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Screening Bioactive Molecules In Tetrahymena thermophila Using Phagocytosis Determined By Flow Cytometry

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SCREENING BIOACTIVE MOLECULES IN *TETRAHYMENA THERMOPHILA*
USING PHAGOCYTOSIS DETERMINED BY FLOW CYTOMETRY

A Thesis

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Master of Science

by

Santosh E. Gummidipundi

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The Designated Thesis Committee Approves the Thesis Titled

SCREENING BIOACTIVE MOLECULES IN *TETRAHYMENA THERMOPHILA*
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Santosh E. Gummidipundi

APPROVED FOR THE DEPARTMENT OF BIOLOGICAL SCIENCES

SAN JOSÉ STATE UNIVERSITY

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Dr. John Boothby	Department of Biological Sciences
Dr. Joshua Mackie	Department of Biological Sciences
Dr. Shannon Bros	Department of Biological Sciences

ABSTRACT

SCREENING BIOACTIVE MOLECULES IN *TETRAHYMENA THERMOPHILA*
USING PHAGOCYTOSIS DETERMINED BY FLOW CYTOMETRY

by Santosh E. Gummidipundi

The evaluation of molecules that have potentially bioactive significance is an expanding field, but current screening assays are expensive and time consuming. Mammalian cell-based assays require aseptic techniques, complex media and long generation times. This thesis reports on an assay that incorporates *Tetrahymena* phagocytic ingestion and flow cytometry to evaluate several potentially bioactive molecules. Several factors that influenced optimal and reproducible results were analyzed. Seven potentially bioactive molecules (serotonin, norepinephrine, dopamine, morphine, colchicine, mexiletine and procainamide) were evaluated to determine their effect on phagocytosis by *Tetrahymena*. Assay results indicated that the phagocytic rate was unaffected by serotonin, norepinephrine, dopamine, and morphine but inhibited by colchicine, mexiletine and procainamide. The analysis of the data indicated that acclimation time, temperature, cell concentration, and cell wash media affected assay consistency. This assay can be used to screen for bioactive molecules.

ACKNOWLEDGEMENTS

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CHAPTER I
GENERAL INTRODUCTION

With new sources of molecular diversity being discovered at a fast pace,[1] there is a

need for screening assays that are fast, economical and reproducible. This thesis presents

results from an assay based on *Tetrahymena thermophila* phagocytic ingestion first

described by Buduma *et al.* that fits the above criteria.[2] *Tetrahymena*-based phagocytic

ingestion assay results have shown that *Tetrahymena* demonstrates reactivity to opiates,[3]

hormones[4] and sodium channel blockers.[2]

Current screening assays using cultured mammalian cells are limited by low

throughput and high cost. Despite these limitations, *in vitro* mammalian cell culture is

often used for drug discovery. High throughput screening with mammalian cells has

complex culture requirements. Mammalian cell culture density cannot go higher than

10^6 cells/mL and cell doubling times range from 18-36 h. A highly developed aseptic

technique is required for mammalian cell culture, and providing for the cells' fastidious

nutritional requirements is expensive. Some mammalian cells do not respond to a wide

array of molecules. In cancer drug screening, for example, the National Cancer

Institute's Developmental Therapeutics Program requires using 60 different cell lines.

Once a molecule has produced positive results in a mammalian cell assay, the assay is

repeated again over a five dose range.[5]

Natural products may provide an enormous source of bioactive molecules. The

T. thermophila phagocytic ingestion assay described in this thesis is simple, economical

and fast. *Tetrahymena* have simple culture requirements. Media for culturing

Tetrahymena usually consist of a protein digest, with or without glucose, and an iron supplement. *Tetrahymena* are routinely subcultured every week or two, and culture health can be easily determined by monitoring motility, cellular morphology and cell counts. With a generation time of 2 h, *Tetrahymena* can be cultured overnight for experiments the following day. Since phagocytosis experiments can be run within 6 min, experimental data can be obtained and analyzed within a day. *Tetrahymena*'s multi-molecule sensitivity[2–4] reduces the time required to repeat experiments for a single biomolecule preparation using several different cell lines. This is especially important when screening natural products that may have complex biochemical compositions.

Tetrahymena (**Fig. 1**) is a useful model organism because it is ubiquitous,[6] extensively studied and easily cultured. In 1923, Lwoff established pure cultures of *Tetrahymena*, and since that time they have been used in numerous physiological, biochemical and genetic studies.[7,8] Nearly 25% of the papers found in *Proceedings of the Society of Protozoologists* between 1950 and 1954 were concerned with or contained work done with *Tetrahymena*.[9] A Web of Science search for "*Tetrahymena*" yielded 3,295 publications between 2000 and 2015, which indicates sustained scientific focus.

Tetrahymena thermophila is a commonly used model organism.[10] *Tetrahymena thermophila* is easily propagated by incubation in the dark for 48 h at 30°C, and reaches cell densities as high as 4.2×10^5 cells/mL. The process of phagocytosis involves ingestion of particles by a cell and the subsequent formation of a phagosome in which the particles are degraded by enzymatic components and released. The phagocytic response may provide a means of screening for molecules that influence cellular activities.

Modulation of phagocytosis in *T. thermophila* by bioactive molecules has been studied using light microscopy. Phagocytic ingestion assays have been used to evaluate the cellular response to molecules that modulate human physiology such as serotonin, norepinephrine, dopamine, morphine, colchicine and diphenhydramine.[2–4,11]
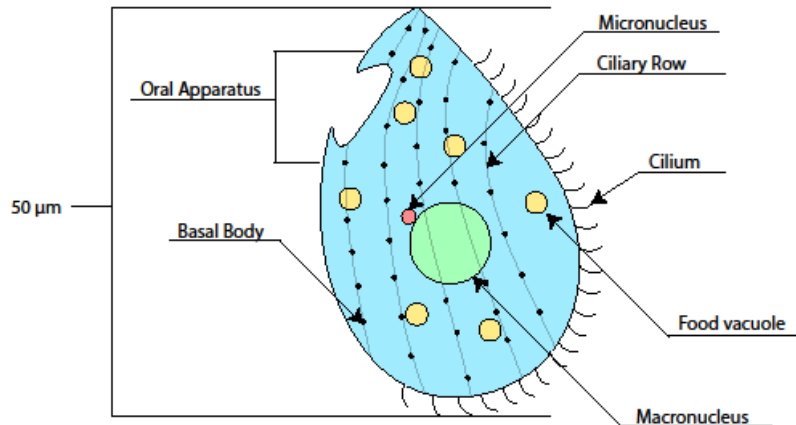
Figure 1. Anatomy of *Tetrahymena thermophila.*

Phagocytic ingestion in *T. thermophila* begins with ligand binding to cell receptors. Potentially bioactive molecules (PBMs) may exert their effect by binding to receptors, most likely on the cell surface.[12] Increased cellular concentrations of secondary messengers occur after ligand binding and may be altered by changes in ionic concentration and membrane potential. Ion channel blockers can regulate the movement of these ions by blocking the channels. Secondary messenger concentration can affect phagosome formation.[13] Both ligand binding and ion channel blocking alter phagocytic activity in *T. thermophila*. Several bioactive molecules were evaluated in this study that could potentially affect phagocytosis.

Tetrahymena thermophila has serotonin-like receptors that modulate cellular activities, including phagocytosis,[4] cilia regeneration[14] and increased cell division[15] by serotonin. In humans and higher animals, serotonin is primarily found in the GI tract and the CNS and is the chemical mediator of the feeling of happiness. The serotonergic system was studied in *Tetrahymena pyriformis,* and serotonin was found in concentrations higher than other biogenic amines, suggesting that it may play a role in cellular signaling events such as cilia regulation and responses to temperature.[16] *Tetrahymena pyriformis* produces enzymes to synthesize and degrade serotonin, implying similarities to higher animals.[17] Understanding the role of serotonin in cellular responses of *Tetrahymena* may lead to methods for screening PBMs with serotonin-like properties.

