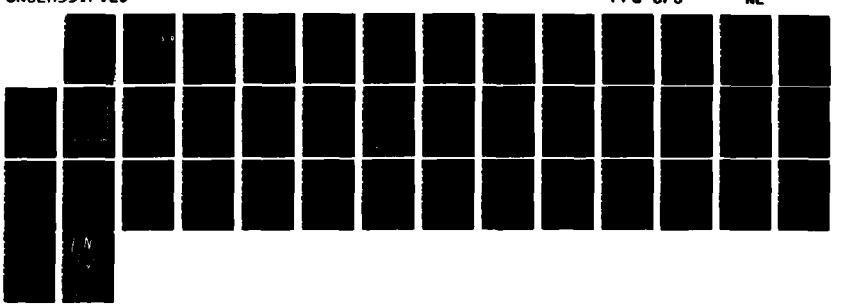
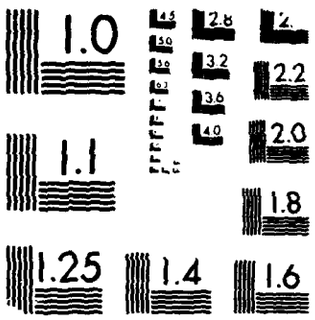
AD-A192 132 CHEMOTHERAPY AND BIOCHEMISTRY OF LEISHMANIA(U) 1/1
MASSACHUSETTS UNIV AMHERST L L NOLAN DEC 86
DAMD17-81-C-1198

UNCLASSIFIED F/G 6/5 NL





(4)

AD_____

CHEMOTHERAPY AND BIOCHEMISTRY OF LEISHMANIA

ANNUAL REPORT

LINDA L. NOLAN, Ph.D.

DECEMBER 1986

Supported by

U.S. ARMY MEDICAL RESEARCH AND DEVELOPMENT COMMAND
Fort Detrick, Frederick, Maryland 21701-5012

Contract No. DAMD17-81-C-1198

University of Massachusetts
Amherst, Massachusetts 01003

SECURITY CLASSIFICATION OF THIS PAGE

REPORT DOCUMENTATION PAGE

Form Approved OMB No. 0704-0188

1a. REPORT SECURITY CLASSIFICATION: UNCLASSIFIED	1b. RESTRICTIVE MARKINGS
2a. SECURITY CLASSIFICATION AUTHORITY	3. DISTRIBUTION/AVAILABILITY OF REPORT: Approved for public release; distribution unlimited
2b. DECLASSIFICATION/DOWNGRADING SCHEDULE	
4. PERFORMING ORGANIZATION REPORT NUMBER(S)	5. MONITORING ORGANIZATION REPORT NUMBER(S)
6a. NAME OF PERFORMING ORGANIZATION: University of Massachusetts; 6b. OFFICE SYMBOL (If applicable)	7a. NAME OF MONITORING ORGANIZATION
6c. ADDRESS (City, State, and ZIP Code): Amherst, Massachusetts 01003	7b. ADDRESS (City, State, and ZIP Code)
8a. NAME OF FUNDING/SPONSORING ORGANIZATION U.S. Army Medical Research & Development Command; 8b. OFFICE SYMBOL (If applicable)	9. PROCUREMENT INSTRUMENT IDENTIFICATION NUMBER: DAMD17-81-C-1198
8c. ADDRESS (City, State, and ZIP Code): Fort Detrick, Frederick, Maryland 21701-5012	10. SOURCE OF FUNDING NUMBERS

PROGRAM ELEMENT NO.	PROJECT NO.	TASK NO.	WORK UNIT ACCESSION NO.
61102A	3M1 61102BS10	AF	080

11. TITLE (Include Security Classification)

(U) Chemotherapy and Biochemistry of Leishmania

12. PERSONAL AUTHOR(S)

Linda L. Nolan

13a. TYPE OF REPORT	13b. TIME COVERED	14. DATE OF REPORT (Year, Month, Day)	15. PAGE COUNT
Annual Report	FROM 1/1/86 TO 12/31/86	December 1986	38

16. SUPPLEMENTARY NOTATION

17. COSATI CODES			18. SUBJECT TERMS (Continue on reverse if necessary and identify by block number)
FIELD	GROUP	SUB-GROUP	Leishmania, mode of action of sinefungin, DNA and RNA replication
06	13		
06	03		

19. ABSTRACT (Continue on reverse if necessary and identify by block number)

A comparison of the enzymes of the pathogenic protozoa to those of man is of fundamental importance to the search for much needed chemotherapeutic agents. Nucleic acid metabolism in trypanosomatids is unique in several ways: (1) they lack the ability to synthesize purines de novo, depending entirely on the salvage pathway for their supply of purine nucleotides; (2) many of the enzymes involved in nucleic acid biosynthesis either have unusual substrate specificities or unusual subcellular localizations; (3) a large proportion of the DNA which is produced is incorporated into a unique organelle known as the kinetoplast; and (4) the DNA polymerase isolated from these organisms demonstrates major differences from its mammalian counterpart.

There is very little information concerning the DNA and RNA polymerases of Leishmania spp.

Our aim has been the isolation and characterization of the DNA and RNA polymerase of

20. DISTRIBUTION/AVAILABILITY OF ABSTRACT	21. ABSTRACT SECURITY CLASSIFICATION
☐ UNCLASSIFIED/UNLIMITED ☒ SAME AS RPT. ☐ DTIC USERS	Unclassified

22a. NAME OF RESPONSIBLE INDIVIDUAL	22b. TELEPHONE (Include Area Code)	22c. OFFICE SYMBOL
Mary Frances Bostian	301/663-7325	SGRD-RMI-S

DD Form 1473, JUN 86 *Previous editions are obsolete.* SECURITY CLASSIFICATION OF THIS PAGE

Leishmania mexicana and search in vivo and in vitro for inhibitors of these enzymes for chemotherapeutic exploitation.

Sinefungin, a natural nucleoside isolated from cultures of Streptomyces incarnatus and Streptomyces griseolus, is structurally related to S-adenosylhomocysteine and S-adenosylmethionine. Sinefungin has been shown to inhibit the development of various fungi and viruses, but its major attraction to date resides in its potent antiparasitic activity. This compound has been reported to display antiparasitic activity against malarial, trypanosoma, and leishmanial species. Very little is known about the antiparasitic mode of action of sinefungin. We have found that S-adenosylmethionine is capable of reversing the inhibitory growth effects of sinefungin in Leishmania mexicana, and that dATP was capable of reversing the inhibitory effects of the drug on DNA polymerase activity when measuring pyrophosphate release. However, when incorporation of [^{3}H]dTTP was used to measure DNA polymerase activity, no inhibition could be observed. The inhibition of DNA polymerase activity by sinefungin occurred only during the initial stages of purification of this enzyme, and inhibition by aphidicolin, a known DNA polymerase α inhibitor, paralleled the inhibition by sinefungin. Neither sinefungin or aphidicolin inhibited partially purified DNA polymerase α. S-adenosylmethionine synthetase was partially purified and sinefungin at levels active in vivo had no significant effect. In collaboration with Dr. William Hanson, University of Georgia, we found that sinefungin was significantly suppressive against both Leishmania donovani and L. braziliensis panamensis infections in hamsters when compared to glucantime.

TABLE OF CONTENTS

LIST OF FIGURES & TABLES

RESUME OF PROGRESS

During the last year we have:

1. Continued our isolation and characterization of the DNA polymerase of *Leishmania mexicana* WR 227.

2. Continued our studies on the mode of action of sinefungin.

3. Isolated the RNAs of Formycin B (an antiparasitic agent) treated leishmanial cells in order to elucidate the compounds mode of action.

4. Partially isolated and characterized S-adenosylmethionine synthetase, an enzyme important in the methylation of protein, carbohydrates, lipids and nucleic acids.

SUMMARY

Sinefungin, a natural nucleoside isolated from cultures of Streptomyces incarnatus and Streptomyces griseolus, is structurally related to S-adenosylhomocysteine and S-adenosylmethionine. Sinefungin has been shown to inhibit the development of various fungi and viruses, but its major attraction to date resides in its potent antiparasitic activity. This compound has been reported to display antiparasitic activity against malarial, trypanosoma and leishmanial species. Very little is known about the antiparasitic mode of action of sinefungin. We have found that S-adenosylmethionine is capable of reversing the inhibitory growth effects of sinefungin in Leishmania mexicana, and that dATP was capable of reversing the inhibitory effects of the drug on DNA polymerase activity when measuring pyrophosphate release. However, when incorporation of [^{3}H]dTTP was used to measure DNA polymerase activity, no inhibition could be observed. The inhibition of DNA polymerase activity by sinefungin occurred only during the initial stages of purification of this enzyme, and inhibition by aphidicolin, a known DNA polymerase inhibitor, paralleled the inhibition by sinefungin. Neither sinefungin or aphidicolin inhibited partially purified DNA polymerase α. S-adenosylmethionine synthetase was partially purified and sinefungin at levels active in vivo, had no significant effect. Sinefungin was significantly suppressive against both Leishmania donovani and L. braziliensis panamensis infections in hamsters when compared to glucantine.

MOLECULAR TARGET OF ANTILEISHMANIAL ACTION OF SINEFUNGIN

Sinefungin, a natural nucleoside isolated from cultures of Streptomyces incarnatus and Streptomyces griseolus, is structurally related to S-adenosyl-homocysteine (SAH) and S-adenosylmethionine (SAM) (Fig.1). Sinefungin has been shown to inhibit the development of various fungi (9,10,11) and viruses (11,23,27,32) but its major attraction to date resides in its potent antiparasitic activity (2,6,7,9,19,23-25,31). This compound displays antiparasitic activity against malarial parasites (31), Trypanosoma (6) and Leishmania species (2,3,22,23). Recently, it was reported that sinefungin inhibits protein methylases in Leishmania (25), but its major site of inhibition has been reported to involve DNA synthesis (3). Thymidine uptake into the cells was not affected. Uridine incorporation was inhibited to a much lesser extent while leucine incorporation was unaffected. It was shown that nuclear and kDNA synthesis was inhibited without inhibition of nucleoside phosphorylation, but accompanied by an increase in nucleoside triphosphate levels (3).

In an attempt to elucidate the molecular mode of action of sinefungin in leishmanial parasites we determined which compounds could reverse its inhibitory action. Because sinefungin is an analog of SAM, we studied its action on S-adenosylmethionine synthetase. In recent years SAM has been found to affect the methylation and the properties of such macromolecules as nucleic acids, proteins, phospholipids and carbohydrates (5,8).

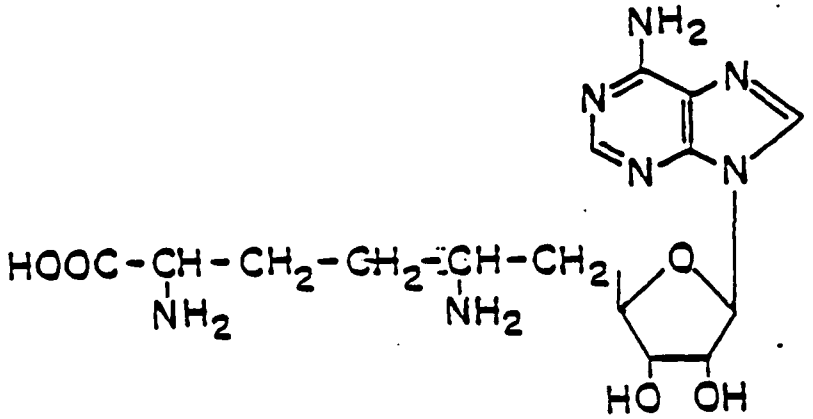
In an attempt to correlate the antileishmanial activity of this molecule with inhibition of DNA synthesis, we studied the effect of this compound on DNA polymerase. We compaired the activity of sinefungin in leishmania-infected hamsters to glucantime, a drug which is used in the treatment of leishmaniasis.

MATERIALS AND METHODS

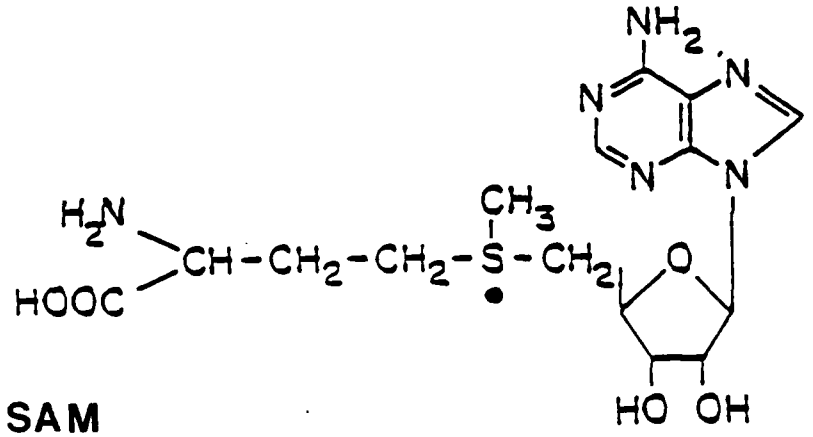
Sinefungin was generously provided by Dr. Malka Robert-Gero, Institut de Chimie des Substances Naturelles C.N.R.S., Gif-sur-Yvette, France. Aphidicolin was supplied by A.H. Todd of Imperial Chemical Industries, England. All other chemicals were of the highest purity and were obtained from Sigma Chemical, Co., except soybean trypsin inhibitor, aprotinin and leupeptin which were crude grade. [^{3}H]TTP (45 Ci mmole^{-1} and [^{14}C]-adenosylmethionine (40 Ci mmole^{-1}) were purchased from Amersham. Heparin-Sepharose CL-6B, cellulose phosphate, and denatured DNA cellulose were obtained from Pharmacia Fine Chemicals.

