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Isolation of plasma membranes of *Trypanosoma vivax* and studies of adenosine metabolism in different cell fractions

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ABSTRACT: This study was designed to isolate the plasma membrane of *Trypanosoma vivax* and investigate how labelled adenosine is incorporated into other purines (bases, nucleosides and necleotides) in the membrane fraction, intact cells, cell homogenate and supernatant fraction.

Determination of marker enzyme activities during the purification of plasma membranes of *T. Vivax* showed that though adenosine triphosphatase (ATPase) activity was present, no sensitivity to oubain or oligomycin was observed. Activities of 5-nucleotidase, acid phosphatase and glucose-6-phosphatase were detected on the isolated membranes.

Analysis of the metabolic products of adenosine in intact cells, cell homogenate, supernatant fraction and isolated plasma membranes showed that radioactive adenosine was incorporated into adenine, hypoxanthine, inosine and total nucleotides in the following order of decreasing radioactivity: inosine < hypoxanthine < nucleotides < adenine.

Our data suggest that *T. vivax* has multiple routes of adenosine metabolism and the route taken depends on the concentration of adenosine.

In vitro assay of enzyme activities provided evidence for the presence of purine nucleoside phosphorylase and adenosine deaminase (among other enzymes of purine salvage) in *T. vivax.*

Key Words: Trypanosomiasis; *Trypanosoma vivax*; Adenosine metabolism; Plasma membranes.

Introduction

Chemotherapy for human and African trypanosomiasis is unreliable because of resistance, refraction and toxic as well as adverse side effects (Enanga *et al*., 1998). Eflornithine which is known to be one of the best drugs currently available has limitations of high cost (TDR, 1991-92; TDR, 1998). In spite of the numerous interdisciplinary research efforts aimed at solving the problem of trypanosomiasis, the problem remains intractable. One of the reasons is that our knowledge of the biochemistry of the trypanosomes is very limited. It is not an exaggeration to state that better understanding of the chemical nature of the parasites remains the key to the development of rational chemotherapy.

It is currently known that trypanosomes have multiple genes which code for the Variant Surface Glycoprotein (VSG). Therefore they may swith coat either by replacing the VSG gene in the active expression site by a different one or by activating another expressing site with concomitant silencing of the previously active one (Borst *et al*., 1997).

The basic knowledge that nucleic acids are the building blocks of genes should be a source of challenge to biochemists and molecular biologists in terms of sorting out the events at the molecular level which are expressed in proteins. This thought formed the basis of our earlier studies on adenosine transport in *Trypanosoma vivax*. (Okochi *et al*., 1983, 1995). Moreso, when it is known that adenosine is an important substrate for purine nucleotide synthesis in blood stream – forms of trypanosomes (james and Born, 1980). This is because this group of trypanosomes are known to lack the ability to synthesize the purine rind *de novo* (Little and Oleson, 1951; Williamson and Rolls, 1952; Fernandes and Castellani, 1958; Jaffe and Gutteridge, 1975).

This nutritional deficiency makes this study attractive, since like all functional cells, these species of trypanosomes, need a balanced supply of nucleotides as precursors of DNA, RNA and nucleotide coenzymes. The complete dependence on exogenously supplied purines for their nucleotide synthesis could make them vulnerable to attack by active anti-metabolites.

Among our findings in our earlier studies (1983, 1995) of adenosine transport is the inhibition of transport by some nucleoside analogues (coformycin and formycin A), which serve as pointers to the mechanism of metabolism. We have, in the previous studies, gained some knowledge about how adenosine gets into *T. vivax* cells but we have no idea about what happens to denosine beyond uptake level.

Our objectives in the present study, therefore, are:

1. to isolate and purify the plasma membrane of *T. vivax*
2. to investigate the metabolism of adenosine both on the membrane surface and the intracellular space, as well as in intact cells, in order to identify possible metabolic differences between the parasites and their mammalion host.

Materials and Methods

($U^{-14}C$) adenosine, adenine, inosine, hypoxanthine and ($2,5',8^{-3}H$) adenosine were purchased from Radio-chemical center, Amersham. Purine bases, nucleosides and nucleotides as well as all reagents (which were of analytical grade) were products of sigma chemical company.

Some of the coformycin used in these studies was kindly provided by Professor H. Umezawa of the Institute of Microbial Chemistry, Tokyo and Professor Hiroshi Oya of the Department of Parasitology, Juntendo University School of Medicine, Tokyo. The rest of the chemical was purchased from Meiji Seika Chemical Company, Tokyo.

Preparation of Trypanosomes

Heavily infected mice were sacrificed by scission of the jugular vein under ether anaesthesia and blood was collected in heparinized tubes. The parasites were isolated by column chromatography on DEAE-cellulose (type DE-52) according to the method of Lanham (1968), using cold phosphate-saline-glucose (PSG) buffer, pH 8.0 *T. vivax* cells were collected from the filtrate by centrifugation and washed twice with PSG buffer.

