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Lesly Ann. Temesvari

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Studies in Trehalose Metabolism in *Dictyostelium discoideum*:
The Role of Trehalose in Stress Management and
the Purification of Trehalase

by

Lesly Ann Temesvari

A Dissertation
Submitted to the Faculty of Graduate Studies and Research
Through the Department of Biological Sciences
in Partial Fulfillment of the Requirements for
the Degree of Doctor of Philosophy at the
University of Windsor

Windsor, Ontario, Canada

1993
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ABSTRACT

by

Lesly Ann Temesvari

The levels of the disaccharide trehalose, in the
dormant stages of various slime molds, were determined.
Trehalose comprised 7.1%, 2.4%, 3.2%, and 3.4% of the dry
weight of Dictyostelium mucoroides macrocysts,
Polysphondylium pallidum microcysts, and Dictyostelium
discoideum spores (strains AC4 and SG1), respectively. In
the vegetative amoebae of D. discoideum, and P. pallidum,
additional roles for this storage carbohydrate were
examined. It was found that trehalose could also act as a
general stress manager; its levels increasing to those
observed in dormant structures, when amoebae were exposed to
heat (30°C), cold (4°C) and heavy metals. Trehalose-6-
phosphate synthetase and trehalase activities remained
unchanged in vegetative amoebae during stress and recovery
from stress indicating that regulation of trehalose
accumulation occurs in a pathway other than a trehalose
futile cycle. It was further observed that glycogen levels
decreased rapidly during stress regimes. This would result
in the accumulation of glucose monomeric units and would
drive trehalose synthesis in an equilibrium dependant manner
(substrate level). Substrate level regulation of stress-
induced trehalose accumulation was supported by the finding
that the stress response was unaltered in the presence of the protein synthesis inhibitor, cycloheximide.

The inhibitory effects of three trehalose analogs on \textit{D. discoideum} lysosomal trehalase were examined. Validamycin A, MDL-25,637, and castanospermine were found to be potent, reversible, inhibitors of the enzyme \textit{in vitro}, with \( K_i \) values of \( 10^{-8} \) M, \( 10^{-7} \) M, and \( 10^{-4} \) M, respectively. In addition, Validamycin A and MDL 25,637 were shown to be time dependent inhibitors of trehalase. A trehalase-specific affinity resin was developed by covalently coupling Validamycin A to Sepharose. The resin was used to purify \textit{D. discoideum} trehalase to homogeneity in a two step procedure. Due to the high specificity of trehalase for its substrate, Validamycin A-Sepharose has broad applications in the rapid purification of trehalases from a variety of species origins.
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I would like to thank Dr. H.B. Fackrell for moral support and the use of his computer facilities at several stages of this study. I am also grateful to Dr. D.D.S. Thomas and Dr. A.H. Warner for technical and logistical assistance during my tenure.

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LIST OF ABBREVIATIONS

ABTS  2,2'-Azino-di-(3-ethyl-benzthiazoline-6-sulphonate)

5'AMP  adenosine 5'-monophosphate

APS  ammonium persulfate

BzPFR  N-benzoyl-L-prolyl-L-phenylalanyl-L-arginine 4 nitroanilide

cAMP  cyclic adenosine monophosphate

ddCPXX  Dictyostelium discoideum cysteine proteinase of XX kDa molecular weight

ddH₂O  double distilled water

D₂O  deuterium oxide

DTT  dithiothreitol

EDTA  ethylenediaminetetraacetic acid

gelatin-SDS-PAGE  SDS-PAGE using gelatin co-polymerized with acrylamide

G6P  glucose-6-phosphate

GP1  glycogen phosphorylase 1

GP2  glycogen phosphorylase 2

GS-agar  glucose-salts agar

hsp(s)  heat shock protein(s)

hsp70  heat shock protein of 70 kDa molecular weight

IEF  isoelectric focusing

LDH  lactate dehydrogenase

L-P agar  lactose-peptone agar

MES  2[N-morpholino]-ethanesulfonic acid

NAD+  β-nicotinamide adenine dinucleotide (oxidized form)
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
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<tbody>
<tr>
<td>NADH</td>
<td>β-nicotinamide adenine dinucleotide (reduced form)</td>
</tr>
<tr>
<td>PEP</td>
<td>phosphoenolpyruvate</td>
</tr>
<tr>
<td>pI</td>
<td>isoelectric point</td>
</tr>
<tr>
<td>PK</td>
<td>pyruvate kinase</td>
</tr>
<tr>
<td>PMSF</td>
<td>phenylmethylsulfonyl fluoride</td>
</tr>
<tr>
<td>psi</td>
<td>pounds per square inch</td>
</tr>
<tr>
<td>rpm</td>
<td>revolutions per minute</td>
</tr>
<tr>
<td>SDS-PAGE</td>
<td>sodium dodecyl sulfate polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>TEMED</td>
<td>N,N,N',N'-tetramethylethylenediamine</td>
</tr>
<tr>
<td>TRIS</td>
<td>Tris (hydroxymethyl) aminomethane</td>
</tr>
<tr>
<td>U</td>
<td>units</td>
</tr>
<tr>
<td>UDPG</td>
<td>uridine 5'-diphosphoglucone</td>
</tr>
<tr>
<td>UDP-glucose</td>
<td>uridine 5'-diphosphoglucone</td>
</tr>
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CHAPTER I

The Accumulation of Trehalose in Cellular Slime Mold Cells in Response to Temperature, Heavy Metal and Developmental Stress
I.A. INTRODUCTION

The Dictyostelids, commonly known as the cellular slime molds, are a small yet extraordinary group of organisms. Within their short life cycles they can display properties which are common to both animals and plants (fungi). While the Dictyostelids were discovered over a century ago, today they are of particular interest as laboratory models for investigations in cellular and developmental biology.

I.A.1. Life Cycle of Dictyostelium discoideum

Of all of the cellular slime molds, Dictyostelium discoideum has received the most attention. The wild-type strain of D. discoideum, NC-4, was first described by Raper (1935). The life cycle of D. discoideum is unique and displays both a unicellular vegetative phase and a multicellular developmental phase (Figure I-1).

In nature, during vegetative growth, amoebae feed on the bacteria of decaying leaf material. While nutrients are available these free living amoebae grow and multiply by binary fission. The developmental program, characterized by aggregation, pseudoplasmodium migration and culmination, ensues upon depletion of the peripheral food source.

At the onset of the developmental program, amoebae form aggregates of approximately $10^5$ cells. Intricate "animal-like" cell to cell communication orchestrates the formation of these aggregates. Centrally located amoebae secrete
Figure I-1. The life cycle of *Dictyostelium discoideum* (after Cotter *et al.*, 1992). The following stages are outlined: (a) spore dispersal; (b) spore germination; (c) vegetative growth; (d) nutrient starvation to preaggregation time, time 0-5 hours; (e) beginning of aggregation, time 6 hours; (f) middle aggregation, time 8 hours; (g) late aggregation, time 9 hours; (h) tipped aggregation, time 11 hours; (i) standing slug, time 13 hours; (j) initiation of pseudoplasmodium (slug) migration, time 16 hours; (k) end of short slug migration period, time 18 hours; (l) reestablishment of vertical polarity in preparation for culmination, time 19 hours (m) initiation of culmination, time 20 hours; (n) early culmination, time 21 hours; (o) middle culmination, time 22 hours; (p) culmination complete, time 24 hours; and (q) fruiting body and spore maturation, time 1 to 10 days.
3',5'-cyclic adenosine monophosphate, (cAMP) which acts as a chemoattractant for neighbouring amoebae and in turn stimulates them to release their own cAMP (Bonner, 1967; Barkley, 1969).

The aggregates become encased in a sheath of cellulose containing glycoproteins which have been secreted by the individual cells of the structure (pseudoplasmodium). The slug or pseudoplasmodium topples over and migrates horizontally in search of additional nutrients.

In the absence of food the slug rounds up upon itself and enters the culmination stage. During this process the anterior cells of the slug enter the fatal pathway of differentiation from pre-stalk cells to stalk cells whereas members of the posterior region differentiate into the pre-spore pathway of development leading to the formation of spores (George et al., 1972). The pre-stalk cells elongate horizontally, secrete cellulose and vacuolate forming a rigid structure which supports the mature dormant spores. The life cycle is complete with the "plant-like" (fungal) release of spores and the subsequent germination of these spores, releasing vegetative amoebae into the environment.

*D. discoideum* has a wealth of properties which make it amenable to laboratory studies. In the laboratory, it can be cultured easily on synthetic media with host bacteria such as *Escherichia coli* (Cotter and Raper, 1968a). Its life cycle is short (4 days) and synchrony from unicellular
existence to multicellular development is easily maintained. Unlike many other eukaryotic organisms, the haploid nature of *D. discoideum* makes it particularly useful for investigations in genetics. In addition the organism has become popular in laboratories committed to studies in molecular biology, developmental biology, cell to cell communication, cellular differentiation and phagocytosis (lysosome biology).

A wealth of mutants of *D. discoideum* derived from the wild type strain, NC-4, have been isolated. Several of these mutants have been employed in this study. The mutant Ax3, can be cultured axenically in rich medium, i.e. in the absence of bacteria (Loomis, 1971). This mutant is particularly useful for biochemical studies as it can be grown in large quantities. The spontaneously germinating mutant, SG1 was isolated by virtue of its ability to germinate in the absence of external activation treatments (Cotter and Dahlberg, 1977).

I.A.2. Alternative Dormant Structures: Microcysts

Under certain conditions, the amoebae of some species of the *Dictyostelids* do not embark on the developmental pathway leading to sorocarps but rather, round up upon themselves individually forming microcysts. The microcysts are dormant structures or transient resting stages of individual cells. It is believed that both sub-optimal
culture conditions and genetic (species specific) components
govern the choice by certain species to form microcysts.

Microcysts are spherical in nature and are formed by an
initial rounding up of the cell, followed by the deposition
of a fibrous outer layers consisting of a dense inner wall
and a loose outer wall (Hohl et al., 1970). These walls
contain large amounts of cellulose and glycogen-like
particles, in addition to lipids and proteins (Toama and
Raper, 1967b). Germination of these structures require no
external activation (Cotter and Raper, 1968b).

The dictyostelid, Polysphondylium pallidum, readily
forms microcysts and often these structures dominate their
vegetative cultures. The influence of the culture
environment on microcyst formation in different strains of
P. pallidum has been well documented. It has been
determined that conditions of low humidity, and the presence
of light, and high concentrations of carbohydrates, KCl,
phosphate and ammonia often favour the formation of
microcysts over sorocarps (Raper, 1960; Hohl and Raper,
1963; Toama and Raper, 1967a,b; Hohl et al., 1970; Githens
and Karnovsky, 1973; Lonski 1976; Choi and O'Day, 1982,
1984). Microcyst differentiation has also been employed as a
model system for biochemical studies of cellular
differentiation (O'Day, 1972; 1973a,b,c; 1974; 1976; 1979).
P. pallidum (strain WS320) was used in this study.
I.A.3. Alternative Dormant Structures: Macrocyts

In addition to the asexual dormant constructions (spores and microcysts), many of the species of the Dictyostelids can produce sexual fructifications known as macrocysts. Macrocyts are formed by cell fusion among amoebae of appropriately paired mating types of heterothallic strains or among cells of self-compatible homothallic strains.

The ultrastructure of macrocyst formation has been documented (Filosa and Dengler, 1972). During early sexual development, small amoeboid cells (gametes) appear in the culture (O'Day et al., 1987; Lydan and O'Day 1988). At 10 hours into sexual development, gamete fusions occur resulting in the appearance of a cell type distinguishable by its increased cytoplasmic volume and two nuclei (O'Day et al., 1987; Lydan and O'Day, 1988). Nuclear fusion and zygote (giant cell) formation occur at approximately 18 hours into the sexual development program. (McConachie and O'Day, 1987). Following nuclear fusion, zygote giant cells are observed to draw amoebae to their surface via cAMP-mediated chemoattraction and primary cellulosic wall synthesis begins (O'Day, 1979). The resulting aggregate is known as a pre-cyst. The giant zygote ingests the surrounding cells in the pre-cyst, enclosing each of the internalized cells in a vacuole (endocyte). When complete ingestion of the surrounding cells is achieved, secondary
and tertiary cellulose layers are deposited around the giant cell. The macrocyst then enters a period of dormancy dependent upon environmental and genetic factors (see Raper, 1984). The environmental conditions which govern macrocyst formation in several species of the Dictyostelids are well documented. It has been determined that conditions of darkness, high humidity (free water) and low phosphate, along with small quantities (ppm) of magnesium, nitrate, sulphate, chloride and calcium (Hirschy and Raper, 1964; Weinkauf and Filosa, 1965; Nickerson and Raper, 1973; O'Day, 1979; Chagla et al., 1980; Lydan and O'Day, 1988) enhance the formation of macrocysts. The first slime mold species to be isolated and described was Dictyostelium mucoroides (Brefeld, 1869). D. mucoroides, strain Dm7, is a well-characterized homothallic macrocyst former, and was used in this study.

I.A.4 Trehalose in the Life Cycle of the Cellular Slime Molds

The non-reducing disaccharide, trehalose (α-D-glucopyranosyl α-D-glucopyranoside), is the most widely distributed disaccharide in fungi (Elbein, 1974). Trehalose levels are negligible in rapidly growing vegetative cells but increase dramatically in resting cells or spores (reviewed by Thevelein, 1984). For this reason the sugar is believed to fulfil an important energy storage function in
fungi.

In *Dictyostelium discoideum*, trehalose may account for up to 5.5% of the dry weight of the dormant spore (Ceccarini and Filosa, 1965). Trehalose has also been shown to accumulate in microcysts of *P. pallidum* (Klein *et al.*, 1992). Prior to this study, trehalose accumulation in macrocysts of cellular slime molds has not been reported.

The following is an overview of carbohydrate metabolism during sporulation and spore germination in *D. discoideum*. At the onset of development amoebal glycogen stores are mobilized and the newly acquired carbon units are used for trehalose anabolism. Trehalose accumulates in both pre-stalk cells and pre-spore cells during the differentiation program. At the culmination stage (18 hours), trehalose can account for up to 1.5% of the dry weight of both of the cell types. As described previously, at the termination of the differentiation program trehalose can account for up to 5.5% (Ceccarini and Filosa, 1965; Ceccarini, 1967; Jackson *et al.*, 1982) of the dry weight of the dormant spore, whereas stalk cell trehalose will have been converted to cellulose for structural integrity. Total carbohydrate remains constant during development.

During germination, the stored trehalose is rapidly mobilized and the resulting glucose is used for energy (Cotter and Raper, 1970). In *D. discoideum* the large quantities of trehalose in dormant spores and their
depletion during germination was recently confirmed by
carbon-13 nuclear magnetic resonance (n.m.r.) studies (Klein
et al., 1990).

I.A.5. Trehalose and Stress

Trehalose may also serve as a protective agent during
dormancy, enhancing the resistance of dormant fungal spores
to adverse environmental conditions. Emmanitoff and Wright
(1979) demonstrated a positive correlation between trehalose
levels and thermal resistance of D. discoideum spores.
Similar findings have been shown for spores of Phycomyces
(Van Laere, 1986) and for bacterial spores of Streptomyces
griseus (Martin et al., 1986).

Interestingly, trehalose is also prevalent in non-
sporulating prokaryotes, where its role appears to be that
of a "general stress manager". In other words, trehalose
appears to counteract various stresses, especially osmotic
stress, which are not related to dormancy. It is suggested
that in Escherichia coli, osmotic pressure is
counterbalanced with the uptake and/or the biosynthesis of a
number of organic osmolytes including trehalose (Larson
et al., 1987). Trehalose may also serve as the predominant
osmolyte in various cyanobacteria (Mackay et al., 1984) and
in the halophilic eubacterium Ectothiorhodospira halochloris
(Galinski and Herzog, 1990).

The number of eukaryotic organisms investigated which
employ trehalose as a stress protectant is rather small. Hottiger et al. (1987a) showed that trehalose metabolism could be influenced by heat stress in vegetative cells of Saccharomyces cerevisiae. Heat induced accumulation of trehalose has also been reported for Schizosaccharomyces pombe (De Virgilio et al., 1990), Neurospora crassa (Neves et al., 1991) and Artemia franciscana (Clegg and Jackson, 1992). As well, it has been shown that trehalose accumulation can be induced in S. cerevisiae by a wide variety of other stress regimens including heavy metal stress (Hottiger et al., 1989).

I.A.6. Purpose of This Study

In view of these data, it was of interest to examine the "general stress managing" role of trehalose in D. discoideum and P. pallidum. Trehalose levels were monitored in both organisms during heat treatment and cold treatment, and in D. discoideum during heavy metal exposure and osmotic shock. In addition, the possibility that trehalose might accumulate in other dormant structures of the cellular slime molds was investigated.
I.B. MATERIALS AND METHODS

I.B.1. Growth of the Organism - *D. discoideum* and *P. pallidum* Amoebae

Vegetative amoebae of the cellular slime molds, *D. discoideum*, strain Ax3 (ATCC 28368) (gift from Dr. R.L. Dimond) and *P. pallidum*, strain WS320 (ATCC 44834) were employed in this study. Vegetative amoebae were grown axenically in TM Medium (Free and Loomis, 1974) which was composed of 10.0 g trypticase peptone (BBL), 5.0 g yeast extract (Difco), 10.0 g glucose (Sigma), 0.35 g Na₂HPO₄ (Sigma) and 1.2 g KH₂PO₄ (Sigma) in 1.0 L of double distilled water (ddH₂O). The pH of the medium was adjusted to 6.5. The medium (50 mL) was dispensed in 125 mL Erlenmeyer flasks and autoclaved at 121°C, 15 psi, for 15 minutes. Cooled, sterile TM medium was inoculated with 5.0 mL of a previously grown log phase culture containing 5 X 10⁶ cells/mL. To ensure uniform suspension and aeration of amoebae in the medium, cultures were shaken on a rotary shaker (Labline) at 165 rpm at 23°C until the log phase cultures reached 5 X 10⁶ cells/mL (approximately 48 hours).

I.B.2. Sample Preparation

Amoebae were treated with various stress regimens in the presence of nutrient medium as described below and intracellular trehalose levels were monitored.
Accumulated intracellular trehalose in control and treated cells was isolated by ethanol extraction as reported previously by Cotter and Niederpruem (1971). At specified time intervals, 5 mL samples were removed from culture flasks and filtered through pre-weighed GF/C glass microfibre filters (Whatman). Filters containing cells were baked for 12 hours at 80°C and the dry weight of the cells was determined (Demoss and Bard, 1957). Extraction of trehalose was achieved by washing the filters twice with 10 mL volumes of 80% ethanol (v/v) at 80°C for 30 minutes. This treatment solubilizes trehalose and glucose but will not solubilize glycogen. Ethanol extracts were air dried at 45°C and re-dissolved in 200 µL of 0.1 M acetate buffer, pH 5.5.

I.B.3. Measurement of Trehalose Accumulation During Stress

The assay for trehalose was performed by methods modified from a previously reported procedure (Chan and Cotter, 1980). Assays were carried out in a total volume of 200 µL of reaction mixture in microtitre wells. Trehalose was quantified using electrophoretically purified trehalase from D. discoideum at a concentration of 100 Units/mL (See CHAPTER III). Glucose reagent was prepared by dissolving 45 Units/mL of glucose oxidase (Sigma), 7.5 Units/mL of peroxidase (Sigma), and 2.3mM 2,2'-Azino-di-(3-ethyl-benzthiazoline-6-sulphonate (ABTS, Boehringer Mannheim) in
25 mL of 0.1 M acetate buffer, pH 5.5. Trehalose extracts of 50 μL were added to 50 μL of trehalase preparation; 100 μL of glucose reagent were added to each well and the contents were mixed and incubated on a shaker unit at 23°C for 30 minutes. In addition, known concentrations of trehalose were treated as above to generate a trehalose standard curve ranging from 10 nmoles to 100 nmoles per reaction.

The purified trehalase liberates glucose from the extracted or control trehalose during the 30 minute incubation period. The glucose then participates in the following reactions:

\[
\text{Glucose} + \text{H}_2\text{O} + \text{O}_2 \xrightarrow{\text{glucose oxidase}} \text{Gluconic Acid} + \text{H}_2\text{O}_2
\]

\[
\text{H}_2\text{O}_2 + \text{reduced ABTS} \xrightarrow{\text{peroxidase}} 2\text{H}_2\text{O} + \text{oxidized ABTS}
\]

The optical density was measured at 405 nM using a Bio-Tek microtitre plate reader. Appropriate controls were employed to determine the level of glucose, which promotes colour development, in each sample. The final trehalose content was determined after adjustment for background glucose in the samples.

I.B.4. Temperature Shock

Cells were heat shocked by transferring cultures growing at 23°C to a 30°C shaking water bath. This raised
temperature was maintained for 2 hours after which cells were allowed to recover from the stress at 23°C for 2 hours. Similarly, cold shock was achieved by transferring cells growing at 23°C to an environment of 4°C. Cultures were maintained at this temperature for 2 hours before being allowed to recover at 23°C for 2 hours.

I.B.5. Heavy Metal Stress

Heavy metal containing compounds were introduced to cultures of *D. discoideum* amoebae to final concentrations as given: 0.8 mM CoCl$_2$ for 45 minutes; 5 mM CuSO$_4$ for 60 minutes; 1 mM ZnSO$_4$ for 45 minutes; 100 mM CdCO$_3$ for 45 minutes or 250 μM CdCl$_2$ for 2 hours. As well, control salts were introduced into cultures of *D. discoideum* amoebae to final concentrations as given: 1mM (NH$_4$)$_2$SO$_4$ for 45 minutes; 500 μM NaCl for 45 minutes; 250 μM (Na)$_2$CO$_3$ for 45 minutes; or 1.6 mM KNO$_3$ for 45 minutes.

I.B.6. Osmotic Shock

To confer osmotic shock upon the cells the osmotic potential of the surrounding medium was changed by the addition of various solutes to obtain appropriate concentrations. Amoebae of *D. discoideum* were exposed to 25 mM, 50 mM, 75 mM or 100 mM phosphate (pH 6.5; Na$_2$HPO$_4$:KH$_2$PO$_4$, 3:1) for 1 hour; 1 M NaCl or 5 M KCl for 4 hours.
I.B.7. Measurement of Trehalose in other Dormant Stages of the Cellular Slime Molds

Spores of *D. discoideum*, strain AC4 (from the collection of Raper; not listed in ATCC) and strain SG1 (ATCC 44849) were produced by the methods of Glaves and Cotter (1989). These strains form fruiting bodies when grown in association with *Escherichia coli B/r* on Adams (1959) glucose-salts (GS) agar. This medium was prepared by adding 1.0 g NH₄Cl (Sigma), 3.0 g KH₂PO₄ (Sigma), 6.0 g Na₂HPO₄ (Sigma), 0.13 g MgSO₄ (Sigma) and 15.0 g agar (Gibco) to 1.0 L of ddH₂O. The medium was autoclaved at 121°C, 15 psi, for 15 minutes and then combined with a sterile glucose (Sigma) solution (40.0 g/100 mL) to give a final glucose concentration of 0.4 %. Cooled medium (50°C) was dispensed into sterile plastic Petri plates (100 X 15 mm) and allowed to solidify at 23°C. Plates were stored in the cold (4°C) until used.

Spores were aseptically transferred by loop into 10-20 mL of sterile ddH₂O to a final concentration of 10⁷ spores/mL. One loopful of *E. coli B/r* was added to the spores, and the suspension was mixed on a Vortex mixer. 1:5 - 2.0 mL of spore/bacteria mixture were dispensed evenly onto the surface of glucose-salts agar plates and incubated at 23°C for 4 - 6 days.

Induction of macrocysts of *D. mucoroides*, strain DM7 (ATCC 42609), was performed by the methods of North and
Cotter (1991a). The medium employed was 0.1% Lactose-Peptone (L-P) agar. This medium was prepared by adding 1 g lactose (Sigma), 1 g Bacto-peptone (Difco), and 15 g agar (Gibco BRL) to 1.0 L of ddH$_2$O. The medium was autoclaved at 121°C, 15 psi, for 15 minutes. Cooled (50°C) medium was dispensed into sterile plastic Petri plates (100 x 15 mm) and allowed to solidify at 23°C.

