleosides were determined in a complete growth medium. Washed 18-hour cells were used in the experiments and the incorporation of 8-^{14}C-labelled purine bases and nucleosides followed as described in 'Materials and Methods' (VII).

The validity of the chilling technique as a method for terminating purine transport experiments was tested in the following variation of a typical adenosine incorporation experiment. Two minutes after mixing the cell suspension with the adenosine-containing solution, one portion of the mixture was chilled and centrifuged (control), while a second portion was chilled and placed in an ice-water bath at 0°C. The remainder of the cell suspension was incubated at 30°C for an additional 15 minutes. At that time a portion of the cell suspension was chilled, and both it, and the portion which had been held at 0°C for 15 minutes, were centrifuged. It was found that the sample held at 0°C for 15 minutes incorporated an additional 11.2% (0.75%/minute) over the control value, while the sample held at 30°C incorporated an additional 284%

(or 18.9%/minute). Assuming a 6-minute (maximum) holding period (for chilling and centrifugation) in a typical experiment, the maximum amount of adenosine that would be taken up during the experimental holding period would be in the order of 4% of the amount previously incorporated. Since this value is small, and was shown to be consistent in several experiments, it was disregarded in the calculations.

As can be seen in Table I, adenosine uptake is greater than the uptake of inosine and hypoxanthine, and these differences appear to correlate with the relative effectiveness of these compounds as growth inhibitors (Fig.2). However, other non-inhibitory purine derivatives (in particular, guanosine) enter M. sodonensis at rates equal to, or greater than, those of the bacteriostatic compounds. This suggests that transport is not an important determinant of inhibitory capability, although the rate of transport (or, in other words, the rate at which the intracellular concentration of the compound or its inhibitory derivative is increased) may be a factor in modifying the inhibition which is effected. This does not, however, explain the specific susceptibility of M. sodonensis to the inhibitory compounds, as opposed to the other, readily-transportable but non-inhibitory, purine derivatives. For this reason, the investigation of the mechanism for growth inhibition was subsequently directed towards an examination of other metabolic events associated with growth inhibition.

TABLE I

UPTAKE OF PURINE BASES AND NUCLEOSIDES BY M. SODONENSIS

^{14}C-Permeant	Disappearance of ^{14}C from supernatant (mμ moles/mg of cells/10 min.)
Adenosine	15.5
Inosine	5.6
Hypoxanthine	2.4
Guanosine	16.4
Guanine	3.7
Adenine	3.3
Xanthosine	1.9

Uptake was determined by measuring the disappearance of radioactivity from the supernatant over a 10-minute interval in cultures in BN medium supplemented with the appropriate ^{14}C-labelled purine base or nucleoside at a concentration of 0.2 mM and a specific activity of 25 μc/m mole.

III. The Metabolism of Adenosine by M. sodonensis

In order to elucidate the role of adenosine as a bacteriostatic agent it was necessary to conduct an investigation into the fate of adenosine in cultures of M. sodonensis and to examine the possible routes by which adenosine could be metabolized. Consequently, the events occurring in the supernatant and in cell fractions of adenosine-supplemented cultures were investigated, as were the activities of a number of enzymes that might be involved in adenosine metabolism.

1. Changes occurring in the culture supernatant.

Washed 18-hour cells grown in BN-medium were inoculated into fresh BN-medium containing 0.5 mM ^{14}C-labelled adenosine (25 µc/m mole) to a cell density of 4.8 mg cell protein/ml and incubated at 30°C. At specific time intervals, 0.25 ml samples of the culture were taken, centrifuged in a Beckman Microfuge to remove cells, and the supernatant analyzed by paper chromatography, using the n-butanol/NH_4OH solvent system ('Materials and Methods', XVII). Spots located with U.V. light and by scanning with a Nuclear Chicago Actigraph III strip scanner were cut out, eluted with 0.1N HCl, and quantitated spectrophotometrically.

The results of these experiments, illustrated in Figs. 5 and 6, show that as adenosine disappeared from the supernatant, first inosine and then hypoxanthine were detectable. As the incubation time was increased, the inosine concentration rose to a maximum and then decreased until, at the end of the experiment, hypoxanthine was the only purine

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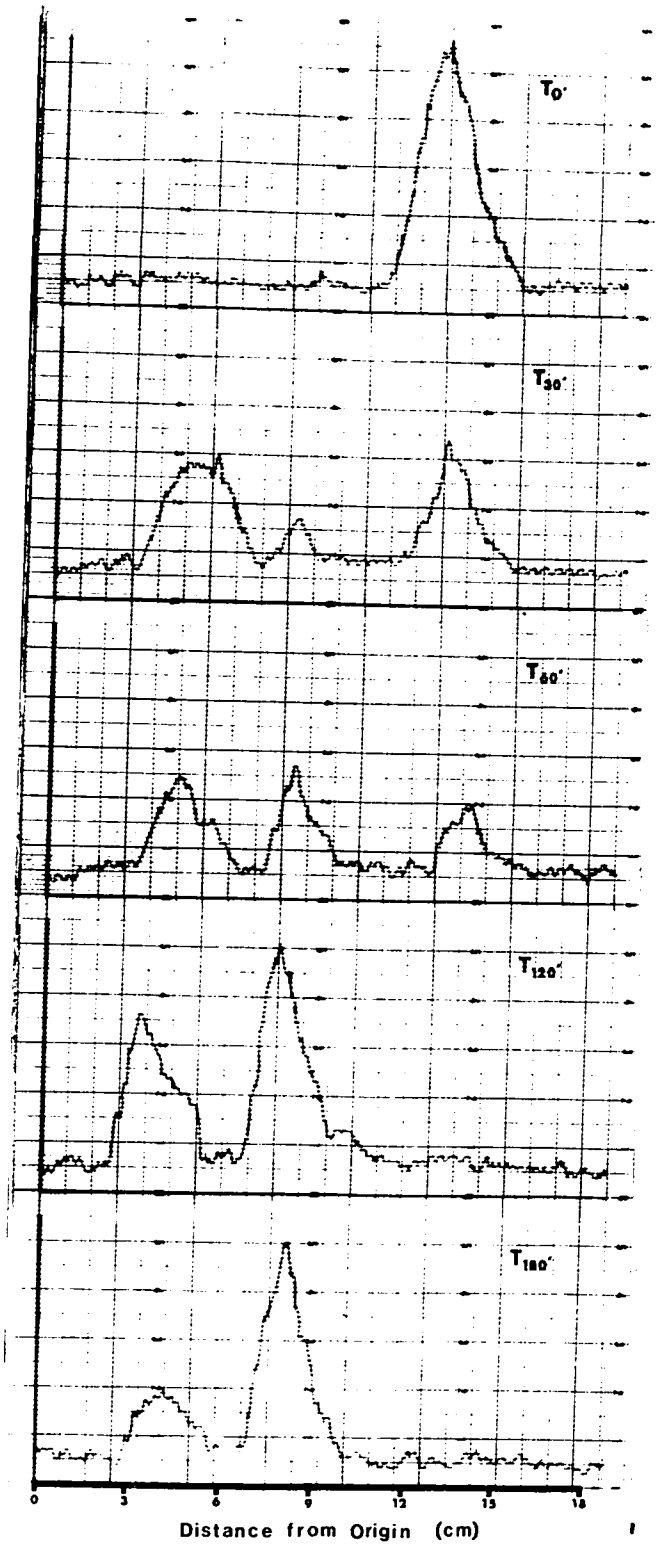

$T_{0°}$
$T_{30°}$
$T_{60°}$
$T_{120°}$
$T_{180°}$
0
3
6
9
12
15
18
Distance from Origin (cm)

FIGURE 5

TIME-DEPENDENT DISAPPEARANCE OF ^{14}C-ADENOSINE AND APPEARANCE OF ^{14}C-INOSINE AND HYPOXANTHINE IN THE SUPERNATANT OF ADENOSINE-SUPPLEMENTED CULTURES OF M. SODONENSIS

Five ml samples of culture grown in 0.5 mM ^{14}C-adenosine (specific activity, 25 µc/m mole) were centrifuged at 43,000 x g at the designated times and 100 µl were spotted on Whatman No. 1 filter paper and chromatographed with the n-butanol:20% NH_4OH (83:17) solvent system. Compounds were located with U.V. light and radioactive spots detected by scanning with a Nuclear Chicago Actigraph III strip scanner.

Adenosine	13 cm from origin
Inosine	4.5 cm from origin
Hypoxanthine	8 cm from origin

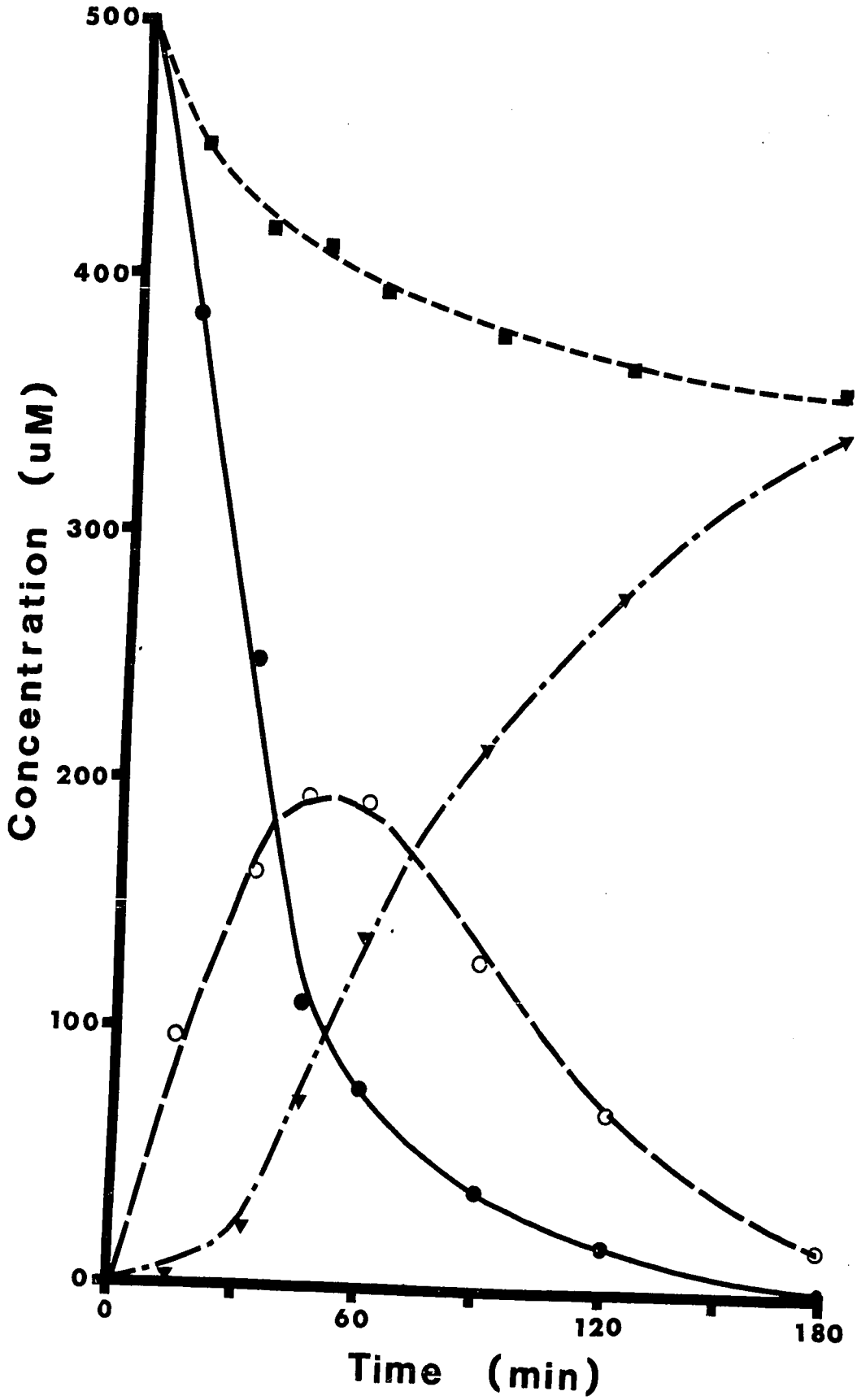
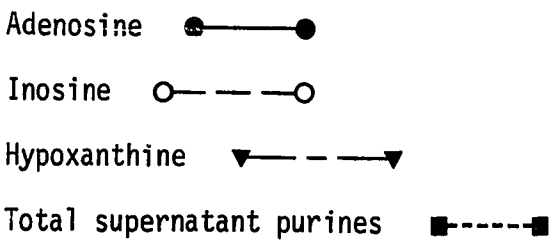

500
400
300
200
100
0
Concentration (uM)
0
60
120
180
Time (min)

FIGURE 6

TIME-DEPENDENT CHANGES IN THE CONCENTRATIONS OF ADENOSINE, INOSINE, AND HYPOXANTHINE IN SUPERNATANTS OF 0.05 mM ADENOSINE-SUPPLEMENTED CULTURES OF M. SODONENSIS

Five-ml samples of the culture were centrifuged at 43,000 x g at the designated times and 100 µl of the supernatant were spotted on Whatman No. 1 filter paper and chromatographed with the n-butanol:20% NH_4OH solvent system. Spots were located under U.V. light, cut out and eluted from the paper with 0.1 N HCl and quantitated spectrophotometrically.



present in the supernatant in a significant concentration. At no time was adenine detected in the supernatant. The data indicate that adenosine is deaminated very rapidly in the cells and that the inosine thus formed can either escape from the cells or be further catabolized with the release of hypoxanthine. The appearance and apparent accumulation of hypoxanthine in the culture supernatant suggests that the reduced growth rates observed during the recovery phase of cells grown in BN-medium supplemented with high initial adenosine concentrations (Fig. 3) could be a reflection of the presence of hypoxanthine in the culture supernatant, since hypoxanthine was previously shown to be a partially effective bacteriostatic agent in this system (Fig. 2).

2. Distribution of adenosine derivatives in the culture.

In order to ascertain whether or not adenosine metabolism in M. sodonensis was typical, prior to a more complete investigation of the distribution of adenosine derivatives within the cells, a simple fractionation of a culture exposed to 0.5 mM ^{14}C-adenosine (specific activity, 25 μc/m mole) for various periods of time was carried out. At the designated time intervals, 4 ml samples were removed, centrifuged and the cell pellet washed with distilled water. The pooled "supernatant" fraction was saved. The pellet was extracted twice, for 30 minutes at 0°C each time. with 2 ml of cold 5% TCA to obtain the "TCA-soluble" and "TCA-insoluble" fractions. Radioactivity in the three fractions was determined by liquid scintillation counting ('Materials and Methods'. VIII).

