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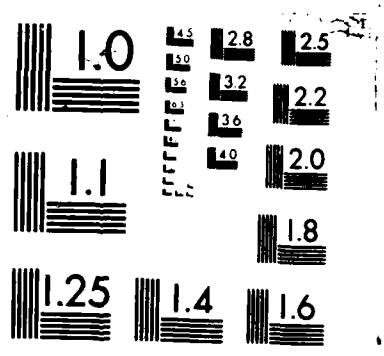

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BIOCHEMISTRY OF TRYPANOSOMATIDAE OF IMPORTANCE IN AFRICA

ANNUAL SUMMARY REPORT

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DECEMBER 1981

Supported by

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

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

A comparison of the enzymes of pathogenic protozoa to those of man is of fundamental importance to the search for much needed chemotherapeutic agents. The enzymes involved in purine salvage are of particular interest because most pathogenic protozoa lack the ability to synthesize purines de novo and consequently are obligate salvagers of preformed purines.

This project involves a comparison of purine and pyrimidine metabolism of Trypanosoma and Leishmania. Comparisons of their biochemistry will be made

within the parasitic group and to their host cells.

Basic information regarding metabolic capacities of these organisms will be obtained. Attention will be given to the mechanisms by which these organisms absorb nutrients from their environment. These mechanisms involve enzymes excreted into their surroundings, enzymes located on the cell surface, and enzymes located within the cell.

Enzyme and transport mechanisms which exhibit differences from those of host cells will offer targets for chemotherapeutic exploitation. Inhibitors will be sought which will affect these target systems. Those inhibitors which are trypanocides and/or leishmanicides will then be tested in an appropriate animal system.

Experiments to date have demonstrated that Leishmania donovani strain 2S of Dwyer is capable of transporting the purine bases, adenine, guanine and hypoxanthine and the nucleoside adenosine.

Adenine deaminase a possible chemotherapeutic target because of its uniqueness in some trypanosomatids, has been partially purified from this organism and purine analogs will be tested for inhibition.

SUMMARY

A comparison of the enzymes of pathogenic protozoa to those of man is of fundamental importance to the search for much needed chemotherapeutic agents. The enzymes involved in purine salvage are of particular interest because most pathogenic protozoa lack the ability to synthesize purines de novo and consequently are obligate salvagers of preformed purines.

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Foreword

Citations of trade names in this report does not constitute an official Department of the Army endorsement or approval of the use of such items.

In conducting the research described in this report, the investigator(s) adhered to the "Guide for the Care and Use of Laboratory Animals," prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council (DHEW Publication No. (NIH) 78-23, Revised 1978).

Although this report is formally designated as an annual report, because of it being submitted in conjunction with application for renewal of the contract, it, in fact, represents only three months work (October 1, 1981 - December 31, 1981). The research proposal submitted for the contract proposed the following areas of investigation for the first year: (I) Characterization of the purine and pyrimidine enzymes, and transport systems of Leishmania donovani, Trypanosoma gambiense and T. rhodesiense. Special attention will be given to the mechanisms by which these organisms absorb nutrients from their environment. These enzymes involve enzymes excreted into their surroundings, enzymes located on the cell surface, and enzymes located within the cell. (II) Parasite enzymes differing from corresponding mammalian enzymes will be tested in vitro with enzyme-specific chemical inhibitors. Chemicals active in the in vitro inhibition of parasite enzymes will also be examined using an appropriate laboratory animal model system, or susceptible cell line.

During the past three months I have set up my laboratory at the University of Massachusetts, and have begun investigations on purine transport in Leishmania donovani strain 2S of Dwyer.

MATERIALS AND METHODS

Organism. To date the organism used has been Leishmania donovania strain 2S of Dwyer.

Media. For convenience to the reader and for ease of future reference, compositions of the various media are given in detail. All amounts are for one liter, final volume.

Brain Heart Infusion (BHI): BBL brain heart infusion, 37g; 100 ml heat inactivated (56°C 30 minutes) fetal or newborn calf serum; 2.5 mg hemin, filter sterilized.

RE 1X (Steiger and Black)

Components per liter:

A)	8.0g	NaCl
	400 mg	KCl
	200 mg	$MgSO_4 \cdot 7H_2O$
	60 mg	$Na_2HPO_4.2H_2O$
	60 mg	KH_2PO_4
	2.0g	glucose
B)	200 mg	L-arginine
	100 mg	L-histidine
	100 mg	L-isoleucine
	300 mg	L-leucine
	250 mg	L-lysine. HCl
	50 mg	L-methionine
	100 mg	L-phenylalanine
	300 mg	L-proline
	400 mg	L-threonine
	50 mg	L-tryptophan
	50 mg	L-tyrosine
	100 mg	L-valine
C)	300 mg	L-glutamine
	1.0 g	$NaHCO_3$
	14.25 g	HEPES (=60 mM)
	20 mg	adenosine
D)	1 mg	D-biotin
	1 mg	choline chloride
	1 mg	folic acid
	2 mg	i-inositol
	1 mg	niacinamide
	1 mg	D-pantothenic acid (hemi-calcium salt)
	1 mg	pyridoxal.HCl
	0.1 mg	riboflavine
	1 mg	thiamine.HCl
E)	2.5 mg	haemin

Culture of organism: Stock cultures of L. donovani were maintained in BHI medium.

