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# PURIFICATION AND CHARACTERIZATION OF MYCOBACTERIUM SMEGMATIS ADENOSINE DEAMINASE ENZYME

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**ABSTRACT**
Purification and characterization of Mycobacterium smegmatis adenosine deaminase enzyme was carried out by usual methods such as purification by heat treatment, ammonium sulphate precipitation, ion exchange chromatography and gel filtration chromatography. The enzyme was purified up to 112 folds, showed homogeneity and its molecular weight was found to be approximately 45 KDa on SDS PAGE. The enzyme had slightly higher affinity to adenosine and than 2'deoxyadenosine as substrate. The Km values for adenosine and 2' deoxyadenosine as substrate were $1.2 \times 10^{-5}$ M and $1.4 \times 10^{-5}$ M respectively. It has maximum activity in alkaline medium i.e. between PH 8 – 8.5. Sulfhydryl reagents showed mild inhibitory effect whereas heavy metals markedly suppressed the enzyme activity. ATP and AMP regulate the enzyme activity at their respective microbial concentration. Xanthine also suppressed the enzyme activity by 48% but inosine and urate had no effect.

## INTRODUCTION

Mycobacterium Smegmatis is non-pathogenic fast growing acid fast and saprophytic bacteria. It is strictly aerobic in nature and has resemblance with the pathogenic strain Mycobacterium Tuberculosis, the causative agent of human tuberculosis and most of the protein cross reacts with antibodies against proteins from other microbacterias. Adenosine deaminase (amino hydrolase EC 3.5 – 4.4) converts adenosine into inosine with the removal of ammonia. The enzyme is distributed both in animal and microbial Systems. Interest in this enzyme first centered on its congenital deficiency in patients with Severe Combined Immunodeficiency (SCID) syndrome an autosomal recessive disorder in which both thymus derived and bone marrow derived lymphocytes are sparse [1-3]. SCID is due to accumulation of dATP which allosterically Inhibits ribonucleotide reductase and thereby deplets dCTP , a DNA Precursor of cell . Adenosine deaminase activity was studied in mycobacterial infections and high level in serum and peripheral blood Lymphocutes of pulmonary tuberculosis, leprosy and cerebrospinal fluid of tuberculous meningitis cases was observed. It is because of active participation of T- lymphocyte mediated immune response. This high level of enzyme tried to use as a diagnostic marker of tuberculosis patiens[3-7]. Whether this high level of enzyme in patients is totally from human origin or is also from mycobacterial origin, is not studied so far. The present study was undertaken for purification and characterization of adenosine deaminase. Thus it Was planned that these studies would give not only an insight in metabolic activity of the mycobacteria also might provide useful information regarding its role in cell growth, proliferation and early diagnosis and treatment of mycobacterial infection. In this study The purification protocol is followed as sonication of the cells, heat treatment,ammonium sulphate precipitation, ion exchange chromatography and gel filtration chromatography[8].

## MATERIAL AND METHOD:

Materials, chemicals used are of AR quality and purchased From M/s Sigma Chemical Company (USA), BDH, SRL,and Ranbaxy ( India).

Mycobacteria smegmatis TMC 1546 is originally obtained From the Trudeau Mycobactrial Culture Collections, Denver Colorado, USA.

**Cell culture, harvesting and maintenance**

The cells of mycobacterium smegmatis is grown on shake culture using Youmans and Karlson liquid culture medium[9]. In this media $NH_4 ( SO_4 )_2$ is used as nitrogen source in place of L- Aspargin at equimolar basis to get the maximum yield of enzyme[10.] The first subculture was used ( 0.1 ml of log phase cells) to inoculate for further subculture. The inoculum used had an option density between 2.0 and 3.0 and mid log phase cells were used for metabolic studies. The cells were harvested by centrifugation at 5000 rpm at 4ºC and washed twice with chilled distilled water and again centrifuge it. Cells dried between the folds of Whatman No. 1 filter paper and stored at -20ºC until further use. The mycobacterial culture was maintained as stock on Lowenstain Jenesen( LJ) medium and sub-cultured after every two to three month. The LJ agar slants wee stored at -20ºC after sufficient growth had been achieved [11] .

25 gm of mid log phase cells were taken with 50 ml 50 mM Tris HCL buffer pH 7.6 in ice bath and sonicated by using Vibronic sonicator (50 Khz at 250Volts). The cell extract was centrifuged at 1800 xg for one hour at 4ºC

and supernatant was used for enzyme assay by spectrophotometric method[12] and protein estimation by Lowrys et al[13]. Ethylene glycol (20% of the total volume) was added to the supernatant.

**Heat treatment**

The supernatant fluid containing 20% ethylene glycol was heated for 8 minute at 60ºC with continuous shaking allowed to cool and centrifuged at 18000xg for 45 minutes to remove precipitated matter. The supernatant was used for estimation of protein content and ADA activity.