Cell Culture Conditions for Enzyme Isolations: Promastigotes of Walter Reed Strain #227 were used in these experiments. This strain has been previously identified as Leishmania mexicana amazonensis (J. Decker-Jackson and P. Jackson, personal communication), and was obtained from the Leishmania Section of the Walter Reed Army Institute of Research. Promastigotes were grown in Brain Heart Infusion Medium (BHI)

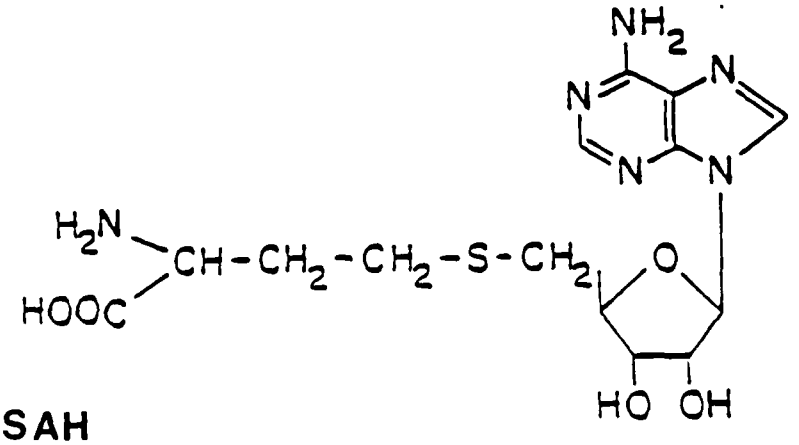
Fig. 1. Sinefungin and Related Compounds



sinefungin



SAM



SAH

containing 37g BHI (Difco) L^{-1} water, 10% heat inactivated serum and 26 ug hemin ml^{-1}. Cells were grown at 26°C in 2000 ml wide Fernbach flasks containing 250 ml of BHI. Cells were harvested after 4 days during the exponential growth phase. The cell density was 4-6 x 10^7 cells ml^{-1}.

Inhibition and Reversal Studies: Promastigotes of L. mexicana were grown in the defined medium of Steiger and Steiger (30) supplemented with 5% heat inactivated calf serum. When specific purines were being tested for reversal of the inhibitory action of sinefungin, the purine source (adenosine) was omitted and the test purine was added to the medium. The leishmania were initially grown in BHI and when in log phase (2 x 10^6 cells/ml) these cells were used as an inoculum (0.5 ml) and asceptically transferred to 4.5 ml of Steiger and Steiger medium in 14.5 cm x 1.5 cm test tubes. The compound to be tested was added and the cells were incubated in a slanted position at 26° C. Optical density readings at 660 nm were taken every 24 hrs. for a total of 96 hrs. Growth experiments were done in duplicate.

Purification of Leishmanial DNA polymerase: Cells (1.5 liters) were harvested in 250 ml centrifuge bottles and centrifuged at 12,000 rpm for 10 min. The cells were washed twice in buffer containing 50 mM Tris-HCl (pH 7.5). The cell pellet (usually 4-6 g wet wt.) was suspended in lysis buffer (8-12 ml) containing 10 mM Tris-HCl (pH 7.5), 20% glycerol, 1 mM dithiothreitol, 1 mM EDTA, 1.0 M HCl and 0.3% Triton X-100. This cell suspension was diluted 1:100 with the following stock solution of protease inhibitors: soybean trypsin inhibitor (4.8 mg ml^{-1}), aprotinin (4.8 mg ml^{-1}) and leupeptin (2 mg ml^{-1}). Isolated cells were homogenized at 4°C with a Teflon homogenizer or sonicated 5 times for 10 sec with 2 min. cooling intervals. This was carried out with a Braun-Sonic 2000 ultrasonic disruptor at 4°C. The suspension was centrifuged at 18,000 x g for 1 hr. at 4°C. This supernatant fluid was subjected to protamine sulfate treatment, dialysis and column chromatography. DNA was removed by adding sufficient 2% protamine sulfate to the crude enzyme to result in a 1:10 dilution. The precipitate from the protamine sulfate step was removed by centrifugation at 18,000 x g for 15 min at 4°C. The supernatant was dialyzed against 2 L of standard buffer which consisted of 20 mM Tris-HCl (pH 7.5), 1 mM dithioerythritol, 1 mM EDTA, and 20% glycerol. The precipitate that formed after dialysis was removed by centrifugation at 18,000 x g for 20 min at 4°C.

Chromatography: The supernatant was applied to a Heparin-Sepharose column (8.5 cm x 1.3 cm) equilibrated with 0.1 M KCl in standard buffer. The column was washed with 20 mM Tris-HCl pH 7.5 buffer until the absorbance at 280 nm was less than 0.1. The DNA polymerase was then eluted with 0.5 M KCl in standard buffer. Active fractions were pooled and protease inhibitors were added as described. The pooled fractions were dialyzed overnight against 2 L of standard buffer containing 0.1 M KCl. After dialysis more protease inhibitors were added as described above, and the pooled fractions were applied to a cellulose-phosphate

column (7.0 cm x 1.3 cm) equilibrated with 0.1 M KCl in standard buffer. The column was washed with the same buffer until the absorbance at 280 nm was less than 0.1. The DNA polymerase was then eluted with 0.35 M KCl in standard buffer. The active fractions were pooled and protease inhibitors were added. The fractions were dialyzed against 2 L of standard buffer overnight. The pooled dialyzed fractions were applied to a denatured DNA cellulose column (13.0 cm x 1.0 cm) equilibrated with standard buffer. The column was washed first with 15 ml of standard buffer, and the enzyme was eluted using 15 ml step gradients (0.1 M KCl, 0.25 M KCl) in the same buffer.

Protein Assays: Protein concentrations were either determined by the dye-binding method (Bio-Rad Labs) or by a modified method. The modified method was performed in 96 well microplates by adding 80 ul of Bio-Rad dye and 20 ul of a column fraction. The plate was then read in a Dynetech 580 microplate reader at 575 nm.

DNA Polymerase Radioactive Assay: Enzymes were assayed for activated DNA dependent activity in a reaction mixture containing 10 mM Tris-HCl (pH 7.5), 41.6 uM each of dATP, dCTP, dGTP and [methyl-^{3}H]dTTP at 200-300 cpm pmol^{-1}, 5 mM $MgCl_2$, 100 ug ml^{-1} bovine serum albumin and 50 ug ml^{-1} activated calf thymus DNA. Assays were for 30 min at 30°C. Compounds tested for inhibition were preincubated 15 min at 30°C with the enzyme to be tested. Assays were routinely carried out in a final volume of 50 ul. Reactions were terminated by pipetting 10 ul of a solution containing 2.5% SDS and 0.15 M sodium pyrophosphate (18). The reaction mixture was then pipetted onto DEAE-cellulose discs (Whatman DE81) followed by immersion in 0.5 M Na_2HPO_4. The filters were washed 5 times for 5 min each in this buffer, twice in distilled H_2O, twice in 95% EtOH, once in ether, and air-dried. The discs were counted in Fisher Scint Verse II in a Beckman scintillation counter.

One unit of activity is defined as the incorporation of 1 pmole of dTTP into DNA in 30 min under standard assay conditions.

DNA Polymerase Spectrophotometric Assay: This assay is described in Sigma Technical Bulletin No. 7275 issued August 1983. This bulletin describes the spectrophotometric determination of pyrophosphate and the assay of DNA polymerase.

Isolation and Assay of S-Adenosylmethionine Synthetase: Using the method of Hoffman and Kunz (14), we optimized our enzyme assay for L. Mexicana 227 promastigotes. Methionine adenosyltransferase activity was measured at 35°C in 100 ul of a standard assay mixture containing the following components: 150 mM KCl, 20 mM $MgSO_4$, 5 mM dithiothreitol, 50 mM Tris pH 7.5, 5 mM ATP, and 10 uM L-[^{14}C]-methionine. The cationic [^{14}C]-adenosylmethionine formed was isolated by spotting 80 ul portions of reaction mixtures on 2.3 cm discs of Whatman P81 cellulose phosphate cation-exchange paper, removing unreacted methionine by collecting and

washing discs three times in a beaker of cold 0.1 M ammonium formate, pH 3.0, followed once with 95% ethanol, and once with ether. [^{14}C]-Adenosylmethionine was quantified by liquid scintillation counting of dried discs under 5 ml Fisher Scint Verse II.

S-Adenosylmethionine synthetase was isolated by suspending 8 g of pelletted L. Mexicana 227 cells in buffer containing 50 mM Tris pH 7.5, 10 mM $MgSO_4$, 1 mM EDTA, and 1 mM DTT. The cells were sonicated 3x for 15 seconds, and the cell suspension was centrifuged at 4°C for 90 min at 40,000 x g in a SW-55 Ti rotor. The cell extract (5.3 ml) was applied to a DEAE-cellulose column and the above buffer was passed through the column until the absorbance at 280 nm was less than 0.1. The enzyme was then eluted with a linear gradient of KCL (0-.3 M) in a volume of 80 ml.

Mutagenicity Testing: The Ames Salmonella test for detecting carcinogens and mutagens was performed as previously described (1) in the presence and absence of mammalian liver S9 fraction. The strains used included TA 1535, TA 1538, TA 98 and TA 100. Sinefungin at levels from 2-2000 ug/ml was used in the test system and results were compared to a known mutagen aflatoxin (0-0.3 ug/ml).

Animals

Male golden hamsters (Mesocricetus auratus) weighing approximately 60 gm were obtained from Harlan Sprague Dawley (Madison, Wisconsin), housed in plastic hamster cages on double screened stainless steel racks, and given Rodent Blox (Wayne, Eatonton, GA) and tap water ad libitum. The hamsters were maintained in a climate controlled room at a temperature of 72°F in the presence of 12 hrs of light and 12 hrs of darkness each day.

Testing Procedure: Leishmania donovani

Each hamster was infected with amastigotes of L. donovani (WR378) obtained from heavily infected donor hamster spleens. Splenic suspensions were prepared by grinding the spleen in sterile saline in a Ten Broeck tissue grinder and diluting with saline so that 0.2 ml contained approximately 10×10^6 amastigotes. Each experimental hamster was infected via the intracardiac injection of 0.2 ml of the amastigote suspension.

Prior to initiation of treatment, initial body weights of hamsters were recorded to serve as a basis to determine weight changes during treatment and to determine the quantity of drug to be used. Treatment was initiated on Day 3 post infection and continued through Day 6. Control groups received either vehicle only or one of three dosage levels of the reference compound, Glucantime, twice daily via the intramuscular route. Sinefungin was administered at three dosage levels via the intramuscular route twice daily. Hamsters were observed during this period for such clinical signs of toxicity as death, nervous disorders, roughened hair coat, and sluggish activity.

One day following completion of treatment (Day 7), hamsters were weighed, killed with CO_2, livers removed and weighed, and liver

impressions made for enumeration of amastigotes. Subsequently, the total numbers of parasites per liver (29), percent weight change, and percent amastigote suppression were calculated for all experimental groups using an IBM-XT microcomputer.

Testing Procedure: Leishmania braziliensis panamensis

In preparation for infection with L. braziliensis panamensis (WR539) and weekly during the experiment, the hair was clipped on the dorsal tail head and a commercial dipilatory agent (Nair, Carter-Wallace, Inc., New York, NY) was applied to the area to remove the remaining hair. Each hamster was inoculated via the intradermal route with 15×10^6 promastigotes near the base of the tail using a 0.25 ml glass syringe equipped with a 30 gauge x 1/2 inch needle. Promastigotes were grown in Schneider's Drosophilia Media (Gibco, Grand Island, NY) supplemented with 10% fetal bovine serum (Gibson, Grand Island, NY), harvested by centrifugation, and diluted using Schneider's Drosophilia Medium without fetal bovine serum so that each 0.05 ml contained 15×10^6 parasites.

Treatment was begun on Day 19 post infection and continued through Day 22 being administered twice daily via the intramuscular route at the desired dosage levels. Hamsters were weighed prior to initiation of treatment for volume of dosage determination and to serve as a basis for the determination of weight change for indication of drug toxicity. Groups of hamsters receiving vehicle only or one of three dosage levels of Glucantime were included as controls. Final weights were recorded for each group of animals one day following completion of treatment (Day 23).