The distribution of adenosine derivatives among the three fractions is shown in Fig. 7. Their accumulation in the 5% TCA-soluble fraction and subsequent incorporation into the 5% TCA-insoluble fractions is qualitatively as might be expected if adenosine were being metabolized according to the normal pathways that have been described (i.e. through the nucleotide pool into the nucleic acid components of the cells).

In a similar type of experiment, the incorporation of label from ^{14}C-adenosine into the 5% TCA-insoluble fraction over a one-hour interval was compared with the incorporation from ^{14}C-guanosine under identical conditions. The 5% TCA-soluble fraction was further treated to obtain alkali-labile (RNA-derived ribonucleotides) and alkali-stable (DNA) fractions as described in 'Materials and Methods' (VIII), and the incorporation of label into those fractions was also determined by liquid scintillation counting. The results given in Table II indicate that the total incorporation of label into the TCA-insoluble fraction is lower from adenosine than from guanosine. In addition, there may be a disproportionate incorporation of adenosine derivatives into the RNA fraction as compared with the DNA fraction, or a disproportionate inhibition of DNA synthesis in adenosine-grown cells as compared with guanosine-grown cells. This would be in agreement with the observations made by Neidhardt (1963) on adenosine-inhibited A. aerogenes.

These results suggest two possible sites for inhibition by adenosine or its derivatives. Either a step in the synthesis of deoxyribonucleotides and therefore, ultimately, of DNA, could be specifically inhibited, or

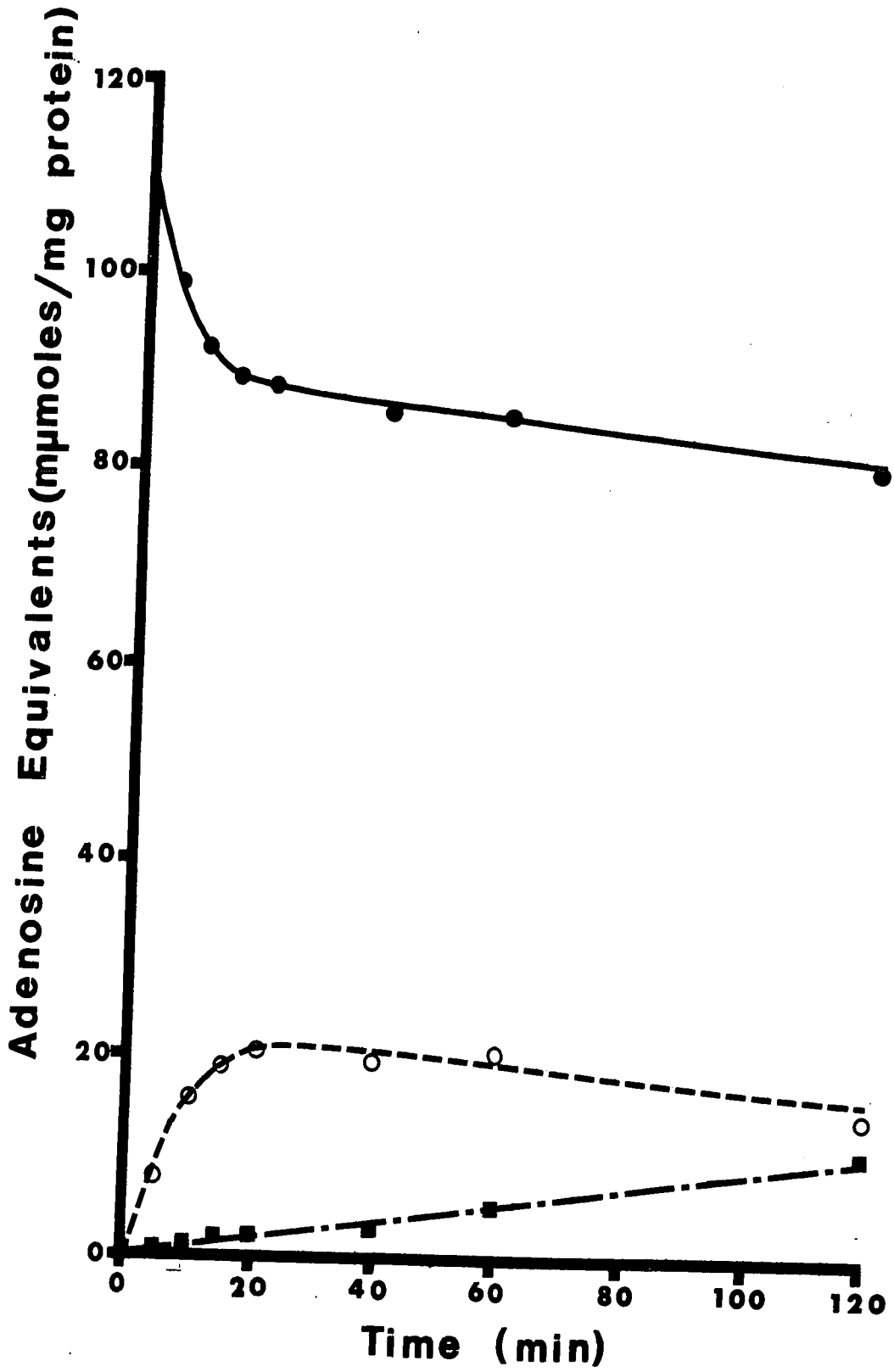

Adenosine Equivalents(mμmoles/mg protein)
120
100
80
60
40
20
0
0
20
40
60
80
100
120
Time (min)

FIGURE 7

CHANGES IN THE DISTRIBUTION OF ^{14}C-LABELLED ADENOSINE DERIVATIVES WITH TIME IN FRACTIONS OF A CULTURE OF M. SODONENSIS

BN-medium was supplemented with 0.5 mM ^{14}C-labelled adenosine (specific activity 26 µc/m mole). Portions of the culture were fractionated at the designated times into supernatant, 5% TCA-soluble and 5% TCA-insoluble fractions and the radioactive material in the three fractions was measured.

Supernatant fraction ●———●

5% TCA-insoluble fraction ■— – —■

5% TCA-soluble fraction O-----O

TABLE II

DISTRIBUTION OF ^{14}C-LABELLED DERIVATIVES OF ADENOSINE OR GUANOSINE IN THE 5% TCA-INSOLUBLE CELL FRACTION AFTER 60 MINUTES INCUBATION

Exogenous Nucleoside (0.5 mM)	Concentration of derivatives in 5% TCA-Insoluble fraction (n moles/mg cell protein).				
	Total	Alkali Labile (RNA)	Alkali Stable (DNA)	Ratio:	Alkali labile / Alkali stable
Adenosine	5.2	4.86	0.34		14.3
Guanosine	39.5	25.20	14.3		1.76

The 5% TCA-insoluble fractions of cells incubated for 60 minutes in the presence of 0.5 mM ^{14}C-labelled adenosine (specific activity, 26 µc/m mole) or guanosine (specific activity, 23 µc/m mole) were fractionated into alkali labile (containing 2' and 3'-nucleoside-monophosphates derived from RNA) and alkali stable (containing DNA) fractions, and radioactivity in the fractions was determined.

energy-yielding catabolism in the cells could be interfered with at some step, resulting also in a reduction of total nucleic acid synthesis. If the latter hypothesis is valid, one would expect to see a disproportionate inhibition of DNA synthesis as compared to RNA synthesis, since de novo deoxyribonucleotide biosynthesis involves more ATP-requiring steps.

3. Effect of exogenous adenosine and guanosine on the acid-soluble nucleotide pool of M. sodonensis.

The overall experiment was carried out as follows: washed 18-hour cells were incubated in BN-medium in the presence and absence of 1.0 mM adenosine or 1.0 mM guanosine. At various intervals, 0.3 ml samples of adenosine-supplemented cultures were centrifuged to remove cells and the supernatants assayed enzymatically for residual adenosine ('Materials and Methods', XIV 3, a, i). In addition, 50 µl of the supernatant from both adenosine and guanosine-supplemented cultures were chromatographed on paper using the n-butanol/NH_4OH solvent system in order to determine other supernatant purine derivatives. At 15 minutes after inoculation and at those times when 50% and less than 0.1% of the original adenosine was remaining in the culture supernatant (as determined enzymatically) the cultures were acidified with 0.1 volumes of ice-cold 70% $HClO_4$, and rapidly cooled in an ice bath. Extraction of the nucleotide pool, desalting with charcoal, fractionation into nucleotide and non-nucleotide fractions, and quantitation of adenine and guanine nucleotides were carried out as described in 'Materials and Methods' (IX and XVII, 2, 6b).

(a) Bases and nucleosides in the intracellular acid-soluble pool.

In view of the necessity to acidify cultures of M. sodonensis prior to extraction of the acid-soluble pool (see below), intracellular non-phosphorylated purine derivatives could not be determined directly. However, these could be determined indirectly by taking the difference between their concentrations in the supernatant of the culture and their total concentrations in the culture. Therefore, 0.3 ml samples of the culture, obtained immediately prior to acidification, were centrifuged to remove cells, chromatographed on paper using the n-butanol/NH_4OH solvent system, and the spots located with U.V. light eluted with 0.1 NHCl and quantitated spectrophotometrically. The values obtained were compared with those obtained by similarly treating samples of the neutralized acid-soluble extract (prior to charcoal desalting procedures). Equivalent samples of the neutralized extract were chromatographed, as were samples equivalent to 40 times the amount of supernatant that had been chromatographed, in order to ensure that compounds present in small amounts would also be detected. The difference between the concentration of purine derivatives in the neutralized extract and in the culture supernatant were taken as a

measure of the intracellular concentrations. The value for each compound was determined by averaging the values from 6 different determinations.

The only non-phosphorylated purine derivatives which could be detected in cells from adenosine-supplemented cultures were inosine (8.5 mμ moles/mg cell protein), hypoxanthine (11.32 mμ moles/mg cell protein), and adenosine (less than 1.0 mμ mole/mg cell protein). These data suggest that adenosine is not functioning directly as the inhibitor since it is present intracellularly in such low concentrations, but rather that it functions as the most effective extracellular precursor of the intracellular inhibitor. Exogenous inosine and hypoxanthine also produce growth inhibition, but are less effective than adenosine, possibly as a result of their comparatively slow rates of transport into the cells (as was previously discussed).

(b) Purine nucleotides in the intracellular acid-soluble pool.

i) Analysis of the nucleotide extraction technique

Before the results of these experiments are presented, a brief discussion of some of the problems encountered in the analysis of the nucleotide pool and the techniques which were devised to

circumvent them is in order. For example, washing cells of M. sodonensis even at low temperatures (-7°C), prior to extraction with $HClO_4$ permitted rapid degradation of nucleoside triphosphates, generally the predominant components of intracellular nucleotide pools, to below detectable levels. This breakdown could be controlled by acidifying the culture with 0.1 volume of 70% $HClO_4$ prior to centrifugation. However, this procedure introduced the new problem of desalting the nucleotide extracts - a necessary preliminary step to ion-exchange chromatography. The $HClO_4$ can easily be removed by neutralizing the extract in the cold with KOH and centrifuging the $KClO_4$ precipitate which forms. However, the medium salts, which are necessary for optimum adenosine incorporation and which had been removed in the initial procedure by washing the cells prior to acid extraction, remained in the nucleotide extracts.

A variety of techniques were unsuccessfully employed in attempts to desalt the nucleotide mixtures. Sephadex G-10 was unsuccessful because of retardation of nucleoside monophosphates by the gel; Biogel P-2 had unsuitable exclusion limits.

Even recycling the extracts through high-capacity columns two and three times failed to give adequate separation of the salts from the nucleotides.

Attempts were made to adsorb the nucleotides onto anion exchange resins in the hope that the salt problem could be circumvented by washing the the columns with large volumes of water, and subsequently eluting the nucleotides with salt or pH gradients. Although good gradient elution systems were devised for the separation and quantitation of of synthetic nucleotide mixtures with PEI cellulose and Dowex 1 (formate) resins, it was not possible to use these techniques when salts were associated with the mixtures of nucleotides (the normal situation for cell extracts), since the salts interfered with the binding of nucleotides to the resins. Desalting with a mixed resin consisting of equal parts of Dowex 1 (OH^-) and Dowex 50 (H^+) was also unsuccessful. An example of the separation of salt-free nucleotides which is possible with PEI-cellulose column chromatography is illustrated in Fig. 8.