Preparation of the Plasma Membrane buffer (PMB) according to Voorheis et al., (1979).

The plasma membrane buffer was prepared with 2mm N-tris (hydroxymethyl) methyl-2-aminoethane sulfonic acid (Tes), 15mm KCl, 1mm ethyleneglycol-bis-(beta-amino ethyl ether) N.N-tetraacetic acid (EGTA), 0.1mm phenyl-methyl sulfonyl fluoride (PMSF) and 1mm B-mercaptoethanol. The stock solution of the buffer (which was ten times the concentrations mentioned above) was prepared by dissolving Tes, KCl and EGTA in 80ml deionized water. The pH was adjusted to 7.5 with 1N HCl or 1N KOH (prepared with deionized water), then made up to 100ml and stored frozen till use.

The PMSF was also prepared as a stock solution, dissolved in acetone and stored frozen separately. The working PMB solution was prepared by adding 10ml of the stock solution to 80ml of deionized water. In order to dissolve the PMSF in the working buffer, 10ml of deionized water was heated to boiling in a 150ml capacity beaker. Mixing with magnetic stirrer, 1ml of stock PMSF was added rapidly in the boiling

water. The acetone boiled off and the diluted buffer (90ml), was added quickly. The buffer was then cooled and stored frozen till needed. PMSF protects the membranes from being degraded by proteases (Voorheis *et al*., 1979).

Preparation of Plasma Membranes

The method for isolating the membrane was as described by Voorheis *et al*., (1979). *T. vivax* cells were suspended in 10ml ice-cold plasma membrane buffer (PMB), pH 7.5 and diluted with 30ml ice-cold deionized water in a prechilled beaker. This was then homogenized in a tight-fitting Dounce homogenizer. Immediately after homogenization, 2ml of 3M KCl was added. The homogenate was then centrifuged at 4°C at 750 x g for 15 minutes. The resultant pellet was discarded while the supernatant was spun down again for 30 minutes at 7,500 x g. The pellet resulting from this was washed once with PMB, pH 7.5 and suspended in 19ml of same buffer. 3ml of this fraction was removed for assay of membrane marker enzyme activities. The remainder was treated with deoxyribonuclease (DNAse), (0.1mg/10ml) for 5 minutes at room temperature. At the end of incubation, an aliquot was removed for the assay of marker enzyme activities.

The remaining portion of the DNAse treated material was again spun down at the same speed and resuspended in 2ml of 40% sucrose solution prepared in PMB, pH 7.5. 0.5ml of this was layered on discontinuous sucrose gradient of 40%, 50% and 60% (each layer being 2ml). Centrifugation was carried out at 86,000 x g for 3 hours at 4°C in Beckman ultracentrifuge (with SW 60 Ti rotor). At the end of centrifugation, the membrane fraction was collected in the 50% layer and diluted with 40ml OMB. This was washed by centrifugation at 7,500 x g for 15 minutes. The washing was repeated once and the membrane material was finally suspended in 3ml PMB and used for metabolic assays.

Preparation of cell fractions for metabolic studies

Freshly isolated and washed *T. vivax* cells were suspended in Tris-HCl buffer, pH 7.5, containing 10mM 2-mercaptoethanol (Krenitsky *et al*., 1968). This suspension was homogenized with the Karl-Kolb potter homogenizer, under ice, at 1,200 cycles/min. for 3 minutes. An aliquot of the homogenate was saved for metabolic studies while the remaining was spun down at 17,000 x g for one hour. The resulting supernatant was used for the required experiments.

Assay of marker enzyme activities in the isolated membrane fractions

The marker enzymes were assayed according to the methods outlined by Voorheis *et al*., 1979.

ATPase Activity

Total ATPase activity was assayed by the method of Tansky and Shorr (1953), by the determination of Pi liberated from ATP (45mM) in a buffer, pH 7.5, containing 7.5mM $MgCl_2$, 0.75 EDTA, 30mM histidine, 30mM glycyl-glycine, 1.5M NaCl and 0.3M KCl. To this was added 0.2mll of the post sucrose membrane preparation (0.2ml of the post sucrose membrane preparation) (0.35mg protein). The total reaction mixture was 0.6ml. Oubain and oligomycin-sensitive ATPases were measured in the presence of 1mM oubain and 80µg oligomycin respectively.

5'-Nucleotidase Assay

5'-nucleotidase was assayed by measuring Pi liberated from AMP in a reaction mixture containing the nucleotidase buffer, pH 7.2, consisting of 50mM tris-HCl, 100mM KCl, 5mM $MgCl_2$. 0.1M AMP and 0.2ml of membrane preparation in a total, volume of 0.6ml.