Vegetative amoebae from a stock culture of *D. mucoroides* were transferred to 200 mL of sterile ddH$_2$O. *E. coli*, strain B/r (2 loopfuls) was added to the suspension. Aliquots (10 mL) of the suspension were dispensed onto 0.1% L-P agar plates. The plates were incubated at 23°C, in the dark. Macrocysts began to form after 4 days. Macrocysts were harvested after 9 days and used for trehalose estimations.

Induction of microcysts of *P. pallidum*, strain WS320 (ATCC 44834), was performed by modified methods of Cotter and Raper (1968b). A 0.1 mL mixture of *P. pallidum* amoebae and *Escherichia coli*, strain B/r, was spread on dry plates of glucose-salts (GS) agar. Plates were incubated at 23°C in the dark for 10 days. Microcysts, estimated to be 5 days of age, were dislodged from the surface of the agar with a stream of distilled water. Trehalose extractions from all dormant forms of the slime molds and measurements of sugars were performed as above.
I.B.8. Statistical Analysis

All data were analyzed using Student's 2 sample T-test. Results of the stress treatments were obtained using 3 trials except in the case of osmotic shock with phosphate where 4 trials were performed. Significance was accepted at the p<0.05 level.
I.C. RESULTS

I.C.1. Temperature Stress

The relationship between trehalose content and heat stress in *D. discoideum* was determined by transferring logarithmically growing amoebae from 23°C to 30°C and maintaining the cells at this higher temperature for 2 hours. This regimen has been shown to induce the maximal heat shock response in *D. discoideum* (Loomis and Wheeler, 1980). As depicted in Figure I-2 trehalose levels increased significantly (*t_s=5.742, p<0.01*), by a factor of 18-fold over control cells during the heat shock program. Following heat shock treatment, cells were shifted back to 23°C and allowed to recover. The trehalose content of the cells diminished rapidly such that no trehalose was detectable after 2 hours of recovery.

The effects of cold shock on trehalose accumulation in *D. discoideum* were also examined. Trehalose levels were observed to increase 25-fold over the level found in control cells 2 hours after logarithmically growing cells were shifted from 23°C to 4°C (Figure I-3). The observed increase was statistically significant (*t_s=2.899, p<0.05*). As well, trehalose was rapidly degraded during a 2 hour recovery period at 23°C. The *P. pallidum* response to heat and cold shocks, was similar to those of *D. discoideum* with intracellular
Figure I-2. Trehalose accumulation during heat stress in vegetative amoebae of *D. discoideum*. Heat stress experiments consisted of a 2 hour incubation at 30°C (2 h) followed by a 2 hour recovery period at 23°C (3 h, 4 h). The first hour after the initiation of recovery is indicated by (R^+). In all cases (each n=3), mean trehalose levels which had accumulated after 2 h of treatment were significantly higher than those of control cells (0 h). Analysis by Student's 2-sample T-test (p<0.05). (□) control; (■) treatment; (□) recovery; (nd) trehalose not detectable.
Figure I-2
Figure I-3. Trehalose accumulation during cold stress in vegetative amoebae of *D. discoideum*. Cold stress experiments consisted of a 2 hour incubation at 4°C (2 h) followed by a 2 hour recovery period at 23°C (3 h, 4 h). The first hour after the initiation of recovery is indicated by (R+). In all cases (each n=3), mean trehalose levels which had accumulated after 2 h of treatment were significantly higher than those of control cells (0 h). Analysis by Student's 2-sample T-test (p<0.05). (□) control; (■) treatment; (☑) recovery; (nd) trehalose not detectable.
trehalose levels increasing significantly from non-detectable concentrations to 0.5% (tₛ=4.677, p<0.01) and 0.42% (tₛ=8.400, p<0.01) of the dry weight to the cells, respectively (Figure I-4,5).

I.C.2. Heavy Metal Stress

The effects of heavy metals on the accumulation of intracellular trehalose are shown in Figure I-6. All heavy metals employed in this study induced intracellular accumulation of trehalose in D. discoideum amoebae. The most significant increase in trehalose was observed when the test salt CdCl₂ was added to a final concentration of 250 μM to the media of logarithmically growing cells. In this instance, trehalose levels were approximately 12-fold higher than those of control cells (tₛ=2.518, p<0.02). Increases of intracellular trehalose of approximately 11.5-fold (tₛ=2.993, p<0.02), 5.8-fold (tₛ=2.410, p<0.05) and 4.3-fold (tₛ=2.225, p<0.02) were observed for the test salts Co(NO₃)₂ (0.8mM), ZnSO₄ (1mM) and CdCO₃ (250μM) respectively.

It was important to examine the relative contribution of both the cation (heavy metal) and anion constituents of the test salts. To achieve this, control salts containing the anion (non-heavy metal) portion of each salt were used as a stress agent. The salts (NH₄)₂SO₄ (1mM), NaCl (500μM), NaCO₃ (500μM) and KNO₃ (1.6 mM) were applied as controls for
Figure I-4. Trehalose accumulation during heat stress in vegetative amoebae of *P. pallidum*. Heat stress experiments consisted of a 2 hour incubation at 30°C (2 h) followed by a 2 hour recovery period at 23°C (3 h, 4 h). The first hour after the initiation of recovery is indicated by (R+). In all cases (each n=3), mean trehalose levels which had accumulated after 2 h of treatment were significantly higher than those of control cells (0 h). Analysis by Student's 2-sample T-test (p<0.05). (□) control; (■) treatment; (□) recovery; (nd) trehalose not detectable.
Figure I-4
Figure I-5. Trehalose accumulation during cold stress in vegetative amoebae of *P. pallidum*. Cold stress experiments consisted of a 2 hour incubation at 4°C (2 h) followed by a 2 hour recovery period at 23°C (3 h, 4 h). The first hour after the initiation of recovery is indicated by (R+). In all cases (each n=3), mean trehalose levels which had accumulated after 2 h of treatment were significantly higher than those of control cells (0 h). Analysis by Student's 2-sample T-test (p<0.05). (☐) control; (■) treatment; (□) recovery; (nd) trehalose not detectable.
Figure I-6. Trehalose accumulation in vegetative amoebae of *D. discoideum* during heavy metal exposure. Trehalose levels increased in amoebae when treated with i) 250 μM CdCl₂ for 45 min; ii) 0.8 mM Co(NO₃)₂ for 45 min; iii) 1 mM ZnSO₄ for 45 min; iv) 250 μM CdCO₃ for 45 min; and v) 5 mM CuSO₄ for 1 h. The levels of trehalose which accumulated during each treatment (each n=3) were significantly higher than those levels found in control amoebae (n=36). Analysis by Student's 2-sample T-test (p<0.05). (□) control; (■) treatments.
Figure I-6

The graph shows the trehalose (% dry weight of amoebae) for different heavy metal treatments. The treatments include CONTROL, CdCl₂, Co(NO₃)₂, ZnSO₄, CdCO₃, and CuSO₄. The y-axis represents the trehalose levels, and the x-axis represents the different heavy metal treatments.
the heavy metal compounds ZnSO₄, CdCl₂, CdCO₃ and Co(NO₃)₂ respectively. These control salts did not affect the levels of intracellular trehalose (data not shown).

I.C.3. Osmotic Stress

To test the effects of osmotic stress on the accumulation of intracellular trehalose, non-metabolizable solutes were added into the medium of logarithmically growing D. discoideum amoebae. The data in Figures I-7, 8, 9 show that the levels of trehalose were not dramatically affected in a statistically significant manner by 5M KCl ($t_5=0.864$, $p>0.05$), or by 1M NaCl ($t_5=0.374$, $p>0.05$).

Similarly trehalose levels did not increase in the presence of phosphate at concentrations of 25mM ($t_5=0.000$, $p>0.05$); 50mM ($t_5=0.070$, $p>0.05$); 75mM ($t_5=0.691$, $p<0.05$) or 100 mM ($t_5=0.204$, $p>0.05$).

I.C.4. Trehalose in Other Dormant Stages of Slime Molds

Trehalose levels in the dormant stages of various slime molds were determined. These values which were obtained by the enzymatic methods of this study are presented in Table I-1. The trehalose content of D. discoideum spores, strains AC4 and SG1, was 3.2% and 3.4% respectively. Trehalose comprised 7.1% of the dry weight of 5 day old macrocysts of D. mucoroides. For P. pallidum microcysts, 2.3% of their dry weight could be attributed to trehalose (Table I-1).
Figure I-7. Lack of trehalose accumulation during osmotic stress in vegetative amoebae of *D. discoideum*. Amoebae were treated for 4 hours with 5 M KCl (n=3). Analysis by Student's 2-sample T-test (p>0.05). (□) control; (■) treatments.
Figure I-7
Figure I-8. Lack of trehalose accumulation during osmotic stress in vegetative amoebae of *D. discoideum*. Amoebae were treated for 4 hours with 1 M NaCl (n=3). Analysis by Student's 2-sample T-test (p>0.05). (□) control; (■) treatments.
Figure I-8

Trehalose (% Dry Weight of Amoebae)

Time (Hours)

0  4
Figure I-9. Lack of trehalose accumulation during osmotic stress in vegetative amoebae of *D. discoideum*. Amoebae were treated for 1 hour with 25mM to 100 mM phosphate (n=4). Analysis by Student's 2-sample T-test (p>0.05). (□) control; (■) treatments.
Figure I-9

![Graph showing the effect of phosphate concentration on trichloroacetic acid precipitable Trehalose (% Dry Weight of Amoebae). The x-axis represents Phosphate Concentration (mM) with values 10 (control), 25, 50, 75, and 100. The y-axis represents Trehalose (% Dry Weight of Amoebae) with a scale from 0 to 2.]
Table 1. Levels of Trehalose in the Dormant Forms of Various Cellular Slime Molds.

<table>
<thead>
<tr>
<th>Organism (Strain)</th>
<th>Dormant Form</th>
<th>Trehalose (% Dry Weight of Dormant Structure)(^a)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>D. discoideum</em> (AC4)</td>
<td>Spore</td>
<td>3.2</td>
</tr>
<tr>
<td><em>D. discoideum</em> (SG1)</td>
<td>Spore</td>
<td>3.4</td>
</tr>
<tr>
<td><em>D. mucoroides</em> (DM7)</td>
<td>Macrocyst</td>
<td>7.1</td>
</tr>
<tr>
<td><em>P. pallidum</em> (WS32O)</td>
<td>Microcyst</td>
<td>2.3</td>
</tr>
</tbody>
</table>

\(^a\)Trehalose quantified using electrophoretically purified trehalase (see Methods).
I.D. DISCUSSION

*D. discoideum* amoebae exhibit, as do most other organisms, increased thermotolerance in response to heat shock (Loomis and Wheeler, 1980). A small set of proteins, termed heat shock proteins (hsp's), is induced in the presence of heat and their tenure is transient (Lindquist, 1986). These characteristic proteins are believed to be responsible for conferring heat resistance upon the cell. The current study shows that in response to heat stress regimens, the amoebae of *D. discoideum* accumulate substantial levels of trehalose; the level of carbohydrate persists until the stress is removed, and then the disaccharide is rapidly degraded during the post-stress recovery period. Moreover, the results obtained in this study are consistent with the results of Hottiger *et al.* (1987a), which show a positive correlation between intracellular trehalose accumulation, and hsp synthesis and thermoprotection in *S. cerevisiae*. The similarity between the behaviour of trehalose and heat shock proteins during stress is indeed interesting and leads us to believe that trehalose may also play a role in thermotolerance in *D. discoideum*.

Trehalose has many attributes which potentiate its role in cellular thermoprotection. *In vitro* studies have shown that trehalose can protect protein structure and enzyme activity during desiccation (Carpenter *et al.*, 1987). More
importantly, trehalose has been shown to be well suited to protect complex membrane systems against \textit{in vivo} dehydration (Crowe \textit{et al.}, 1983). \textit{In vitro}, trehalose can expand phospholipid membranes, and increase the fluidity of such membranes (Lee \textit{et al.}, 1986). All such actions are thought to occur via the hydroxyl groups of the sugar molecule, which bind to the polar head groups of phospholipids, replacing water and maintaining their integrity during desiccation.

The favourable characteristics of trehalose which establish protection from desiccation may also play an important role in thermoprotection during heat stress. Desiccation affects intracellular hydration, and specifically the individual water molecules associated with proteins and other macromolecules. Since water putatively has a stabilizing effect on molecules due to hydrophilic/hydrophobic interactions, the removal of water (desiccation) may destabilize macromolecules. The hydroxyl groups on trehalose can replace water molecules and, because of the sturdiness afforded by the structure of the sugar, they add greater stability to associated macromolecules. It is conceivable that desiccation-like phenomena could occur during heat stress and thus the above actions of trehalose might support the idea that trehalose may protect amoebae during heat shock. Other polyols and carbohydrates have been shown to elicit the same effect \textit{in vitro} but are
apparently less efficient than trehalose (reviewed by Van Laere, 1989).

Low temperature induction of stress proteins has been demonstrated in prokaryotes such as *Escherichia coli* (Jones et al., 1987). Thus far, in *D. discoideum*, the cold shock response has been shown to mimic the heat shock response only with respect to the expression of ubiquitin genes (Muller-Taubenberger et al., 1988) and a developmentally regulated membrane protein gene, P8A7 (Maniak and Nellen, 1987). The expression of the ubiquitous heat shock marker protein, hsp70, is not affected by low temperature treatment (Maniak and Nellen, 1987). Trehalose levels in *D. discoideum* can also be manipulated using cold shock. Trehalose levels were observed to increase when cells were shifted to a cold environment and decreased during a two hour recovery period at 23°C. The fact that cold shock induces the accumulation of trehalose but not hsp70 leads to the notion that the sugar may have a more fundamental role in stress management than the heat shock proteins themselves.

The phenomenon of trehalose accumulation in response to temperature stress may be widespread among the slime molds. The responses to heat and cold shocks by *P. pallidum*, were similar to those of *D. discoideum* with intracellular trehalose levels increasing approximately from non-detectable amounts to 0.5% and 0.42% of the dry weight of
cells respectively.

Since the characteristics of heat shock in a wide variety of organisms are induced by other stresses the influence of another environmental condition on trehalose accumulation was tested. Heavy metals are well known non heat inducers of heat shock proteins in *S. cerevisiae* (Lindquist, 1986). In *D. discoideum*, cadmium has been shown to induce ubiquitin gene expression (Muller-Taubenberger et al., 1988). All heavy metals employed in this study induced intracellular accumulation of trehalose in *D. discoideum* amoebae. The most significant increase in trehalose, in response to heavy metal exposure, was obtained when CdCl₂ was used. Other heavy metals employed were less efficient in stimulating trehalose accumulation but still induced a significant increase in intracellular trehalose content. As well, salts containing only the anion portion of the heavy metal salt did not produce accumulation of trehalose. Therefore, the accumulation of trehalose can be attributed solely to the heavy metal.

The adaptation to osmotic stress is fundamental to the survival of an organism. In prokaryotes, an osmoregulatory action of trehalose has been suggested. In *E. coli* osmotic pressure is observed to be counterbalanced with the uptake and/or biosynthesis of the following three organic osmolytes: betaine, glutamic acid and trehalose (Larson et al., 1987). In some cyanobacteria, trehalose may serve as
an osmolyte (Mackay et al., 1984) and in the halophilic eubacterium *Ectothiorhodospira halochloris* trehalose is synthesized in certain osmotically unfavourable environments (Galinski and Herzog, 1990). It has been thought that in the majority of fungi, the dominant osmolites were alternative polyols, and in most cases, glycerol (Brown, 1978; Kelly and Budd, 1990). More recently, trehalose has been shown to act as a eukaryotic osmolyte; accumulating in response to salt stress in a number of yeast species (Meikle et al., 1991).

From this study, it appears that the role of trehalose as a osmoregulatory substance in *D. discoideum* is undefined. No significant accumulation of trehalose was observed when osmotic stress was imposed upon cells via the increase of non-metabolizable solute concentrations in the surrounding media.

This study is the first to demonstrate that trehalose accumulates in all of the dormant structures of the *Dictyostelids*. Moreover, trehalose accumulation in *D. discoideum* during stress is comparable to that observed during multicellular development. The greatest stress-related accumulation was observed under cold stress where trehalose levels comprised 1.9% of the dry weight of cold shocked amoebae. This appears to be significantly less than the contribution of trehalose to the dry weight of dormant spores which has been reported by Ceccarini and Filosa.
(1965) to be 5.5%, and slightly less than the value of 2.2% which has been reported by Klein et al. (1990). The amount of trehalose in other dormant spores of slime molds presented in this study also appear to be significantly higher than the trehalose which accumulates in vegetative amoebae during stress. It is important to note that the dry weight of individual amoebae may decrease by 50% during development; this decrease in dry weight results mainly from a loss in protein content (see Loomis, 1975). Thus it is probable that the total quantity of trehalose synthesized per vegetative amoeba under stress or per developing spore, microcyst or macrocyst is similar.

It is well documented that in the fungal kingdom trehalose is synthesized under starvation conditions, and in some instances this synthesis has been shown to be driven at the expense of glycogen breakdown (Kane and Roth, 1974). The large amount of energy used to transform a viable storage compound such as glycogen to trehalose indicates that the latter must have beneficial features in order to serve as the ideal, or at least the preferred, reserve carbohydrate. In this study, trehalose accumulated under a variety of adverse conditions and one valuable role of trehalose may indeed be stress management.
CHAPTER II

Trehalose Metabolism During Temperature Stress
in Vegetative Amoebae of Dictyostelium discoideum
II.A. INTRODUCTION

Prior to this study, trehalose was not known to accumulate substantially in the vegetative phase of the life cycle of *Dictyostelium discoideum*. Thus, studies involving the metabolism of this disaccharide in amoebae of this organism have never been undertaken. By contrast, the synthesis of trehalose during differentiation of *D. discoideum* and its subsequent accumulation in the dormant structures is well documented (Ceccarini and Filosa, 1965; Klein *et al.*, 1990). Carbohydrate metabolism during sporulation and germination in *D. discoideum* has been described in CHAPTER I. Trehalose metabolism during these two phases of the life cycle have also been documented (reviewed by Wright and Killick, 1975).

II.A.1. Enzymes Governing Trehalose Metabolism during the *D. discoideum* Life Cycle

Several enzymes govern the metabolism of trehalose during sporulation and spore germination. These include two major enzymes; trehalose-6-phosphate synthetase and trehalase, and two additional enzymes; glycogen phosphorylase and UDP-glucose pyrophosphorylase.

II.A.2. Trehalose-6-phosphateSynthetase

Trehalose-6-phosphate synthetase catalyses the formation of trehalose-6-phosphate from UDP-glucose and
glucose-6-phosphate (Roth and Sussman, 1968). Trehalose-6-
phosphate is the immediate precursor to the neutral sugar
trehalose (Cabib and Leloir, 1958). The in vivo regulation
of this enzyme has been the subject of controversy.

Roth and Sussman (1968) reported that synthetase
activity was not detectable during the first 5 hours of
development; increased linearly from 5 to 16 hours; and
declined rapidly at 18 hours into differentiation. They
concluded that the net accumulation of trehalose in pre-
stalk and pre-spore cells was due to the increase in
synthetase activity observed between the aggregation and
culmination stages (5 to 16 hours) of
development.

Killick and Wright (1972a, 1972b, 1974) later reported
that synthetase activity was in fact detectable during the
first 5 hours of development; increased 3-fold during the
first 10 hours; and remained at this higher level from 10
hours to the completion of differentiation. In addition, it
was concluded that the 3-fold increase in synthetase activity
could not completely account for the rapid rate of synthesis
of trehalose and the quantities of the sugar which
accumulated during development. Computer modelling of
carbohydrate metabolism during D. discoideum sporulation
(Wright and Killick, 1975) suggested that a dramatic
increase in the substrates for trehalose-6-phosphate
synthase, (altering the kinetic constants of the synthetic
reaction) must also accompany the increase in synthetase activity in order to account for the substantial trehalose accumulation observed. This type of regulation was termed substrate level control.

Another historical aspect of trehalose-6-phosphate synthetase is worth recounting. The sensitive enzyme extraction, stabilization and measurement techniques perfected by Killick (1979) have revealed that the enzyme is present during the vegetative phase of the life cycle. Its function during this phase of growth is presently unknown.

II.A.3. Trehalase

The enzyme trehalase which catalyses the hydrolysis of trehalose to glucose monomeric units is also important in trehalose metabolism. A detailed review of the *D. discoideum* trehalases is presented in CHAPTER III. Its activity is required both during differentiation and during spore germination. Trehalase activity was reported to appear during the terminal sorocarp formation (Roth and Sussman, 1966; Killick and Wright, 1972a). It was also determined that this enzyme, often referred to as a developmental trehalase was specific to stalk cells (Jefferson and Rutherford, 1976). It has been postulated that the role of developmental trehalase is hydrolysis of pre-stalk cell trehalose, which provides the glucose
monomeric units required for cellulose anabolism.

A spore specific trehalase (vegetative) has also been described (Cotter and Raper, 1970) and the role of this enzyme is believed to be hydrolysis of trehalose for energy production during the germination program. Similar to trehalose-6-phosphate synthetase, trehalase persists throughout the vegetative phase of the life cycle, where its function is presently unknown.

II.A.4. Trehalose Futile Cycling

The presence of trehalose-6-phosphate synthetase and trehalase in vegetative amoebae of D. discoideum suggests that a futile cycle between trehalose and glucose may exist in these organisms. Basal amounts of trehalose may be synthesized by trehalose-6-phosphate synthetase and subsequently degraded by the vegetative form of trehalase. In this manner, amoebae would be poised for stress and development by a delicate balance of trehalose synthesis and hydrolysis. Any signal of stress and/or development could upset this balance by "down-regulating" trehalase and/or "up-regulating" the synthetase such that an accumulation of trehalose ensues. Futile cycling between trehalose and glucose has been shown to exist in S. cerevisiae (Hottiger et al., 1987).
II.A.5. UDP-glucose Pyrophosphorylase and Glycogen Phosphorylase

Two additional enzymes, glycogen phosphorylase and UDP-glucose pyrophosphorylase, are also known to be involved in trehalose metabolism during sporulation (see Wright and Killick, 1975). At the onset of development, glycogen phosphorylase mobilizes intra-amoebal glycogen stores resulting in an increase in intracellular glucose-1-phosphate. This compound serves as the immediate precursor of the two substrates for trehalose-6-phosphate synthetase. The glucose-1-phosphate is converted to UDP-glucose by UDP-glucose pyrophosphorylase. Glucose-1-phosphate is also converted to glucose-6-phosphate by a simple isomerization reaction. Both the activities of glycogen phosphorylase (Rutherford and Cloutier, 1986) and UDP-glucose pyrophosphorylase (Wright and Killick, 1975) are present during the vegetative phase of the life cycle of D. discoideum.

II.A.6. Trehalose Metabolism during Stress in Alternative Systems

Several studies have explored the mechanism of trehalose accumulation in yeast during stress. Over the past few years, the results of these investigations have been the subject of controversy.

Hottiger et al. (1987b) reported that the accumulation
of trehalose during heat shock in *Saccharomyces cerevisiae* was due to an increase in the anabolic enzyme trehalose-6-phosphate synthetase. Similar results were reported for desiccation induced trehalose accumulation in the same organism (Marino et al., 1989). The heat induced increases in trehalose and trehalose-6-phosphate synthase activity in *S. cerevisiae* were reported to be cycloheximide sensitive by Panek et al. (1990). In another system, *Schizosaccharomyces pombe*, accumulation of the sugar and activation of the anabolic enzyme appeared to be cycloheximide insensitive (De Virgilio et al., 1990).

In addition, Hottiger et al. (1987b) reported that the activity of the synthetase in *S. cerevisiae* decreased rapidly during recovery from heat shock, and it was this decline which contributed to observed disappearance of intracellular trehalose content during this cooling period. In contrast to this, Marino et al. (1989) reported that synthetase activity remained high during recovery from desiccation (rehydration).

Studies of trehalose metabolism during stress in *S. cerevisiae* are further complicated by the fact that two different trehalases have been shown to exist in this organism (Londesborough and Varimo, 1984; reviewed by Thevelein, 1984). Neutral trehalase has a pH optimum of approximately 7.0 and is often referred to as a regulatory trehalase by virtue of its rapid activation in a cAMP-
dependent manner. The acid trehalase has a pH optimum of 5.5 and is considered non-regulatory as there is no evidence for rapid control of this enzyme by phosphorylation.