Although desalting of mixtures of nucleic acid precursors is easily accomplished by the use of activated charcoal, this procedure was initally avoided because of the irreversible retention of

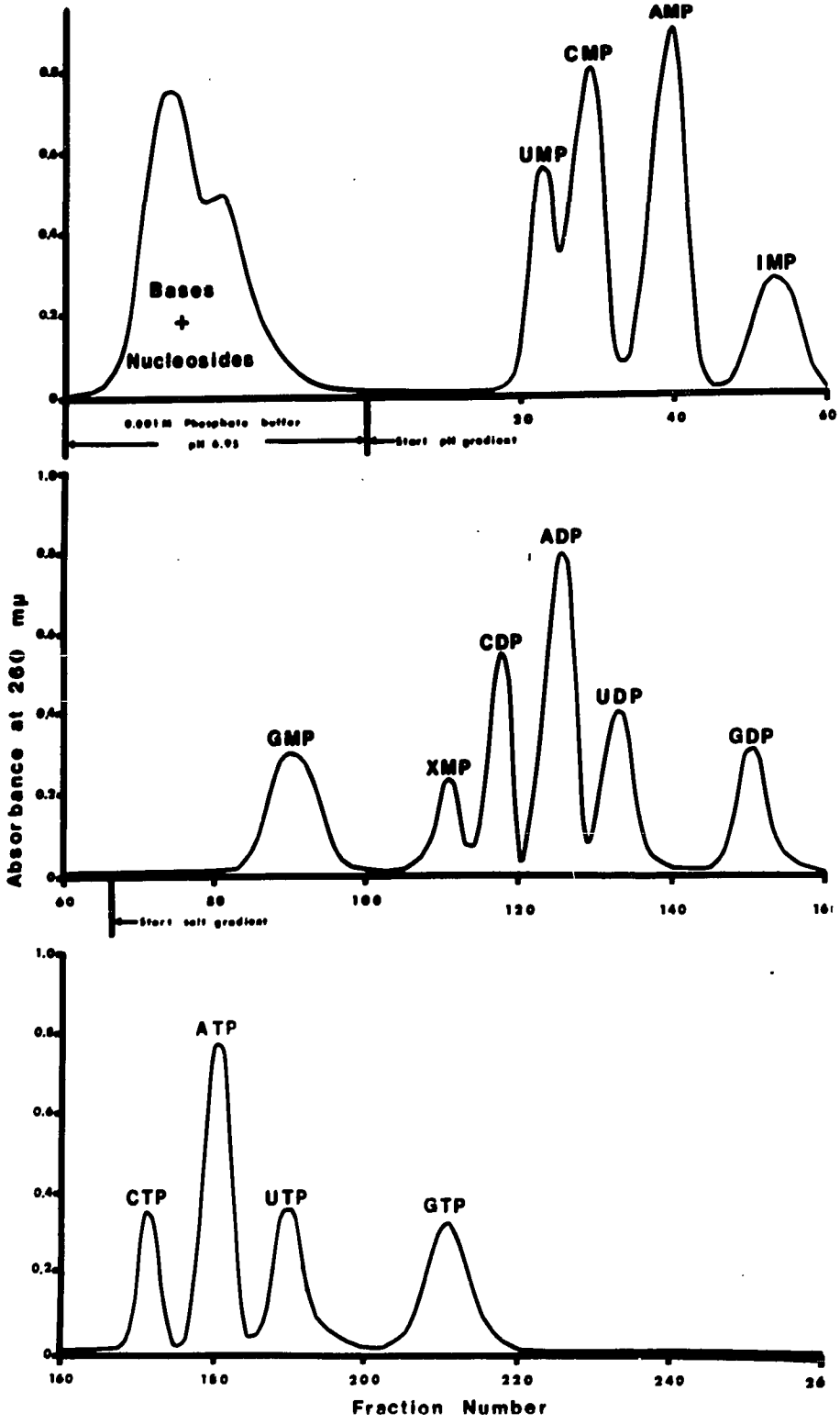

AMP
CMP
UMP
IMP
Bases
+
Nucleosides
0.001 M Phosphate buffer
pH 6.95
Start pH gradient
ADP
CDP
UDP
GMP
XMP
GDP
Absorbance at 260 mμ
Start salt gradient
ATP
CTP
UTP
GTP
Fraction Number

FIGURE 8

ELUTION PROFILE OF A SYNTHETIC MIXTURE OF NUCLEOTIDES SEPARATED BY PEI-CELLULOSE COLUMN CHROMATOGRAPHY

Column effluent was collected in 3 ml fractions with an automatic fraction collector.

See 'Materials and Methods' for details of preparation of resin, packing of column, and elution of nucleotides.

guanine derivatives on charcoal, and because of the known lability of nucleoside triphosphates, particularly ATP, in alkaline solutions such as those required to elute nucleic acid precursors from charcoal. Since the purine nucleotides are of particular interest in this investigation, these disadvantages seemed considerable. However, the failure of the other techniques necessitated a re-examination of the activated charcoal technique and the procedure outlined in 'Materials and Methods' (IX, 2) employing "partially-deactivated" charcoal was devised. Although the disadvantages noted above were not eliminated, they were minimized and reproducible recoveries of guanine derivatives and nucleoside triphosphosphates were obtained.

The partial deactivation of the charcoal necessitated the repeated passage of solutions of nucleic acid precursors through the charcoal bed in order to ensure complete adsorption. As well, approximately 2 1/2 times as much charcoal was required in order to adsorb a given quantity of bases, nucleosides and/or nucleotides. The distinct and essential advantage achieved by the octanol treatment, however, was the reproducibly higher

recovery from the charcoal of adsorbed guanine derivatives, which are less readily and inconsistently eluted from untreated charcoal. In a series of four experiments wherein the recoveries of adsorbed ^{14}C-guanosine were compared using octanol-treated and untreated charcoal, it was found that 81.1% ± 3.4% of the added guanosine was recovered from the treated charcoal, as opposed to 51% ± 14.8% from untreated charcoal.

The eluting solvent was pre-cooled and the filtrates rapidly frozen as they were collected in order to minimize the aforementioned breakdown of nucleoside triphosphates in the alkaline eluting solution. In control experiments in which the elution sequence was carried out at room temperature, less than 15% of the added ATP was recovered as ATP. Under the experimental conditions described in 'Materials and Methods' (IX, 2) 53% of the added ATP was recovered as ATP, while 39% was recovered as ADP and 8% as AMP. It was also noted that when a solution of adenosine in the NH_4OH/water/ethanol eluting solvent was passed through a Millipore membrane filter, there was little, if any, adsorption of adenosine onto the filter as compared with the significant adsorption noted when aqueous solutions

of adenosine were filtered.

Thus it was possible to standardize the extraction procedure and to predict (and allow for in the calculation) the losses which occurred at the various steps of the procedure. In the following experiments, all nucleotide concentrations are corrected for the experimentally determined breakdown of nucleoside triphosphates to di- and mono-phosphates, and all values for guanine derivatives are additionally corrected, assuming 81% recovery after adsorption onto charcoal.

ii) Adenine and guanine nucleotides in the acid-soluble pool.

Since adenosine is apparently a precursor of the intracellular inhibitor, it was thought that an investigation into the effects of adenosine on the purine nucleotide levels in the intracellular pool might provide a clue to the identity of the inhibitory compound. As the changes which result in growth inhibition are a consequence of the presence of adenosine in the culture supernatant, the effects of a non-inhibitory purine (guanosine) were also investigated. The experiment was designed so that changes in intracellular purine nucleotide

concentrations could be related to residual concentrations of exogenous adenosine or guanosine (added initially at 1.0 mM). Samples of the culture supernatants were assayed for residual purine bases and nucleosides as described above, and purine nucleotides were extracted from 100 ml of culture with 6% $HClO_4$ at 15 minutes, 45 minutes, and 150 minutes, as previously described. Nucleotides were purified by the charcoal adsorption / Dowex 50 (H^+) fractionation procedures ('Materials and Methods' IX, 2; and XVII, 2, b) and quantitated after chromatography on PEI-cellulose thin-layer plates ('Materials and Methods', XVII, 1, e). Figure 9 illustrates a typical separation of nucleotides obtained by PEI-cellulose thin-layer chromatography. The results of these experiments are compiled in Tables III, IV and V.

The data in Table III show that there is a marked increase in the concentrations of adenine nucleotides in adenosine-grown cells and of guanine nucleotides in guanosine-grown cells, as compared with the concentrations of those compounds in cells grown in BN-medium. Inosine nucleotides were not detected in the extracts from adenosine-supplemented cultures.

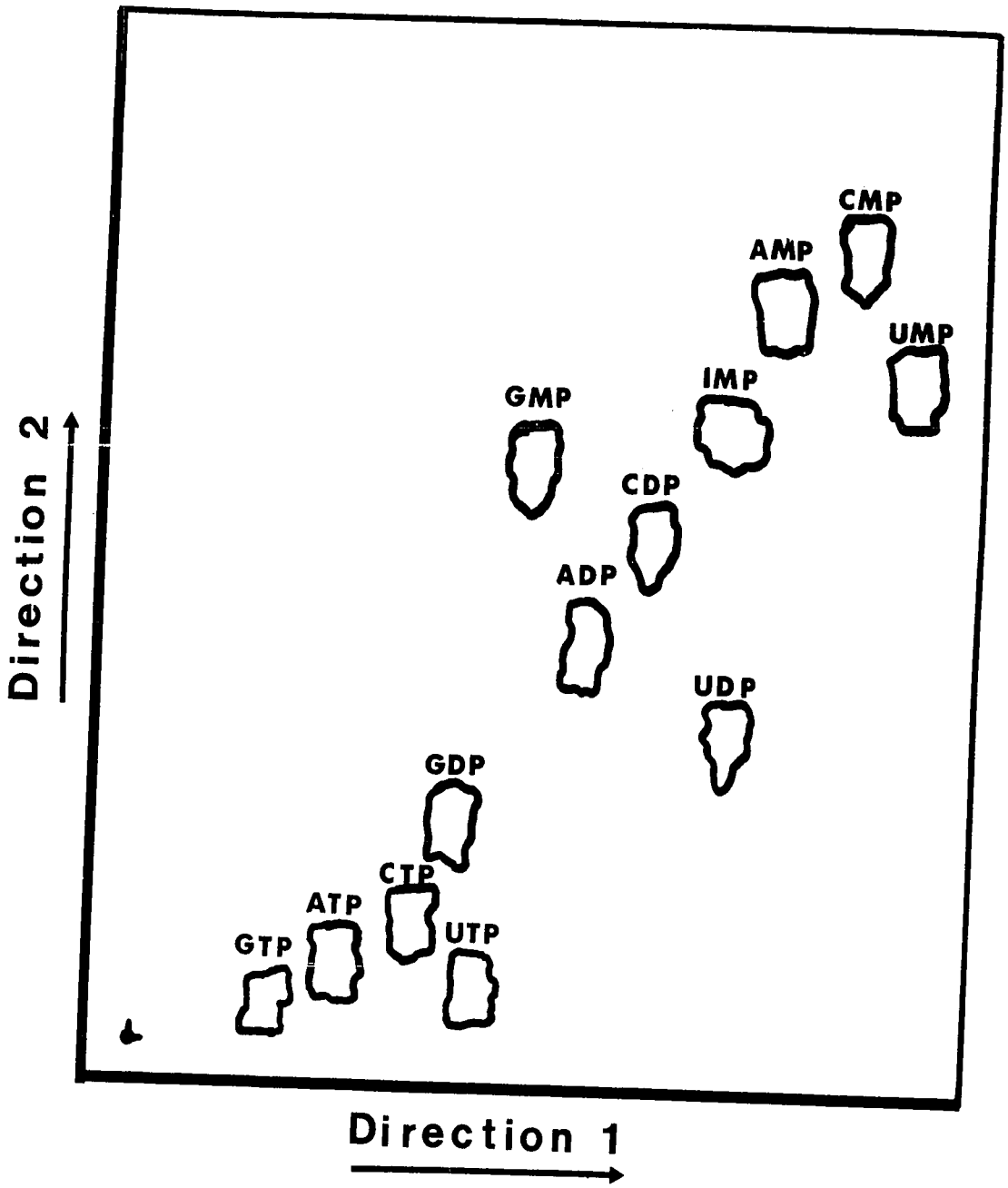

CMP
AMP
UMP
IMP
GMP
CDP
ADP
UDP
GDP
CTP
ATP
UTP
GTP
Direction 2
Direction 1

FIGURE 9

SEPARATION OF PURINE AND PYRIMIDINE NUCLEOTIDES BY PEI-CELLULOSE THIN-LAYER CHROMATOGRAPHY

A stepwise gradient was employed. In each direction, the solvent was changed as the front reached the indicated distance from the origin.

Direction 1: 0.75 M LiCl, to 2.5 cm; 0.95 M LiCl, to 9.0 cm; 1.4 M LiCl, to 16.0 cm.

Direction 2: 0.5 M $HCOONH_4$, pH 3.4, to 2.0 cm; 0.85 M $HCOONH_4$, pH 3.4, to 5.0 cm; 2.0 M $HCOONH_4$, pH 3.4, to 9.0 cm; 4.0 M $HCOONH_4$, pH 3.4, to 16 cm. Nucleotides were located with U.V. light.

TABLE III

PURINE NUCLEOTIDES IN THE ACID SOLUBLE FRACTIONS OF CELLS GROWN IN THE PRESENCE OF 1.0 mM ADENOSINE OR GUANOSINE

	Incubation Conditions			
	BN Medium	BN Medium + 1.0 mM Adenosine		
Nucleotide		15 min	45 min	150 min
AMP	4.08(a)	12.21(a)	11.12(a)	7.14(a)
ADP	6.34	11.32	9.56	6.43
ATP	34.53	38.41]	37.21	35.07
Total	45.05	61.94	57.89	48.64
		(137.5)*	(128)*	(108)*
GMP	1.09	1.99	1.62	1.49
GDP	1.31	2.28	1.91	1.91
GTP	8.99	9.88	10.22	9.71
Total	11.39	14.15	13.75	13.1
		(124)*	(120.7)*	(115)*

Nucleotides were extracted from cells with 6% $HClO_4$. Charcoal-desalted extracts were chromatographed by PEI-cellulose thin-layer chromatography. Spots detected by U.V. light were eluted and quantitated spectrophotometrically.

Contd. ....

TABLE III (Contd.)

Nucleotide	Incubation Conditions			
	BN Medium	BN Medium + 1.0 mM Guanosine		
		15 min	45 min	150 min
AMP	4.08(a)	7.83(a)	4.88(a)	4.47(a)
ADP	6.34	8.71	7.40	7.02
ATP	34.53	37.63	37.08	36.91
Total	45.05	54.17 (120.3)*	49.36 (109.6)*	48.40 (107.5)*
GMP	1.09	2.94	2.38	2.06
GDP	1.31	1.91	2.72	2.52
GTP	8.99	10.55	11.41	11.18
Total	11.39	16.21 (142.3)*	16.41 (144)*	15.76

(a) Nucleotide concentrations expressed as mμ moles/mg cell protein.

* Values in brackets represent the percentage of total adenine or guanine nucleotides compared with the totals obtained from cells in BN medium.

In particular, the concentrations of the nucleoside mono- and di-phosphates are most affected. AMP and ADP concentrations are greatly elevated at 15 minutes and 45 minutes, but have markedly declined by 150 minutes. This decrease correlates closely with the disappearance of adenosine from the culture supernatant (Table IV). In the same cells, the guanine nucleotide levels are also elevated, but not to the same extent as are the adenine nucleotide concentrations.

In guanosine-grown cells, the guanine nucleotide concentrations are elevated even at 150 minutes (Table III). This correlates with the observation (Table V) that guanosine persists in the culture supernatant throughout the course of the experiment. As well, in guanosine-grown cells, the adenine nucleotide level is considerably elevated at 15 minutes, but has declined to near normal levels at 45 minutes and 150 minutes. This suggests that the control of the conversion of GMP to AMP, which is normally exerted by adenine nucleotides,was overwhelmed during the early stages of the incubation, but was ultimately able to re-establish its effect.

TABLE IV

CONCENTRATIONS OF ADENOSINE, INOSINE AND HYPOXANTHINE IN THE SUPERNATANTS OF ADENOSINE-SUPPLEMENTED CULTURES OF M. SODONENSIS FROM WHICH NUCLEOTIDE POOLS WERE EXTRACTED.