Acid Phosphatase Activity

Acid phosphatase activity was determined by estimating the quantity of Pi released from p-nitrophenol phosphate (5mM) in an assay mixture consisting of 50mM acetate buffer, pH 4.3, 0.3m. deionized water and 0.2ml of membrane. The method of Tansky and Shorr (1953) was used to estimate the activities of all

the marker enzymes based on the quantity of inorganic phosphate released per unit of time per milligram protein.

Studies on the pathways of adenosine metabolism in T. vivax.

A. Analysis of Metabolites of adenosine metabolism in intact cells, and isolated membranes:

For the experiment with whole cells, 0.2ml suspension of freshly isolated trypanosomes (4mg protein) were incubated in 0.2ml phosphate-saline-glucose (PSG) buffer, pH 8.0, containing 0.5uCi ($U^{-14}C$) adenosine (specific activity 549 uCi/umol) and 50uM unlabelled adenosine in a total volume of 400ul. The incubation period varied from 0 – 10 minutes.

The same buffer mixture was used in the other assays using cell homogenate (2.6mg protein), supernatant fraction (7 x 10^{-2}mg protein) and isolated membranes (3.5 x 10^{-}mg protein), respectively. The total reaction volume for both the cell homogenate and the supernatant fractions were 400ul while that of isolated membrane was 300ul. Incubation period for each assay was 30 minutes and all incubations were carried out at 37°C. Each reaction was stopped with 0.1ml of 5% per chloric acid, after which 0.1ml of 0.05m K_2CO_3 was added (Manander and Van Dyke, 1957).

B. Assay for some enzymes of Adenosine Metabolism:

(i) Assay for purine nucleoside phosphorylase activity:

Cell homogenate and isolated membranes were the sources of the enzyme (protein). Two types of assays were carried out. In the assay for the foreward reaction of the enzyme, inosine was the substrate while hypoxanthine was the substrate for the reverse reaction. The assay mixture in the presence of cold inosone contained 100ul of 50uM Tris-HCl, pH 7.5, 10mM 2-merca-ptoethanol 100uM cold inosine, varying concentrations of inorganic phosphate (0-5mM), 0.1ml of cell homogenate (2.58mg protein), 1.0uCi ($U^{-14}C$) inosine (specific activity 575 uCi/mmol), in a final volume of 200ul. The cold inosine (100uM) and 2-mercaptoethanol were dissolved in the buffer before its pH was adjusted to 7.5.

When the enzyme source was the membrane fraction 200ul of membrane preparations was used (7 x 10^{-3}mg protein) in a total volume of 300ul. Incubation was for 10 minutes at 70°C. The reaction was stopped with 0.1ml of 5% perchloric acid after which the same volume of 5% KOH was added.

In the second experiment in which hypoxanthine was the substrate, the assay procedure was the same except that the reacting mixture contained 100uM unlabelled hypoxanthine, 0.5uCi ($U^{-14}C$) hypoxanthine (specific activity 55mCi/mmol), varying concentrations of ribose-l-phosphate (0 – 29mM).

The formation of hypoxanthine from inosine in the absence of inorganic phosphate was also determined using cell homogenatae, supernatant and membrane preparation. The reaction medium consisted of 0.2ml of 50uM Tris-HCl buffer, pH 7.5, 50uM cold inosine, 0.1ml of protein material and 0.5 uCi inosine, (specific activity 575uCi/mmol). Other conditions for assay are as already reported above.

(ii) Assay for adenosine deaminase activity

The enzyme activity was determined by measuring the amount of radioactive adenosine incorporated into inosine in 10 minutes of incubation period, in the presence of coformycin. The assay mixture consisted of 0.1ml 50uM tris-HCl buffer, pH 7.5, containing 10mM 2-mercaptoethanol, 0.5uCi (2,5', 8^{-3H}) adenosine (specific activity 41Ci.mmol), 50uM cold adenosine, varying concentration of coformycin (0-1.25mg) and 0.1ml of the supernatant extract (0.35mg protein), in a total volume of 200ul. The protein extract was preincubated in coformycin for 10 minutes before the enzyme assay was started with the addition of the labelled substrate. Other conditions of assay remain as reported for hypoxanthine.

Chromatographic analysis of the assay products

The metabolic products resulting from the experiments described above were analysed by chromatography. Each mixture was centrifuged after stopping the reaction and 20ul of the supernatant was applied on thin layer plates coated with solica gel GF along with a standard mixture of adenine, adenosine,

hypoxanthine, inosine and AMP. The plates were developed in 86% (v/v) acqueous n-butanol with 5% by volume of concentrated ammonia solution added to the bottom of the tank (Lederer and Lederer, 1957). The resulting sports were viewed under UV light, marked out carefully, scrapped into scintillating vials for the determination of radioactive content in each product. The same procedure was employed in the separation of all other products of adenosine metabolism reported in this study.