Unexpectedly, the levels of neutral trehalase in *S. cerevisiae* (Hottiger et al., 1987) and *S. pombe* (De Virgilio et al., 1990; 1991b), were observed to increase along with trehalose-6-phosphate synthetase during heat stress. Recently, these conclusions were refuted by De Virgilio et al. (1991b), whereby it was suggested that the increase in regulatory trehalase activity during stress was due to inappropriate sampling and artificial *in vitro* activation of the enzyme. By contrast, neutral trehalase activity has been observed to decrease during anhydrobiotic stress (desiccation) in *S. cerevisiae* (Marino et al., 1989).

Finally, a recent study by Winkler et al. (1991) reported that neither trehalose-6-phosphate, neutral trehalase nor acid trehalase could be observed to increase during temperature stress in *S. cerevisiae*. Rather, it was suggested that a dramatic accumulation in the substrates for trehalose-6-phosphate synthetase (UDP-glucose and glucose-6-phosphate) was responsible for the observed increase in intracellular trehalose (substrate level control).

II.A.7. Purpose of This Study

The goal of this study was to provide insight into the metabolic regulation of trehalose accumulation during stress
in *D. discoideum*. The levels of the critical enzymes thought to be involved in trehalose metabolism, namely trehalose-6-phosphate synthetase and trehalase were examined during heat and cold shock in the presence or absence of the protein synthesis inhibitor, cycloheximide. *D. discoideum* provides a simple system in which to study the role of trehalase in stress because it exhibits only acid trehalase (non-regulatory) activity (Gupta, 1987). In addition, the possibility that glycogen may serve as the source of glucose monomeric units required for trehalose anabolism was investigated. Finally, the existence of a trehalose futile cycle was tested by monitoring trehalose levels during hypersecretion of trehalase.
II.B. MATERIALS AND METHODS

II.B.1. Temperature Stress

Temperature stress was imparted on Ax3 amoebae of *D. discoideum* as described in CHAPTER I. Briefly, amoebae were heat shocked by shifting cultures growing at 23°C to a 30°C shaking water bath. This raised temperature was maintained for 2 hours after which cells were allowed to recover from the stress at 23°C for 2 hours. Similarly, cold shock was achieved by shifting cells growing at 23°C to an environment of 4°C. Cultures were maintained at this temperature for 2 hours before being allowed to recover at 23°C for 2 hours.

At specified time intervals 1 mL samples were removed from the culture flasks and analyzed for trehalase activity, trehalose-6-phosphate synthetase activity, and glycogen content (see below). In addition, stressed amoebae were analyzed for their trehalose content as described in the previous chapter.

II.B.2. Measurement of Protein

Protein was determined according to the methods of Bradford (1976) using the Bio-Rad Protein Assay. The assay was standardized using bovine serum albumin (Sigma).

II.B.3. Measurement of Trehalase Levels in Stressed Amoebae

Stressed and unstressed amoebae (6.5 X 10⁶ cells) were collected by centrifugation in an Eppendorf microcentrifuge
(Model 5414) at 2000 X g, for 5 minutes at 4°C. The spent
growth medium (supernatant) was retained as a source of
extracellular or secreted trehalase. Cells were washed free
of spent growth medium by repeated centrifugation (3 times)
in the presence of ice cold 0.1 M sodium acetate (pH 5.5).
The amoebae were resuspended in the above buffer and quickly
frozen in an ethanol-ice bath. Frozen cells were stored at
-20°C.

To prepare cell free extracts the amoebae were allowed
to thaw at 23°C and the suspension was centrifuged in an
Eppendorf microcentrifuge (Model 5414) at
16,000 X g, for 30 minutes at 4°C. The resulting
supernatant material served as a source of intracellular
trehalase for all subsequent manipulations.

The assay for trehalase was performed by methods
modified from a previously reported procedure
(Chan and Cotter, 1980). Trehalase was quantified using
trehalose (Sigma) as the substrate. Glucose liberated from
trehalose hydrolysis by trehalase was monitored by a glucose
detection reagent as described in CHAPTER I.

Assays were carried out in a total volume of 200 µL of
reaction mixture in 96 well microtitre plates. Trehalase
preparations of 50 µL were added to 50 µL of a 0.05 M
solution of trehalose in a microtitre well; 100 µL of
glucose reagent was added to each well and the contents were
mixed and incubated on a shaker unit at 23°C for 30 minutes.
In addition, known concentrations of glucose were incubated with glucose reagent, under identical conditions, to generate a glucose standard curve ranging from 10 nmoles to 100 nmoles of glucose per reaction. On occasion, it was necessary to dilute the trehalase preparation prior to use, to ensure that the amount of product formed was within this glucose standard range.

Oxidation of chromogen, and thus amount of glucose in a sample, was monitored spectrophotometrically at 405 nm using a Bio-Tek microtitre plate reader. Appropriate controls were employed to determine the level of background glucose, which promoted colour development, in each sample. The final enzyme units were determined after adjustment for background glucose in the samples. One unit of trehalase activity was defined as one μmole of glucose released per minute. Specific activity was defined as enzyme units per mg of protein.

II.B.4. Measurement of Trehalose-6-Phosphate Synthetase Levels in Stressed Amoebae

Stressed and unstressed amoebae (6.5 X 10^6 cells) were collected by centrifugation in an Eppendorf microcentrifuge (Model 5414) at 2000 X g, for 5 minutes at 4°C. Cells were washed free of spent growth medium by repeated centrifugation (3 times) in the presence of cold (4°C) 50 mM MES (2[N-morpholino]-ethanesulfonic acid)-NaOH (pH 6.5).
The amoebae were resuspended in the above buffer and quickly frozen in an ethanol-ice bath. Frozen cells were stored at 
-20°C.

To prepare cell free extracts the amoebae were allowed to thaw at 23°C and the suspension was centrifuged in an Eppendorf microcentrifuge (Model 5414) at 16,000 X g, for 30 minutes at 4°C. The resulting supernatant material served as a source of trehalose-6-phosphate synthetase for all subsequent manipulations.

The assay for trehalose-6-phosphate synthase was performed by the methods of Killick (1979). The assay was carried out at 23°C in a 400 μL reaction mixture that contained 25 mM glucose-6-phosphate (G6P, Sigma), 10 mM uridine 5'-diphosphoglucose (UDPG, Sigma), 400 mM KCl (Sigma), 62.2 mM MgCl₂ (Sigma), 0.25 M β-nicotinamide adenine dinucleotide, reduced form (NADH, Sigma), 4 units of pyruvate kinase (PK, Sigma), 40 units of lactate dehydrogenase (LDH, Boehringer Mannheim), and 2.5 mM phosphoenolpyruvate (PEP, Sigma). The solution was allowed to equilibrate at 23°C, for 10 minutes prior to the addition of cell free extract (synthase). The components of the assay mixture participated in the following series of reactions:
G6P + UDPG $\xrightarrow{\text{SYNTETASE}}$ trehalose-6-phosphate + UDP

UDP + PEP $\xrightarrow{\text{PK}}$ UTP + pyruvate

pyruvate + NADH $\xrightarrow{\text{LDH}}$ NAD$^+$ + lactate

Oxidation of NADH was followed monitored spectrophotometrically (340 nm) using a Beckman DU-64 Spectrophotometer. NADH oxidation was quantified using 6.22 X $10^3$ as the molar extinction coefficient. Controls using boiled cell free extracts were included to correct for endogenous NADH oxidation. No endogenous NADH oxidation was detected in any of the assays. One unit of trehalose-6-phosphate synthase activity was defined as 1 µmole of product released per minute at 23°C. Specific activity was expressed as enzyme units per mg of protein.

II.B.5. Measurement of Glycogen Levels in Stressed Amoebae

Stressed and unstressed amoebae (6.5 X $10^6$ cells) were collected by centrifugation in an Eppendorf microcentrifuge (Model 5414) at 2000 X g, for 5 minutes at 4°C. Cells were washed free of spent growth medium by repeated centrifugation (3 times) in the presence of cold (4°C) 0.1 M sodium acetate (pH 5.5). The amoebal pellets were quickly frozen in an ethanol-ice bath and stored at -20°C.

The assay for glycogen was performed by methods modified from a previously reported procedure (Clegg and Jackson, 1992). To induce cell lysis, the frozen amoebae
were allowed to thaw at 23°C. The suspension was extracted twice with 80% (v/v) ethanol for 30 minutes at 80°C. This procedure solubilized glucose and trehalose but not glycogen. Ethanol-insoluble material containing glycogen was collected by centrifugation in an Eppendorf microcentrifuge (Model 5414) at 16,000 x g, for 30 minutes in the cold. Ethanol-insoluble pellets were treated with 200 μL of 50% (w/v) KOH, for 2 hours at 95°C. Cell debris was removed from the alkaline-solubilized glycogen by centrifugation in an Eppendorf microcentrifuge (Model 5414) at 16,000 x g, for 30 minutes at 4°C. Absolute ethanol (2 volumes) was added to the resulting supernatents (glycogen), and the samples were boiled to facilitate reprecipitation of pure glycogen. Purified glycogen appeared as a pellet of "glassy" beads. The glassy pellets were neutralized with 50 μL of 1 N HCl. The neutralized glycogen was treated with 200 μL of 4 N HCl for 2 hours, in a boiling water bath to facilitate its hydrolysis to glucose monomers. Hydrolysed glycogen was neutralized with 200 μL of 4 N NaOH and analyzed for glucose content by the glucose detection assay of Chan and Cotter (1980). Estimates of glycogen content were reported as nmoles of glucose equivalents per mg of protein.

II.B.6. Hypersecretion of Trehalase

Secretion of lysosomal enzymes by D. discoideum is
developmentally and nutritionally regulated. Addition of non-metabolizable substrates, such as sucrose, promotes the secretion of lysosomal enzymes, including trehalase (Crean and Rossomando, 1979; Seshadri et al., 1986).

Axenically grown Ax3 cells were harvested in mid-log (6.5 x 10^6 cells/mL) by centrifugation in an IEC clinical centrifuge, at setting 6, at 23°C. The amoebae were washed free of spent growth medium by repeated centrifugation in the presence of 0.1 M sodium phosphate (pH 6.5). The cells were resuspended to their original concentration in sterile TN medium, containing 0.1 M sterile sucrose. The sucrose had been dissolved in HPLC grade H_2O and filter sterilized using a disposable 0.2 μm syringe tip membrane filtration unit (Nalgene) prior to addition to TM medium. Sucrose supplemented cultures were shaken for 6 hours over which time samples were collected and analyzed for trehalose content and intracellular and extracellular trehalase activities as described previously.

II.B.7. Temperature Stress in the Presence of Cycloheximide

Protein synthesis was blocked in Ax3 amoebae of D. discoideum using the protein synthesis inhibitor, cycloheximide. Amoebae were treated with cycloheximide, at a final concentration of 200 μg/mL, for 1 hour, at 23°C prior to heat and cold shock. The cycloheximide conditioned cells were heat shocked or cold shocked as described
previously. At specified times, 1 mL samples were removed from the culture flasks and analyzed for trehalose levels, glycogen levels, trehalase activity, and trehalose-6-phosphate synthetase activity as described above.

II.B.8. Statistical Analysis

All data were analyzed using Student's 2-sample T-test. Results of each of the stress experiments were obtained from 3 trials. Significance was accepted at the p<0.05 level.
II.C. RESULTS

II.C.1. Temperature Stress and Trehalose Metabolism

In order to elucidate the mechanism of trehalose accumulation during temperature stress, analyses of the enzyme for trehalose anabolism, trehalose-6-phosphate synthetase, and of the enzyme for trehalose catabolism, trehalase, were undertaken.

As seen in Figure II-1, trehalose-6-phosphate synthetase activity remained unchanged during the 2 hour stress regime (heat or cold) as compared to the room temperature control. Likewise, synthetase levels remained constant during the 2 hour recovery period at 23°C. Although slight fluctuations in trehalose-6-phosphate synthetase activity between control and treated cells could be observed, none of these differences were statistically significant for heat shock and recovery from heat shock (1 hour, \( t_s = 0.118, p > 0.05 \); 2 hours, \( t_s = 0.162, p > 0.05 \); 3 hours, \( t_s = 0.318, p > 0.05 \); 4 hours, \( t_s = 0.291, p > 0.05 \)) and for cold shock and recovery from cold shock (1 hour, \( t_s = 0.035, p > 0.05 \); 2 hours, \( t_s = 0.516, p > 0.05 \); 3 hours, \( t_s = 0.272, p > 0.05 \); 4 hours, \( t_s = 0.293, p > 0.05 \)).

Since the enzyme trehalase is known to be secretory (lysosomal) in nature (Seshadri et al., 1986), it was necessary to analyze both intracellular and extracellular levels of activity during stress.

Extracellular (secreted) trehalase concentrations were
Figure II-1. Trehalose-6-phosphate synthetase levels remain unchanged during temperature stress in vegetative amoebae of D. discoideum. The temperature stress regime was carried out as described in the Materials and Methods. The first hour after initiation of recovery is indicated by (R+). Amoebae were heat shocked at 30°C (■) or cold shocked at 4°C (□). Control cells (□) were held at 23°C for 4 hours. During heat shock and recovery from heat shock (each n=3) or cold shock and recovery from cold shock (each n=3), mean trehalose-6-phosphate synthetase activity remained unchanged as compared to control cells (p>0.05). Analysis by Student's 2-sample T-test (see text).
not affected in a statistically significant manner by the 2 hour heat shock or cold shock treatments nor were the levels of secreted enzyme affected by the 2 hour recovery period (Figure II-2). In other words, trehalase was neither "hyposcreted" nor "hypersecreted" at any time during temperature stress or recovery from temperature stress. When analyzed by Student's 2-sample T-tests, it was found that the levels of secreted trehalase in treated cell cultures did not differ significantly from control cell cultures during the heat shock and recovery from heat shock (1 hour, \( t_s = 0.227, p > 0.05 \); 2 hours, \( t_s = 0.189, p > 0.05 \); 3 hours, \( t_s = 0.202, p > 0.05 \); 4 hours, \( t_s = 0.153, p > 0.05 \)) or during the cold shock and recovery regimes (1 hour, \( t_s = 0.384, p > 0.05 \); 2 hours, \( t_s = 0.251, p > 0.05 \); 3 hours, \( t_s = 0.158, p > 0.05 \); 4 hours, \( t_s = 0.70, p > 0.05 \)).

Figure II-2 demonstrates that intracellular trehalase activity also remained unchanged during the 2 hour stress regime (heat or cold) as compared to room temperature control cells. Likewise, intracellular trehalase levels remained constant during the 2 hour recovery period at 23°C. Although slight differences in intracellular and extracellular cellular trehalase activity between control and treated cells could be observed, none of these fluctuations were statistically significant for heat shock and recovery from heat shock (1 hour, \( t_s = 0.094, p > 0.05 \); 2 hours, \( t_s = 0.080, p > 0.05 \); 3 hours, \( t_s = 0.085, p > 0.05 \);
Figure II-2. Intracellular and Extracellular trehalase levels remain unchanged during temperature stress in vegetative amoebae of *D. discoideum*. The temperature stress regime was performed as described in the Materials and Methods. The first hour after initiation of recovery is indicated by (R+). Amoebae were heat shocked at 30°C ( ■ ) or cold shocked at 4°C ( □ ). Control cells ( □ ) were held at 23°C for 4 hours. During heat shock and recovery from heat shock (each n=3), or cold shock and recovery from cold shock (each n=3), mean intracellular and extracellular trehalase activity remained unchanged as compared to control cells (p>0.05). Analysis by Student's 2-sample T-test (see text).
Figure II-2

**INTRACELLULAR**

% INTRACELLULAR

TIME (hours)

**EXTRACELLULAR**

% EXTRACELLULAR

TIME (hours)
4 hours, \( t_5 = 0.066, \ p > 0.05 \) or during the cold shock and recovery regimes (1 hour, \( t_5 = 0.191, \ p > 0.05 \); 2 hours, \( t_5 = 0.122, \ p > 0.05 \); 3 hours, \( t_5 = 0.062, \ p > 0.05 \); 4 hours, \( t_5 = 0.027, \ p > 0.05 \)).

II.C.2. Temperature Stress and Glycogen Levels

In order to discern the possible source of glucosyl residues for the anabolism of trehalose during stress, the levels of glycogen were examined during heat shock and cold shock. As depicted in Figure II-3, glycogen levels decreased after 1 hour of heat stress and the levels continued to decline significantly \( (t_5 = 3.104, \ p < 0.05) \) over the second hour of the treatment. During recovery from heat stress the glycogen content of the cells remained lower than those of control cells. The effects of cold shock on intracellular glycogen content in \( D.\ discoideum \) amoebae were also examined. Glycogen levels were observed to decrease approximately 7.5 fold below the levels found in control cells, 2 hours after logarithmically growing cells were shifted from 23°C to 4°C (Figure II-3). The observed decreases were statistically significant both at 1 hour \( (t_5 = 4.224, \ p < 0.05) \) and at 2 hours \( (t_5 = 02.868, \ p < 0.05) \) into the cold shock treatment. As in heat shock, glycogen levels remained lower than control cell levels during the 2 hour recovery period at 23°C.
Figure II-3. The decrease in glycogen levels during temperature stress in vegetative amoebae of D. discoideum. The temperature stress regime was performed as described in the Materials and Methods. The first hour after initiation of recovery is indicated by (R⁺). Amoebae were heat shocked at 30°C (■) or cold shocked at 4°C (□). Control cells (□) were held at 23°C for 4 hours. For both heat shock and cold shock (each n=3), mean glycogen levels declined significantly from those of control cells (p<0.05). Analysis by Student's 2-sample T-test (see text).
Figure II-3

GLYCOGEN
(nmoles glucose eq./mg protein) x 10^3

TIME (hours)

R1
II.C.3. Trehalose Levels during Hypersecretion of Trehalase

The possibility that trehalose levels could be affected by laboratory (sucrose) induced hypersecretion of trehalase was investigated. Amoebae which were treated with 0.1 M sucrose, over-secreted trehalase such that 90% of the total activity was found extracellularly after 6 hours of incubation (Figure II-4). Trehalose levels remained unchanged during the hypersecretion of trehalase (2 hour, \( t_s = 0.225, p > 0.05 \); 4 hours, \( t_s = 0.016, p > 0.05 \); 6 hours, \( t_s = 0.137, p > 0.05 \)).

II.C.4. Effect of the Protein Synthesis Inhibitor, Cycloheximide, on the Temperature Stress Response in D. discoideum Amoebae

In order to determine whether or not protein synthesis was required for the induction of the temperature stress response, various facets of the stress response were examined in the presence of cycloheximide. Amoebae were treated with cycloheximide, at a final concentration of 200 \( \mu g/mL \), for 1 hour, at 23°C prior to heat and cold shock. The cycloheximide conditioned cells were heat shocked or cold shocked and analyzed for trehalose levels, trehalose-6-phosphate synthetase activity, trehalase activity and glycogen levels.

As demonstrated in Figure II-5 trehalose levels were unaffected by cycloheximide alone. During heat shock,
Figure II-4. Lack of trehalose accumulation during increased secretion of trehalase in vegetative amoebae of *D. discoideum*. Amoebae were treated for 6 hours with 0.1 M sucrose to induce the hypersecretion of trehalase (A) over which time intracellular trehalose levels remained unchanged (B) (n=3). Analysis by Student's 2-sample T-test (p>0.05).
Figure II-5. The effect of the protein synthesis inhibitor, cycloheximide on the temperature stress response in vegetative amoebae of D. discoideum: Trehalose. Amoebae were treated with cycloheximide at a final concentration of 200 μg/mL for 1 hour, at 23°C prior to heat and cold shock. Trehalose levels were measured prior to addition of cycloheximide (Control A) and after 1 hour of incubation in cycloheximide (Control B). Temperature stress and recovery from temperature stress were performed as described in the Materials and Methods. The first hour after initiation of recovery is indicated by (R^+). Amoebae were heat shocked at 30°C ( ■ ) or cold shocked at 4°C ( ☐ ). control cells ( □ ) were held in cycloheximide at 23°C for 4 hours.
trehalose levels in cycloheximide conditioned cells increased over control cells at 1 hour into the treatment and continued to increase during the second hour of the heat shock. The trehalose content of recovering cells diminished rapidly such that no trehalose was detectable after 2 hours of recovery. Similarly, protein synthesis was not required for trehalose accumulation during cold shock.

As seen in Figure II-6, trehalose-6-phosphate synthetase in cycloheximide conditioned amoebae remained unchanged in the presence of cycloheximide and during the 2 hour stress regime (heat or cold) as compared to the room temperature control. Likewise, synthetase levels remained constant during the 2 hour recovery period at 23°C. Although slight fluctuations in trehalose-6-phosphate synthetase activity between control and treated cells could be observed, none of these differences were statistically significant for heat shock and recovery from heat shock (1 hour, t_s=0.028, p>0.05; 2 hours, t_s=0.047, p>0.05; 3 hours, t_s=0.293, p>0.05; 4 hours, t_s=0.456, p>0.05) or for cold shock and recovery from cold shock (1 hour, t_s=0.519, p>0.05; 2 hours, t_s=0.522, p>0.05; 3 hours, t_s=0.765, p>0.05; 4 hours, t_s=0.944, p>0.05).

Extracellular (secreted) trehalase concentrations were not affected in a statistically significant manner by cycloheximide alone, the 2 hour heat shock or cold shock treatments nor the 2 hour recovery period (Figure II-7).
Figure II-6. The effect of the protein synthesis inhibitor, cycloheximide on the temperature stress response in vegetative amoebae of *D. discoideum*: Trehalose-6-phosphate synthetase. Amoebae were treated with cycloheximide, at a final concentration of 200 μg/mL, for 1 hour, at 23°C prior to heat and cold shock. Trehalose-6-phosphate synthetase was measured prior to the addition of cycloheximide (control A) and after 1 hour of incubation in cycloheximide (control B). Temperature stress and recovery from temperature stress were performed according to the Materials and Methods. The first hour after initiation of recovery is indicated by (R+). Amoebae were heat shocked at 30°C ( ■ ) or cold shocked at 4°C ( □ ). Control cells ( □ ) were held in cycloheximide at 23°C for 4 hours. During heat shock and recovery from heat shock (each n=3), or cold shock and recovery from cold shock (each n=3), in the presence of cycloheximide mean intracellular trehalose-6-phosphate synthetase activity remained unchanged as compared to control cells (p > 0.05). Analysis by Student's 2-sample T-test (see text).
Figure II-6

SYNTHASE ACTIVITY

(μmoles product/min./mg protein)

TIME (hours)

0 1 2 3 4

A B R↓
When analyzed by Student's 2-sample T-tests, it was found that the levels of secreted trehalase in treated cell cultures did not differ significantly from control cell cultures during the heat shock and recovery from heat shock (1 hour, $t_s=0.156, p>0.05$; 2 hours, $t_s=0.344, p>0.05$; 3 hours, $t_s=0.154, p>0.05$; 4 hours, $t_s=0.057, p>0.05$) or during the cold shock and the recovery regime (1 hour, $t_s=0.234, p>0.05$; 2 hours, $t_s=0.309, p>0.05$; 3 hours, $t_s=0.334, p>0.05$; 4 hours, $t_s=0.055, p>0.05$).