**Ammonium sulphate precipitation (33% to 65%)**

11.64 g ammonium sulphate( 19.4g / 100ml) was added to the supernatant to bring 35% saturation. After allowing to stand for 30minutes, it was centrifuged at 18000 xg for 45 minutes and the precipitated proteins were discarded. The supernatant was brought to 65% saturation by adding 11.04 gm ammonium sulphate and centrifuged at 18000 xg for 45 minutes. The precipitated was dissolved in a small volume of 50 mM Tris HCL buffer, pH 7.6 containing 20% of ethylene glycol and dialyzed at 4ºC against the same buffer. This 65% fraction was used for subsequent purifications enzyme and protein estimation.

**Ion exchange chromatography**

4gm of DEAE cellulose 52 (whatmann 10) preactivated ion exchanger was dissolved in tris HCL buffer ( 2M, pH 7.6) and washed twice with same buffer of 50mM( pH 7.6) . A column of ion Exchanger prepared and equilibrated with Tris HCL buffer, 50mm (pH 7.6) containing 20% ethylene glycol. The ammonium sulphate precipitated protein was dialyzed against tris HCL buffer (50 mm, ph 7.6 containing 20% ethylene glycol ). The dialysed protein sample was charged to ion exchange column and unbound protein was washed with tris HCL buffer (pH 7.6, 50mm, 20% ethylene glycol). The proteins were eluted with sodium chloride gradient (0.1 -1m ) using gradient mixer. Fractions of 3 ml were collected at a flow rate of 20 ml per hour and monitored for protein and ADA activity. Fractions containing maximum enzyme were pooled and concentrated by dialysis bags kept in PEG 15000 -20000 MW.

**Gel filtration chromatography**

Sephadex G-150 was allowed to swell in tris HCL buffer ( 0.2 m, pH 7.6) for 24 hours. After deareation the slurry was poured into two different size columns (1.5 x 22 cm and 2.5 x 90cm).the columns were equilibrated with eluting buffer ( 50 mM, pH 7.6, 0.15M NaCl and 20% ethylene glycol) and void volume was determined by using 0.2% blue dextran ( 0.5 ml) through the Column. The dialysed fraction obtained from ion exchange Chromatography loaded on the small column ( 1.5 x 22cm) and eluted with eluting buffer, fractions of 1 ml volume were collected at a flow rate of 10ml/ hour and fractions containing higher activity were pooled and concentrated using PEG 15000 – 2000 MW. The concentrated fraction loaded in the second column

(12.5 x 90 cm size) and eluted with buffer at a flow rate of 30 ml /hour, enzyme activity and protein content is estimated in fractions.

**Characterization of enzyme**

Enzyme was characterized by determining its optimum pH, temperature, molecular weight, substrate specificity, Km value, effect of substrate analog and inhibitors[8] as described method by using different range of temperature, pH, different inhibitors, substrate analogs, nucleosides and nucleotides.

Molecular weight of adenosine deaminase was determined by using sodium dodecyl sulphate polyacrylamide slab gel electrophoresis ( SDAS PAGE) with different marker protein [14] were denatured in 0.01 m phosphate buffer pH 7.0 at 100ºC for 5 minutes. The electrophoresis was carried out for two hours at 30 A current at 4ºC and gel was stained with commassie blue for 4 hours and destained mechanically in methanol and acetic acid ( 5:7:5 v/v) mixture. The mobilities of the different marker proteins were plotted against the log values of their molecular weights.

**Enzyme assay**

Colorimetric method[15]**.** This method based on estimation of ammonia.

liberated from adenosine by the action of Adenosine deaminase enzyme ammonia is determined by the Chaney and Marbach modification of the Berth lot reaction, in which ammonia forms an intensely blue indophenol with sodium hypochlorite and phenol in alkaline solution , sodium nitroprusside acts as catalyst. The ammonia concentration is directly proportional to the absorbance of the indophenol. The reaction catalyzed by ADA is topped at the end of the incubation period by the addition of the phenol nitroprusside solution.

The reaction mixture containing 1ml phosphate buffer (pH 7.4, 50 mm) adenosine ( 21 mm) and enzyme ( 50 µl) was incubated for one hour at 34ºC in water bath. The reaction was stopped by adding 3ml alkaline nitroprisside followed by addition of 5µl enzyme to the blank and 3 ml of alkaline hypochlorite colouring reagent. Mixed and incubated for 30 m at 37ºC and extinction were measured against distilled water as blank at 628nm. 1ml of ammonium sulphate solution ( 75 µm ammonia) was used as standard. One unit of enzyme is defined as the amount of enzyme that liberates 1 µm of ammonia per minute under the assay conditions.