One week following completion of treatment (Day 29) lesions were measured using a quantitated template which determined the diameter of each lesion in square millimeters.

Mean lesion size, percent weight change, and percent suppression were calculated for all groups using an IBM-XT microcomputer.

RESULTS

Antileishmanial effect of sinefungin compaired to aphidicolin. The antileishmanial activity of sinefungin has been reported for a number of species, but not for Leishmania mexicana (23). Compared to previous reports, L. mexicana appears to be the most sensitive strain tested. Fig. 2 shows the sensitivity of this strain to sinefungin in the medium of Steiger & Steiger. In our growth experiments the average concentration of sinefungin producing 50% inhibition was 5 nM. We compared this activity to a known DNA polymerase α inhibitor aphidicolin. As shown in Figure 3, 2 um aphidicolin produced 50% growth inhibition in L. mexicana.

Since SAM and SAH are structurally related to sinefungin these compounds were tested both for reversal of inhibition by sinefungin and tested for their growth effects on L. mexicana. It was found that SAH (0-100 uM) had no effect on the growth of the parasite and could not

Figure 2. Growth Inhibition of L. mexicana By Sinefungin

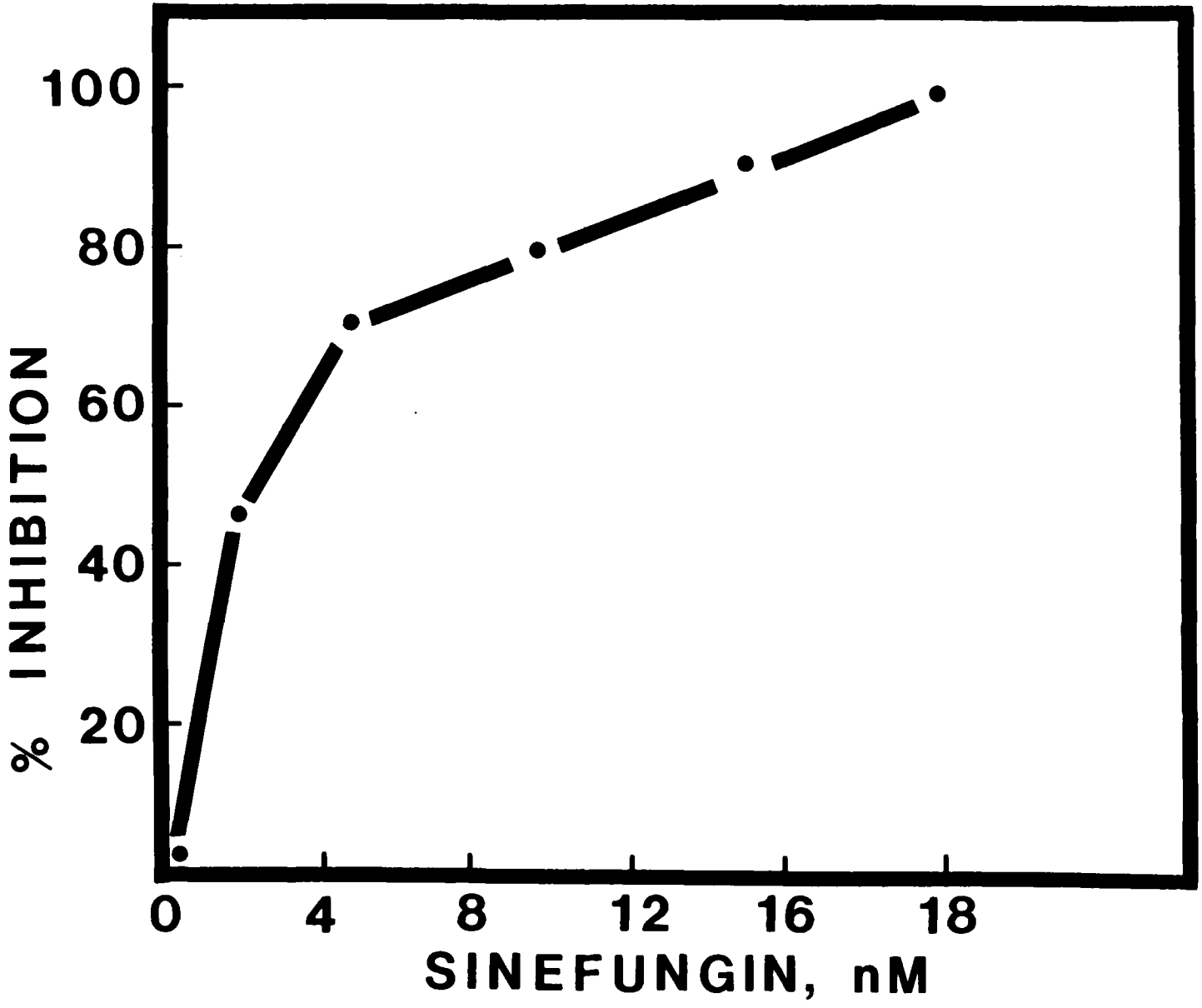
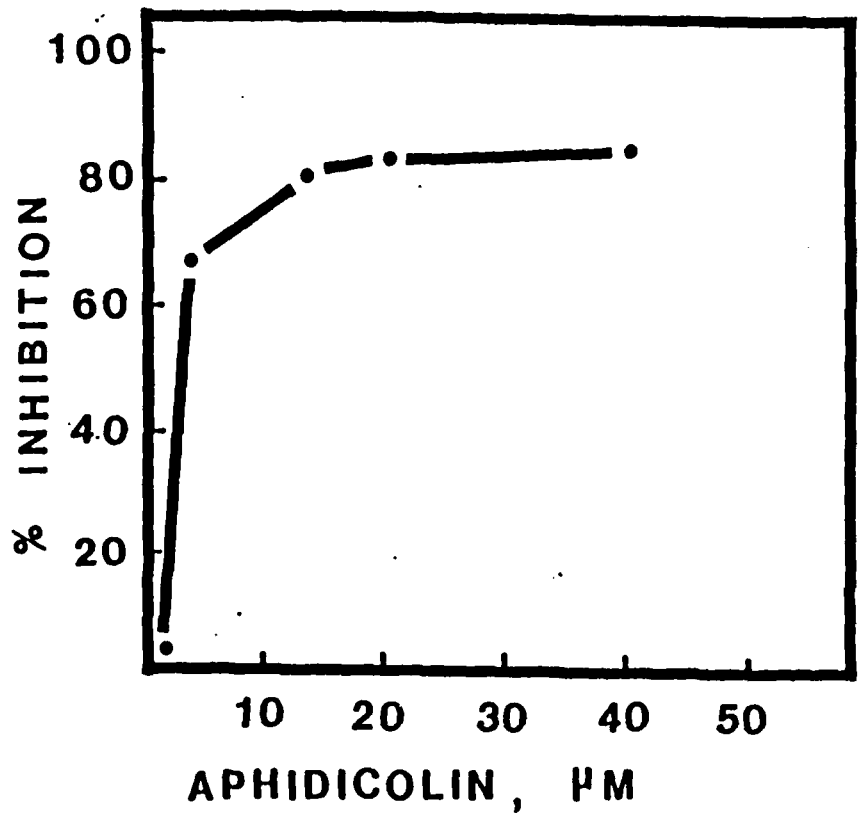


Figure 3. Growth Inhibition of L. mexicana by Aphidicolin



reverse growth inhibition by sinefungin. SAM (at 60 uM) was found to both stimulate growth of the parasite when added alone to the medium and was able to completely reverse the inhibitory action of sinefungin (at 5 nM). SAM is one of nature's most versatile molecules, serving as a methyl donor and precursor in the biosynthesis of polyamines which influence nucleic acid biosynthesis. Therefore, we tested the following compounds for reversal of growth inhibition by sinefungin: putrescine, spermine, spermidine, methionine, folic acid, thymidine, and adenosine. None of these compounds were able to reverse growth inhibition by sinefungin in L. mexicana.

The effect of sinefungin on S-adenosylmethionine synthetase. The results of our reversal experiments suggested that this enzyme was a possible target for the inhibitory action of sinefungin since SAM, the product of this reaction, could reverse inhibition but methionine, the precursor, could not. This enzyme catalyzes the following reaction L-methionine + ATP ---> S-adenosyl-methionine + PP_i + P_i. This enzyme also appeared to be a logical target since its inhibition would inhibit DNA synthesis. This is because SAM, the product, is necessary for the methylation of DNA.

The isolation procedures described eliminated 99% of the starting protein and resulted in a 430 fold increase in activity of the enzyme (data not shown). Assay for DNA polymerase by procedures described showed that polymerase activity co-eluted with S-adenosylmethionine synthetase. Further chromatography of SAM synthetase (data not shown) on Phenyl-sepharose, CL-6B sepharose and Sephadex G-150, resulted in an 80% reduction in activity of the enzyme and DNA polymerase activity no longer eluted with this enzyme. It was found that sinefungin was slightly inhibitory (14%) at concentrations up to 1 mM, depending on temperature, ATP concentration and the addition of the product of the reaction, SAM.

Effect of sinefungin on DNA polymerase activity utilizing the spectrophotometric assay. Using a coupled assay system as described by Sigma technical bulletin 7275, we first determined that sinefungin at the levels being used had no effect on the coupling assay system. Since sinefungin most closely resembles dATP compared to the other deoxynucleotide triphosphates we varied its concentration in the presence of 0.5 uM sinefungin. As shown in Fig. 4 using this assay system dATP had a Km of 55.5 uM and sinefungin inhibited the enzyme with a Ki of 15 nM. SAM and SAH (up to 40 uM), which are structurally related to sinefungin, showed no inhibition. Complete reversal of inhibition by sinefungin was obtained by varying dATP concentrations (Fig. 5). To ensure that we were indeed observing DNA polymerase activity a number of known DNA polymerase inhibitors were tested in this assay system. N-ethylmaleimide, KCl, ethidium bromide, berenil and aphidicolin were found to be inhibitory. Aphidicolin at 20 uM inhibited the enzyme activity by 50% which correlates reasonably well with its in vivo antileishmanial activity (Fig. 3). Since it is well documented that N-ethylmaleimide and aphidicolin are specific DNA polymerase inhibitors, and since we were able to inhibit activity completely with higher concentrations of these compounds, it was assumed that it was DNA polymerase α that was being assayed. We were able to

Fig. 4. A. Inhibition of DNA Polymerase

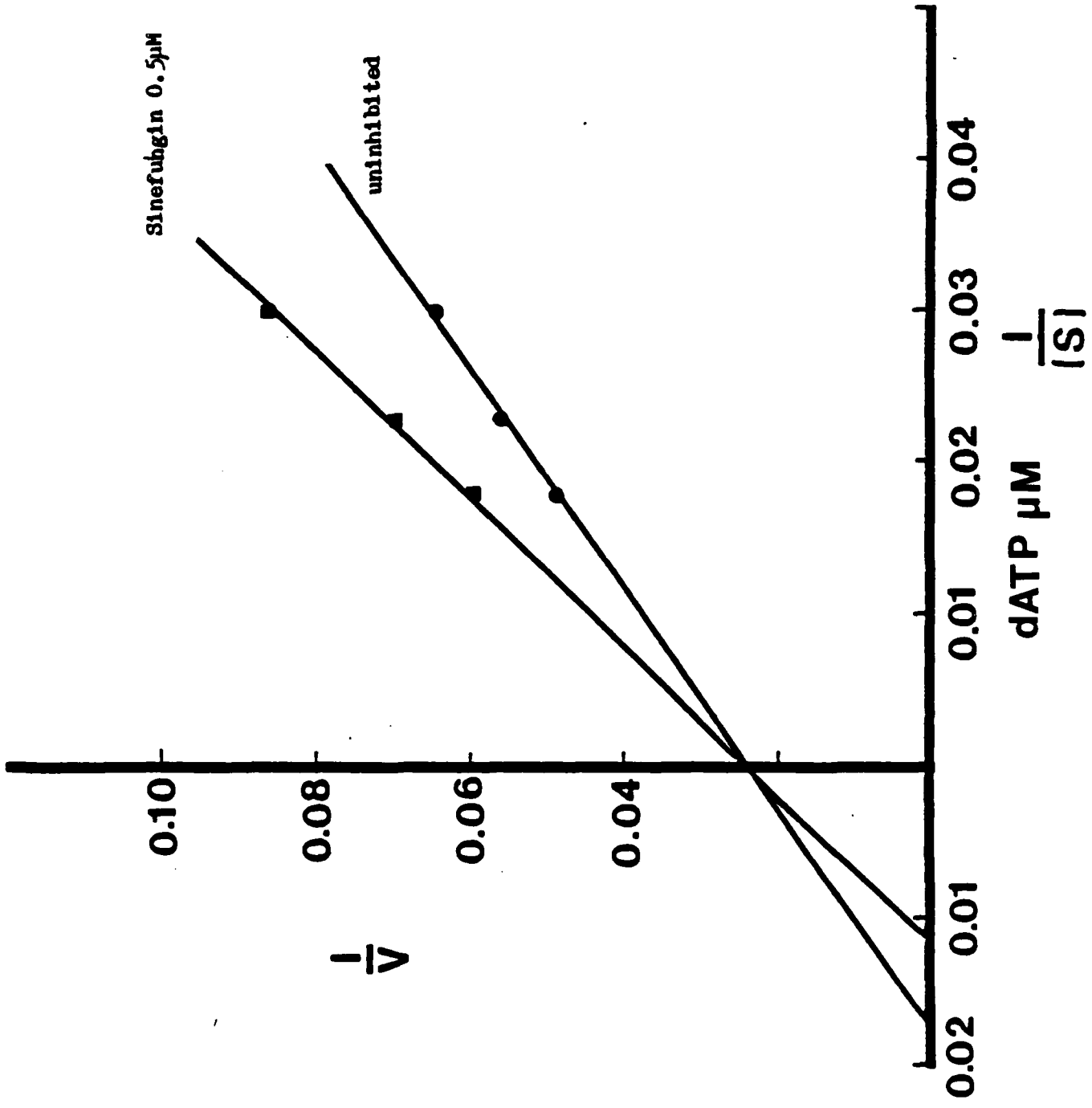


Fig. 4. B. Dixon Plot of Increasing Concentrations of Sinefungin in the Presence of dATP.

