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CALCIUM, CALCIUM CHANNEL BLOCKERS, AND AGING IN THE ROTIFER ASPLANCHNA BRIGHTWELLI, GOSSE

Alison J. McTavish

A Thesis

in

The Department

of

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ABSTRACT

CALCIUM, CALCIUM CHANNEL BLOCKERS, AND AGING IN THE ROTIFER *ASPLANCHNA BRIGHTWELLI*, GOSSE

Alison J. McTavish

This study was undertaken to test the theory that intracellular calcium accumulation influences aging. It was found that calcium ions tend to accumulate in the cells of the rotifer *Asplanchna brightwelli* as it ages. The results show that the 1,4-dihydropyridine calcium channel blocker nifedipine prevents this age-related accumulation when administered to rotifers at a concentration of 1.0 uM. This drug also decreased both the activity level and the phototactic response of the animals. A range of nifedipine concentrations from 0.1 uM to 5.0 uM was found to significantly increase the lifespan of these rotifers; this prevention of age-associated calcium accumulation is correlated with increased lifespan. These findings indicate that there is an age-related decline in calcium homeostasis, and appear to support the theory that calcium ion regulation is a vital factor in aging tissues.

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INTRODUCTION

This study was designed to test the calcium theory of aging (Khachaturian, 1984). This theory will be explained more fully after a brief discussion of biological aging.

Aging

Aging has been described as "the post-reproductive changes which result in a decreased survival capacity for an organism" (Strehler, 1959). These changes may be characterized as a series of unavoidable and necessarily deleterious cellular events which lead to the disintegration and, ultimately, to the death of the organism. Recent gerontological research has focused on attempts to explain these degenerative changes at the cellular and molecular level in the hope of elucidating the mechanism behind the aging process.

Although a completely satisfactory explanation of aging has yet to be found, several theories have been proposed which attempt to explain various aspects of aging. Some theories explain aging in terms of cellular decay: according to the free radical theory, aging is a result of the loss of cellular integrity that occurs when toxic free radicals attack plasma membranes (Harman, 1956). Other theories suggest that aging is closely linked to the genetic

material: aging is either "programmed" in the DNA or results from mutation or macromolecular damage to DNA (Hall, 1984).

Calcium Theory of Neural Aging

Calcium is the basic regulator of intracellular function in all living organisms (Ebashi, 1988). As such it is responsible for the control of a large number of biochemical processes within cells (Carafoli, 1987). Since calcium ions can efficiently signal at very low intracellular concentrations, it is vital that calcium homeostasis be rigidly maintained: any fluctuation in cytoplasmic calcium concentration results in disruption of intracellular signalling (Carafoli, 1987).

In healthy cells a number of systems exist to maintain calcium homeostasis. Excess calcium entering via calcium channels can be pumped out by the membrane calcium-ATP exchange protein, or can be sequestered in large protein complexes such as calmodulin, or in organelles such as the mitochondria and endoplasmic reticulum (Gibson and Peterson, 1987).

Recent research, however, has shown that maintenance of this delicate calcium balance becomes more difficult as the cell ages, and this finding has led Khachaturian (1984) to propose the theory that calcium plays an important role in the aging of neural systems. He posits that the cellular

mechanisms that regulate ion concentration play a crucial role in neural aging; and that cytoplasmic calcium regulation failure may account for age-related changes in cell function including neurotransmitter synthesis and energy metabolism, which ultimately result in cell death (Khachaturian, 1984).

Michaelis (1987) notes that a " substantial body of evidence" now exists linking aging and altered calcium homeostasis in neural regions. Several researchers have found that calcium ion levels in the cytosol tend to increase with age (Leslie, 1987; Michaelis, 1987; Trabucchi and Govoni, 1987). It is clear, however, that the cell's ability to sequester influxing calcium in the mitochondria, endoplasmic reticulum, and in calcium-calmodulin complexes, declines with age (Gibson and Peterson, 1987). This finding may partially explain the elevated level of calcium in the cytosol of older cells (Leslie, 1987).

Elevated intracellular calcium has been shown to be toxic to cells. Seisjo (1981) found that if the concentration of calcium in a cell remains high for too long, cell death results. Trump et al. (1984) found that increased calcium in the cytosol was an important factor in the initiation of cell death following cell injury. Cells which normally have large calcium fluxes across their membranes (such as nerve, muscle and sensory cells) are particularly sensitive to alterations in calcium

availability. Gibson and Peterson (1987) therefore note that artificial maintenance of the calcium equilibrium " may ameliorate some of the consequences of aging."

A potential method of reducing calcium fluxes and thus artificially maintaining homeostasis could be to limit the entry of calcium into cells at the cell membrane level. This should prevent the build-up of potentially toxic levels of calcium in the cytoplasm. Since most calcium enters the cell through ion channels in the cell membrane, blockade of these channels with calcium channel blocking drugs might be expected to help restore the intracellular calcium balance during aging. If calcium build-up is associated with aging and cell death, prevention of calcium entry from this source could potentially lead to increased lifespan.

Rotifers

The organism chosen to test the hypotheses described above was the short-lived rotifer Asplanchna brightwelli.

Rotifers, as Clement (1984) points out, represent "a biological model which is without parallel in the animal kingdom". Asplanchna brightwelli reproduces by diploid parthenogenesis (King, 1972) and as a result all offspring are genetically identical clones. Furthermore, growth is almost perfectly eutelic: all rotifers contain approximately 1000 highly specialized cells (Clement, 1984). The relatively simple culture methods and small size of rotifers

allows both easy maintenance and experimentation with a large number of individuals. The short lifespan of A. brightwelli (approximately 5 days) makes this rotifer particularly useful for longitudinal lifespan studies.

Ion Channels

Ion channels are integral membrane proteins that have hydrophilic pores through which ions can flow (Hodgkin and Huxley, 1952). Since electrical activity in excitable cells depends upon transient changes in ionic gradients across the cell membrane, Hodgkin and Huxley suggested that ion channels may have evolved to facilitate these gradient fluctuations: gradients are altered when ions pass back and forth through channels.

The idea that channels can regulate ion flow implies two properties: firstly that channels are selective; and secondly that they are gated (Stevens, 1979). Channel selectivity means that only a particular ion species may pass through a channel - for example, only divalent cations can flow through calcium channels while all other ions are excluded (Keynes, 1979). Channel gating allows an even greater degree of control since it means that the channel can open and close, thus blocking or promoting ion flow respectively (Stevens, 1984).

The gating property of channels has proved to be an important focal point in research: classes of organic

chemicals which modulate gating activity have led to the identification, purification and characterization of ion channels at the molecular level (Catteral, 1982; Tanabe et al, 1987; Leung et al, 1987; Lai et al, 1988). Catteral (1982) notes that neurotoxins such as tetrodotoxin, which inhibits ion transport, were the "essential tools" for the isolation of the sodium channel. Similar work on calcium channels using ion-transport-inhibiting channel blockers (Miller 1985) and high affinity calcium channel radioligands (Reuter et al, 1985) has been equally successful.

It is only of late that the universality of calcium channels in the animal kingdom has been fully appreciated. Hagiwara (1983) notes that it is difficult to find a tissue that does not have calcium channels. Among mammals these channels can be found in the membranes of all types of excitable tissue (Hagiwara and Byerly, 1981). In the invertebrate animals, calcium channels have been identified in many organisms from Paramecia (Wehner and Hildebrand, 1985) and tunicates (Miyazaki et al, 1972), to molluscs (Hagiwara, 1974) and insects (Washio, 1972). Based on these findings, the assumption that calcium channels exist in rotifers appears to be sound.

Calcium Channel Blockers

Calcium channel blockers are a fairly diverse group of chemicals. Each blocker belongs to one of several classes

depending on their chemical structure. Each class of blocker has been found to bind to a unique site on the calcium channel (Miller, 1985). For example, 1,4-dihydropyridine blockers have been found to bind to a specific polypeptide on the calcium channel, the amino-acid sequence of which has recently been elucidated (Tanabe et al, 1987).

Recently several new dihydropyridine derivatives have been isolated. These new drugs, in contrast to the dihydropyridines described above, have been found to increase the permeability of excitable cells to calcium ions (Hess et al, 1983). It has been found that these drugs modify the voltage-dependent gating of calcium channels thus blocking the channels open.

Dihydropyridines appear to be particularly useful in labeling voltage-sensitive calcium channels. These channels are known to open and close in response to small changes in voltage across the plasma membrane, and are commonly found on the muscle cell membranes of invertebrates. These voltage -sensitive channels are normally of three types, either T, L or N (Reuter and Porzig, 1985). The L-type channel produces a long-lasting calcium current, and is the only one of the three sensitive to dihydropyridines. The dihydropyridine nifedipine has been identified particularly as a highly potent and selective antagonist of the L-type

channels (Fleckenstein, 1970).

Nervous System of the Rotifer

Since the binding of calcium channel blockers has not yet been tested on rotifers, the site(s) of action of these drugs is not documented. However, the ultrastructure of the rotifer nervous system is well documented, and it seems likely that the blockers bind to channels on the excitable membranes of muscles and neurons, as they do in other invertebrates (Hagiwara, 1983).

In most organisms, calcium currents are coupled with effector functions. In ciliates calcium channels play a vital role in movement and orientation by allowing the flow of a regenerative calcium current (Wehner and Hildebrand, 1985), while in ctenophores calcium currents are essential for muscle contraction (Bilbaut et al, 1988). There is also evidence of calcium channels in muscle tissue from the nematode Ascaris (Weisblat et al, 1976). If calcium channel distribution follows this pattern in rotifers, one likely site of action of the calcium channel blockers is at the level of effector organs.

In rotifers, sensory information is received from a variety of mechanoreceptors, chemoreceptors and photoreceptors which take the form of modified dendrites (Clement, 1983). This information travels via afferent neurons to the brain, an organ of no more than 200 cells (Ware, 1971). According to Clement (1983) the brain

consists of two layers: an inner group of axons surrounded by perikarya and epithelial cells. From the brain effector neurons lead to muscles, glands (Clement, 1977), and epithelia (Clement, 1983). Ware (1971) notes that there are also about 50 interneurons in A. brightwelli. Clearly, therefore, the neural tissue is a possible binding site for calcium channel blockers.