Figure II-7 also demonstrates that intracellular trehalase activity also remained unchanged during the 2 hour stress regime (heat or cold) as compared to the room temperature control. Likewise, intracellular trehalase levels remained constant during the 2 hour recovery period at 23°C. Although slight differences in cellular trehalase activity between control and treated cells could be observed, none of these fluctuations were statistically significant for heat shock and recovery from heat shock (1 hour, $t_s=0.035, p>0.05$; 2 hours, $t_s=0.147, p>0.05$; 3 hours, $t_s=0.041, p>0.05$; 4 hours, $t_s=0.016, p>0.05$) or during cold shock and the recovery regime (1 hour, $t_s=0.067, p>0.05$; 2 hours, $t_s=0.063, p>0.05$; 3 hours, $t_s=0.080, p>0.05$; 4 hours, $t_s=0.016, p>0.05$).
Figure II-7. The effect of the protein synthesis inhibitor, cycloheximide on the temperature stress response in vegetative amoebae of D. discoideum: Trehalase. Amoebae were treated with cycloheximide, at a final concentration of 200 μg/mL, for 1 hour, at 23°C prior to heat and cold shock. Glycogen was measured prior to the addition of cycloheximide (control A) and after 1 hour of incubation in cycloheximide (control B). All temperature stress experiments on cycloheximide conditioned amoebae were performed according to the Materials and Methods. The first hour after initiation of recovery is indicated by (R¹). Amoebae were heat shocked at 30°C (■) or cold shocked at 4°C (□). Control cells (□) were also conditioned with cycloheximide and held at 23°C for 4 hours. During heat shock and recovery from heat shock (each n=3), or cold shock and recovery from cold shock (each n=3), in the presence of cycloheximide, mean intracellular and extracellular trehalase activity remained unchanged as compared to control cells (p > 0.05). Analysis by Student's 2-sample T-test.
Figure II-7

% INTRACELLULAR

TIME (hours)

% EXTRACELLULAR

TIME (hours)
As depicted in Figure II-8, stress induced glycogen declines were not altered by the presence of cycloheximide and glycogen decreased during heat stress to levels which were significantly lower than control levels at 2 hours ($t_s=3.168$, $p<0.05$). During recovery from heat stress glycogen content of the cycloheximide conditioned cells remained lower than those of control cells.

The effects of cold shock on intracellular glycogen content in cycloheximide treated *D. discoideum* amoebae were also examined. Glycogen levels were observed to fall below the levels found in control cells, 2 hours after logarithmically growing cells were shifted from 23°C to 4°C (Figure II-8). The observed decreases were statistically significant both at 1 hour ($t_s=2.861$, $p<0.05$) and at 2 hours ($t_s=2.790$, $p<0.05$) into the cold shock treatment. As in heat shock, glycogen levels remained lower than control cell levels during the 2 hour recovery period at 23°C.
Figure II-8. The effect of the protein synthesis inhibitor, cycloheximide on the temperature stress response in vegetative amoebae of *D. discoideum:* Glycogen. Amoebae were treated with cycloheximide, at a final concentration of 200 μg/mL, for 1 hour, at 23°C prior to heat and cold shock. Intracellular and extracellular trehalase was measured prior to the addition of cycloheximide (control A) and after 1 hour of incubation in cycloheximide (control B). All temperature stress experiments on cycloheximide conditioned amoebae were performed according to the Materials and Methods. The first hour after initiation of recovery is indicated by (R+). Amoebae were heat shocked at 30°C (■) or cold shocked at 4°C (□). Control cells (□) were also conditioned with cycloheximide and held at 23°C for 4 hours. For both heat shock and cold shock, in the presence of cycloheximide (each n=3), mean glycogen levels declined significantly from those of control cells (p < 0.05). Analysis by Student's 2-sample T-test.
Figure II-8

GLYCOGEN (nmole glucose eq./mg protein) x 10^3

TIME (hours)

A, B, R1
II.D. DISCUSSION

In order to determine if a futile cycle between trehalose and glucose existed and in order to gain insight the mechanics of trehalose accumulation in the vegetative cells of *D. discoideum*, the activities of the enzymes directly involved in trehalose metabolism, namely trehalose-6-phosphate synthetase and trehalase, were examined.

To determine whether a direct increase in trehalose-6-phosphate synthetase activity was responsible for the observed temperature dependent accumulation of trehalose, the activity of this enzyme was monitored during heat shock and cold shock. Only slight fluctuations in trehalose-6-phosphate synthetase activity were observed during stress and therefore an increase in synthetase activity (by enzyme activation or *de novo* synthesis), does not explain the net accumulation of trehalose during stress.

The catabolic portion (trehalase) of the putative trehalose cycle was also examined to determine if it played a role in trehalose accumulation during stress. Trehalase is known to be secretory in nature, therefore, in theory its intracellular levels can be reduced by at least two ways: (1) direct temperature sensitive deactivation of existing enzyme or temperature sensitive reduction of its *de novo* synthesis; or, (2) hypersecretion via exocytosis from the amoebae. Down-regulation of this enzyme in either of these two manners would result in an unbalanced futile cycle in
which trehalose anabolism might predominate.

Extracellular trehalase levels remained unchanged during stress, indicating that the enzyme was neither hypersecreted nor hyposecreted during the unfavourable temperature treatments. Likewise, intracellular trehalase levels remained unchanged during the stress regimes. Thus it was concluded that the intracellular increase in trehalose content during stress was not due to an alteration in either of the two trehalose metabolizing enzymes.

It is well documented that the addition of non-metabolizable substrates, such as sucrose, to the surrounding medium promotes the secretion of *D. discoideum* lysosomal enzymes, including trehalase (Crean and Rossomando 1979; Seshadri et al., 1986; Klein et al., 1989). This property of the secretory system of *D. discoideum* was exploited to further test the existence of a trehalose futile cycle. Hypersecretion of trehalase was induced by the addition of sucrose to actively growing cultures of *D. discoideum*. This situation is representative of "down-regulation" of trehalase by increased exocytosis. Since trehalose levels failed to increase under these conditions, it was concluded that a constitutive trehalose futile cycle, governed in part by trehalase, does not exist.

In the fungal kingdom, it has been documented that trehalose is synthesized during periods of starvation and development, and this synthesis is often driven at the
expense of glycogen breakdown (Kane and Roth, 1974). Likewise, in *D. discoideum*, a critical step in differentiation is the hydrolysis of glycogen which provides the precursors for the synthesis of certain developmental end products including cellulose and trehalose (Wright and Dahlberg, 1967; Cleland and Cloe, 1968; Wright et al., 1968; see CHAPTER I).

To discover whether stress induced accumulation of trehalose was also accompanied by the mobilization of glycogen, the levels of this storage polysaccharide were monitored during heat and cold shock. It was found that the glycogen levels declined rapidly during both heat and cold stress. Glycogen hydrolysis during heat stress was recently reported in the brine shrimp, *Artemia franciscana* (Clegg and Jackson, 1992) and for *Neurospora crassa* (Neves et al., 1991).

The enzyme responsible for the hydrolysis of glycogen in *D. discoideum* is glycogen phosphorylase (GP). This enzyme exists in two developmentally regulated forms (Rutherford et al., 1988). The first form, a 92 kD protein (GP1), is present during vegetative growth and early development (Rutherford and Cloutier, 1986; Cloutier and Rutherford, 1987; Brickey et al., 1990). The second, a 107 kD protein (GP2), is found exclusively in late development (Cloutier and Rutherford, 1987). GP1 is likely to be responsible for glycogen hydrolysis during stress in *D.*
discoideum, as it is the only form of the enzyme present in vegetative amoebae (Rogers et al., 1992).

The action of this enzyme on intra-amoebal levels of glycogen during temperature shifts would result in an increase in the intracellular levels of glucose-1-phosphate. As described previously, this metabolite is easily converted to the two substrates of trehalose-6-phosphate synthetase: UDP-glucose and glucose-6-phosphate. An intracellular accumulation of these metabolites would in fact drive the trehalose-6-phosphate synthetase reaction in the direction favouring intracellular trehalose accumulation. This is similar to substrate level control which is partially responsible for the accumulation of trehalose during development (Wright and Killick, 1975).

In summary, the activities of trehalose-6-phosphate synthetase and trehalase were not significantly changed during heat shock at 30°C or cold shock at 4°C. Furthermore, the increase in trehalose levels during stress were insensitive to the protein synthesis inhibitor cycloheximide. Therefore it is proposed that it is not an alteration in synthesis, activation, degradation or secretion of any of these enzymes that is responsible for trehalose accumulation during stress, but a temperature induced shift in the equilibrium of the synthetase reaction in favour of the production of trehalose.

It has been reported that part of the heat shock
response in *Neurospora crassa* (Habel et al., 1991) and *Histoplasma capsulatum* (Patriarca et al., 1992) is characterized by a reduction in oxygen uptake and a decline in mitochondrial function. In theory, this would result in an increase in intracellular adenosine 5'-monophosphate (5'-AMP). Interestingly, during vegetative growth, *D. discoideum* GP1 is absolutely dependant on the presence of 5'-AMP for activity (Rutherford and Cloutier, 1986). It is conceivable that anaerobic-like phenomena occur in *D. discoideum* during temperature stress. Therefore, under these circumstances, the accumulated 5'-AMP could unmask the activity of GP1 and initiate the cascade to trehalose accumulation.

It would be expected that a mere shift in the kinetic constants could easily be reversed. While the levels of trehalose declined rapidly during recovery from stress glycogen levels were not restored. It is likely that the glucose units generated from trehalose hydrolysis during recovery are preferentially used for the production of energy rather than restored in the form of glycogen. Post-stress requirement for energy is necessary for the rectification of some irreversible effects of temperature stress such as protein denaturation (Lindquist, 1986).

The role of the lysosomal enzyme in *D. discoideum* has been thought to be hydrolysis of the major storage carbohydrate during spore germination and for cellulose
formation in stalk cells during development. There has been a long standing question as to the reason trehalase is present throughout vegetative growth. Perhaps the enzyme is necessary to hydrolyse the trehalose in bacteria which normally serve as the nutrient source of amoebae. The results of this study suggest that trehalase may have an additional role in recovery from stress; i.e. trehalase may remove a predominant stress metabolite, namely trehalose, once a particularly harsh environment improves. The disappearance of trehalose during recovery from heat and cold shock must be attributed to trehalase. The enzyme is preformed as recovery occurred even in the presence of cycloheximide. The role of trehalase in recovery from stress is further supported by the fact that trehalase is considered to be fairly heat resistant, and can withstand heat treatments of as high as 50°C for 5 minutes (Ceccarini, 1965).

While the results of this study are thought provoking, they do not provide definitive conclusions regarding the role of trehalase during stress or recovery from stress. Studies at the molecular level are required to determine the role of trehalase during stress. Molecular biological approaches to problems are often initiated with the purification of the protein or proteins of interest. For this reason purification of vegetative trehalase was undertaken (CHAPTER III).
CHAPTER III

Affinity Purification of Vegetative Trehalase from

Dictyostelium discoideum
III.A. INTRODUCTION

As described previously, the disaccharide, trehalose, has been isolated from a variety of organisms including bacteria, yeast, filamentous fungi and insects (Elbein, 1974). In these organisms, trehalose serves as an endogenous energy reserve for a variety of physiological purposes. In addition to providing energy for a number of cellular processes, the results of this study suggest that trehalose may also serve as an intracellular stress manager.

Trehalose is hydrolyzed to its glucose monomeric units by the enzyme trehalase. The enzyme has been isolated from bacterial, fungal, yeast, plant, insect and mammalian systems. The enzyme displays diverse properties and serves a variety of functions.

III.A.1. Widespread Occurrence of Trehalases

In prokaryotes, trehalases have been isolated from a wide variety of systems including Ectothiorhodospira halochloris (Herzog et al., 1990) and Escherichia coli (Boos et al., 1987). In these organisms, trehalase is periplasmic in location and induced by conditions of high osmolality. The enzyme is required for the hydrolysis of the osmoprotectant, trehalose, during recovery from stress. In certain strains of E. coli, a constitutive cytoplasmic trehalase may also be present and its function is believed to be that of a digestive enzyme necessary for bacterial
growth on trehalose. (Gutierrez et al., 1989)

Trehalases have also been identified in invertebrates. In *Artemia salina*, a pH dependant trehalase has been shown to be important in the mobilization of trehalose during development (Hand and Carpenter, 1986). As well, trehalase has been shown to serve as a digestive enzyme in the green mussel, *Perna viridis* L (Teo and Lim, 1991).

The trehalases are very important in the fungal and insect kingdoms. Trehalases have been isolated from every fungal species examined to date (reviewed by Thevelein, 1984). Recently, new fungal trehalases have been isolated from the conidia of the filamentous fungus, *Humincola grisea* var. *thermoidea* (Zimmerman et al., 1990) and from vegetative cells of *Schizosaccharomyces pombe* (De Virgilio et al., 1991b). As well, the enzyme has been found in all insects where it can occur in both soluble and particulate forms (Gilby et al., 1967; Yamagawa, 1971; Talbot et al., 1975; Sumida and Yamashita, 1977; Jahagirdar et al., 1990). In fact, both forms of trehalase often occur simultaneous in the same insect tissue or organ. The role of trehalase in insects is mobilization of their major energy carbohydrate, trehalose. The importance of trehalases to insects and fungi is reflected in numerous recent studies on trehalase inhibitors and trehalase-targeted insecticides and fungicides (Asano et al., 1987; Nakayama et al., 1991; Murao et al., 1991; Ando et al., 1991; Asano et al., 1990)
Trehalases have been identified in a wide variety of plant tissue culture cell lines, including *Selaginella lepidophylla*, wheat callus, jack pine, white spruce and alfalfa (Kendall *et al.*, 1990). Trehalase has also been isolated from the peribacterioid space, of *Glycine max* root nodules (Kinnbacker and Werner, 1991). The role of trehalase in plants is highly speculative and it has been suggested that trehalase may be responsible for protection against infection by fungi, insects or bacteria.

Trehalases are ubiquitous in mammalian systems and occur as intrinsic glycoproteins of the small intestine and renal brush border membranes (Takesue *et al.*, 1986). Mammalian trehalases have been shown to have medical importance. Urinary levels of trehalase have been used as indicators of kidney injury during chronic cadmium exposure (Iwata *et al.*, 1988). The trehalase content of human amniotic fluid has been shown to be a useful prenatal marker for cystic fibrosis (Szabo *et al.*, 1989). As well, human anomalies where trehalase is absent from the intestinal brush border membranes have been documented (Bergoz, 1971). The absence of intestinal trehalase can result in severe gastroenteritis upon the ingestion of trehalose or trehalose containing foods (mushrooms).

III.A.2. *D. discoideum* Trehalases

The fungal trehalases can be divided into two groups
(reviewed by Thevelein, 1984). The neutral trehalases function optimally at pH 6.0 to 7.5, and can be activated by cAMP-dependent protein phosphorylation. These trehalases are often referred to as regulatory trehalases. The acid trehalases or non-regulatory trehalases have pH optima which lie between 5.5 and 5.7 and can not be activated in a cAMP-dependent manner.

While both types of trehalases occur in *S. cerevisiae* (Londesborough et al., 1984) only acid trehalase has been found to occur in *D. discoideum* (Seshadri et al., 1986). The enzyme persists throughout the life cycle of the organism, where it is found maximally during periods of vegetative growth. The function of vegetative trehalase is unknown, however, the results of this study suggest that the enzyme may be required to hydrolyse trehalose during recovery from stress.

Trehalase is released into the surroundings during early development and remains low during most of the developmental program. Trehalase activity reappears during terminal sorocarp formation (Roth and Sussman, 1966; Killick and Wright, 1972a) and it has been determined that this enzyme, referred to as a developmental trehalase, is specific to stalk cells (Jefferson and Rutherford, 1976). It is assumed that the role of this developmental trehalase is to hydrolyse trehalose which has accumulated in pre-stalk cells during early development, providing the glucose
monomeric units required for cellulose anabolism.

A spore specific trehalase (vegetative) has also been found in *D. discoideum* (Cotter and Raper, 1970) and the role of this enzyme is believed to be hydrolysis of trehalose for energy provision during the germination program.

It has been determined that vegetative and developmental trehalase are isozymes differing in molecular weight (Killick, 1983) and charge (Seshadri et al., 1986). In *D. discoideum* vegetative and developmental trehalases have been referred to as Form I and Form II trehalases, respectively.

III.A.3. Purpose of This Study

Despite the ubiquitous nature of trehalases and the biological importance of trehalose, trehalase has been purified from relatively few sources. In addition, trehalase purification schemes have been effective enough to undertake studies at the molecular level in only a few cases (Boos et al., 1989; Ruf et al., 1990).

Trehalases of *D. discoideum* are particularly interesting due to their diverse functions throughout the life cycle. Form I (vegetative) is likely to be involved in nutrition, recovery from stress and spore germination. Form II (developmental) is involved in stalk cell formation. Therefore the motivation behind this study was to develop a rapid purification scheme for *D. discoideum* trehalase.
Form I enzyme was chosen for this study due to its interesting relationship to stress management in this organism.

III.A.4. N-containing Analogs of Trehalose

The purification scheme developed in this study employed an affinity purification step using N-containing analogs of the natural substrate trehalose. A historical overview of such analogs is presented below.

III.A.5. Validamycins

The Validamycins (A-G) were discovered by virtue of their antibiotic nature against rice sheath blight Rhizoctonia solani (Iwasa et al., 1970; Horii et al., 1972; Kameda et al., 1986). In all cases the Validamycins were isolated from cultures of Streptomyces hygroscopicus. Asano et al. (1987) and Kameda et al. (1987) later reported that the structure of these compounds resembled trehalose and that their mode of anti-fungal action was due to their inhibitory action on trehalases. It was also reported that the Validamycins were highly specific for trehalase, exhibiting negligible effects on β-glucosidases, α-glucosidases or pectinases. The Validamycins are equally effective against trehalases from rat, rabbit, yeast, Mycobacterium and insect larva (Spodoptera litura) with IC$_{50}$ values ranging from $10^{-5}$ M to $10^{-10}$ M (Kameda et al., 1987;
Asano et al., 1990). The most potent of the Validamycins, Form A (Figure III-1), has been formulated into a commercial fungicide known as "Solocol." Validamycin A was employed in this study.

III.A.6. Castanospermine

Castanospermine is a plant indolizidine alkaloid isolated from the Castanospermum australe (Hohenschuts et al., 1981) (Figure III-1). The compound is highly toxic to mammals and its ingestion can lead to severe gastroenteritis and death. Castanospermine is a potent competitive inhibitor of β-glucosidase and β-cerebroside (Saul et al., 1983) as well as an inhibitor of α-glucosidase (Saul et al., 1984). Castanospermine has recently been shown to be an inhibitor of porcine trehalase (Salleh and Honek, 1990).

III.A.7. MDL 25,637

Information about the compound MDL 25,637 (Figure III-1) is very limited. MDL 25,637 is a synthetic analog of trehalose (Dr. E.H. Bohme, personal communication) formulated by the Marion Merrell Dow Research Institute (Cincinnati, Ohio, USA). MDL 25,637 has recently been shown to be a potent competitive inhibitor of porcine trehalase (Salleh and Honek, 1990).
Figure III-1. Chemical structures of α,α-trehalose and N-containing trehalose analogs.
Figure III-1

Trehalose

MDL 25,637

R = β-D-glucose
Validamycin A

Castanospermine
III.B. MATERIALS AND METHODS

III.B.1. Growth of the Organism - *D. discoideum* Amoebae

The axenic mutant of *D. discoideum*, strain Ax3 (ATCC 28368) (gift from Dr. R.L. Dimond) was used as a source of vegetative trehalase (Form I). Vegetative amoebae were grown axenically in TM Medium (Free and Loomis, 1974) which was composed of 10.0 g trypticase peptone (BBL), 5.0 g yeast extract (Difco), 10.0 g glucose (Sigma), 0.35 g Na₂HPO₄ (Sigma) and 1.2 g KH₂PO₄ (Sigma) in 1.0 L of double distilled water (ddH₂O). The pH of the medium was adjusted to 6.5. Medium (400 mL) was dispensed in 1 L Erlenmeyer flasks and autoclaved at 121°C, 15 psi, for 15 minutes. Cooled sterile TM medium was inoculated with 5.0 mL of a previously grown log phase culture containing 1 X 10⁶ cells/mL. To ensure uniform suspension and aeration of amoebae in the medium, cultures were shaken on a rotary shaker (Labline) at 165 rpm at 23°C until the log phase cultures reached 1 X 10⁶ cells/mL (approximately 48 hours).

III.B.2. Growth of the Organism - *D. discoideum* Sorocarps

The matrix of the haploid wild type strain of *D. discoideum*, strain NC4 (ATCC 28245) served as a source of developmental trehalase (Form II). This strain forms fruiting bodies when grown in association with *Escherichia coli* B/r on Adams (1959) glucose-salts agar. This medium was prepared by adding 1.0 g NH₄Cl (Sigma), 3.0 g KH₂PO₄
(Sigma), 6.0 g Na₂HPO₄ (Sigma), 0.13 g MgSO₄ (Sigma) and 15.0 g agar (Gibco) to 1.0 L of ddH₂O. The medium was autoclaved at 121°C, 15 psi, for 15 minutes and then combined with a sterile glucose (Sigma) solution (40.0 g/100 mL) to give a final glucose concentration of 0.4 %. Cooled medium (50°C) was dispensed into sterile plastic Petri plates (100 X 15 mm) and allowed to solidify at 23°C. Plates were stored in the cold (4°C) until used.

Fruiting bodies of D. discoideum, strain NC4-H, were prepared according to the methods of Cotter and Raper (1968). Spores were aseptically transferred by loop into 15 - 20 mL of sterile ddH₂O to a final concentration of 10⁷ spores/mL. One loopful of E. coli B/r was added to the spores, and the suspension was mixed on a Vortex mixer. 1.5 - 2.0 mL of spore/bacteria mixture was dispensed evenly onto the surface of glucose-salts agar plates and incubated at 23°C for 4 - 6 days.

III.B.3. Preparation of Crude Extracellular Vegetative Trehalase

As one of the long term goals of this work is N-terminal amino acid sequencing of trehalase it was necessary to work exclusively with secreted trehalase. This ensured minimal contamination with pre-processed intracellular forms of the enzyme which may still harbour the N-terminal signal sequence common to all lysosomal enzymes of Dictyostelium
discoideum (Freeze et al., 1983). The use of extracellular enzyme also represents a purification of 20-fold, as less than 5% of proteins are normally secreted during log phase growth (Dimond et al., 1981).

Log phase amoebae were separated from spent TM medium by centrifugation in a Sorvall RC2-B centrifuge (GS-A rotor), at 3000 X g, for 30 minutes at 4°C. The supernatant fraction represents crude extracellular (secreted) vegetative trehalase.

III.B.4. Preparation of Crude Developmental Trehalase

Dormant spores are suspended in a mucoid droplet of extracellular material (matrix) and are supported above the substratum by a column of dead stalk cells. The matrix surrounding the spores is a rich source of many extracellular enzymes including Form II trehalase. The use of matrix enzyme also represents a purification of at least 5-fold, as less than 20% of proteins are normally secreted during the sporulation process (Seshadri et al., 1986).

Dormant spores and matrix were collected from 4 - 6 day old sorocarps by passing a wet microscope slide 2 - 3 mm above the agar surface. The collected material was deposited into 10 mL of ddH₂O. Dormant spores were separated from matrix by centrifugation in an IEC clinical centrifuge, at 500 X g, for 5 minutes, at 23°C. The supernatant fraction represents crude developmental
trehalase.

III.B.5. Inhibition of Proteolysis

To inhibit proteolysis, a cocktail of protease inhibitors was included in all subsequent manipulations of the crude trehalase preparations (Form I and Form II) described above. This cocktail of protease inhibitors did not inhibit trehalase activity. The stock protease inhibitor mixture contained leupeptin (5 mg/mL) (Sigma), phenylmethylsulfonyl fluoride (PMSF) (80 mg/mL) (Sigma), ethylenediamine tetraacetic acid (EDTA) (20 mg/mL) (Sigma), and 2,2'-dithiodipyridine (5 mg/mL) (Fluka). The cocktail was added to the crude trehalase preparation in a final concentration of 2 % (v/v).

III.B.6. Ammonium Sulphate Precipitation

Both vegetative and developmental crude trehalase preparations were concentrated with ammonium sulphate. Solid ammonium sulphate (Sigma) was added to the crude extracellular fractions of trehalase to a final concentration of 80 % (w/v). The salted extract was stirred overnight at 4°C and the precipitate that formed was collected by centrifugation in a Sorvall RC2-B centrifuge (GS-A rotor), at 13,000 X g, for 60 minutes at 4°C. The precipitate was solubilized in 0.1 M acetate buffer (pH 5.5) and was dialysed (molecular weight cut off - 12 kD)
overnight (4°C), against 400 volumes of the same buffer. After dialysis the sample was clarified by centrifugation in a Sorvall RC2-B (SS-34 rotor), at 30,000 X g, for 30 minutes at 4°C. Concentrated protein was stored at -20°C.

