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CHEMOTHERAPY AND DRUG TARGETING IN THE TREATMENT OF LEISHMANIASIS

ANNUAL REPORT

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Leishmaniasis, a disease caused by protozoan parasites of the Lesihsmania spp., is one of the major public health problems currently affecting humanity. Therapeutic agents for this disease is either ineffective or toxic. The purpose of this work is to aid in the development of an effective, non-toxic treatment for leishmaniasis.

The objective of this research was to isolate and characterize unique leishmanial enzymes (DNA polymerase and S-adenosylsynthetase) for the purpose of chemotherapeutic exploitation.

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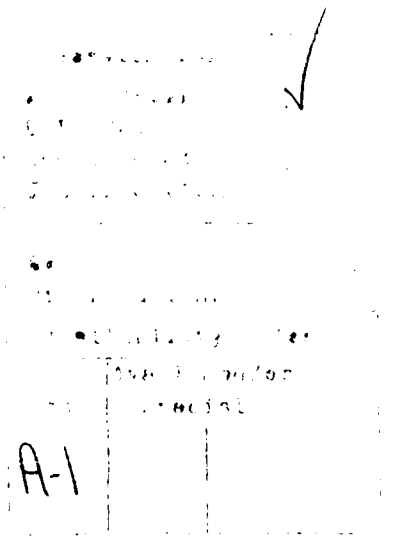


INTRODUCTION

Species of the parasitic protozoan genus *Leishmania* are the causative agents of a wide variety of human cutaneous, mucocutaneous, and visceral diseases. These organisms (lower eukaryotes) reside throughout their digenetic life cycles in different environments. The extracellular, flagellated promastigote forms reside in the alimentary tract of their sandfly vector hosts, and the obligate intracellular amastigote form exists within the phagolysomal system of macrophages in their mammalian hosts. How these organisms transform, survive, and respond to signals within their infected hosts is unknown.

Most of the major metabolic pathways in the parasitic protozoa have been reported to be similar to those of the mammalian host except for nucleic acid metabolism. This pathway is unusual in several ways. *First*, it lacks the ability to synthesize the purine *de novo*, making them entirely dependent on the salvage pathway for their supply of purine nucleotides. *Second*, many of the enzymes involved in nucleic acid biosynthesis either have unusual substrate specificities or unusual subcellular localizations. *Third*, a large proportion of the DNA which is produced is incorporated into a unique organelle known as the kinetoplast. Kinetoplast DNA, the mitochondrial DNA of *Leishmania* and related parasitic protozoa, has a remarkable structure. It consists of networks of thousands of interlocked DNA circles, and each cell has one network within its single mitochondrion. Nothing is known either about the function of kinetoplast minicircles or the reason that these molecules are interlocked, together with maxicircles, in an enormous network. Nor is it known why these parasitic protozoa, alone among eukaryotes, have their mitochondrial DNA organized in this unusual way. *Fourth*, the major DNA polymerase isolated from the parasitic protozoa has

been shown to have different characteristics than its mammalian counterpart and to be immunologically distinct.

The presence of multiple DNA polymerases in eukaryotic cells is a well established fact. The use of specific inhibitors has helped to characterize nuclear and organelle DNA polymerases. The DNA polymerase involved in the repiication of the nuclear genome is strongly inhibited by aphidicolin regardless of the source of the enzyme. Other eukaryotic DNA polymerases, like the β-polymerase involved in DNA repair, as well as the chloroplastic and mitochondrial polymerases, are not affected by this drug.

Since the first description of a DNA polymerase in an animal cell 28 years ago, an immense body of information has been accumulated on eukaryotic DNA polymerases, their classification, prevalence, evolution, physical and catalytic properties, and roles in DNA metabolism in vivo. Animal cell DNA polymerases are distinctive from prokaryotic and viral polymerases and have been classified into alpha (α), beta (β), gamma (γ), and delta (δ) by cellular, physical, and enzymological parameters, as well as by their different responses to selective inhibitors.

Determining the roles of the four mammalian DNA polymerases is a fundamental problem in biology. Most studies to date conclude that DNA polymerase α is the primary polymerase responsible for nuclear DNA replication. Polymerase β is established to contain intrinsic 3' to 5' exonuclease (proofreading) activity. Butylphenyldeoxyguanosine triphosphate (BuPdGTP) and monoclonal antibodies directed against polymerase α have been shown to discriminate the activities of α and β.

Chang, et. al. (1980) [1] reported that extracts of bloodstream forms of Trypanosoma brucei showed that both DNA polymerase α and DNA polymerase β activities were present. The detection of DNA polymerase α in T. brucei demonstrated the presence of this enzyme in unicellular organisms. Chang also

stated that DNA polymerase α was present in L. mexicana [1]. They found the DNA polymerases in T. brucei to be immunologically distinct from host enzymes, and suggested that the structural differences between the parasite and the host enzymes could be exploited for the development of agents to combat parasitic diseases. Dube, et. al. [2] reported on the detection and characterization of DNA polymerase α in T. brucei and found that specific antisera that cross-reacted with mammalian DNA polymerase α from different species failed to cross-react with the trypanosomal polymerase.

Solari, et. al. [3] reported the surprising finding that Trypanosoma cruzi DNA polymerase (predominate form) failed to be inhibited by aphidicolin. Holmes, et. al. [4] reported that a related organism, Crithidia fasiculata, had two types of DNA polymerase activity, the α-type reported as DNA polymerase A and a β-type reported as DNA polymerase B. The response of the C. fasiculata A enzymes to inhibitors and utilization of poly (rA)-oligo (dT) showed these enzymes to be markedly different from mammalian DNA polymerases. Aphidicolin had no effect on either the DNA polymerase A enzymes or on DNA polymerase B, at concentrations of up to 250 μM. If their observations are correct, these lower eukaryotes will be truly unique in that their major mode of DNA replication is clearly different from that in mammals. The recent excitement about aphidicolin began with the demonstration that it is a specific, direct inhibitor of animal DNA polymerase α but is without effect on polymerases β or γ. Using aphidicolin as a tool, it has been shown that DNA polymerase α is clearly the principal polymerase required for DNA replication in all animals and plants studied.

Except for the brief report by Chang, et. al. [1], to our knowledge no one has characterized or performed kinetic studies with the DNA polymerases in Leishmania spp. We have begun studies to isolate the polymerases of L. mexicana for the purpose of elucidating key differences in DNA synthesis and its regulation by

DNA and DNA polymerase binding proteins and other cellular modulators (i.e., hormones, prostaglandins, polyamines) between the parasitic protozoa and higher eukaryotes. This information will not only provide basic information on the evolution of DNA replication and regulation, but may provide information on how specific signals in the parasite's environment modify its morphology and biochemistry. Elucidation of key differences between parasite and host will offer targets for chemotherapeutic exploitation.

In contrast to the findings of Holmes, et. al. [4], Solari, et. al. [3], and Dube, et. al. [2], we have found that aphidicolin is inhibitory both to growth and to DNA polymerase (in vitro) activity of L. mexicana. We have found that inhibition is dependent on the purification of the enzyme. In crude preparations of DNA polymerase, aphidicolin (20 μM) inhibits over 50%, but as the enzyme is purified it is not inhibited at all or at 10-100x the concentration used in crude preparations. Foster, et. al. [5] have reported that resistance of adenoviral DNA replication to aphidicolin is dependent on the 72-kilodalton DNA-binding protein. This protein protects the DNA polymerase of the virus from inhibition by aphidicolin. However, this protein does not appear to protect host cells infected with the virus from inhibition by aphidicolin. The possibility exists that in vitro a similar type protein protects the DNA polymerase of parasitic protozoa. Although aphidicolin has been shown to inhibit DNA synthesis (of parasitic protozoa) in vivo more efficiently than ethidium bromide and berenil, this laboratory has been the first to demonstrate inhibition of DNA polymerase in vitro under certain conditions.

Binding of proteins to DNA and DNA polymerase is fundamental to the mechanism of the control of gene expression in both prokaryotic and eukaryotic cells. Knowledge of the specific molecular features of DNA recognized by complementary features of the three-dimensional structure of DNA-binding

proteins is still in its infancy. The binding proteins form stoichiometric complexes which modulate subsequent enzymatic transformations.

Prokaryotic binding proteins have been shown to be essential in initiation and elongation in DNA replication and in DNA repair and recombination. Binding proteins isolated from calf thymus, Ustilago maydis, mouse ascites cells, and other eukaryotic sources have been shown to stimulate DNA polymerase activity.

S-Adenosylmethionine Studies

The essentiality of *S*-adenosylmethionine (SAM) in transmethylation reactions and polyamine biosynthesis provides a rationale for the development of methionine antileishmanial agents. Recent studies indicating that polyamine biosynthesis is essential for parasitic replication suggest that these organisms may be particularly susceptible to growth inhibition by methionine analogs.

In recent years, SAM has been found to affect the methylation and the properties of such macromolecules as nucleic acids, proteins, phospholipids, and carbohydrates. SAM is also a precursor to the polyamines (Fig. 1). The naturally occuring polyamines putrescine, spermidine, and spermine are organic cations widely distributed in both prokaryotic and eukaryotic organisms. Polyamine synthesis increases and polyamine levels rise when the growth rate is maximal. Growth appears to be related to and dependent upon polyamine biosynthesis.

Since our previous studies have indicated that one of the most potent antileishmanial agents to date, Sinefungin, a SAM analog, interferes indirectly with nucleic acid metabolism, we have begun studies to determine the significance and uniqueness of the parasitic *S*-Adenosylmethionine synthetase (the enzyme which produces SAM).

Fig. 1

$H_2N(CH_2)_4NH_2$ $H_2N(CH_2)_4NH_4$

Putrescine Spermidine

$H_2N(CH_2)_3NH(CH_2)_4NH(CH_2)_3NH_2$

Spermine

Stuctures of the common polyamines.

SPECIFIC AIMS OF CURRENT YEAR

* To isolate and characterize the
    (1) DNA polymerases
    (2) *S*-Adenosylmethionine synthetases
of *Leishmania mexicana*, a parasitic protozoan.