Dopamine is a monoamine neurotransmitter found to have phagocytic-modulating activity in *T. thermophila,* and a dopamine receptor has been reported.[4,18] In the brains of vertebrates, dopamine is involved in cognition, sleep and motivation. Dopamine is part of the galactokinase regulation system in *T. thermophila.*[19] The presence of a D1 class receptor on the cell surface of *T. thermophila* was confirmed using competition between rhodamine SKF-38393 (a dopamine agonist) and rhodamine SCH-23390 (a dopamine antagonist) fluorescent probes. These findings indicated that *T. thermophila* may have a specific sensitivity to dopamine, which could be useful in detecting antagonistic molecules.

Opiates were found to produce phagocytic-modulating activity in *T. thermophila,* and a putative receptor has been reported.[3,20] Opiates are psychoactive substances that bind to opiate receptors, causing decreases in the sensation of pain. Some opiates of importance

include heroin, morphine, leu-enkephalin and β-endorphin.[21] A putative opiate receptor

in *T. pyriformis* was described by O'Neill *et al.* using binding assays and partial protein

digestions.[20] Receptor binding was displaced by various opiate subtypes and reversible

by naloxone, an opiate receptor antagonist. The putative opiate receptor was similar to

the opiate receptor found in rat brain.[20] Endogenous opiate-like molecules called β-

endorphins were found in *T. thermophila* and validated by HPLC and immunoblotting.

These purified endogenous molecules had the same effect as exogenous compounds

when applied to cultures of *T. thermophila*.[22] Because the binding of morphine had a

much stronger inhibition of phagocytosis than β-endorphin, Chiesa *et al.* speculated that

there is a μ-class opiate receptor in *T. thermophila*.[23] They also found that the opiate

antagonist naloxone reversed the inhibitory effect of morphine on phagocytic activity.

   Tetrahymena secondary messengers form a signal cascade when an agonist binds to a

cell surface receptor, eventually resulting in a physiological response.[21] Secondary

messengers, including nucleotides, nucleotide cyclases, protein kinases, and ions have

been implicated in modulating motility and phagocytosis.

   Motility is modulated in response to changes in ionic concentration. Schultz and

Schönborn[24] reported that the increase of extracellular [K^+] and subsequent

depolarization reduced forward swimming speeds. When cells were acclimatized to a

16mM [K^+] medium and moved to a [K^+]-free medium (hyperpolarized), forward

swimming speeds increased two-fold.[24] Ionic changes as well as hormonal stimulation

regulated cAMP levels. Diluting extracellular [K^+] (hyperpolarization) stimulated

intracellular cAMP generation. Conversely, potassium channel blockers such as

tetraethylammonium, Cs^+, and quinine, inhibited cAMP generation. Increased

concentrations of Ca^{+2} increased motility and cAMP generation.[24]

Adenylate cyclase catalyzes the formation of cAMP, which regulates physiological

processes in *Tetrahymena.*[21] Activation of adenylate cyclase occurs via G_s activation

subsequent to ligand-receptor binding. Csaba and Lantos found that the cAMP derivative

dibutyril cAMP increased phagocytosis activity.[25] Buduma *et al.* reported that

diphenhydramine, a sodium channel blocker, inhibited phagocytosis of fluorescent

polystyrene beads.[2] These findings suggest that secondary messengers play an important

role in regulating phagocytosis.

Elucidating the effects of specific bioactive molecules on phagocytosis in

Tetrahymena may lead to an efficient analysis of complex mixtures in natural products.

By comparing phagocytosis rates of the natural products alone to the phagocytosis rates

with the inclusion of antagonists with known activity, the molecule class of unknown

molecules present in the preparation could be pinpointed.

This thesis reports on the effect of several bioactive molecules on phagocytosis

activity in *T. thermophila* detected by flow cytometry, using a modification of the method

of Boothby *et al.*[26] and Buduma *et al.*[2] This assay delivers consistent results with

minimum preparation and execution time. It is economical, requiring minimum labor,

equipment and reagents. Much of the economy, consistency, and time savings is due to

the use of flow cytometry. Compared with previous microscopic methods using India

ink[27] or fluorescent latex beads,[28] flow cytometry is faster and can analyze larger cell

populations. Flow cytometry acquires data on hundreds of cells in a few seconds and facilitates rapid data analysis.

One goal of this study was to evaluate the effect of several bioactive molecules on phagocytic activity in *T. thermophila*. A range of hormone, opiate, and ion blocker pilot molecules with known biological activity in *Terahymena* were studied. This work may lead to a consistent, economical, and potentially high throughput screening assay to identify similar molecules of unknown biological activity.

A second goal of this study was to define the most critical variables in the assay. Understanding the critical assay variables should be useful in refining this assay for high throughput applications.

CHAPTER II

OPTIMIZATION OF A *TETRAHYMENA THERMOPHILA* PHAGOCYTIC
INGESTION ASSAY

OPTIMIZATION OF A *TETRAHYMENA THERMOPHILA* PHAGOCYTIC INGESTION ASSAY

S.E. Gummidipundi[1]

[1]Department of Biological Sciences
San José State University
San José, CA 95192

Corresponding Author:
Santosh Gummidipundi, San José State University, Department of Biological Sciences, One Washington Square, San José, CA 95192.
Email: santoshgummidipundi@gmail.com

Abstract

The development of high throughput, cheap and consistent assays with the potential for detecting bioactive molecules is essential. The protozoan *Tetrahymena thermophila* has been used to study physiological responses to pharmaceutically active molecules, such as hormones and opiates. We evaluated the variability of an assay using ingestion of fluorescent polystyrene beads by *T. thermophila* as measured using flow cytometry. Assays that varied in acclimation time, cell wash medium, cell concentration, cell strain and temperature were evaluated by post hoc analysis. Analysis of variance (ANOVA) was used to determine the statistical significance of variations in phagocytosis data. These analyses indicated that the assay conditions of no acclimation time, a temperature of 30°C and Neff's modified wash medium resulted in the lowest assay variability. A

cell concentration of 10,000-20,000 cells/mL and use of the cell wash medium proteose peptone proved to be the most effective. Cell wash medium had no effect at a cell concentration of 250,000 cells/mL. Assays performed with no acclimation time, a temperature of 30°C, Neff's modified medium as wash medium, and a cell concentration of 10,000 – 20,000 cells/mL had optimal phagocytosis.

Introduction

There is a need for an economical, quick, simple, high throughput technique for screening potentially bioactive molecules (PBMs). Methods involving mammalian cell culture are expensive and time consuming. The assay results described here using phagocytic modulation in *Tetrahymena thermophila* can be quantified rapidly using flow cytometry.

Phagocytosis is the process by which particles in the environment are ingested by the cell, resulting in the formation of a phagosome. Subsequent enzymatic degradation in the phagosome is followed by release from the cell.[1] This phagocytic event is modulated in *Tetrahymena* in response to external stimuli such as hormones, opiates and ion channel blockers. These stimuli also elicit physiological responses in humans.[2–4] This cross-organism reactivity is potentially a result of similarities between human and *T. thermophila* target molecules. These targets may include receptors, ion channels and tubulin. De Jesus and Renaud[3] and Quinones-Maldonado and Renaud[2] found that various opiates and hormones had the ability to modulate phagocytosis in *T. thermophila*. Buduma *et al*. observed that diphenhydramine, a known sodium channel blocker, caused a decrease in phagocytic activity in *T. thermophila*.[4] Colchicine, a natural product and an inhibitor of microtubule polymerization, is a classic inhibitor of phagocytosis in *Tetrahymena*.[5] Sensitivity to various bioactive molecules is important to evaluate because of their potential for human therapies. Using phagocytosis as a model for cellular responses may lead to detecting bioactive molecules in a quick, controlled and repeatable manner.

To evaluate assay reliability, sources of variation must be determined. This study analyzed assay variation due to acclimation time, cell wash medium, cell concentration (confounded with cell strain) and temperature.