**Spectrophotometric method**

This method is based on the decrease in optical density of assay mixture at 265 nm due to the conversion of adenosine to inosine. The amount of product formed was calculated by taking into molar extinction coefficient of adenosine as $8.5 \times 10^{-3}$. Reaction mixture contains 980 µl of Tris HCL buffer ( pit 7.6 80 mm, )10µl of buffered adenosine (5mM) and 10 µl of enzyme. Optical density was measured after every minute for 5 minutes and average decrease in optical density was calculated . One unit of enzyme was defined as the amount of enzyme catalyzing the deamination of 1µm of adenosine per minute[4,12 .]

**RESULTS AND DISCUSSION**

The enzyme was purified 111.71 fold with total activity 123.33 and Specific activity 41.11 units per milligram of protein (Table 1). Single band was observed on S.D.S. PAGE electrophoresis (Fig. 1A and 1B) having approximately 45KDa molecular weight. Different molecular weights of ADA from different sources like Azotobacter vinelandii Micrococcus sodoneisis, Escherichia coli, were reported as 66 KDa 120 KDa, and 29 KDa respectively[8,17,18.] Molecular weight of human

RBC, ADA was reported As 38 KDa[16] while from rabbit intestine had molecular weight of 26.5 KDa and 32KDa for small and large forms respectively. No isoenzyme has been reported from microbial sources. The optimum pH was found to be between 8 to 8.5 (Fig.2). the optimum pH of ADA from various had been reported. In Azotobactor vinelandii it was 7.2[8] .E. coli ADA showed a broad range of pH between 6.9 to 8.5 [17]. In human tissue optimum pH for ADA activity was shown to be 7.4 for large and small forms, 5.5 in case of intermediate form of the enzyme[16] . The optimum temperature for the enzyme was found to be 50ºC when the assay was performed for 5 minutes (Fig.3).

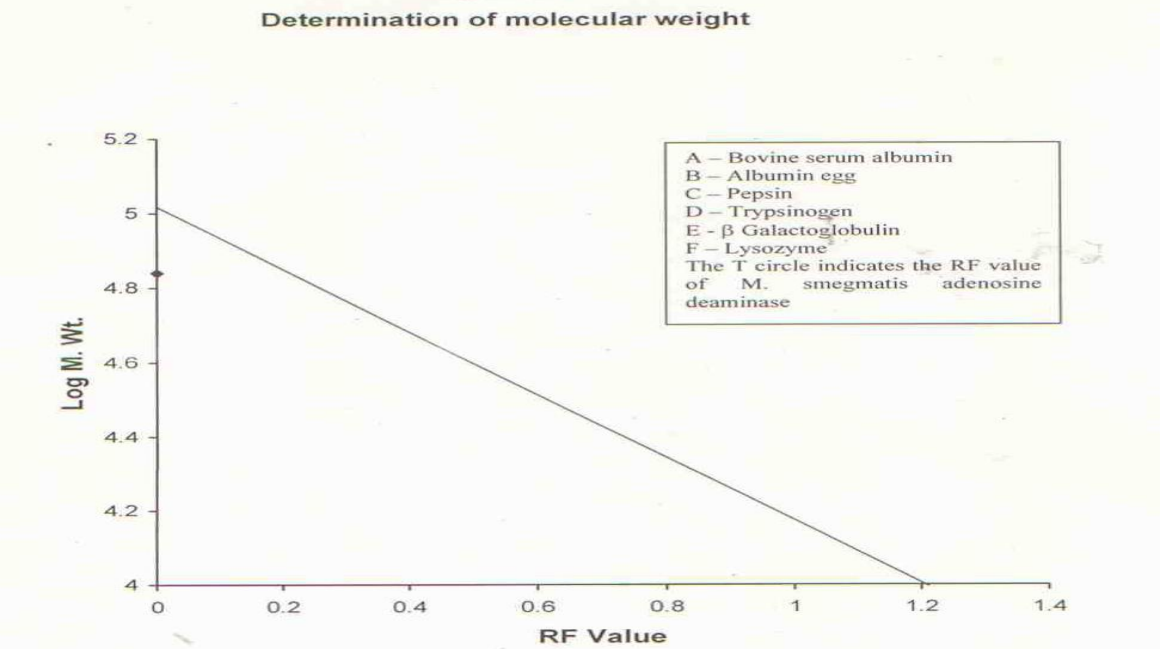


**Figure 1A-** Determination of molecular weight:- Determination of molecular weight (Plot of log molecular weight versus RF value).Calibration curve for sodium dodecyl sulphate polyacrylamide gel eletrophoresis of marker protein and adenosine deaminase.