Marker enzymes of the isolated membranes

Table 1 is a representation of the results obtained from the assay of marker enzyme activities of the isolated membranes. There was adenosine triphosphatase – (ATPase) activity which was not inhibited by either oubain or oligomycin.
5-nucleotidase, glucose-6-phosphatase and acid phosphatase activities were detected in all fractions including the post-sucrose membrane fraction.

Metabolic Products of adenosine in whole cells and the different cell fractions

Table 2 shows the pattern of radioactivity recorded in intact cells of *T. vivax* at different intervals. The incorporation of radio-activity into the different metabolites in decreasing order was observed to be inosine > hypoxanthine > nucleotide > adenine.

When the metabolic products of adenosine were analysed in the cell homogenate, supernatant fraction and isolated membranes (Table 3), it was observed that, in all cases, the highest level of metabolite was recorded in isonine just like in the whole cells (Table 1).

Assay for adenosine deaminase activity

Table 4 is a representation of the data obtained when the incorporation of radioactive adenosine into inosine was examined in the presence of coformycin (concentration varying from 0 – 125ug). It was observed that at low concentrations (1 – 11.6ug coformycin), there was 13% - 17% stimulation. But at higher concentration (31 – 125ug coformycin), an inhibtion level of between 16% - 33% was observed. In intact cells of *T. vivax*, Table 5, there was consistent inhibitory effect by between 25% - 30%.

Assay for purine nucleoside phosphorylase activity in the cell homogenate

When hypoxanthine was employed as the substrate for the enzyme assay (Fig. 1), it was found that in the presence of ribose-l-phosphate (1-29uM), inosine formation was stimulated by a maximum of 16%.

When the enzyme activity was assayed in the presence of varying concentrations of inorganic phosphate (0-5mM) and inosine (1.0uCi, specific activity 575mCi/mmol) in cell homogenate (Fig. 2) or isolated membranes (Fig. 3), it was observed (for the cell homogenate), that the conversion of inosine to hypoxanthine (Fig. 2) was stimulated to a maximum of 73% of 1mM inorganic phosphate (Pi). Increase in the concentration of pI above 1.5mM resulted in a sharp drop in enzyme activity. The formation of nucleotides from inosine was also stimulated to a maximum of 32% at 2.5mM Pi (Fig. 2). At 5mM Pi, a sharp drop was observed in the level of nucleotide formed from inosine. In the case of the isolated membranes (Fig. 3), maximum increases in the levels of hypoxanthine and nucleotide formed were 38% and 20% respectively, at Pi concentration of 1mM. Further increase in the concentration of Pi resulted in a drop in activity in each case.

Table 6 is a representation of the data obtained when the formation of hypoxanthine from inosine was determined in the absence of Pi. The percentage radioactive inosine incorporated into hypoxanthine were 24%, 25% and 28% for cell homogenate, the supernatant fraction and isolated membranes, respectively.

Table 1: Marker Enzyme Activities at various stages of Membrane Isolation: Activities are expressed as nmol Pi/min/mg Protein.

Marker Enzyme	Homogenate	Pre-DNAse Treatment*	Post DNAses	Post Sucrose Membranes
Total ATPase	0.033	1.6	1.7	5.14
Oubain-sensitive ATPasde	0.029	1.6	1.9	5.14
5-nucleotidase	0.089	4.9	5.18	5.84
Acid Phosphatase	0.0125	0.406	0.488	2.28
Glucose-6-phosphatase	0.02	-	-	2.85

Marker enzyme activities at different stages of membrane purification. The enzyme activities were determined by measuring the quantity of inorganic phosphate liberated from the appropriate substrate for each enzyme as described in the text, using the method of Tansky and Shorr (1953).

Table 2: Products of Adenosine Metabolism in whole cells of *T. vivax* in a time course Assay

Time (MINS.)	Products (CPM/mg protein x 10^3)			
	Adenine	Hypoxanthine	Inosine	Total Nucleotides
1	10.125	67.084	81.258	13.500
3	10.125	58.725	91.384	16.706
5	8.606	69.357	113.400	18.484
7.5	11.902	63.540	86.827	14.125
10	9.614	59.996	107.584	13.241

Products of adenosine metabolism in whole cells of *T. vivax* in a time course assay. Details of assay conditions are reported in the text.