Materials and Methods

Test Procedure and Variables

A modification of the phagocytic ingestion assay described by Buduma *et al.*[4] was used in this study. Briefly, *Tetrahymena thermophila* strains SB1969 (a gift from Dr. Eduardo Orias, University of California, Santa Barbara, CA) and SB210 (Tetrahymena Stock Center, Cornell, Ithaca, NY) were inoculated into a protein-digested medium and incubated for 24-48 h at 30°C. The cultures were centrifuged, washed with a cell wash medium (proteose peptone, HEPES or Neff's modified medium) and adjusted to an appropriate cell concentration using a hemocytometer. A 450 μL aliquot was then placed into flow cytometry culture tubes in preparation for addition of a control or a drug solution and approximately 1,375,000 polystyrene fluorescent beads, (Spherotech, Inc., Lake Forest, IL). The reaction tubes were then incubated for various periods (1, 2, 3, 4, 5, 6, 10 or 15) of time before the addition of formalin (1% methanol-free) to stop phagocytosis and fix the cells. Acquisition and analysis were performed on a Becton-Dickinson FACSCalibur using CellQuest™ Pro software (BD Biosciences, San José, CA). Phagocytosis percentages were calculated according to quadrant statistics (SSC vs. FL-1) on *Tetrahymena*-gated cells (FSC vs SSC). These data were used to derive the number of *Tetrahymena* that had ingested one or more fluorescent polystyrene beads.

To determine if starving the cells had an impact on bead consumption, the time period between the introduction of the cells and the polystyrene bead suspension (referred to as acclimation time) was varied (0,10, 20, 30, 45, 60 and 120 minutes).

This study evaluated the effect on phagocytosis rate of the following media: proteose peptone (PP) (% w/v proteose peptone (BD, Sparks, MD) supplemented with 0.003% Fe-EDTA), HEPES (10 mM), Neff's modified medium (NMM) (0.25% w/v proteose peptone (BD, Sparks, MD), 0.25% w/v yeast extract (Difco Laboratories, Detroit, MI) and 0.5% w/v D-Glucose (J.T. Baker Chemicals, Phillipsburg, NJ) supplemented with 33.3 μM $FeCl_3$). To evaluate the effect of all concentration phagocytic rates were determined for three cell concentrations (250,000 cells/mL, 20,000 cells/mL and 10,000 cells/mL). Elevated (30°C water bath) and room temperatures (20-24°C) were used to determine the effect of temperature on the phagocytic response.

Data Manipulation and Analysis

Experiments were designed to examine one variable at a time, but some subsets of conditions were combined to evaluate potential interactions between factors. Because there was an insufficient number of cases for some combinations, three variables were pooled: the number of acquired events (250 and 500 were pooled and compared to 1500 number of acquired events), acclimation time (10, 20, 30, 45, 60, and 120 minutes were pooled and compared to 0 minutes), and cell concentration (10,000 and 20,000 cells/mL were pooled and compared to 250,000 cells/mL). Cell strain and cell concentration were confounded, so differences involving these variables could not be independently determined. To meet the assumption of equality of variance for analysis of variance

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(ANOVA),[6] the angular transform of the dependent variable, percent phagocytosis was used in all analyses. Four combinations were tested:

1.   To examine the effects of cell strain (or cell concentration) on phagocytotic rates, cases were restricted to experiments using cell wash medium (PP or HEPES), an assay temperature of 30°C, an acclimation time of 0 minutes, 250-500 acquired events and cell strains SB210 and SB1969. These data were analyzed using a two-way ANOVA, with cell strain (or cell concentration) and cell wash medium as factors.

2.   To determine if cell wash medium affected phagocytotic rates under assay conditions different from those associated in the above paragraph, cases were restricted to having cell wash medium as a factor with two levels (PP and NMM), a cell concentration between 10,000 and 20,000 cells/mL (or cell strain SB210), an assay temperature of 24°C, an acclimation time of 0 minutes and 250-500 acquired events. A one-way ANOVA was used to determine if cell wash medium affected phagocytosis.

3.   To determine if temperature affected phagocytosis, cases were restricted to those with a PP cell wash medium, a cell concentration of 250,000 cells/mL, an acclimation time of 0 minutes and 250-500 acquired events. These cases were analyzed using a one-way ANOVA to determine if temperature affected phagocytosis.

4.   To determine if cell wash medium and acclimation time affected phagocytosis, cases were restricted to those with cell wash medium as a factor with two levels

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(PP and NMM), a cell concentration of 10,000-20,000 cells/mL (or cell strain

SB210), an assay temperature of 24°C and 250-500 acquired events. These cases

were analyzed using a two-way ANOVA to determine if cell wash medium and

acclimation time affected phagocytosis.

Results and Discussion

Examination of results revealed that cell strain and cell concentration were confounded. Cell strain SB1969 was used in experiments when the cell concentration was 250,000 cells/mL. Cell strain SB210 was used in experiments where cell concentration was between 10,000 and 20,000 cells/mL. Therefore, any variation due to cell strain or cell wash medium could not be independently determined.

Cell wash media appeared to have a strong influence on phagocytosis and appeared to be linked to cell concentration or the cell strain, (given 250-500 acquired events and no acclimation time). At a temperature of 24°C, a cell concentration of 10,000 – 20,000 cells/mL (or cell strain SB210), a one-way ANOVA (**Table 1**) showed that phagocytosis was significantly ($p = 0.001$) greater using NMM cell wash medium rather than PP (**Fig 1**). A two-way ANOVA (**Table 2**) showed that the effect of cell wash medium (PP or HEPES) on phagocytosis varied with different cell concentrations (10,000-20,000 cells/mL and 250,000 cells/mL) or cell strain at a temperature of 30°C. The significant ($p = 0.010$) cell concentration (10,000- 20,000 cells/mL, 250,000 cells/mL)*cell wash medium (PP, HEPES) interaction indicated that phagocytosis with a *T. thermophila* cell concentration of 250,000 cells/mL (or cell strain SB1969) did not appear to be greatly affected by the type of cell wash medium. However, phagocytosis in the lower cell concentration (10,000-20,000 cells/mL or cell strain SB210) was clearly greater in the PP medium (**Fig 2**).

Selection of cell wash medium for *T. thermophila* was important for phagocytic activity. HEPES (considered a non-nutritive medium) contributed to lower phagocytosis,

presumably due to its lower nutritional value. Proteose peptone contains high protein but low glucose. Conversely, NMM has more glucose and resulted in higher observed phagocytic activity. Cohn and Morse found that the continued availability of glucose was necessary for the continued ingestion of bacteria by human leucocytes.[7] When *T. thermophila* was incubated for an extended time, phagocytic rate decreased, suggesting that allowing *T. thermophila* no acclimation time prior to the introduction of fluorescent beads led to higher phagocytic rates.

Since cell concentration and cell strain were confounded, determination of their individual effects on phagocytosis was not possible. However, there is evidence that differences in cell concentration rather than strain predominate.[8] Controlling for the interaction of strain and cell to bead ratio was addressed in later studies using appropriate experimental controls. The effect of drug to cell ratio was addressed by using a constant concentration of cells and appropriate drug dilutions.

Table 1. One-way ANOVA to determine the effects of cell wash media (PP, NMM) on phagocytosis. The dependent variable was the angular transform of percent phagocytosis. The factor was cell wash medium. Temperature was 24°C, acclimation time was 0 and the number of acquired events was 250-500. Bold type indicates significance.

Source	Type III Sum of Squares	df	Mean Square	F	p value
Cell wash medium	**0.350**	**1**	**0.350**	**11.047**	**0.001**
Error	4.500	142	0.032		
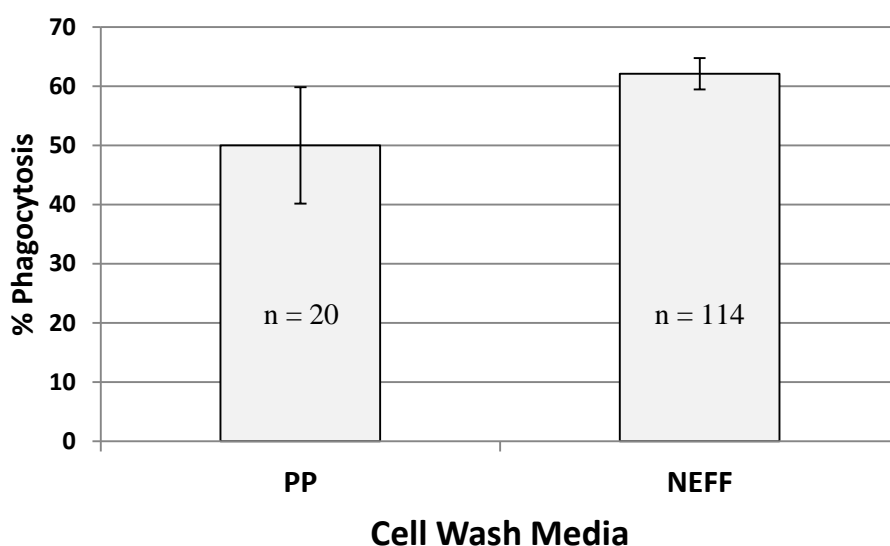
Figure 1. Significant cell wash medium effect from one-way ANOVA to determine the effect of cell wash medium (PP and NEFF) on % phagocytosis. Percent phagocytosis was transformed (angular) for analysis. Error bars represent 95% confidence level.