	Concentration of Exogenous Purine Derivative (mM)			
Incubation Time (Min)	Adenosine	Inosine	Hypoxanthine	Total
0	1.120	-	-	1.120
15	0.84	0.121	0.041	1.1002
45	0.572	0.194	0.198	0.964
90	0.250	0.149	0.495	0.894
135	0.107	0.129	0.553	0.789
150	0.014	0.047	0.683	0.744

Adenosine was determined enzymatically with adenosine deaminase. Inosine and hypoxanthine were determined spectrophotometrically after elution from paper chromatograms developed with n-butanol:20% NH_4OH (83:17).

TABLE V

CONCENTRATIONS OF GUANINE AND GUANOSINE IN THE SUPERNATANTS OF GUANOSINE-SUPPLEMENTED CULTURES OF M. SODONENSIS FROM WHICH THE NUCLEOTIDE POOLS WERE EXTRACTED

	Concentration of exogenous purine derivative (mM)		
Incubation Time (Min)	Guanosine	Guanine	Total
0	1.150	-	1.150
15	0.906	0.093	0.999
45	0.776	0.141	0.917
90	0.631	0.169	0.800
135	0.375	0.138	0.513
150	0.232	0.122	0.354

Guanine and guanosine were determined spectrophotometrically after elution from paper chromatograms developed with n-butanol: 20% NH_4OH (83:17).

The data in Table III further suggest that the control by guanine nucleotides of the AMP to GMP interconversion is less effective than is the control over the GMP to AMP interconversion, since guanine nucleotides are present at elevated levels in both adenosine and guanosine grown cells, while adenine nucleotides are maintained at elevated levels only in the presence of exogenous adenosine.

This information suggests that intracellular adenine nucleotides are of considerable importance in relation to the observed bacteriostatic effect of purine derivatives on the growth of M. sodonensis, since the growth inhibitors (adenosine, inosine and hypoxanthine) are all precursors of purine nucleotides and, in particular, of adenine nucleotides. It is evident that if the adenine nucleotides are involved in the inhibitory mechanism, those exogenous purine derivatives which can function most effectively as nucleotide precursors will produce a more marked effect on cell growth. Since the rate of transport into the cells is one factor which could limit the effectiveness of a compound as a nucleotide precursor, and since the effectiveness of adenosine, inosine and hypoxanthine as inhibitors appears to be

related to their uptake, the adenine nucleotides are the primary candidates for being directly implicated in the mechanism for inhibiting growth.

4. Enzymes involved in adenosine metabolism.

Cell-free extracts of M. sodonensis were assayed, as described in 'Materials and Methods' (XI), for enzyme activities associated with purine metabolism in order to determine the pathways by which purine nucleotides could be synthesized from adenosine in these cells. The results of these experiments, outlined in Tables VI, VII and VIII, show that both adenosine and guanosine are readily converted to the corresponding nucleotides, and that the ready interconversion of purine nucleotides can occur. Although activities for several of the enzymes were not detectable in cell-free extracts from BN-grown M. sodonensis, these activities may be inducible under the appropriate conditions. However, this aspect of the problem was not investigated, since other results (reported below) demonstrated that the intracellular events which occurred when M. sodonensis was inoculated into adenosine-supplemented BN medium and which ultimately resulted in the growth inhibition, were initiated without a noticeable lag after the cells came into contact with exogenous adenosine. This suggested that induction of new enzyme activities was not required before before inhibition-initiating events could occur. In addition, the enzyme activities which were present constituitively in M. sodonensis could account for the rapid synthesis of

TABLE VI

ACTIVITIES IN CELL-FREE EXTRACTS OF 18-HOUR, BN-GROWN *M. SODONENSIS* OF ENZYMES INVOLVED IN PURINE BASE AND NUCLEOSIDE CATABOLISM

Enzyme	Activity (μmoles/hr.mg protein)*
Adenosine deaminase	37.98
Guanosine deaminase	Not detectable
Hydrolytic purine nucleosidase activities	
Substrate	
Adenosine	Not detectable
Guanosine	0.44
Inosine	2.58
Purine nucleoside phosphorylase activities	
Substrate	
Adenosine	Not detectable
Guanosine	1.08
Inosine	9.12
Adenase	Not detectable
Guanase	Not detectable

*Protein was determined by a modification of the procedure of Lowry *et al.* (1951).

TABLE VII

ACTIVITIES IN CELL-FREE EXTRACTS OF 18-HOUR, BN-GROWN *M. SODONENSIS* OF ENZYMES INVOLVED IN PURINE NUCLEOTIDE SYNTHESIS

Enzyme	Activity (mμ moles/hr/mg protein)*
Purine nucleoside phosphokinase activity	
Substrates	
Adenosine	0.042
6-(methylmercapto) purine ribonucleoside	0.544
Inosine	Not detectable
Purine nucleoside-5'-monophosphate pyrophosphorylase activities	
Substrates	
Adenine	57.0
Hypoxanthine	552.0
Guanine	1,332.0

Protein was determined by a modification of the procedure of Lowry, *et al.* (1951).

TABLE VIII

ACTIVITIES IN CELL-FREE EXTRACTS OF 18-HOUR, BN-GROWN *M. SODONENSIS* OF ENZYMES INVOLVED IN PURINE NUCLEOTIDE INTERCONVERSIONS

Enzyme	Activity (mμ moles/h/mg protein)*
AMPS synthetase-lyase	1.6
AMP deaminase	40.8
IMP dehydrogenase	72.0
XMP aminase	3.6
GMP reductase	Not detectable

* Protein was determined by a modification of the procedure of Lowry, *et al.* (1951).

adenine nucleotides, the proposed intracellular inhibitors, from adenosine.

The reactions leading to the synthesis of adenine nucleotides from adenosine in M. sodonensis can be summarized as illustrated in Fig. 10. The data supporting this proposed sequence are as follows: first, adenine formation from adenosine could not be demonstrated either in vivo (Fig. 5) or in vitro (Table VI); second, purine nucleoside phosphokinase activity was very slow for adenosine and not detectable for inosine (Table VII). The higher activity with 6-(methylmercapto) purine ribonucleoside suggests that adenosine deaminase activity masks the phosphokinase just as it masks the nucleosidase activities.

The inability to demonstrate IMP in the nucleotide pool of cells grown in the presence of adenosine does not necessarily negate this proposed pathway. Although feed-back inhibition by adenine nucleotides of adenylosuccinate synthetase has been reported in E. coli by Wyngaarden and Greenland (1963) and Benson, et al. (1970), if this control is reduced or absent in M. sodonensis, the synthesis of adenine nucleotides from the adenosine-derived precursors would be facilitated and IMP levels would not necessarily be elevated under the influence of exogenous adenosine. Adenylosuccinate synthetase activity could not be detected by itself in cell-free extracts of M. sodonensis (although the combined activities of the synthetase/lyase enzymes (Table VIII) could be demonstrated, and this aspect of the investigation was not pursued.

The previously observed anomaly that, although adenine is a direct precursor of adenine nucleotides and is transported into the cells at a faster rate than hypoxanthine (Table I) and although adenine

FIGURE 10

PROPOSED SEQUENCE OF REACTIONS FOR THE SYNTHESIS OF ADENINE NUCLEOTIDES FROM EXOGENOUS ADENOSINE BY *M. SODONENSIS*

1. Adenosine	Adenosine deaminase	Inosine + NH_3
2. a) Inosine + water	Hydrolytic purine nuclosidase	Hypoxanthine + Ribose
and/or		
b) Inosine + inorganic phosphate	Purine nucleoside phosphorylase	Hypoxanthine + Ribose-1-phosphate
3. Hypoxanthine + 5'-phosphoribosyl-1-pyrophosphate	Purine nucleoside-5'-monophosphate pyrophosphorylase; Mg^{++}	Inosine-5'-monophosphate
4. Inosine-5'-monophosphate + Aspartic acid	Succinyl-adenosine-5'-monophosphate synthetase; Mg^{++} ATP	Succinyl-adenosine-5'-monophosphate
5. Succinyl-adenosine-5'-monophosphate	Succinyl-adenosine-5'-monophosphate lyase	Adenosine-5'-monophosphate + fumarate

is an active bacteriostatic agent in other systems but is not inhibitory to growth of M. sodonensis, can be explained by the data in Table VII. It is apparent that adenine is a poor substrate for purine nucleoside-5'-monophosphate pyrophosphorylase, as compared with guanine and hypoxanthine, and as such, is probably a poor precursor, in vivo, of the inhibitory adenine nucleotides.

The failure to demonstrate GMP reductase activity in vitro in in M. sodonensis can be attributed to an excessive lability of the enzyme, since this activity was demonstrated indirectly during the analysis of the nucleotide pool (Table III).

IV. Reversal of Adenosine-Induced Bacteriostasis.

In these experiments, a number of normal metabolites were incorporated (at the concentrations indicated below) into BN-medium and 1.0 mM adenosine-supplemented BN-medium, in order to investigate their effects on cell growth and their abilities to reverse the adenosine-induced bacteriostasis. Media were inoculated with washed 18-hour BN-medium-grown cells and cultures were incubated at 30°C with vigorous aeration. The absorbance of the culture at 600 mμ was determined after 24 hours and total cell protein determined from a previously prepared standard curve ('Materials and Methods', V).

None of the purine or pyrimidine nucleosides tested individually at 1.0 mM concentrations had any effect on the growth inhibition (Table IX). However, when a number of vitamins were tested at 1 μg/ml

TABLE IX

EFFECTS OF ADDED PURINE AND PYRIMIDINE NUCLEOSIDES ON ADENOSINE-INDUCED BACTERIOSTASIS IN *M. SODONENSIS*

	Cell yield (µg cell protein/ml/24 hours)*	
Addition (1.0 mM)	BN-medium	BN-medium + adenosine (1.0 mM)
0	650	132
Guanosine	690	128
Cytidine	700	120
Uridine	600	114
Thymidine	580	108
5-Methylcytidine	670	130

* Cell protein was determined by a modification of the procedure of Lowry, *et al.* (1951) for insoluble protein.

in a similar manner (Table X), thiamine HCl, alone among the vitamins, completely reversed the adenosine-induced growth inhibition.

The pyrimidine precursor (B_1-pyrimidine) and the thiazole precursor (B_1-thiazole) of thiamine, were also tested, at 1 µg/ml, for their respective abilities to eliminate the adenosine-induced inhibition. Although B_1-thiazole had no protective properties (Table XI), B_1-pyrimidine was at least as effective as thiamine, on a weight basis, in reversing the growth inhibition. By adding sub-optimal concentrations of B_1-pyrimidine and thiamine to adenosine-supplemented medium (Fig. 11), it was possible to demonstrate that on a molar basis, the pyrimidine precursor was just as effective as thiamine in reversing adenosine-induced growth inhibition. B_1-thiazole did not enhance the effect of B_1-pyrimidine (Fig. 11).

These observations suggest that the growth inhibition which is observed when M. sodonensis is grown in an adenosine-supplemented medium is the direct consequence of thiamine starvation resulting from an impairment, under those conditions, of the cells' ability to synthesize the pyrimidine precursor of the vitamin.

V. Inhibition of Thiamine Biosynthesis During Growth in Adenosine-Supplemented Medium

In order to test the hypothesis suggested above, it was necessary to follow thiamine biosynthesis in cells growing in the presence and in the absence of exogenous adenosine. The experiments also included an

TABLE X

EFFECT OF ADDED VITAMINS ON ADENOSINE-INDUCED BACTERIOSTASIS IN M. SODONENSIS

Addition (1 µg/ml)	Cell Yield (µg cell protein/ml/24 hrs)*	
	BN-medium	BN-medium + adenosine (1.0 mM)
0	770	170
Vitamin A	760	150
α-Lipoic acid	760	175
Vitamin B_{12}	780	180
Riboflavin-5-PO_4	785	185
Folic acid	780	170
Calcium pantothenate	775	180
β-Carotene	755	175
Pyridoxine HCl	760	190
Niacin	770	185
p-Aminobenzoic acid	765	190
Thiamine HCl	790	785

Cell protein was determined by a modification of the procedure of Lowry, et al., (1951) for insoluble protein.

TABLE XI

EFFECTS OF ADDED THIAMINE AND THIAMINE PRECURSORS ON ADENOSINE-INDUCED BACTERIOSTASIS IN M. SODONENSIS

Addition (1 µg/ml)	Cell Yield (µg cell protein/ml/24 hrs)*	
	BN-medium	BN-medium + adenosine (1.0 mM)
0	770	170
Thiamine HCl	780	775
B_1-pyrimidine	775	780
B_1-thiazole	750	165

* Cell protein was determined by a modification of the procedure of Lowry et al. (1951) for insoluble protein.