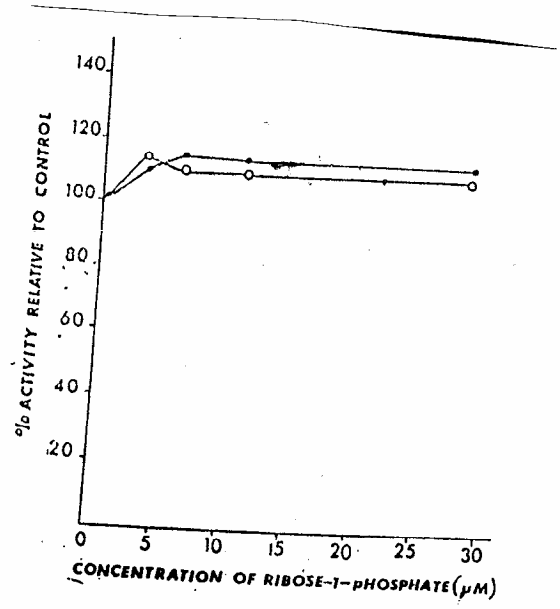


Fig. 1: Effect of ribose-l-phosphate on the conversion of hypoxanthine to inosine (●) and nucleotide (o) in the cell homogenate of *T. vivax*. The assay was carried out in the presence of varying concentrations of ribose-l-phosphate (1-29μM).

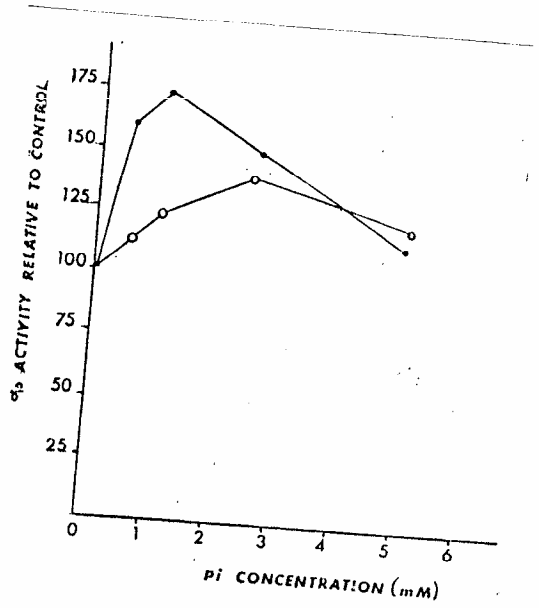


Fig. 2: Effect of inorganic phosphate on the conversion of inosine to hypoxanthine (●) and nucleotide (o) in cell homogenate. Inorganic phosphate was varied from 1-5mM.

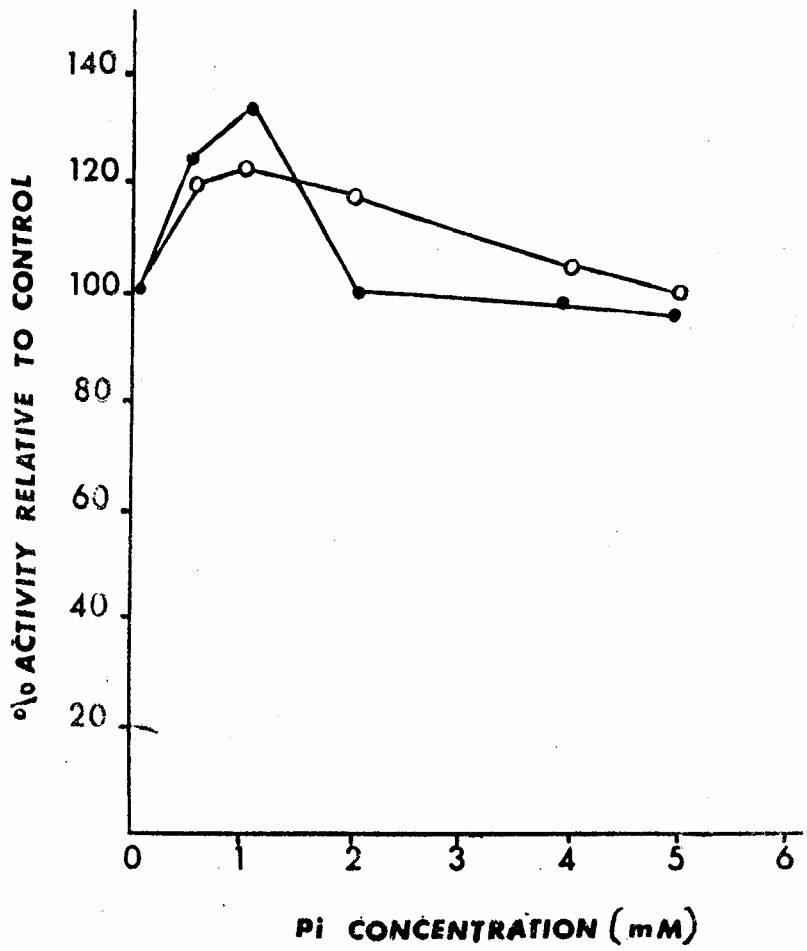


Fig. 3: Effect of inorganic phosphate on the conversion of inosine to hypoxanthine (●) and nucleotide (O) in isolated membranes. Inorganic phosphate concentration was as in Figure 2.