Possible Effects of Dihydropyridine Binding

Miller (1987) found that the binding of micromolar concentrations of the dihydropyridine nifedipine to rat brain synaptosomes led to the inhibition of neurotransmitter release from the synaptosomes. In vivo binding would thus lead to reduced acetylcholine release and muscle relaxation (Miller 1985). If this pattern is also followed in rotifers, the relaxation of muscles should lead to a reduction in the movement of the organism.

Grotta et al. (1986) attempted to administer dihydropyridines to neurons immediately prior to, and during cell injury. They found that the administration of this drug was able to improve cell function, probably by delaying the cytotoxic events usually initiated by a build-up of calcium. Several other researchers have reported similar results (Steen et al, 1984).

As noted above, Trump et al. (1984) also found an increased level of calcium in cells following cell injury.

One type of injury these researchers discussed was plasma membrane damage caused by lipid peroxidation, a phenomenon known to increase with age in rotifers (Sawada, personal communication). The age-related increase in lipid peroxidation could therefore lead to an increase in intracellular calcium in the cells of this organism. This could be an important factor in the death of the cells, and ultimately in the death of the rotifer itself. Administration of dihydropyridines to A. brightwelli may be able to delay the increase in intracellular calcium thus delaying both cell death and the death of the organism.

Aims of This Study

The experiments described here were designed with the following aims: 1. to determine whether the level of intracellular calcium increases with age in the cells of the rotifer; 2. to determine whether the administration of the calcium channel blocker nifedipine decreases calcium ion transport thus preventing a build-up of calcium ions; 3. to see if calcium channel blockade results in decreased activity in the organism; and 4. to determine whether this drug can consequently increase rotifer lifespan.

MATERIALS AND METHODS

The short-lived rotifer Asplanchna brightwelli (clone B461), originally obtained from Dr. J. Gilbert, Dartmouth College, Hanover, New Hampshire, was the organism used in this study.

Culture Methods

The food source of this rotifer species, Paramecium caudatum, was cultured in a cerophyll infusion medium first described by Bridger (1970), and subsequently modified by Sawada (1983).

Every 7 days 1900 ml of distilled water, 0.12 g calcium carbonate (Anachemia, Montreal) and 14.0 ml of a phosphate buffer solution (see appendix) were placed in a 4000 ml Erlenmeyer flask and brought to a boil. To the boiling mixture, 1.5 g of cerophyll (Ward's Natural Science Establishment, Mississauga, Ontario) suspended in 86 ml of distilled water, was added. The suspension was allowed to boil for 10 more minutes with constant stirring, after which the flask was placed in an ice bath until the contents cooled to room temperature. Once cooled the suspension was filtered through Whatman #3 filter paper and then sterilized by autoclaving for 20 minutes.

After removal from the autoclave, the sterile solution

was left to cool to room temperature. The cooled medium was innoculated with a generous loopful of E. coli K12, originally obtained from Dr. E. Newman, Concordia University, and subsequently maintained at 5°C on dextrose agar (Difco Laboratories, Detroit, Michigan). The newly innoculated medium was incubated at 37°C for 24 hours, after which it was allowed to cool to room temperature. One hundred ml of medium containing Paramecium caudatum (originally obtained from Boreal Laboratories Ltd., Mississauga, Ontario) from the previous week's Paramecium culture were then innoculated into the cooled medium.

Preparation and Storage of Stock Cultures

The rotifers were maintained in 250 ml of medium in 500 ml Erlenmeyer flasks. Every three days new stock cultures were prepared by transferring approximately 40 rotifers into fresh medium. All rotifer cultures were stored at room temperature away from direct sunlight.

Maintenance of Sterility

Throughout all culturing procedures, sterile techniques were employed. Further, all glassware, pipettes and culture dishes were either sterilized by autoclaving for 20 minutes or were purchased sterile.

To ensure that medium was not contaminated due to failure of sterile techniques, 5 ml samples were plated on

dextrose agar (Difco Laboratories, Detroit, Michigan), incubated at 37°C for 24 hours and examined for the presence of contaminating bacteria. Contamination was rarely found. In these rare instances, contaminated medium was discarded.

Experimental Methods

To begin any experiment, each well of a 24 well tissue culture dish (No.76-063-05; Flow Laboratories, McLean, Virginia) was filled with 2.5 ml of fresh medium. Rotifers were then taken from newly replenished stock culture flasks, placed in a Petri dish and examined under a dissecting microscope. Adult rotifers were selected and individually segregated into each well of the prepared culture dish using a micropipette (Finipipette Ky, Helsinki, Finland) on a 10 uL setting.

The culture dish was placed in an incubator at 19°C and each well was checked every hour for offspring. Newborn rotifers were collected and placed immediately into new experimental culture dishes. Consequently the age of rotifers at the start of any experiment was accurate to within one hour.

Lifespan Data Collection

The wells of each culture dish were examined every six hours until the onset of senescence. During this

examination, offspring were counted and removed, and the viability of the rotifers was checked. To ensure a constant food supply, 1.0 ml of medium was removed from each well and was replaced by 1.0 ml of either fresh control or fresh experimental medium every six hours.

After the onset of senescence - usually characterized by slowing of movement and increasing body wall transparency - the wells were examined every three hours to better determine the exact time of death. Rotifers were considered to be dead when no movement was observed even after agitation with a Pasteur pipette.

Paramecia Counts

To ensure that all experimental rotifers had access to an equal amount of food, the number of Paramecia in randomly chosen experimental wells was counted twice daily using a method described by Sawada (1983).

Paramecium medium samples of 100 uL were taken from wells, and stained using a toxic 2 uL dose of 0.1 ug/ml neutral red. The samples were then placed on a 35 x 10 mm Petri dish with 2 mm grids (No.3160; Costar, Cambridge, Massachusetts). Counts were made using a dissecting microscope. Two hundred Paramecia per 100 uL was considered to be an adequate food supply (Sawada, 1983).

Nifedipine Experiments

Nifedipine (Lot N-7634) was obtained from Sigma Chemical Company, St. Louis, Missouri. Stock solutions of the chemical were prepared by dissolving 0.346 g of nifedipine in 100 ml of 95% ethanol. These 0.01 M stock solutions were refrigerated until required for an experiment.

Medium for experiments was prepared by dissolving known amounts of nifedipine stock solution in measured volumes of fresh Paramecium medium. A range of concentrations from 0.01 uM to 10 uM was prepared in an attempt to establish the optimal non-toxic concentration of the chemical. This procedure was carried out 30 minutes before each experiment.

Rotifers of known age were then transferred into wells of new tissue culture dishes containing either medium with various nifedipine concentrations or control medium. The culture dishes were incubated at 19 ^{o}C throughout the experiment and lifespan data was collected as described above.

Determination of Activity

The activity level of rotifers in both control and experimental groups was determined in two ways: by counting movement across the squares of a grid; and by counting the number of organisms moving towards light in a

fixed period of time.

In the first set of experiments control and nifedipine-treated rotifers were placed as usual in individual wells of a tissue culture dish. The dish was placed on a transparent plastic sheet upon which was drawn a 0.5 mm grid network. The rotifers were observed under a dissecting microscope, and the number of grids they traversed in a 1 minute time period was recorded (Beavais and Enesco, 1985).

In the second set of experiments, movement of the rotifers depended upon their positive phototactic response (Clement and Wurdak, 1983). Sixty rotifers were placed in the middle chamber of a rectangular three-chambered box with dimensions 12 cm x 5 cm x 3 cm. All sides of the box were blackened except for a small slit at one end at which a beam of light could be placed. The animals were dark-adapted for ten minutes after which the light turned on and the partitions between the chambers removed. The rotifers were left for a further ten minutes; the partitions were then replaced, and the rotifers in each chamber counted (Cornillac et al, 1983).

Calcium Ionophore Experiments

Calcium ionophore C-9275 was obtained from Sigma Chemical Company, St. Louis, Missouri. Since the chemical tends to disaggregate fairly soon after dissolution, stock

solutions were not prepared until required. The ionophore was dissolved in 95% ethanol and the resulting solution was dissolved in fresh Paramecium medium to obtain concentrations of 0.1 and 1.0 uM.

Rotifers were segregated into individual wells of the tissue culture dishes and were exposed throughout their lifespan to these ionophore concentrations. Lifespan data were collected as previously described.

Uptake of Channel Blockers

In order to determine that nifedipine could bind to Paramecium, and could thus be ingested by A. brightwelli, the uptake of radioactive calcium into control and nifedipine-exposed Paramecia was measured over predetermined time intervals.

Paramecium caudatum was raised in calcium-free medium for one week. At the end of the week the medium was separated into two cultures. To the first culture 25 uL of 0.01 M stock nifedipine solution was added and the volume was adjusted so that the final nifedipine concentration was 1.0 uM. The second culture was untreated. Both cultures were left at room temperature for three hours and were then filtered twice with Nitex mesh to ensure the removal of any contaminating algae.

Following filtration, 28.2 ml samples of the two cultures were separately placed into each of ten plastic

conical centrifuge tubes (five control and five experimental) and 1.8 ml of 45-calcium chloride (112000 cpm) was added to each tube. The Paramecia in the five pairs of tubes were exposed to the radioactive calcium for 1, 10, 30, 60, and 90 minutes. The organisms were observed throughout the exposure to ensure that they were alive and thus transporting calcium.

After the 45-$CaCl_2$ treatment the samples were filtered through 8 uM millipore SC type filters (Millipore Corporation, Bedford, Massechusetts) and rinsed twice with buffer. The filters were placed in liquid scintillation vials and placed in the freezer to induce rupturing of the cell membranes. Two ml of scintillation fluid was added to each vial which was then vortexed for 30 seconds. The vials were counted for radioactivity in a liquid scintillation counter (Nuclear Chicago, Mark III).

Uptake of Calcium by Rotifers

Two groups of rotifers were used for this set of experiments. The control group was raised in normal medium while the experimental group was raised in medium containing 1.0 uM nifedipine. During all experimental procedures the control and experimental rotifers were kept in phosphate buffer and phosphate buffer with 1.0 uM nifedipine (see Appendix 1) respectively.

Control and experimental rotifers were cultured and separated into three age groups: 0.5 day, 3 days and 5 days old. The 0.5 day-old samples each comprised 100 rotifers while three and five day old samples contained 50 rotifers. In all, 12 samples were analysed. Each experiment in the following section was repeated twice.