* To focus on detecting possible chemotherapeutic exploitable differences between these parasitic enzymes and mammalian host enzymes.

Progress on the Isolation and
Characterization of the *L. mexicana* DNA polymerases

Materials and Methods

Materials

Promastigotes of *Leishmania mexicana amazonensis* (Walter Reed strain 227) were obtained from the Leishmania Section of the Walter Reed Army Institute of Research. Sodium α-oxomethylenediphosphonate (carbonildiphosphonate, COMDP), and the 2-arylaminopurine deoxyribonucleoside 5'-triphosphates BuAdATP and BuPdGTP were generously provided by Dr. G. Wright, Pharmacology Dept., University of Massachusetts Medical School, Worcester, MA, USA. Sodium Suramin was purchased from Miles Pharmaceuticals (West Haven, CT, USA). [*methyl*-^{3}H]dTTP (spec. act. 20.5 Ci/mmol), and [*methyl*-^{3}H]dGTP (spec. act. 11.1 Ci/mmol) were obtained from New England Nuclear (Boston, MA, USA). Bio Safe II liquid counting cocktail was purchased from RPI (Mount Prospect, IL, USA). Cellulose phosphate (P11) and GF-C glass fiber filter disks were from Whatman (Clifton, NJ, USA). Sephacryl S-200 HR, PD-10 column, blue dextran, poly (rA)-p$(dT)_{10}$, poly (rC)-p$(dG)_{12-18}$, poly (dA), poly (dC), oligo $(dT)_{12-18}$, oligo $(dG)_{12-18}$, catalase, and ferritin were from Pharmacia LKB Biotechnology (Piscataway, NJ, USA). Centricon-10 microconcentrator was from Amicon (Danvers, MA, USA). Enzyme grade ammonium sulfate and G4 glass fiber filter disks were obtained from Fisher Scientific (Fair Lawn, NJ, USA). Benzamidine hydrochloride was from Eastman Kodak (Rochester, NY, USA). Molecular weight standards for gel filtration were from Bio Rad Laboratories (Richmond, CA, USA). Calf thymus activated DNA, bovine serum albumin, cytochrome C, yeast alcohol dehydrogenase, and all other reagents and laboratory chemicals were obtained from Sigma (St. Louis, MO, USA) and were of the highest purity available.

Methods

Isolation of a *Leishmania mexicana* HMW DNA polymerase

Leishmania mexicana 227 promastigotes were grown as previously described [6]. An 18.5 g (wet weight) *L. mexicana* cell pellet was resuspended in 22 ml of 200 mM potassium phosphate buffer, pH 7.4, 1 mM benzamidine hydrochloride, 1 mM dithiothreitol, 1% (v/v) dimethylsulfoxide (Me_2SO), 1 mM EDTA (buffer A) and sonicated three times for 15 seconds each at an output of 160 watts on a Braunsonic 2000 sonicator. A mixture of protease inhibitors was immediately added after sonication to a final concentration of 48µg/ml trypsin inhibitor, 48µg/ml aprotinin, 20µg/ml leupeptin and 1 mM phenylmethyl-sulfonyl fluoride (PMSF) . The sonicate was centrifuged at 45 000 x *g* for 90 min at 2°C. The supernatant (18 ml, fraction I, crude extract) was filtered through a layer of glass wool to remove the lipid layer. A 2% (w/v) protamine sulfate solution prepared fresh in buffer A (1.8 ml) was added dropwise to precipitate DNA. The extract was stirred for 30 min at 4°C and centrifuged at 27,000 x *g* for 15 min at 4°C. The supernatant (fraction II, 15.8 ml) was dialyzed overnight with one buffer change against 2 l of 50 mM potassium phosphate buffer (pH 7.4) containing 1 mM benzamidine hydrochloride, 1 mM EDTA, 1 mM dithiothreitol, 1% (v/v) Me_2SO, and 10% (v/v) glycerol (buffer B). The dialyzed sample was loaded on a 5 x 1.5 cm cellulose phosphate (P11) column equilibrated with buffer B at a flow rate of 0.5 ml/min. The column was washed with 5 column volumes of buffer B, followed by 5 column volumes of 0.15 M KCl in the same buffer. DNA polymerase was eluted with four column volumes of 0.4 M KCl in buffer B to give fraction III. Fraction III was made 60% ammonium sulfate saturation and centrifuged at 30 000 x *g* for 10 min at 4°C. The pellet was resuspended in 50 mM Tris buffer (pH 7.5) containing 1 mM dithiothreitol, 50 mM KCl, and 20% (v/v) glycerol, and the protease inhibitors mix (buffer C). The sample was dialyzed overnight against buffer C and loaded on a 1.5 x 1.7 cm denatured DNA cellulose

column at a flow rate of 0.5 ml/min. DNA polymerase activity was eluted from the column with 0.15 M KCl in buffer C. Active fractions were pooled (Fraction IV) and dialyzed against 50 mM Potassium Phosphate (pH 7.4), 1 mM dithiothreitol, 50% (v/v) glycerol, and stored at -70°C for up to 8 months.

Molecular weight determination

The molecular weight of the DNA polymerase enzyme was estimated from the activity profile of the fractions of a Sephacryl S-200 HR gel filtration column. An aliquot of Fraction IV was dialyzed by ultrafiltration in a Centricon 10 microconcentrator against 50 mM Hepes pH 7.5, 1 mM DTT, 0.5 M NaCl, 10% Glycerol (buffer D) and loaded on a 1 x 92 cm column equilibrated in buffer D at a flow rate of 0.4 ml/min. The column was washed with buffer D and 1 ml fractions were collected and assayed for DNA polymerase activity. Thyroglobulin (670 kDa), gamma globulin (158 kDa), ovalbumin (44 kDa), myoglobin (17 kDa), and cyanocobalamin (1,350 Da) were used as standards. The molecular weight was independently estimated from sedimentation coefficient analysis using a standard curve generated by plotting the logarithm of the molecular weight vs. the logarithm of the sedimentation coefficient. Aliquots (0.25 ml) of fraction III DNA polymerase were layered on top of preformed 5 to 20 % (w/v) sucrose solution density gradients prepared in 25 mM potassium phosphate buffer (pH 7.4), 1 mM dithiothreitol, and 150 mM KCl. The gradients were centrifuged at 40 000 rpm for 16 h at 4°C in a Beckman sw 41 Ti rotor. Gradients were fractionated by displacement from the bottom with a 50% (w/v) sucrose solution containing 2 mM potassium hydrogen phthalate as an endpoint marker. Fractions (0.375 ml) were collected and assayed for activated DNA-dependent DNA polymerase activity. Sedimentation coefficients were calculated according to Martin and Ames [7] using cytochrome C, bovine serum albumin, alcohol dehydrogenase, and ferritin as standards.

Enzyme Assays

Activated DNA dependent enzyme activity was assayed in a final volume of 65µl for 30 min at 35°C. The reaction mixture contained 20 mM 4-morpholineethanesulphonic acid (Mes) (pH 6.7), 50µM each of dATP, dCTP, dGTP, 1 mM dithiothreitol, 8 mM $MgCl_2$, 125 µg/ml activated calf thymus DNA, 100µg/ml bovine serum albumin, 10µL of enzyme sample, and 40 nM [*methyl*-^{3}H]dTTP at 350-400 CPM/pmole. The reaction was stopped by spotting 50µL on GF-C or G4 glass filter disks numbered with a laundry marker pen and collecting the filters in 5% (w/v) trichloroacetic acid, 1% (w/v) sodium pyrophosphate, and processed for counting as previously described [8]. The radioactivity on the dry disks was determined by counting in Bio-Safe II liquid counting cocktail in a liquid scintillation counter. One unit of DNA polymerase is defined as the incorporation of 1 pmol of [*methyl*-^{3}H]dTMP into acid insoluble material in 30 min at 35 °C. Unless otherwise noted, all assays were done in duplicate and using activated DNA as the template.

Synthetic template-primer assays

The ability of the DNA polymerase to utilize synthetic template-primer was assayed in the assay mix described above. Poly (dA)-oligo $(dT)_{12-18}$ and poly (dC) -oligo $(dG)_{12-18}$ (base ratio 10:1) were prepared as described [9]. For the assays, 50-100µg/mL of synthetic template-primer replaced activated DNA and dNTP. For the poly (dC) -oligo $(dG)_{12-18}$ and poly (rC) -p$(dG)_{12-18}$ assays, 36µM [*methyl*-^{3}H]dGTP (350 CPM/pmole) was the labelled nucleotide. For the poly (rA)-p$(dT)_{10}$ and poly (rC) -p$(dG)_{12-18}$ assays, 0.5 mM $MnCl_2$ replaced $MgCl_2$.

Drug inhibition studies

Fraction IV enzyme was assayed for inhibition of activity by different compounds. Each compound was tested by including it in the assay mix at the

appropriate concentration. The enzyme, drug, and assay mix were incubated in ice for 30 minutes and then assayed as described above.

Arachidonic, Linolenic, and Linoleic acids were made as 100 mM stocks in 97% ethanol, diluted with 50 mM Tris pH 9.6 to 50 mM and then brought to the desired concentration in the assay with 10 mM Tris pH 7.5 so that the final concentration of ethanol (EtOH) in the assay was no more than 0.5%. Care was taken to flush the reaction vials and the concentrated stocks with Argon to prevent oxidation of the fatty acids. The unused 100 mM stocks were stored under Argon on a dessicator at -30 ^{o}C until used again. A 0.5% (v/v) EtOH control was included in the assays when testing linoleic, linolenic, and arachidonic acids against the DNA polymerases. Aphidicolin was prepared as a 10 mM stock in Me_2SO and diluted accordingly so that the final concentration of Me_2SO in the assay was lower than 0.04% (v/v). Other compounds were made into aqueous buffer solution stocks and diluted to the final concentrations in the assay with water.

Thermal stability of the DNA polymerase

The stability of the enzyme was tested at both low and high protein concentrations. Approximately 67 units (250µl) of the Fraction IV DNA polymerase were incubated at 45 ^{o}C and duplicate 10µl aliquots were removed every 5 min and assayed as described using poly (dA) -oligo $(dT)_{12-18}$ as the template. Fraction IV (approx. 67 units) was adjusted to 1 mg of protein with BSA in a total volume of 250 µl, incubated at 45 ^{o}C and aliquots removed every 5 minutes and assayed as described above.