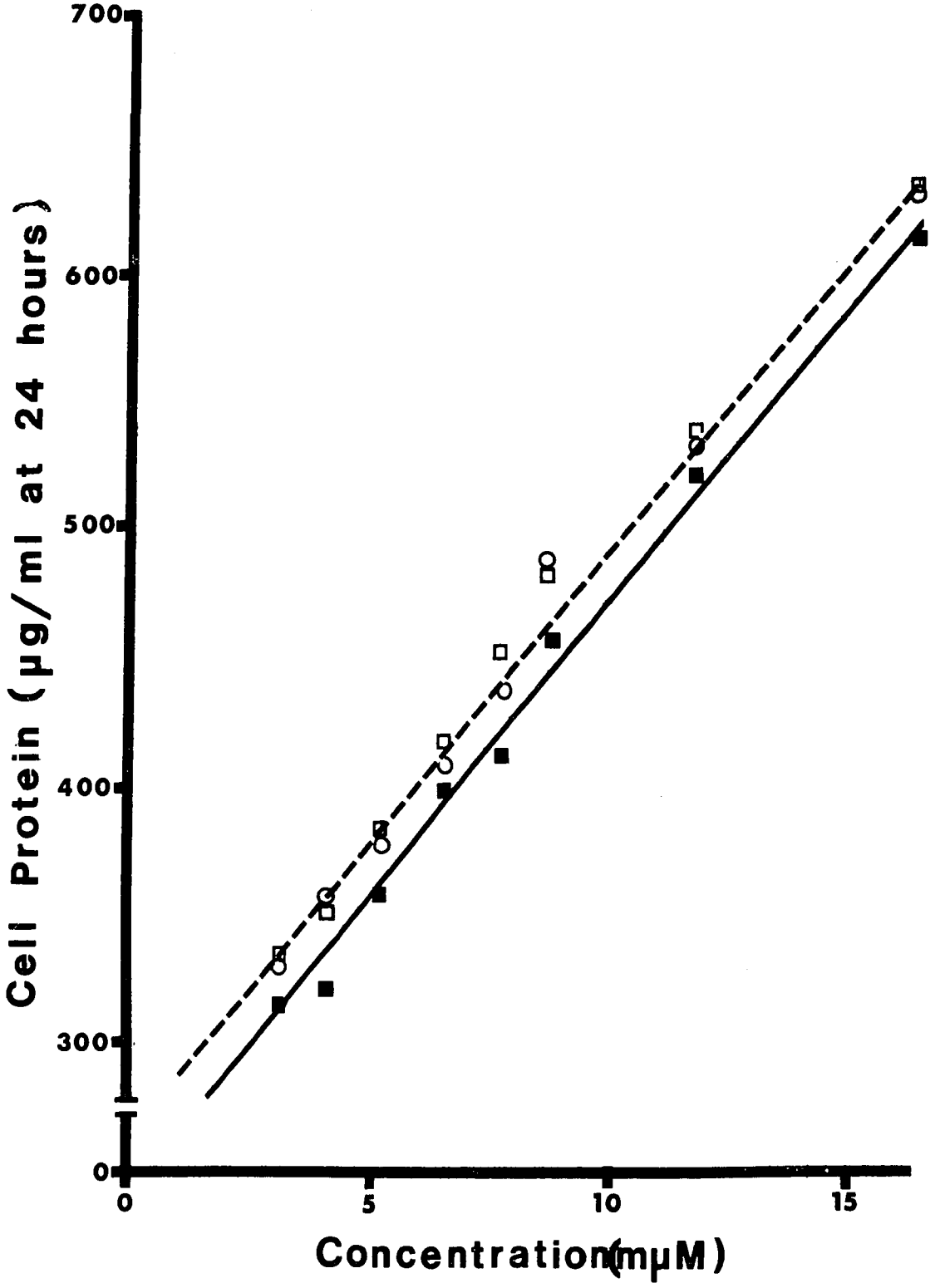
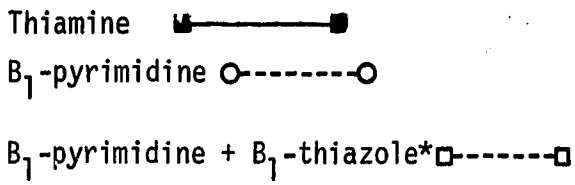

700
600
500
400
300
0
0
5
10
15
Cell Protein (µg/ml at 24 hours)
Concentration (mµM)

FIGURE 11

RELATIVE EFFECTIVENESS OF THIAMINE, B_1-PYRIMIDINE AND B_1-PYRIMIDINE + B_1-THIAZOLE IN REVERSING ADENOSINE-INDUCED BACTERIOSTASIS IN M. SODONENSIS

Cell protein was determined by a modification of the procedure of Lowry, et al., (1951) for insoluble protein.



*(B_1-pyrimidine and B_1-thiazole were both added at the concentration designated in the figure.)

investigation of thiamine biosynthesis in guanosine-supplemented medium as an additional control.

Washed 18-hour BN-grown cells were inoculated into BN-medium and into BN-medium supplemented with 0.1 mM adenosine, 1.0 mM adenosine or 1.0 mM guanosine and incubated at 30°C. At various intervals, 10 ml samples of the culture were centrifuged at 24,000 x g for 10 minutes, and the cell pellet washed twice with sterile 1% NcCl. The pellet was re-suspended in 5 ml of 0.12 N HCl and hydrolyzed in an autoclave. The hydrolysate was subsequently treated with Takadiastase and assayed for thiamine with L. viridescens as described in 'Materials and Methods' (X: 1, 2).

Takadiastase treatment, which converts thiamine phosphates to free thiamine, was included in the procedure to increase the efficiency and accuracy of the assay, since thiamine phosphates were shown to be less effective than free thiamine in supporting growth of the assay organism. Thiamine pyrophosphate was only 35% as effective as free thiamine in this respect.

The efficiency of the Takadiastase treatment was tested by incubating known amounts of thiamine pyrophosphate with Takadiastase under the conditions described in 'Materials and Methods' (X) and chromatographing the cold acid soluble products of the reaction, using the i-propanol:formic acid:urea solvent system ('Materials and Methods', XVII). Spots were eluted with 0.01 N HCl and the absorbance at 267 mμ measured. Free thiamine production was calculated using the known

extinction coefficient at 267 mμ for thiamine of 9.0×10^3/M. Results indicated greater than 97% conversion of thiamine pyrophosphate to thiamine. An assay of the Takadiastase powder for contaminating thiamine indicated that there was less than 0.03 mμg of thiamine/mg of dry powder.

The results of the experiments on thiamine biosynthesis in M. sodonensis are illustrated in Fig. 12. They indicate that biosynthesis of thiamine is almost completely inhibited in the presence of 1.0 mM adenosine. In contrast, in the absence of exogenous purine nucleosides, thiamine biosynthesis begins quite early in the growth phase. In the presence of 0.1 mM adenosine, thiamine biosynthesis is inhibited for approximately 8 to 9 hours, after which time, biosynthesis is initiated at a slightly accelerated rate. In the presence of 1.0 mM guanosine, the onset of thiamine biosynthesis is also delayed, but begins much sooner (at 4 hours) than it does in 0.1 mM adenosine-supplemented cultures.

These results are further illustrated in Fig. 13 in which thiamine content is related to cell density. In cells growing in BN-medium there is a short lag before rapid thiamine synthesis occurs (about 4 hours), although thiamine is synthesized at a reduced rate during this time (Fig. 12). However, in the presence of guanosine or adenosine, thiamine biosynthesis is shut off immediately upon the exposure of the cells to the nucleoside, since the dilution of thiamine among the cells occurs at a rate which approximates the growth rate of the organism at that time (Fig. 1, Fig. 3). In the case of guanosine-supplemented cultures, thiamine synthesis begins at 4 hours. Since this occurs before the end of the

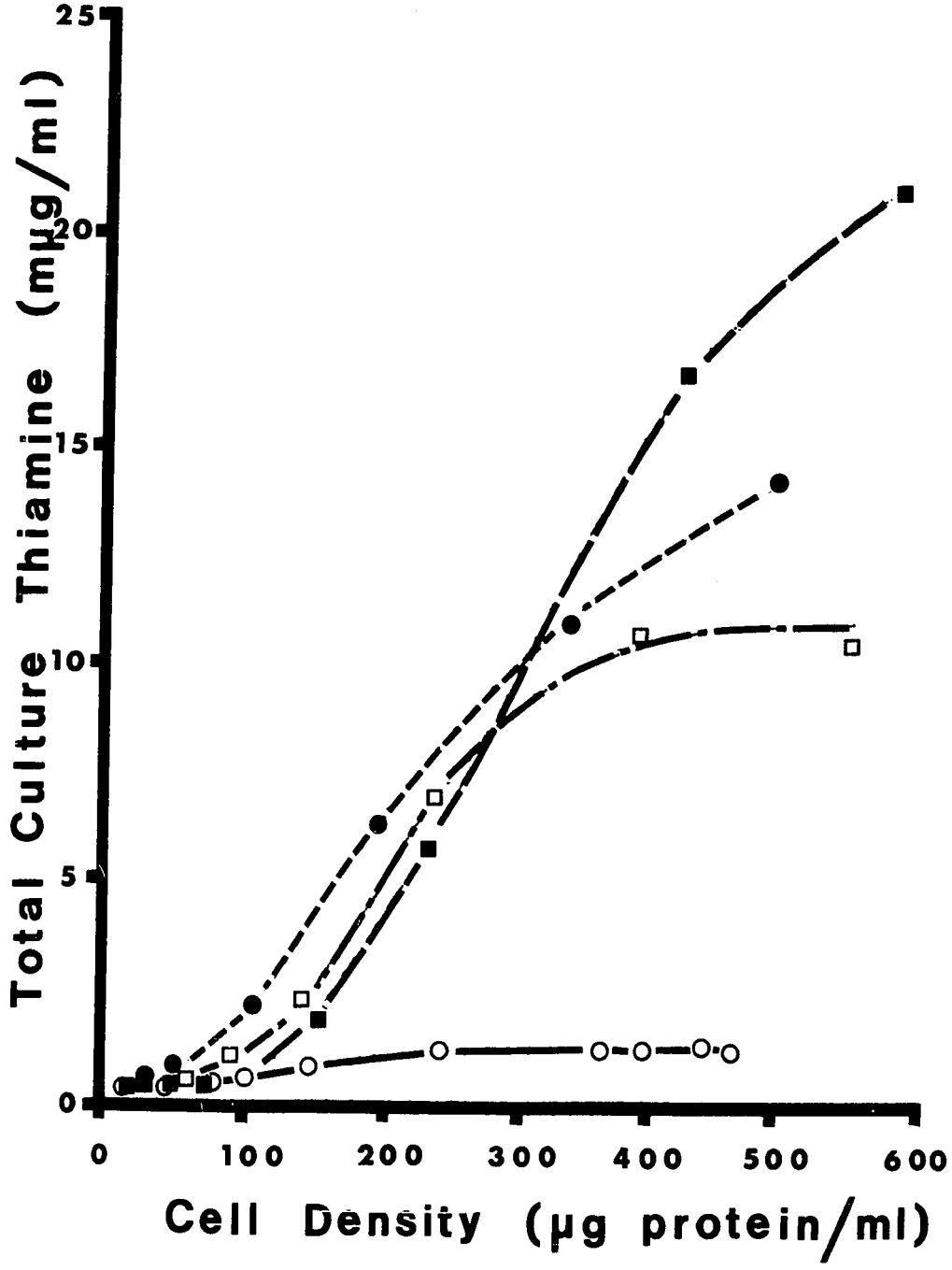

Total Culture Thiamine (mμg/ml)
25
20
15
10
5
0
0
100
200
300
400
500
600
Cell Density (μg protein/ml)

FIGURE 12

TOTAL THIAMINE CONTENT OF CELLS GROWN IN THE PRESENCE AND ABSENCE OF ADENOSINE OR GUANOSINE

Thiamine was determined at various times by microbiological assay after acid extraction from M. sodonensis grown in the designated media.

BN-medium ●-----●

BN-medium + 1.0 mM adenosine ○———○

BN-medium + 0.1 mM adenosine ■— — —■

BN-medium + 1.0 mM guanosine □— – —□

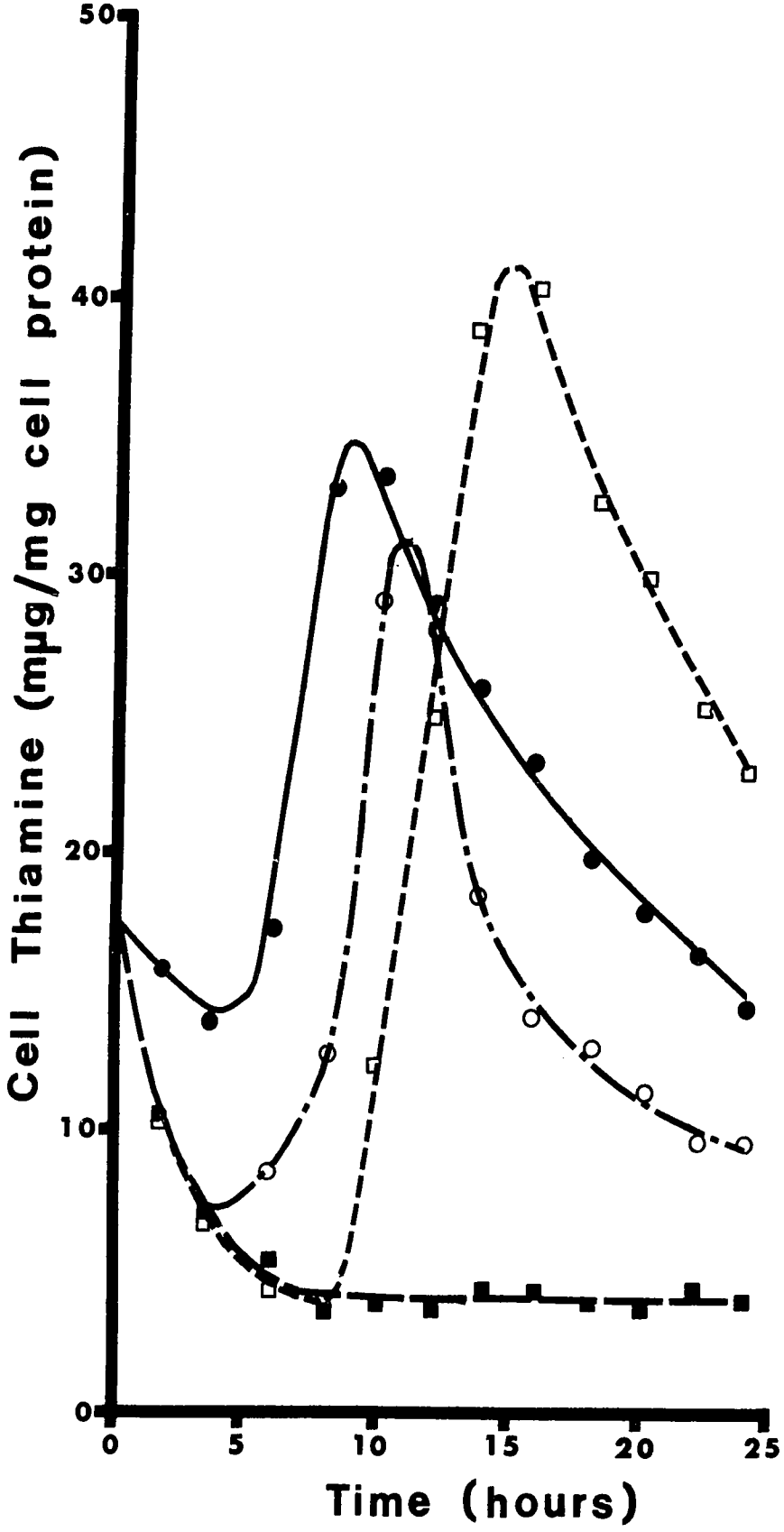

Cell Thiamine (mμg/mg cell protein)
50
40
30
20
10
0
0
5
10
15
20
25
Time (hours)

FIGURE 13

THIAMINE CONTENT (ng/mg CELL PROTEIN) OF CELLS GROWN IN THE PRESENCE AND ABSENCE OF ADENOSINE OR GUANOSINE

Thiamine was determined at various times by microbiological assay after acid extraction of M. sodonensis grown in the designated media.

Cell protein was determined by a modification of the procedure of Lowry et al., (1951) for insoluble protein.