1. Calcium uptake by whole rotifer homogenates

Rotifers in each sample were homogenized in 100 uL of phosphate buffer or phosphate buffer with nifedipine. Following homogenization 300 uL of 0.25% trypsin in normal saline (Gibco Laboratories, Grand Island, New York) was added and the samples were placed in a shaker bath at room temperature for 30 minutes. The samples were then centrifuged for 15 minutes at 1500 g and the pellets resuspended in 100 uL of the appropriate phosphate buffer. A Bradford's protein assay (Bradford, 1976) was run on 20 uL of each suspension.

After the protein concentrations in each sample had been established, 200 ug of rotifer protein was taken from each sample and placed in separate tubes. The volume of each was then made up to 60 uL with appropriate amounts of buffer. The samples were left to incubate with 103 uL of $^{45}CaCl_2$ (100,000 cpm) for 20 minutes.

After incubation, each sample was separately placed in a sterile syringe and filtered through an 8 uM millipore

filter. The samples were then washed twice with 10 ml of either phosphate or phosphate-nifedipine buffer. The filters were then placed in liquid scintillation vials with 2.0 ml of scintillation fluid, vortexed for 30 seconds, and placed in a liquid scintillation counter.

2. Calcium uptake by rotifer membrane vesicles

Rotifer membranes were isolated using a dextran polyethelene glycol (PEG) aqueous 2-phase system (Burnette and Till, 1981). The membrane fragments obtained from this procedure tend to reseal cytoplasmic face out due to the hydrophobicity of the broken phospholipid ends. The following chemicals were mixed in a separatory funnel and left overnight at 4 ^{o}C : 20 g of 20% dextran, 10.3 g of 30% PEG, 33.3 ml of 0.22 M sodium phosphate buffer (pH 7.0) and 17.9 ml of distilled water.

Rotifers (separated according to age and treatment as above) were homogenized in 150 uL of phosphate buffer (see appendix) and centrifuged at 11700 g for 20 minutes. The pellets were resuspended in 1.5 ml of PEG and vortexed. Following the addition of 1.5 ml of dextran, the samples were again vortexed and centrifuged at 8000 g for 15 minutes. The interface solution was taken from these samples and placed in new tubes containing 1.0 ml fresh dextran and 1.0 ml fresh PEG and again vortexed. These tubes were centrifuged at 8000 g for 15 minutes. The

interface solutions from this centrifugation were diluted four times with the phosphate buffer thus disrupting the layers. The samples were centrifuged at 12000 g for a further 20 minutes. Bradford's protein assays were performed on small fractions of the pellets obtained.

Samples containing 25 ug of each of the pellets were suspended in 250 uL of Tes/triethanolamine (pH 7.4). The following chemicals were then added to these suspensions: 100 uL of 1.25 M sucrose, 50 uL of 0.001 M ouabain, 50 uL of 0.05 M magnesium chloride and 50 uL of 0.06 M ATP (Tris salt), for a final volume of 0.5 ml. To start the reaction, 110 uL 45-$CaCl_2$ was added and the samples were incubated for 30 minutes.

Following incubation 1.0 ml of a mixture containing 50 mM Tes/triethanolamine buffer, 0.25 M sucrose and 5 mM EGTA was added to the samples to stop the reaction. The samples were then filtered through 8uM millipore SC type filters as previously described, rinsed twice and the filters placed in vials with 2.0 ml of scintillation fluid. Radioactivity was counted in a liquid scintillation counter.

ATP-dependent calcium uptake into the vesicles was determined by subtracting the value obtained in the absence of ATP from that obtained in its presence (Minami and Penniston, 1987).

Statistical Analysis

All lifespan data was analysed using a one-way analysis of variance followed by a post-hoc Tukey test (Sokal and Rohlf, 1981). Other data was analysed using a post-hoc Dunnett's test and two-way analysis of variance (Zar, 1984).

RESULTS

The initial experiments were performed to establish the normal values for the lifespan of Asplanchna brightwelli. These values were then compared to data obtained from rotifers treated with varying concentrations of the calcium channel blocker nifedipine. The nifedipine concentration producing the largest increase in rotifer lifespan was thus determined and was used in all subsequent experiments. All experiments reported were repeated at least twice.

Effect of Nifedipine

A. brightwelli was exposed to a range of nifedipine concentrations in order to determine the drug's effect on rotifer lifespan. Table 1 shows the data from one of three replicate experiments. A range of nifedipine concentrations from 0.1 uM to 10 uM is presented. A one way analysis of variance followed by a post-hoc Tukey test showed that concentrations of nifedipine between 0.1 and 5 uM produced a significant increase in rotifer lifespan compared to the untreated controls ($F(4, 115) = 10.462$, $p<0.001$). Higher doses of the drug had either no effect on lifespan or significantly shortened lifespan. Similar results were obtained in all replicate experiments.

The data in Table 1 indicates that the increase in lifespan observed in the rotifers was primarily due to an

Table 1

THE EFFECT OF NIFEDIPINE ON THE LIFESPAN AND PREREPRODUCTIVE PERIOD OF A. BRIGHTWELLI

(n=24)

Nifedipine concentration	Prereproductive period (days ± S.E.M.)	Mean lifespan (days ± S.E.M)
0	2.49 ± 0.06	5.10 ± 0.25
0.1 uM	3.37 ± 0.30 a	6.28 ± 0.12 a
0.5 uM	3.34 ± 0.32 a	6.34 ± 0.14 a
1.0 uM	3.24 ± 0.20 a	6.10 ± 0.13 a
5.0 uM	2.99 ± 0.24 a	5.97 ± 0.24 a
10.0 uM	2.52 ± 0.36	5.31 ± 0.21

a Significantly different from control

Prereproductive period: $F(4, 115) = 11.476$, $p<0.001$

Lifespan: $F(4, 114) = 10.462$, $p<0.001$

increase in the prereproductive period. This increase was found to be significant compared to the untreated controls ($F(4, 115) = 11.476$, $p<0.001$).

Survivorship curves for the control and experimental groups are displayed in Figure 1. The curves for rotifers treated with 0.5 uM and 1.0 uM doses of nifedipine show a clear shift to the right of the control group indicating their relatively longer lifespan. The data show that rotifer lifespan was maximally enhanced over a range of concentrations (0.1 uM - 5.0 uM). Since there was no significant difference between these four groups, a concentration of 1.0 uM was selected for all subsequent experiments.

Fecundity

The fecundity of rotifers treated with nifedipine is presented in Table 2. The results show that there was no significant difference in the number of offspring produced at any nifedipine concentration except those at which rotifer lifespan was reduced ($F(5, 138) = 0.5619$, $p>0.5$). The reproductive periods of all rotifers also show no significant difference. This finding may suggest that the treated rotifers remained at least as healthy as controls throughout the experiments.

Figure 1. Survivorship curves of rotifers exposed to different concentrations of nifedipine compared to untreated control. (n=24)

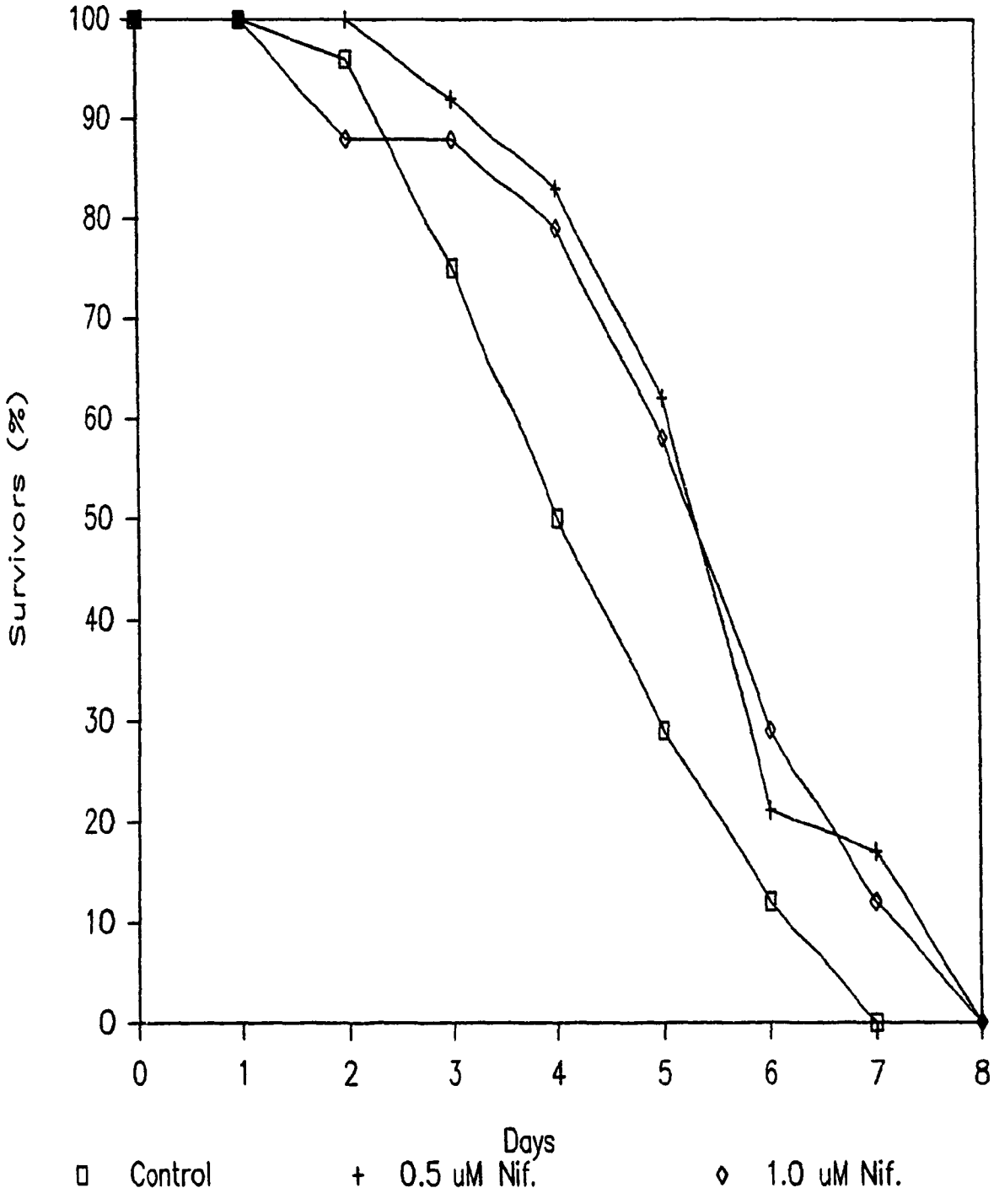

100
90
80
70
60
50
40
30
20
10
0
Survivors (%)
0
1
2
3
4
5
6
7
8
Days
Control
0.5 uM Nif.
1.0 uM Nif.