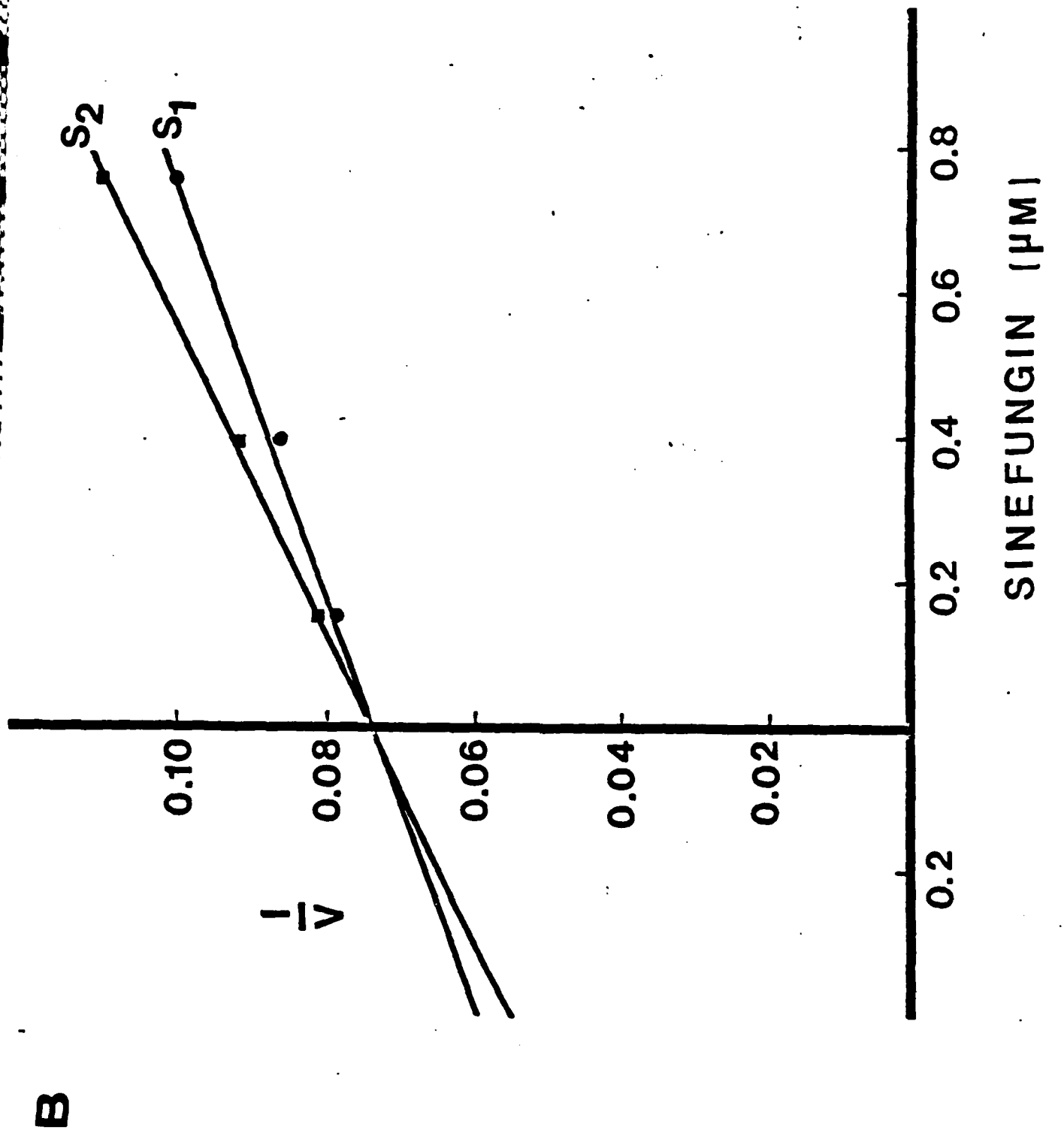
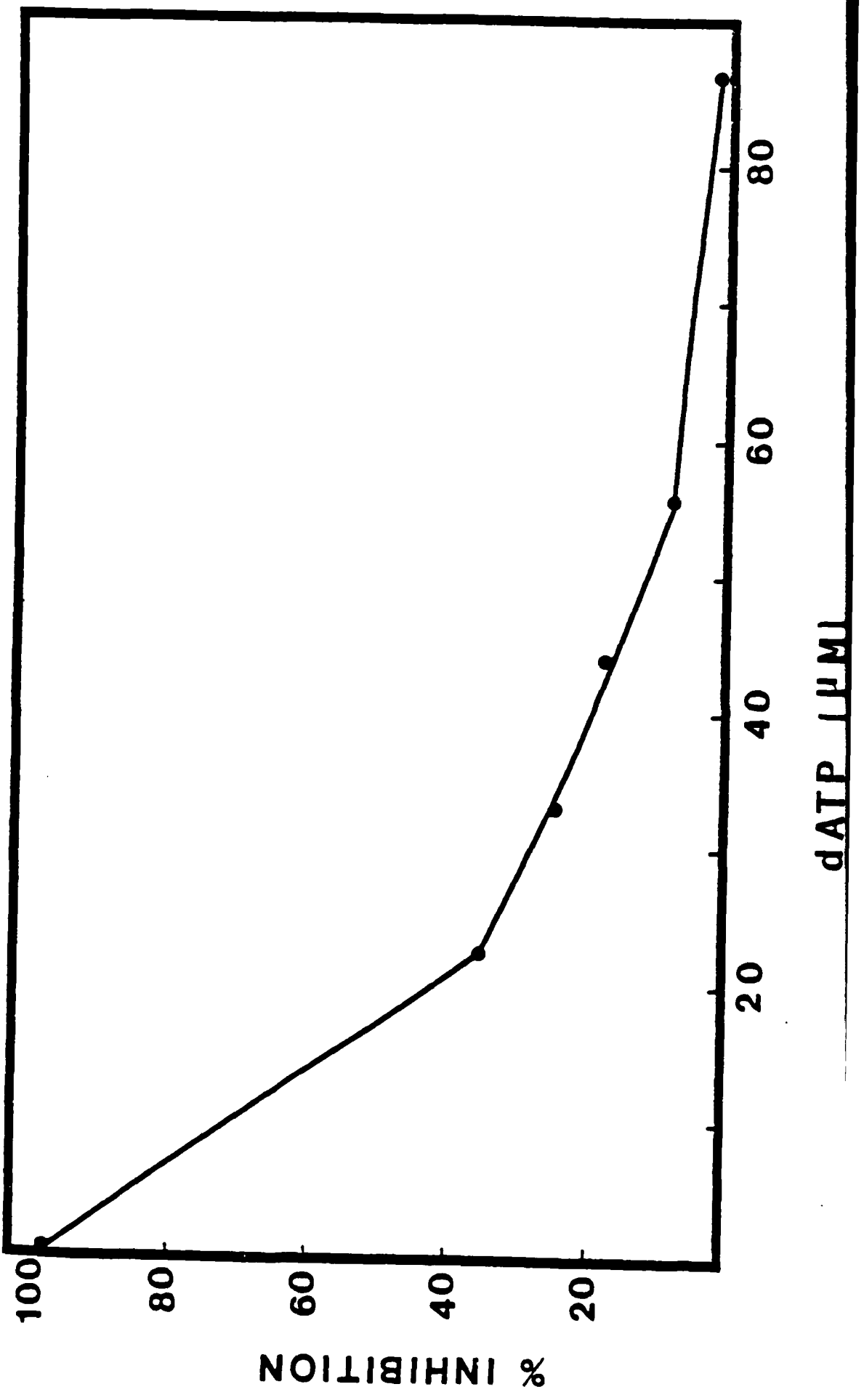


Fig. 5.. Reversal of DNA Polymerase Inhibition by dATP.



detect DNA polymerase activity using this assay until the enzyme was purified further using denatured DNA cellulose (Table 1). After this point in purification, we could not detect any inhibition by either sinefungin or aphidicolin using concentrations up to 200 uM. Fearing that perhaps the assay system was at fault, we decided to utilize a radioactive assay system used by most investigators in the field.

Effect of sinefungin and aphidicolin on L. mexicana DNA polymerase utilizing a radioactive assay. Table 1 shows the purification scheme obtained for the isolation of DNA polymerase with the activity quantitated by use of [^{3}H]TTP. Much to our surprise, at all steps we could not detect any inhibition of activity by sinefungin or aphidicolin. Neither could we detect inhibition by sinefungin utilizing [^{3}H]dATP as the isotope in the assay. Other investigators utilizing this assay technique have reported similar results using aphidicolin as a tool to detect DNA polymerase in trypanosomes (28) and in related protozoan Crithidia fasciculata (15). These findings have produced a great amount of interest because aphidicolin was demonstrated to be a specific direct inhibitor of animal DNA polymerase α but is without effect on polymerases β or γ (16). Using aphidicolin as a tool, it has been shown that DNA polymerase is clearly the principal polymerase required for DNA replication in all animals and plants studied (14).

Effect of sinefungin in the Salmonella/Mammalian Mutagenicity Test. Sinefungin drastically inhibits DNA synthesis (3). We found that growth inhibition can be reversed with SAM only when added to the medium at the same time as sinefungin. If SAM is added hrs later after the addition of sinefungin, complete reversal does not occur. These results indicated that sinefungin may be interferring with the methylation of DNA, a function which has been implicated in regulating gene expression (5,8). To determine if sinefungin might be a possible mutagen the Ames test was performed using the strains described in Methods in the presence and absence of mammalian S-9 liver fraction. The latter is required for the activation of several known mutagens and carcinogens (1). Aflatoxin produced 600-2000 revertants. In the presence of sinefungin the number of revertants was lower than the number of spontaneous revertants (data not shown).

Antileishmanial effect of sinefungin in hamsters. Sinefungin significantly suppressed growth of both L. donovani (Table 2) and L. b. panamensis (Table 3) in hamsters. A comparison of the suppressive activity of sinefungin with that of glucantime against L. donovani indicates that sinefungin was approximately 4 times more effective than the reference compound. The ED_{50} of sinefungin was approximately 4 mg/kg body wt as compared to 16 mg Sb/kg body wt for glucantime. The activity of sinefungin was approximately 30 fold greater against L. b. panamensis than was glucantime. The ED_{50} for sinefungin was approximately 22 mg/kg body wt while that of glucantime was 660 mg Sb/kg body wt.

Table 1.
Purification Scheme for DNA Polymerase

Purification Step	Total Volume (ml)	Total Protein (mg)	Units* $(ml)^{-1}$	Total Activity (units)	Specific Activity (units mg^{-1})	Purification (-fold)
Lysed cells	40.8	1448.4	6.70	273.18	0.189	
Crude extract (15,000 rpm, 1 hr)	38.5	1068.38	7.7	296.45	0.277	1.47
Protamine Sulfate Supernatant	37.5	441.0	15.29	573.36	1.3	6.88
Heparin-Sepharose	14.5	41.62	119.77	1736.67	41.73	220.79
Cellulose Phosphate	10.2	4.38	26.58	271.14	61.82	327
DNA Cellulose	3.1	0.108	29.08	90.15	834.72	4416.51

*One unit of activity is defined as the incorporation of 1 pmole of dTMP into DNA in 30 min under standard assay conditions. Assay conditions are described in Methods.

Table 2 Comparison of the supressive activity of Glucantime and Sinefungin against Leishmania donovani in the golden hamster.