Protein Determination

Protein concentration was determined according to the modified dye binding method of Redinbaugh and Campbell [10] using bovine serum albumin as the standard.

Results

Isolation of a DNA polymerase from *L. mexicana*

The DNA polymerase was isolated and purified more than 800 fold using a modification of the method developed for the *Chrithidia fasciculata* DNA polymerases [4]. A summary of the purification procedure is shown in Table 1. The most significant step of the isolation procedure was the cellulose phosphate column which resulted in over a 600 fold purification. The activity of the DNA polymerase was low at the early stages of the purification, namely the crude extract and the protamine sulfate supernatant. Treatment of the crude extract with Triton X-100 or polyethyleneimine did not result in an increase of enzyme activity (data not shown). Assay of the resuspended protamine sulfate pellet showed that no enzyme activity was lost to precipitation. The activity of the enzyme was sharply increased after cellulose phosphate chromatography (Table 1), indicating the possibility that an endogenous DNA polymerase inhibitor [11] was removed at this step. Development of the cellulose phosphate column using steps of increasing KCl concentration yielded a DNA polymerase fraction with a specific activity that was comparable to the specific activity obtained using linear gradients of increasing KCl concentration to elute the enzyme from the column (data not shown). A large decrease in the total number of units resulted after precipitation of the enzyme with ammonium sulfate and dialysis (Fraction IV, Table 1). This loss is a result of the ammonium sulfate precipitation, a decrease in the stability of the enzyme due to the decreased protein concentration and the length of dialysis at 4 ^{o}C. However, we determined this to be the most effective way to concentrate the enzyme prior to affinity chromatography.

Chromatography of fraction IV on a Sephacryl S-200 HR gel filtration column resulted in a peak of activity eluting with an apparent molecular weight of 145kDa. Sedimentation of fraction III DNA polymerase on a 5% to 20% (w/v) sucrose solution density gradient showed a peak of enzyme activity sedimenting at

6.8 S (Figure 2). An apparent molecular weight of 130 kDa was calculated for this enzyme from the density gradient data assuming a globular shape. As previuosly reported by Chang [1], we detected another DNA polymerase activity present in the cellulose phosphate fraction (Fraction IV) that had a different pH optimum, sedimented at 3.4 S and at a low molecular weight in gel filtration (see below).

The enzyme fraction obtained after denatured DNA Cellulose (Fraction IV) was used for all subsequent experiments. With activated calf thymus DNA as the template, the optimum divalent metal ion was found to be Mg^{+2} at a concentration of 8 mM (Figure 3). The enzyme activity was lower when Mn^{+2} was present and activated DNA was the template (Figure 3). The optimum pH for DNA polymerase activity was found by assaying the enzyme with buffers having different useful pH ranges. The highest DNA polymerase activity was obtained when the buffer used was MES pH 6.7 (Figure 4). Exposure of the enzyme to increasing concentrations of KCl or NaCl during the assay resulted in 50% inhibition by 95 mM KCl or 80 mM NaCl. A small increase in activity (maximum of 15% over the control) was observed when the KCl or NaCl concentration was between 1-10 mM in the assay mix (Figure 5).

Effect of DNA polymerase inhibitors

The denatured DNA Cellulose enzyme fraction (Fraction IV) was tested for inhibition by *N*-ethylmaleimide (NEM), a specific mammalian DNA polymerase inhibitor [12], by incubating enzyme and NEM at final concentrations of 1-15 mM in the assay mix for 30 min at 0 ^{o}C. The enzyme was then assayed as described using activated DNA as the template. At the lowest concentration tested (1 mM) NEM inhibited the *L. mexicana* DNA polymerase 78%, with 94% inhibition at a concentration of 5 mM. Aphidicolin, a known mammalian DNA polymerase α inhibitor [13], did not inhibit the Fraction IV enzyme at the concentration range tested (1-400μM). An analog of dTTP, 2',3'-dideoxythymidine-5'-triphosphate (d_2TTP) to

which mammalian DNA polymerase α is slightly resistant, was inactive against the *L. mexicana* DNA polymerase at a d_2TTP to dTTP ratio of five. At a concentration of 600 μM, carbonyldiphosphonate (COMDP), a selective inhibitor of mammalian DNA polymerase δ[14] resulted in 50% inhibition of the DNA polymerase. This PP_i analog inhibited the calf thymus DNA polymerase α 50% at 300μM, whereas only 40μM was required for 50% inhibition of the DNA polymerase from the same source [14]. Two specific DNA polymerase α inhibitors, BuPdGTP and BuAdATP were tested against the *L. mexicana* DNA polymerase and found to be less effective than with the mammalian DNA polymerase α [9]. At 600μM, BuPdGTP inhibited the enzyme 95%, whereas BuAdATP inhibited it 90% at 400 μM. In contrast, the Chinese hamster ovary and Hela DNA polymerases α are both sensitive to these inhibitors in the nanomolar range [9]. Arachidonic, Linolenic, and Linoleic acids were all inhibitory to the *L. mexicana* DNA polymerase, with IC_{50} of 200, 195, and 190μM , respectively (Figure 6). The antiprotozoal compound Sodium Suramin was the strongest inhibitor of the DNA polymerase among all compounds tested. A summary of the concentration giving 50% inhibition (IC_{50}) for the compounds tested against the *L. mexicana* DNA polymerase is shown in Table 2. Table 3 shows compounds that were found to be inactive against the enzyme.

Synthetic Template-Primer Assays

Unlike most mammalian DNA polymerase α forms, the *L. mexicana* DNA polymerase can copy poly (rA)-$(dT)_{10}$ as efficiently as activated DNA (Table 4). The DNA polymerase will incorporate [*methyl*-^{3}H]TTP 9.5 times more efficiently than [*methyl*-^{3}H]dGTP when activated DNA is the template. This difference is likely due to the template source since the activity of the enzyme was about the same with both labelled nucleotides in the presence of poly (dA) -oligo $(dT)_{12-18}$ or poly (dC) -oligo $(dG)_{12-18}$ (Table 4).

Thermal stability of the DNA polymerase

Incubation at 45 ^{o}C causes inactivation of the DNA polymerase at high or low protein concentrations. As shown in Figure 7, after 5 min of incubation in the absence of BSA there was only 60% of the activity remaining, with only 7% activity remaining after 30 min. When BSA was present, essentially no difference was observed, with 76% of the initial activity remaining after the first 5 min of incubation. Under both conditions of low and high protein concentration less than 10% of the initial activity remains after 30 min of incubation (Figure 7).

Table 1. PARTIAL PURIFICATION OF *L. mexicana* DNA POLYMERASE. Enzyme fractions were assayed using Poly (dA)- oligo $(dT)_{12-18}$ as described in Materials and Methods.

Fraction	Protein (mg)	Units (U)	Specific Activity (U/mg)
I Crude extract	360.3	2400	6.7
II Protamine Sulfate	437.0	2400	5.5
III Cellulose phosphate	45.8	61440	1340.3
IV Den. DNA Cellulose	3.3	18426	5533.3

Table 2. IC_{50} OF COMPOUNDS INHIBITORY TO THE *L. mexicana* DNA POLYMERASE. Each assay was done in duplicate as described in Materials and Methods.

Name	Concentration	%Inhibition	IC_{50}
Suramin	0.7-105 μM	0-100	8 μM
Ethidium Bromide	15-100 μM	32-92	25 μM
Berenil	5-100 μM	18-81	40 μM
Hemin	1.5-306 μM	8-100	90 μM
BuPdGTP	100-400 μM	45-93	125 μM
Pentamidine	10-500 μM	21-72	125 μM
BuAdATP	150-600 μM	45-97	150 μM
Linoleic Acid	10-500 μM	13-100	190 μM
Linolenic Acid	25-1000 μM	7-100	195 μM
Arachidonic Acid	10-1000 μM	25-100	200 μM
Spermine	5-500 μM	0-75	250 μM
COMDP	150-900 μM	11-84	600 μM
N-Ethylmaleimide	1.25-15 mM	78-100	< 1 mM
Phosphomycin	50-1000 μM	26-51	1 mM

Table 3. COMPOUNDS INACTIVE AGAINST THE *L. mexicana* DNA POLYMERASE

Inhibitor	Concentration	Max. % Inhibition
Acyclovir	5-100μM	0
Aphidicolin	5-100μM	6
AraCTP	10-500μM	0
AZT	5-100μM	0
ddC	10-500μM	25
ddI	5-500μM	22
ddTTP	1-500μM	0
Garlic Extract	2.25-45μg/ml#	18
Phosphonoacetic Acid	10-2000μM	35
Sinefungin	5-500μM	21

Table 4. TEMPLATE SPECIFICITY OF THE *L. mexicana* DNA POLYMERASE. Assay conditions were as described in Materials and Methods.

TEMPLATE-PRIMER	pmoles labelled nucleotide/30 min
With [*methyl*-^{3}H]dTTP:	
Activated DNA	9.5
poly (rA) -$(dT)_{10}$	8.8
poly (dA) -oligo $(dT)_{12-18}$	30.6
With [*methyl*-^{3}H]dGTP:	
Activated DNA	1.0
poly (rC) -$(dG)_{12-18}$	1.8
poly (dC) -oligo $(dG)_{12-18}$	26.5

Figure 2. Sucrose solution density gradient sedimentation analysis of the L. mexicana DNA polymerase. A 1.5 ml aliquot of Fraction III was adjusted to 25 mM potassium phosphate buffer (pH 7.4), 1 mM dithiothreitol, 150 mM KCl by washing in a centricon-10 ultrafiltration unit at 4 ^{o}C. Two hundred and fifty microliters were layered on top of preformed gradients as described in materials and methods. The arrows show the elution of the sedimentation standards, (1) cytochrome C, 1.7 S; (2) bovine serum albumin, 4.31 S; (3) yeast alcohol dehydrogenase, 7.6 S; (4) ferritin, 17.6 S.