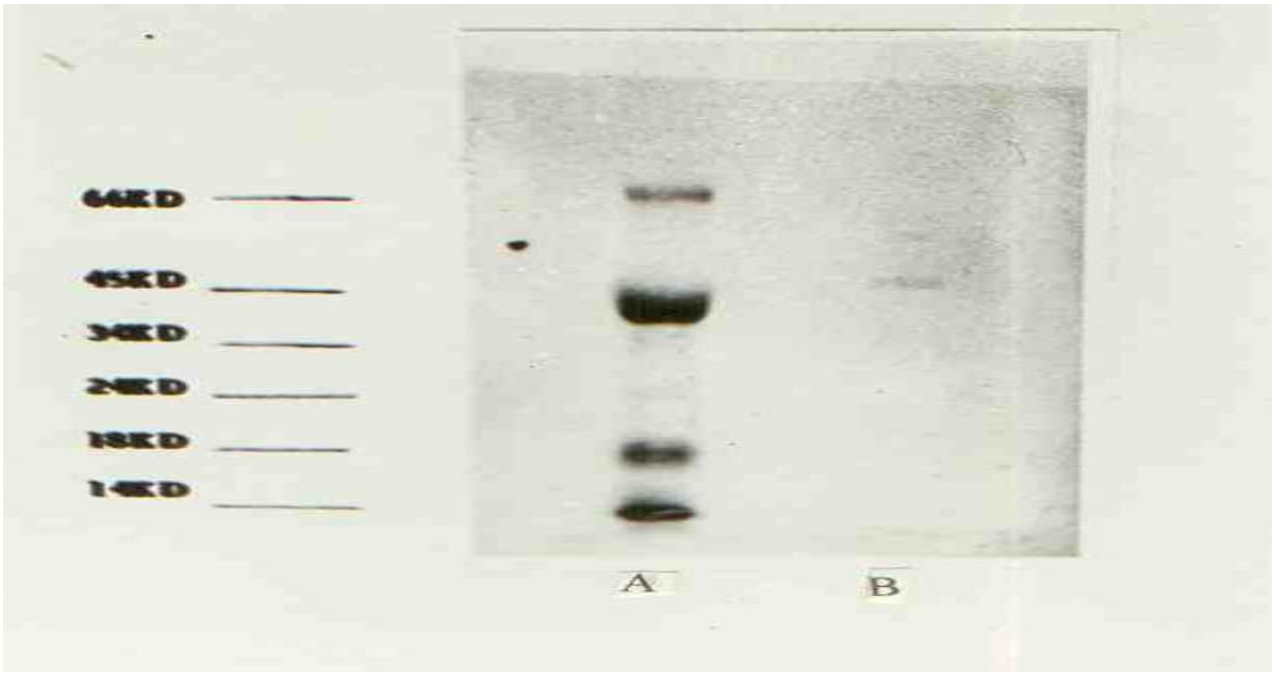


**Figure 1B:-** SDS-POlyacrylamide gel electrophoresis of purified M. smegamatis ADA with marker proteins with different molecular weight using commassie brilliant blue staining.

| | | | | |
|---|---|---|---|---|
| Lane A- | BSA | - 66 KDa | Egg albumin | -45 KDa |
| | Pepsin | -3.47 KDa | Trypsinogen | -24 KDa |
| | β-galactoglobulin | - 18.4 KDa | Lysozyme | - 14.3 KDa |

Lane B- Purified Adenosine Deaminase – approx. 45 KDa.

**TABLE .1** Result of purification of M. smegmatis adenosine deaminase

| Step | Total Protein ( mg) | Total activity (unit) | Specific activity units/ mg of protein | Purification fold |
|---|---|---|---|---|
| Crude extract | 950 | 350 | 0.368 | 0 |
| Heat treatment | 108 | 200 | 1.80 | 4.89 |
| 35%-65%ammonium Sulphate precipitation | 34.4 | 160 | 4.65 | 12.63 |
| DEAE cellulose Chromatography | 22.9 | 155 | 7.07 | 19.21 |
| Sephadex G-150 ( 1.5 X 2mm) | 8.0 | 145.80 | 18.23 | 49.54 |
| Sephadex G-150 (2.5 X90mm) | 3.0 | 123.33 | 41.11 | 111.71 |