Compound	Total Mg/Kg*	No. Animals	No. Deaths	% Wt. Change	Mean No. Parasites Per Liver (X 10^6)	Percent Suppression
Vehicle Control	-	6	0	-10	525	-
Glucantime	416	6	0	-12	1	100
	52	6	0	-10	131	75
	13	6	0	- 7	278	47
Sinefungin	104	6	0	-10	31	94
	52	6	0	-10	77	85
	6.5	6	0	- 6	206	61

*Dosage levels of Glucantime based on percent antimony. That of Sinefungin based on total weight of the compound.

Sinefungin is approximately 4 times more potent than Glucantime at ED_{50}.

Table 3 Comparison of the suppressive activity of Glucantime and Sinefungin on Leishmania braziliensis panamensis in the golden hamster.

Compound	Total Mg/Kg*	No. Animals	No. Deaths	% Wt. Change	Mean Lesion Area (mm^2)	Percent Suppression
Vehicle Control	-	6	0	- 6.8	121	-
Glucantime	4128	6	0	3.8	17	86
	2064	6	0	- 2.1	26	79
	832	6	0	- 2.2	44	63
Sinefungin	208	6	0	-10.6	42	66
	104	6	0	- 9.9	26	79
	26	6	0	- 7.0	50	59

*Dosage levels of Glucantime based on percent antimony. That of Sinefungin based on total weight of the compound.

Sinefungin is approximately 30 times more potent than Glucantime at ED_{50}.

Although treatment of hamsters with sinefungin did not result in significant loss of body weight, the hair coats of hamsters receiving this compound appeared slightly roughened suggesting some toxicity. Additional work would be required in order to draw valid conclusions regarding toxicity of sinefungin.

DISCUSSION

In our particular strain of L. mexicana sinefungin has been shown to be one of the most antileishmanial compounds tested in our in vivo promastigote test system. Utilizing an in vivo hamster test system, sinefungin was shown to be 4-5 times more effective against L. donovani and 12-13 times more effective against L. braziliensis panamanensis than glucantime. When compared to all compounds tested utilizing the hamster test system, sinefungin was the most effective compound against L. braziliensis panamanensis.

In vivo, sinefungin has been reported to cure mice infected with various Trypanosoma species, with no toxic side effects (6). Also, no toxic side effects were observed in mice treated with L. donovani and L. tropica, and the potency of sinefungin relative to sodium stibogluconate, a known antileishmanial drug, was reported to be 73 times greater (22). Although sinefungin has been shown to inhibit DNA synthesis in leishmania (3), we have shown that it is not mutagenic in the Ames test. Nearly 90% of all carcinogens tested utilizing this method have been found to be mutagenic (1).

The mode of action of sinefungin appears to involve inhibition of DNA replication. The replication of DNA is enzymatically complex and requires many activities in addition to the DNA polymerase. A difficulty in analyzing the role of eucaryotic DNA polymerases is caused by the lack of in vitro systems containing all the components needed for replication. Biochemical characterization of eucaryotic DNA replication is still in its infancy.

The precursor for DNA synthesis is a nucleoside triphosphate, which loses two phosphate groups in the reaction. It appears from the results presented here that sinefungin and aphidicolin interfere with a pyrophosphatase activity which is associated with the DNA polymerase at certain stages of purification. This pyrophosphatase activity is inhibited by both aphidicolin and sinefungin using the spectrophotometric assay. It is interesting to note that when this activity is lost during purification; a dramatic increase in the specific activity is observed utilizing the [^{3}H]TMP incorporation assay (Table 1, 13-fold increase). Because of the complexity of the reaction catalyzed by DNA polymerase, it is impossible at this time to estimate the exact relevance of our findings. However, the results presented here and in previously published reports on the nature of DNA polymerase α in parasitic protozoa are compatible with the following interpretation. Solari et al. (28) and Holmes et al. (15) reported the isolation of a DNA polymerase in parasitic protozoa which was resistant to inhibition by aphidicolin, yet

the growth of the parasites were strongly inhibited by aphidicolin. Solari et al. reported that DNA synthesis in vivo was inhibited by aphidicolin. These investigators found the isolated DNA polymerase -like failed to cross-react with mammalian DNA polymerase α from different species (15,24). These investigators have suggested that the DNA polymerase α of these parasites are immunologically distinct from host enzyme, and that the structural differences between the parasite and host enzymes could be exploited for the development of agents to combat parasitic diseases. Our results suggest that in the case of L. mexicana that an aphidicolin-sensitive activity is associated with DNA polymerase activity, and that this activity is lost early in purification. The enzymatic activity associated with aphidicolin inhibition fails to be detected utilizing the commonly-used radioactive assays for DNA polymerase activity. Sinefungin also appears to inhibit this pyrophosphatase activity. The reported in vitro studies showing that sinefungin drastically inhibits DNA synthesis (3) suggests that this enzyme is crucial for in vivo DNA replication. It is interesting to note that S-adenosylmethionine (SAM) synthetase has tripolyphosphatase activity, and in our early purification steps, this enzyme co-eluted with DNA polymerase. The observation that only SAM could reverse the in vivo growth inhibition of sinefungin suggests that perhaps SAM synthetase is somehow involved in regulating DNA synthesis via methylation of the DNA. Since neither aphidicolin or sinefungin inhibited partially purified SAM synthetase, the possibility exists that these compounds inhibit DNA replication only when SAM synthetase and DNA polymerase are together.The observation that dATP was able to reverse inhibition by sinefungin in the in vitro DNA polymerase spectrophotometric assay suggests that this compound, which is sterically similar to sinefungin, prevented the inhibitor from binding at the appropriate site. Future studies will be directed on the purification and characterization of this sinefungin- and aphidicolin-sensitive pyrophosphatase activity and studies will be conducted on the elucidation of this activity on DNA replication.

The data presented substantiates the report that DNA replication is inhibited by sinefungin in leishmanial parasites (3) and the site of action appears to be unique in these lower eucaryotes.

Since the DNA polymerase of the Trypanosomatidae has been shown to be biochemically and immunologically distinct from its mammalian counterpart, it is a rational target for chemotherapy. Sinefungin has been shown to be antiparasitic at levels not toxic to mammalian cells and its major mode of action appears to interfere with DNA replication. Sinefungin appears to have potential as an antiparasitic drug, and further elucidation of its mode of action will provide a framework for the synthesis of an ever more selective drug.

DNA POLYMERASE AND RNA POLYMERASE STUDIES

Research has centered on (1) further isolation and characterization of the DNA polymerases of Leishmania mexicana WR 227 (2) and the incorporation of [H^3]-Formycin B into the RNA's of this organism. DNA

polymerase of leishmania is being studied because it has many different characteristics from mammalian DNA polymerase and provides a rational chemotherapeutic target. The mode of action of Formycin B is being studied because it is a potent leishmanicide, and its exact leishmanicidal action has not been fully elucidated.

To ascertain if the leishmanial DNA polymerase is similar to that which was partially isolated from Trypanosoma brucei, we followed the isolation procedures of Marcus et al.(20) Our results to date suggest that the leishmanial DNA polymerase is similar to that reported for T. brucei. A single peak of DNA polymerase activity from extracts of L. mexicana was obtained by DEAE-cellulose and phosphocellulose ion-exchange chromatography. This was resolved into two peaks differing in KCL concentration utilizing a DNA-agarose column. Both peaks resolved on DNA agarose have similar characteristics (so far tested) and appear to be -like as evidenced by their ability to be inhibited by N-ethylmaleimide. Experiments are now in progress to test template requirements, effect of polyamines and nucleotide analogues.

INHIBITION STUDIES

DNA polymerase was isolated as depicted in Table 1. Various levels of glucantine (0-3 mg/ml) were tested against the enzyme (Fig. 6). As shown in this figure, glucantine at 3.75 mg/ml (9.86mM) gave 50% inhibition of the enzyme in a typical assay. However, when the nucleotide concentration or activated DNA varied, the enzyme inhibition by glucantine changed. Fig. 7 shows that varying the nucleotide concentration from 12.5uM - 50uM in the assay could change the inhibition by glucantine by as much as 28%. Fig. 8 shows that varying the amount of activated DNA which serves as a template in the assay also influenced inhibition by glucantine. In Fig. 8 activated DNA varied from 10-50ug/ml and the greatest inhibition occurred using 10ug/ml activated DNA. Using the same level of glucantine but with varying concentrations of DNA, the % inhibition varied by as much as 36%.

These studies suggest that glucantine acts indirectly on the DNA polymerase by interacting with the template and nucleotide concentration. Varying the enzyme concentration in these experiments had an insignificant effect (not shown). We are currently trying to relate our findings to an in vivo cellular situation. Our present assay contains high levels of DNA and nucleotides, which would require high levels of glucantine for inhibition. Further experiments will be done reducing the nucleotide levels and template DNA.

Sangivamycin - Inhibition of DNA Polymerase

Sangivamycin, which is a natural nucleoside isolated from Streptomyces, has been one of the few natural nucleosides exhibiting antileukemic activity that has been chosen for clinical trials. We have shown that it inhibits the DNA polymerase of L. mexicana 227. Fig. 9 shows that 45uM inhibits the DNA polymerase 50%. As shown in this report, Sangivamycin also inhibits S-adenosylmethionine synthetase in the uM range, and we have shown previously that it is a potent growth inhibitor of promastigotes.

Figure 6. **DNA POLYMERASE INHIBITION BY GLUCANTINE**

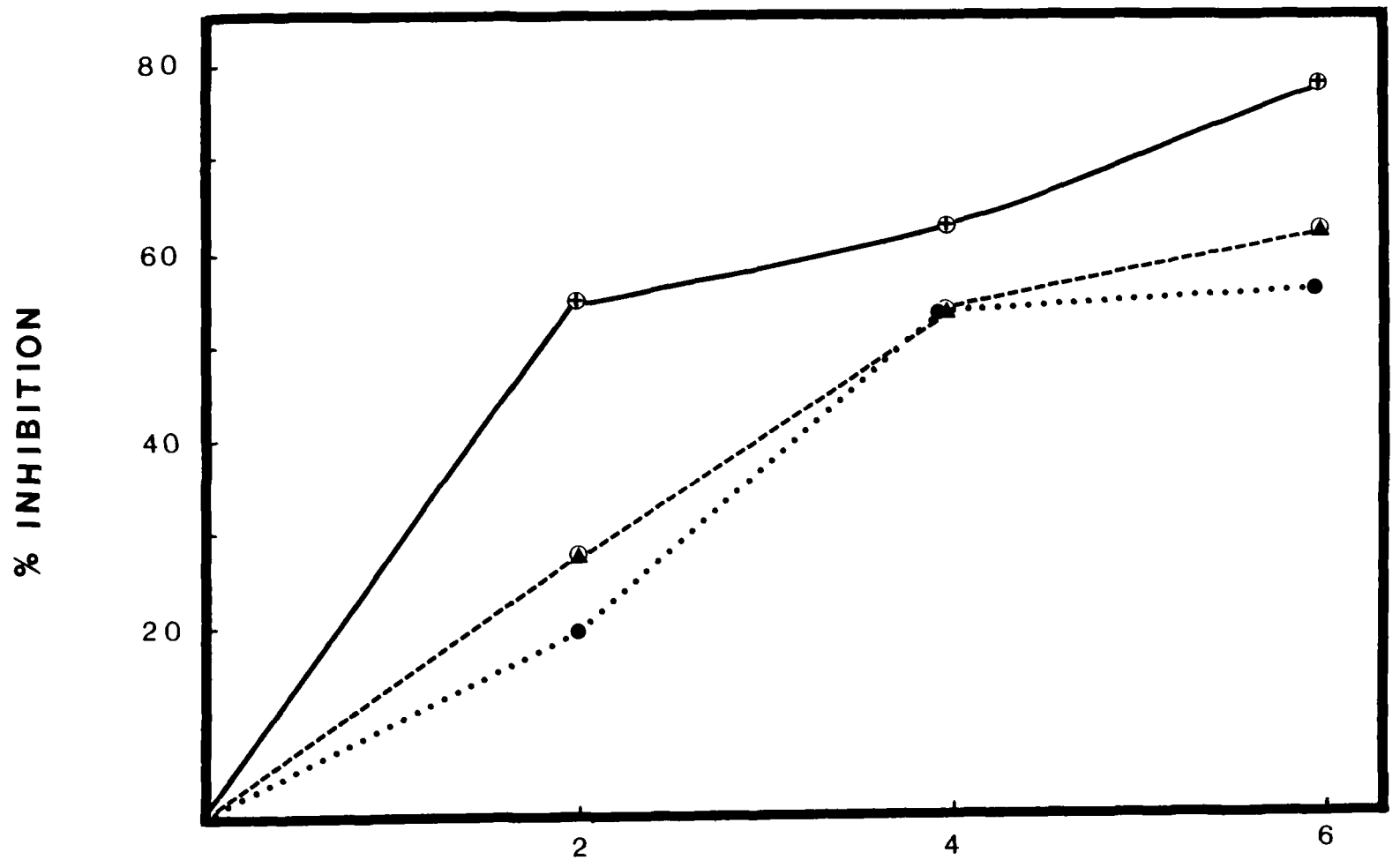


Figure 7. DNA in the Presence of Varying Concentrations of dNTP.