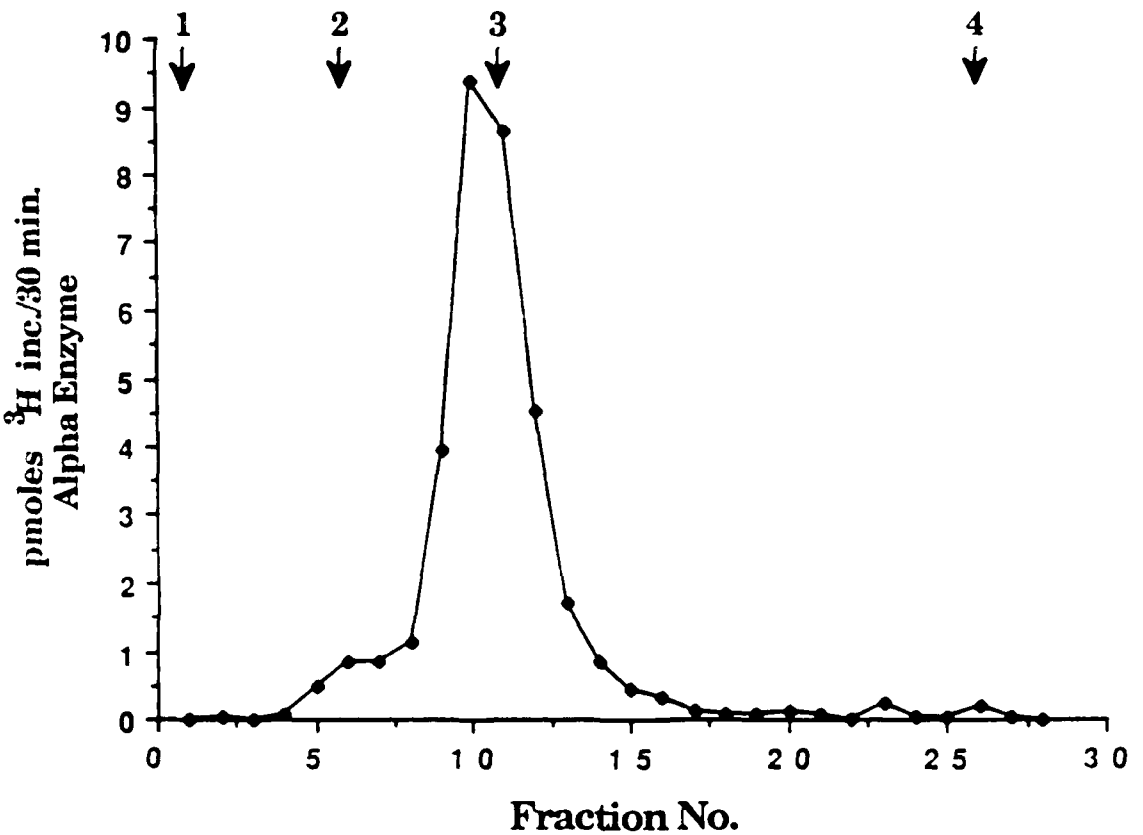


Figure 3. Optimum metal ion ($Metal^{+2}$) concentration for the L. mexicana DNA polymerase. (■) $MgCl_2$; (□) $MnCl_2$. The concentrations shown are the final concentration in the assay. Assay conditions were as described in Materials and Methods.

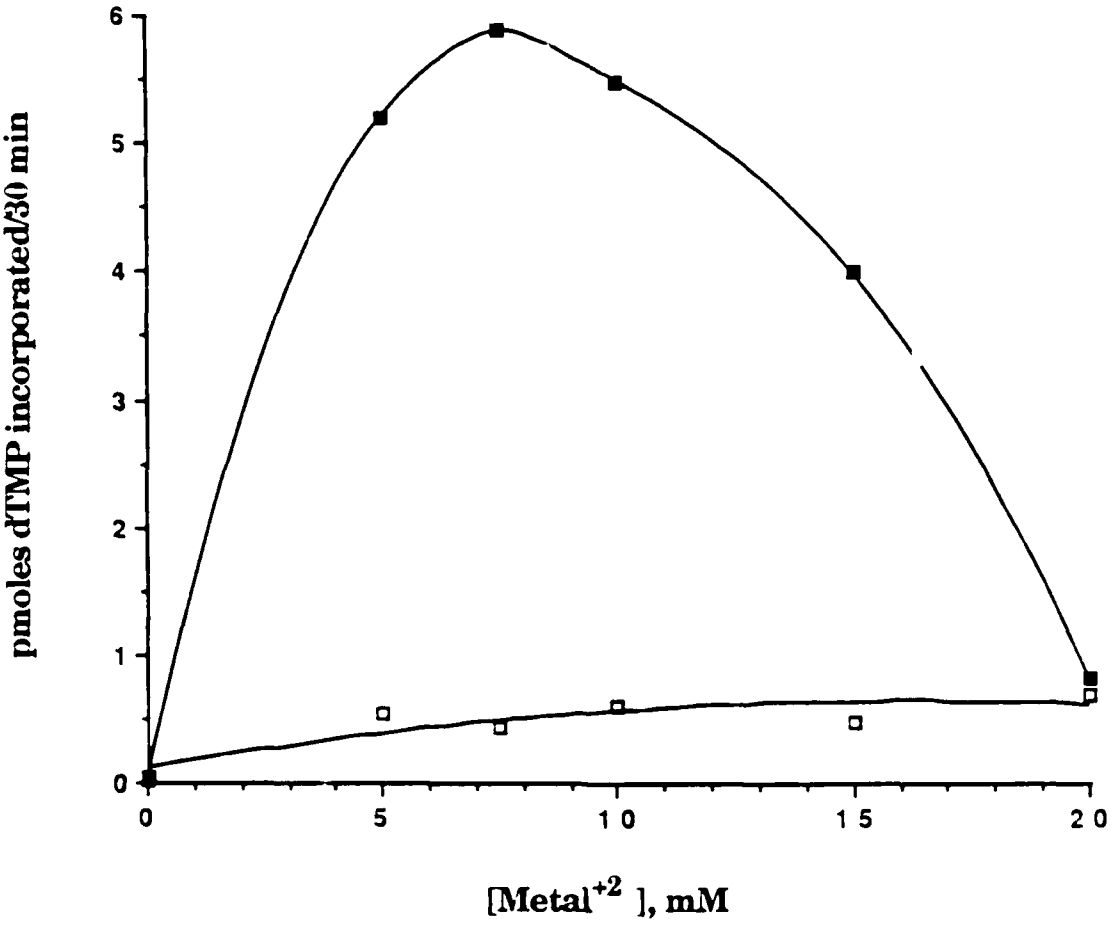


Figure 4. Optimum pH for the L. mexicana DNA polymerase. Buffers used were substituted in the assay mix at a final concentration of 20 mM. Symbols for the buffers are (-○-) MES buffer, pH 5.5-6.7; (-□-) potassium phosphate, pH 6.0-8.0; (··△··) Tris buffer, pH 7.2-8.6; (··■··) Ammediol buffer, pH 8.7-9.7.

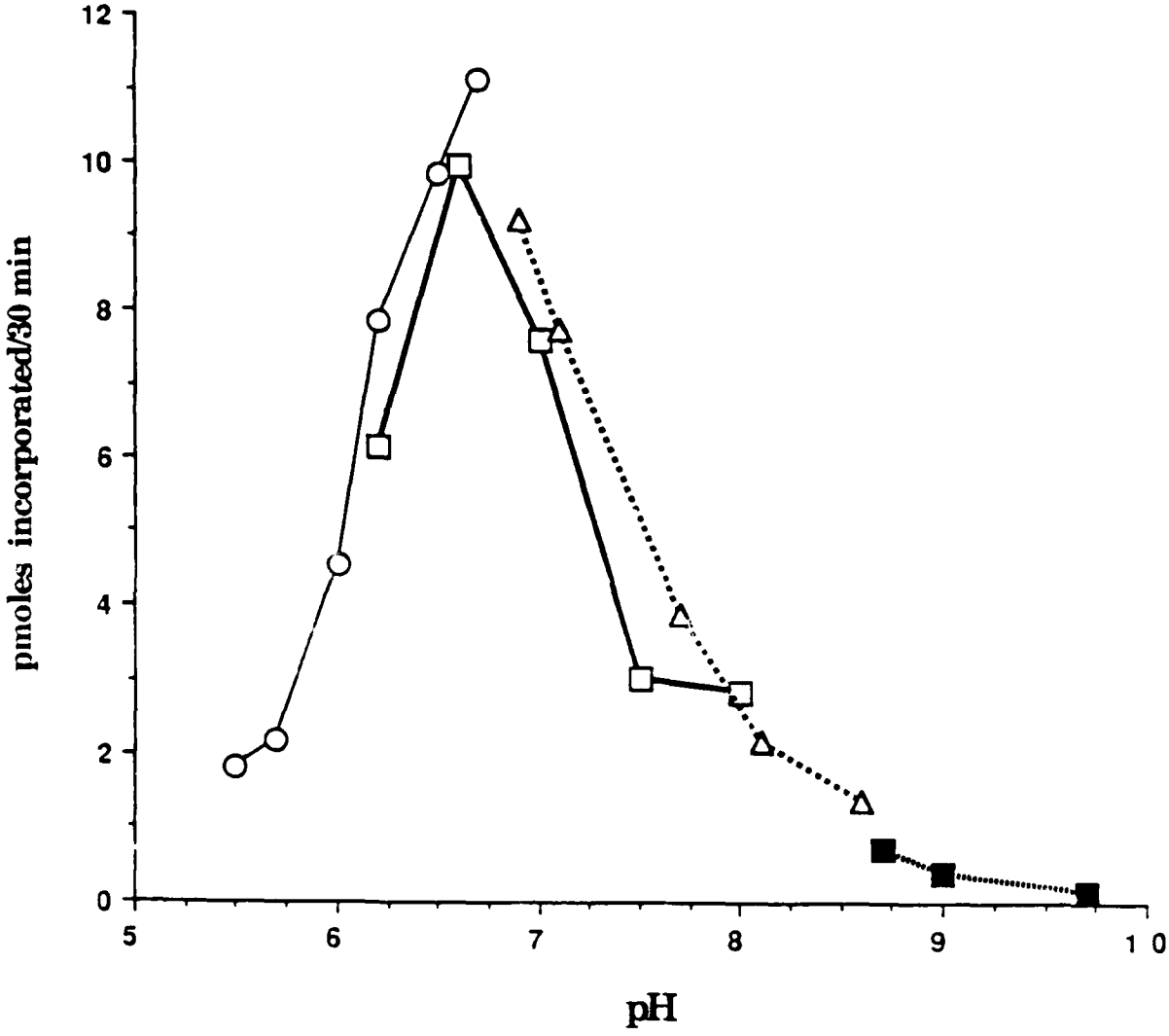


Figure 5. Effect of KCl and NaCl on the activity of the L. mexicana DNA polymerase. Concentrations shown are the final concentration in the assay. Assay conditions were as described in Materials and Methods.