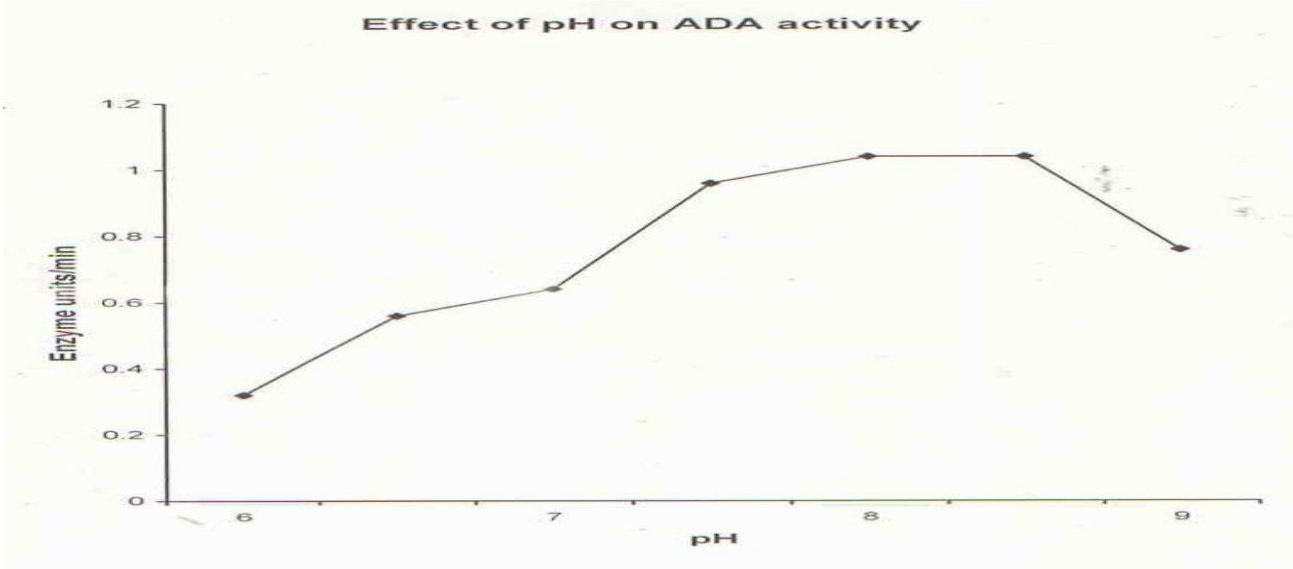


**FIGURE 2** – Effect of pH on ADA activity:- The enzyme assay was carried out by spectrophotometric method at pH 6 to 9 of Tris HCL buffer (50 mM). The assay mixture contained 5 mM adenosine, 1 unit enzyme in Tris HCL buffer (50mM).

**TABLE 2.** Study of substrate specificity of adenosine deaminase

| Substrate | Aelative velocity | Activity unit / min |
|---|---|---|
| Adenosine | 1 | 0.89 |
| 2'Deoxyadenosine | 1 | 0.88 |
| Adenine | 0 | 0.0 |
| Guanosine | 0 | 0.0 |
| Cytidine | 0 | 0.0 |
| Formycin A | 0 | 0.0 |

The assay mixtire of 1ml contained 0.90 unit of enzyme in 50 mM Tris HCL buffer pH 7.6 and subatrate / analog 5mM. the enzyme assay was carrid out by spectrophometric method .

**TABLE 3.** Study of effect of substrate analog on adenosine deaminase activity

| Substrate | Activity | % of inhibition |
|---|---|---|
| Adenosine (control) | 0.94 | control |
| Adenine | 0.917 | 2 |
| Guanosine | 0.6 | 36 |
| Cytidine | 0.917 | 2 |
| Formycin A | 0.89 | 5 |

Assay mixture of one ml contains 0.95 unit/min of enzyme in Tris HCL buffer pH 7.6 with 10 µl of 5 mM adenosine as substrate analog ( 5 mM ). Enzyme assay was carried out by spectrophotometric method.