Table 3: Metabolites of adenosine in cell fractions of *T. vivax*: Activities expressed as CPM/mg protein x 10^3

Products formed	Cell Fractions Used		
	Homogenate	Supernatant	Isolated Membranes
Total Nucleotides	2.97	63.0	130
Inosine	26.0	340.0	780
Hypoxanthine	6.3	107.5	237
Adenine	2.9	52.1	137

The products of adenosine metabolism analyzed in the cell homogenate, supernatant fraction and isolated membranes. The cell homogenate had a protein level of 2.6mg while those of supernatant and membranes were 7 x 10^{-2}mg and 3.5 x 10^{-3}mg respectively.

Table 4: Effect of Coformycin on the formation of inosine from adenosine in *T. vivax* Sup. Extract

Concentration of Coformycin (µg)	Inosine formed (cpm/mg protein)	% Inhibition	% Stimulation
0 (Control)	11783	-	-
1.25	13831	-	17
11.6	13299	-	13
31.5	9953	16	-
125.0	10387	12	-
250.0	9680	18	-
500	7971	32	-
1000	8280	30	-
1250	7960	33	-

Assay for adenosine deamniase activity in the presence of different concentration of coformycin (0 – 125 µg). The assay mixture is as described in the text. The supernatant fraction used had a protein level of 3.5 x 10^{-2}mg. The protein was preincubated in coformycin for 10 minutes before the experiment was started with the addition of labelled substrate.

Table 5: Effect of coformycin in whole cells of *T. vivax*

Concentration Coformycin (µg)	Inosine formed (cpm/mg protein)	% Inhibition	% Stimulation
0	51025	-	-
150	35750	30	-
200	41775	18	-
250	35225	31	-
300	38275	25	-
350	36625	28	-

Determination of the effect of coformycin on the metabolism of adenosine in intact cells of *T. vivax*. The cells were preincubated in the presence of coformycin for 10 minutes before the assay was started. Assay period lasted for 10 minutes.

Table 6: Metabolites of Inosine metabolism in the absence of Pi: Activities expressed as cpm/mg protein x 10^3

	Homogenate	Supernatant	Isolated Membrane
Nucleotides	24.5	28.1	715.2
Hypoxanthine	7.6	12.9	282.4
Adenosine	<.1	1.69	31.7
Adenine	<.1	.690	14.6

Activities recorded in nucleotides, hypoxanthine, adenosine and adenine when radio-active inosine was incubated in cell homogenate, supernatant fraction and isolated membrane of *T. vivax* without Pi. The assay procedure is as described in the text.

Discussion

The marker enzyme activities determined during the purification of the *T. vivax* membranes are shown in Table 1. Though ATPase activity was present in the isolated membranes, no sensitivity to oubain or oligomycin was observed. Oligomycin-sensitive ATPase serves as a specific mitochondrial marker but blood stream forms of trypanosomes are known to lack fully functional mitochondria (Opperdoes *et al*., 1977). Therefore, lack of sensitivity to oligomycin is consistent with that concept.

Oubain inhibition is an indicator of the presence of Na^+/K^+ ATPase activity. In our earlier studies (Okochi *et al*, 1983), it was observed that oubain inhibited adenosine transport in intact cells of *T. vivax* and it was concluded that such inhibition could be on the sodium pump, and implication that Na^+/K^+ ATPase activity is present. Indeed, when the level of Na^+ content in the buffer medium was reduced by 82% transport activity was decreased by 55%.

In current study, inhibition of ATPase in isolated membrane was not detected. The possible explanation to this observation is that in the previous experiment, the asymmetrical orientation of the enzyme was satisfied because the closed membrane structure was definitely maintained in the whole cells of *T. vivax*. However, in the isolated membrane, this asymmetry may not have been satisfied if the membrane did not reseal. In that case, the transport function of ATPase is likely absent while the basic enzymatic activities leading to ATP hydrolysis remain.

Oubain-sensitive ATPase activity has been reported to be present in *T. brucei* (Voorheis *et al*, 1979 and Rovis and Baekkeskov, 1980).

The activity of 5-nucleotidase was also detected in this study. It is an enzym,e involved with the dephosphorylation of AMP and IMP to form purine nucelosides. Its activity is well associated with the external surface of several types of mammalian cells (Frick and Loweinstein, 1978), as well as some species of trypanosomatids (Hunt and Ellar, 1974; Voorheis *et al*, 1979 and Steiger *et al*, 1980). Also the functional asymmetric deposition of the enzyme on the membrane surface has been identified in *L. denovani* (Gottlieb and Dwyer, 1981). Since the specific activity increased with higher purification in this study, it is reasonable to suggest that this enzyme could be part of the isolated membrane. This is an appropriate location where it can operate as an instrument for extracting adenosine from circulating adenosine monophosphate (AMP) of the host, particularly, if it can also function as a translocase (frick and Loweinstein, 1978) which facilitates the uptake of adenosine into the cell. This function would be crucial for this parasite since it belongs to the group of blood-stream forms of African trypanosomes that lack the ability to make the amino substitution on the C_6 of purine ring and must therefore salvage its purine nucleotides from the host (James and Born, 1980).