Table 2

THE EFFECT OF DIFFERENT CONCENTRATIONS OF NIFEDIPINE ON THE FECUNDITY OF A. BRIGHTWELLI

(n=24)

Nifedipine concentration	Mean # offspring ± S.E.M.
0	9.16 ± 0.63
0.1 uM	8.95 ± 0.58
0.5 uM	8.79 ± 0.48
1.0 uM	8.62 ± 0.54
5.0 uM	8.87 ± 0.49
10.0 uM	7.96 ± 0.55

No significant difference between groups.

$F(5, 138) = 0.5619$, $p>0.5$

Paramecia Counts

During all lifespan experiments the number of Paramecia in randomly chosen wells was counted. Table 3 shows a representative count. One-way analysis of variance showed that there was no significant difference in the number of Paramecia in control or experimental wells ($F(28, 81) = 2.780$, $p>0.05$). This indicates that all rotifers had access to an equal supply of food.

Effect of Ethanol

The nifedipine used in all experiments was dissolved in 95% ethanol. Since ethanol has been found to have free radical scavenging capability, ethanol controls were run to ensure that lifespan was not affected.

Table 4 shows the mean lifespan of rotifers treated with ethanol compared to control and nifedipine-treated animals. A one way analysis of variance followed by a post-hoc Tukey test showed that there was no significant difference between the groups ($F(2, 69) = 0.201$, $p>0.5$). At the concentrations used here, ethanol does not affect rotifer lifespan.

Determination of Activity

The experiments in this section were designed to determine the activity level of control and nifedipine-treated rotifers. The experimental animals were exposed to

Table 3

THE DENSITY OF PARAMECIA IN RANDOMLY SAMPLED WELLS OF ROTIFERS EXPOSED TO NIFEDIPINE (NUMBER OF PARAMECIA PER 100 MICROLITERS OF MEDIUM)

(n=3)

	# Paramecia / 100 uL medium ± S.E.M.			
Age (days)	Control	0.1 uM Nifed.	0.5 uM Nifed.	1.0 uM Nifed.
1	24.6 ± 3.1	30.0 ± 5.6	21.0 ± 2.9	23.0 ± 3.6
2	25.3 ± 3.2	36.3 ± 0.2	25.3 ± 1.2	24.6 ± 2.8
3	32.3 ± 4.3	23.3 ± 2.1	36.3 ± 2.6	31.0 ± 1.7
4	36.3 ± 3.5	34.6 ± 2.6	36.6 ± 2.4	33.3 ± 3.2
5	35.6 ± 1.7	35.6 ± 5.1	32.6 ± 2.2	37.3 ± 3.8
6	29.6 ± 2.9	35.0 ± 3.7	33.3 ± 1.7	29.3 ± 0.7
7	36.6 ± 5.9	30.6 ± 4.1	38.6 ± 2.8	33.6 ± 1.4

No significant difference between groups.

$F(28, 81) = 2.780$, $p>0.05$

Table 4

THE EFFECT OF ETHANOL ON THE LIFESPAN OF A. BRIGHTWELLI

(n=24)

Ethanol concentration	Mean lifespan (days ± S.E.M.)
0	5.38 ± 0.22
10 uM	5.52 ± 0.18
100 uM	5.58 ± 0.19

No significant difference between groups.

$F(2, 69) = 0.201$, $p>0.5$

1.0 uM nifedipine.

In the first experiment, the number of grids that control and experimental rotifers traversed in a one minute time period was counted. Table 5 displays the mean movement count each day throughout the lifespan of the organism. It can be seen that activity declines significantly late in life ($F(5, 24) = 3.959$, $p<0.05$). One way analysis of variance showed that the nifedipine-treated rotifers had a similar decrease in movement with increasing age ($F(6, 28) = 10.142$, $p<0.001$). Figure 2 indicates the general decrease in rotifer activity with age and clearly shows the decreased activity of the rotifers administered the calcium channel blocker compared to controls.

The second activity-measuring experiment made use of the positive phototactic response of rotifers. Sixty 3-day old rotifers were placed in the central chamber of a three-chambered rectangular dish and were left to migrate towards a beam of light placed at one end of the dish. The experiment was repeated using rotifers exposed to nifedipine since birth.

Table 6 shows the mean number of control and treated rotifers found in each chamber following a 10 minute exposure to the light. A one-way analysis of variance showed that significantly fewer nifedipine-treated rotifers moved towards the light source ($F(2, 12) = 20.557$, $p<0.01$). The bar graph in Figure 3 illustrates the number of rotifers

Table 5

THE EFFECT OF NIFEDIPINE ON THE ACTIVITY LEVEL OF A. BRIGHTWELLI (EXPRESSED AS THE MUMBER OF GRIDS TRAVERSED IN A ONE MINUTE PERIOD)

(n=5)

	# Grids ± S.E.M.	
Age (days)	Control	1.0 uM Nifedipine
1	17.0 ± 2.74	19.6 ± 1.38
2	18.7 ± 3.09	15.6 ± 1.09
3	19.5 ± 2.28	14.2 ± 2.31 a
4	20.0 ± 2.69	11.2 ± 1.86 a
5	16.0 ± 2.37 a	6.8 ± 1.03 a
6	9.3 ± 1.30 a	6.0 ± 0.98 a
7	-	5.0 ± 1.11 a

a Significantly different from day 1 count

Control: $F(5, 24) = 3.959$, $p<0.05$

Nifedipine: $F(6, 28) = 10.142$, $p<0.001$

Figure 2. Movement curves of A. brightwelli exposed to different concentrations of nifedipine. (Movement expressed in mean number of squares transversed per minute). (n=24)

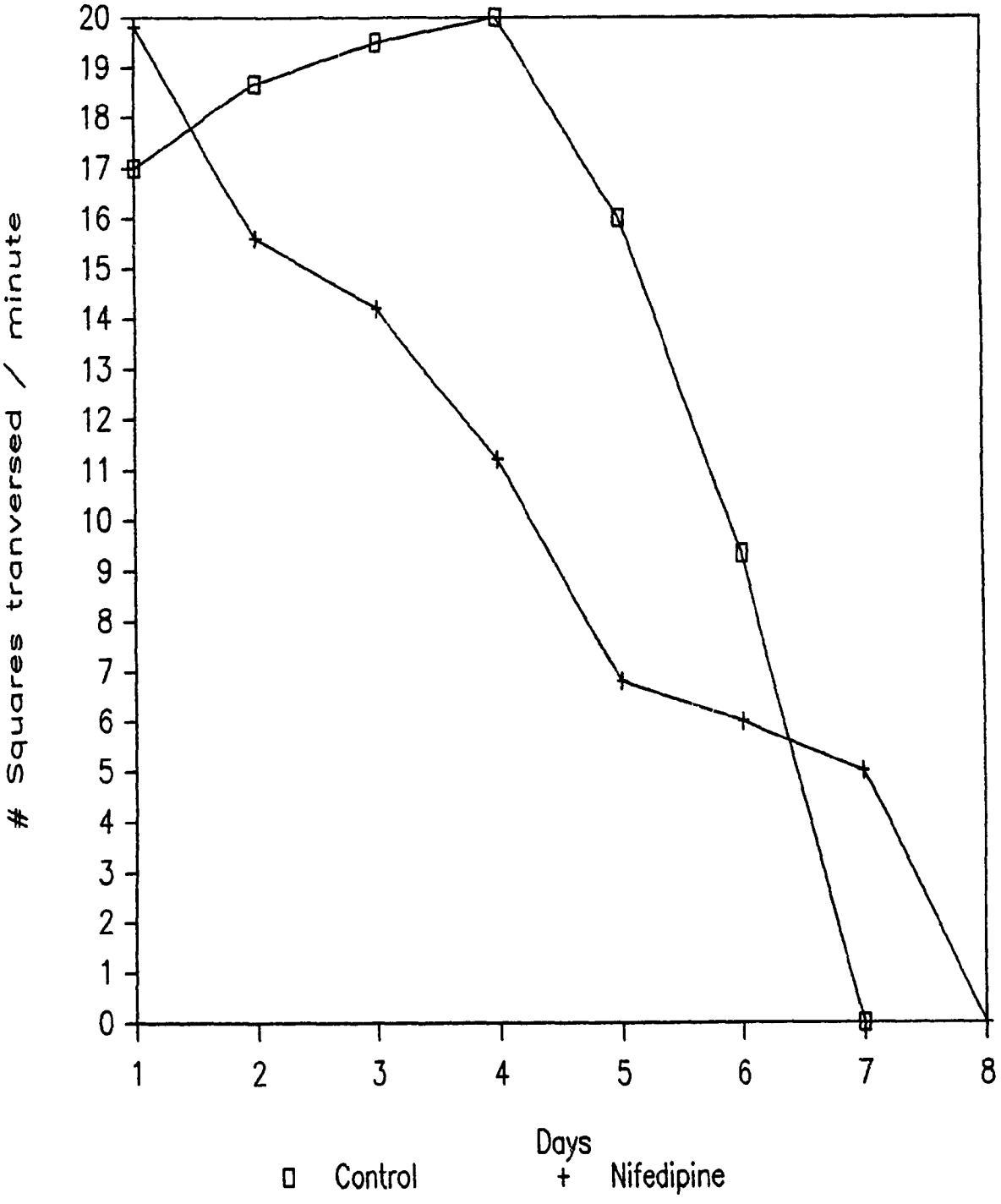

Squares tranversed / minute
20
19
18
17
16
15
14
13
12
11
10
9
8
7
6
5
4
3
2
1
0
1
2
3
4
5
6
7
8
Days
Control
Nifedipine