Table 2. Two-way ANOVA to determine the effects of cell concentration (10,000-20,000, 250,000 cells/mL) and cell wash media (PP, HEPES) on phagocytosis. The dependent variable was the angular transform of percent phagocytosis. The factors were cell strain and cell wash medium type. Temperature was 30^0C, acclimation time was 0 and the number of acquired events was 250-500. Bold type indicates significance.

Source	Type III Sum of Squares	df	Mean Square	F	p value
Cell Strain	0.001	1	0.001	0.020	0.887
Cell Wash Medium	**0.515**	**1**	**0.515**	**10.726**	**0.001**
Cell Strain * Cell Wash Medium	**0.328**	**1**	**0.328**	**6.839**	**0.010**
Error	8.019	167	0.048		

As demonstrated in other studies,[4,9] temperature modulates phagocytosis. A one-way
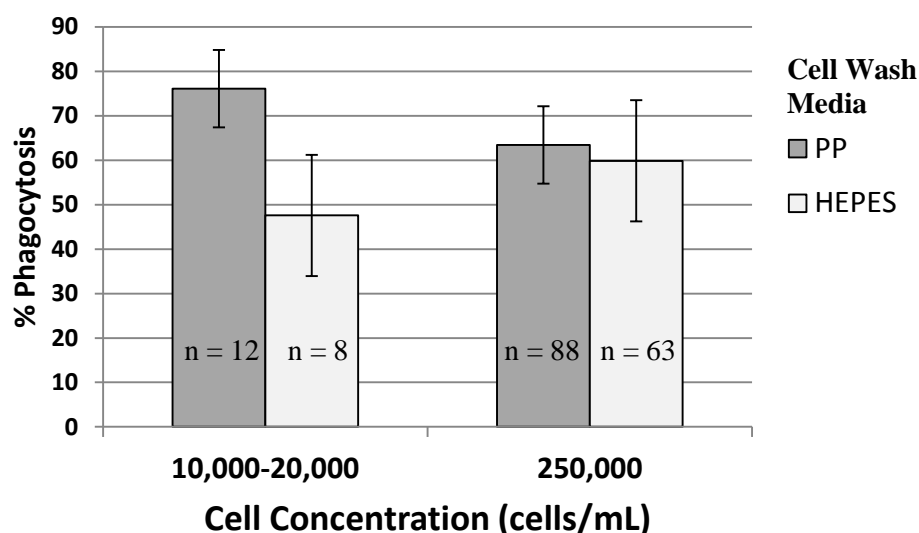
Figure 2. Significant cell wash medium vs cell concentration interaction from a two-way ANOVA to determine the effects of cell concentration (10,000-20,000, 250,000 cells/mL) and cell wash media (PP, HEPES) on % phagocytosis. Percent phagocytosis was transformed (angular) for analysis. Error bars represent 95% confidence level.

ANOVA indicated that an elevated temperature produced the highest phagocytosis in the

higher cell concentration (250,000 cells/mL) (**Fig 3**). A temperature of 30°C produced a

significantly (p < 0.001) higher phagocytic response than at 24°C with the nutritive cell

wash medium PP and 0 minutes of acclimation time (**Table 3**). Kinetic energy,

movement of biomolecules and their associated metabolic processes increase with

increasing temperature. Phagocytosis may be one such metabolic process that is

increased. Duhra *et al.* stated that higher temperature caused efficient RNA

transportation during protein synthesis, leading to faster generation of cilia.[9] Increased

ciliation results in faster and more efficient phagocytosis.[9] Their result agreed with this

study and with Buduma *et al.,* who found that increased temperature resulted in increased

phagocytosis.[4]

Table 3. One way ANOVA to determine the effect of temperature (24°C, 30°C) on phagocytosis. The dependent variable was the angular transform of percent phagocytosis. The independent factor was temperature. Cell wash medium is PP, acclimation time was 0, cell concentration was 250,000 cells/mL and the number of acquired events was 250-500. Bold type indicates significance.

Source	Type III Sum of Squares	df	Mean Square	F	p-value
Temperature	**0.303**	**1**	**0.303**	**16.171**	**< 0.001**
Error	1.873	100	0.019		
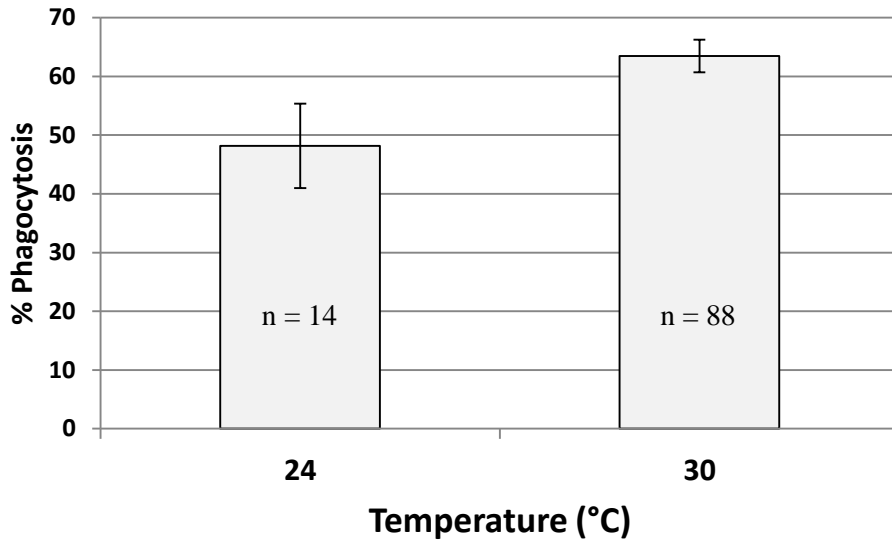
Figure 3. Significant temperature effect from one-way ANOVA to determine the effect of temperature (24°C,30°C) on % phagocytosis. Percent phagocytosis was transformed (angular) for analysis. Error bars represent 95% confidence level.

Allowing cells to acclimate to a new cellular medium decreased phagocytosis (**Table 4**). The non-significant (p = 0.083) acclimation time (0, 10-120 min)*cell wash media (NEFF, PP) interaction in two-way ANOVA showed that acclimation time and cell wash medium affected the rate of phagocytosis independently. As in prior experiments, phagocytosis was significantly (p = 0.047) greater in NMM than in PP (**Fig 4**). The highly significant (p < 0.001) acclimation time effect showed that phagocytosis was greater when there was no acclimation period (**Fig 5**). Introducing the cells directly into the phagocytic assay produced higher phagocytosis rates than if the cell were first acclimated.