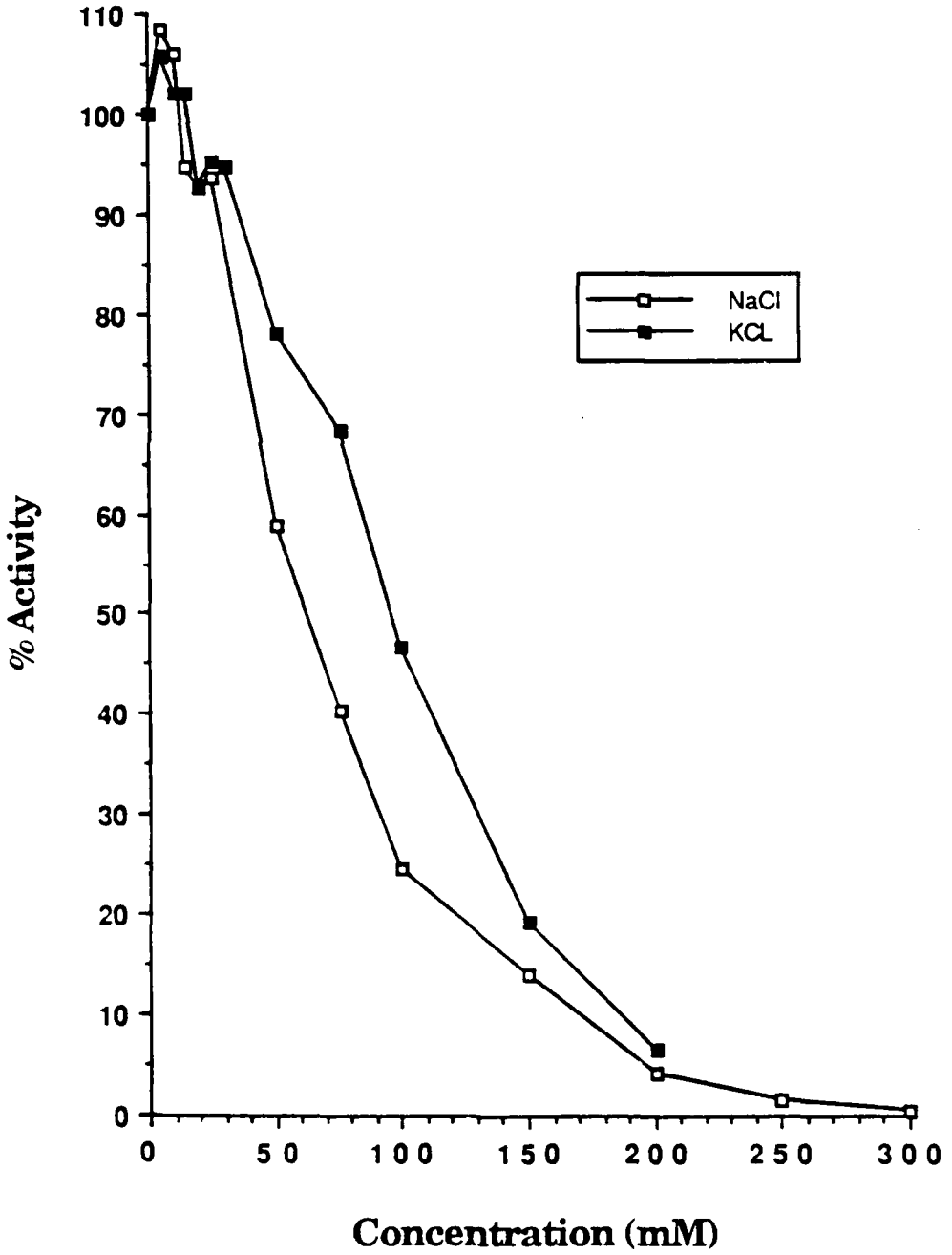


Figure 6. Inhibition of L. mexicana DNA polymerase activity by fatty acids. (—■—) Arachidonic acid, (- -□- ·) Linoleic acid, (—○—) Linolenic acid. The enzyme was incubated with each fatty acid on ice for 30 minutes and then assayed as described in materials and methods.

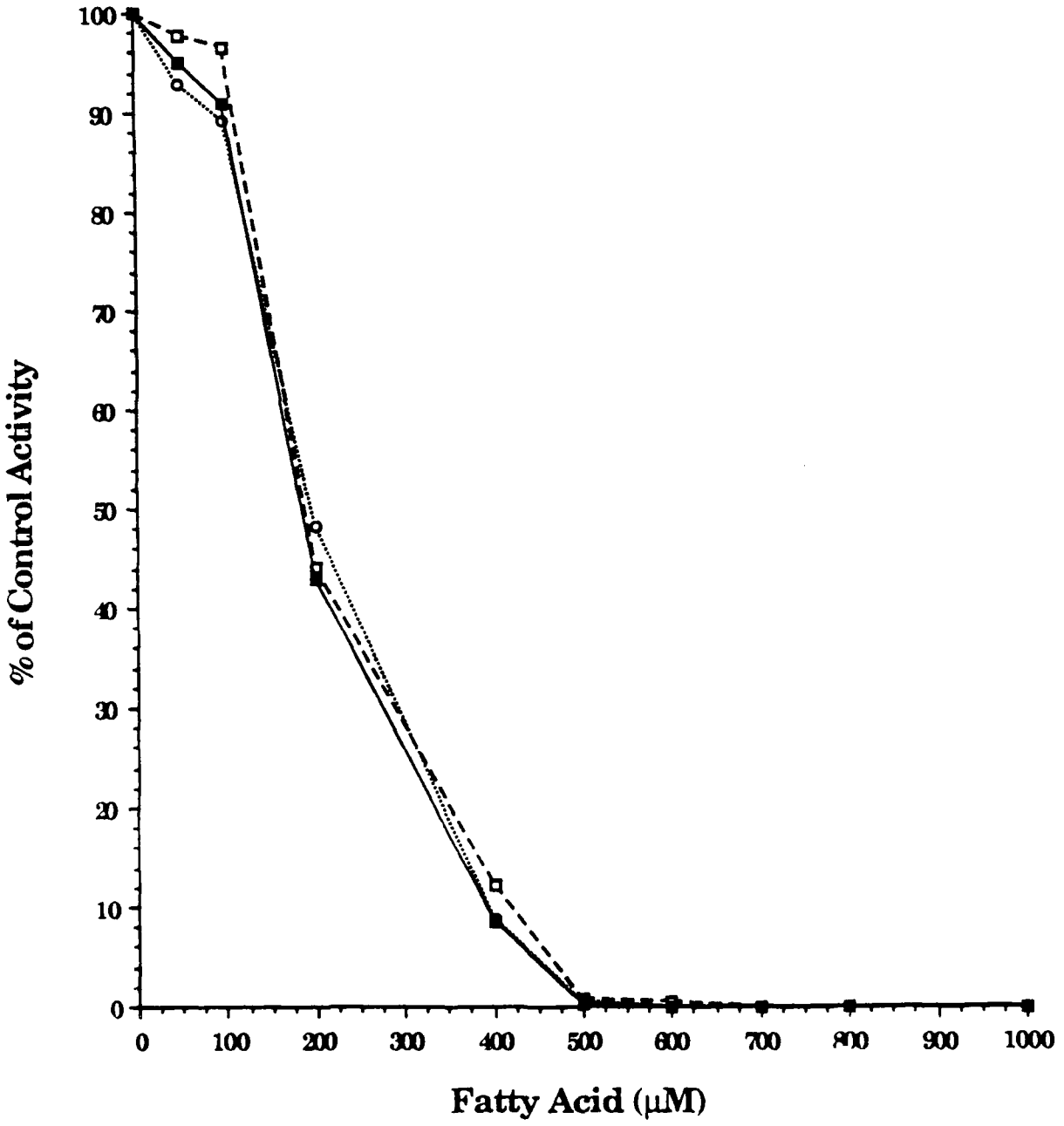
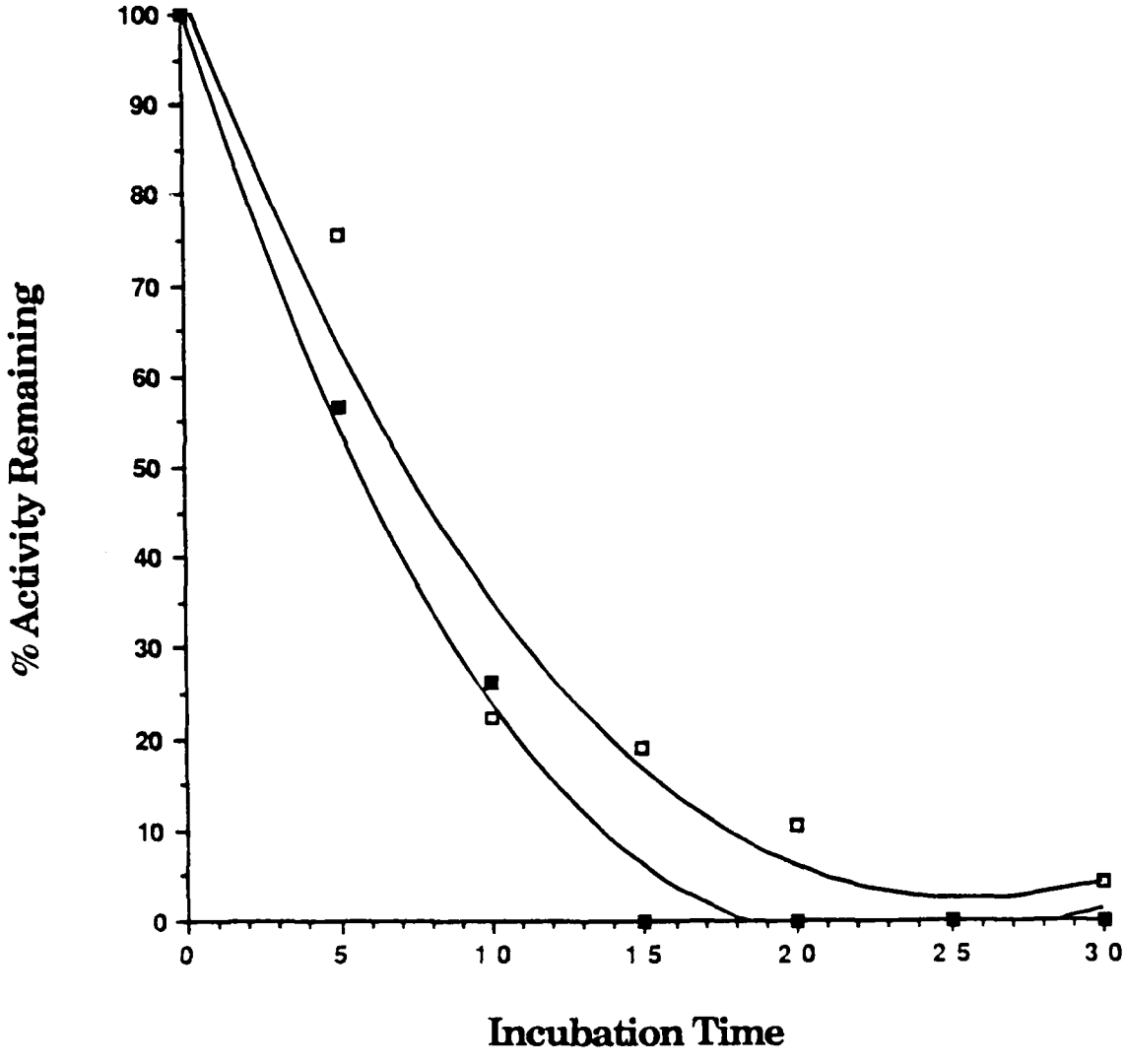