Table 6

THE EFFECT OF NIFEDIPINE ON THE MOVEMENT OF A. BRIGHTWELLI FROM THE INTERMEDIATE CHAMBER OF A THREE-CHAMBERED BOX LIT AT ONE END

(n=5)

	Mean # Rotifers ± S.E.M.	
Chamber	Control	Nifedipine
Light	46.2 ± 2.59	21.0 ± 3.33
Intermediate	11.6 ± 2.92 a	35.2 ± 4.01
Dark	2.2 ± 0.99 a	3.8 ± 1.01 a

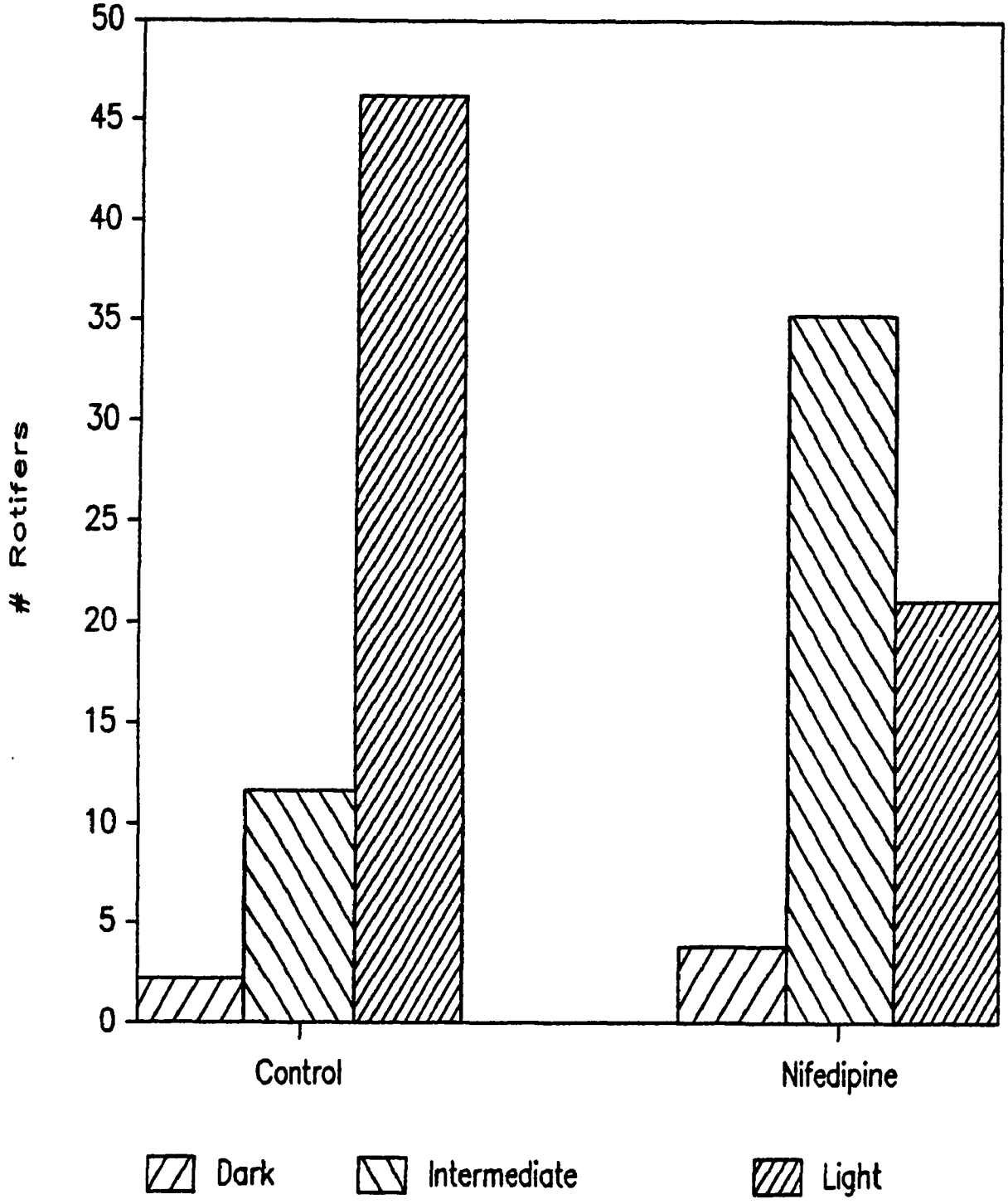
a Significantly different from light

Control: $F(2, 12) = 79.19$, $p<0.01$

Nifedipine: $F(2, 12) = 20.557$, $p<0.01$

Figure 3. Bar graph comparing the movement of control and nifedipine-treated A. brightwelli from the intermediate chamber of a three-chambered box lit from one end. (n=5)

Nifedipine concentration = 1 micromolar


50
45
40
35
30
25
20
15
10
5
0
Rotifers
Control
Nifedipine
Dark
Intermediate
Light

found in each chamber at the end of the experiment. It clearly shows that most treated rotifers remained in the central chamber where they were initially placed.

The results of these experiments indicate that nifedipine significantly reduces the activity level and the phototactic response of rotifers exposed to the drug throughout their lifespan.

Effect of Calcium Ionophore

Table 7 shows the effect of calcium ionophore C-9275 on the lifespan of rotifers. This large molecule causes an increase in the membrane's permeability to calcium. At both ionophore concentrations presented, the lifespan of A. brightwelli was significantly shortened ($F(2, 69) = 260.511$, $p<0.001$). Neither of the treated groups lived beyond the prereproductive period and thus produced no offspring.

Uptake of Calcium Ion by Paramecia

The experiments in this section were performed to determine if nifedipine could bind to Paramecia and could thus be ingested by the rotifers as they consume their prey. The binding of nifedipine was determined by the loss of the organism's ability to take up calcium ions. Paramecium protein was measured using a standard Bradford's assay (see Appendix 2).

Table 7

THE EFFECT OF CALCIUM IONOPHORE C-9275 ON THE LIFESPAN OF *A. BRIGHTWELLI*

(n=24)

Ionophore concentration	Mean lifespan (days ± S.E.M.)
0	5.13 ± 0.16
0.1 M	1.55 ± 0.15 a
1.0 M	0.92 ± 0.11 a

a Significantly different from control

$F(2,69) = 260.511$, $p<0.001$

Table 8 compares the mean concentration of calcium ion moving across 1 ug of Paramecia membranes in the course of 90 minutes in control and nifedipine-treated animals. One way analysis of variance showed that the calcium uptake of animals exposed to 1.0 uM nifedipine did not significantly increase over the 90 minutes ($F(2, 9) = 2.987$, $p>0.05$), while that of the control animals increased dramatically ($F(2, 9) = 69.760$, $p<0.01$). Figure 4 clearly illustrates the contrast in the rate of calcium ion entry into control and nifedipine-treated Paramecia. The calcium ion blockade indicated by these results suggests that nifedipine binds to Paramecia and may thus be available for rotifer ingestion.

Uptake of Calcium Ion by Rotifers

In this group of experiments, the effect of calcium channel blockers and age on the uptake of calcium by rotifers of 3 different age groups was examined. Firstly the calcium ion uptake by dispersed rotifer cells was examined; and secondly the ATP-dependent calcium uptake by isolated rotifer membrane vesicles was determined. In both experiments the rotifer protein was measured using a standard Bradford's assay. The protein recovery was found to be reasonably consistant from sample to sample. The data is presented in Tables 9 and 10.

Table 8

THE EFFECT OF NIFEDIPINE ON THE MEAN UPTAKE OF CALCIUM ION BY PARAMECIUM CAUDATUM OVER 90 MINUTES (MEAN UPTAKE IN NANOMOLES OF CALCIUM PER MICROGRAM OF PARAMECIUM PROTEIN)

(n=3)

	nmol Calcium / ug Protein ± S.E.M.	
Time (minutes)	Control	Nifedipine
10	1.00 ± 0.14	0.30 ± 0.08
30	1.41 ± 0.16 a	0.45 ± 0.04
60	2.74 ± 0.02 a	0.38 ± 0.02
90	2.76 ± 0.08 a	0.46 ± 0.05

a Significantly different from 10 min. sample

$F(2,12) = 10.142$, $p<0.05$

Figure 4. Bar graph comparing rate of calcium uptake of control and nifedipine-treated P. caudatum over 90 minutes. (Rate expressed in nanomoles of calcium per minute per microgram of Paramecium protein). (n=3)