The acid phosphatase activity was also found to increase with more purification of the membrane faction. Its activity is ubiquitous in trypanosomes, having been detected in the lysosomes, cristernae-like structures in the cytoplasm as well as the golgi apparatus (Langreth and Balber, 1975). However, an enrichmentof the enzyme activity in the plasma membrane of some trypanosomal pecies has previously been reported (Hunt and Ellar, 1979). The presence of this activity in isolated membranes is associated with certain components of the trypanosomatid which are not detached from the membranes during the isolation and purification processes. These include the flagella pocket as well as other subcellular membrane systems other than the flagella pocket (Steigner *et al*, 1980) and the subpellicular microtubules which are connected to the membranes (Voorheis *et al*, 1979).

The source of the glucose-6-phosphatase activity detected in this study is not clear but it has been previous suggested that such activity could originate from the membrane lining the flagella pocket (Seed, 1967). Similar findings has been reported for *T. brucei* (Rovis and Baekkeskov (1980).

Analysis of Metabolic Products

Analysis of products of adenosine metabolism in different cell fractions showed that the intermediate products for nucleotide synthesis in this parasite include inosine, hypoxanthine and adenine (tables 2 and 3) with inosine forming the major product. The presence of inosine as a metabolite of adenosine metabolism in trypanosome has been associated with the presence of either the amino hydrolase activity or that of 5'-nucleotidase and AMP deaminase (Fish *et al*, 1982, Ogbunede and Ikekiobi, 1983). In this study, the formation of inosine from adenosine has been monitored in the presence of coformycin – a well known inhibitor of adenosine deaminase (Agarwal and Parks, 1975; Argawal *et al*., 1975; Synder and Henderson, 1973). This is an indication that adenosine deaminase is p[resent in *T. vivax*.

Ogbunude and Ikediobi (1983) failed to detect adenosine deaminase in *T. vivax*, probably because they did not use coformycin in their own studies as we did in the present work. Our suggestion that adenosine deaminase is present in *T. vivax* is made on the strength of the documented evidence that coformycin inhibits adenosine deaminase as already discussed above. In the absence of coformycin, it will be difficult to differentiate between the activity inhibited by coformycin and the activities of aminohydrolase, 5'-nucleotidase and AMP deaminase respectively.

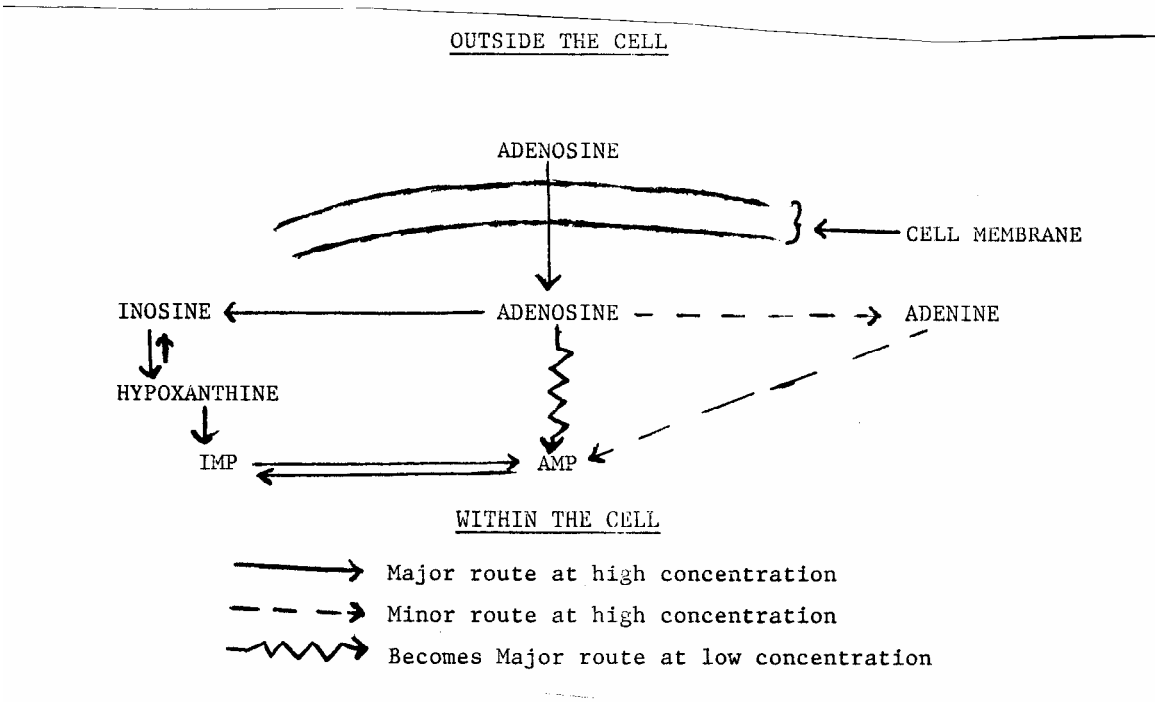
The metabolism of Adenosine to Inosine was inhibited up to 30% by coformycin both in intact cells and in the supernatant fraction (Table 4 and 5). But since the level of inhibition did not exceed 30% at the highest concentration of coformycin (1250ug) it is plausible to suggest that the deaminase exists, probably, along with aminohydrolase and 5'-nucleotidase as has previously been reported by the other workers –