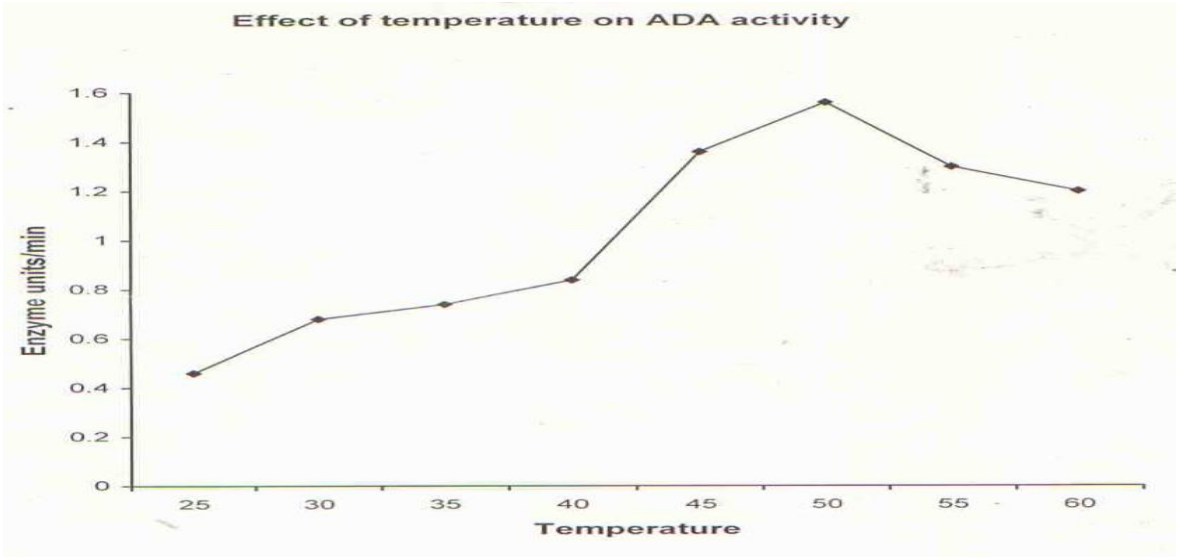


**FIGURE 3.** Effect of temperature on ADA activity: - The enzyme assay was carried out by spectrophotometric method at temperatures ranging from 25°C to 60°C for 5 minutes. The assay mixture (1 ml) contains 5 mM adenosine, 0.75 unit enzyme in Tris HCL buffer (50 mM; pH 7.6).

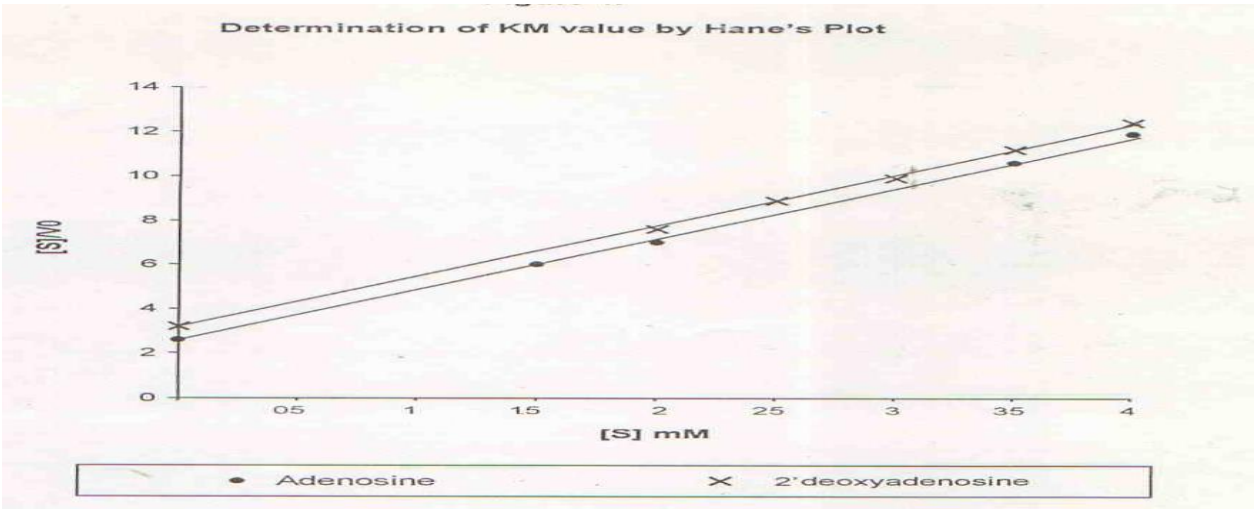


**FIGURE 4.** Determination of KM value by Hane`s Plot :- Hanes Plot of adenosine as a function of adenosine and 2`deoxyadenosine concentration Vs [s] / Vo.

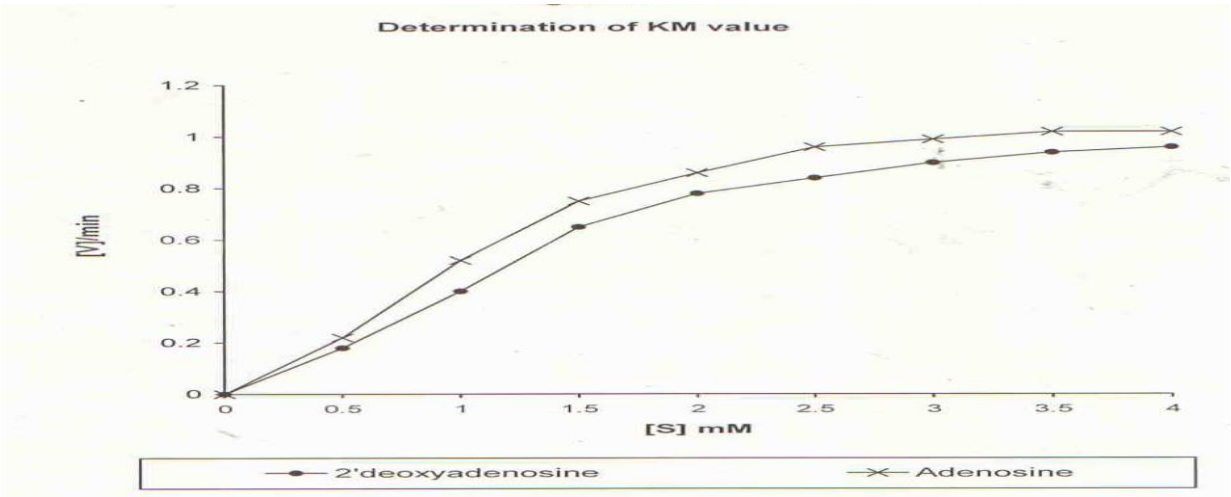


**FIGURE 5.** Determination of Km value :- The assay mixture contains 10µml of enzyme in Tris HCL (50 mM; PH 7.6) and adenosine / deoxyadenosine substrate 0.5 to 4 mM. Enzyme assay was carried out by spectrophotometric method.