Nifedipine concentration = 1 micromolar

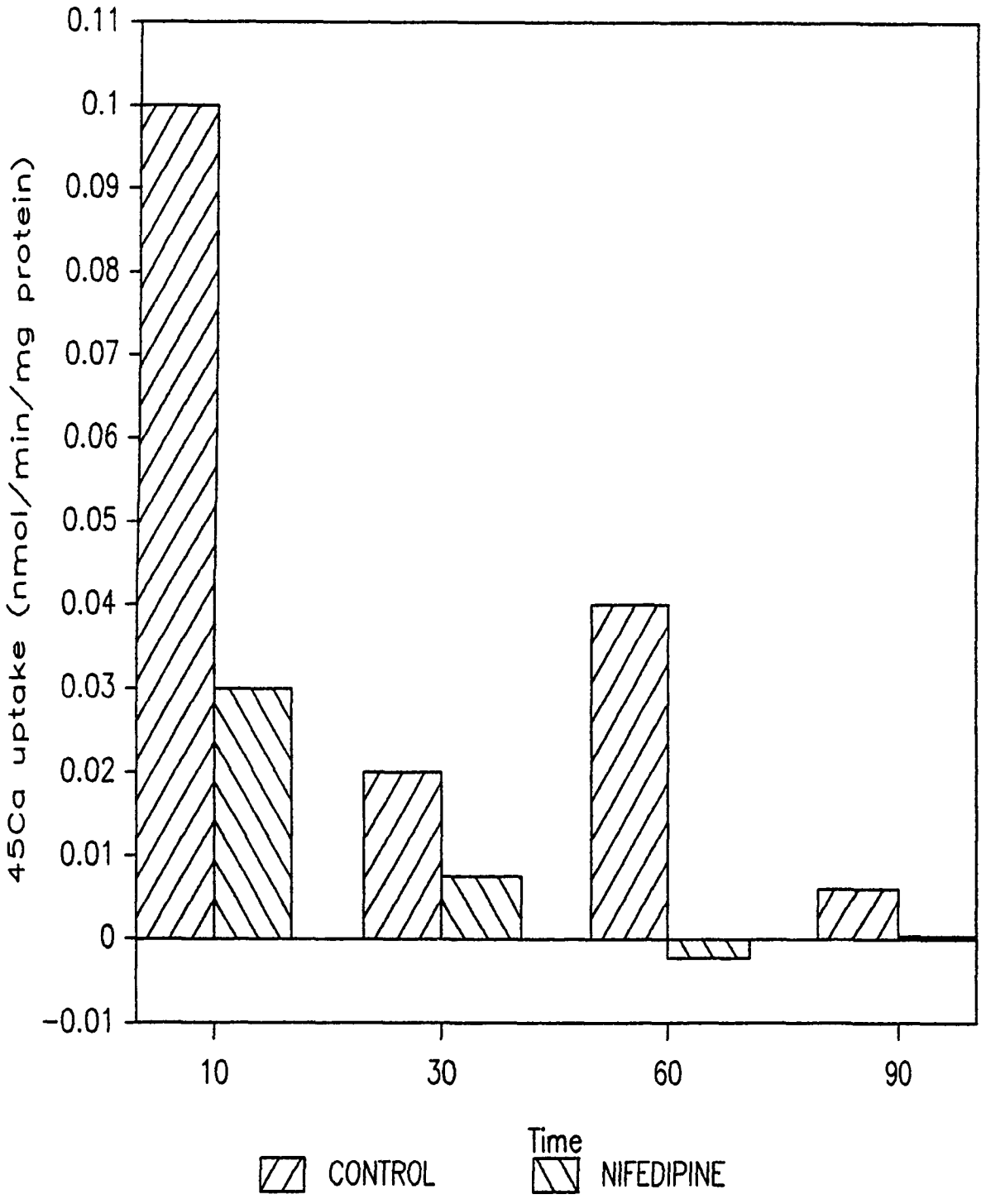

0.11
0.1
0.09
0.08
0.07
0.06
0.05
0.04
0.03
0.02
0.01
0
-0.01
45Ca uptake (nmol/min/mg protein)
10
30
60
90
Time
CONTROL
NIFEDIPINE

Table 9

MEAN RECOVERY OF PROTEIN FROM DISPERSED ROTIFER CELLS OF DIFFERENT AGES (PROTEIN CONCENTRATION IN MICROGRAMS PER MICROLITER OF PHOSPHATE BUFFER)

(n=3)

		ug Protein / uL Buffer ± S.E.M.	
Age (days)	# Rotifers	Control	Nifedipine
0.5	50	3.17 ± 0.13	3.19 ± 0.03
3	50	3.27 ± 0.93	2.39 ± 0.27
5	50	5.14 ± 0.47	3.61 ± 0.68

Table 10

MEAN PROTEIN RECOVERY FROM ISOLATED MEMBRANE VESICLES FROM ROTIFERS OF DIFFERENT AGES (PROTEIN CONCENTRATION IN MICROGRAMS PER MICROLITER OF PHOSPHATE BUFFER)

(n=3)

		ug Protein / uL Buffer ± S.E.M.	
Age (days)	# Rotifers	Control	Nifedipine
0.5	50	0.61 ± 0.27	0.41 ± 0.17
3	50	0.35 ± 0.04	0.47 ± 0.02
5	50	1.22 ± 0.54	1.08 ± 0.46

1. Calcium uptake by dispersed rotifer cells

The mean calcium ion uptake in one minute by one milligram of rotifer cells is shown in Table 11. A one way analysis of variance test performed on this data, followed by a post-hoc Dunnett's test showed that there was a significant increase in the rate of calcium uptake in rotifers with increasing age ($F(2, 5) = 64.334$, $p<0.01$). This same test performed on the nifedipine data showed no significant increase in calcium ion uptake with age ($F(2, 5) = 2.58$, $p>0.05$). A two way analysis of variance revealed that the combined effect of age and nifedipine treatment was highly significant: at 0.5 days the difference in calcium uptake by control and experimental animals is very small, while the difference in the 5 day old rotifers is large (see Appendix 4). The bar graph in Figure 5 clearly illustrates these differences in calcium uptake at the three age groups examined.

2. Calcium uptake by isolated rotifer membranes

In the previous experiment it was found that the uptake of calcium ion by rotifer cells greatly increased with age and was greatly reduced by nifedipine treatment. This finding suggested that the mechanism to pump calcium ions out of the cells may also have been affected by age or by nifedipine treatment. The results from the ATP assay are, however, are less clear.

Table 11

THE EFFECT OF NIFEDIPINE ON THE MEAN UPTAKE OF CALCIUM ION BY DISPERSED CELLS OF A. BRIGHTWELLI AT THREE DIFFERENT AGES (MEAN UPTAKE IN NANOMOLES OF CALCIUM PER MINUTE PER MILLIGRAM OF ROTIFER PROTEIN)

(n=3)

	nmol Calcium / Min / mg Protein ± S.E.M.	
Age (days)	Control	Nifedipine
0.5	0.04 ± 0.01	0.02 ± 0.001
3	0.08 ± 0.02 a	0.03 ± 0.005
5	0.54 ± 0.05 a	0.05 ± 0.01

a Significantly different from 1 day old

$F(2,5) = 64.334$, $p<0.01$

Figure 5. Bar graph comparing the uptake of calcium ion by the dispersed cells of control and nifedipine-treated rotifers of three different age groups. (Mean uptake expressed in nanomoles of calcium per minute per milligram of rotifer protein). (n=3)

Nifedipine concentration = 1 micromolar

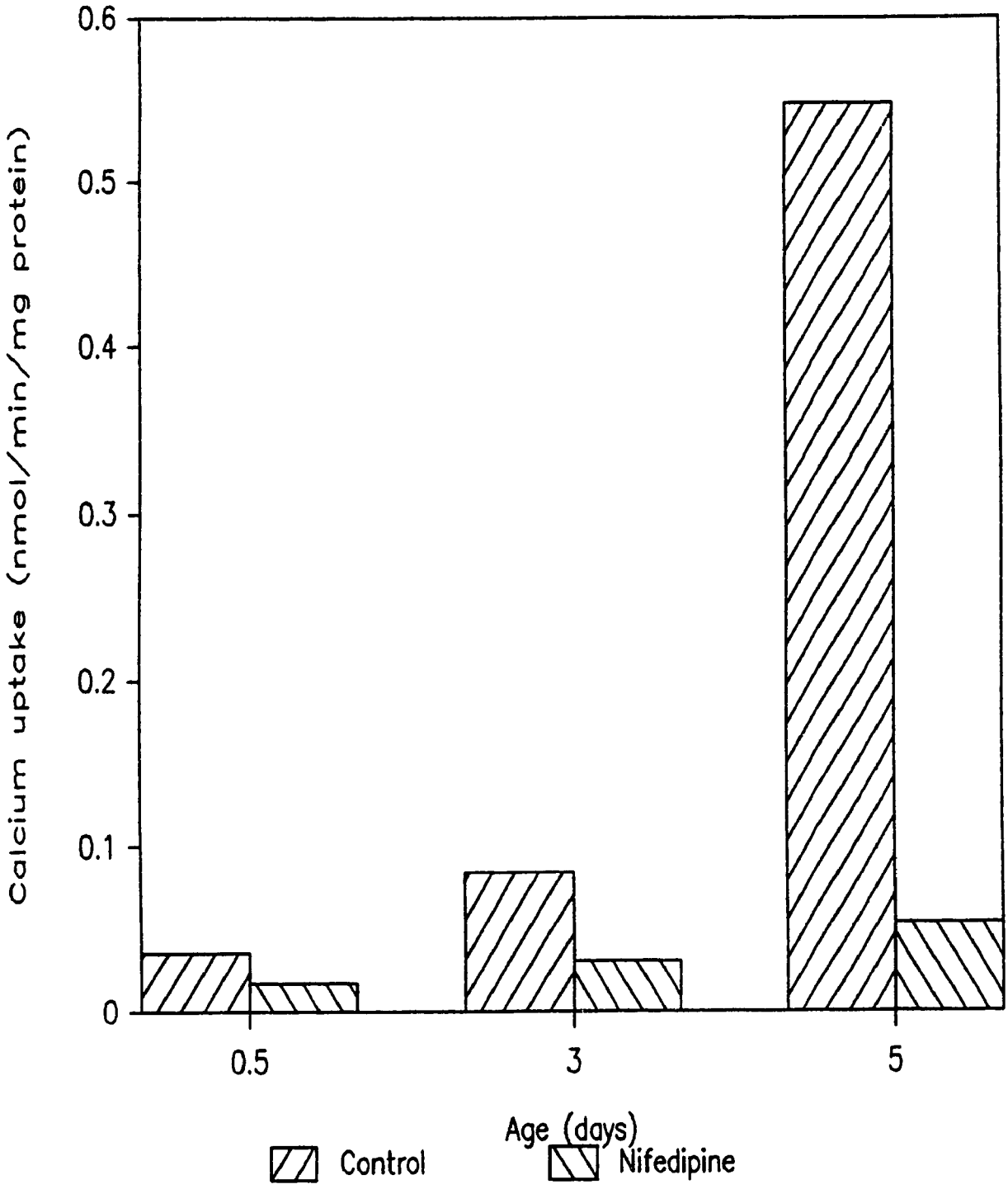

0.6
0.5
0.4
0.3
0.2
0.1
0
Calcium uptake (nmol/min/mg protein)
0.5
3
5
Age (days)
Control
Nifedipine

Table 12

THE EFFECT OF NIFEDIPINE ON THE MEAN ATP-DEPENDENT UPTAKE OF CALCIUM ION BY MEMBRANE VESICLES OF A. BRIGHTWELLI AT THREE DIFFERENT AGES (MEAN UPTAKE IN NANOMOLES OF CALCIUM PER MINUTE PER MILLIGRAM OF ROTIFER PROTEIN)