Table 4. Two-way ANOVA to determine the effect of acclimation time (0 minutes, 10-120 minutes) and cell wash media (PP, NMM) on phagocytosis. The dependent variable was the angular transform on percent phagocytosis. The independent factors were acclimation time and cell wash medium. Temperature was 24°C, cell concentration was 10,000 – 20,000 cells/mL, the number of acquired events was 250-500 and cell wash medium was either PP or NMM. Bold type indicates significance.

| Source	Type III Sum of Squares	df	Mean Square	F	p-value
Acclimation time	**1.359**	**1**	**1.359**	**60.576**	**< 0.001**
Cell wash medium	**0.090**	**1**	**0.090**	**4.001**	**0.047**
Acclimation time * Cell wash medium	0.017	1	0.017	0.766	0.383
Error	3.141	140	0.022		
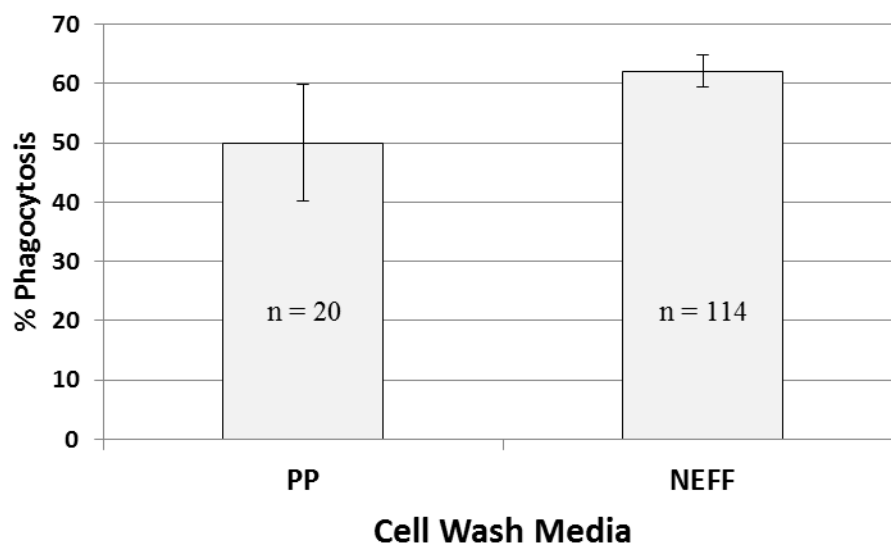
Figure 4. Significant cell wash medium effect from two-way ANOVA to determine the effect of cell wash media (PP, NEFF) on % phagocytosis. Percent phagocytosis was transformed (angular) for analysis. Error bars represent 95% confidence level.
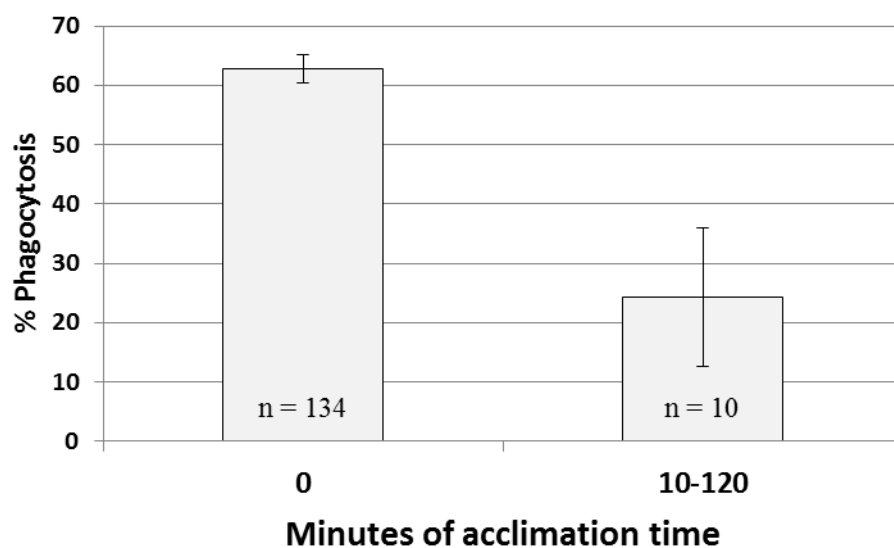
Figure 5. Significant acclimation time effect from two-way ANOVA to determine the effect of acclimation time (0, 10-120 min) on % phagocytosis. Percent phagocytosis was transformed (angular) for analysis. Error bars represent 95% confidence level.

In summary, the optimal conditions for the *T. thermophila* phagocytic assay appeared to be an incubation temperature of 30°C, use of NMM as a nutritive cell wash medium and no acclimation time. These results also suggested that a cell concentration of 10,000 – 20,000 cells/mL using proteose peptone was preferred, although cell concentration was confounded with cell strain. This combination provided consistent results with a relatively high level of phagocytosis.

This phagocytic ingestion assay should be an effective tool for evaluating potentially bioactive molecules. The assay is economical, fast and sensitive to multiple molecules, with the potential for high throughput automation. Using *T. thermophila* as an assay organism to screen for molecules is cost effective because the cell media and labor required to maintain cells are minimal.[10] The organism exhibits sensitivity to a range of molecule classes, making it an assay organism that has the potential for detecting

multiple targets.[3–5] The assay, when scaled to perform flow cytometry in a multi-well format,[11] may have the ability to screen for hundreds of molecules in a few hours. When adapted commercially this assay may provide a viable option for screening the increasing array of diverse molecules for pharmaceutical activity.

Acknowledgements

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CHAPTER III
SCREENING BIOACTIVE MOLECULES IN *TETRAHYMENA THERMOPHILA*
USING PHAGOCYTOSIS DETERMINED BY FLOW CYTOMETRY

SCREENING BIOACTIVE MOLECULES IN *TETRAHYMENA THERMOPHILA* USING PHAGOCYTOSIS DETERMINED BY FLOW CYTOMETRY

S.E. Gummidipundi[1]

[1]Department of Biological Sciences
San José State University
San José, CA 95192

Corresponding Author:
Santosh Gummidipundi, San José State University, Department of Biological Sciences, One Washington Square, San José, CA 95192.
Email: santoshgummidipundi@gmail.com

Keywords:
Tetrahymena thermophila, serotonin, dopamine, norepinephrine, morphine, colchicine, sodium channel blocker, flow cytometry

Abstract

   Tetrahymena thermophila is a useful model for studying eukaryotic cell responses because of its ease of culture and sensitivity to specific molecule classes. The effect of several bioactive molecules on phagocytosis of fluorescent microbeads in *T. thermophila* was determined using flow cytometry. Morphine, serotonin, dopamine, and norepinephrine had no effect, whereas colchicine and the sodium channel antagonists mexiletine and procainamide inhibited phagocytosis. This model system may be useful in evaluating compounds for biological activity.

Introduction

New sources of molecular diversity are being discovered every day from natural products.[1] Sifting through these molecules to find pharmaceutically active candidates for drug development is a challenge, prompting the development of economical, fast and reliable screening assays. Current assay formats such as *in vitro* mammalian cell culture are expensive and slow. We have addressed these limitations by adapting phagocytosis in *Tetrahymena thermophila* analyzed with flow cytometry as a way to screen for bioactive molecules. *Tetrahymena thermophila* is a widely studied protozoan that exhibits phagocytic modulation in response to specific molecule classes.[2] Its short generation time of 2 h[3] makes it possible to prepare cultures overnight and perform experiments the next day. Phagocytosis experiments using fluorescent microbeads take a few minutes, and analysis of hundreds of cells for the presence of ingested microbeads can be completed in several seconds.

Tetrahymena thermophila responds to specific molecular classes by inhibition or stimulation of phagocytosis.[4–7] Quiñones-Maldonado and Renaud,[5] De Jesus and Renaud,[4] and Bozzone[6] reported *T. thermophila* was sensitive to serotonin, norepinephrine, dopamine, morphine and colchicine. Quiñones-Maldonado and Renaud found that serotonin, norepinephrine, and dopamine upregulated phagocytosis in *T. thermophila*. De Jesus and Renaud, found that opiates such as morphine inhibited phagocytosis. Clinically, these molecules are important because hormones such as serotonin and dopamine are chemical mediators of emotion. Opiates serve as analgesics and are implicated in drug abuse.

The human sodium channel blockers mexiletine and procainamide are important in the treatment of cardiac arrhythmia. Diphenhydramine, a sodium channel blocker, significantly inhibits phagocytosis in *T. thermophila*.[4] The effect of human sodium channel blockers on physiological responses in *T. thermophila* has not been well documented. The significant effect of diphenhydramine on phagocytosis and the lack of evidence pointing to a common sodium channel blocking mechanism led us to explore the effect of mexiletine and procainamide on phagocytosis in *T. thermophila*.

This study reports on the effect of several bioactive molecules on *T. thermophila* ingestion of fluorescent polystyrene beads as determined by flow cytometry using a modification of the method of Buduma *et al.*[7] Data on the effect of serotonin, norepinephrine, dopamine, morphine, mexiletine, procainamide and colchicine on phagocytosis are presented. The ease of *Tetrahymena* culture and experimental manipulation, the specificity of the phagocytic response to a range of bioactive molecules, and the speed of flow cytometric analysis may lead to adaptation of this assay to high throughput format.