BN-medium ●——●

BN-medium + 1.0 mM adenosine ■— — —■

BN-medium + 0.1 mM adenosine □-------□

BN-medium + 1.0 mM guanosine O— – —O

second generation time, growth inhibition as a result of thiamine deprivation is not detected. However, in cultures supplemented with 0.1 mM adenosine, thiamine synthesis does not begin until approximately 9 hours after exposure to the inhibitor (that is, early in the fourth generation). As a result, the growth inhibition is observed (Fig. 3). A comparison of Figure 3 with Figure 13 also shows that the renewal of thiamine biosynthesis at 9 hours correlates with the initiation of the recovery phase of growth in cultures initially supplemented with 0.1 mM adenosine. In the presence of 1.0 mM adenosine, thiamine biosynthesis, as well as cell growth, was inhibited throughout the course of this experiment.

In order to determine the residual adenosine and hypoxanthine concentrations at the times when the recovery phases were being initiated in 0.1 mM and in 1.0 mM adenosine-supplemented cultures of M. sodonensis, the following experiment was conducted. Washed 18-hour BN-grown cells were inoculated into BN-medium supplemented with either 0.1 mM or 1.0 mM adenosine and incubated at 30°C with vigorous aeration. At various times, 5 ml samples of the culture were obtained and centrifuged to remove the cells. Aliquots of the supernatant were assayed enzymatically for adenosine and hypoxanthine with adenosine deaminase and xanthine oxidase, respectively, as described in 'Materials and Methods' (XIV; 3, i and ii). The results of these experiments, given in Table XII, indicate that adenosine is exhausted from the culture supernatants just prior to the times when the recovery phases in these

TABLE XII

RESIDUAL ADENOSINE AND HYPOXANTHINE CONCENTRATIONS IN THE SUPERNATANTS OF CULTURES SUPPLEMENTED WITH 0.1 mM or 1.0 mM ADENOSINE

	Concentration of exogenous purine derivative (mM)			
	BN-medium + 1.0 mM adenosine		BN-medium + 0.1 mM adenosine	
Incubation Time (hrs)	Adenosine	Hypoxanthine	Adenosine	Hypoxanthine
0	1.06	-	0.099	-
3	1.01	> 0.01	-	-
4	-	-	0.073	0.006
5	0.95	0.03	0.033	0.020
8	0.89	0.05	0.005	0.040
10	-	-	> 0.001	0.060
20	-	-		
24	0.19	0.56		
30	0.11	0.64		
33	0.05	0.72		
36	> 0.01	0.78		

Adenosine and hypoxanthine were determined enzymatically with adenosine deaminase and xanthine oxidase respectively, as described in 'Materials and Methods'.

cultures are initiated (Fig. 3). In 0.1 mM adenosine-supplemented cultures this time is between 9 and 10 hours after inoculation, whereas in 1.0 mM adenosine-supplemented cultures, this occurs between 34 and 36 hours after inoculation. In 1.0 mM adenosine-supplemented cultures, initiation of thiamine biosynthesis is also observed at approximately 35 hours, but these observations are not as dramatic as those seen at 10 hours in 0.1 mM adenosine-supplemented cultures. In the former case thiamine synthesis occurs at a relatively slow rate, which is probably a reflection of the presence of the significant concentrations of hypoxanthine, a partially effective growth inhibitor, in the supernatant at that time.

Thus it is possible to correlate the initiation of the recovery phase in M. sodonensis with the disappearance of adenosine from the culture supernatant, with the onset of thiamine biosynthesis, and with the return to near-normal levels of the adenine nucleotide concentrations in the intracellular pool. As well, the correlation between high intracellular adenine nucleotide levels and inhibition of thiamine biosynthesis is apparent in both adenosine- and guanosine-grown cells. The latter observation supports the earlier conclusion that adenine nucleotides played a critical role in the mechanism of purine-induced bacteriostasis.

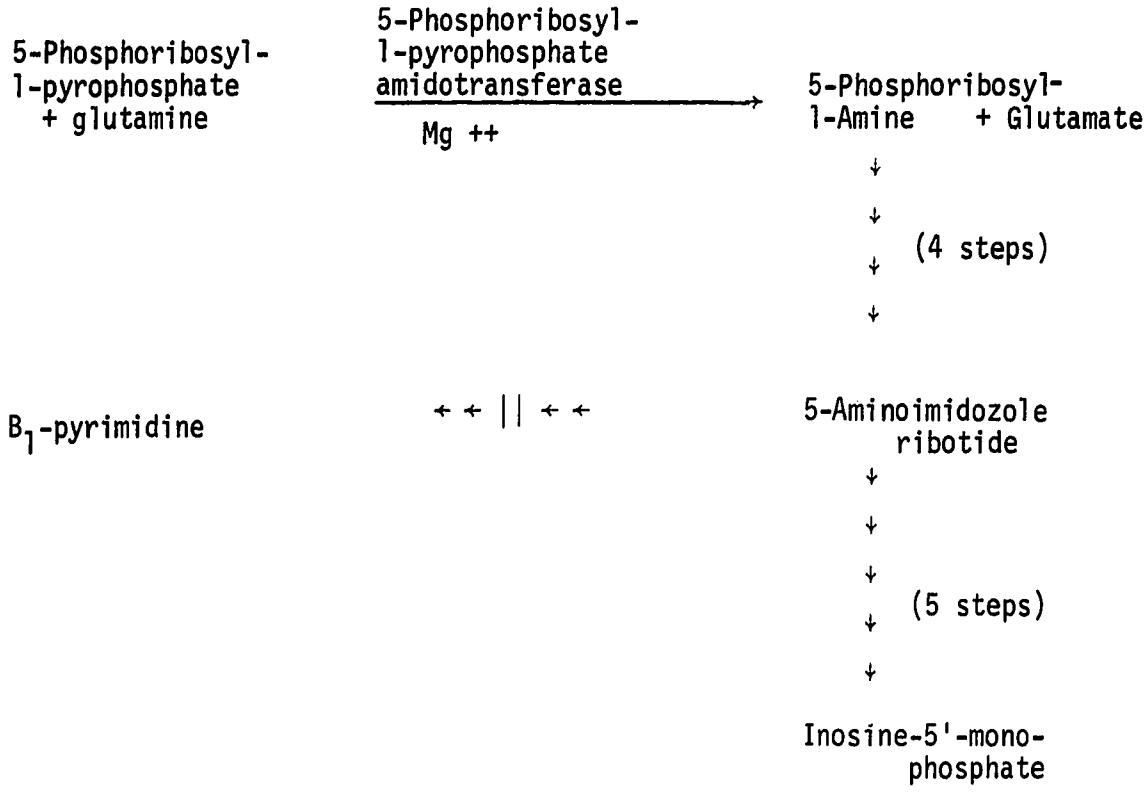
VI. Investigation of PRPP Amidotransferase Activity

Newell and Tucker (1967 and 1968b) have conclusively demonstrated that the B_1-pyrimidine precursor of thiamine is synthesized in a pathway

which branches off the pathway for de novo purine biosynthesis at the level of 5-aminoimidazole ribotide (AIR) (Fig. 14). As a result of these findings, it is possible to postulate a mechanism whereby adenosine-induced inhibition of thiamine biosynthesis is effected. Since this mechanism involves feed-back inhibition, by adenine nucleotides, of de novo purine biosynthesis, and since PRPP amidotransferase has been implicated, as the first enzyme in the pathway, in the control of de novo purine biosynthesis, attempts were made to isolate and purify the enzyme in order to investigate the effects of a variety of purine nucleotides on its activity. Purification sequences were based initially on the methods (and several modifications of the methods) of Hartman and Buchanan (1957), Wyngaarden and Ashton (1959), Hartman (1963), Neirlich and Magasanik (1964), Rottman and Guarino (1964), and Hill and Bennett (1969). Extracts were prepared from cells broken in a Hughes Press, from sonicated cells and from lysozyme-digested cells in the presence of both high and low ionic strength buffers, and in the presence and absence of thiol reagents, chelating reagents, organic solvents (glycerol) and a variety of buffers (phosphate, Tris, glycyl-glycine and malate). PRPP amidotransferase activity was not detected in crude extracts and this was attributed to the presence of a highly active glutaminase in cell preparations which rapidly degraded the glutamine in the reaction mixture. The addition of glutamine in a large excess did not overcome this problem. As a result, attempts were made to further purify the enzyme. The techniques employed included

FIGURE 14

PROPOSED INTERRELATIONSHIP OF THE PATHWAYS FOR DE NOVO BIOSYNTHESIS OF PURINE NUCLEOTIDES AND B_1-PYRIMIDINE IN M. SODONENSIS



$(NH_4)_2$ SO_4 fractionation, Sephadex gel filtration, DEAE-cellulose column chromatography, protamine sulfate precipitation and acid precipitation as well as precipitation by dialysis and heat denaturation. Most of these fractionation procedures were carried out at 0°C and in the presence and absence of the protective agents noted above for the extraction procedures.

The enzyme in these cells is extremely labile and, in spite of the efforts to overcome this problem, only very low levels of activity could be detected by any of the procedures employed. Furthermore, the lack of reproducibility in the recovery of activity did not permit confidence to be placed in the quantitative data which was obtained. The purification procedure outlined in 'Materials and Methods' (XII) was the most successful and reproducible, but the enzyme preparation, at all stages of the purification, was still highly unstable. As a result, it has not been possible to obtain reliable data for the in vitro control, by purine nucleotides, of PRPP amidotransferase activity from M. sodonensis.

Similar difficulties with the enzyme from pigeon liver (Hartman and Buchanan, 1958); Rowe and Wyngaarden, 1968), from chicken liver (Reem, 1968; Hartman, 1963; Hartman and Buchanan, 1958), from adenocarcinoma cells (Hill and Bennett, 1969) and from Bacillus subtilis (Rottman and Guarino, 1969) have been reported. However, the enzyme from avian sources appears to be more amenable to handling than do the enzymes from other sources and most of the information

concerning this reaction has been obtained through the investigation of the avian enzymes. The only reported purification of PRPP amidotransferase from a bacterial source has been from B. subtilis (Rottman and Guarino, 1964). This enzyme was also reported to be highly unstable, with a half-life of 4 hours at 0°C.

VII. Effects of Exogenous Adenosine and Guanosine on the De Novo Biosynthesis of Purines In Vivo

Since the control of PRPP amidotransferase could not be studied directly, the in vivo effect of exogenous adenosine on the de novo biosynthesis of purines was investigated. The effect of exogenous guanosine was also investigated as an additional control. De novo purine biosynthesis was measured by following the incorporation of ^{14}C-glycine into the purine fraction of the cells.

The concentration for optimum transport of exogenous glycine was determined as outlined in 'Materials and Methods' (XIII) and was found to be 1.0 mM or greater. An arbitrary concentration of 6.0 mM glycine, a clear excess, was chosen for use in the following experiments.

Washed 18-hour BN-grown cells were incubated at 30°C with 2-^{14}C-glycine (6.0 mM; specific activity, 10 μc/m mole) in BN-medium and in BN-medium supplemented with either 1.0 mM adenosine or 1.0 mM guanosine. At the designated times, 5 ml samples of the culture were frozen at -60°C. Subsequently the frozen samples were thawed, mixed

with 3 ml of 1.0N H Cl and hydrolyzed in an autoclave at 121°C (15 psi) for one hour. Cooled hydrolysates were passed through activated charcoal to adsorb solubilized nucleic acid components. The charcoal was washed extensively with distilled water and the adsorbed components eluted with 10% pyridine in 50% ethanol. Radioactivity in the eluents was determined by liquid scintillation spectrometry.

The results, summarized in Fig. 15, show that in BN-medium, de novo purine biosynthesis proceeds at the rate of 0.79 mμ moles/mg cell protein/minute. In the presence of 1.0 mM guanosine, the rate is 0.22 mμ moles/mg protein/minute. However, in the presence of 1.0 mM adenosine, the rate of de novo purine biosynthesis is less than 0.02 mμ moles/mg cell protein/minute. This latter value represents an inhibition of greater than 97% of the activity of the de novo purine pathway. This finding supports the hypothesis that adenosine exerts its inhibitory effect on cell growth by causing changes in the levels of intracellular adenine nucleotides that lead directly to a marked inhibition of de novo purine biosynthesis, which, in turn, leads to an inhibition of de novo thiamine biosynthesis, the primary cause of the observed growth inhibition.

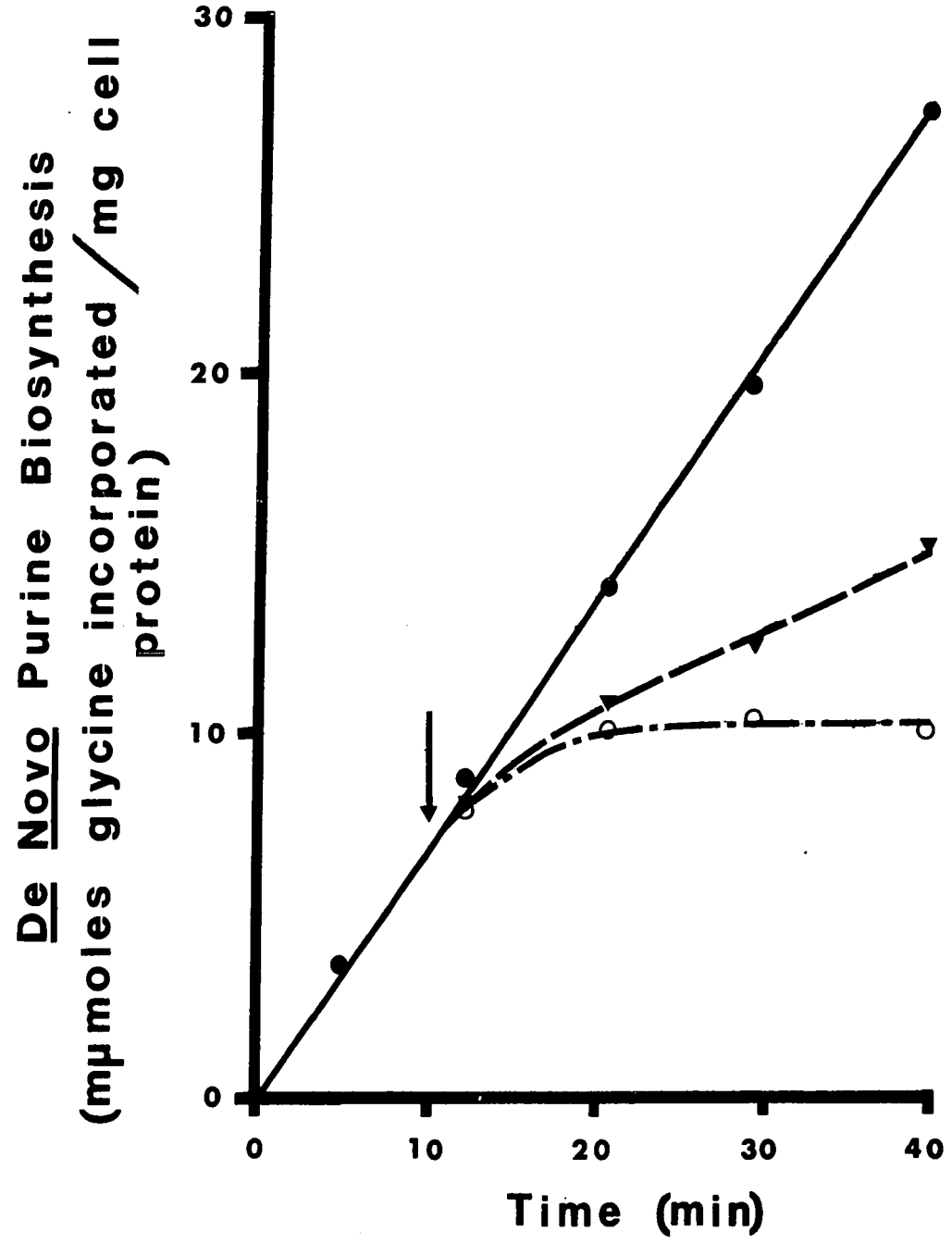

De Novo Purine Biosynthesis
(mμmoles glycine incorporated / mg cell protein)
30
20
10
0
0
10
20
30
40
Time (min)

FIGURE 15

EFFECTS OF EXOGENOUS ADENOSINE OR GUANOSINE ON THE *DE NOVO* BIOSYNTHESIS OF PURINE NUCLEOTIDES IN *M. SODONENSIS*

Purine nucleotide synthesis was measured by determining the incorporation of ^{14}C-glycine into charcoal adsorbable components of acid-hydrolysates of *M. sodonensis*. ^{14}C-labelled glycine (6.0 mM; specific activity, 10 μc/m mole) was added to the culture and the incorporation into the cells terminated at the designated times by rapid freezing (-60°C).* Radioactivity in the charcoal adsorbable fraction of acid hydrolysates of the samples was determined after elution from the charcoal with 10% pyrimidine in 50% ethanol.