Figure 7. Thermal inactivation of the L. mexicana DNA polymerase. Aliquots of Fraction IV (250µl each) were incubated at 45°C for up to 30 min in the presence (▫) or absence (■) of BSA. The lines were fitted using a third order polynomial equation. The assay conditions were as described in Materials and Methods.



Isolation of a LMW DNA polymerase from *L. mexicana.*

A LMW DNA polymerase from *L. mexicana* has been isolated and partly characterized using the same purification protocol as described above for the HMW DNA polymerase.

Enzyme Assays

Activated DNA dependent enzyme activity was assayed in a final volume of 65 µl for 30 min at 35 °C. The reaction mixture contained 20 mM CHES (pH 9.0), 50µM each of dATP, dCTP, dGTP, 1 mM dithiothreitol, 12 mM $MgCl_2$, 125µg/ml activated calf thymus DNA, 100µg/ml bovine serum albumin, 10µL of enzyme sample, and 40 nM [*methyl*-^{3}H]dTTP at 350-400 CPM/pmole. The reaction was stopped by spotting 50µl on GF-C or G4 glass filter disks numbered with a laundry marker pen and collecting the filters in 5% (w/v) trichloroacetic acid, 1% (w/v) sodium pyrophosphate, and processed for counting as previously described [8]. The radioactivity on the dry disks was determined by counting in Bio-Safe II liquid counting cocktail in a liquid scintillation counter. One unit of DNA polymerase is defined as the incorporation of 1 pmol of [*methyl*-^{3}H]dTMP into acid insoluble material in 30 min at 35 °C.

Results

The LMW enzyme eluted from the denatured DNA Cellulose column at higher ionic strength (0.3 M) than the HMW enzyme (0.15 M). Assay of the DNA cellulose fractions indicate that this LMW enzyme constitutes about 20 % of the total DNA polymerase activity detected. Chromatography on a Sephacryl S-200 HR column resulted in the enzyme eluting with an apparent molecular weight of 45 kDa. Sucrose solution density gradient analysis showed this enzyme to have a sedimentation coefficient of 3.4 S. The buffer and pH optimum were determined as CHES 9.0.

Exposure of this LMW DNA polymerase to the sulfhydril blocking reagent *N*-ethylmaleimide showed this enzyme to be partly resistant, with 14 % detectable activity at a concentration of 15 mM NEM. Resistance to NEM is a characteristic of the mammalian DNA polymerase β. This LMW enzyme was also active in the presence of NaCl and KCl. The IC_{50} of NaCl and KCl were determined to be 165 and 180 mM, respectively. The dideoxy analog of Thymidine triphosphate, ddTTP, was inhibitory to the enzyme with an IC_{50} of 6μM. Similar to the HMW enzyme, Suramin was the most potent inhibitor of the LMW DNA polymerase. A summary of active compounds against the LMW DNA polymerase is shown in Table 5. Table 6 shows a comparison of the effect of BuPdGTP and BuAdATP on the DNA polymerases of HeLa cells and *L. mexicana* cells.

TABLE 5. Compounds inhibitory to the LMW DNA polymerase.

COMPOUND	IC_{50}
Suramin	3μM
ddTTP	6μM
Linoleic Acid	20μM
Linolenic Acid	20μM
Arachidonic Acid	20μM
BuAdATP	< 50μM
Berenil	50μM
Hemin	60μM
AraCTP	500μM
COMDP	> 1000μM

Table 6. Comparison of Enzyme Sources

Source	BuPdGTP	BuAdATP
HeLa polα	1 nM	< 10 nM
L. mexicana polα	125μM	150μM
HeLa polβ	Resistant	100μM
L. mexicana polβ	160μM	< 50μM

Progress on the
Partial Purification and Characterization of Two Isozymes of
S-Adenosylmethionine synthetase

Two isoforms of *S*-Adenosylmethionine (SAM) synthetase, α and β, have been isolated and partially purified from *Leishmania mexicana* 227 cells. The purification procedures are as follows: the crude enzymes were isolated by suspending 18 g of pelletted wet cells in 18 ml buffer A containing 20 mM potassium phosphate pH 7.5, 0.1 mM EDTA, 1 mM DTT, 0.02% NaN_3. The cells were sonicated 3x for 15 seconds, and then protease inhibitors were added. The broken cell suspensions were centrifuged at 4° C for 90 minutes at 18,000 rpm in a SS-34 rotor (39,000 x *g*). The supernatant fluid was passed through glass wool to remove lipids, then fractionated with ammonium sulfate (75% saturation). The precipitated proteins were dissolved in 13 ml of buffer A and then dialyzed at 4° C against 200 volumes of the same buffer. The dialyzed enzyme (18 ml) was applied to a DEAE-cellulose column and the above buffer was passed through the column until 280 nm-absorbing material in the eluate was negligible. Enzyme was eluted using a 230 ml linear gradient of 0-0.4 M KCl in buffer A. A typical elution pattern of *S*-Adenosylmethionine synthetase is shown in Figure 8. Fractions with activity of 50% of maximum or greater were kept. The pooled DEAE-cellulose eluate (75 ml) was brought to 75% saturation with solid ammonium sulfate. The precipitate was collected by centrifugation (39,000 x *g*, 30 min.) and redissolved in a minimal volume (3 ml) of buffer B containing 20 mM potassium phosphate pH 7.0, 0.2 M KCl, 1 mM DTT, 0.1 mM EDTA, 20% (v/v) glycerol, 0.02% NaN_3. The enzyme suspension was concentrated and dialyzed by Centricon-10 using buffer B, then chromatographed on a column (1.5 x 75 cm) of Sephacryl S-200. As Figure 9 shows, two forms (α and β) of SAM-synthetase were eluted. The purification procedures,

involving ammonium sulfate fractionation, DEAE-cellulose chromatography, and Sephacryl S-200 gel filtration result in 221-fold and 215-fold purification of SAM-synthetase α and β, respectively (Table 7).

Thermal stability of *S*-Adenosylmethionine synthetase α and β has also been studied. Sephacryl S-200 purified isozymes were concentrated by Centricon-10 and placed at low temperatures. Both α and β isozymes are stable for 6 days at -70° C, -20° C, and 4° C (Table 8). When the isozymes were stored at -70° C for 4 months, the α enzyme lost 15% of activity, and the β enzyme lost 31%. Greater loss of activity was observed when both isozymes were stored at -20° C for 4 months. Heating to 45° C resulted in a loss of activity of 17% and 66% for the α and β enzymes, respectively. No activity remained for the α enzyme when heating at 75° C for 30 minutes, and for the β enzyme when heating at 55° C for 30 minutes. These data indicate that the β enzyme is more heat labile than the α enzyme. Both isozymes were stored in concentrated form at -70° C, as they are more labile in diluted form.

The apparent molecular masses, estimated by Sephacryl S-200 gel filtration, are 91 kDa for α and 44 kDa for β using thyroglobulin (670 kDa), γ-globulin (158 kDa), ovalbumin (44 kDa), myoglobin (17 kDa), and cyanocobalamin (1.35 kDa) as marker proteins. A pH optimum of 8.0 was found for α SAM synthetase and for the β enzyme a broad pH optimum of between 8.2 and 9.0 was found (Figure 10 and Figure 11). The effect of divalent cations on the enzyme's activity have been studied. Maximal activity is obtained with Mg^{2+} for both α and β enzymes. Mn^{2+} can replace Mg^{2+} for both α and β enzymes with lower relative activity. The effect of other divalent cations such as Ca^{2+} and Fe^{2+} on α and β isozymes are distinct. Ca^{2+} gives no activity to the α enzyme while it gives 52% relative activity to the β enzyme. Fe^{2+} gives 30% relative activity to the α enzyme while it gives only 2% relative activity to the β enzyme (Figure 12). The effect of methionine and ATP concentration on the reaction rate of *S*-Adenosylmethionine synthetase α and β has

been investigated. Both α and β enzymes follow Michaelis-Menten kinetics with apparent K_m values of 1.4 mM and 0.8 mM for ATP and 1.1 mM and 0.2 mM for L-methionine.