Materials and Methods

Cell culture and test procedure

Tetrahymena thermophila SB210 (*Tetrahymena* Stock Center, Cornell University, Ithaca N.Y.) stock cultures were maintained on 2% w/v proteose peptone (BD, Sparks, MD) supplemented with 0.003% Fe-EDTA. Cells were prepared for experimental work by transferring 500 µL of stock culture into 50 mL of working medium (0.25% w/v proteose peptone (BD, Sparks, MD), 0.25% w/v yeast extract (Difco Laboratories,

Detroit, MI), and 0.5% w/v D-Glucose (J.T. Baker Chemicals, Phillipsburg, NJ) supplemented with 33.3 µM $FeCl_3$. After incubating for 24-48 h at 30˚C in the dark in working medium, cells were washed once, adjusted to a concentration of 80,000 cells/mL in fresh medium using a hemocytometer, and set to incubate for 1 h to acclimatize cells before adding 450 µL aliquots of the adjusted *Tetrahymena* cell culture to 5 mL tubes. Then, either 10 µL or 60 µL of potentially bioactive molecule (PBM) or control solution was added, followed immediately by 100 µL of 2.19 µm (approximately 634,333) fluorescent polystyrene beads (Spherotech, Inc., Forest, IL). Each PBM control and drug concentration was performed in 6 replicates. When competitive assays were conducted on selective PBMs, pre-addition of different concentrations of sodium bicarbonate was performed 10 s prior to addition of PBM. After 6 min incubation, 450 µL of 1% methanol-free formalin was added to halt phagocytic uptake and fix the cells. Samples were vortexed before analysis.

Potentially bioactive molecule solutions

Serotonin hydrochloride, norepinephrine hydrochloride, dopamine hydrochloride

(0.0001 mM to 10 mM), mexiletine hydrochloride and procainamide hydrochloride (0.1

mM to 10 mM) and colchicine (0.001 to 10 mM) were prepared in 0.2 µm filter sterilized

Millipore water.

Solubility of all test drugs was determined using UV–vis spectroscopy (NanoDrop

ND-1000, Thermo Scientific, Waltham, MA) by examining absorption of molecules in

UV-vis spectrum (220-750 nm). Molecules exhibiting absorbance near ultraviolet ranges

were considered solubilized while those exhibiting a signal in the far visual spectrum

(700 nm) were considered insoluble.

Flow cytometry and data analysis

Flow cytometry acquisition and analysis were performed on a Becton-Dickinson

FACSCalibur using CellQuest™ Pro (BD Biosciences, San Jose, CA). *Tetrahymena*-

gated cells were selected based on cell complexity and cell size. Quadrant statistics were

used to calculate the percentage of *Tetrahymena* that had ingested at least one fluorescent

bead as indicated on side scatter vs FL2 dot plots. For each PBM being evaluated, a

corresponding set of controls containing identical solvent condition (minus PBM) was

run.

Results were not included in the analysis if the standard deviation of the controls

exceeded 5% of the mean value of the replicas or mean proportion of cells that had

ingested at least one fluorescent bead was less than 50%. For cultures demonstrating

phagocytic inhibition, viability was determined by checking motility using a bright field

light microscope. For each sample, the number of non-motile cells in ten random visual

fields were counted. Non-motile cells were never observed, indicating that inhibition of

phagocytosis was not related to viability in this study.

The data are expressed as phagocytosis factor (PF), where:

$$\text{Eq. (1). PF} = \frac{\text{\% of cells in test solution that have phagocytized one or more beads}}{\text{\% of cells in control solution that have phagocytized one or more beads}}$$

Statistical significance was determined by analysis of variance (ANOVA)[8] comparing

different treatment levels (various concentrations of PBM as well as control) and post hoc

analysis (Scheffe's post hoc method comparing treatment levels to the non-dose control)

using SPSS version 22 (IBM, Armonk, NY). The independent variable was drug

concentration and the dependent variable was PF. Statistical significance ($p < 0.050$) in

this study implied that the test molecule elicited a phagocytosis response that was

significantly different than control values.

Results and Discussion

Serotonin, norepinephrine, dopamine, and morphine had little or no effect on phagocytosis when compared to controls (**Fig. 1**). ANOVAs indicated that phagocytosis results from serotonin cultures did not differ significantly from control cultures (**Table 1**). Phagocytosis results from cultures treated with norepinephrine (p = 0.003), dopamine (p = 0.004) and morphine (p = 0.001) were statistically significant compared control cultures according to ANOVAs (**Table 2A-4A**). Phagocytosis results from cultures treated with dopamine (p = 0.031) and norepinephrine (p = 0.040) were statistically significant at 10^{-7} M, and increased phagocytosis by 4% and 2% respectively compared to the control cultures (**Table 2B, 3B**). The effect of morphine was statistically insignificant at all concentrations by Scheffe's post hoc analysis (**Table 3B**).

1 A
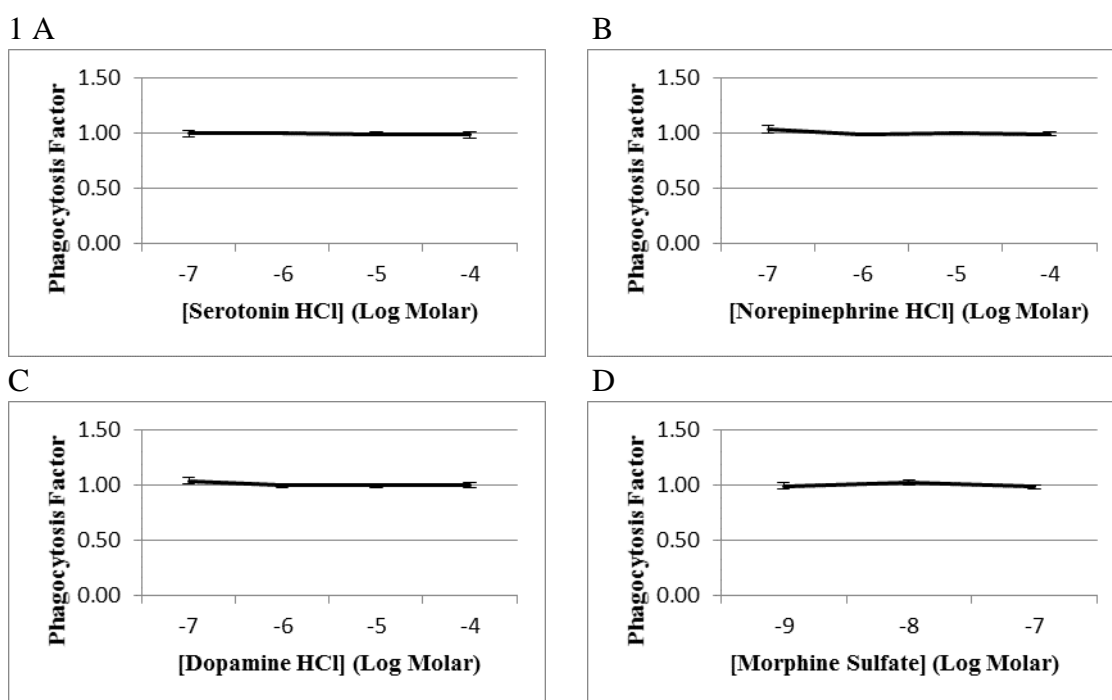
Figure 1 Phagocytosis factor vs log molar A) serotonin hydrochloride, B) norepinephrine hydrochloride, C) dopamine hydrochloride and D) morphine sulfate salt pentahydrate. Error bars represent plus or minus one standard deviation.

Table 1. One-way ANOVA comparing control and concentrations of serotonin hydrochloride (10^{-4} M, 10^{-5} M, 10^{-6} M, and 10^{-7} M). The Within Group measured internal variance at each concentration (6 replicates per level).

	Sum of Squares	df	Mean Square	F	p value
Between Log Molar Concentrations of Serotonin Hydrochloride	0.001	4	0.000	0.462	0.763
Between Log Molar Concentrations of Serotonin Hydrochloride	0.013	25	0.001		
Total	0.014	29			

Table 2A. One way ANOVA comparing control and concentrations of norepinephrine hydrochloride (10^{-4} M, 10^{-5} M, 10^{-6} M, and 10^{-7} M). The Within Group measured internal variance at each concentration (6 replicates per level). Bold type indicates significance.