(n=3)

	nmol Calcium / Min / mg Protein ± S.E.M.	
Age (days)	Control	Nifedipine
0.5	0.02 ± 0.01	0.05 ± 0.01
3	0	0.11 ± 0.05
5	0.17 ± 0.08	0.06 ± 0.05

No significant difference between groups

Control: $F(2,6) = 2.935$, $p>0.05$

Nifedipine: $F(2,6) = 0.431$, $p>0.05$

Figure 6. Bar graph comparing the ATP-dependent uptake of calcium ion by the membrane vesicles of control and nifedipine-treated rotifers of three different age groups. (Mean uptake expressed in nanomoles of calcium per minute per milligram of rotifer protein). (n=3)

Nifedipine concentration = 1 micromolar

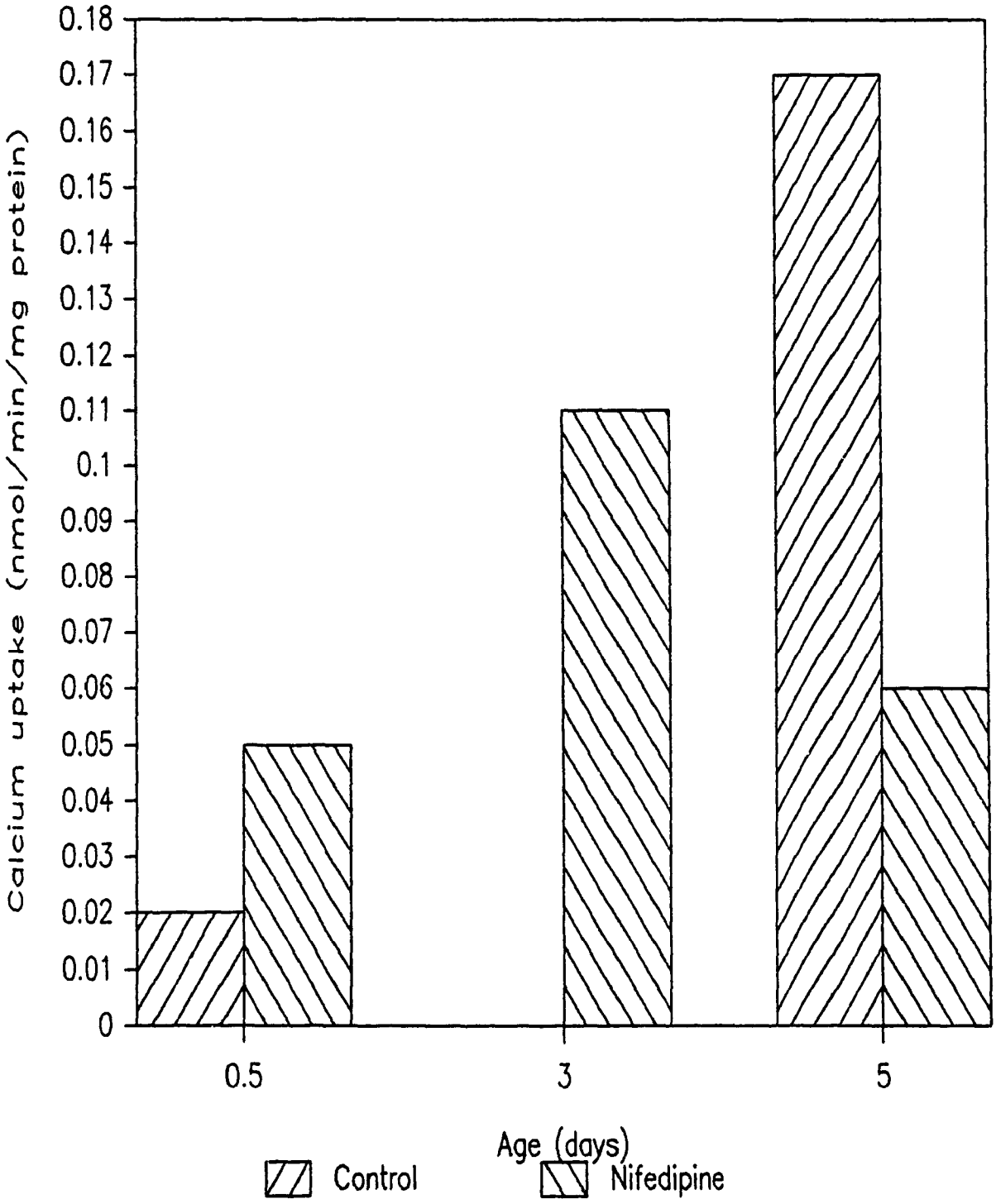

0.18
0.17
0.16
0.15
0.14
0.13
0.12
0.11
0.1
0.09
0.08
0.07
0.06
0.05
0.04
0.03
0.02
0.01
0
Calcium uptake (nmol/min/mg protein)
0.5
3
5
Age (days)
Control
Nifedipine

Table 12 shows the mean ATP-dependent calcium uptake in one minute by one milligram of isolated rotifer membrane vesicles. One way analysis of variance tests performed on both sets of data showed no significant difference with age in either the control or experimental groups ($F(2, 6) = 2.935$, $p>0.05$; ($F(2, 6) = 0.431$, $p>0.05$). Two-way analysis of variance revealed that there was no significant interaction between age and nifedipine treatments (see Appendix 4). Although these results indicate that there does not seem to be any change in the activity of the membrane ATP-dependent calcium pump with age, Figure 6 indicates that there is a trend towards increased calcium pump activity in aging rotifers. The rotifers treated with nifedipine showed almost no change in calcium pump activity from the lowest age group to the highest.

DISCUSSION

The results reported here show that there is an age-related accumulation of calcium ions in the cells of the rotifer Asplanchna brightwelli. The exposure of this rotifer to the calcium channel blocker nifedipine appears to prevent this calcium build-up, and results in an increase in the lifespan of the organism.

Calcium Accumulation and Free Radicals

Experiments conducted by Sincock (1974, 1975) and Lansing (1942) suggested that rotifers tend to accumulate calcium as they age. These authors, however, did not speculate on why this cation should accumulate or why it should be toxic.

More recently, several authors have reported that high intracellular calcium concentrations are a common finding in aging, necrotic and ischemic cells (Farber, 1981; Siesjo, 1981; Khachaturian, 1984). According to Khachaturian (1984) Seisjo's (1981) work on ischemia provides an important link between intracellular calcium accumulation and the free radical theory of aging.

Free radicals are unstable molecules which lack an electron in their outer orbital. These molecules are formed as intermediates in the normal functioning of enzyme systems

within cells. They gain stability by removing electrons from other molecules such as membrane polyunsaturated fatty acids. The radicals produced by the breakdown of these fatty acids form bonds producing large polymers that are of no use to the cell.

Siesjo (1981) suggests that ischemia, like aging, results in a build-up of toxic free radicals that cause lipid peroxidation damage to plasma membranes resulting in an increased calcium influx. Similar findings have been reported by Farber (1981) and Trump et al. (1984). These authors found that carbon tetrachloride-induced lipid peroxidation of the membranes of cells in culture resulted in an accumulation of calcium and, ultimately, in cell death.

It seems likely that lipid peroxidation of aging rotifer membranes is an important factor in the calcium ion accumulation reported here. Sawada (personal communication) has found that there is a dramatic increase in lipid peroxidation of the plasma membranes of A. brightwelli as the rotifer ages. The attendant membrane damage and calcium influx may be an important trigger for the initiation of cell death.

Calcium and Cell Death

Farber (1981) suggests that the common morphology of dead cells implies that the causative mechanisms may be

similar in all cells. He notes that most dead cells contain a large amount of calcium. This accumulation could conceivably have occurred simply because of the large electrochemical and concentration gradients across the cell membrane. However, experiments have shown that tissue culture cell death particularly involves altered calcium homeostasis, since other ions and molecules present in high concentrations in the medium did not similarly accumulate (Farber, 1981).

Several mechanisms have been proposed to explain the involvement of calcium in cell death. According to Grotta et al. (1986), the pressure placed upon organelles, particularly the mitochondria, to sequester the influxing calcium may result in the disruption of normal cellular function. In the case of the mitochondria this could result in the compromising of respiration in favor of sequestration, and a decrease in the availability of ATP for the entire cell.

The build-up of free radicals with age has been well documented (Hall, 1984). The membrane damage which results may cause a disruption of the permeability barrier to calcium ions (Farber, 1981). Siesjo (1981) suggests that calcium may activate phospholipase enzymes which in turn cause further membrane breakdown, the generation of more free radicals, and finally, cell death.

In A. brightwelli, an organism with only about 1000

cells (Ware, 1984), and in which almost no active cell division occurs, the death of a small number of cells is likely to have more serious consequences for the overall well-being of the rotifer than would a similar level of cell death in a larger invertebrate or mammal. The blocking of calcium entry may therefore be an important factor in delaying cell death.

The Effect of Nifedipine

The results show that administration of the calcium channel blocker nifedipine to the rotifers stopped the age-related influx of calcium into rotifer cells and increased the lifespan of the organism. Again this finding is in agreement with experiments performed on ischemic cells. It has been found that 1,4-dihydropyridine calcium channel blockers can prevent neural cell damage in rats and in dogs (Steen et al., 1983; Grotta et al., 1986). These authors note that untreated cells quickly accumulate calcium and die.

The result of preventing calcium influx can be explained in light of the reported effect of intracellular calcium accumulation. Grotta et al. note that the blockade of calcium channels may preserve mitochondrial function in neurons by freeing these organelles from sequestering activities. Furthermore, if calcium is involved in the activation of phospholipases in rotifers, channel blockade

could conceivably delay this activation and thus prevent additional free radical formation. Further experimentation is required to establish whether or not this is the case.

Activity Level

If, as Khachaturian (1984) suggests, calcium accumulation-related cell death is paticularly important in neural regions, the loss of either brain cells or interneurons in the rotifer could result in a serious decrease in brain-coordinated functions such as muscle contraction and sensory function. Loss of neurons and / or neuro-muscular connections may be a factor in the decrease in activity seen as a consequence of age in rotifers.

The decrease in rotifer activity observed in organisms treated with nifedipine cannot be definitively explained. The effect may, however, indicate that neurotransmitter release was affected by the channel blockade.