We have also studied the inhibitory effect of dimethylsulfoxide and some amino acid analogues on activities of both α and β *S*-Adenosylmethionine synthetase. The results are reported in Table 9 and Table 10. Among the compounds assayed, tripolyphosphate, which is a powerful inhibitor of SAM synthetase from mammalian sources, is most active. L-cis-AMB, a methionine analogue, which was characterized as an inhibitor of both normal and tumor-derived rat liver SAM synthetase is also an effective inhibitor of both the α and β isozymes. Cycloleucine, a known inhibitor of *S*-Adenosylmethionine synthetase from many sources, is less effective, with a higher IC_{50} value. On the other hand, dimethylsulfoxide, a known stimulator to mammalian SAM synthetase, gives a significant inhibition on α, and less on the β enzyme (Table 9). Other compounds tested, showing inhibition at higher concentrations or showing no inhibition, are listed in Table 10.

Table 7. Summary of purification of S-Adenosylmethionine Synthetase α and β from Leishmania mexicana 227 promastigotes.

	Protein (mg)	Total Activity (units)	Specific Activity (units/mg)	Yeild (%)	Purification (n-fold)
(1) Crude Extract	504	17073	33.75	100	1
(2) Ammonium Sulfate Fractionation	495	83952	170	492	5
(3) DEAE - Cellulose	39	95625	2450	560	73
(4) Sephacryl S-200					
SAM - Synthetase α	3	22342	7450	131	221
SAM - Synthetase β	1	7398	7250	43	215

Table 8. Thermal Stability of S-Adenosylmethionine Synthetase α and β.

Storage Conditions	% Original Activity[a]	
	Isoform α	Isoform β
Freezing (-70°C)		
6 days	100	100
4 months	85	69
Freezing (-20°C)		
6 days	100	100
4 months	77	57
Refrigeration (4°C)		
6 days	100	100
Heat (°C)[b]		
45	87	34
55	58	0
65	4	--
75	0	--

[a] Relative to activity when frozen at -70°C for up to 6 days.

[b] Samples were heated for 30 minutes at indicated temperature.

Table 9. Inhibition of *S*-Adenosylmethionine synthetase α and β by substrate and product analogues and dimethylsulfoxide.

Compound[a]	IC_{50} (mM), Isoform α	IC_{50} (mM), Isoform β
Tripolyphosphate	0.120	0.098
L- *cis*- AMB[b]	0.125	0.10
Cycloleucine	2.84	2.98
Dimethylsulfoxide	9% (v/v)	22% (v/v)

[a] All compounds commercially available except where noted.

[b] L- *cis* -AMB (L-2-amino-4-methoxy-*cis* -but-3-enoic acid) is a gift from Dr. J.R. Sufrin

Table 10. Compounds tested showing Low inhibition or No inhibition of *Leishmania mexicana* *S*-Adenosylmethionine synthetase.

Compound[a]	Concentration (mM)	Maximum Inhibition, α(%)	Maximum Inhibition, β(%)
Methapyrilene HCl[b]	0.1	28	10
5-Azacytidine	2.5	0	0
DL-Homocysteine	10.0	42	33
DL-Homoserine	50	38	39
L-Homocysteine Thiolactone	50	16	51
O-Methyl-DL-serine	50	8.6	25
S-Adenosyl-L-homocysteine	1.0	4	0

[a]All compounds commercially available except where noted.

[b]Methapyrilene hydrochloride, an antihistamine, is a gift from Dr. W. Lijinsky

Figure 8. Chromatography of S-Adenosylmethionine Synthetase on DEAE-Cellulose

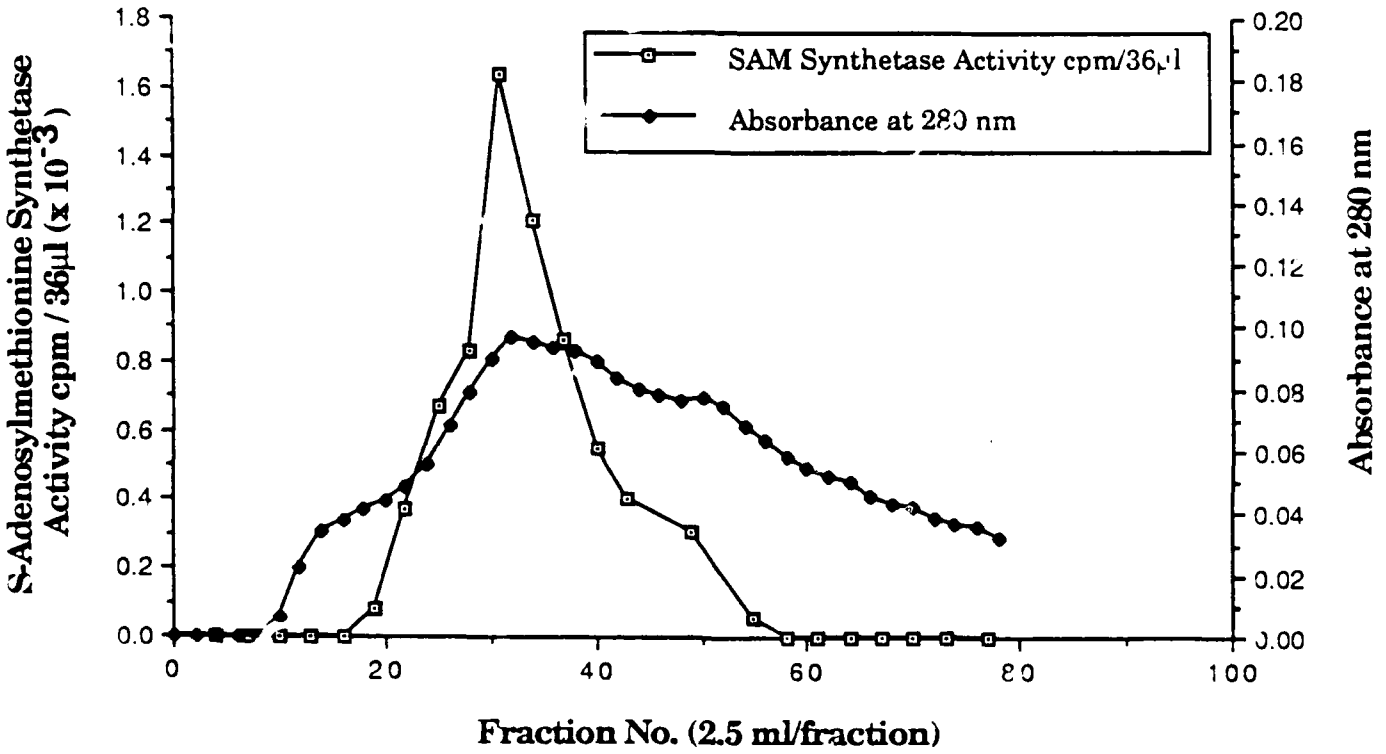


Figure 9. Elution Pattern of S-Adenosylmethionine Synthetase α and β from Sephacryl S-200 HR