	Sum of Squares	df	Mean Square	F	p value
Between Log Molar Concentrations of Norepinephrine hydrochloride	**0.008**	**4**	**0.002**	**5.487**	**0.003**
Within Log Molar Concentrations of Norepinephrine hydrochloride	0.009	25	0.000		
Total	0.017	29			

Table 2B. Post hoc analysis using Scheffe's method. The control mean is being compared to each concentration of norepinephrine hydrochloride (10^{-4} M, 10^{-5} M, 10^{-6} M, and 10^{-7} M). Concentration of epinephrine hydrochloride was the independent variable and phagocytosis factor was the dependent variable.
Bold type indicates significance.

(I) Control	(J) Log Molar Concentration Norepinephrine hydrochloride	Mean Difference (I-J)	Std. Error	p-value	95% Confidence Interval Lower Bound	Upper Bound
Control	**10^{-7} M**	**-0.03833**	**0.01115**	**0.040**	**-0.0754**	**-0.00013**
	10^{-6} M	0.00667	0.01115	0.985	-0.0304	0.0437
	10^{-5} M	-0.00500	0.01115	0.995	-0.0420	0.0320
	10^{-4} M	0.00500	0.01115	0.995	-0.0320	0.0420

Table 3A. One way ANOVA comparing control and concentrations of dopamine hydrochloride (10^{-4} M, 10^{-5} M, 10^{-6} M, and 10^{-7} M). The Within Group measured internal variance at each concentration (6 replicates per level). Bold type indicates significance.

	Sum of Squares	df	Mean Square	F	p-value
Between Log Molar Concentrations of Dopamine hydrochloride	**0.008**	**4**	**0.002**	**5.012**	**0.004**
Within Log Molar Concentrations of Dopamine hydrochloride	0.010	25	0.000		
Total	0.018	29			

Table 3B. Post hoc analysis using Scheffe's method. The control mean is being compared to each concentration of dopamine hydrochloride (10^{-4} M, 10^{-5} M, 10^{-6} M, and 10^{-7} M). Concentration of dopamine hydrochloride was the independent variable and phagocytosis factor was the dependent variable. Bold type indicates significance.

(I) Control	(J) Log Molar Concentration Dopamine Hydrochloride	Mean Difference (I-J)	Std. Error	p-value	95% Confidence Interval Lower Bound	Upper Bound
Control	**10^{-7} M**	**-0.04167**	**0.01169**	**0.031**	**-0.0805**	**-0.0028**
	10^{-6} M	0.00333	0.01169	0.999	-0.0355	0.0422
	10^{-5} M	-0.00167	0.01169	1.000	-0.0405	0.0372
	10^{-4} M	-0.00500	0.01169	0.996	-0.0438	0.0338

Mexiletine, procainamide, and colchicine inhibited phagocytosis in a dose dependent manner (**Fig. 2**). ANOVAs indicated that mexiletine (p = 0.001), procainamide (p = 0.001), and colchicine (p = 0.001) culture results were significantly different from controls (**Table 5A-7A**). The effects of colchicine and mexiletine were statistically significant at all concentrations as indicated by Scheffe's post hoc analysis, while the effects of procainamide were significant only at one concentration. Colchicine at 10^{-2} M (p = 0.001) (**Table 5B**) caused a 95% inhibition in phagocytosis compared to controls (**Fig. 2**). The effect of mexiletine was significant at all concentrations, with the highest concentration of 10^{-2} M (p = 0.010) (**Table 6B**) exhibiting 84% inhibition of phagocytosis compared to controls. Scheffe's post hoc analysis indicated that the effect of procainamide was significant only at the highest concentration of 10^{-2} M (p = 0.001) (**Table 7B**) showing inhibition of 27% compared to the controls.

Table 4A. One way ANOVA comparing control and concentrations of morphine sulfate salt pentahydrate (10^{-7} M, 10^{-8} M, and 10^{-9} M). The Within Group measured internal variance at each concentration (6 replicates per level). Bold type indicates significance.

	Sum of Squares	df	Mean Square	F	p-value
Between Log Molar Concentrations of Morphine Sulfate Salt Pentahydrate	**0.005**	**3**	**0.002**	**3.846**	**0.025**
Within Log Molar Concentrations of Morphine Sulfate Salt Pentahydrate	0.008	20	0.000		
Total	0.013	23			

Table 4B. Post hoc analysis using Scheffe's method. The control mean is being compared to each concentration of morphine sulfate salt pentahydrate (10^{-7} M, 10^{-8} M, and 10^{-9} M). Concentration of morphine sulfate was the independent variable and phagocytosis factor was the dependent variable.

(I) Control	(J) Log Molar Concentration Morphine Sulfate Salt Pentahydrate	Mean Difference (I-J)	Std. Error	p-value	95% Confidence Interval Lower Bound	Upper Bound
Control	10^{-9} M	0.00500	0.01171	0.980	-0.0307	0.0407
	10^{-8} M	-0.02333	0.01171	0.295	-0.0590	0.0124
	10^{-7} M	0.01500	0.01171	0.656	-0.0207	0.0507

2 A
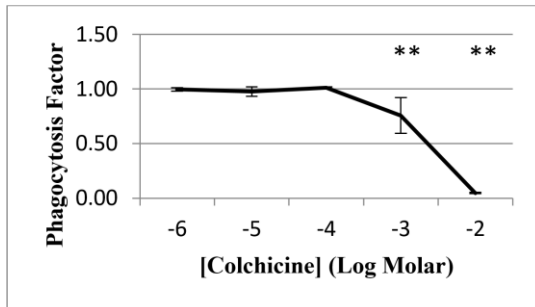
B
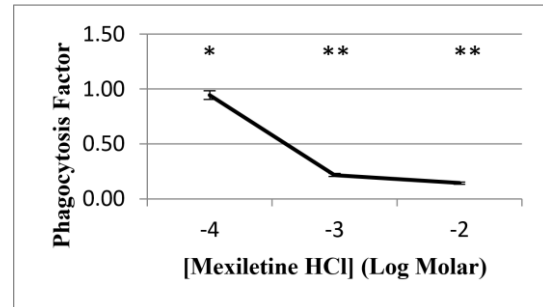
C
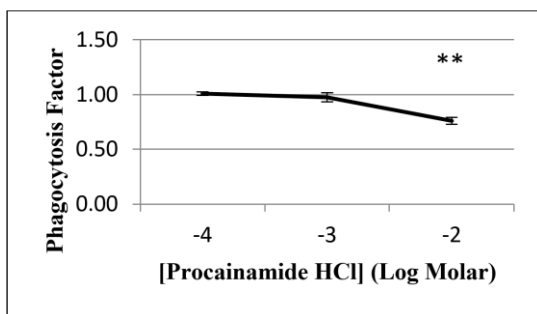
Figure 2. Phagocytosis factor vs log molar A) colchicine, B) mexiletine hydrochloride, C) procainamide hydrochloride. Error bars represent plus or minus one standard deviation. Asterisks indicate treatment levels that were significantly different from the control treatment (Scheffe's post hoc test):*, $p < 0.05$;**, $p < 0.001$.

These data showed that phagocytosis in *T. thermophila* was not modulated by

serotonin, norepinephrine, dopamine or morphine. Quiñones-Maldonado and Renaud

reported that serotonin, norepinephrine, and dopamine stimulated phagocytosis, while De

Jesus and Renaud have reported that morphine inhibited phagocytosis.[4,5] However, we

found no effect. These discrepancies could be due to differences in cell wash media, cell

strain, particle type, and the methods used for measurement.[4,5,9] For instance, previous

studies used tantalum beads rather than fluorescent polystyrene beads and a visual

determination of phagocytosis using light microscopy. Their sample size was 100 cells in

each of three replicas, whereas using flow cytometry we analyzed over 1500 cells in each

of six replicas. Thus, a greater power to detect inhibition could be expected using flow

cytometry.

Table 5A. One way ANOVA comparing control and concentrations of colchicine (10^{-2} M, 10^{-3} M, 10^{-4} M, 10^{-5} M, and 10^{-6} M). The Within Group measured internal variance at each concentration (6 replicates per level). Bold type indicates significance.