III.B.7. Measurement of Protein

Protein was determined according to the methods of Bradford (1976) using the Bio-Rad Protein Assay. The assay was standardized using bovine serum albumin (Sigma). Due to the presence of Tween-20, affinity purified trehalase was quantified using the Bio-Rad Detergent Compatible (DC) Protein Assay. DC protein assays were standardized using bovine gamma globulin (Bio-Rad) in 0.3% (v/v) Tween-20.

III.B.8. Measurement of Trehalase Activity

The assay for trehalase was performed by methods modified from a previously reported procedure (Chan and Cotter, 1980) and was described in detail in CHAPTERS I and II.

III.B.9. Measurement of Cysteine Proteinase Activity

The assay for cysteine proteinase activity was performed according to the methods of Achstetter et al., (1981). Cysteine proteinase was quantified using the synthetic substrate N-benzoyl-L-prolyl-L-phenylalanyl-L-arginine 4-nitroanilide (BzPFR) (Sigma). A 10mM stock
solution (in ddH₂O) of this substrate was maintained at -20°C. Prior to cysteine proteinase determination a substrate cocktail containing 1.0 mL of 0.1 M sodium phosphate buffer (pH 6.0), 10 μL of stock substrate and 10 μL 0.1 M dithiothreitol (DTT) (Sigma) was prepared.

Assays were carried out in a total volume of 165 μL in 96 well microtitre plates. Protein preparations of 15 μL were added to 150 μL of substrate cocktail in a microtitre well. The contents of the well were mixed and incubated on a microtitre plate shaker for 30 minutes at 23°C. The release of 4-nitroaniline was monitored spectrophotometrically at 405 nm using a Bio-Tek microtitre plate reader. In addition, the optical densities of known concentrations of p-nitroaniline (Sigma) were determined to establish a standard curve which ranged from 10 - 50 nmoles of product per reaction well.

III.B.10. Analytical Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE)

Analytical SDS-PAGE was performed using the conventional discontinuous buffer system described by Laemmli (1970).

Mini SDS-PAGE gels were produced using the Bio-Rad Mini Protean II Electrophoresis System. The 7.5 % (acrylamide polymer) separating gel consisted of the following components:
i) 4.85 mL ddH₂O

ii) 2.5 mL 1.5 M Tris-HCl (pH 8.8) (Sigma)

iii) 2.5 mL Acrylamide (Bio-Rad) monomer solution (30% T)

iv) 100 µL 10% (w/v) SDS (Sigma)

v) 50 µL 10% (w/v) Ammonium Persulphate (APS) (Sigma)

Following a degassing period, of the mixture above, under house vacuum, 10 µL of N,N,N',N'-tetramethylethylenediamine (TEMED) (Sigma) was added to initiate polymerization. The solution was poured into gel formers and overlayed with ddH₂O. The dimensions of the separating gels were 8.0 cm in width, 5.0 cm in height and 0.75 mm or 1.0 mm thick.

After polymerization (1 hour) a 4% (acrylamide polymer) stacking gel was formed above the separating gel. The techniques employed to cast the stacking gel were identical to those of the separating gel. The stacking gel consisted of:

i) 6.1 mL ddH₂O

ii) 2.5 mL 0.5 M Tris-HCl (pH 6.8)

iii) 1.3 mL Acrylamide monomer solution (30% T)

iv) 100 µL 10% (w/v) SDS

v) 50 µL 10% (w/v) APS

vi) 10 µL TEMED (added after degassing)

A 10 - 15 well slot former was inserted into stacking gels
to facilitate the formation of sample wells during the polymerization process.

Protein preparations (4 parts) were added to 1 part of a 5X sample buffer and loaded into preformed wells. The 5X sample buffer consisted of:

i) 2.5 mL 2-Mercaptoethanol (Sigma)
ii) 5.0 mL Glycerol (Sigma)
iii) 2.5 mL 0.5 M Tris-HCl (pH 6.8)
iv) 1.0 g SDS
v) 0.4 mg Bromophenol Blue (Sigma)

Typically 10 to 30 µg of protein (20 - 25 µL) were loaded into each well.

Electrophoresis was carried out at 4°C, in a running buffer which consisted of Tris (25 mM), Glycine (192 mM) (Sigma) and SDS (0.1% w/v). The constant current applied was 12 mA and 18 mA while the sample travelled through the stacking gel and through the separating gel respectively. This low current was required to minimize heat production and sample denaturation. Electrophoretic runs were stopped when the bromophenol blue tracking dye was approximately 1 mm from the bottom of the gel (2 - 4 hours). After electrophoresis, gels were stained for total protein using the Bio-Rad silver stain kit or stained for trehalase activity (see below).
III.B.11. Detection of Trehalase Activity in situ After Electrophoresis

After electrophoresis gels were washed for 1 hour, in a 2.5\% (v/v) solution of Triton-X-100 (Sigma). This procedure removed the SDS from the gel and facilitated the renaturation of the separated proteins in situ (North et al., 1988). The renaturation gels were placed on sheets of absorbent filter paper (Whatman No. 1) which had been saturated with a solution of trehalose (0.05 M) and the glucose detection reagent of Chan and Cotter (1980). Additional trehalose solution and glucose reagent were pipetted onto the surface of the gel. After 5 to 30 minutes of incubation at 23°C, trehalase was revealed as a brilliantly stained green band of activity.

III.B.12. Preparative SDS-PAGE and Electrophoretic Purification of Vegetative Trehalase (Form I)

Preparative SDS-PAGE was performed using the conventional discontinuous buffer system described by Laemmli (1970).

SDS-PAGE gels were produced using the Bio-Rad Protean II Electrophoresis System. The 7.5\% (acrylamide polymer) separating gel consisted of the following components:

i) 19.6 mL ddH$_2$O

ii) 10.6 mL 1.5 M Tris-HCl (pH 8.8)

iii) 11.0 mL Acrylamide monomer solution (30% T)
iv) 410 µL 10% (w/v) SDS

v) 200 µL 10% (w/v) Ammonium Persulphate (APS)

Following a degassing period, of the mixture above, under house vacuum, 10 µL of TEMED was added to initiate polymerization. The solution was poured into gel formers and overlayed with ddH₂O. The dimensions of the separating gels were 14 cm in width, 16 cm in height and 1.5mm thick.

After polymerization (3 hours) a 3% (acrylamide polymer) stacking gel was formed above the separating gel. The techniques employed to cast the stacking gel were identical to those of the separating gel. The stacking gel consisted of:

i) 13.0 mL ddH₂O

ii) 5.0 mL 0.5 M Tris-HCl (pH 6.8)

iii) 2.0 mL Acrylamide monomer solution (30% T)

iv) 205 µL 10% (w/v) SDS

v) 200 µL 10% (w/v) APS

vi) 10 µL TEMED (added after degassing)

A single well slot former was inserted into stacking gels to facilitate the formation of a single large preparative sample well during the polymerization process.

Ammonium sulphate concentrated trehalase (Form I) preparations (4 parts) were diluted with 1 part of the 5X sample buffer described above. Typically 2 to 4 mg of protein (2.5 mL) were loaded into each preparative well.

Electrophoresis was carried out at 4°C, in a running
buffer which consisted of Tris (25 mM), Glycine (192 mM) and SDS (0.1% w/v). The constant current applied was 40 mA and 60 mA while the sample travelled through the stacking gel and through the separating gel, respectively. This low current was required to minimize heat production and sample denaturation. Electrophoretic runs were stopped when the bromophenol blue tracking dye was approximately 1 cm from the bottom of the gel (4 - 6 hours).

After electrophoresis the entire gel was renatured by incubation in 2.5 % (v/v) Triton-X-100 at 23°C for 2 hours. Following renaturation, a 1 cm vertical strip was excised from each side of the gel and stained for trehalase activity as described above. The trehalase stained gel strips were re-aligned with the original gel; the trehalase location was predicted in the unstained gel; and excised.

Protein was eluted from the excised gel strip using an ISCO Electrophoretic Concentrator (Model 1750). The gel slices were positioned in the large well of a two-well sample cup. A 0.04 M Tris-Acetic acid buffer (pH 8.6) was placed in the electrode chambers of the elution apparatus. A 0.01 M Tris-Acetic acid buffer (pH 8.6) was placed in the inner chambers of the elution apparatus and in the sample cup over the gel slices. Electroelution was conducted for a period of 12 hours at a constant power setting of 3 W. Concentrated electroeluted protein was recovered in 200 µL of the inner chamber buffer from the smaller well of the
sample cup.

Electroeluted protein was then analyzed by analytical SDS-PAGE and Gelatin-SDS-PAGE and used in enzyme kinetic studies.

III.B.13. Gelatin-SDS-PAGE

The analysis of cysteine proteinases using gelatin-SDS-PAGE was performed using the methods of North et al. (1988). Mini gelatin-SDS-PAGE gels were produced using the Bio-Rad Mini Protean II Electrophoresis System.

The 7.5 % (acrylamide polymer) separating gel consisted of the following components:

i) 3.85 mL ddH₂O

ii) 2.5 mL 1.5 M Tris-HCl (pH 8.8)

iii) 2.5 mL Acrylamide monomer solution (30% T)

iv) 100 μL 10% (w/v) SDS

v) 50 μL 10% (w/v) Ammonium Persulphate (APS)

vi) 1.0 mL 2% (w/v) Swine gelatin (proteinase substrate) (Sigma)

Following a degassing period of the mixture above, under house vacuum, 10 μL of TEMED was added to initiate polymerization. The solution was poured into gel formers and overlayed with ddH₂O. The dimensions of the separating gels were 8.0 cm in width, 5.0 cm in height and 0.75 mm or 1.0 mm thick.

After polymerization (1 hour) a 4% (acrylamide polymer)
stacking gel was formed above the separating gel as described for analytical SDS-PAGE above.

Protein preparations (4 parts) were diluted with 1 part of the 5X sample buffer described above. Typically 10 to 30 µg of protein (20 - 25 µL) were loaded into each well.

Electrophoresis was carried out at 4°C, in a running buffer which consisted of Tris (25 mM), Glycine (192 mM) and SDS (0.1% w/v). The constant current applied was 12 mA and 18 mA while the sample travelled through the stacking gel and through the separating gel respectively. This low current was required to minimize heat production and sample denaturation. Electrophoretic runs were stopped when the bromophenol blue tracking dye was approximately 1 mm from the bottom of the gel (2 - 4 hours).

After electrophoresis, gels were washed in 2.5% (v/v) Triton-X-100 for 1 hour to remove SDS and facilitate the renaturation of proteins in situ. Cysteine proteinases were specifically activated by incubating the renatured gels at 23°C in 0.1 M acetate buffer (pH 4.0), supplemented with 1.0 mM DTT. Activation required 16 hours. During the activation period, the various cysteine proteinase species hydrolyzed the gelatin substrate network in their immediate vicinity. Visualization of cysteine proteinase bands was accomplished by staining gels for 1 hour in a Coomassie Blue staining solution which consisted of 0.25% (w/v) Coomassie Brilliant Blue R-250, 50% (v/v)
methanol and 10% (v/v) glacial acetic acid. Following staining, gels were partially destained in 40% (v/v) methanol and 10% (v/v) glacial acetic acid until good contrast between the proteinase bands (clearing) and the background colour (blue) was obtained.

III.B.14. Preparative Isoelectric Focusing (IEF)

It was necessary to completely desalt protein preparations prior to preparative IEF on the Rotofor System (Bio-Rad). Dialysis against HPLC grade H₂O efficiently removed excess salt which ensured that the nominal pH range of the ampholyte would extend over the full length of the focusing chamber. Desalting also allowed for maximum voltage application without detrimental overheating of the sample during focusing.

Approximately 45 mL of ammonium sulphate concentrated trehalase preparations (Form I and Form II) were dialysed against 400 volumes of HPLC grade H₂O, at 4°C, overnight. A volume of 2.0 mL of a 40% (w/v) ampholyte solution (pH range 3-5) and 0.1 mL of a 40% (w/v) ampholyte solution (pH range 3-10) were added to the desalted preparation. The protein/ampholyte mixture was diluted to 51 mL with HPLC grade H₂O and clarified by centrifugation in a Sorvall RC2-B centrifuge (SS-34 rotor), at 30,000 x g, 4°C, for 30 minutes. The entire clarified mixture was loaded onto the Rotofor system. The cathode (-) assembly chamber was filled
with the basic electrolyte 0.1 M NaOH and the anode (+) assembly chamber was filled with the acidic electrolyte 0.1 M H$_3$PO$_4$.

Focusing on the Rotofor system required 6 hours at 12 W constant power at 4°C. The initial conditions were 558 V and 21 mA. At equilibrium the electrophoretic conditions were 1000 V and 12 mA. All 20 fractions were collected and their pH value measured. The trehalase activity in each fraction was also measured. In addition, aliquots of the fractions were analyzed by Gelatin-SDS-PAGE for cysteine proteinase activity.

III.B.15. Sources of Amino Sugar Inhibitors of Trehalase

In the search for the appropriate ligand for the affinity purification of trehalase, three N-containing compounds which are structural analogues of the natural substrate, trehalose, were analyzed.

Validamycin A was obtained from 2 sources. Solocol, a fungicide/insecticide manufactured by Takeda Chemical Industries Ltd. (Tokyo, Japan), was kindly provided by Dr. H. W. Platt (Agriculture Canada, Charlottetown, P.E.I.). Solocol is a 3 % (w/v) commercial formulation of Validamycin A which also contains wetting agents (soaps) and copper salts. Validamycin A was purified from this pesticide by ion exchange chromatography. Since the amount of Validamycin A obtained in this manner was insufficient to
construct an affinity column, the details of this purification procedure are reported elsewhere (Appendix A). Crystalline Validamycin A (93.5 % pure) (100 mg) was kindly provided by Mr. N. Sugita of Takeda Chemical Industries Ltd. (Tokyo, Japan). This second source of high quality Validamycin A was used in all subsequent experiments.

A second trehalose analogue, MDL 25,637 (50 mg), was kindly provided by Dr. E. H. W. Bohme of the Marion Merrell Dow Research Institute (Cincinnati, Ohio, USA). The third amino sugar of interest, castanospermine, was purchased from Sigma Chemicals (St. Louis, Missouri, USA).

III.B.16. Inhibition of Trehalase by Amino Sugars (K_i)

Inhibition of trehalase activity by Validamycin A, MDL 25,637, or castanospermine was carried out using enzyme which had been partially purified by preparative electrophoresis. The total enzyme activity 2 mUnits in the 210 μL reaction mix. The substrate trehalose was used at concentrations of 1mM, 2mM, 3mM and 11 mM. Enzyme assays were carried out in the absence of inhibitors or in the presence of a range of concentrations of Validamycin A (1 X 10^{-9} M to 10 X 10^{-8} M), MDL 25,637 (1 X 10^{-8} M to 10 X 10^{-8} M) or castanospermine (3 X 10^{-5} M to 10 X 10^{-5} M).

None of the amino sugar inhibitors, at the highest concentration used in these studies, interfered with the glucose detection system.
To 100 μL of reagent in a microtitre plate well, the appropriate amounts of trehalose (in 50 μL) and inhibitor (in 10 μL) were added and mixed. At time zero, the enzyme (in 50 μL) was mixed into the well and the change in absorbance (405 nm) was monitored every 3 seconds for 15 minutes using a Bio-Tek Microplate Autoreader (Model EL309). This procedure was performed in triplicate for all of the combinations of substrate and inhibitor. Data were collected directly from the plate reader with the aid of the computer program, COLLECT (developed by Dr. H. B. Fackrell, University of Windsor).

In addition, enzyme kinetic studies were carried out using trehalase which had been preincubated with the amino sugar inhibitors. For these studies, a range of concentrations of Validamycin A (0.5 X 10⁻⁹ M to 10 X 10⁻⁹ M), MDL 25,637 (1 X 10⁻⁸ M to 10 X 10⁻⁸ M) or castanospermine (3 X 10⁻⁵ M to 10 X 10⁻⁵ M) and 2 mU of trehalase were incubated with shaking for 30 or 60 minutes. The reaction was started by the simultaneous addition of trehalose and the glucose detection reagent. Optical density of the reaction mixture was monitored for 15 minutes as described above. The optical densities of completed reactions (30 minutes) were also recorded.

The initial velocity (V₀) of each reaction was determined using the computer program ANALYSE (developed by Dr. H.B. Fackrell, University of Windsor). The program
calculated the derivative of the reaction curve. Apparent $K_m$ and $V_{max}$ were estimated by Lineweaver-Burk plots of reciprocal velocity vs. reciprocal substrate concentration. $K_i$ was estimated using a secondary plot of apparent $K_m$ vs. inhibitor concentration. The computer program SYSTAT (Version 5.01) was used to generate regression statistics and to assess the linearity of the Lineweaver-Burk and secondary plots. Statistical analyses of enzyme kinetic plots are reported in Appendix B.

III.B.17. Inhibition of Trehalase by Amino Sugars (Reversibility)

Investigations to determine the reversibility of inhibition by amino sugars and the reactivation time of the inhibited enzyme upon dilution were performed. Trehalase (0.03 Units) was incubated with Validamycin A ($1 \times 10^{-6}$ M), MDL 25,637 ($1 \times 10^{-5}$ M), and castanospermine ($1 \times 10^{-4}$ M) for 60 minutes at $23^\circ$C. Exhaustive dialysis against 400 volumes of 0.1 M acetate buffer (pH 5.5) was carried out with the pre-inhibited enzyme for 18 hours. Trehalase activity was recorded at 0, 2, 6 and 18 hours into dialysis. As a control, uninhibited trehalase was monitored under similar experimental conditions.

III.B.18. Preparation of the Affinity Support

Validamycin A was coupled to epoxy-activated Sepharose
6B (Sigma) according to the recommendations of the manufacturer. Epoxy-activated Sepharose 6B (0.5 g) dried beads were swollen for 15 minutes in 10 mL of HPLC grade H_2O. The H_2O was removed by filtration in a sintered glass funnel and the gel was washed and drained 5 times with 10 mL volumes of HPLC grade H_2O. Washed and drained activated Sepharose 6B was suspended in a round bottom flask containing, for each 1 mL of swollen gel, 15 mL of 0.1 M sodium borate (pH 9.0) and 20 mg of Validamycin A. Since there are 20 μmoles of available epoxide groups for 1 mL of swollen gel, this combination represented a molar ratio of Validamycin A to coupling groups of 1.5:1.

The flask was stoppered and the mixture was incubated in a 32°C shaking H_2O bath for 18 hours. At 18 hours, the buffer was removed by filtration, and the filtrate (post-coupling buffer) was retained to estimate the amount of Validamycin A which had been successfully coupled to the gel matrix (see below). The retentate (gel) was further washed with 5 volumes of HPLC grade H_2O. Unreacted epoxide groups were blocked by incubating the gel for 12 hours at 4°C with 1 M ethanolamine/HCl buffer (pH 9.0). The blocking solution was removed by filtration and the gel was washed, alternating five times between 0.1 M sodium acetate (pH 4.0) containing 0.5 M NaCl and 0.1 M sodium borate (pH 9.0) containing 0.5 M NaCl. The substituted gel was packed into a siliconized pasteur pipette, resulting in a column of 1.2
mL (0.5 cm X 4.8 cm) substituted-gel bed size. Before use, the column was equilibrated with the appropriate buffer (see below).


The inhibitory activity of Validamycin A on porcine trehalase is well documented (IC$_{50}$=10$^{-8}$ M) (Kendall et al., 1990). This information was pivotal to the estimation of the extent of substitution of the Validamycin A-Sepharose adsorptive matrix.

In general, the inhibitory activity of a range of dilutions (10 fold to 100,000 fold) of post-coupling buffer on porcine trehalase was examined. To 100 µL of glucose detection reagent in a microtitre plate well, 50 µL of a 0.05 M trehalose solution and 10 µL of diluted post-coupling buffer were added and mixed. As a control, 10 µL of 0.1 M sodium borate buffer was used instead of post-coupling buffer. At time zero, 0.1 units of porcine trehalase (Sigma) were mixed into the well. The contents were incubated on a microtitre plate shaker unit at 23°C, for 30 minutes, at which time the optical density (405 nm) was recorded. The percentage of trehalase inhibition was calculated by comparing the optical density of samples containing post-coupling buffer to the optical density of samples containing pristine borate buffer. Since it is
known that the particular dilution of post-coupling buffer which inhibits porcine trehalase by 50% (IC$_{50}$) must contain Validamycin A in a concentration of 10$^{-8}$ M, the amount of Validamycin A remaining in the undiluted post-coupling buffer (and thus the amount of Validamycin A which was bound to Sepharose) could be determined.

III.B.20. Affinity Purification of Vegetative Trehalase (Form I)

Unless otherwise stated, all procedures associated with the affinity purification of trehalase were performed at 4°C. The flow rate of the column was consistently 0.2 mL per minute.

Ammonium sulphate concentrated trehalase (approximately 10 mg) in a volume of 20 mL was applied to the affinity column which had been equilibrated with 0.3 M sodium acetate buffer, containing 0.3 % (v/v) of the detergent Tween-20 (Sigma). Material passing through the column was collected and immediately reapplied to the affinity adsorbent at least 3 times. After sample application and cycling, the column was washed with the starting buffer (usually 150 to 200 column volumes), until no protein could be detected by the BioRad DC Protein Assay. The extensive washing of the affinity matrix facilitated the complete removal of unbound or non-specifically bound material prior to elution. To elute bound trehalase, the column was washed with 15 mL of
an alkaline desorption buffer which consisted of 0.1 M sodium borate (pH 10.0) and 0.5 M NaCl. Fractions of 1 mL were collected and dialysed for 12 hours against 400 volumes of 0.01 M sodium acetate (pH 5.5). The desalted fractions were analyzed for protein content and trehalase activity. The fractions which demonstrated trehalase activity were pooled and concentrated by centrifugation (2000 X g, 45 minutes) using the Millipore Ultrafree-MicroCentrifuge filter unit (molecular weight cut off 5 kD). Concentrated purified trehalase was analyzed by SDS-PAGE for homogeneity.
III.C. RESULTS

Previously, vegetative trehalase from Dictyostelium discoideum was partially purified in a 6-step procedure which utilized conventional protein purification techniques (Gupta and Cotter, 1990). The authors reported that the enzyme behaved anomalously throughout the purification regime, and displayed multiple molecular weights ranging from 30 kD to 162 kD. In an attempt to maximize the first step in a new purification scheme for trehalase the characteristics of molecular mass and charge were investigated.

III.C.1. Molecular Mass – Preparative Electrophoresis

As reported by Gupta and Cotter (1990), trehalase exhibited aberrant molecular weight characteristics when subjected to gel filtration chromatography. It was suggested that this behaviour was due to the presence of post-translational carbohydrate additions on the enzyme. Preparative SDS-PAGE (partially denaturing) was chosen as an alternative purification step which would exploit molecular mass for the separation of proteins.

Vegetative (Form I) trehalase preparations which had been concentrated by ammonium sulphate were subjected to SDS-PAGE under partially denaturing conditions (without heating), on 16 cm gels. Following electrophoresis, the gels were treated with Triton-X-100 to facilitate
renaturation of the separated proteins and trehalase activity was detected in situ as described in the Materials and Methods. The region of the gel which exhibited trehalase activity (42kD) was excised and the protein was electroeluted. Electrophoretically purified trehalase was examined for trehalase activity, protein content and was subjected to further SDS-PAGE to detect trehalase activity and protease activity in situ.

Figure III-2 is a photograph of a SDS-PAGE gel (silver stain), of concentrated and electrophoretically purified trehalase preparations. Electroelution of trehalase-specific bands resulted in a partial purification of the enzyme and, as is evident from the gel, a substantial amount of protein could be removed from the trehalase preparation by this single step (see Table III-1 also). When the same preparations were stained for trehalase activity it was seen that the partially purified enzyme retained stainable activity and displayed this activity in the 42 kD region of the gel (Figure III-3). In other words, the aberrant molecular weight characteristics previously reported for this enzyme were not observed when preparative electrophoresis was employed. The results of electrophoretic purification of trehalase are summarized in Table III-1.