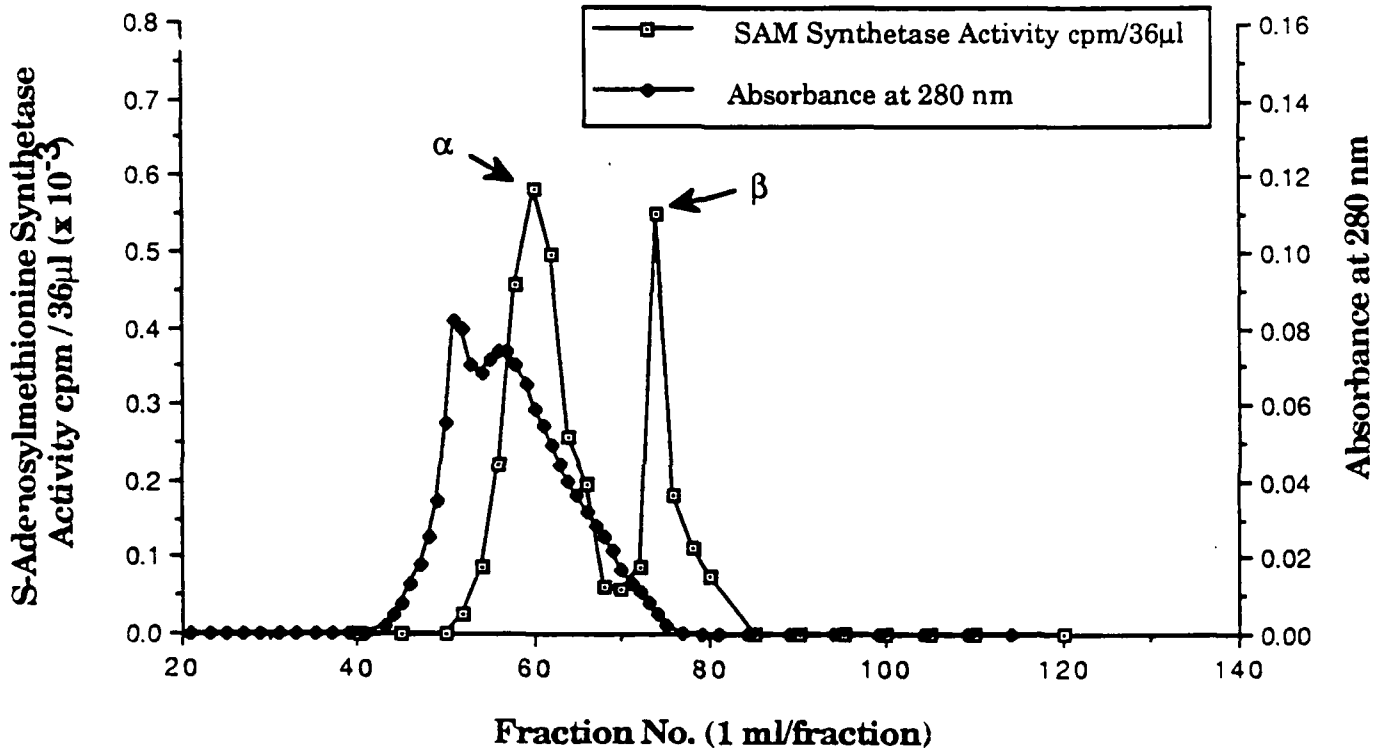


Figure10. pH Optimum of α S-Adenosylmethionine Synthetase

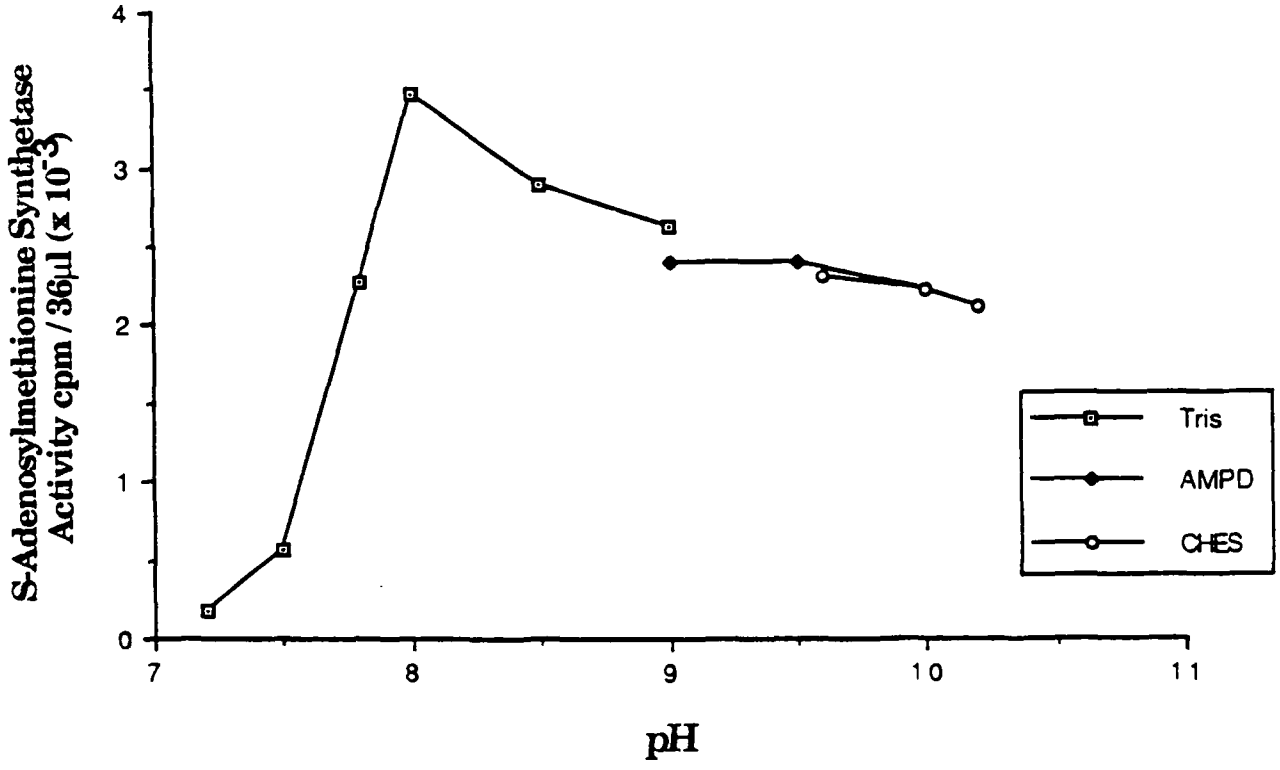


Figure 11. pH Optimum of β S-Adenosylmethionine Synthetase

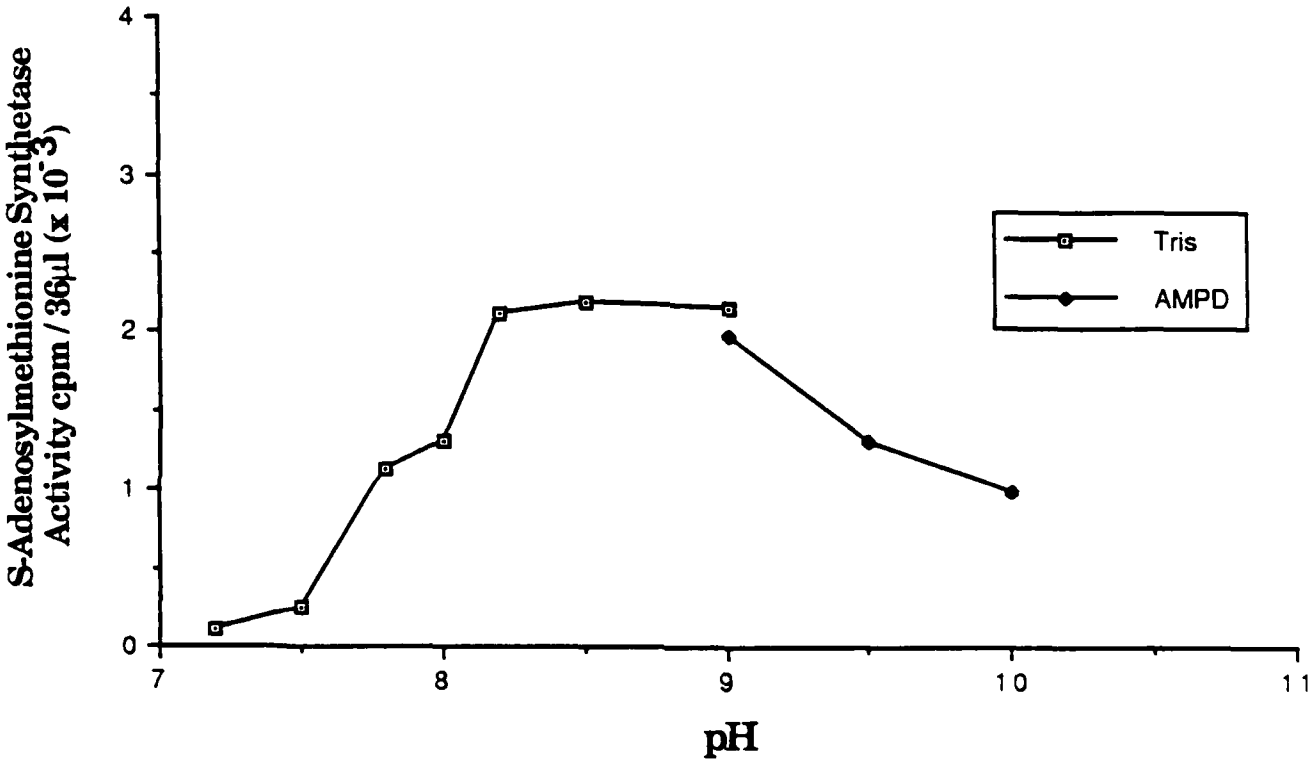
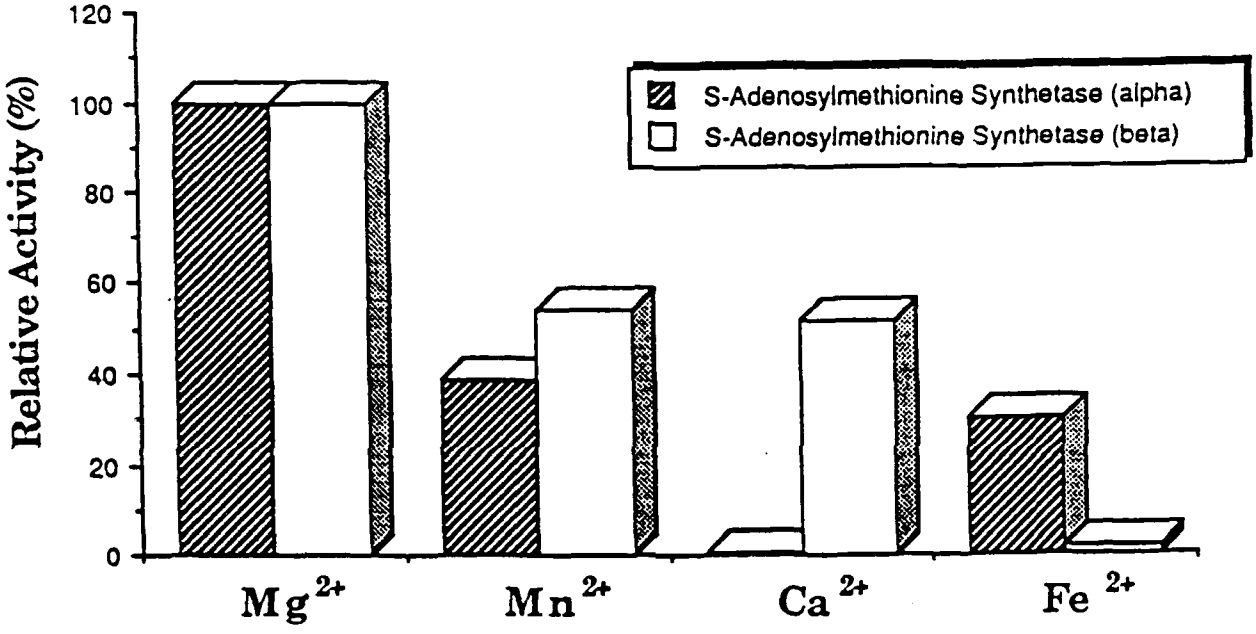


Fig.12. Effect of Divalent Cations on α and β S-Adenosylmethionine Synthetase Activity



Assay in the presence of 20mM of each cation.

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