	Sum of Squares	df	Mean Square	F	p-value
Between Log Molar Concentrations of Colchicine	**4.324**	**5**	**0.865**	**184.229**	**0.001**
Within Log Molar Concentrations of Colchicine	0.141	30	0.005		
Total	4.465	35			

Table 5B. Post hoc analysis using Scheffe's method. The control mean is being compared to each concentration of colchicine (10^{-2} M, 10^{-3} M, 10^{-4} M, 10^{-5} M, and 10^{-6} M). Concentration of colchicine was the independent variable and phagocytosis factor was the dependent variable. Bold type indicates significance.

(I) Control	(J) Log Molar Concentration Colchicine	Mean Difference (I-J)	Std. Error	p-value	95% Confidence Interval Lower Bound	95% Confidence Interval Upper Bound
Control	10^{-6} M	0.00333	0.03956	1.000	-0.1375	0.1441
	10^{-5} M	0.02333	0.03956	0.996	-0.1175	0.1641
	10^{-4} M	-0.01000	0.03956	1.000	-0.1508	0.1308
	10^{-3} M	**0.24167**	**0.03956**	**0.001**	**0.1009**	**0.3825**
	10^{-2} M	**0.95167**	**0.03956**	**0.001**	**0.8109**	**1.0925**

Cell wash medium may have been a critical parameter. A previous study[4] used Tris-

HCl as the wash buffer (which does not have added nutrients), while we used nutrient

rich Neff's Modified Medium. A starvation effect could explain the differences in

results. In preliminary studies, glucose-deficient media (proteose peptone and HEPES

buffer) led to lower levels of phagocytosis overall (data not shown).

**Table 6A.** One way ANOVA comparing control and concentrations of mexiletine hydrochloride (10^{-2} M, 10^{-3} M, and 10^{-4} M). The Within Group measured internal variance at each concentration (6 replicates per level). Bold type indicates significance.

	Sum of Squares	df	Mean Square	F	p-value
Between Log Molar Concentrations of Mexiletine hydrochloride	**3.774**	**3**	**1.258**	**2175.197**	**0.001**
Within Log Molar Concentrations of Mexiletine hydrochloride	0.012	20	0.001		
Total	3.786	23			

Table 6B. Post hoc analysis using Scheffe's method. The control mean is being compared to each concentration of mexiletine hydrochloride (10^{-2} M, 10^{-3} M, and 10^{-4} M). Concentration of mexiletine hydrochloride was the independent variable and phagocytosis factor was the dependent variable. Bold type indicates significance.

(I) Control	(J) Log Molar Concentration Mexiletine hydrochloride	Mean Difference (I-J)	Std. Error	p-value	95% Confidence Interval Lower Bound	Upper Bound
Control	**10^{-4} M**	**0.05333**	**0.01388**	**0.010**	**0.0110**	**0.0957**
	10^{-3} M	**0.77500**	**0.01388**	**0.001**	**0.7327**	**0.8173**
	10^{-2} M	**0.85833**	**0.01388**	**0.001**	**0.8160**	**0.9007**

Colchicine, mexiletine, and procainamide significantly inhibited phagocytosis. The inhibition may reflect actions of these molecules at downstream stages in the signal cascade. Buduma *et al.*[7] found that diphenhydramine, a known sodium-channel blocker, inhibited phagocytosis. Mexiletine and procainamide are known sodium channel blockers in humans. Their effect in *Tetrahymena* was previously not known and evidence of sodium channel blocking activity in *Tetrahymena* has not been previously documented. There may be several reasons for the lack of evidence supporting sodium

channel blocking activity. There may be a different ion channel involved, possibly in the form of voltage gated potassium channels and/or sodium-potassium pumps in *T. thermophila*.[10] The possible implication of voltage gated potassium channels and sodium-potassium pumps as a central element in signaling implies at least two possible mechanisms: 1) downstream signaling linkages between potassium and sodium ions, or 2) cross-reactivity between sodium channel blockers, potassium channels or sodium-potassium pumps. The effect of ionic concentration and ion channel blockers on modulating phagocytosis in *Tetrahymena* requires further study.

This phagocytosis assay in *T. thermophila* provides a robust model system for studying the effects of potentially bioactive molecules. *Tetrahymena*'s sensitivity to diverse molecules and the rapid acquisition and analysis of large datasets using flow cytometry increase potential applications of this assay. If adapted to a multi-well plate format and automated by robotics, assay volumes, cost, time, and human error could be further reduced. One could also take advantage of *T. thermophila* responses to a variety of molecule classes. Rather than repeated evaluations for each molecule class as in other assay formats, the use of specific competitors/inhibitors could elucidate the mechanism of action in one experiment. An example of this could be the use of a competitive assay with a potential opiate and naloxone, an opiate antigonist.[4] These advantages make *T. thermophila* an attractive option in screening solutions of unknown composition for biomolecular activity.

Table 7A. One-way ANOVA comparing control and concentrations of procainamide hydrochloride (10^{-2} M, 10^{-3} M, and 10^{-4} M). The Within Group measured internal variance at each concentration (6 replicates per level). Bold type indicates significance.

	Sum of Squares	df	Mean Square	F	p-value
Between Log Molar Concentrations of Procainamide hydrochloride	**0.250**	**3**	**0.083**	**101.393**	**0.001**
Within Log Molar Concentrations of Procainamide hydrochloride	0.016	20	0.001		
Total	0.266	23			

Table 7B. Post hoc analysis using Scheffe's method. The control mean is being compared to each concentration of procainamide hydrochloride (10^{-2} M, 10^{-3} M, and 10^{-4} M). Concentration of procainamide hydrochloride was the independent variable and phagocytosis factor was the dependent variable. Bold type indicates significance.

(I) Control	(J) Log Molar Concentration Procainamide hydrochloride	Mean Difference (I-J)	Std. Error	p-value	95% Confidence Interval Lower Bound	Upper Bound
Control	10^{-4} M	-0.01000	0.01654	0.946	-0.0604	0.0404
	10^{-3} M	0.02333	0.01654	0.584	-0.0271	0.0738
	10^{-2} M	**0.23833**	**0.01654**	**0.001**	**0.1879**	**0.2888**

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CHAPTER IV
CONCLUSION

The use of flow cytometry and *T. thermophila*'s multiple molecule class sensitivity

should be advantageous in screening for bioactive molecules. This phagocytic ingestion

assay is a quick, economical and potentially high throughput technique. It has possible

applications in screening many molecule classes for bioactivity and overcomes

limitations of current assay formats, such as mammalian cell culture. Some of these

limitations include the fastidious nutritional requirements of mammalian cells and

complex culture curation.

Seven bioactive molecules were evaluated using this phagocytic ingestion assay. The

human sodium channel blockers mexiletine, procainamide, and colchicine elicited strong

inhibition of phagocytosis. These data indicate that the inhibiting effect of sodium

channel blockers is consistent with Buduma *et al.*'s results using diphenhydramine.[2]

Depression of phagocytosis by colchicine has also been reported.[11] Conversely, in our

study, the opiate morphine, and hormones serotonin, norepinephrine, and dopamine had

no significant effect on phagocytosis. Discrepancies in phagocytic measurement between

our studies and those of Quiñones-Maldonado and Renaud[4], and De Jesus and Renaud[3]

may be explained by differences in assay variables. Some of these parameters include

cell wash media, acclimation time, cell strain, the method of measurement and sample

size.

This study showed that temperature, cell concentration (or cell strain), cell wash

media, and acclimation time are important factors in *T. thermophila* ingestion assay

performance. The analysis suggested that optimum conditions for the assay are a temperature of 30°C, an intermediate *T. thermophila* cell concentration relative to beads (e.g. 10,000-20,000 cells/mL), a cell wash medium rich in glucose (NMM) and no acclimation time. Further work should be done to explore additional levels for each variable. Varying the concentration of glucose in cell wash medium could result in higher rates of phagocytosis, and evaluating cell concentrations between 10,000-20,000 cells/mL could determine optimal concentrations of *Tetrahymena* and beads.

   The results of this study indicate that the *T. thermophila* phagocytic ingestion assay shows promise for screening bioactive molecules. Exploring the scope of molecule classes to which *T. thermophila* is sensitive may be beneficial. Characterizing the effect of antibiotics and anti-cancer agents on phagocytosis, for instance, may expand the relevance of this assay into new therapeutic areas. Selectively inhibiting specific molecular components could lead to a better understanding of the cellular mechanisms involved in phagocytosis.

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