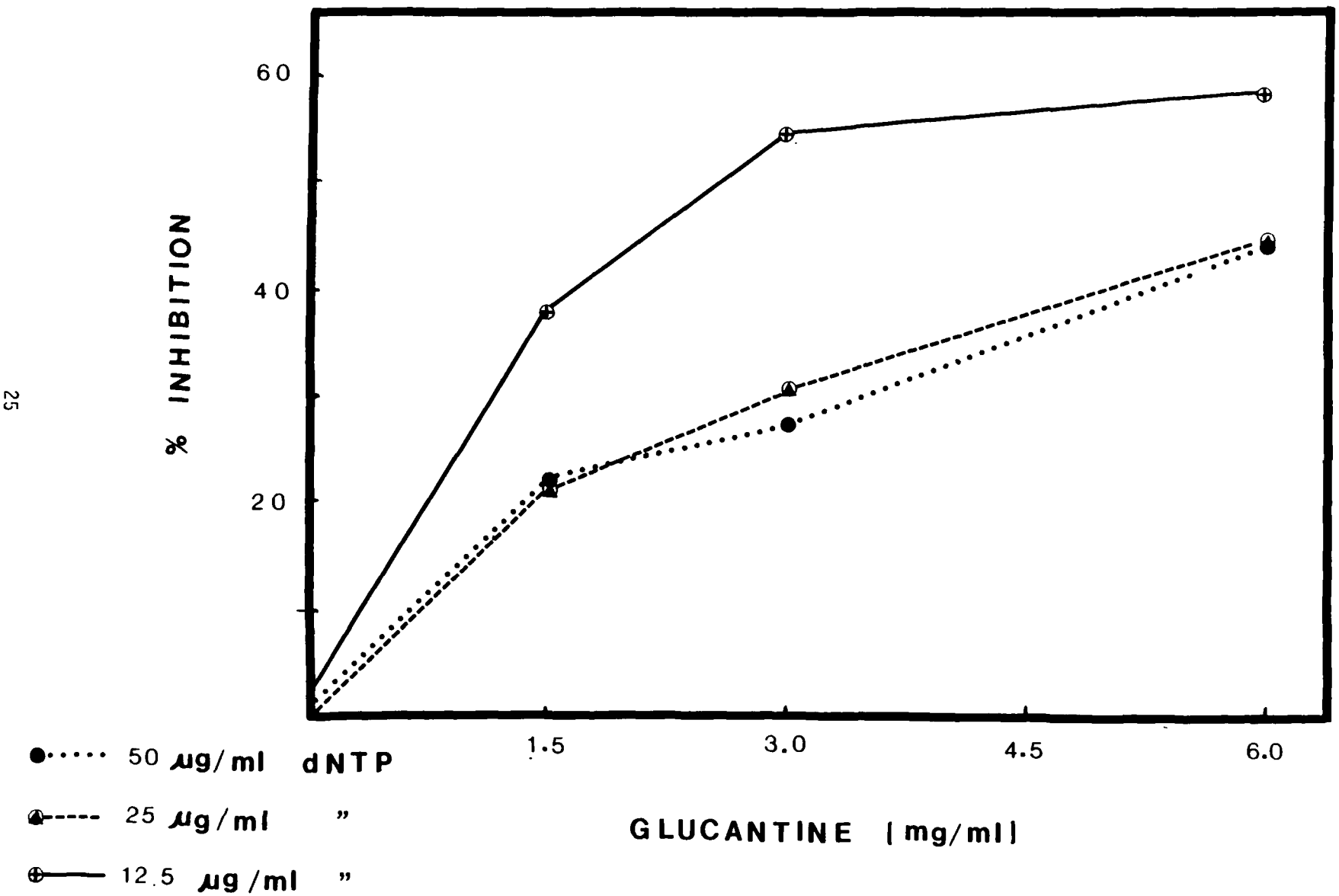


Figure 8. DNA Polymerase Inhibition by Glucantine in the Presence of Varying Concentrations of Activated DNA.

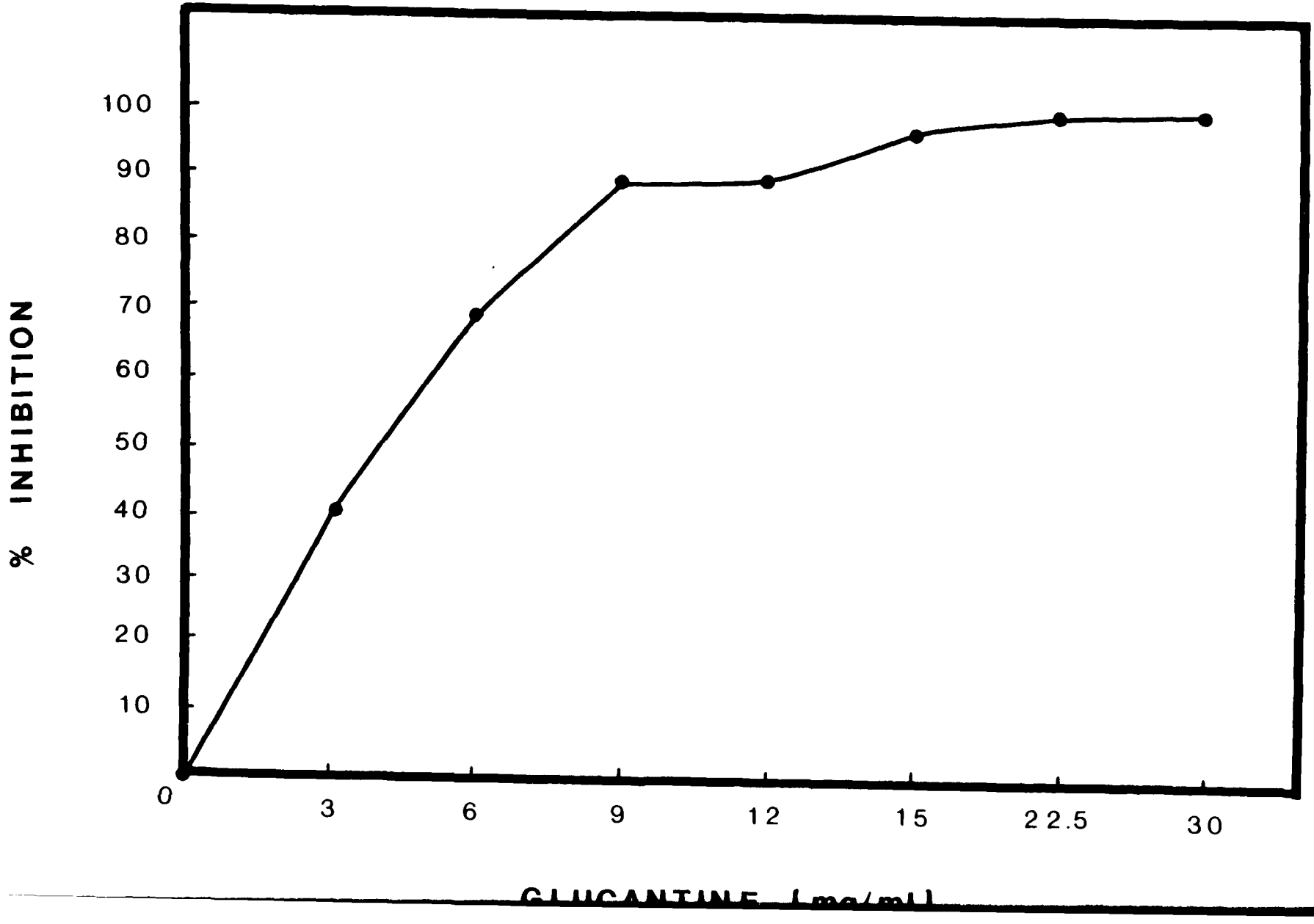
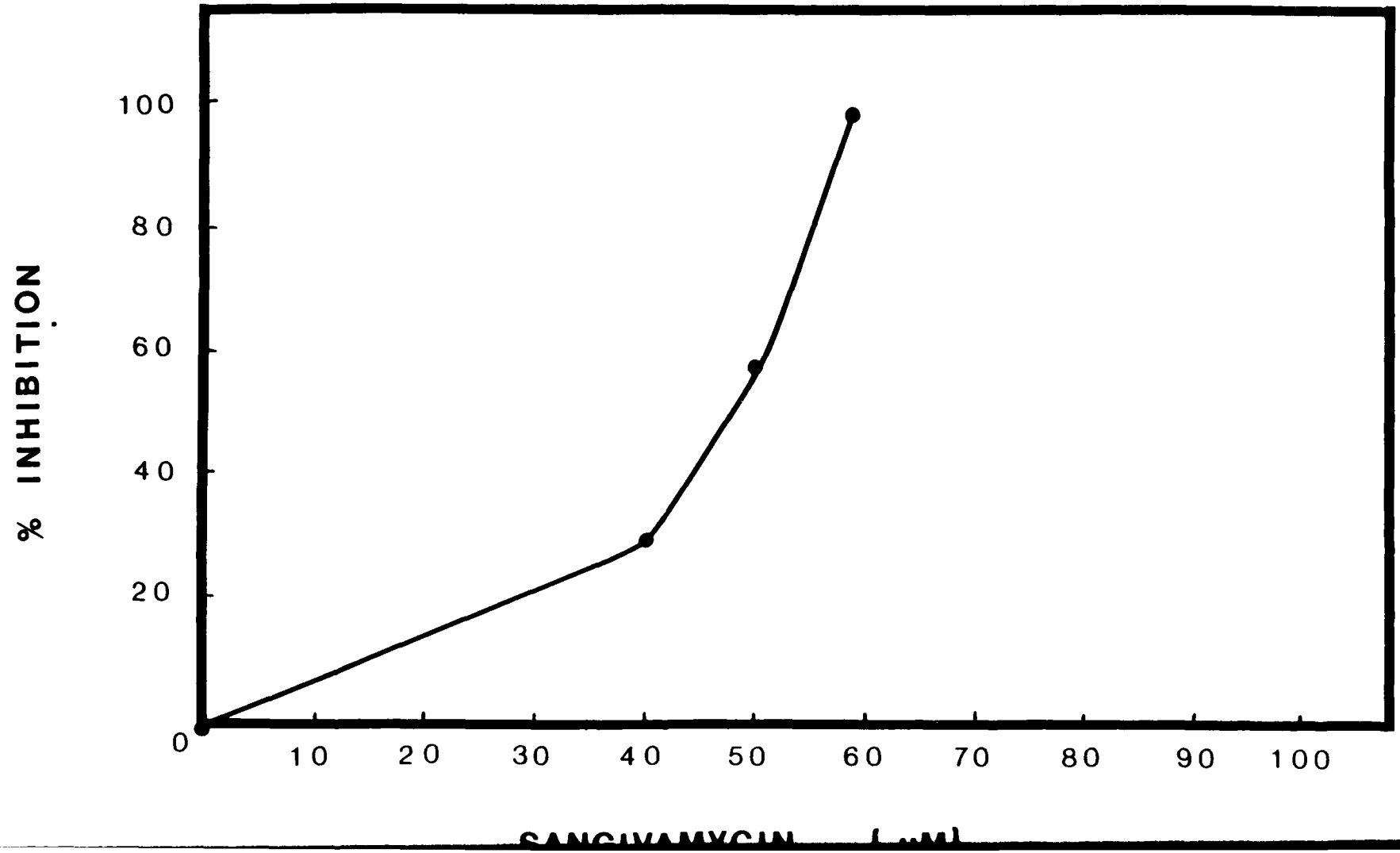


Figure 9.

DNA POLYMERASE INHIBITION BY SANGIVAMYCIN



We are presently trying to do enzyme kinetics with this compound, and are collaborating with a group at the University of Massachusetts Medical School who have agreed to test this compound on human DNA polymerase.

INCORPORATION OF FORMYCIN B AND ITS METABOLITES INTO THE RNAs OF LEISHMANIA MEXICANA

In experiments to elucidate the mechanism of action of Formycin B (FoB), we isolated total tRNA and mRNA by a sucrose gradient (15%-30%) and demonstrated that [H^3]-FoB metabolites were incorporated into all RNA species.

In a previous publication, Biochemistry International 9(2), 207-218, we demonstrated for the first time that RNA of leishmanial cells exposed to Formycin B was defective in its ability to function in the translational process. We now have isolated mRNA, rRNA and tRNA from [H^3]-Formycin B exposed cells and have demonstrated for the first time that it is converted to Formycin A and preferentially incorporated into the mRNA as opposed to the other RNAs. This data substantiates the hypothesis that <u>one</u> of the targets of Formycin B toxicity in leishmania is via defective mRNA translational capabilities. Substantial amounts of Formycin B as well as Formycin A were found in the mRNA and rRNA. Our washing and isolation procedures were quite extensive, and the possibility exists that Formycin B nucleotides are incorporated into these RNAs. If the incorporation of Formycin B nucleotides takes place and alters RNA function, this would help explain Dr. Buddy Ullman's mutant data suggesting that Formycin B compounds are toxic (17).