**TABLE 4.** Study of inhibitors on adenosine deaminase activity

| Inhibitors | Concentration mM | Activity/ unit/min | % of inhibition |
|---|---|---|---|
| No. inhibitor (control) | - | 1.03 | 0.0 |
| *Metal ions:* | | | |
| $Zn^{+2}$ | 0.6 | 0.491 | 51.0 |
| $Hg^{+2}$ | 0.008 | 0.508 | 50.0 |
| $Ag^{+}$ | 0.08 | 0.508 | 50.0 |
| $Pb^{+2}$ | 0.5 | 0.489 | 51.0 |
| $Mg^{+2}$ | 0.5 | 1.030 | 0.0 |
| $Ca^{+2}$ | 0.5 | 0.050 | 0.0 |
| *Sulfhydryl reagents* | | | |
| Iodacetamide | 1 | 0.874 | 15.0 |
| N N'ethylnaleimide | 1 | 0.851 | 17.0 |
| P-hydroxymercuri- benzoic acid | 1 | 0.79 | 23.0 |

Assay mixture contained 5mm adenosine, 1 unit enzyme in 50 mM Tris HCL buffer pH 7.6 and inhibitor with concentration mentioned in the table. The enzyme assay was carried out by spectrometric method.

**TABLE 5.** Inhibitory effect of nucleotides on ADA

| Conc. (mM) | Percentage of inhibition | | | |
|---|---|---|---|---|
| | AMP | ADP | ATP | IMP |
| 1 | 36 | - | - | 0 |
| 2 | 80 | 29 | 21 | 0 |
| 3 | 100 | 33 | - | 0 |
| 4 | - | 42 | 45 | 0 |
| 5 | - | 100 | 0 | 0 |
| 6 | - | - | 71 | 0 |
| 7 | - | - | - | 0 |
| 8 | - | - | 100 | 0 |

Assay mixture contained 5mm adenosine, 1unit enzyme in 50 mM Tris HCL buffer ph 7.6 and nucleotides with concentration mentioned in the table. The enzyme assay was carried out by spectrometric method.