BN-medium ●——●

BN-medium + 1.0 mM guanosine ▼— — —▼

BN-medium + 1.0 mM adenosine O— - —O

*(1.0 mM adenosine or guanosine were added to ^{14}C-glycine supplemented cultures in BN-medium at 10 minutes.)

GENERAL DISCUSSION

Purine-induced bacteriostasis was initially observed in A. aerogenes in 1954 (Brooke and Magasanik), and has since been reported by a number of other investigators (Yara, 1956, for S. typhimurium; Neidhardt, 1963 for A. aerogenes; Moyed, 1964, for A. aerogenes; and de Repentigny, 1968 for S. aureus). However, the mechanism by which the growth inhibition is effected has for the most part gone unexplained. This study of the effects of adenosine on the growth and metabolism of M. sodonensis represents a more complete investigation of the phenomenon and provides a framework into which the results of other investigators may be fitted.

The initial observation was that the inclusion of adenosine at concentrations of greater than 0.1 mM in the synthetic medium routinely used for the culturing of M. sodonensis resulted in an inhibition of the growth of that organism. The inhibition could not be detected until near the end of the second generation cycle and it was clear that ten-fold differences in the concentration of adenosine did not influence the time at which the inhibition became evident. Similarly, Moyed (1964) indicated that adenosine-induced inhibition of the growth of A. aerogenes was invariably observed just after the end of the first division cycle, regardless of the initial adenosine concentration (100 - 1,000 µg/ml).

The second observation was that during the inhibited phase, the cell mass increased in a non-exponential manner. Likewise, Neidhardt has reported that the growth of adenosine-inhibited A. aerogenes is linear, and replotting Moyed's (1964) data for A. aerogenes on a linear scale confirms this observation. These findings suggest that the synthesis of an essential, stable, recycling catalyst is being inhibited when these organisms are grown in the presence of adenosine.

From the data presented, it is obvious that in the presence of inhibitory concentrations of adenosine, thiamine biosynthesis is rapidly reduced. As cell growth continues, the existing intracellular thiamine pool is diluted among the newly-formed cells until, near the end of the second generation cycle, the thiamine content becomes limiting (at approximately 5 mμgms thiamine/mg cell protein) and the growth rate of the cells decreases. Adenosine-induced growth inhibition could be prevented by including either thiamine or its pyrimidine precursor in the medium, and this result supports the conclusions of Moyed (1969) and of Newell and Tucker (1966a), that exogenous adenosine inhibits the biosynthesis of thiamine and its pyrimidine precursor in A. aerogenes and S. typhimurium, respectively.

Growth during the inhibited phase is probably limited by the availability of one or more of those substances whose synthesis involves a thiamine-catalyzed reaction. Since it may be assumed that the synthesis of the rate-limiting metabolite continues at a uniform rate in the presence of a fixed amount of thiamine, the cell mass will

increase at a rate determined by the rate of synthesis of the limiting metabolite and the growth rate will approximate a linear function.

Thiamine is essential for the synthesis of citrate from pyruvate and oxaloacetic acid and under conditions whereby thiamine was limiting, Kreb's cycle activity would certainly be depressed. This occurrence would have the dual effect of reducing the availability of Kreb's cycle intermediates for subsequent metabolism, as well as reducing the rate of ATP synthesis which is dependent on the interconversion of those intermediates (i.e. oxidative phosphorylation). The possible consequences of an interference with energy metabolism were discussed earlier with reference to the disproportionate synthesis of RNA as compared to DNA under the influence of exogenous adenosine. Indeed, either limiting the availability of Kreb's cycle intermediates or interfering with energy metabolism in any way would be enough to induce bacteriostasis.

On the basis of direct thiamine assays alone, one cannot distinguish between the effects which are possible under inhibited conditions. First, thiamine biosynthesis could be completely inhibited and the only available thiamine that which is recycled, implying that thiamine catabolism does not occur in M. sodonensis; or second, thiamine biosynthesis could proceed, but at a reduced rate equal to the rate of thiamine catabolism. In either case, the thiamine content of the culture would remain constant during the inhibited phase, as the data indicate. Although oxidative catabolism of thiamine has been described by Neal (1968) in soil isolates, and thiaminase activity in Bacillus thiaminolyticus has been reported (Wong and Airth, 1967), thiamine turnover in

M. sodonensis was not investigated, since the observation that B-pyrimidine was equally as effective as thiamine in preventing the inhibition suggested that the site of inhibition was more closely associated with B_1-pyrimidine metabolism than with the metabolism of thiamine itself. This conclusion is further supported by the observation that the B_1-thiazole precursor, which might be expected to have the same preventive effects as B_1-pyrimidine, if the mechanism for thiamine depletion involved the catabolism of thiamine, per se, was not able to prevent the adenosine-induced growth inhibition.

This brings to mind a third possibility which could explain the observed reduction of thiamine biosynthesis in the presence of adenosine. Should adenosine be degraded so that a compound comprised of a pyrimidine ring with an N - C or N - C - N substituent on C_5 of the ring be formed, such a compound might compete with B_1-pyrimidine for the available B_1-thiazole and thus form a metabolically inactive "pseudo-thiamine" moiety. By adding B_1-pyrimidine one might shift the equilibrium of the competition enough to satisfy the cells' requirements for thiamine. This possibility is not favoured for several reasons. First, B_1-pyrimidine is equally as effective on a molar basis as thiamine in preventing adenosine-induced inhibition whereas it might be reasonable to assume that it would be less effective if it were involved in a competition reaction with an analogue. Second, one might expect that guanosine as well as adenosine could be degraded in such a way as to produce the hypothetical competitive analogue of B_1-pyrimidine, but, as has been demonstrated, guanosine

has no inhibitory effect on M. sodonensis. Therefore, the first proposed mechanism (i.e. inhibition of B_1-pyrimidine biosynthesis with little or no catabolism of thiamine) is favoured.

Other investigators have also shown that the addition of thiamine, in concentrations similar to those required by thiamine auxotrophs, to a purine-inhibited culture of A. aerogenes results in the elimination or immediate reversal of the inhibition (Neidhardt, 1963; Brooke and Magasanik, 1954; Moyed, 1964). Furthermore, Moyed (1964) was able to show that the B_1-pyrimidine precursor of thiamine was just as effective as thiamine in eliminating the adenosine-induced inhibition of A. aerogenes, whereas the B_1-thiazole precursor had no protective effect. Moyed, (1964) also demonstrated that in the presence of adenosine, A. aerogenes was unable to synthesize either the B_1-pyrimidine or thiamine itself and suggested that either a pseudo-feedback inhibition of B_1-pyrimidine biosynthesis, or a competition between adenosine (or a derivative of adenosine) and one of the intermediates in the pathway for B_1-pyrimidine biosynthesis for an enzyme site, might explain how this inhibition was produced. Subsequently, Newell and Tucker (1966a) demonstrated that growing S. typhimurium in the presence of adenosine led to a de-repression of thiamine and B_1-pyrimidine biosynthesis. This de-repression was shown to be the result of a decrease in intracellular thiamine concentration rather than of the accumulation of a B_1-pyrimidine precursor, as would be expected if the competition mechanism postulated by Moyed were correct. These results argue also against the possibility that the mechanism by which B_1-pyrimidine

synthesis is inhibited in the presence of adenosine involves a competition between adenosine (or one of its derivatives) and a precursor of B_1-pyrimidine for the active site of an enzyme.

Investigations by Newell and Tucker (1967, 1968a, 1968b) established the fact that the pyrimidine precursor of thiamine was synthesized by a pathway that branched off the pathway for de novo purine biosynthesis at the level of 4-amino-imidazole ribotide (AIR). Their results clarified the confusion that had arisen as a result of earlier findings (Yara, 1956) with respect to the thiamine requirements of mutants of S. typhimurium blocked at various stages of the pathway for de novo purine biosynthesis, including some apparently blocked after AIR. This latter case was shown to be a phenotypic effect rather than an actual block in the pathway leading to B_1-pyrimidine synthesis. The later step mutants (blocked after AIR) appeared to accumulate AICAR, which, like adenosine, could inhibit B_1-pyrimidine biosynthesis and thereby give these mutants the same phenotype as true B_1-requiring mutants. Knowing that B_1-pyrimidine is synthesized in a pathway which is common, in part, with the early reactions of the de novo purine pathway, one can postulate that changes in the activity of the de novo purine pathway might be reflected in variations in the synthesis of B_1-pyrimidine and thiamine.

Feedback inhibition of de novo purine biosynthesis occurs at the level of 5-phosphorylribose-1-pyrophosphate (PRPP) amidotransferase, the first reaction in the purine pathway (Henderson and Khoo, 1965; McCollister et al., 1964; Caskey, et al., 1964; Rottman and

Guarino, 1964; Wyngaarden and Ashton, 1959; Nierlich and Magasanik, 1954; Hill and Bennett, 1969; Hartman, 1963). In vitro investigations of the sensitivity of PRPP amidotransferase to inhibition by purine ribonucleotides indicates that the regulation of this enzyme in vivo is a complex process in which a number of purine ribonucleotides can participate. The enzyme model proposed by Caskey, et al. (1964) contains at least two sites (one for binding of adenine nucleotides and one for guanine nucleotides), one of which may participate in the binding of PRPP. A third site might also be present specifically for the binding of PRPP. The feedback inhibitions that they observed could be the result of competition for the PRPP binding site in the case of a two-site enzyme, or a result of spatial distortion of the substrate binding site (or sites) as a result of the interaction of inhibitors with the enzyme in the case of an enzyme with three or more binding sites. The latter possibility appears to be somewhat more likely, since in certain instances, the effect of adding two inhibitors is greater than the effect of either inhibitor when present at the same total concentration, and greater than the sum of the fractional inhibitions of either inhibitor alone. In this case, it appears that the binding of one inhibitor increases the inhibitory effect of the binding of the second inhibitor.

Wyngaarden and Ashton (1959) demonstrated that in pigeon liver, PRPP amidotransferase is inhibited by ATP ($K_i = 3.7 \times 10^{-5}$), ADP ($K_i = 3.9 \times 10^{-5}$), GMP ($K_i = 8.6 \times 10^{-5}$), AMP ($K_i = 9.0 \times 10^{-5}$), IMP ($K_i = 1.8 \times 10^{-4}$) and GDP ($K_i = 3.8 \times 10^{-4}$). The inhibitions in all

cases were competitive with respect to PRPP binding and non-competitive with respect to glutamine binding. Similar inhibitory effects by ribonucleotides on the activity of the enzyme from Adenocarcinoma 755 cells were reported by Hill and Bennett (1969), but more complex kinetics than those of Wyngaarden and Ashton (1959) were observed.

Although these authors have had some success in studying the in vitro control of PRPP amidotransferase, the majority of the reports in the literature deal with in vivo investigations of the problem of the control of de novo biosynthesis of purines.

Burns (1964), working with yeast mutants, demonstrated that adenine would prevent accumulation of AIR and correlated changes in nucleotide pool levels of IMP with inhibition and stimulation of AIR accumulation. He observed that when pool IMP levels increased in response to the addition of adenine, AIR accumulation was inhibited. He interpreted his results as indicating that IMP played a more important role than other purine nucleotides in the control of de novo purine biosynthesis. Henderson and Khoo (1965), working with whole Ehrlich ascites tumor cells, however, provided strong evidence that adenine nucleotides were more potent inhibitors of de novo purine biosynthesis than were hypoxanthine nucleotides. This evidence was based on the use of a mutant which was blocked at adenylosuccinate synthetase and thus was unable to convert IMP to AMP. Previous experiments (reported in the same paper) illustrated that conversion of purine bases to ribonucleotides was an absolute prerequisite to

inhibition of the de novo purine pathway, since mutants lacking adenine and guanine-hypoxanthine phosphoribosyl transferase activities did not exhibit inhibition of de novo purine biosynthesis in the presence of these bases. When mutants which contained the purine phosphoribosyl transferases but which were blocked at adenylosuccinate synthetase were fed adenine or guanine, the de novo purine pathway was inhibited as under normal conditions. However, if these cells were fed hypoxanthine, the inhibition was markedly reduced. This reduction was attributed, for the most part, to the inability of these cells to form AMP from the IMP derived from hypoxanthine and this evidence was the basis for the claim that AMP was the more potent feedback inhibitor.