In brief, <u>L. mexicana</u> promastigotes were exposed to [H^3] Formycin B, 8 um for 5 hrs., harvested, washed and lysed. mRNA, tRNA and rRNA were isolated as described in Methods in Enzymology, vol. XII, pp. 581-596. The RNAs were digested to the nucleotides by the method of Randerath and Gubta (26) and analyzed by HPLC by the method of Hartwick et al. (13), Figs. 10 and 11 show the results of this analysis. The major peak eluted corresponds to Formycin A (FoA) and the minor peak Formycin B (FoB). Based on ^3H counts/ g RNA, 93% of the counts represented FoA in mRNA, 3.6% in tRNA and 3.3% in rRNA. Of the total counts found in mRNA, 16% were represented by FoB and 84% by FoA. In tRNA, 10% of the counts eluted with FoB, and 90% with FoA. For rRNA, 19% of total counts in rRNA eluted with FoB, and 81% with FoA. This data demonstrates that Formycin B and its metabolites are preferentially incorporated into mRNA.

ISOLATION AND CHARACTERIZATION OF S-ADENOSYLMETHIONINE SYNTHETASE

As previously discussed, since the structure of sinefungin is similar to that of S-adenosyl-homocysteine (SAH) and that of S-adenosyl-methionine (SAM), the possibility existed that sinefungin may be acting as an inhibitor in enzymatic reactions in which these compounds are involved. Our previous data suggested that perhaps S-adenosyl-methionine synthetase

Fig. 10. Incorporation of Formycin B into mRNA.

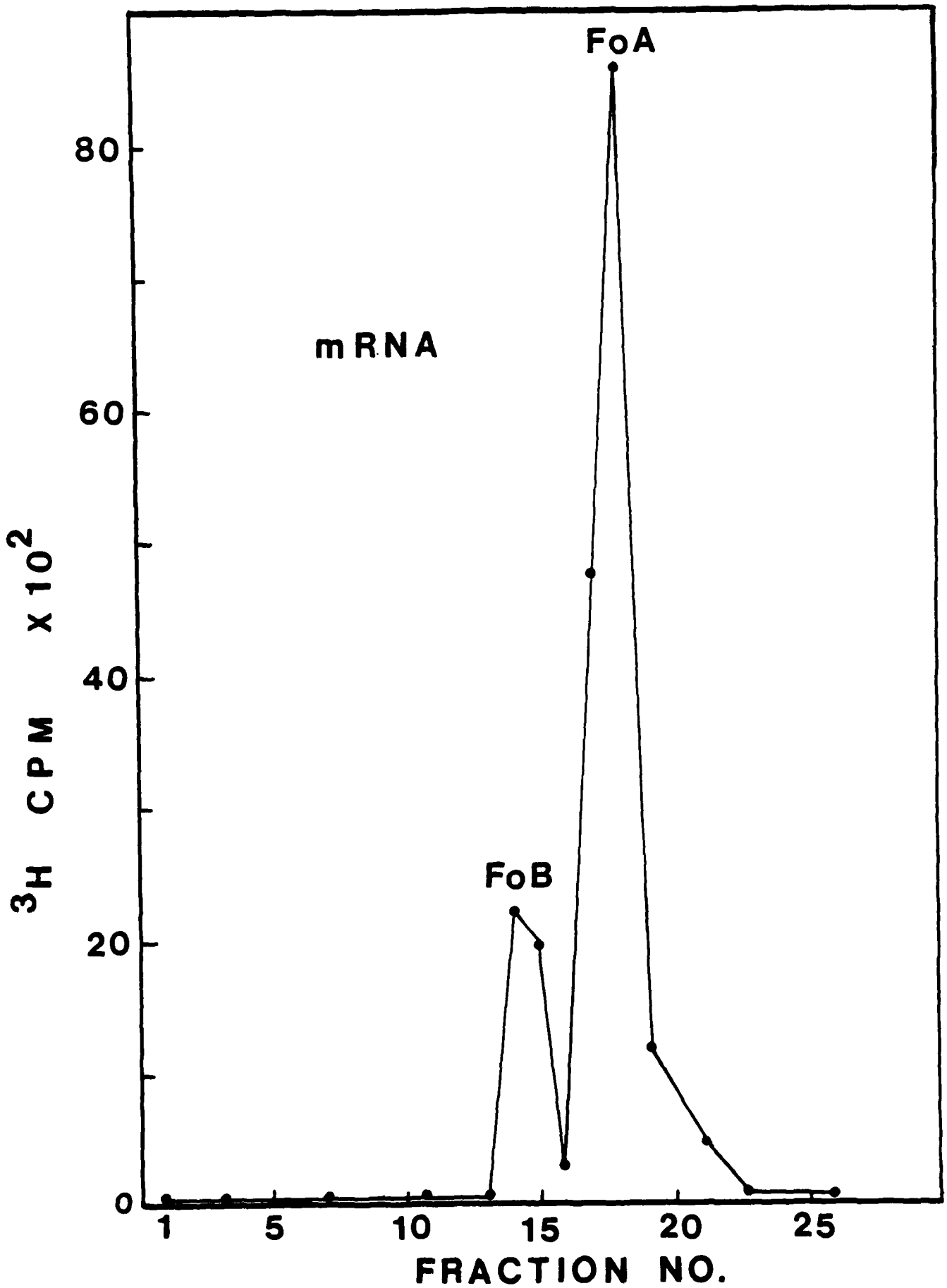


Fig. 11. Incorporation of Formycin B into tRNA and rRNA.

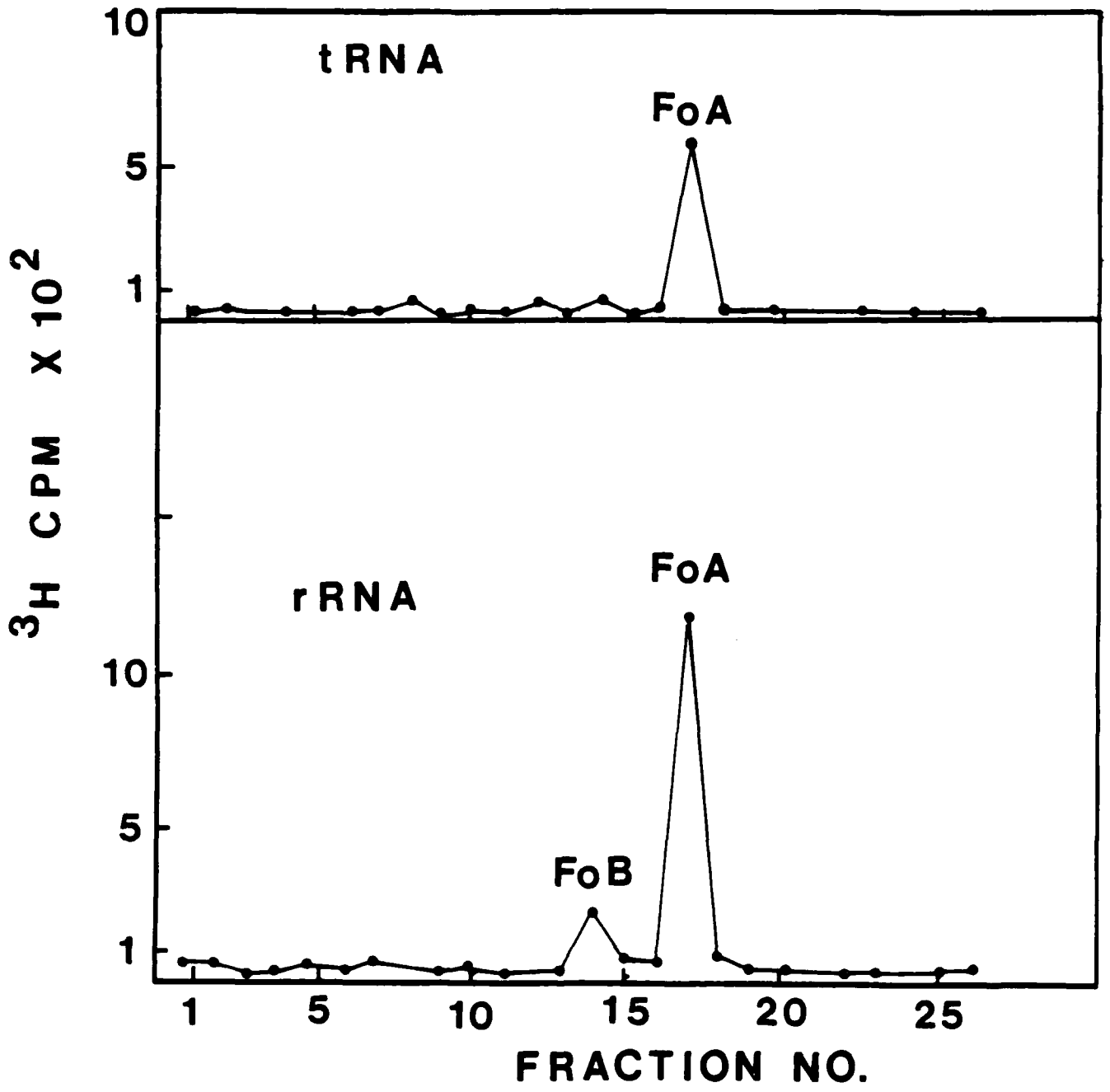
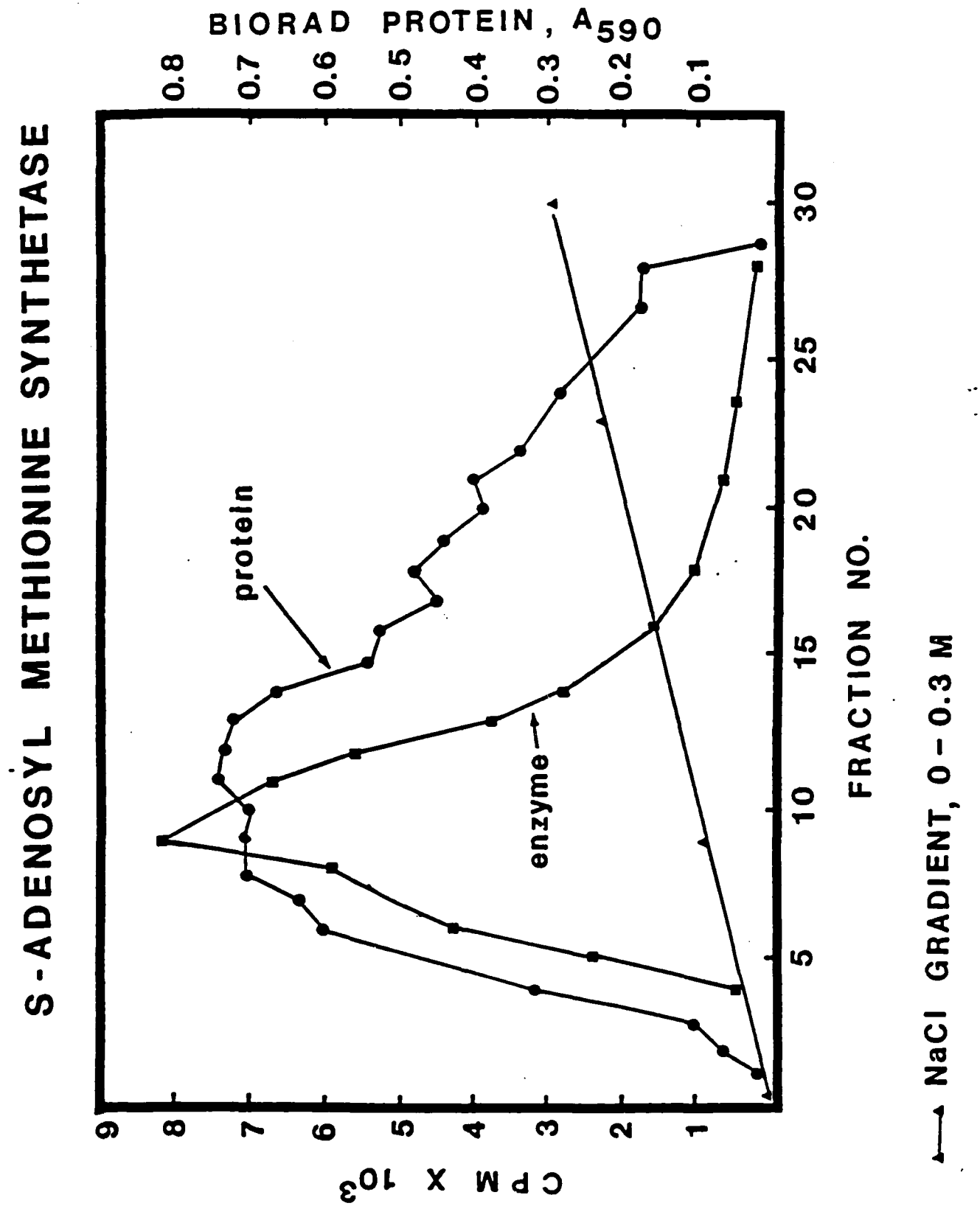


Figure 12. Chromatography of S-Adenosylmethionine on DEAE Cellulose



was a target enzyme for inhibition by sinefungin. This enzyme catalyzes the following reaction L-methionine + ATP ---> S-adenosyl-methionine + PPi + Pi. In mammalian cells there are three separate enzymes catalyzing this reaction and SAH is a feedback inhibitor of the reaction. Since we found that SAM can reverse the inhibition by sinefungin in L. mexicana 227 promastigotes, but methionine cannot, this enzyme seemed a logical target to investigate. Also, we had previously found that DNA synthesis in Leishmania was severely inhibited by sinefungin. Inhibition of S-adenosyl-methionine synthetase could account for the inhibition of DNA synthesis because SAM the product is necessary for the methylation of DNA.