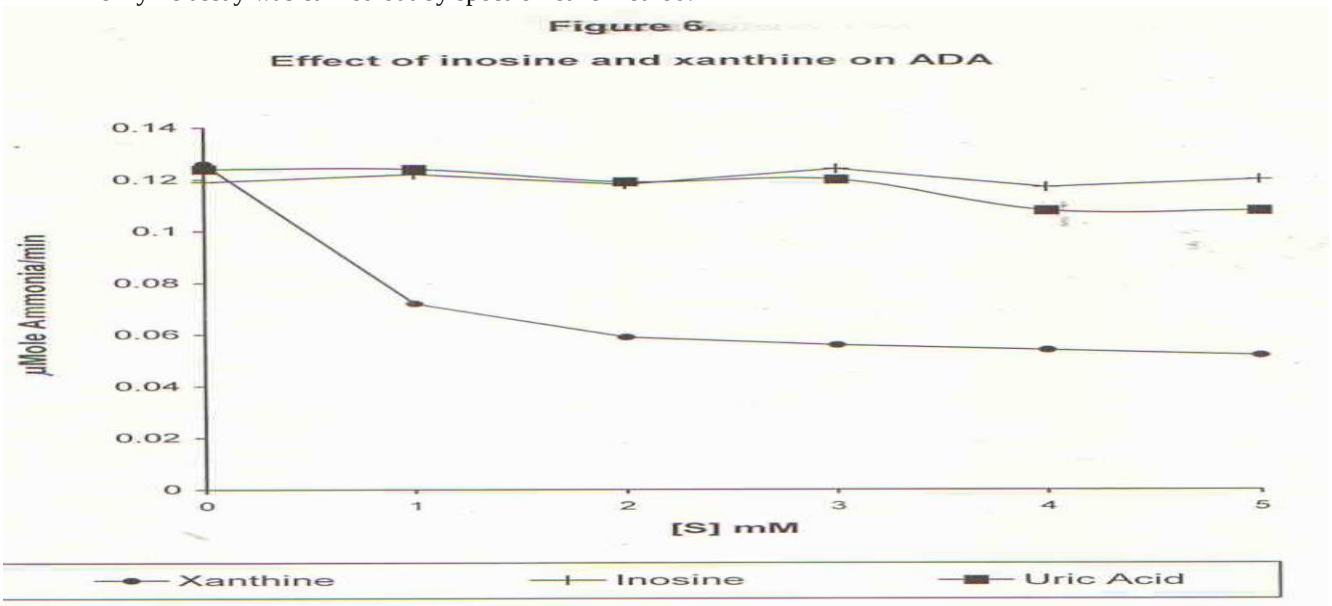


**FIGURE 6.** Effect of inosine and xanthine on ADA :- The assay mixture contained 5 mM of adenosine, 10µl enzyme and different concentrations of inosine, xanthine and uric acid. The assay was carried out by colorimetric method.

The substrate specificity of ADA was studied by using different substrate analogs in bacteria as well as in human system by different workers. Adenosine deaminase from Micrococcus sodonesis utilized 3'deoxyadenosine,

2'3'diacetyl adenosine and 6'chloropurineriboside as substrate besides adenosine and 2'deoxyadenosine[17] .
E. coli ADA utilized adenosine 2'deoxyadenosine, 6'methylaminopurine ribonucleoside, adenosine arabinoside, 3'deoxyadenosine and 2'3'di-deoxyademosine as substrate[18]. ADA from bovine intestine and chicken duodenum utilized 2, 6 diaminopurine riboside, 6-chloropurine riboside,
3, deoxyadenosine and adenosine a substrate[19]. Present study showed that M. smegmatis ADA can utilized adenosine as well as 2'deoxyadenosine as substrate( Table 2). Km value of human ADA for adenosine was reported to $5.2 \times 10^{-5}$ M[16]. Bovine brain ADA showed substrate specificity for Adenosine as well as 2'deoxyadenosine with Km $4 \times 10^{-5}$ M and $5.2 \times 10^{-5}$ M respectively [20].
The Km values of M. smegmatis ADA for adenosine and 2' deoxyadenosine as substrate were found to be $1.2 \times 10^{-5}$ M and $1.4 \times 10^{-5}$ M respectively indicating not much difference in the affinity of the enzyme for these two substrate In contrast Km values for E.coli ADA were reported 7.5 x M for 2'deoxyadenosine[18]. This showed that KM values varies with respect to sources of ADA and M. smegmatis ADA has high affinity for adenosine and 2'deoxyadenosine as compared to ADA from other sources. ( Fig.4,5)
When substrate analog were added in the reaction mixture (containing adenosine as substrate), it was found that only gaunosine inhibited the enzyme activity (Table 3). Like adenosine deamonase from E.coli, Azotobacter Vinelandii, Micrococcus sodonesis, Chicken duodenum enzyme and human erythrocyte[17] M. smegmatis, adenosine was also inhibited by heavy metals.( Table 4).
Studying the effect of adenine nucleotides on ADA activity of A. vinelandii[8] showed that ATP at 5 mM concentration had 71% inhibition and at physiological concentration of ATP (approximately 4 mM) there was 50% inhibition of the enzyme, but AMP as well as IMP did not show any inhibitory effect. In the present study with 4 mM ATP and ADP concentration similar effect was obtained with ADA of M. smegmatis, whereas with AMP complete inhibition of the enzyme was observed at 3mM concentration (Table 5).
Further the effects of metabolites of adenosine such as inosine, xanthine and uric acid on ADA were analyzed. Xanthine showed a 48% inhibition at 2 mM, whereas marginal inhibition (14%) was observed with uric acid. Inosine which is the product of ADA did not show any inhibition effect on the enzyme indicating no product inhibition on ADA activity of M. Smegmatis (Fig. 6.).
Inosine, the product of ADA with oxygen at $6^{th}$ position has no inhibition effect on ADA. Guanosine and xanthine which contain oxygen at $6^{th}$ position of purine ring like inosine inhibited ADA ( up top 50%), this could be due to the amino and oxy groups present at 2nd position ,whereas uric acid which has an extra oxy group at $8^{th}$ position as compared to xanthine is having only marginal inhibitory effect. AMP, ADP and ATP inhibits M.smegmatis ADA activity by 100% at 3mm, 5mm and 8mm concentration respectively. As compared to adenosine, these nucleotides posses one, two or three extra phosphate groups. As the number of phosphate groups attached to adenosine is increases and indicate that phosphate groups impair the binding of these nucleotides to the enzyme.
In view of the role of ADA in cell division and increased activity was seen at mid log phase of growth of M.smegmatis, further work using 2 amino or 2 oxy derivatives of purine and AMP may prove useful in developing drugs that might inhibit ADA and thereby mycobacterial growth.

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**Abbreviations**

SCID – Sever Combined Immunodeficiency Syndrome
ADA - Adenosine Deaminase
TMC – Trudeau Mycobacterial Culture