(Fish *et al*, 1982). In other words, the partial inhibition is an indication of the presence of alternative pathways of adenosine metabolism.

As in the case of inosine, the presence of adenine and hypoxandthine among the metabolic products of adenosine would suggest that the purine nucleoside phosphorylase activity ios present in *T. vivax*. *In vitro* assay of the enzyme activity was carried out with freshly prepared cell homogenate. In the presence of inorganic phosphate (0-5mM), a maximum of 73% stimulation was observed in the formation of hypoxanthine from inosine (Fig. 2). In the reverse reaction, the formation of inosine from hypoxanthine in the presence of ribose-l-phosphate was stimulated slightly (11-16%), (Fig. 1). These results indicate that reversible phosphorolysis does occur in this microorganism. But considering the level of stimulation in each case (73% when inosine was the substrate and 16% when hypoxanthine was substrate) it appears that inosine is the favoured substrate. In addition, it was observed that hypoxanthine was formed even in the absence of exogenous inorganic phosphate (Table 6), suggesting that (cleaving) deriboxylating or hydrolytic activity is also present. These activities are known to be characteristics of purine nucleoside phosphorylase in Ehrlich ascite tumor cells (Pinto and Tonster, 1966) and for human erythrocytes (Krenitsky *et al*, 1968). The activity of purine nucleoside phosphorylase has been reported for *T. congolense* (Ogbunede and Ikediobi, 1983), while purine nucleoside hydrolase activity has been reported for *T. vivax* (Ogbunede and Ikediobi, 1983) for *T. brucei*, *T. gamniense* and *L. donovani* (Fish *et al* 1982, and Lafon *et al*, 1982).

The cumulative information arising from our studies of adenosine metabolism is that *T. vivax* has multiple routes of utilizing adenosine for nucleotide synthesis, depending on the concentration of adenosine available in the cell. It is well known that different types of mammalian cells, when confronted with low concentrations of adenosine, convert most of it into nucleotides by direct phosphorylation by adenosine kinase (Synder and Hunderson, 1973; Holmsen and Rozenberg, 1968; Meyekens and Williams, 1971 and Gotto *et al*, 1964). On the other hand, if the concentration of adenosine is high, most of it is deaminated to inosine which is subsequently converted to hypoxanthine, though the inosine/hypoxanthine pathway is not the major route of nucleotide synthesis under such conditions. Our results are consistent with this observation. We have used a relatively high concentration of adenosine in this study (50 μM – 10mM) and this is much more than the level of adenosine in the blood (10^{-7}M, Sherman 1979). A close examination of the pattern of incorporation of radioactive adenosine into its different metabolites (Tables 2 and 3) shows that the level of nucleotide formed does not relate directly to the level of inosine, rather it is more closely related to that of adenine. Inosine could then function as a "safety tank" to safeguard the cell from the overload of the more toxic adenosine (Hirshom *et al*, 1970 and Green and Chanm, 1973).

On the basis of the findings made under the conditions of the present studies, a general scheme for the mechanism of adenosine metabolism in this parasite is suggested as presented below:



This sketch does not lose slight of the fact that the activities of the salvage pathway enzymes were detected on the isolated membranes. What it is depicting is the fact that adenosine is not metabolized while in transit across the membrane. As reported in our earlier studies on adenosine transport (Okochi *et al*, 1983) coformycin inhibited adenosine transport after a time lag. This suggests that the transport process precedes the metabolic process. This in effect shows that the activity of the salvage pathway enzymes detected in the isolated membranes (Tables 3 and 6, Fig. 3) are located on the cytoplasmic side of the membrane therefore they do not constitute part of the transport complex. However, they may function in the regulation of intracellular concentration of adenosine in this micro organism. Definitely, more work is needed to define their rolkes more clearly before suitable antimetabolites targetted against them can be deployed.

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