Since proteases are often hazardous contaminants in the preparations of purified proteins, electrophoretically
Figure III-2. SDS-PAGE analysis of two trials of electrophoretically purified trehalase. Sample preparation and electrophoresis were carried out as described in the Materials and Methods. Following electrophoresis the gel was silver stained.

Lane 1: Ammonium sulphate precipitated trehalase preparation.
Lane 2: Electrophoretically purified trehalase (trial 1).
Lane 3: Electrophoretically purified trehalase (trial 2).
Lane 4: Molecular Weight Markers (Apparent MW in kDa).
Figure III-2
Figure III-3. SDS-PAGE analysis of two trials of
electrophoretically purified trehalase. Sample preparation
and electrophoresis were carried out as described in the
Materials and Methods. Following electrophoresis the gel
was stained for trehalase activity.

Lane 1: Ammonium sulphate precipitated trehalase
(positive control).
Lane 2: Electrophoretically purified trehalase (trial 1).
Lane 3: Electrophoretically purified trehalase (trial 2).
Figure III-3
Table III-1. Purification of Trehalase from Myxamoebae of D. discoideum (Form I) by Preparative SDS-PAGE.

<table>
<thead>
<tr>
<th>Purification Step</th>
<th>Total Protein&lt;sup&gt;a&lt;/sup&gt; (mg)</th>
<th>Total Activity&lt;sup&gt;b&lt;/sup&gt; (x 10&lt;sup&gt;3&lt;/sup&gt;)</th>
<th>Specific Activity&lt;sup&gt;c&lt;/sup&gt; (x 10&lt;sup&gt;3&lt;/sup&gt;)</th>
<th>Fold Purity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude Extract</td>
<td>47.9</td>
<td>26.2</td>
<td>0.5</td>
<td>20X</td>
</tr>
<tr>
<td>Ammonium Sulfate Precipitate</td>
<td>4.9</td>
<td>7.4</td>
<td>1.5</td>
<td>60X</td>
</tr>
<tr>
<td>Preparative SDS-PAGE</td>
<td>0.2</td>
<td>0.3</td>
<td>1.5</td>
<td>60X</td>
</tr>
</tbody>
</table>

<sup>a</sup> Determined by the method of Bradford (1976)

<sup>b</sup> Values in the column should be multiplied by 10<sup>3</sup>

<sup>c</sup> Specific activity expressed in μmoles product/min./mg protein

**Final Products**

Protein: 0.2 mg

% Yield of Activity: 1.1%
purified trehalase was subjected to Gelatin SDS-PAGE to
determine if any contaminating proteases were present. It
was found that a single cysteine protease at 42 kD (ddCP42)
and on occasion two cysteine proteases at 42 kD and 41 kD
(ddCP42 and ddCP41) co-eluted during electrophoretic
purification of trehalase (Figure III-4).

III.C.2. Molecular Charge - Preparative IEF

To investigate the possibility of exploiting charge as
a means of purifying trehalase, preparative IEF was
performed using the Rotofor system (Bio-Rad). Rotofor
separations are often affected adversely by high
concentration of salts in the protein preparations (> 10.0
mM). Therefore, it was necessary to desalt all concentrated
trehalase preparations by dialysis prior to IEF.

III.C.3. Preparative IEF of Vegetative Trehalase

The results of IEF of D. discoideum vegetative
trehalase are shown in Figure III-5. The volume of
fractions 3-18 was 1.7 mL, of fractions 2 and 19 was 2.5 mL
and of fractions 1 and 20 was 3.2 mL. (The anomalies in
fraction volume are due to the Rotofor apparatus structure).
The pH ranged from 2 to 14, and was linear in the range of 3
to 6. A summary of the results of preparative IEF of
vegetative trehalase are found in Table III-2.

Trehalase activity was detected by the colourimetric

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Figure III-4. Gelatin SDS-PAGE analysis of electrophoretically purified trehalase from D. discoideum. Sample preparation and electrophoresis were carried out according to the methods of North et al. (1988).

Lane 1: Electrophoretically purified trehalase (trial 1).
Lane 2: Electrophoretically purified trehalase (trial 2).
Lane 3: Molecular Weight Markers (Apparent MW in kDa).
Figure III-4
Figure III-5. Examination of crude vegetative trehalase by preparative IEF. The pH range of the ampholyte solution used was 3 to 5. Preparative IEF was carried out for 6 hours, at 12 watts constant power at 4°C. Shown are the analyses of the 20 10 Rotofor fractions for pH (---), protein content (○), and trehalase activity (●).
Figure III-5
Table III-2. Purification of Trehalase from Myxamoebae of *D. discoideum* (Form I) by Preparative IEF.

<table>
<thead>
<tr>
<th>Purification Step</th>
<th>Total Protein (mg)</th>
<th>Total Activity (x 10^3)</th>
<th>Specific Activity (x 10^3)</th>
<th>Fold Purity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude Extract</td>
<td>391.0</td>
<td>36.3</td>
<td>0.09</td>
<td>20X</td>
</tr>
<tr>
<td>Ammonium Sulfate Precipitate</td>
<td>10.0</td>
<td>16.1</td>
<td>1.6</td>
<td>340X</td>
</tr>
<tr>
<td>Preparative IEF^a</td>
<td>0.7</td>
<td>1.3</td>
<td>1.8</td>
<td>400X</td>
</tr>
</tbody>
</table>

^a Pooled fractions 1-4 (See Figure II-5)
^b Determined by the method of Bradford (1976)
^c Values in column should be multiplied by 10^3
^d Specific activity expressed in μmoles product/min./mg protein

Final Products

Protein: 0.7 mg
% Yield of Activity: 3.5%
method described in the Materials and Methods and was found predominantly in fractions 1 through 4 (not focussed). Approximately 66% of the total recovered trehalase activity was present in fraction 1. The isoelectric point (pI) of vegetative trehalase was estimated to be ≤ 2.0.

III.C.4. Gelatin SDS-PAGE of Rotofor Purified Vegetative Trehalase

Gelatin SDS-PAGE of the Rotofor fractions revealed contaminating proteases which co-migrated with trehalase in the acidic pH range (Figure III-6). Of particular interest was the fact the vegetative ddCP42 and ddCP41 co-migrated with trehalase. This indicated that these proteases are likely to have the same pI as trehalase in addition to having a similar molecular weight as the enzyme.

III.C.5. Preparative IEF of Developmental Trehalase

The results of IEF of *D. discoideum* developmental trehalase are shown in Figure III-7. The volume of fractions 3-18 was 2 mL, of fractions 2 and 19 was 3 mL and of fractions 1 and 20 was 4 mL. The pH ranged from 2 to 14, and was linear in the range of 3 to 5.

Trehalase activity was detected by the colourimetric method described in the Materials and Methods and was found to be concentrated in fractions 7 through 10. Approximately 40% of the total recovered trehalase activity was present in
Figure III-6. Gelatin SDS-PAGE analysis of Rotophor purified vegetative trehalase from *D. discoideum*. Sample preparation and electrophoresis was carried out according to the methods of North *et al.* (1988). The pH of each Rotophor fraction can be determined from Figure III-5. The apparent molecular weight (in kDa) of major proteinases was estimated according to North *et al.* (1990).

Lanes 1-10: Rotophor Fractions (1-10)

Lanes 11-20 Rotophor Fractions (11-20)
Figure III-6

1 2 3 4 5 6 7 8 9 10

11 12 13 14 15 16 17 18 19 20
Figure III-7. Examination of crude developmental trehalase by preparative IEF. The pH range of the ampholyte solution used was 3 to 5. Preparative IEF was carried out for 6 hours, at 12 watts constant power at 4°C. Shown are the analyses of the 20 Rotofor fractions for pH (--), protein content (O), and trehalase activity (●).
fraction 9. The pI of developmental trehalase was estimated to be 4.2. The results of preparative IEF of developmental trehalase are summarized in Table III-3.

III.C.6. Gelatin SDS-PAGE of Rotofor Purified Developmental Trehalase

Gelatin SDS-PAGE of all 20 Rotofor fractions revealed a great abundance of contaminating proteases which focused in the same pH range as did trehalase (Figure III-8). Of particular interest was the fact that a developmental ddCP42 focused with trehalase. This indicated that a developmental form of this protease exists and is likely to have the same pI as developmental trehalase in addition to having a similar molecular weight as the enzyme.

III.C.7. Rationale for the Use of Affinity Chromatography for the Purification of Trehalase

Since it was revealed that neither molecular size nor charge could be exploited for the separation of trehalase from ddCP42 or ddCP41, it was necessary to design a purification method based on trehalase-specific features. Trehalase is known to be highly specific for its substrate (Gupta, 1987), therefore the feature of substrate-specificity was chosen as the basis of a rapid purification scheme employing affinity chromatography.

The search for an appropriate affinand for the
Table III-3. Purification of Trehalase from Spore Matrix of *D. discoideum* (Form II) by Preparative IEF.

<table>
<thead>
<tr>
<th>Purification Step</th>
<th>Total Protein(^b) (mg)</th>
<th>Total Activity(^c) (x 10^3)</th>
<th>Specific Activity(^d) (x 10^3)</th>
<th>Fold Purity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude Extract</td>
<td>75.0</td>
<td>90.0</td>
<td>1.2</td>
<td></td>
</tr>
<tr>
<td>Ammonium Sulfate Precipitate</td>
<td>9.0</td>
<td>13.0</td>
<td>1.4</td>
<td>1.2X</td>
</tr>
<tr>
<td>Preparative IEF(^a)</td>
<td>3.0</td>
<td>0.1</td>
<td>0.04</td>
<td>na(^e)</td>
</tr>
</tbody>
</table>

\(^a\) Pooled fractions 7-10 (See Figure II-7)  
\(^b\) Determined by the method of Bradford (1976)  
\(^c\) Values in this column should be multiplied by \(10^3\)  
\(^d\) Specific activity expressed in \(\mu\)moles product/min./mg protein  
\(^e\) Specific activity decreased

**Final Products**

Protein: 3.0 mg  
% Yield of Activity: 0.1%
Figure III-8. Gelatin SDS-PAGE analysis of Rotophor purified developmental trehalase from *D. discoideum* spore matrix. Sample preparation and electrophoresis was carried out according to the methods of North et al. (1988). The pH of each of the Rotophor fractions can be determined from Figure III-7. The apparent molecular weight (in kDa) of the major proteinases was estimated according to North and Cotter (1991b).

Lanes 1-10: Rotophor Fractions (1-10)
Lanes 11-20: Rotophor Fractions (11-20)
purification of *D. discoideum* trehalase lead to the survey of 3 non-metabolizable trehalose analogs. These 3 amino-substituted sugars were Validamycin A, MDL 25,637 and castanospermine. Each potential inhibitor was examined for the following aspects: 1) the ability of the compound to interfere with the enzyme based glucose detection system used throughout this study; 2) the ability of the compound to act as a competitive inhibitor of vegetative trehalase; and, 3) the time dependence and reversibility of the inhibitory action on trehalase. The results of the analyses of the three potential affinands are presented below.

III.C.8. Glucose Detection in the Presence of Validamycin A

Glucose detection was unimpeded by the presence of Validamycin A at a concentration of 1 X 10⁻⁸ M. This concentration was chosen as it represented the highest concentration of Validamycin A used throughout the survey. As shown in Figure III-9, a range of molar quantities of glucose was easily detectable in the presence of Validamycin A. No significant differences in absorbance at 405 nm could be detected between samples with or without Validamycin A at any concentration of glucose (all p>0.05)

III.C.9. Trehalase Activity in the Presence of Validamycin A

Figure III-10 depicts the Lineweaver-Burk analysis of trehalase activity in the presence of a range of
Figure III-9. The effect of Validamycin A on the glucose
detection system of Chan and Cotter (1980). Known amounts
of glucose (20 nmoles to 100 nmoles) were combined with
glucose detection reagent in the presence (■) or
absence (□) of 1 x 10⁻⁸ M Validamycin A. After 1 hour of
incubation (23°C) the optical density (405 nm) of the
mixtures were recorded. Values are presented as a mean of
the optical density ± s.e.m. (n=3). For any concentration
of glucose, no significant difference in optical density
could be detected between samples with and without
Validamycin A (p > 0.05). Analysis by Student's 2-sample T-
test.
Figure III-9

[Graph showing the relationship between glucose concentration (nmoles) and absorbance]
Figure III-10. Lineweaver-Burk plot of trehalase activity in the presence of Validamycin A. Values are presented as means of reciprocal enzyme activity (n=3) plotted as a function of reciprocal substrate concentration. Linear regressions were performed using the computer program SYSTAT (Version 5.01). Tests for significance of each regression were performed using ANOVA (SYSTAT, Version 5.01). All linear regressions were significant (p < 0.001) (see Appendix B for statistical analyses of enzyme kinetic plots). Each reaction contained 0.2 μg of protein. A $K_m$ of 1.0 mM and a $V_{max}$ of 6000 nmoles/min./mg of protein were calculated from the set of reactions which did not contain Validamycin A (control).
Figure III-10

\[
\frac{1}{V} \text{ [mole/sec.]}
\]

\[
\frac{1}{S} \text{ [mM]}
\]

- $1 \times 10^8$
- $6 \times 10^6$
- $2 \times 10^6$
- $1 \times 10^6$

control
concentrations of Validamycin A. Validamycin A behaved as a competitive inhibitor of *D. discoideum* trehalase. When compared to the control (uninhibited) maximum velocity of 6000 nmoles of glucose/min./mg of protein, $V_{\text{max}}$ remained essentially unchanged in the presence of Validamycin A, ranging from 5100 nmoles of glucose released/min./mg of protein to 6100 nmoles of glucose released/min./mg. The $K_m$ was determined to be 1.0 mM. As expected for competitive inhibitors, the apparent $K_m$ ($K_m'$) increased in the presence of increasing concentrations of Validamycin A.

To determine the inhibitor constant ($K_i$) for Validamycin A, $K_m'$ was plotted as a function of inhibitor concentration (Figure III-11). $K_i$ was determined to be 1.6 x $10^{-8}$ M.

**III.C.10. Preincubation of Trehalase with Validamycin A**

Figure III-12 illustrates the relationship between the exposure time of trehalase to Validamycin A and the amount of enzyme inhibition imparted by Validamycin A. It was observed that as the inhibitor pre-treatment time increased, the amount of inhibition caused by Validamycin A also increased.

A Lineweaver-Burk analysis was performed for trehalase which had been preincubated with $6 \times 10^{-9}$ M Validamycin A for 1 hour (Figure III-13). Under these circumstances, mixed inhibition of trehalase activity by Validamycin A was
Figure III-11. Secondary plot of competitive inhibition of trehalase activity by Validamycin A. Shown are the values for apparent $K_m$, calculated from Figure III-10 plotted against Validamycin A concentration. The linear regression was performed using the computer program SYSTAT (Version 5.01). The test for significance of the regression was performed using ANOVA (SYSTAT, Version 5.01). The regression line was significant at the 95% confidence limit ($p<0.1$) (see Appendix B for statistical analysis). An apparent $K_i$ of $1.6 \times 10^{-8}$ M was calculated for Validamycin A.
Figure III-12. Time dependent inhibition of trehalase by Validamycin A. Enzyme and inhibitor were preincubated for 0 (○), 30 (●), or 60 (□) minutes at 23°C before the addition of 50 μL of a 0.05 M solution of trehalose and 100 μL of glucose detection reagent. Values are presented as a mean ± s.e.m. (n=3) of percent of uninhibited trehalase. Total uninhibited enzyme activity (100 %) was 0.03 units. This value remained unchanged during an analogous 30 and 60 minute 23°C incubation in the absence of Validamycin A.
Figure III-12

\[\text{CONC. OF VALIDAMYCIN A (M \times 10^9)}\]

\[% \text{RESIDUAL ACTIVITY}\]
Figure III-13. Lineweaver-Burk plot of trehalase preincubated with $6 \times 10^{-9}$ M Validamycin A. Values are presented as means of reciprocal enzyme activity ($n=3$) plotted as a function of reciprocal substrate concentration. Linear regressions were performed using the computer program SYSTAT (Version 5.01) Tests for significance of each regression were performed using ANOVA (SYSTAT, Version 5.01). All linear regression lines were significant ($p<0.001$) (see Appendix B for statistical analyses of enzyme kinetic plots). Each reaction contained 0.2 $\mu$g of protein. Upon preincubation, Validamycin A behaved as an mixed inhibitor of trehalase activity.
Figure III-13

Graph showing enzymatic activity plotted on a double reciprocal (Lineweaver-Burk) plot. The x-axis represents the reciprocal of the substrate concentration (1/S [mM]⁻¹), and the y-axis represents the reciprocal of the initial velocity (1/V [nmol/sec x 10⁻⁴]⁻¹).

- The data points for uninhibited conditions are indicated with open circles.
- The data points for a preincubated condition are indicated with solid squares.

Key:
- 6 x 10⁻⁶ (preincubated)
- 6 x 10⁻⁴

157
observed. The $K_m'$ of trehalase increased and the $V_{max}$ of trehalase decreased. This type of inhibition is also known as mixed inhibition.

III.C.11. Reversibility of Validamycin A Inhibition of Trehalase

An investigation to determine if trehalase could be reactivated after treatment with Validamycin A was performed. Dialysis of the inhibited enzyme resulted in restoration of nearly 100% of the original enzyme activity within 18 hours (Figure III-14).


Glucose detection was unimpeded by the presence of castanospermine at a concentration of $1 \times 10^{-4}$ M. This concentration was chosen as it represented the highest concentration of castanospermine used throughout the survey. As shown in Figure III-15, a range of molar quantities of glucose was easily detectable in the presence of castanospermine. No significant differences in absorbance at 405 nm could be detected between samples with or without castanospermine (all $p>0.05$).
Figure III-14. Reversibility of Validamycin A inhibition of trehalase activity. Trehalase (0.03 units) was preincubated with $1 \times 10^{-6}$ M Validamycin A for 60 minutes at 23°C. Exhaustive dialysis against 400 volumes of 0.1 M acetate buffer (pH 5.5) was carried out with pre-inhibited (A) or uninhibited (inset(B)) enzyme in the cold for 18 hours. Samples were taken at specified times and trehalase activity was measured. Values are presented as a mean ± s.e.m. (n=3) of the percent residual trehalase activity as compared to uninhibited trehalase.
Figure III-14

% RESIDUAL ACTIVITY

DIALYSIS TIME (hours)

TREHALASE ACTIVITY (nmoles glucose/min/mg protein)

DIALYSIS TIME (hours)
Figure III-15. The effect of castanospermine on the glucose detection system of Char and Cotter (1980). Known amounts of glucose (20 nmoles to 100 nmoles) were combined with glucose detection reagent in the presence (■) or absence (□) of $1 \times 10^{-4}$ M castanospermine. After 1 hour of incubation (23°C) the optical density (405 nm) of the mixtures were recorded. Values are presented as a mean of the optical density ± s.e.m. (n=3). For any concentration of glucose, no significant difference in optical density could be detected between samples with and without castanospermine (p>0.05). Analysis by Student's 2-sample T-test.
Figure III-15

![Graph showing absorbance vs. glucose concentration.](image-url)
III.C.13. Trehalase Activity in the Presence of Castanospermine

Figure III-16 depicts the Lineweaver-Burk analysis of trehalase activity in the presence of a range of concentrations of castanospermine. Castanospermine behaved as a competitive inhibitor of D. discoideum trehalase. When compared to the control (uninhibited) maximum velocity of 6000 nmoles/min./mg of protein, $V_{\text{max}}$ remained essentially unchanged in the presence of castanospermine, ranging from 5700 nmoles/min./mg of protein to 6250 nmoles/min./mg of protein. As expected for competitive inhibitors, the apparent $K_m$ ($K_m'$) increased in the presence of increasing concentrations of castanospermine.

To determine the inhibitor constant ($K_i$) for castanospermine, $K_m'$ was plotted as a function of inhibitor concentration (Figure III-17). $K_i$ was determined to be $1 \times 10^{-4}$ M.

III.C.14. Preincubation of Trehalase with Castanospermine

Figure III-18 illustrates the relationship between the exposure time of trehalase to castanospermine and the amount of enzyme inhibition imparted by the compound. It was observed that as the inhibitor pre-treatment time increased, the amount of inhibition caused by castanospermine was only slightly increased.

A Lineweaver-Burk analysis was performed for trehalase
Figure III-16. Lineweaver-Burk plot of trehalase activity in the presence of castanospermine. Values are presented as means of reciprocal enzyme activity (n=3) plotted as a function of reciprocal substrate concentration. Linear regressions were performed using the computer program SYSTAT (Version 5.01). Tests for significance of each regression were performed using ANOVA (SYSTAT, Version 5.01). All linear regressions were significant (p<0.001) (see Appendix B for statistical analyses of enzyme kinetic plots). Each reaction contained 0.2 μg of protein. A $K_m$ of 1.0 mM and a $V_{max}$ of 6000 nmoles/min./mg of protein were calculated from the set of reactions which did not contain castanospermine (control).
Figure III-16

Graph showing a plot of $1/V$ vs $1/S$ with different concentrations of substances labeled as $1 \times 10^4$, $7 \times 10^4$, $5 \times 10^4$, $3 \times 10^4$, and control.
Figure III-17. Secondary plot of competitive inhibition of trehalase activity by castanospermine. Shown are the values for apparent $K_m$ calculated from Figure III-16 plotted against castanospermine concentration. The linear regression was performed using the computer program SYSTAT. The test for significance of the regression was performed using ANOVA (SYSTAT, Version 5.01). The regression line was significant at the 95% confidence limit ($p<0.05$) (see Appendix B for statistical analysis). An apparent $K_i$ of $1 \times 10^{-4}$ M was calculated for castanospermine.
Figure III-17

CONC. OF CASTANOSPERMINE (M x 10^-6)
Figure III-18. Test for time dependent inhibition of trehalase by castanospermine. Enzyme and inhibitor were preincubated for 0 (○), 30 (●), or 60 (■) minutes at 23°C before the addition of 50 μL of a 0.05 M solution of trehalose and 100 μL of glucose detection reagent. Values are presented as a mean ± s.e.m. (n=3) of percent of uninhibited trehalase. Total uninhibited enzyme activity (100 %) was 0.03 units. This value remained unchanged during an analogous 30 and 60 minute 23°C incubation in the absence of castanospermine.
Figure III-18

% RESIDUAL ACTIVITY

CONC. OF CASTANOSPERMINE (M x 10⁻³)
which had been preincubated with $5 \times 10^{-5}$ M castanospermine for 1 hour (Figure III-19). Preincubation of trehalase with castanospermine did not result in any changes in inhibitor behaviour.

III.C.15. Reversibility of Castanospermine Inhibition of Trehalase

An investigation to determine if trehalase could be reactivated after treatment with castanospermine was performed. Dialysis of the inhibited enzyme resulted in restoration of nearly 100% of the original enzyme activity within 6 hours (Figure III-20).


Glucose detection was unimpeded by the presence of MDL 25,637 at a concentration of $1 \times 10^{-7}$ M. This concentration was chosen as it represented the highest concentration of MDL 25,637 used throughout the survey. As shown in Figure III-21, a range of molar quantities of glucose was easily detectable in the presence of MDL 25,637. No significant differences in absorbance at 405 nm could be detected between samples with or without MDL 25,637 (all $p>0.05$).