Initially using the method of Hoffman and Kunz (14), we optimized our enzyme assay for L. Mexicana 227 promastigotes. Methionine adenosyltransferase activity was measured at 35°C in 100 ul of a standard assay mixture containing the following components: 150 mM KCL_{14} 20 mM $MgSO_4$, 5 mM dithiothreitol, 50 mM Tris pH 7.5, 5 mM ATP, and 10 uM L-[^{14}C]-methionine. The cationic [^{14}C]-adenosylmethionine formed was isolated by spotting 80 ul portions of reaction mixtures on 2.3 cm discs of Whatman P81 cellulose phosphate cation-exchange paper, removing unreacted methionine by collecting and washing discs three times in a beaker of cold 0.1 M ammonium formate, pH 3.0, followed once with 95% ethanol, and once with ether. [^{14}C]-Adenosylmethionine was quantified by liquid scintillation counting of dried discs under 5 ml Fisher Scint Verse.

S-Adenosylmethionine synthetase was isolated by suspending 8 g of pelletted L. mexicana 227 cells in buffer containing 50 mM Tris pH 7.5, 10 mM $MgSO_4$, 1 mM EDTA, and 1 mM DTT. The cells were sonicated 3x for 15 seconds, and the cell suspension was centrifuged at 4°C for 90 min at 31,000 rpm in a SW-55 T rotor. The cell extract (5.3 ml) was applied to a DEAE-cellulose column and the above buffer was passed through the column until the absorbance at 280 nm was below 0.1. The enzyme was then eluted with a linear gradient of KCL (0-.3 M) in a volume of 80 ml. As Figure 12 shows, only one form of S-adenosylmethionine synthetase was eluted. Characterization of this enzyme showed that KCL and DTT were absolutely necessary. Optimal activity of the enzyme required 150 mM KCL and 5 mM ATP_3 The enzyme activity was completely destroyed by the addition of 10^{-3} M N-ethylmaleimide and p-chloromercuriphenyl sulfonic acid, and the absence of DTT reduced the activity by 70%. This suggests that the enzyme has S-H groups necessary for activity of the enzyme.

The isolation procedures described eliminated 99% of the starting protein and resulted in a 430 fold increase in activity of the enzyme. Assay for DNA polymerase by procedures already reported, showed that polymerase activity co-eluted with S-adenosylmethionine synthetase.

Further chromatography on Phenylsepharose, CL-6B sepharose and Sephadex 150, resulted in a great reduction in activity of the enzyme. We are now investigating ways to stabilize this enzyme after purification.

We have begun studies on inhibition of this enzyme and have found that sinefungin (up to 1 mM) and glucantine (up to .04 g/ml) can inhibit this enzyme up to 46% depending on temperature, ATP concentration and the addition of the product of the reaction SAM. Our results on inhibition studies will be reported when more conclusive data is available.

INHIBITION STUDIES WITH S-ADENOSYLMETHIONINE SYNTHETASE

The following compounds were tested for inhibition against the enzyme. These compounds are either purine or amino acid analogs or are antiparasitic agents whose mode of action is unknown.

Compounds Tested Which Were Inhibitory

	Conc. Giving 50% Inhibition
Sangivanmycin	41 uM
Mercaptopurine riboside-5-phosphate	105 uM
S-adenosylhomocysteine	0.83mM
Diminazine Acetate	1.67mM
DFMO	2 mM
Cystosine B-D-arabinofuranoside	2.05mM
Formycin triphosphate	2.08mM
Cycloleucine	2.5 mM
Deaza SIBA	3.45mM
Tunicamycin	3.79mM
4-Aminoprazolopyrimidine	4.65mM
Allopurinol ribotide	4.17mM
Formycin B-monophosphate	6.78mM
N^6-cyclopentyladenosine	8.33mM
Cordycepin	10 mM
Glucantine	5 mg/ml ~ 13.16 mM

Compounds Tested at 0.5mM Showing No Inhibition

6-Methylmercaptopurine riboside

5'-o-trityladenosine

5`-Deoxy-5'methylthioadenosine

6-Methylmercaptopurine

6-Methylthio-2-hydroxypurine

Neoplanocin A

Neoplanocin D

Aristeromycin

Amphotercin

Isobutylaminoadenosine

5'-Deoxymethylthiadenosine

N^6-Methyl-2'-deoxyadenosine

5'-S-isobutyl-5'-deoxyadenosine

References

1. Ames, B.N., J. McCann and E. Yamasaki. 1975. Methods for detecting carcinogens and mutagens with the Salmonella/mammalian-microsome mutagenicity test. Mutation Res. 31:347-364.

2. Bachrach, U., L.F. Schnur, J. El-On, C.L. Greenblatt, E. Pearlman, M. Robert-Gero, and E. Lederer. 1980. Inhibitory activity of sinefungin and SIBA on the growth of promastigotes and amastigotes of different species of Leishmania. FEBS Lett. 121:287-291.

3. Blanchard, P., N. Dodic, J.L. Fourrey, M. Geze, F. Lawrence, H. Malina, P. Paolantonacci, M. Vedel, C. Tempete, M. Robert-Gero, and E. Lederer. 1986. Sinefungin and derivatives: synthesis, biosynthesis and molecular target studies in Leishmania, p. 435-446. In R.T. Borchardt, C.R. Creveling, and P.M. Ueland (ed.), Biological methylation and drug design. The Humana Press.

4. Chiang, P.K., and G.L. Cantoni. 1977. Activation of methionine for transmethylation. 1977. J. Biol. Chem. 252:4506-4513.

5. Chisholm, R.L. 1982. Methylation and developmental regulation of gene expression. TIBS:421-417.

6. Dube, K.D., G. Mpimbaza, A.C. Allison, E. Lederer, and L. Robis. 1983. Antitrypanosomal activity of sinefungin. J. Trop. Med. Hyg. 32:31-33.

7. Dube, D.K., R.O. Williams, G. Seal and S.C. Williams. 1979. Detection and characterization of DNA polymerase from Trypanosoma brucei. Biochem. Biophys. Acta 561:10-16.

8. Drahousky, D. and T.L.J. Boehm. 1980. Enzymatic DNA methylation in higher eukaryotes. Int. J. Biochim. 12:523-528.

9. Ferrante, A., I. Ljungstrom, G. Huldt, and E. Lederer. 1984. Amoebocidal activity of the antifungal antibiotic sinefungin against Entamoeba histolytica. Trans. R. Soc. Trop. Med. Hyg. 78:837-839.

10. Fuller, R.W., and R. Nagarajan. 1978. Inhibition of methyl transferases by some new analogs of S-adenosylhomocysteine. Biochem. Pharmacol. 27:1981-1983.

11. Gordee, R.S., and T.F. Butler. 1973. A9145, a new adenine containing antifungal antibiotic. II. Biological activity. J. Antibiot. 26:466-467.

12. Hamill, R., and M. Hoehn. 1973. A9145, a new adenine containing antifungal antibiotic. I. Discovery and isolation. J. Antibiot. 26:463-465.

13. Hartwick, A. 1979. Analysis of RNA by HPLC. J. Liq. Chromat. 2(5):715-744.

14. Hoffman, J.L., and G.L. Kunz. 1977. Differential activation of rat liver methionine adenosyltransferase isozymes by dimethylsulfoxide. Biochem. Biophys. Res. Commun. 77:1231-1236.

15. Holmes, A.M., E. Cheriathundam, A. Kalinski and L.M.S. Chang. 1984. Isolation and partial characterization of DNA polymerases from Crithidia fasciculata. Mole. and Biochem. Parasitol. 10:195-205.

16. Ikegami, S., T. Taguchi, and M. Ohashi. 1978. Aphidicolin prevents mitotic cell division by interfering with the activity of DNA Polymerase- . Nature (Lond.) 275:458-460.

17. Iovannisci, D.M., D. Goebel, K. Allen, K. Kaui, B. Ullman. 1984. Purine Transport in Leishmania. J. Biol. Chem. 259(3):14617-14623.

18. Lowe, P.A., D.A. Hager, and R.R. Burgess. 1979. Purification and Properties of the Subunit of Escherichia Coli DNA-Dependent RNA Polymerase. Biochem. 18:1344-1352.

19. Li, A.W., R.A. Singer, and G.C. Johnston. 1985. Effects of sinefungin on rRNA production and methylation in the yeast Saccharomyces cerevisiae. Arch. Biochem. Biophys. 240:613-620.

20. Marcus, S.L., G. Lipshik, G. Trueba, and C.J. Bacchi. 1980. The DNA Polymerases of Trypanosoma Brucei. Biochem. Biophys. Res. Commun. 93(4):1027-1035.

21. Mudd, S.H. 1962. Activation of methionine for transmethylation. J. Biol. Chem. 238:2156-2160.

22. Neal, R.A., S.L. Croft, and D.J. Nelson. 1985. Antileishmanial effect of allopurinol ribonucleoside and related compounds, formycin B, sinefungin and lepidine WR 6026. Trans. R. Soc. Trop. Med. Hyg. 79:85-122.

23. Paolantonacci, P., F. Lawrence and M. Robert-Gero. 1985. Differential effect of sinefungin and its analogs on the multiplication of three Leishmania species. Antimicrob. Agents Chemother. 28:528-531.

24. Paolantonacci, P., F. Lawrence, F. Lederer and M. Robert-Gero. 1986. Protein methylation and protein methylases in Leishmania donovani and Leishmania tropica promastigotes. Mol. & Biochem. Purasitol. 21:47-54.

25. Pugh, C.S.G., R.T. Borchardt, and H.O. Stone. 1978. Sinefungin, a potent inhibitor of virion mRNA (guanine-7)-methyltransferase, mRNA (nucleoside-2'-)-methyltransferase, and viral multiplication. J. Biol. Chem. 253:4075-4077.

26. Randerath, K., R.C. Gubta. 1979. Digestion of RNA. Fed. Proc. 38:499-505.

27. Robert-Gero, M., A. Pierre, M. Vedel, J. Enouf, F. Lawrence, A. Raies, and E. Lederer. 1980. Analogues of S-adenosylhomocysteine as *in vitro* inhibitors of transmethylases and *in vivo* inhibitors of viral oncogenesis and other cellular events, p. 61-74. *In* U. Brodbeck (ed.), Enzyme inhibitors 1980. Verlag Chemie, Weinheim, Federal Republic of Germany.

28. Solari, A., D. Tharaud, Y. Repetto, J. Aldunate, A. Morello and S. Litvak. 1983. *In vitro* and *in vivo* studies of *Trypanosoma cruzi* DNA polymerase. Biochem. Int. 7:147-157.

29. Stauber, L.A. 1958. Host resistance to the Kartoum strain of *Leishmania donovani*. The Rice Institute Pamphlet XLVC):80-96.

30. Steiger, R.E. and E. Steiger. 1977. Cultivation of *Leishmania donovani* and *Leishmania braziliensis* in defined media: Nutritional requirements. J. Protozool. 24:437-441.

31. Trager, W., M. Tershakovec, P.K. Chiang, and G. Cantoni. 1980. *Plasmodium falciparum*: antimalarial activity in culture of sinefungin and other methylation inhibitors. Exp. Parasitol. 50:83-89.

32. Vedel, M., F. Lawrence, M. Robert-Gero, and E. Lederer. 1978. The antifungal antibiotic sinefungin as a very active inhibitor of methyltransferases and of the transformation of chick embryo fibroblasts of Rous sarcoma virus. Biochem. Biophys. Res. Commun. 85:371-376.

DISTRIBUTION LIST

12 copies	Director Walter Reed Army Institute of Research Walter Reed Army Medical Center ATTN: SGRD-UWZ-C Washington, DC 20307-5100
1 copies	Commander US Army Medical Research and Development Command ATTN: SGRD-RMI-S Fort Detrick, Frederick, Maryland 21701-5012
12 copies	Defense Technical Information Center (DTIC) ATTN: DTIC-DDAC Cameron Station Alexandria, VA 22304-6145
1 copy	Dean School of Medicine Uniformed Services University of the Health Sciences 4301 Jones Bridge Road Bethesda, MD 20814-4799
1 copy	Commandant Academy of Health Sciences, US Army ATTN: AHS-CDM Fort Sam Houston, TX 78234-6100

END

DATE

FILMED

5-88

DTIC