Miller (1985) found that the application of calcium channel blockers to rats resulted in a decrease in acetylcholine release and muscle relaxation. Since the presence of acetylcholine in rotifers has been established (Nogrady and Alai, 1983), the results reported here suggest that nifedipine treatment may interfere with acetylcholine release thus slowing movement.

The finding that nifedipine decreases the rotifers' ability to move towards light also suggests an effect on the

neurons and / or muscles of these organisms. Cornillac (1982) found that A. brightwelli is positively phototactic. This response has been clearly explained by Clement (1984) Light striking the cerebral eye of this rotifer results in the initiation of a nervous signal in a single sensory neuron which leads to the brain. This signal is the transmitted via motor neurons to the muscles of the cingulum (Clement, 1977). The contraction of these muscles results in the movement of the rotifer towards the light source.

Treatment of rotifers with nifedipine may interfere with the phototactic response in two ways. Firstly, the rotifers may simply respond more slowly because the muscles of the cingulum are more relaxed. Alternatively, nifedipine may bind to the calcium channels of neurons along the intervening sensory pathway thus blocking nervous transmission before the signal reaches the muscles of the cingulum.

ATP-Dependent Calcium Efflux

Michaelis et al. (1984) found that the efficiency of plasma membrane calcium regulating systems, including the ATP-dependent calcium pump, decreased only slightly with increasing age. Bertrand et al. (1975) found no change in the activity of the pump in the membranes of muscle sarcoplasmic reticulum with increasing age.

Gibson and Peterson (1987) report that ATPase activity

appears to be reduced during aging at normal cytosolic calcium concentrations, but maintained at physiologically high calcium concentrations. Paradoxically, the concentration of calcium accumulating in rotifer cells as they age appears to be physiologically high. This may account for the lack of change in ATPase pump activity reported here. These results, however, are preliminary. The isolation of rotifer plasma membranes is difficult due to the small size of the organisms. Use of a larger volume of membranes may produce clearer results.

It has been established that the efficiency of calcium buffering systems within cells decreases with age. Large proteins such as calmodulin, which complexes calcium, become less efficient with age (Gibson and Peterson, 1981). Mitochondria become less able to sequester calcium as they age (Victoria and Satrustegui, 1986; Gibson and Peterson, 1987). It is clear, therefore, that maintaining the operation of the ATPase pump at a constant rate as the cell ages will not prevent the intracellular calcium concentration from ultimately reaching toxic levels.

Effect of Calcium Ionophore

Farber (1981) has reported that the treatment of cells in culture with calcium ionophores results in rapid cell death. Ionophores work by causing the cell membrane to become freely permeable to a specific ion. The results show

that calcium ionophore C-9275 caused a dramatic decrease in the lifespan of treated rotifers at all concentrations tested. It is possible that the chemical caused an early intracellular accumulation of calcium and massive cell death in the rotifer. However, since this ionophore is highly unstable, it may simply have been toxic to the organism. Calcium uptake studies, or the administration of calcium channel agonist drugs, such as Bay K 8644, may clarify this finding.

Dietary Restriction

Dietary restriction has been found to increase the lifespan of A. brightwelli (Verdone-Smith and Enesco, 1981). The finding that nifedipine-treated rotifers were less active suggested that the animals may have been less able to obtain food from the medium, undergo dietary restriction and thus live longer. This explanation does not seem likely since steps were taken to ensure that all rotifers had adequate access to food.

The Paramecia counts indicate that all rotifers had access to a plentiful food supply. Ingestion of Paramecia by rotifers depends upon the rotifer touching the prey (Pennak, 1978). Prey density was such that rotifers were continually surrounded by food. Experiments performed by Verdone-Smith and Enesco (1981) indicate that the lifespan of rotifers undergoing dietary restriction was specifically

lengthened in the reproductive period. Rotifers in the present study had a significantly longer prereproductive period, while the reproductive period remained the same length as in controls.

Assimilation of Nifedipine

The question of how rotifers assimilate nifedipine has not been fully answered in this study. Since rotifers have a number of sensory receptors in direct contact with the surrounding medium (Clement et al, 1983), nifedipine may be assimilated directly from the environment. The results reported here indicate that nifedipine binds to Paramecia and appears to reduce the flow of calcium ions into the ciliate. Rotifers may thus be able to obtain nifedipine from their diet.

Conclusion

The aims of this study were to determine whether the calcium ion concentration in rotifer cells increases with age; to determine if nifedipine could prevent this increase; and to assess the effect of nifedipine on rotifer activity and lifespan.

As the results indicate, calcium ions tend to accumulate in the cells of aging rotifers. The calcium channel blocker nifedipine not only prevents this accumulation, but also reduces the activity level of the

organism and increases rotifer lifespan.

The findings reported here appear to support Khachaturian's (1984) theory that calcium homeostasis is related to aging. The regulation of intacellular calcium ion concentration appears to play an important role in rotifer aging: the decrease in regulatory efficiency as A. brightwelli ages results in a build-up of calcium ions and, ultimately, in the death of the organism.

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Appendix 1

Stock Buffer Solutions

1. Phosphate buffer

   (culturing of Paramecia and rotifers)

COMPONENT	MOLECULAR WT.	AMOUNT USED
distilled H_2O	-	250.0 ml
NaH_2PO	137.99	34.5 g
NaOH	40.00	4.0 g

Components were mixed in a 500 ml Erlenmeyer flask. 7.0 ml of the buffer were added to each liter of medium.

2. Diluted phosphate buffers

   (homogenization of rotifers)

In all experiments using rotifer tissue homogenates, 7.0 ml of the above buffer were added to a liter of distilled water. To prepare phosphate-nifedipine buffer, 10 uL of a 0.001 M stock nifedipine solution were added to 100 ml of the diluted buffer producing a final nifedipine concentration of 0.00001 M.

Appendix 2

Protein Determination (Bradford, 1976)

COMPONENT	AMOUNT USED
Coomassie Brilliant Blue G-250	100 mg
95% ethanol	50 ml
85% ortho-phosphoric acid	100 ml
H_2O	750 ml

The Coomassie blue dye and ethanol were mixed, the phosphoric acid was added, and the solution was diluted to a final volume of 1 liter with distilled water.

To determine protein levels of samples, 5.0 ml of the above reagent was added to 100 uL of the sample. The solution was then vortexed and the absorption at 595 nm was measured and compared to a standard curve prepared using a 10 ug - 200 ug range of albumin concentrations. The data and standard curves generated are presented in the following pages.

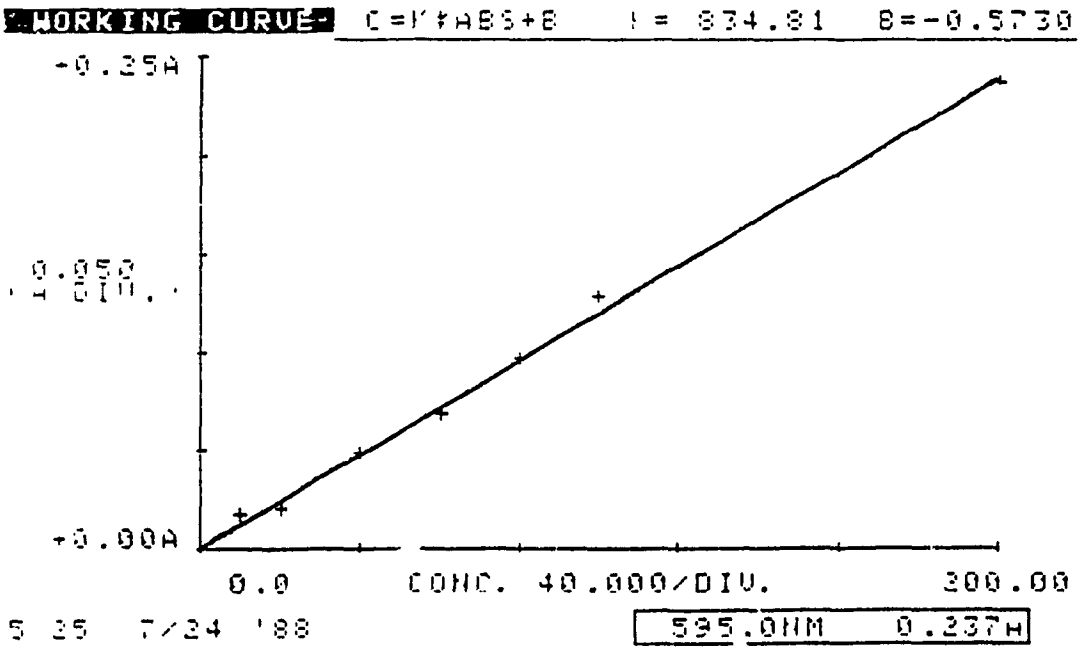
Absorption at 595 nm of Seven Concentrations of Albumin (concentration in micrograms, absorption in Angstroms)

CALIBRATION WORKING CURVE DISPLAY Y/N ?

STD. No.	CONC.	ABS.
1	10.000	0.015
2	30.000	0.044
3	70.000	0.095
4	80.000	0.110
5	100.000	0.135
6	200.00	0.270

Standard Curve

(concentration vs absorption)



Appendix 3

Decay of 45-Calcium Chloride

The radionuclide 45-calcium chloride (specific activity 20 mCi/mg) was used within 5 weeks of the purchase date. Since the product has a half-life of only 164 days, its radioactivity decreased significantly from one week to the next. In calculating the calcium concentrations from liquid scintillation counts, a decay factor (provided by Amersham Canada Ltd., Oakville, Ontario) was used to account for this decrease.

Appendix 4

Summary of 2-Way Analyses of Variance

1. Calcium uptake by whole rotifer homogenates

Source of variation	DF	SS	MS	FS
A (nifedipine)	1	0.116770	0.116770	59.17505
B (age)	2	0.259289	0.129644	65.69902
A x B	2	0.252713	0.126356	64.03275
Error	10	0.019733	0.001973	
Total	15	0.648506		

2. Calcium uptake by rotifer membrane vesicles

Source of variation	DF	SS	MS	FS
A (nifedipine)	1	0.000548	0.000548	0.062971
B (age)	2	0.024958	0.012479	1.432571
A x B	2	0.037185	0.018592	2.134446
Error	12	0.104531	0.008710	
Total	17	0.167223		