III.C.17. Trehalase Activity in the Presence of MDL 25,637

Figure III-22 depicts the Lineweaver-Burk analysis of trehalase activity in the presence of a range of
Figure III-19. Lineweaver-Burk plot of trehalase preincubated with $5 \times 10^{-5}$ M castanospermine. Values are presented as means of reciprocal enzyme activity ($n=3$) plotted as a function of reciprocal substrate concentration. Linear regressions were performed using the computer program SYSTAT (Version 5.01). Tests for significance of each regression were performed using ANOVA (SYSTAT, Version 5.01). All linear regressions were significant ($p<0.001$) (see Appendix B for statistical analyses of enzyme kinetic plots). Each reaction contained 0.2 µg of protein. Upon preincubation, castanospermine behaved as a competitive inhibitor of trehalase activity.
Figure III-19

![Graph showing the relationship between 1/V (in moles/sec. x 10^-1) and 1/S (in mM) for different inhibitor concentrations. The graph includes data points for 5 x 10^-6 (preincubated), 5 x 10^-5, and uninhibited conditions.]
Figure III-20. Reversibility of castanospermine inhibition of trehalase activity. Trehalase (0.03 units) was preincubated with $1 \times 10^{-4}$ M castanospermine for 60 minutes at 23°C. Exhaustive dialysis against 400 volumes of 0.1 M acetate buffer (pH 5.5) was carried out with pre-inhibited (A) or uninhibited (inset (B)) enzyme in the cold for 18 hours. Samples were taken at specified times and trehalase activity was measured. Values are presented as a mean ± s.e.m. (n=3) of the percent residual trehalase activity as compared to uninhibited trehalase.
Figure III-21. The effect of MDL 25,637 on the glucose detection system of Chan and Cotter (1980). Known amounts of glucose (20 nmoles to 100 nmoles) were combined with glucose detection reagent in the presence (■) or absence (□) of 1 x 10⁻⁷ M castanospermine. After 1 hour of incubation (23°C) the optical density (405 nm) of the mixtures were recorded. Values are presented as a mean of the optical density ± s.e.m. (n=3). For any concentration of glucose, no significant difference in optical density could be detected between samples with and without MDL 25,637 (p>0.05). Analysis by Student’s 2-sample T-test.
Figure III-21

![Graph showing absorbance vs. glucose concentration]

Absorbance (a/cm)

0 20 40 60 80 100

Glucose (nmoles)
Figure III-22. Lineweaver-Burk plot of trehalase activity in the presence of MDL 25,637. Values are presented as means of reciprocal enzyme activity (n=3) plotted as a function of reciprocal substrate concentration. Linear regressions were performed using the computer program SYSTAT (Version 5.01). Tests for significance of each regression were performed using ANOVA (SYSTAT, Version 5.01). All linear regressions were significant (p<0.001) (see Appendix B for statistical analyses of enzyme kinetic plots). Each reaction contained 0.2 μg of protein. A $K_m$ of 1.0 mM and a $V_{max}$ of 6000 nmoles/min./mg of protein were calculated from the set of reactions which did not contain MDL 25,637 (control).
Figure III-22
concentrations of MDL 25,637. MDL 25,637 behaved as a competitive inhibitor of D. discoideum trehalase. When compared to the control (uninhibited) maximum velocity of 6000 nmoles/min./mg of protein, $V_{\text{max}}$ remained essentially unchanged in the presence of MDL 25,637, ranging from 5400 nmoles/min./mg of protein to 5800 nmoles/min./mg of protein. As expected for competitive inhibitors, the apparent $K_m$ ($K_m'$) increased in the presence of increasing concentrations of MDL 25,637.

To determine the inhibitor constant ($K_i$) for MDL 25,637, $K_m'$ was plotted as a function of inhibitor concentration (Figure III-23). $K_i$ was determined to be $1.0 \times 10^{-7}$ M.

III.C.18. Preincubation of Trehalase with MDL 25,637

Figure III-24 illustrates the relationship between the exposure time of trehalase to MDL 25,637 and the amount of enzyme inhibition imparted by the compound. It was observed that as the inhibitor pre-treatment time increased, the amount of inhibition caused by MDL 25,637 also increased. A Lineweaver-Burk analysis was performed for trehalase which had been preincubated with $6 \times 10^{-8}$ M MDL 25,637 for 1 hour (Figure III-25). Under these circumstances, mixed inhibition of trehalase activity by MDL 25,637 was observed. The $K_m'$ of trehalase increased and the $V_{\text{max}}$ of trehalase decreased.
Figure III-23. Secondary plot of competitive inhibition of trehalase activity by MDL 25,637. Shown are the values for apparent $K_m$, calculated from Figure III-23 plotted against MDL 25,637 concentration. The linear regression was performed using the computer program SYSTAT (Version 5.01). The test for significance of the regression was performed using ANOVA (SYSTAT, Version 5.01). The regression line was significant ($p<0.01$) (see Appendix B for statistical analysis). An apparent $K_i$ of $1 \times 10^{-7}$ M was calculated for MDL 25,637.
Figure III-23

![Graph showing the relationship between $K_{ir}$ and CONC. MDL 25,637 (M X $10^{-7}$).]
Figure III-24. Time dependent inhibition of trehalase by MDL 25,637. Enzyme and inhibitor were preincubated for 0 (O), 30 (●), or 60 (■) minutes at 23°C before the addition of 50 μL of a 0.05 M solution of trehalose and 100 μL of glucose detection reagent. Values are presented as a mean ± s.e.m. (n=3) of percent of uninhibited trehalase. Total uninhibited enzyme activity (100 %) was 0.03 units. This value remained unchanged during an analogous 30 and 60 minute 23°C incubation in the absence of MDL 25,637.
Figure III-24

% RESIDUAL ACTIVITY

CONC. OF MDL 25,637 (M X 10^4)
Figure III-25. Lineweaver-Burk plot of trehalase preincubated with $6 \times 10^{-8}$ M MDL 25,637. Values are presented as means of reciprocal enzyme activity (n=3) plotted as a function of reciprocal substrate concentration. Linear regressions were performed using the computer program SYSTAT (Version 5.01). Tests for significance of each regression were performed using ANOVA (SYSTAT, Version 5.01). All linear regressions were significant ($p<0.001$) (see Appendix B for statistical analyses of enzyme kinetic plots). Each reaction contained 0.2 µg of protein. Upon preincubation, the MDL 25,637 behaved as mixed inhibitor of trehalase activity.
Figure III-25

\[ \frac{1}{V} \text{ [nmoles/sec.$\times 10^{-1}$]} \]

\[ \frac{1}{S} \text{ [mM]} \]

- $6 \times 10^8$ (preincubated)
- $6 \times 10^4$
- uninhibited
This type of inhibition is also known as mixed inhibition.

III.C.19. Reversibility of MDL 25,637 Inhibition of Trehalase

An investigation to determine if trehalase could be reactivated after treatment with MDL 25,637 was performed. Dialysis of the inhibited enzyme resulted in the restoration of nearly 100% of the original enzyme activity within 6 hours (Figure III-26).

III.C.20. Construction of A Trehalase Affinity Matrix

Kinetic analysis of trehalase activity in the presence of the 2 amino-substituted analogs of trehalose had shown that Validamycin A, exhibiting a $K_i$ of $1.6 \times 10^{-6}$ M was the most potent of the 3 inhibitors. Therefore an affinity matrix was prepared by coupling Validamycin A to epoxy-activated Sepharose 6B via a covalent ether linkage.

Only a small amount of Validamycin A could be coupled to the insoluble matrix due to its scarce availability. 4 x $10^{-5}$ moles of Validamycin A were mixed with 1.2 mL of swollen Sepharose 6B beads in a total volume of 15 mL of sodium borate buffer (pH 9.0). Several original attempts to couple the inhibitor to Sepharose 6B at neutral pH were unsuccessful (data not shown). Coupling of Validamycin A to Sepharose 6B was performed at 32°C, for 18 hours.
Figure III-26. Reversibility of MDL 25,637 inhibition of trehalase activity. 0.03 units of trehalase was preincubated with $1 \times 10^{-7}$ M MDL 25,637 for 60 minutes at 23°C. Exhaustive dialysis against 400 volumes of 0.1 M acetate buffer (pH 5.5) was carried out with pre-inhibited (A) or uninhibited (inset (B)) enzyme in the cold for 18 hours. Samples were taken at specified times and trehalase activity was measured. Values are presented as a mean ± s.e.m. (n=3) of the percent residual trehalase activity as compared to uninhibited trehalase.
Figure III-26

A

% RESIDUAL ACTIVITY

B

TREHALASE ACTIVITY (molecules glucose/mg in 200,000 protein)

DIALYSIS TIME (hours)

DIALYSIS TIME (hours)
Following the 18 hour coupling step, the amount of bound ligand was calculated indirectly by calculating the amount of Validamycin A remaining in the supernatant (post-coupling buffer) over the Sepharose gel matrix. To accomplish this, the inhibitory action of serially diluted supernatant on purified porcine trehalase was examined. It has been reported that the IC\textsubscript{50} of Validamycin A on porcine trehalase is $1 \times 10^{-9}$ M (Kendall et al., 1990). Therefore the dilution which can inhibit porcine trehalase by 50% must contain Validamycin A in a concentration of $1 \times 10^{-8}$ M. From this information the amount of Validamycin A in the original undiluted supernatant and therefore the amount of Validamycin A bound to Sepharose could be determined.

Dilutions of the post-coupling buffer (10 fold to 100,000 fold) were made. Diluted and undiluted post-coupling buffer were incubated with porcine trehalase and the components of the glucose detection system in a total reaction volume of 210 µL. This represented a second dilution of the post-coupling supernatant of 21 fold. Diluted supernatant was unable to inhibit porcine trehalase (data not shown). Undiluted supernatant had low but detectable inhibitory activity against porcine trehalase (15%). Therefore the concentration of Validamycin A in the reaction containing undiluted post-coupling buffer contained Validamycin A in a concentration that was less than $1 \times 10^{-8}$ M. The original supernatant must then contain Validamycin A
in a concentration that was less than $2.1 \times 10^{-7} \text{ M}$ ($1 \times 10^{-8} \text{ M} \times 21$). Less than $2.1 \times 10^{-9}$ moles of Validamycin A remained in solution after the coupling step. This indicated that $>99\%$ of the Validamycin A which had been presented to Sepharose 6B during coupling bound to the matrix (40 μmoles of Validamycin A/mL of swollen matrix).

III.C.21. Affinity Purification of Vegetative Trehalase

Ammonium sulphate concentrated trehalase was applied to the Validamycin A-Sepharose column which had been equilibrated with 60 column volumes of 0.3 M sodium acetate buffer containing 0.3% (v/v) Tween-20.

Desorption of trehalase with trehalose could not be achieved. However, when elution was attempted by washing the column with 0.1 M sodium borate (pH 10.0) containing 0.5 M NaCl trehalase could be desorbed in approximately 10 mL (Figure III-27). It was also observed that all of the detectable cysteine proteinase activity (nitroanilide hydrolysis) was removed from the preparation in approximately 190 mL. No cysteine proteinase activity could be detected by nitroanilide hydrolysis in the specifically desorbed fractions. The results of affinity purification of vegetative trehalase are summarized in Table III-4.

SDS-PAGE (silver stain) of affinity purified trehalase revealed a major protein species of 42 kD and 2 minor protein species of approximately 45 kD and 49 kD.
Figure III-27. Affinity chromatography of vegetative trehalase. A concentrated enzyme extract was passed over a column of Validamycin A immobilized onto Sepharose 6B. After extensive washing with 0.3 M sodium acetate buffer (pH 5.5) supplemented with 0.3% (v/v) Tween-20, trehalase was eluted with 0.1 M sodium borate buffer (pH 10.0) containing 0.5 M NaCl. Elution commenced at the position indicated by the arrows. Separated fractions were analyzed for cysteine proteinase activity (A), trehalase activity (B) and total protein (C).
Figure III-27

A

PROTEASE ACTIVITY (nmolase/mg x 10^(-3)/min)

B

TRYPsinase ACTIVITY (nM/mg)

C

PROTEIN (mg)

FRACTION (mL)
Table III-4. Purification of Trehalase from Vegetative Myxamoebae of *D. discoideum* (Form I) by Affinity Chromatography.

<table>
<thead>
<tr>
<th>Purification Step</th>
<th>Total Protein (mg)</th>
<th>Total Activity (x 10^3) c</th>
<th>Specific Activity (x 10^3) c</th>
<th>Fold Purity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude Extract</td>
<td>219.0^a</td>
<td>38.3</td>
<td>0.2</td>
<td>20X</td>
</tr>
<tr>
<td>Ammonium Sulfate Precipitate</td>
<td>10.0^b</td>
<td>17.8</td>
<td>1.8</td>
<td>180X</td>
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<tr>
<td>Affinity</td>
<td>0.003^b</td>
<td>0.006</td>
<td>2.0</td>
<td>200X</td>
</tr>
</tbody>
</table>

^a Determined by the method of Bradford (1976)  
^b Determined by the Bio-Rad DC Protein Assay  
^c Values in this column should be multiplied by 10^3  
^d Specific activity expressed in μmoles product/min./mg protein

**Final Products**

Protein: 3 μg  
% Yield of Activity: 0.02%
(Figure III-28). When the same sample was stained in situ for trehalase activity, the predominant protein at 42 kD was weakly active (data not shown). Gelatin SDS-PAGE of affinity purified trehalase revealed that a small amount of protease activity contaminated the preparation (Figure III-29). However, this protease activity could only be detected if the gels were incubated in the activation buffer for at least 20 hours.
Figure III-28. SDS-PAGE analysis of affinity purified trehalase. Sample preparation and electrophoresis were carried out as described in the Materials and Methods. Following electrophoresis the gel was silver stained.

Lane 1: Affinity purified trehalase
Lane 2: Molecular Weight Markers (Apparent MW in kDa)
Figure III-29. Gelatin SDS-PAGE analysis of affinity purified trehalase. Sample preparation and electrophoresis was carried out as described in North et al. (1988). The apparent molecular weights (in kDa) were estimated according to North et al. (1990).

Lane 2: Affinity purified trehalase.
Lane 1: Ammonium sulphate precipitate trehalase
   (positive control).
III.D. DISCUSSION

As reported by Gupta and Cotter (1990), trehalase exhibited aberrant molecular weight characteristics when subjected to gel filtration chromatography. It was suggested that this behaviour was due to the post-translational modifications of the enzyme. Therefore preparative SDS-PAGE in partially-denaturing conditions was chosen as an alternative purification step that would exploit the characteristic of molecular weight.

Excision and elution of vegetative trehalase activity after SDS-PAGE resulted in a 3-fold purification of the enzyme. Trehalase activity was consistently evident in the 42 KD regions of large preparative gels as well as in SDS-PAGE mini-gels used in subsequent analyses. Therefore unlike gel filtration chromatography, trehalase did not display aberrant molecular weight characteristics under the partially denaturing conditions of preparative SDS-PAGE.

Gelatin-SDS-PAGE analysis showed that a single contaminating cysteine proteinase, ddCP42 and on occasion an additional proteinase, ddCP41 co-eluted with trehalase. The presence of these proteinases in extracellular preparations of *D. discoideum* vegetative amoebae have been noted previously (North et al., 1988). These proteinases have molecular weights which are essentially identical to trehalase and therefore any protein purification technique which exploits the characteristics of molecular mass for
separation will fail to separate them successfully.

Large amounts of protein could be removed from the trehalase preparation by preparative SDS-PAGE, however the final yield of trehalase activity was unacceptably low. This loss in activity was likely due to proteolytic action by the proximal proteinases during electrophoresis. Although the cysteine proteinase inhibitor 2,2'-dithiodipyridine was included in all stages of the purification, the mode of action of this inhibitor (by formation of disulfide bridges) was likely to be reversed under the reducing conditions of the SDS-PAGE step. For this reason this technique was abandoned.

To investigate the option of exploiting charge as a means of purifying trehalase, studies involving preparative IEF were undertaken. Since previous studies on cysteine proteinases (North et al., 1988) concluded that a developmental form of ddCP42 was non-existent, both the vegetative and developmental forms of trehalase were examined by this technique.

When preparations of vegetative trehalase were subjected to IEF, the enzyme was found in the acidic pH range (pI<2.0). This confirms the previously reported pI for *D. discoideum* vegetative trehalase of less than 2.5 (Gupta and Cotter, 1990). Low pIs are characteristic of *D. discoideum* lysosomal enzymes (Freeze and Muller, 1980; Freeze et al., 1983) therefore the results of this study
also confirm the lysosomal nature of this enzyme.

Interestingly, vegetative ddCP42 and ddCP41 co-migrated with vegetative trehalase indicating that these proteins have the same molecular charge in addition to having similar molecular weights.

When developmental trehalase preparations were subjected to IEF, the enzyme focused at approximately pH 4.2 (pI=4.2). The elevated pI for developmental trehalase has been reported previously (Gapta, 1987). In addition, under extended in vitro activation (> 18 hours), gelatin-SDS-PAGE of the Rotofor fractions revealed a developmental form of ddCP42. As with preparative SDS-PAGE, unacceptably low yields of trehalase activity resulted and this was likely due to proteolysis by the co-focusing proteases.

Since neither size nor charge could be exploited to separate trehalase from at least two of its contaminants ddCP42 and ddCP41, affinity purification of trehalase was pursued. N-containing analogs of glucose, mannose and N-acetylglucosamine have been successfully applied to the affinity chromatography of glycosidases (Hettkamp et al., 1984), mannosidases (Schweden and Bause, 1989) and N-acetyl-β-glucosaminidase (Legler et al., 1991) respectively. These approaches were adopted for the affinity purification of trehalase.

The decision to pursue affinity chromatography for the purification of D. discoideum vegetative trehalase led to a
survey of three nitrogen-containing analogs of the natural substrate trehalose: Validamycin A, castanospermine, and MDL 25,637. Prior to this study, the effects of glycohydrolase inhibitors on *D. discoideum* trehalase have not been reported.

Validamycin A and MDL 25,637 were potent competitive inhibitors of *D. discoideum* vegetative trehalase with $K_i$ values of $1.6 \times 10^{-8} \text{ M}$ and $1 \times 10^{-7} \text{ M}$ respectively. This is in contrast to the action of such compounds on porcine trehalase (Salleh and Honke, 1990) whereas MDL 25,637 displayed more effective inhibition than did Validamycin A.

Castanospermine also behaved as a competitive inhibitor of *D. discoideum* trehalase, however the magnitude of its potency was moderate ($K_i = 1 \times 10^{-4} \text{ M}$) in comparison to the other two inhibitors. These results are not surprising. Of the three inhibitors employed, castanospermine least resembles the natural substrate. Moreover, Saul et al. (1984) reported that the inhibitory action of castanospermine is pH-dependent and the unprotonated form ($pK_a$ of endocyclic N group = 6.04) of the compound is more effective than its protonated counterpart. Since all of the inhibition studies using *D. discoideum* trehalase were performed at the pH optimum of trehalase (5.5) at least half of the castanospermine was protonated rendering it less effective.

Validamycin A and MDL 25,637 also displayed time
dependent inhibition of *D. discoideum* trehalase. In other words the potency of these 2 inhibitors were observed to increase on a tangible time scale.

The competitive natures of Validamycin A and MDL 25,637 were altered during preincubation with enzyme such that the compounds behaved as mixed inhibitors. Mixed inhibition upon preincubation of a competitive inhibitor and an enzyme has been reported for bovine N-acetyl-β-D-glucosaminidase (Legler et al., 1991). Taken together these results suggest that the action of Validamycin A and MDL 25,637 are of a "slow-binding" nature (Schloss, 1988). Castanospermine did not display any of the characteristics of slow binding inhibitors.

It was also observed that all three inhibitors were reversible upon extensive dialysis. Validamycin A-inhibited trehalase preparations displayed the slowest rate of reactivation. Despite the structural and functional similarities between MDL 25,647 and Validamycin A, complete reactivation of enzymes inhibited with the former compound could be observed in one-third of the time required for complete reactivation of Validamycin A-inhibited enzyme.

It is widely accepted that the ideal ligand employed in affinity chromatography should exhibit specific reversible binding affinity for the biomolecule of interest; and, should contain chemically modifiable groups which allow it to be attached to an insoluble matrix.
Validamycin A was chosen for affinity chromatography of *D. discoideum* trehalases as it was the most potent of the three inhibitors and it fulfilled all of the requirements for ideal ligands described above. While MDL 25,637 was a potent, reversible inhibitor of trehalase and contained chemical groups conducive to matrix coupling, the strict specificity of this compound for trehalase has not been documented. In addition the rapid reversible nature of this inhibitor warranted caution. On the other hand, castanospermine lacked potency, specificity and favourable coupling groups thereby fulfilling few of the requirements described above.

The coupling of Validamycin A to Sepharose proved to be a highly efficient process, resulting in an "intra-gel" concentration of inhibitor of 33 μmoles per mL of gel. However, it must be considered that Validamycin A contains three equivalent primary hydroxyl coupling groups and numerous secondary hydroxyl coupling groups. Therefore, this ligand may be bound to the Sepharose matrix in a number of different orientations. In some of these orientations binding by trehalase may be impeded. Trehalase preparations were applied under the conditions of optimum trehalase activity. Greater than 99 % of the activity which was applied to the column actually remained bound.

Free inhibitor could not be used to desorb bound trehalase due to its scarcity. Initial attempts to desorb
bound trehalase with its natural substrate (1M trehalose) were unsuccessful. Substrate mediated desorption of bound enzymes are often slow and lead to dilution of the enzyme. In other words, trehalase may have indeed eluted from the column under these conditions but it may have been too dilute to detect. Another reason for poor competition between free trehalose and coupled Validamycin A may be the fact that trehalase has a higher affinity for Validamycin A than its own substrate. In support of this, trehalose competed poorly with free inhibitor in in vitro preincubation experiments.

A solution to the problem of "super-adsorbed" trehalase was found by employing a highly alkaline desorption buffer (0.1 M sodium borate, pH 10.0) supplemented with 0.5 M NaCl. Under these conditions large quantities of trehalase were desorbed from the column in a relatively small volume. Unfortunately harsh non-specific desorption presents several drawbacks. Proteins eluted in this manner are often irreversibly denatured. While this does not interfere with subsequent molecular biological or immunological studies of the purified enzyme, purification schemes which employ such techniques often appear inefficient due to low yields of enzyme activity. This was the case for the alkaline-facilitated desorption of bound trehalase of this study. Large quantities of protein could be recovered, however the levels of trehalase activity were very low. Addition of 1 M
trehalose to the desorption buffer improved the yield of activity by 10 fold. Secondly, harsh, non-specific desorption of affinity bound proteins can result in contamination of the purified preparation with proteins which had bound tightly but non-specifically to the matrix. In this study two upper molecular contaminants could be observed after SDS-PAGE and silver staining. The contaminants were not visible by Coomassie Brilliant Blue staining indicating that contamination by these species was marginal.

Interestingly, these contaminants were able to bind tightly to the affinity matrix in the presence of a high ionic strength environment and high concentrations of detergent. These species may be other *D. discoideum* glycohydrolases or trehalose-6-phosphate synthase. In support of this, it has been reported that glycohydrolases generally exhibit catalytic versatility (Hehre et al., 1986; Kasumi et al., 1986). On the other hand, Validamycin A has been reported to display absolute specificity for trehalase (Asano et al., 1987).

Contamination of affinity purified trehalase by proteinases was considered to be extremely small. Proteinases were only detectable by extended activation periods (more than 20 hours) of gelatin SDS-PAGE gels. The array of proteinases was not visible by Coomassie Brilliant Blue staining, silver staining of SDS-PAGE gels nor was
their activity detectable by the *in vitro* assay employing nitroanilide hydrolysis. It is believed that the tight binding of these proteinases to this affinity column was not due to an affinity for the ligand but due to an affinity for bound trehalase. This is a drawback of all purification schemes which employ affinity chromatography.

This is the first report of affinity purification of a trehalase. Large amounts of protein were obtained by this technique, however the yields of trehalase activity were compromised by the harsh conditions required for elution. This purification scheme features a greatly reduced number of steps for the partial purification of *D. discoideum* trehalases (Gupta and Cotter, 1990) as well as possibly for the purification schemes of heterologous trehalases (Boos et al., 1987; Ruf et al., 1987; Mittenbuhler and Holzer, 1988; App and Holzer, 1989; Sanker and Sivakami, 1990; Zimmerman et al., 1990). Since Validamycin A displays strict specificity for trehalases it is proposed that "Validamycin A-Sepharose" has potential use in the rapid purification of a variety of trehalases.

III.D.1. Protein Sequencing Anomalies and Future Studies

It is believed that affinity purified *D. discoideum* trehalase is of a purity that is conducive to N-terminal sequencing. Knowledge of N-terminal sequences are critical in molecular biological studies as synthetic oligonucleotide
probes can be deduced and generated from such amino acid sequences.

Several attempts have been made to sequence trehalase by Edman degradation. Such trials were unsuccessful and several explanations for these difficulties will be offered. Original trials revealed that the protein was blocked at the N-terminus rendering it refractory to Edman degradation. Subsequently, a CNBr treatment of what was believed to be 35-60 µg (approximately 800 to 1400 pmoles) of protein generated two "sequence-able" peptides. There is at least one internal methionine within *D. discoideum* trehalase and this is similar to the internal methionine content (one) of the acid trehalase of *S. cerevisiae* (Mittenbuhler and Holzer, 1988).