Transport Assays

All assays reported here were carried out at 0°, 25°, and 37°C. Cells which had grown for three days in BHI medium (250 ml in a low profile flask, 10% inoculum) were reinoculated (10%) into RE-IX medium minus the purine-adenosine. These cells were incubated 24 hrs at 25°C to deplete the cells of purine. The cells were then centrifuged 5,000 xg, washed once in RE-IX minus purine and resuspended in the same buffer minus purine to a density of 2×10^8 cells/ml. The cells were then divided into aliquots of 10 ml and placed in beakers (50 ml) at temperatures 0°, 25° and 37°C for 30 minutes. Radioactive substrate was added with an Eppendorf pipet and at various times aliquots (1 ml) of cell suspensions were pipeted into plastic microcentrifuge tubes (1.5 ml) and sedimented (10 sec) in a Fisher microcentrifuge. When this type of centrifuge is turned on the cells are sedimented and removed from contact with the medium within a fraction of a second. Aliquots (.1 ml) were then pipeted into scintillation vials containing Aquasol and counted in a Beckman Scintillation Counter.

All radioactive purines (50 mCi/mmol) were labeled in the 8 position and were obtained from Amersham/Searle.

Preparation of Adenine Deaminase

Cell extracts were harvested from harvested washed cells (5,000 xg, washed in .05 M Tris pH 7.5). After sonication, the cell deris was removed by centrifugation at 40,000 xg for 1 hour.

Gel filtration of extracts was carried out on Sephadex G-200 columns (2.5 x 35 cm) using either phosphate or Tris buffers, 0.01 M, pH 7.0. Fractions of 3.0 ml were collected. Assays of fractions were carried out spectrophotometrically by measuring the decrease in absorbance at 265 nm (11), the molar difference taken as 8600. The assay mixture contained, in a volume of 1.0 ml, 0.2 µmol of adenine, 0.1 ml of gel filtrate, and 0.01 M phosphate buffer, pH 7.0.

The standard assay mixture contained, in a volume of 0.1 ml, 320 nmol of adenine (300 nmol of unlabeled plus 20 nmol of [8-^{14}C]adenine) phosphate buffer (0.01 M, pH 7.0), and enzyme. The reactions were stopped by the addition of a drop of glacial acetic acid. Reaction mixtures were streaked on Whatman No. 1 paper and chromatographed in n-butanol:acetic acid:water (20:3:7, v/v/v) descending or subjected to paper electrophoresis with formic acid:water (8:300, v/v) as the electrolyte. Strips were examined for radioactivity in a Tracerlab 4" scanner and peaks were identified by comparison with cochromatographed standards. Quantitation was by planimetry.

RESULTS AND DISCUSSION

During the first three months of this project, I have equipped my laboratory with a Biogard Laminar-Flow Hood and CO_2 Incubator in order to work with the trypanosomatids and tissue culture systems which will be used for their propagation. In order to save on expenses rat, hamster and mouse breeding colonies have been started to pass and propagate the trypanosomes. Supplies and equipment necessary for investigations of purine transport and metabolism have been procurred. Presently, I am investigating the purine transport and metabolic capacities of Leishmania donovani strain 2S of Dwyer. On

January 12-13, 1981, I visited Walter Reed Laboratories to obtain cultures of Leishmania spp and Trypanosoma spp. which are of importance to the Army.

Efforts during the first three months have dealt with methods of assaying and characterizing transport capabilities in L. donovani promastigotes of adenine, guanine and hypoxanthine. Time, temperature and concentration of purine substrates have been varied. Preliminary results suggest that at 4°C transport of purines does not take place and no significant differences in transport can be detected between temperatures 25°C and 37°C. Assay times have been carried out to 90 minutes with peak transport occurring in all cases between 15-30 minutes. Adenine, hypoxanthine and adenosine during this time period show uptakes of 30-40 μmoles/ml in L. donovani promastigotes. Guanine because of its very low solubility is more difficult to work with and very low concentrations must be used. At present maximum transport has occurred at 30 minutes with 3 μmoles/ml transported.

Adenine deaminase a possible chemotherapeutic target because of its uniqueness in some trypanosomatids, has been purified 400 fold using heating (5 min. 55°C), ammonium sulfate precipitation (40-80%) and chromatography on G-200 Sephadex (procedure described in Materials and Methods). Purine analogs will be tested for inhibition of this enzyme and promising inhibitors will then be tested for in vivo action in L. donovani.

Transport and enzyme capabilities of L. donovani Khartoum Strain, WR 130 drug strain, L. brasiliensis Murray 209 and WR 063, and Trypanosoma rhodesiense will be investigated this year.

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