The results with M. sodonensis agree more closely with the conclusions of Henderson and Khoo (1965) than with those of Burns (1964), since IMP accumulation was not observed in M. sodonensis. The results, however, differ from those of Henderson, in that guanine nucleotides were not as effective as adenine nucleotides in inhibiting de novo purine biosynthesis. With this exception, the results are in general agreement with the findings of a number of investigators (Gots, 1957; Henderson and Khoo, 1965; Burns, 1964; McFall and Magasanik, 1960) that de novo purine biosynthesis can be drastically inhibited in vivo if certain purine and nucleosides are included in the medium.

Gots (1957) demonstrated direct, immediate feedback inhibition of AICA accumulation in mutants of E. coli when adenine was added at levels of 10-20 µg/ml. The inhibition appeared to be in the range of 90-95% and was immediately reversed if adenine was washed away from

the cells. A plot of percent inhibition of AICA accumulation against the log of the adenine concentration gave a straight line relationship which is what one might expect if the inhibition is dependent on the maintenance of an effective internal depot of inhibitor, taking into consideration the rate of transport and rate of conversion to the inhibitory form (Gots, 1957).

The presence of an internal inhibitor pool which is maintained only in the presence of an exogenous precursor, as postulated by Gots (1957), is in agreement with the findings in M. sodonensis. The absolute requirement for the presence of adenosine in the culture supernatant for maximum growth inhibition to occur has been clearly demonstrated, as has its correlation with the elevation of adenine nucleotide levels and the inhibition of de novo purine nucleotide biosynthesis.

The high level of adenosine deaminase activity which is present in extracts of M. sodonensis suggested that little or no intracellular adenosine would accumulate even in the presence of high exogenous adenosine concentrations, and this was confirmed by direct analysis of the acid-soluble pool of adenosine-grown M. sodonensis. This conclusion is further supported by the observation that inosine is rapidly released into the culture supernatant when cells are incubated in the presence of adenosine. These observations lead one to the conclusion that the role of adenosine as the most potent bacteriostatic purine for M. sodonensis is entirely dependent on its high rate of transport and subsequent rapid catabolism and that the adenine

nucleotides, rather than adenosine per se, function as the primary inhibitors inside the cell. Other investigators who have remarked on the specific requirement for the presence of adenosine in the culture supernatant in order to observe growth inhibition have not investigated this possibility.

The recovery phase observed with M. sodonensis has not been described in other systems. It was shown not to result from the appearance of an adenosine-resistant mutant, but to correlate with the disappearance of adenosine from the culture supernatant. This agrees with the observations of Newell and Tucker in S. typhimurium (1966a) and Manohar (1968) in erythrocytes, that washing the cells free of adenosine relieves inhibition. M. sodonensis is unique, at least among reported systems, in that it is inhibited by adenosine, inosine and possibly hypoxanthine, but not by adenine. Except for the report by Neidhardt (1963) of a slight inhibition of A. aerogenes by very high concentrations of guanosine, no other purines or purine derivatives have been reported to have bacteriostatic properties.

Adenosine has also been shown to be the most effective bacteriostatic natural purine derivative in A. aerogenes (Moyed, 1964), S. typhimurium (Newell and Tucker, 1966a) and S. aureus, Strain Wood 46 (de Repentigny, 1968), although inosine (Moyed, 1964) and adenine (Brooke and Magasanik, 1954); Yara, 1956; Moyed, 1969) have also been reported to be very effective growth inhibitors. It would appear, therefore, that only those compounds which are precursors of adenine nucleotides have a capability for producing growth inhibition.

CONCLUSIONS

In the presence of exogenous adenosine, the intracellular adenine nucleotide levels of *M. sodonensis* are elevated, a condition which leads to feedback inhibition of the *de novo* biosynthesis of purine nucleotides. As a result, the cells are depleted of the intermediates of that pathway, including AIR, a direct precursor of the pyrimidine moiety of thiamine as well as of purine nucleotides. Consequently, the biosynthesis of thiamine is also inhibited. Growth continues at a normal rate until the existing thiamine pool becomes limiting, at which time growth inhibition becomes evident. Adenosine functions as the most effective initiator of this sequence of events in *M. sodonensis* because of its high rates of transport and subsequent catabolism, as compared with other, less potent, bacteriostatic purine derivatives.

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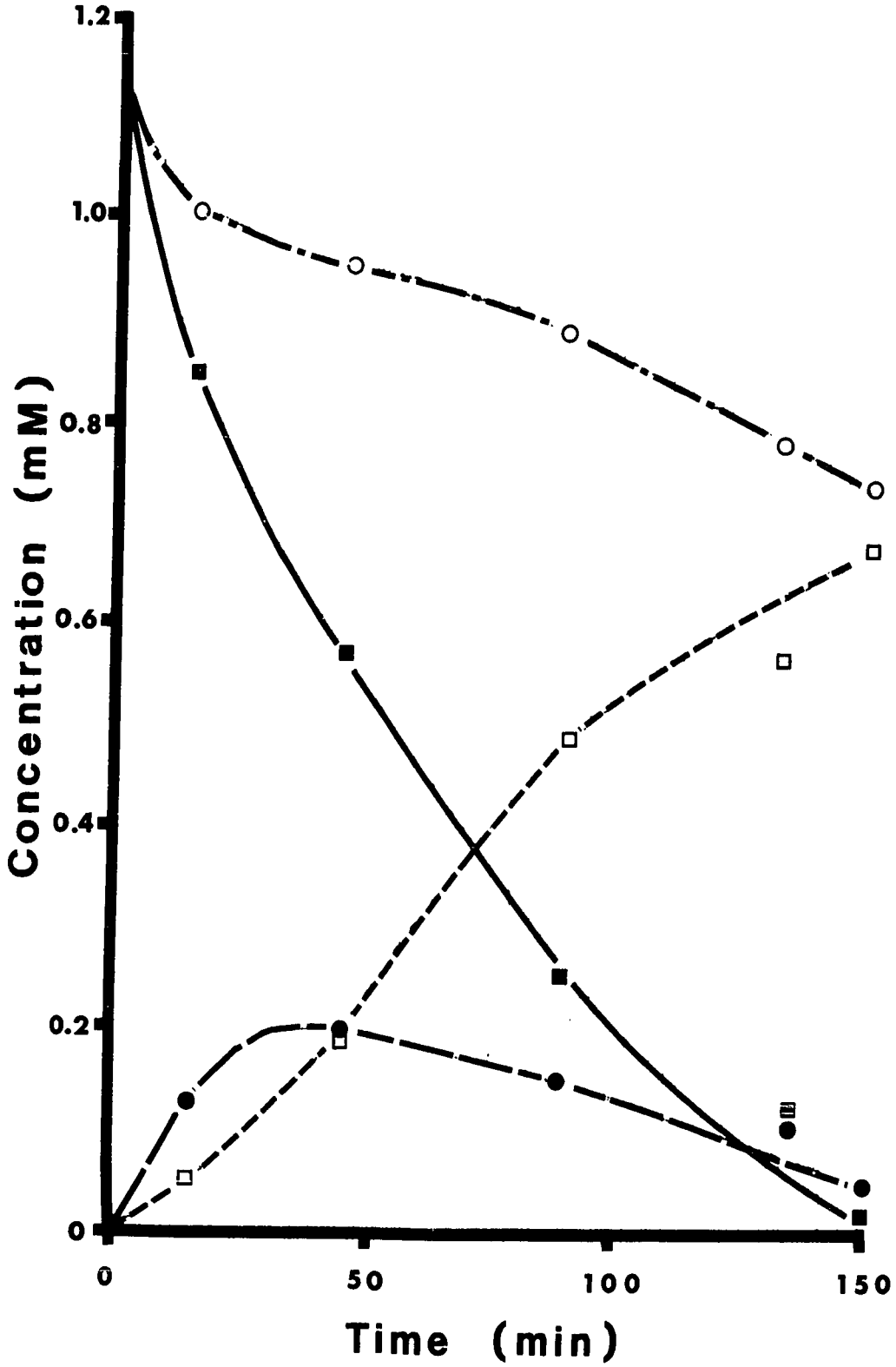
ADDENDUM

TRIVIAL NAMES OF ENZYMES ASSAYED

Trivial Name	Proper Name
Adenase	Adenine aminohydrolase (3. 5. 4. 2.)
Adenine phosphoribosyl-transferase	Adenine phosphoribosyltransferase (AMP: pyrophosphate phospho-ribosyltransferase) (2. 4. 2. 7.)
Adenosine deaminase	Adenosine aminohydrolase (3. 5. 4. 4.)
Adenosine phosphokinase	Adenosine phosphokinase (ATP: adenosine 5'-phosphotransferase) (2. 7. 1.20.)
Adenylosuccinate synthetase	Adenylosuccinate synthetase (IMP: L-aspartate ligase) (6. 3. 4. 4.)
AMP deaminase	AMP aminohydrolase (3. 5. 4. 6.
Glutamic dehydrogenase	Glutamic dehydrogenase (L-glutamate: NAD(P) oxidoreductase (deaminating)) (1. 4. 1. 3.)
Glycinamide ribotide kinosynthase	Phosphoribosyl-glycineamide synthetase (Ribosylamine-5-phosphate: glycine ligase (ADP)) (6. 3. 1. 3.)
GMP reductase	GMP reductase (reduced NADP: GMP-oxidoreductase (deaminating)) (1. 6. 6. 8.)
Guanase	Guanine aminohydrolase (3. 5. 4. 3.)
Hypoxanthine/guanine phosphoribosyltransferase	Hypoxanthine/guanine phospho-ribosyl transferase (IMP/GMP: pyrophosphate phosphoribosyl-transferase) (2. 4. 2. 8.)
IMP dehydrogenase	IMP dehydrogenase (IMP:NAD oxido-reductase) (1. 2. 1.14.)

Trivial Name	Proper Name
PRPP amidotransferase	PRPP amidotransferase (ribosylamine-5-phosphate: pyrophosphate phosphoribosyl transferase (glutamine amidating)) (2. 4. 2.14.)
Purine nucleosidase (hydrolytic)	N-ribosyl purine ribohydrolase (3. 2. 2. 1.)
Purine nucleosidase (phosphorolytic)	Purine nucleosidase (purine nucleoside: orthophosphate ribosyltransferase) (2. 4. 2. 1.)
Xanthine oxidase	Xanthine oxidase (Xanthine: oxygen oxidoreductase) (1. 2. 3. 2.)
XMP aminase	


1.2
1.0
0.8
0.6
0.4
0.2
0
Concentration (mM)
0
50
100
150
Time (min)

APPENDIX A

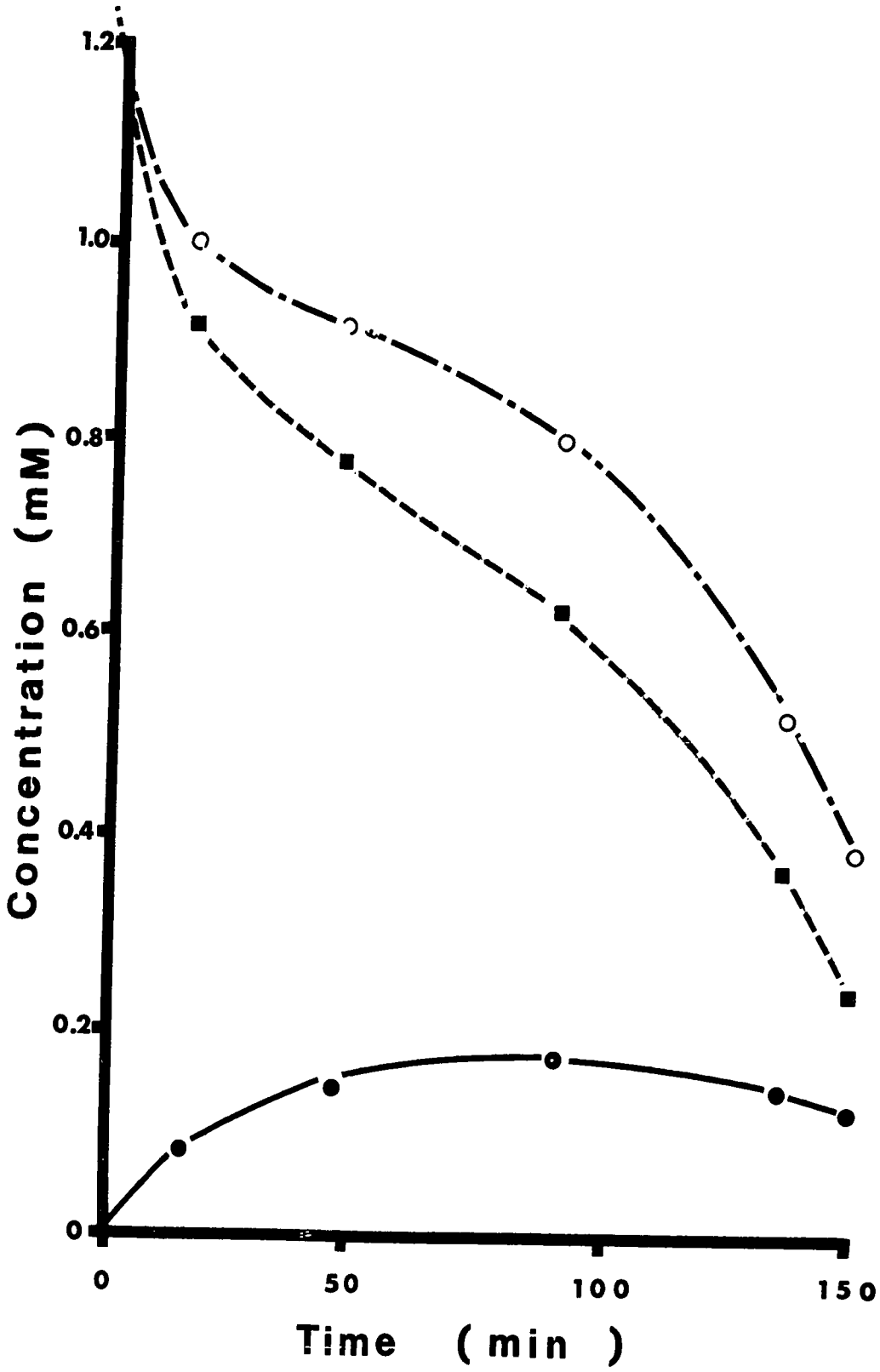
PLOT OF DATA IN TABLE IV.

Adenosine ————————

Inosine — — — —

Hypoxanthine ----------

Total —— – ——


1.2
1.0
0.8
0.6
0.4
0.2
0
Concentration (mM)
0
50
100
150
Time (min)

APPENDIX B

PLOT OF DATA IN TABLE V

Guanine ————————

Guanosine ---------

Total ——— – ———