Interestingly, sequence analysis of these two peptides also revealed that only minute quantities (5-10 pmoles) of trehalase were present. This is in marked contrast to the amount of trehalase which had been predicted by the *in vitro* detergent compatible protein assay. Perhaps trehalase behaves anomalously during *in vitro* protein determinations resulting in an overestimation of the protein. On the other hand, trehalase may have some inherent property or properties which reduce sequencing efficiency, perhaps by inefficient modification of the N-terminal and/or inefficient recovery of modified amino acids following acid hydrolysis.
Overestimation of trehalase quantities and/or inherent properties which result in inefficient sequencing could explain the inability to sequence *D. discoideum* trehalase. The aberrant behaviour of trehalase could also explain the general lack of sequence information for trehalases in the literature. Only rabbit intestinal trehalase has been reported to be sequenced (Ruf et al., 1990).

The inability to sequence *D. discoideum* trehalase may also be due to the predicted extensive post-translation modifications of the enzyme. It is well documented that *D. discoideum* lysosomal hydrolases are extensively modified with N-linked, O-linked and phosphoserine-linked sugars (for review, see Freeze, 1991) and it has been communicated that such glycan-modified amino acids often display reduced or no chromatographic signal during sequencing (Ms. J. Snow, Macromolecular Core Facility, Wayne State University). Although the extent of glycosylation of this enzyme is not known, in a related system (*S. cerevisiae*), it has been reported that glycosyl modifications of acid trehalase may account for 75% of the protein's molecular mass (Mittenbuhler and Holzer, 1988).

Fortunately the protein is of such purity that it may be used in immunological studies. Polyclonal antibodies raised to affinity purified trehalase may be used (in place of synthetic oligonucleotide probes) to screen a *D. discoideum* cDNA expression library. Isolation of the gene
for *D. discoideum* trehalase and subsequent "knock-out" experiments will define conclusively the multi-faceted role of this enzyme throughout the life cycle of this organism.
LITERATURE CITED


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De Virgilio, C.D., Muller, J., Boller, T. and Wiemken, A. 1991b. A constitutive heat shock activated neutral trehalase occurs in Schizosaccharomyces pombe in addition to the sporulation specific acid trehalase. FEMS Microbiol. Lett. 84, 85-90.


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Appendix A

Purification of Validamycin A from Solocol by Ion Exchange Chromatography
Introduction

The Validamycins (A-G) were discovered by virtue of their antibiotic nature against rice sheath blight Rhizoctonia solani (Iwasa et al., 1970; Horii et al., 1972; Kameda et al., 1986). In all cases the Validamycins were isolated from cultures of Streptomyces hygroscopicus. Asano et al. (1987) and Kameda et al. (1987) later reported that the structure of these compounds resembled trehalose and that their mode of antifungal action was due to their inhibitory activity on trehalases.

The most potent of the Validamycins, Form A (See CHAPTER III; Figure III-1A), has been formulated into a commercial fungicide known as "Solocol" (0.3% v/v Validamycin A). In an initial attempt to obtain pure Validamycin A for use as a ligand in the affinity purification of trehalase, the inhibitor was purified from Solocol by anion exchange chromatography.

Materials and Methods

Validamycin A was purified from Solocol by anion exchange chromatography according to the methods of Kendall et al., 1990). QAE Sephadex A-25 resin, which had been equilibrated with 0.2 mM sodium phosphate (pH 7.0), was packed into a column (2 cm X 10 cm) yielding a column bed volume of 10 mL. The packed bed was equilibrated with 200 mL of the same buffer described above prior to each use.
Typically 1 mL of Solocol was loaded onto the column for each column run. Validamycin A was eluted from the column with H₂O in 14 (1 mL) fractions.

Each fraction was analyzed for its ability to interfere with the glucose detection system of Chan and Cotter (1980) and for its inhibitory action on *D. discoideum* vegetative trehalase as described in CHAPTER III. In addition, those fractions which displayed high trehalase inhibitory activity were analyzed for purity by \(^1\text{H}-\text{n.m.r.}\) (performed by Dr. J.H.E. Bailey, Department of Chemistry and Biochemistry, University of Windsor, Windsor, Ontario, Canada). The amount of Validamycin A in those fraction which displayed trehalase inhibitory activity was determined using porcine trehalase as outlined in CHAPTER III.

**Results and Discussion**

QAE Sephadex A-25 was employed for anion exchange chromatographic purification of Validamycin A from Solocol. None of the fractions collected during anion exchange chromatography and therefore, none of the components of the commercial pesticide Solocol interfered with the glucose detection reagent employed throughout this study (data not shown). The greatest inhibitory activity eluted over fractions 4 mL to 7 mL (Figure B-1). These fractions were used in subsequent studies.

To determine the purity of these fractions \(^1\text{H}-\text{n.m.r.}\)
Figure A-1. Anion exchange chromatography of Validamycin A. A volume of 1mL of a 0.3% (w/v) commercial preparation of Validamycin A (Solocol) was loaded onto a QAE-Sephadex A-25 column which had been equilibrated with 0.2 mM sodium phosphate (pH 7.0). The column was then washed with H₂O to facilitate the elution of Validamycin A. The separated fractions were analyzed for their ability to inhibit 0.03 units (100%) of trehalase activity as described in CHAPTER III. Fractions 4-7 exhibited complete inhibition (0% residual activity) of trehalase activity.
Figure A-1.
was performed. The Validamycins contain a vinyl proton (H- 
2', δ 5.995) that resonates downfield from most other sugar 
and alkane protons (Kameda et al., 1986). As depicted in 
Figures A-2 to A-5, fraction 6 appeared to be the most pure 
of the 4 fractions exhibiting inhibition of trehalase 
activity. Despite the strong inhibitory activity of 
fraction 4, the characteristic vinyl proton was not 
detectable. This demonstrates the high potency of this 
trehalase inhibitor.

When Validamycin A was quantified by the porcine 
trehalase method of CHAPTER III it was determined that a 
total of 1.12 μmoles of the inhibitor was present over the 4 
fractions (Figures A-6 to A-9). It was concluded that this 
amount of Validamycin A was insufficient for the 
construction of a trehalase-specific affinity matrix.
Figure A-2. $^1$H-n.m.r. spectrum of affinity purified Validamycin A (Fraction 4). The characteristic vinyl proton (H-2', δ 5.995) is not evident in this fraction.
Figure A-3. $^1$H-n.m.r. spectrum of affinity purified Validamycin A (Fraction 5). The characteristic vinyl proton ($H-2'$, $\delta 5.995$) is evident in this fraction.
Figure A-4. \textsuperscript{1}H-n.m.r. spectrum of affinity purified Validamycin A (Fraction 6). The characteristic vinyl proton (H-2', \( \delta 5.995 \)) is evident in this fraction.
Figure A-5. $^1$H-n.m.r. spectrum of affinity purified Validamycin A (Fraction 7). The characteristic vinyl proton (H-2', δ 5.995) is evident in this fraction.
Figure A-5
Figure A-6. Determination of the quantity of Validamycin A in ion exchange purified inhibitor (Fraction 4). Dilutions of Fraction 4 (10 fold to 100,000 fold) were made. The dilutions were incubated with porcine trehalase (0.1 units) and the components of the glucose detection system in a total reaction volume of 210 μL. This represented a second dilution of the post-coupling supernatant of 21 fold. The appropriate dilution of partially purified Validamycin A which could inhibit porcine trehalase by 50% is indicated by (Δ). Using the reported IC₅₀ value for the action of Validamycin A on porcine trehalase of 10⁻⁸ M (Kendall et al., 1990) the amount of the inhibitor in the undiluted fractions was calculated.
Figure A-6
Figure A-7. Determination of the quantity of Validamycin A in ion exchange purified inhibitor (Fraction 5). Dilutions of Fraction 5 (10 fold to 100,000 fold) were made. The dilutions were incubated with porcine trehalase (0.1 units) and the components of the glucose detection system in a total reaction volume of 210 μL. This represented a second dilution of the post-coupling supernatant of 21 fold. The appropriate dilution of partially purified Validamycin A which could inhibit porcine trehalase by 50% is indicated by (△). Using the reported IC₅₀ value for the action of Validamycin A on porcine trehalase of 10⁻⁸ M (Kendall et al., 1990) the amount of the inhibitor in the undiluted fractions was calculated.
Figure A-7
Figure A-8. Determination of the quantity of Validamycin A in ion exchange purified inhibitor (Fraction 6). Dilutions of Fraction 6 (10 fold to 100,000 fold) were made. The dilutions were incubated with porcine trehalase (0.1 units) and the components of the glucose detection system in a total reaction volume of 210 μL. This represented a second dilution of the post-coupling supernatant of 21 fold. The appropriate dilution of partially purified Validamycin A which could inhibit porcine trehalase by 50% is indicated by (△). Using the reported IC$_{50}$ value for the action of Validamycin A on porcine trehalase of 10$^{-8}$ M (Kendall et al.- 1990) the amount of the inhibitor in the undiluted fractions was calculated.
Figure A-8
Figure A-9. Determination of the quantity of Validamycin A in ion exchange purified inhibitor (Fraction 7). Dilutions of Fraction 7 (10 fold to 100,000 fold) were made. The dilutions were incubated with porcine trehalase (0.1 units) and the components of the glucose detection system in a total reaction volume of 210 μL. This represented a second dilution of the post-coupling supernatant of 21 fold. The appropriate dilution of partially purified Validamycin A which could inhibit porcine trehalase by 50% is indicated by (Δ). Using the reported IC₅₀ value for the action of Validamycin A on porcine trehalase of 10⁻⁸ M (Kendall et al., 1990) the amount of the inhibitor in the undiluted fractions was calculated.
Figure A-9
Appendix B

Statistical Analyses of Trehalase Kinetic Plots
(N-containing Analogs of Trehalose as Inhibitors)
Introduction

Inhibition of trehalase activity by Validamycin A, castanospermine or MDL 25,637 was carried out as described in CHAPTER III. The initial velocity ($V_0$) of each reaction was determined using the computer program ANALYSE (developed by Dr. H.B. Fackrell, University of Windsor). The program calculated the derivative of the reaction curve. Apparent $K_m$ and $V_{max}$ were estimated by Lineweaver-Burk plots of reciprocal velocity vs. reciprocal substrate concentration. $K_i$ was estimated using a secondary plot of apparent $K_m$ vs. inhibitor concentration. The computer program SYSTAT (Version 5.01) was used to generate the regression statistics and to assess the linearity of the Lineweaver-Burk and secondary plots. Statistical analyses of enzyme kinetic plots are reported below.
Lineweaver-Burk Analysis of Reciprocal Trehalase Activity (Velocity) Plotted Against Reciprocal Substrate (Trehalose) Concentration (Regression Statistics and ANOVA)

Inhibitor: No Inhibitor (Control)
Concentration of Inhibitor: N/A

DEP VAR: 1/V, N: 12 MULTIPLE R: .868 SQUARED MULTIPLE R: .753
ADJUSTED SQUARED MULTIPLE R: .729 STANDARD ERROR OF ESTIMATE: 0.007

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ANALYSIS OF VARIANCE

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Lineweaver-Burk Analysis of Reciprocal Trehalase Activity (Velocity) Plotted Against Reciprocal Substrate (Trehalose) Concentration (Regression Statistics and ANOVA)

Inhibitor: Validamycin A
Concentration of Inhibitor: 1 x 10^-10 M

DEP VAR: 1/V, N: 12 MULTIPLE R: .910 SQUARED MULTIPLE R: .829
ADJUSTED SQUARED MULTIPLE R: .812 STANDARD ERROR OF ESTIMATE: 0.008

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250
Lineweaver-Burk Analysis of Reciprocal Trehalase Activity (Velocity) Plotted Against Reciprocal Substrate (Trehalose) Concentration (Regression Statistics and ANOVA)

**Inhibitor: Validamycin A**

**Concentration of Inhibitor:** $2 \times 10^{-9}$ M

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Lineweaver-Burk Analysis of Reciprocal Trehalase Activity (Velocity) Plotted Against Reciprocal Substrate (Trehalose) Concentration (Regression Statistics and ANOVA)

**Inhibitor: Validamycin A**

**Concentration of Inhibitor:** $6 \times 10^{-9}$ M

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Lineweaver-Burk Analysis of Reciprocal Trehalase Activity (Velocity) Plotted Against Reciprocal Substrate (Trehalase) Concentration (Regression Statistics and ANOVA)

Inhibitor: Validamycin A
Concentration of Inhibitor: $1 \times 10^{-8}$ M

\[
\text{DEP VAR: } 1/V, \quad N: 12 \quad \text{MULTIPLE R: } .971 \quad \text{SQUARED MULTIPLE R: } .943 \\
\text{ADJUSTED SQUARED MULTIPLE R: } .937 \quad \text{STANDARD ERROR OF ESTIMATE: } 0.006
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Lineweaver-Burk Analysis of Reciprocal Trehalase Activity (Velocity) Plotted Against Reciprocal Substrate (Trehalase) Concentration (Regression Statistics and ANOVA)

Inhibitor: Validamycin A (pre-incubated)
Concentration of Inhibitor: $6 \times 10^{-3}$ M

\[
\text{DEP VAR: } 1/V, \quad N: 9 \quad \text{MULTIPLE R: } .931 \quad \text{SQUARED MULTIPLE R: } .867 \\
\text{ADJUSTED SQUARED MULTIPLE R: } .848 \quad \text{STANDARD ERROR OF ESTIMATE: } 0.033
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Secondary Plot of $K_a'$ versus Validamycin A Concentration [V]
(Determination of $K_a'$) (Regression Statistics and ANOVA)

DEP VAR: $K_a'$  N: 5  MULTIPLE R: .889  SQUARED MULTIPLE R: .791
ADJUSTED SQUARED MULTIPLE R: .721  STANDARD ERROR OF ESTIMATE: 0.186

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Lineweaver-Burk Analysis of Reciprocal Trehalase Activity (Velocity) Plotted Against Reciprocal Substrate (Trehalose) Concentration (Regression Statistics and ANOVA)

Inhibitor: Castanospermine
Concentration of Inhibitor: 3 x 10^{-5} M

DEP VAR: 1/V, N: 12 MULTIPLE R: .922 SQUARED MULTIPLE R: .851
ADJUSTED SQUARED MULTIPLE R: .836 STANDARD ERROR OF ESTIMATE: 0.007

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Lineweaver-Burk Analysis of Reciprocal Trehalase Activity (Velocity) Plotted Against Reciprocal Substrate (Trehalose) Concentration (Regression Statistics and ANOVA)

Inhibitor: Castanospermine
Concentration of Inhibitor: 5 x 10^{-5} M

DEP VAR: 1/V, N: 12 MULTIPLE R: .972 SQUARED MULTIPLE R: .946
ADJUSTED SQUARED MULTIPLE R: .940 STANDARD ERROR OF ESTIMATE: 0.005

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Lineweaver-Burk Analysis of Reciprocal Trehalase Activity (Velocity) Plotted Against Reciprocal Substrate (Trehalose) Concentration (Regression Statistics and ANOVA)

Inhibitor: Castanosporine
Concentration of Inhibitor: $7 \times 10^{-5}$ M

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Lineweaver-Burk Analysis of Reciprocal Trehalase Activity (Velocity) Plotted Against Reciprocal Substrate (Trehalose) Concentration (Regression Statistics and ANOVA)

Inhibitor: Castanosporine
Concentration of Inhibitor: $1 \times 10^{-4}$ M

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Lineweaver-Burk Analysis of Reciprocal Trehalase Activity (Velocity) Plotted Against Reciprocal Substrate (Trehalose) Concentration (Regression Statistics and ANOVA)

Inhibitor: Castanospermine (pre-incubated)

Concentration of Inhibitor: $5 \times 10^{-5}$ M

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Secondary Plot of K* versus Castanospermine Concentration [C]
(Determination of K*) (Regression Statistics and ANOVA)

DEP: Km N: 4 MULTIPLE R: .946 SQUARED MULTIPLE R: .895
ADJUSTED SQUARED MULTIPLE R: .843 STANDARD ERROR OF ESTIMATE: 0.150

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Lineweaver-Burk Analysis of Reciprocal Trehalase Activity (Velocity) Plotted Against Reciprocal Substrate (Trehalose) Concentration (Regression Statistics and ANOVA)

Inhibitor: MDL 25,637
Concentration of Inhibitor: $1 \times 10^{-8}$ M

DEP VAR: 1/V, N: 12 MULTIPLE R: .974 SQUARED MULTIPLE R: .950
ADJUSTED SQUARED MULTIPLE R: .945 STANDARD ERROR OF ESTIMATE: 0.004

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Lineweaver-Burk Analysis of Reciprocal Trehalase Activity (Velocity) Plotted Against Reciprocal Substrate (Trehalose) Concentration (Regression Statistics and ANOVA)

Inhibitor: MDL 25,637
Concentration of Inhibitor: $2 \times 10^{-8}$ M

DEP VAR: 1/V, N: 12 MULTIPLE R: .977 SQUARED MULTIPLE R: .954
ADJUSTED SQUARED MULTIPLE R: .950 STANDARD ERROR OF ESTIMATE: 0.004

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</tr>
<tr>
<td>RESIDUAL</td>
<td>0.000</td>
<td>10</td>
<td>0.000</td>
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</tr>
</tbody>
</table>

258
Lineweaver-Burk Analysis of Reciprocal Trehalase Activity (Velocity) Plotted Against Reciprocal Substrate (Trehalose) Concentration (Regression Statistics and ANOVA)

Inhibitor: MDL 25,637
Concentration of Inhibitor: $6 \times 10^{-8}$ M

<table>
<thead>
<tr>
<th>DEP VAR: 1/V</th>
<th>N: 12</th>
<th>MULTIPLE R: .991</th>
<th>SQUARED MULTIPLE R: .983</th>
</tr>
</thead>
<tbody>
<tr>
<td>ADJUSTED SQUARED MULTIPLE R: .981</td>
<td>STANDARD ERROR OF ESTIMATE: 0.003</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>VARIABLE</th>
<th>COEFFICIENT</th>
<th>STD ERROR</th>
<th>STD COEF TOLERANCE</th>
<th>T</th>
<th>P(2 TAIL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CONSTANT</td>
<td>5.358</td>
<td>0.002</td>
<td>0.000</td>
<td>0.30E+02</td>
<td>0.000</td>
</tr>
<tr>
<td>1/S</td>
<td>9.417</td>
<td>0.004</td>
<td>0.991</td>
<td>0.100E+01</td>
<td>0.24E+02</td>
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</tbody>
</table>

**ANALYSIS OF VARIANCE**

<table>
<thead>
<tr>
<th>SOURCE</th>
<th>SUM-OF-SQUARES</th>
<th>DF</th>
<th>MEAN-SQUARE</th>
<th>F-RATIO</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>REGRESSION</td>
<td>0.005</td>
<td>1</td>
<td>0.005</td>
<td>0.579E+03</td>
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</tr>
<tr>
<td>RESIDUAL</td>
<td>0.000</td>
<td>10</td>
<td>0.000</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Lineweaver-Burk Analysis of Reciprocal Trehalase Activity (Velocity) Plotted Against Reciprocal Substrate (Trehalose) Concentration (Regression Statistics and ANOVA)

Inhibitor: MDL 25,637
Concentration of Inhibitor: $1 \times 10^{-7}$ M

<table>
<thead>
<tr>
<th>DEP VAR: 1/V</th>
<th>N: 12</th>
<th>MULTIPLE R: .993</th>
<th>SQUARED MULTIPLE R: .986</th>
</tr>
</thead>
<tbody>
<tr>
<td>ADJUSTED SQUARED MULTIPLE R: .984</td>
<td>STANDARD ERROR OF ESTIMATE: 0.003</td>
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</tbody>
</table>

<table>
<thead>
<tr>
<th>VARIABLE</th>
<th>COEFFICIENT</th>
<th>STD ERROR</th>
<th>STD COEF TOLERANCE</th>
<th>T</th>
<th>P(2 TAIL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CONSTANT</td>
<td>5.305</td>
<td>0.002</td>
<td>0.000</td>
<td>0.26E+02</td>
<td>0.000</td>
</tr>
<tr>
<td>1/S</td>
<td>11.883</td>
<td>0.005</td>
<td>0.993</td>
<td>0.100E+01</td>
<td>0.26E+02</td>
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</tbody>
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**ANALYSIS OF VARIANCE**

<table>
<thead>
<tr>
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<th>MEAN-SQUARE</th>
<th>F-RATIO</th>
<th>P</th>
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<tbody>
<tr>
<td>REGRESSION</td>
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<td>0.008</td>
<td>0.694E+03</td>
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<tr>
<td>RESIDUAL</td>
<td>0.000</td>
<td>10</td>
<td>0.000</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Lineweaver-Burk Analysis of Reciprocal Trehalase Activity (Velocity) Plotted Against Reciprocal Substrate (Trehalose) Concentration (Regression Statistics and ANOVA)

Inhibitor: MDL 25,637 (pre-incubated)
Concentration of inhibitor: 6 x 10^{-4} M

DEP VAR: 1/V, N: 9 MULTIPLE R: .966 SQUARED MULTIPLE R: .972
ADJUSTED SQUARED MULTIPLE R: .968 STANDARD ERROR OF ESTIMATE: 0.012

<table>
<thead>
<tr>
<th>VARIABLE</th>
<th>COEFFICIENT</th>
<th>STD ERROR</th>
<th>STD COEF TOLERANCE</th>
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<th>P(2 TAIL)</th>
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</thead>
<tbody>
<tr>
<td>CONSTANT</td>
<td>8.658</td>
<td>0.008</td>
<td>0.000</td>
<td>0.11E+02</td>
<td>0.000</td>
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<tr>
<td>1/S</td>
<td>37.250</td>
<td>0.024</td>
<td>0.986</td>
<td>0.100E+01</td>
<td>0.16E+02</td>
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ANALYSIS OF VARIANCE

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<th>DF</th>
<th>MEAN-SQUARE</th>
<th>F-RATIO</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>REGRESSION</td>
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<td>0.033</td>
<td>0.243E+03</td>
<td>0.000</td>
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<tr>
<td>RESIDUAL</td>
<td>0.001</td>
<td>7</td>
<td>0.000</td>
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</tbody>
</table>

260
Secondary Plot of $K_1$ versus MDL 25,637 Concentration [M]
(Determination of $K_1$) (Regression Statistics and ANOVA)

DEP VAR: $K_1$  N: 5 MULTIPLE R: .984 SQUARED MULTIPLE R: .969
ADJUSTED SQUARED MULTIPLE R: .958  STANDARD ERROR OF ESTIMATE: 0.098

<table>
<thead>
<tr>
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<th>STD COEF TOLERANCE</th>
<th>T</th>
<th>P(2 TAIL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CONSTANT</td>
<td>1.109</td>
<td>0.053</td>
<td>0.000</td>
<td>0.18E-02</td>
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<tr>
<td>[M]</td>
<td>1.138</td>
<td>0.118</td>
<td>0.994</td>
<td>0.100E+01</td>
<td>9.619</td>
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ANALYSIS OF VARIANCE

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<th>MEAN-SQUARE</th>
<th>F-RATIO</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>REGRESSION</td>
<td>0.892</td>
<td>1</td>
<td>0.892</td>
<td>0.925E+02</td>
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<tr>
<td>RESIDUAL</td>
<td>0.029</td>
<td>3</td>
<td>0.010</td>
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</tr>
</tbody>
</table>
VITA AUCTORIS

Name: Lesly Ann Temesvari

Born: July 8, 1965

Place of Birth: Windsor, Ontario, Canada

Education:

University of Windsor
Windsor, Ontario, Canada
1993 Ph.D.

McGill University
Montreal, Quebec, Canada
1987 B.Sc. (Microbiology and Immunology)
Minor (Chemical Engineering)

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1983 O.S.S.H.G.D.

Honours:

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University of Windsor
1992

Predoctoral Fellowship
Natural Sciences and Engineering
Research Council of Canada
1990-1992

Conference Travel Bursary
University of Windsor
1991, 1990

Ontario Graduate Scholarship
1989-1990

Graduate Tuition Bursary
University of Windsor
1988-1989
Abstracts:


