Cholinergic effects on the feeding of *Brachionus calyciflorus* (Aschelmintes Rotifera)

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ABSTRACT

The effect of cholinergic drugs was investigated on the rotifer Brachionus calyciflorus using qualitative behavioural observations and quantitative feeding experiments using the yeast Rhodotorula glutinis labelled with tritiated glucose. Food uptake was measured by scintillation counting.

The qualitative observations uncovered two novel phenomena. One was a selective foot paralysis caused by muscarinic blockers. The other one was an oscillating tachyphylaxis (drug habituation) that was repeated several times within an hour. It was caused by antimuscarinics, ganglionic blockers and neuromuscular blockers alike, but only by some representatives of each group.

The quantitative experiments revealed that acetylcholine inhibited food uptake in a dose-unrelated fashion, but had no other physiological effect. Similarly, the six anticholinergic drugs investigated all acted as feeding inhibitors, but in a statistically highly significant dose-dependent fashion. The agonist-antagonist interactions were, however, not clearcut. The same drugs that produced oscillating tachyphylaxis in the qualitative experiments also produced tachyphylaxis in the feeding

experiments, insofar as food uptake recovered, following a minimum at 10 or 20 minutes exposure to drugs.

It was concluded that the rotifer cholinoceptor is a primitive, nonspecific lock on an ionophore, capable of mediating diverse effects, and that no direct comparison with vertebrate cholinoceptors is justified.

ACKNOWLEDGMENTS

I would like to express my sincere thanks and appreciation to my supervisor, Dr. Thomas Nogrady for his support, interest, and encouragement throughout the course of these studies.

In addition, I am grateful to Scott McMillan for his help in the conduct of the research.

Sincere thanks are also extended to Miss Sau-lai Lui for her excellent typing of this thesis.

TABLE OF CONTENTS

PAGE

LIST OF FIGURES

Figure	Page

LIST OF TABLES

INTRODUCTION

Rotifers are ubiquitous aquatic micrometazoans belonging to the phylum Aschelminthes. They are often the major component of the freshwater plankton biomass and are therefore of great importance in the aquatic food chain. (For summaries see Nogrady., 1978; Pennak, 1978). It is therefore surprising that rotifers have been used to such a minor extent as vectors and models in environmental toxicology.

The use of rotifers in monitoring ecotoxicology has been haphazard in the past (Hueck, 1979; Halbach, 1984). Using a systematic approach and the acquired knowledge of rotifer neuropharmacology, one would hope to establish a rational basis for a rotifer model applicable to specific toxicological problems.

In general, the advantages of the use of rotifers in bioassays can be summarized in the following points:

- Their cosmopolitan and common occurrence and direct involvement in the aquatic food-chain allows for accumulation or metabolic dissipation of environmental toxicants.

- Their rapid parthenogenetic reproduction (2-4 eggs per day, hatching in 24-36 hours) and short life time (5-25 days) allows for the production of large, cloned populations, thus providing a rapid and convenient experimental tool.

- Direct observation of their embryology, genetic alterations and adaptive morphological changes are possible within a short time, due to the transparency and short life of the animals.

- The constancy of cell numbers within each species, due to the lack of mitosis after birth or eclosion, assures a uniform life schedule that is advantageous in statistical evaluation. It also prevents "healing" of chemical lesions, but precludes investigations on carcinogenesis.

The major problem in this investigation was the lack of previous information on specific responses of B. calyciflorus to drugs, except for a brief note on general anesthesia, a rather useless all-or-none response (Marriott et al., 1948). Thus our first problem was to find an appropriate experimental 'handle' suitable for specific, quantitative, and reproducible measurements of specific drug effects.

It was a difficult task to select an experimental system that could reflect the influence of specific drugs in rotifers, without knowing anything about the innervation of different organs and neurotransmission in physiological terms. The only quantitative methodology found in the literature was the series of careful papers by Gilbert and his collaborators (Gilbert et al., 1977, 1978; Starkweather et al., 1977a, b, 1978) on food uptake in B. calyciflorus using labelled algae or yeast. While this method, using my modifications, turned out to be gratifyingly reproducible, resulting in data which was highly significant, it suffers from the major- and inevitable- drawback, that food uptake is an extremely complex phenomenon, composed of locomotion, activity of coronal cilia, screening by trochal cirri, chemoreception, and mastax activity.

Food uptake is, therefore, the final result of many more specific physiological actions. I decided to use the food uptake model only because any other method that would have require lengthy preliminary work that, in itself, could have constituted a thesis in rotifer biology. The rate of change in swimming direction (klinokinesis) was used occasionally.

The purpose of this study was to find out how rotifers respond to low dosages of Ach and how they respond to drugs

that interfere with Ach activity. The questions that I asked in this study were as follows:

1) Do rotifers respond in the same manner as other invertebrates or mammals to cholinergic signals?

2) Do cholinergic drugs stimulate or inhibit the food intake that might lead to increase or decrease in population of rotifers.

The responses of the rotifer that I was able to measure quantitively were effects on food intake and the responses that I could measure only qualitatively were changes in locomotion and behavior of the animals. Ciliarly action is required in the rotifer for food intake; therefore, food intake could be an adequate measure of rotifers to in response to cholinergic drugs.

Qualitatively, I obtained some information on locomotion and behavior of rotifers which served as a measure of acute responses to cholinergic drugs foot most significant of these observations was a specific food paralysis caused by muscarinic antagonists which turned out to be a useful model in subsequent investigations on the adrenergic pharmacology of rotifers as well.

These experiments were also designed to form the informational basis of additional research on ecotoxicological problems caused by insecticides acting on cholinergic neurons. However, I did not perform experiments with

insecticides. Results of the investigation was published in a subsequent paper (Nogrady and Keshmirian, 1986).

BIOLOGY OF ROTIFERS

If one were to designate a single major taxonomic category that is most characteristic of fresh waters, it could only be the phylum Rotatoria. The rotifers are one of the few groups that have unquestionably originated in freshwater, and it is here that they have attained their greatest abundance and diversity. Probably over 2,000 species have been described, but less than 5 percent of these are restricted to marine and brackish waters; only two species occur in the mid-Atlantic. The vast majority of rotifers encountered under natural conditions are females, reproducing parthenogenetically. Thus the offspring are clones. Some recent monographs on rotifer biology and taxonomy are Pennak (1978), Ruttner-Kolisko (1974) and Koste (1978).

My experiments utilized a common planktonic species, Brachionus calyciflorus (Pallas), which can easily be cultured in the laboratory and produce a large number of cloned individuals in a short time. (Fig. 1)

Fig. 1

Photomicrograph of live Brachionus calyciflorus carrying three eggs.

Females of Brachionus calyciflorus are about 200-300 um in length and are common in eutrophic (nutrient-rich) ponds and lakes throughout the world. They are generally planktonic but may also attach themselves by their foot to various substrata and to the air interface. They are suspension feeders; they have cilia on theire corona(head) which produce water currents (which serve for locomotion as well as feeding). The animal removes small particles from these currents, and then transports the particles to the oral canal.

B. calyciflorus may regulate ingestion of suspended particles by at least three mechanisms (Gilbert & Starkweather, 1977).

1. The pseudotrochal cirri on the corona may form a screen over the buccal field, deflecting even very small particles.

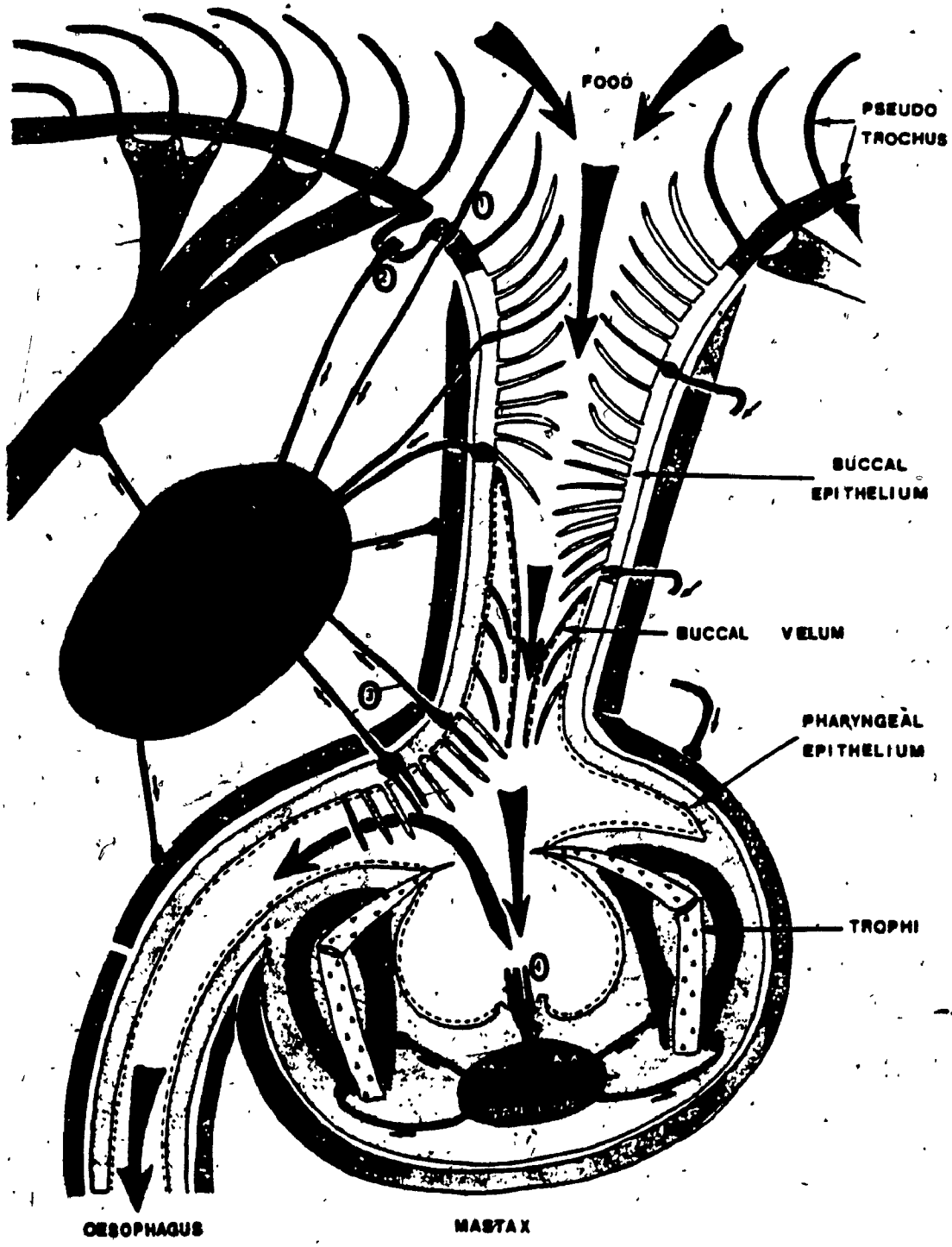
2. The buccal field cilia may reject particles which have been admitted.

3. The oral canal may push particles back into the buccal field for subsequent rejection, presumably triggered by recognition through chemoreceptors.

The following diagram (Figure 2) illustrates the structure of these mechanisms, as well as the complexity of feeding, as outlined in the introduction.

Fig. 2

Diagram and mechanisms of the feeding behavior in Philodina and Brachionus. Between the anterior mechano-receptors (1) and chemoreceptors (2), and the sensory receptors of the mastax (3 and 4), there are some receptors in the buccal epithelium, M1, M2, M3, M4 and M5 are the muscles innervated by the brain or by the mastax ganglion. (reproduced from Clement et al., in Biology of Rotifers, edited by B. Pejler, R. Starkweather and T. Nogrady, 1983, p.103).


FOOD
PSEUDO
TROCHUS
BUCCAL
EPITHELIUM
BUCCAL VELUM
PHARYNGEAL
EPITHELIUM
TROPHI
OESOPHAGUS
MASTAX

AN OUTLINE OF CHOLINERGIC PHARMACOLOGY

Cholinergic agents are drugs that either directly or indirectly produce effects similar to those elicited by acetylcholine. According to their mode of action, cholinergic agonist may be divided into two main classes: direct cholinergic agonists and indirect cholinergic agonists. For a summary of cholinergic pharmacology see Nogrady (1985) pp.119-140.

Acetylcholine

Acetylcholine (ACh) is the only naturally occuring transmitter in the cholinergic neuronal system. The chemical transmitter at all ganglia and at the somatic neuromuscular junction is acetylcholine. Once released into the synaptic cleft, the acetylcholine diffuses to the postsynaptic membrane where it combines with a receptor to produce depolarization, leading to initiation of contraction of the muscle. Also present at the post-synaptic membrane is the enzyme actylcholinesterase, which rapidly hydrolyzes the acetylcholine into acetic acid and choline, thereby terminating its action. Acetylcholine is the transmitter between certain nerve cells and between nerve cells and many kinds of effector cells.

Nerve-nerve transmission is prominent in autonomic ganglia, both sympathetic and parasympathetic. A-Ch is also the transmitter between many cells in the central nervous system.

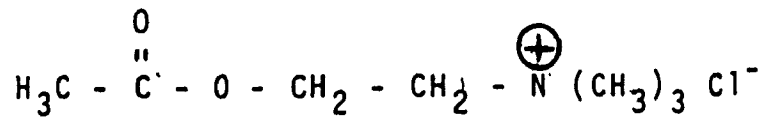
Nerve-effector organ junctions mediate neurotransmission at the neuromuscular junction and the whole somatic motor system depends on them. In addition, acetylcholine is the transmitter at all parasympathetic endings. Acetylcholine is the most widely occuring transmitter in the autonomic nervous system, since it is the product of sympathetic and parasympathetic pre-ganglionic nerves, and of parasympathetic post-ganglionic nerves.

The effects of intravenous acetylcholine in man include flushing, sweating, salivation, lachrymation, increased mucus secretion, and as secondary consequences, nausea, coughing and dyspnea. These are all due to muscarinic actions, no nicotinic actions are noted.

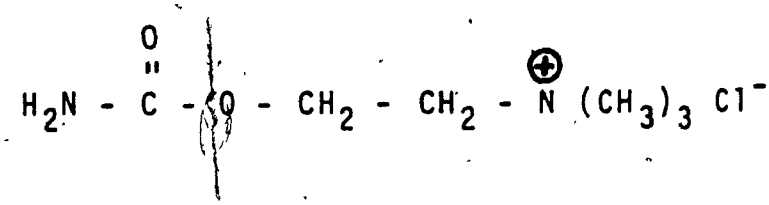
Although acetylcholine is very important as a neurohumoral transmitter, it is not used as a drug since it is poorly absorbed following oral or subcutaneous administration. It is also rapidly broken down by enzymes in the blood and tissues. Only high doses given intra-

venously produce any effect and, even then, only briefly.

The vulnerable ester group in the molecule can be stabilized against esterase attack by replacing the acetic acid moiety with carbamic acid. The carbamic acid ester of choline (Carbachol) is a potent cholinergic agent with both muscarinic and nicotinic activity.



Acetylcholine chloride



Carbachol chloride

It is hydrolyzed more slowly by acetylcholinesterase than is acetylcholine, and can therefore by administered orally.

If a methyl group is attached to the beta-carbon of the choline moiety of acetylcholine, the resulting

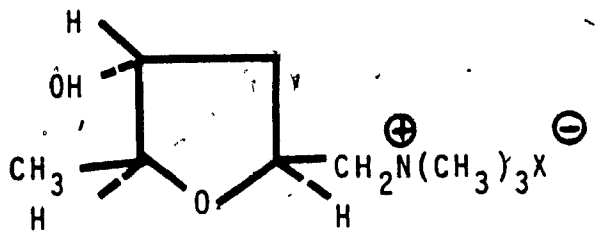
drug, methacholine, loses its nicotinic avtivity but is still prone to be attacked by acetylcholinesterase.

Direct cholinergic agonists

Direct cholinergic agents, also called cholinomimetics and parasympathomimetics are agents with both chemical structure and distances between their polar groups as well as in charge distribution similar to that of acetylcholine.

There are two types of cholinergic receptors which are characteristic for different sites of action. The two types are termed the muscarinic and nicotinic receptors of acetylcholine.

Muscarinic actions are similar to those produced by the alkaloid muscarine, which is present in the mushroom *Amanita muscaria* and is not used clinically. It produces effects similar to the responses to parasympathetic stimulation. The muscarinic actions of acetylcholine are seen on post-ganglionic synapses of organs and smooth muscle, as well as in the CNS.

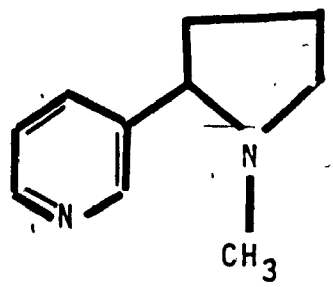


CIS - L (+) - Muscarine

Other cholinomimetics are structurally related to acetylcholine. All are simple onium salts with the general formula $RN^{+}(CH_3)_3$ was studied here.

Natural Alkaloids with Direct Cholinergic Activities

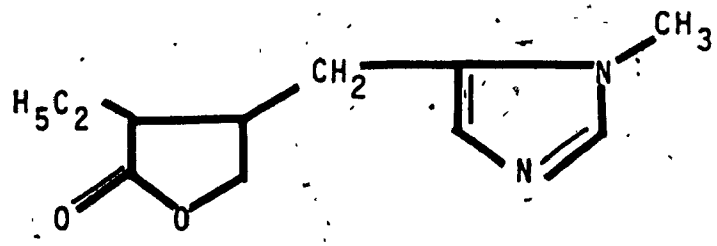
Nicotinic activity is shown by nicotine, an alkaloid from the leaves of tobacco, Nicotiana tabacum and is not used clinically, because of its high toxicity. Nicotinic actions of acetylcholine produces stimulation of sympathetic and parasympathetic ganglia, that is, stimulation of postsynaptic structures within the ganglia so that the postganglionic fibers release transmitter at their peripheral endings. In addition, all neuromuscular end-plates of skeletal muscles are nicotinic receptors.



Nicotine

Both nicotinic and muscarinic agents can be found in this category. Nicotine is used because of its central rather than its ganglionic effects.

Pilocarpine, an alkaloid from Pilocarpus jaborandi or Pilocarpus microphyllus also possess cholinergic activity.



Pilocarpine

It has muscarinic and some nicotinic action.

Indirectly-acting Cholinergic Drugs (cholinesterase blockers).

All cholinesterase blockers inhibit both acetylcholinesterase and plasma cholinesterase. In denervated organs in which no acetycholine is released, inhibition of acetylcholinesterase is without pharmacological effect, that means, cholinesterase blockers appear to be inactive. Chemically, there are two main classes of compounds that inhibit acetylcholinesterase:

a) Carbamate derivatives which are reversible inhibitors.

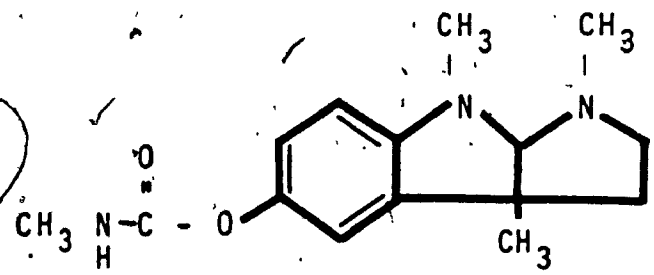
b) Organophosphates, which are nearly irreversible inhibitors since they form stable covalent complexes with acetylcholinesterase.

Reversible Inhibitors

These agents are competitive inhibitors of the cholinesterases, that is, they compete with acetylcholine for the enzymatic binding sites.

Physostigmine

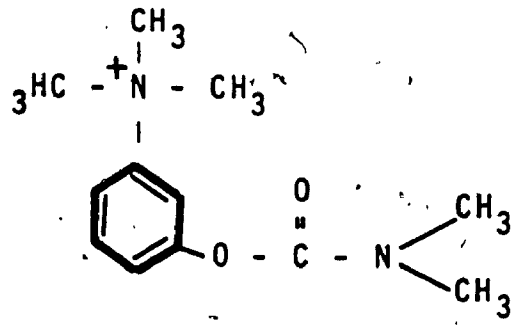
Physostigmine or eserine is an alkaloid extracted from the African calabar bean (Physostigma venenosum). Because of its tertiary amine nature, it penetrates all biological membranes.



Physostigmine (Eserine)

Neostigmine

Neostigmine or prostigmine is a synthetic analog of physostigmine.

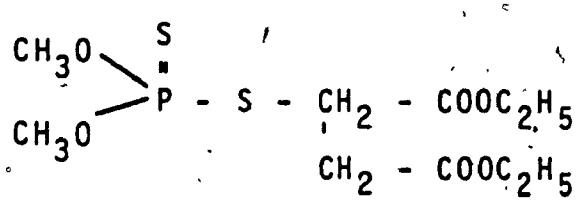


Neostigmine (Prostigmine)

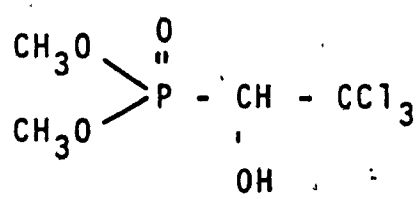
Irreversible Inhibitors (organophosphates and carbamates).

This class of agents, consisting of hundreds of active chemicals, is toxicologically rather than therapeutically important. Nerve gases are included in this category. Both types of compounds are used in enormous quantities as agricultural insecticides.

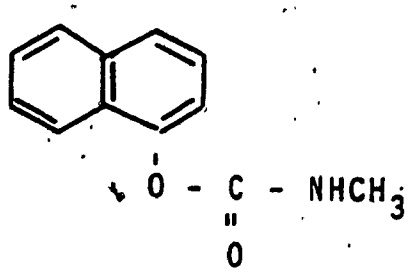
Some examples are:



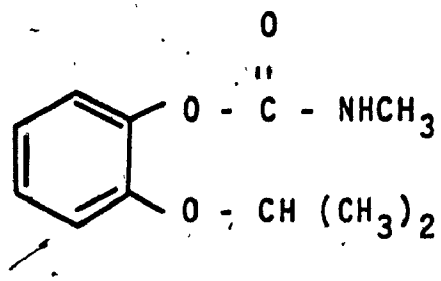
Malathion



Trichlorfon



Sevin



Baygon

Cholinergic Blocking Drugs

Anticholinergics, or cholinergic blocking agents, are drugs that inhibit the activity resulting from acetylcholine. They may act at different sites such as;

a) at the postganglionic terminations of the parasympathetic nervous system; these are called antimuscarinics;

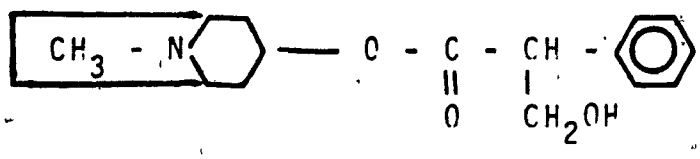
b) at the ganglia of both sympathetic and parasympathetic nervous system. They are known as ganglionic blocking agents; and

c) at the neuromuscular junctions of the voluntary nervous system. These are called neuromuscular blocking agents.

Antimuscarinics

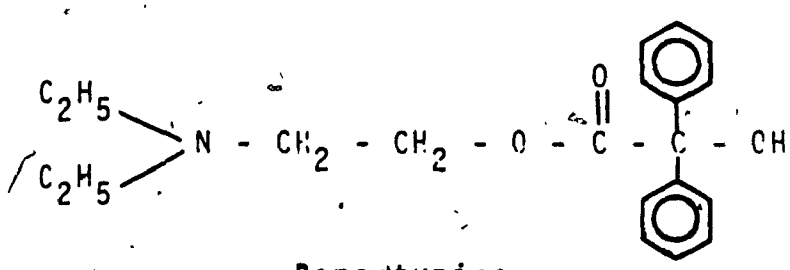
Antimuscarinics are also called parasympatholytics, anticholinergics, atropinics, or parasympathetic blockers. The following examples show the drugs used in this investigation.

Atropine, an alkaloid of Atropa belladonna blocks selectively the muscarinic actions of acetylcholine. It also blocks the muscarinic action of other cholinergic agents. The blocking action of atropine is competitive or reversible, that is; it can be overcome by increasing the acetylcholine concentration at the receptor sites.



Homatropine

Homatropine is a simplified atropine analog, using the mandelate instead of tropinoate ester.



Benactyzine

Benactyzine is one of the many synthetic antimuscarinic agents where the ester part is a bulky group. As many muscarinic agents, it has a tertiary rather than a quaternary nitrogen.

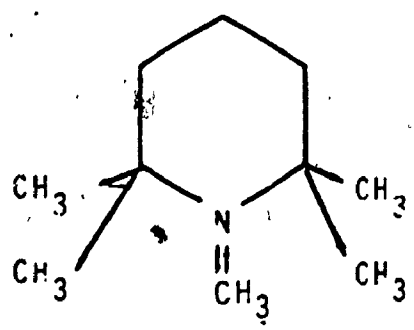
Ganglionic Blocking Agents

Nicotine itself act as an agonist in low concentration, but in high concentrations becomes a ganglionic blocking agent.

Quaternary and tertiary ganglionic blockers used in this investigation are:

$$(CH_3)_3\overset{\oplus}{N} - (CH_2)_6 - \overset{\oplus}{N}(CH_3)_3$$

Hexamethonium



Pempidine (1,2,2,6,6 - pentamethyl-piperidine)

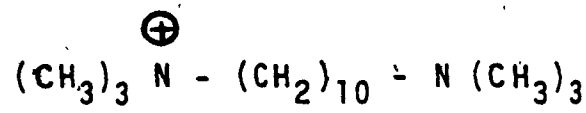
They act on the ganglia of both sympathetic (adrenergic) and parasympathetic (cholinergic) neurons, and therefor show many different pharmacological effects. It is not clear whether rotifers possess ganglia similar to those of higher animals.

Neuromuscular Blocking Agents

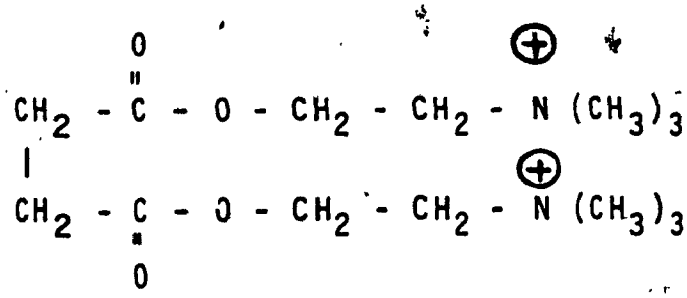
Neuromuscular blocking agents are drugs that bring about voluntary-muscle relaxation and have some points in common with some ganglionic blocking agents. Since their activity is similar to that of curare, they are also called curariform or curarimimetic drugs.

According to their mode of action, neuromuscular blocking agents are classified into three types;

1. Depolarizing blocking agents. They cause depolarization of the membrane of the muscle end plate, similar to that produced by acetylcholine itself, owing to its nicotinic effect, at ganglia and neuromuscular junctions; examples are decamethonium and succinylcholine.



decamethonium



succinylcholine

2. Non-depolarizing competitive blocking agents. It is thought that they compete with acetylcholine for the receptor site at the myoneural end plate but are unable to effect the depolarization characteristic of the natural neuroeffector. The most important example is tubocurarine.

As a result of the receptor occupation by D(+)-tubocurarine, fewer receptors are available to interact with released ACh, and no action potential is triggered. Curariform drugs can be displaced from the receptor and muscle response can be restored, by increasing the concentration of ACh, for example by inhibiting acetylcholinesterase. Tubocurarine is inactive when administered by mouth in human and is always administered intravenously. The action on the neuromuscular junction begins to wear off after about 20 minutes due to redistribution of the drug.

Tubocurarine is used in conjuction with general anaesthesia when prolonged or profound muscle relaxtion is required for the purpose of surgery.

MATERIALS and METHODS

Yeast culture

The yeast *Rhodotorula glutinis* was obtained from The American Type Culture Collection, Baltimore, MD, and cultured on Bacto potato dextrose agar enriched with inorganic salts.

The composition of the medium was the following:

$Ca\ Cl_2$	0.20 g/L
$Mg\ SO_4$	0.50 g/L
$(NH_4)_2\ SO_4$	0.50 g/L
$KH_2\ PO_4$	0.50 g/L
Bacto potato dextrose agar	39 g/L

This was autoclaved for 20 minutes and plated in Petri dishes. *Rhodotorula glutinis* was cultured at ambient temperature for 18-30 hours on agar , forming a dense orange growth. This was then suspended in the rotifer medium.

Rotifer Culture

The culture of *Brachionus calyciflorus* clone S4 was obtained from Dr. J.J. Gilbert (Department of Biology, Dartmouth College, Hanover, N.H.). We thank Dr. Gilbert for the donation of the original *Brachionus* culture. The animals were fed daily with the yeast *Rhodotorula glutinis* at a concentration in excess of 1×10^6 cells/ml, in a modified Woods Hole MBL medium at pH 7.2-7.5 (Table 1). This medium was originally designed to culture algae as well as rotifers, and it was also suitable for the culture of rotifers on yeast. Thus we have seen no need to aclimate rotifers to another inorganic medium. The vitamins are absolutely necessary for the rapid reproduction of *Brachionus*.

Table 1

Composition of modified Woods Hole MBL medium
(Dr. J.J. Gilbert, personal communication)

Compound	mg/L
A. Macronutrients	
$Ca\ Cl_2.2H_2O$	36.76
$Mg\ SO_4.7H_2O$	36.97
$Na\ H\ CO_3$	12.60
$K_2\ H\ PO_4$	4.35
$Na\ NO_3$	42.50
$Na\ Si\ O_3.9H_2O$	28.42
B. Micronutrients	
Na_2 EDTA	4.36
$Fe\ Cl_3.6H_2O$	3.15
$Ca\ SO_4.5H_2O$	0.01
$Zn\ SO_4.7H_2O$	0.022
$Co\ Cl_2.6H_2O$	0.01
$Mn\ Cl_2.4H_2O$	0.18
$Na\ Mo\ O_4.2H_2O$	0.006
$H_3\ B\ O_4$	0.13
C. Vitamins	
Thiamine HCl (B_1)	1.0
Biotin	0.5
Cyanocobalamin (B_{12})	0.1
D. Buffer	
(adjust with phosphate buffer to pH 7.2)	

All cultures were kept at room temperature in a 16 hr/8 hr light-dark cycle, and subcultured every second day. Failure to do so results in overcrowding, and production of males and resting eggs, leading to the extinction of the culture. In order to keep the yeast cells in suspension and accessible to the filter-feeder Brachionus, the cultures were kept in 1 L bottles with a sponge stopper, and the bottles were rotated along their long axis on the instrument shown on Fig. 3, at four revolutions per minute. This is unnecessary if Brachionus is cultured on algae (Chlamydomonas or Euglena) which are flagellates, and therefore mobile, and remain in suspension.

Measurement of Drug Effects

The effects of drugs were quantitated by measuring changes in the food uptake of rotifers per unit time using yeast labelled with 6-^{3}H-glucose (New England Nuclear) as food. To obtain yeast with maximum tritium content, it was labelled with tritiated glucose for 14-16 hours at 37°C. Two cpm of ^{3}H-glucose is added for each yeast cell in 1 ml of water and placed in water bath.

The remaining radioactive glucose in solution was removed by washing the yeast cells a minimum of 5 times with 2.5 - 3.0 mls of distilled water with centrifugation at about 5,000 g for five minutes. When the supernatant showed a radioactivity of less than 28,000 dpm/ml, (a decrease from 2.8 x 10^6 dpm/ml), which is the minimum steady state count, the cells were suspended in 1.0 - 1.5 ml of distilled water and kept at 0^oC. The concentration of the cells was determined using a hemocytometer.

During a 16 hour incubation at 37^oC, an activity between 0.25 and 0.45 dpm/cell was obtained. The labelled yeast suspension can be kept in the refrigerator for several weeks with only a slight decrease in radioactivity.

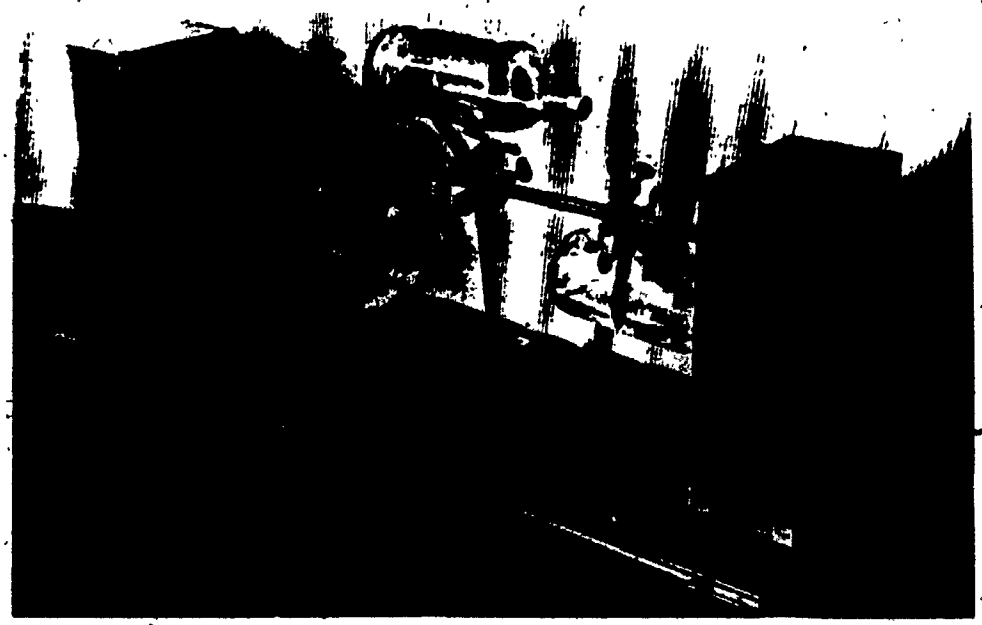
In order to obtain a uniform ingestion rate, a cell density greater than 1 x 10^6 cells/ml was maintained. Gilbert and Starkweather (1977) have reported that below this density a linear increase in ingestion rate is observed. Above this point, a plateau is maintained where a constant ingestion rate occurs. Therefore, all trials were carried out at a concentration of 1 x 10^6 cells/ml or greater. In another paper Starkweather and Gilbert (1978) also state that the egestion of food particles in B. calyciflorus became significant after 20 minutes.

Therefore in all experiments feeding time was maintained at exactly 20 minutes, to avoid reaching a steady state between ingestion and elimination of labelled food.

Gilbert and Starkweather used algae (Euglena) labelled with ^{32}P in their feeding experiments. I decided to use tritiated yeast instead. The half-life of tritium is much longer than that of ^{32}P, it is less dangerous to work with, cheaper, and gave highly reproducible results. Since yeast was used in the feeding experiments, the rotifers were also cultured on yeast. Brachionus grown on algae requires a habituation time of about 30 minutes before it is willing to feed on yeast. Since my experiments were timed very precisely, such uncontrollable acclimation to a different food source was not allowable. Yeast-grown animals will, of course, feed immediately on tritiated yeast.

Fig. 3

Photograph of rotifer culturing flasks which are rotated at 4 rpm in order to keep the yeast in suspension.



Feeding experiments

The experimental cells used were glass tubes with an outside diameter of 0.7 cm and 4.0 cm length. One end of the tubes was closed with a Nitex filter of 75 um pore size using a non-poisonous acrylate glue. The size of Brachionus calyciflorus varies between 120 and 300 µm and is therefore retained by this filter whereas the yeast cells, measuring only 3-5 um, can be washed through the filter at the end of the experiment. In order to perform a series of experiments at once, a multiunit rack was used. This retainer allowed for 14 tubes to be assayed at the same time under identical experimental condition (Fig. 4). All experiments were run in duplicate and replicated.

It is important in feeding experiments to use animals at approximately the same stage of physiological development. Therefore only mature egg-bearing rotifers were used for the experiments, to give a sufficiently high radioactivity. A total of 20 rotifers were assayed per experimental tube. Each assay started with a starvation period to insure that the animals excrete all unlabelled yeast in their digestive tract. Therefore, animals were isolated and placed into medium that did

not contain any yeast. This solution was kept in the small glass vials (see Fig.4) and the whole rack, holding the tubes, could be immersed simultaneously. These vials also contained the appropriate concentrations of drugs, and a pair of blanks for each run. The tubes containing the rotifers were first kept in MBL medium for 30 minutes, and then immersed into the drug solutions for the timed period.

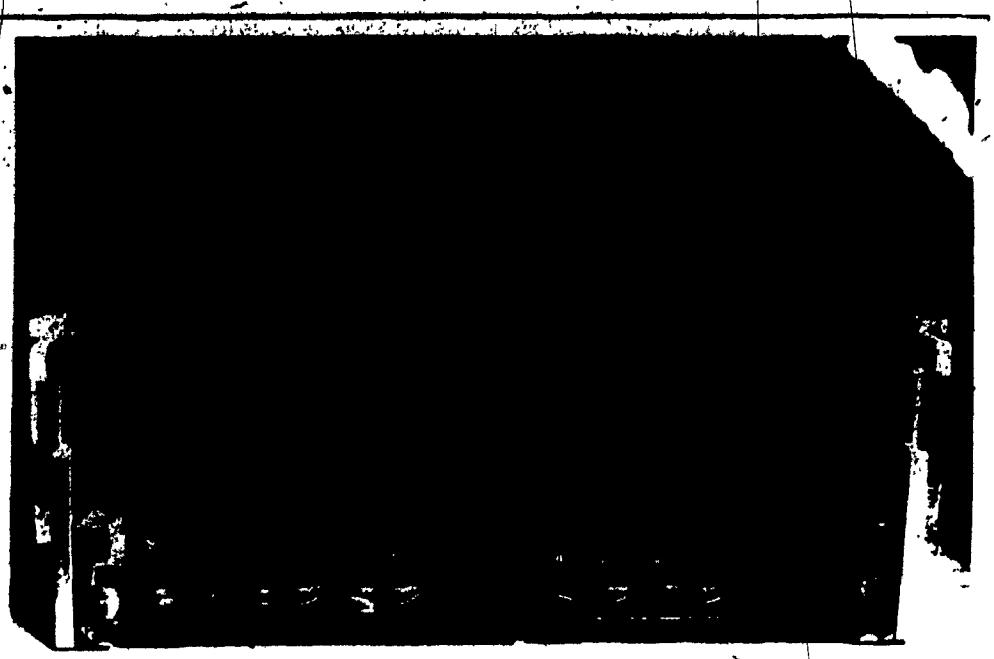
At the end of drug incubation, the rack was lifted. The feeding was done in the 250 ul residual volume held by capillarity in the lifted tube, after adding 25 ul labelled yeast suspension with an Eppendorf pipette and were subsequently incubated for 20 minutes. One control in each experiment contained the labelled yeast and no rotifers, while a second control consisted of rotifers not treated with drug.

At the end of the feeding period, the rotifers were washed with 3x5 ml medium, killed with 95% ethanol, the whole tube was immersed in 1 ml protosol + 1 ml ethanol and heated to 60°C for one hour. After addition of 8 ml scintillation cocktail (Aquasol) the solution was kept at room temperature for one day, to avoid recording chemi-luminescence as scintillation. The vials were subsequently counted in a Beckman 100-C liquid scintillation

counter, and the raw counts corrected for background. (see Appendix).

Fig. 4

A picture of multi-unit rack for assay of drug effects.



Statistical Analysis and Calculations

One-way analysis of variance has been performed on all data obtained. Prior to Anova test, the "Q" test has been performed to reject data below the 90% probability limit.

The limiting value for the ratio of the difference to the range is called the Q value. These values are based on a 90 percent probability level, implying that a result rejected as the outcome of this method of testing has a 90 percent validity of rejection. (Q_{90} value for four number of measurements is 0.76).

All p values are shown on the appropriate tables.

The following is a computer print-out sample for the Anova test: (STATPAK program, Concordia University).

The numbers in group 1 are:
68, 46, 70, 51,
The numbers in group 2 are:
57, 52, 58, 77,
The numbers in group 3 are:
78, 78, 76, 86,

Group	Sum of x	N	Mean	Sum of x*x
1	235	4	58.75	14241
2	244	4	61	15246
2	318	4	79.5	25340

Source	SS	DF	MS	F
Between	1037.17	2	518.586	5.45402
Within	855.75	9	95.0833	
Total	1892.92			

Calculation of food uptake in *B. calyciflorus* was performed using the formula

$$\% \text{ uptake} = \frac{D - C}{MBL - C} \times 100$$

where:

D = dpm count in drug solution

C = dpm count in blank (containing labelled yeast but no rotifers)

MBL = dpm count in control without drug

RESULTS

As mentioned previously, the lack of precedents and information pertaining to rotifer neuropharmacology necessiated that rather extensive qualitative or semiquantitative experiments be carried out in an efficient fashion. These qualitative experiments were valuable in themselves, as they suggested a number of further potential investigations, some of which are being pursued at present in this laboratory. We report the qualitative observations here in the form they were obtained, with the understanding that most of them could be, and were eventually quantitated.

The quantitative experiments, which formed the body of this Thesis, are reported separately for acetylcholine, and the six cholinergic antagonist used. The data are summarized in Table 2 , and graphically represented in Figures 5 to 18. The results for each compound are represented in two graphs, showing the same data in two different ways. The first set of curves are traditional semilogarithmic dose - response curves, showing each <u>incubation time</u> on a separate curve. The second set of curves uses the same data as the previous one, but uses time as the independent variable, and show each <u>concentration</u> on

a separate curve. The second set of curves is perhaps somewhat redundant, as it can be inferred from studing the first set carefully, but it illustrates one of the major, but rather puzzling finding reported here, that of an oscillating tachiphylaxis.

Qualitative observations

Several mature egg bearing animals were isolated and placed in different concentrations ($1x10^{-2}$, $5x10^{-3}$, $1x10^{-3}$ M) of drug solutions. The animals were observed for periods of up to 45 minutes at 5, 10, 15, 20, 30 and 45 minutes. The observations were mainly based on locomotion and behavior of the animals. Any other physiological changes which were not translated into locomotion were excluded from these preliminary observations.

The following drugs were tested:

Acetylcholine: At concentration of $1x10^{-2}$, $5x10^{-3}$, and $1x10^{-3}$ M, B. calyciflorus seemed to behave normally and no changes were observed. It appeared that acetylcholine has no effect on locomotion. Quantitative data on klinokinesis (rate of change of direction) are discussed on page .

Carbachol: At concentration of $1x10^{-2}$ M, locomotion and mastax activity decreased dramatically and immediately and within 20 minutes the animals were dead. At concentration of $5x10^{-3}$ M and $1x10^{-3}$ M the movement decreased but the animals were still alive at both concentrations up to 45 minutes.

Hexamethonium: Locomotion appeared to decrease at concentration of $1x10^{-2}$ M and $5x10^{-3}$ M with increasing incubation time. No changes were observed at $1x10^{-3}$ M.

Pempidine: At concentration of $1x10^{-3}$ M and $5x10^{-3}$ M animals sank to the bottom of observation well. At $1x10^{-2}$ M concentration most of them died within 20 minutes.

Benactyzine: The highest concentration used immobilized almost all animals immediately, but they were still alive and the mastax seemed to be functioning. This

may indicate an independence of the mastax ganglion from other neuronal structures. At a concentration of $5x10^{-3}$ M about 75% of animal died within 10 minutes. Below this concentration periodical changes in the rate of movement become apparent. The immobilized animals recovered and swam about again after 5-10 minutes, and subsequently died. The most peculiar effect of benactyzine was that it produced a postural paralysis of B. calyciflorus. Normally, the animals swam about with their foot retracted into the lorica, extending it only occasionally and placing it against the ventral surface, before retracting it again. Under the influence of benactyzine, the foot becomes rigidly and permanently extended at a 90 degree angle to the body, even if the animal is apparently capable of retracting the foot occasionally. Hydrodynamically, this is an obviously unfavourable posture. Subsequent experiments, performed after the completion of this thesis, have shown that other muscarinic blockers (atropine) and even beta-adrenergic blockers (propranolol) produce this phenomenon. At present, I can offer no explanation for this intriguing pharmacological effect.

Homatropine: The effect of this drug at concentrations below $1x10^{-2}$ M is not significant in terms of movement and behavioral changes. The only slight change observed is that the animals tend to rotate at a higher rate than normal. This, however, was not explored by quantitative measurements. It is interesting to note that the quantitatively measured homatropine effect ($p < .025$) is statistically not significant at lower concentrations.

Decamethonium: This neuromuscular blocker had a very strong initial effect at a concentration of $1x10^{-3}$ M acting within 10 minutes. More than 75% of animals sank to the bottom of dish but were not dead. Mastax activity could be observed but locomotion was extremely slow. This period did not last long; within 30 minutes almost all animals swam again. However, the locomotion appears to be slower than usual but the animals seem to have recovered. This recovery is somewhat similar to that seen with benactyzine and could, perhaps, also be attributed to tachyphylaxis, i.e., an uncoupling of the recognition site of the receptor from the ionophore. Hence one sees normal functioning again, at such high non-physiological drug concentrations. It has been

suggested (Leak and Walker 1981, p.96) that the acetylcholine receptor is really just a "lock" on an ion channel and can be used to mediate any cholinergic response, in a much less differentiated fashion than in higher organism. We shall return to this idea further in the discussion part of this thesis.

d-Tubocurarine: This drug has a very strong effect on locomotion. At concentration $5x10^{-3}$ M and 0 time, 30% of animals fall to the bottom, but at $1x10^{-4}$ M concentration and 0 time there is almost no effect. At 10 minutes and $5x10^{-3}$ M concentration 75% of the animals are found at the bottom of the dish. In general the body appears to be rigid and movements tends to be mostly a rotation along the body axis.

Effect of acetylcholine on benactyzine effects:

1. Keeping benactyzine at $1x10^{-3}$ M, the acetylcholine concentration was varied at $5x10^{-3}$ M, $1x10^{-3}$ M and $1x10^{-4}$ M. Animals were exposed to both drugs at the same time. A preliminary experiment indicated that the effect of antagonist takes precedence over that of the agonist and in some cases these effects are additive, as both result in decrease of feeding. This means that the increasing concentration of agonist did not alter

the antagonist effect as expected. For quantitative data see Fig.15 on page 82.

2. In another experiment, the animals were incubated in benactyzine (antagonist) first and then transfered to acetylcholine (agonist). The concentrations which were used were indentical to the previous experiment, but the results were quite different. To explain it further, animals were exposed to benactyzine at $1x10^{-3}$ M concentration. The animals slowed down and some were sinking to the bottom of the dish. After 15 minutes, they were transfered to a $1x10^{-3}$ M acetylcholine solution, and 50% of the animals recovered in two minutes. The animals also recover slowly if transfered to culture medium, but it took about five minutes until 50% of the animals were swimming again. Furthermore, some animals never recovered in water while all of them revived in ACh solution, Because it is impossible to time "recovery" precisely, attempts to evaluate the difference in recovery between ACh and water were significant only at the .05 level in a Student's t-test. For the same reason, dose-response curves could not be produced in a reliable fashion. Nevertheless, these experiments strongly suggest, that ACh antagonizes the action of benactyzine administered consecutively, while no antagonism could be seen in experiments with simultaneous administration

of the two drugs. It has to be emphasized, that these results pertain to locomotion, not feeding.

Quantitative observations

Acetylcholine

Table 2 and Fig. 5, show that the food uptake is decreased by almost 40% at concentration of $1x10^{-3}$ M. Fig. 6 illustrates that ACh reaches its maximal effect at 20 minutes. Nevertheless, a 20 minutes incubation period does not show any major difference from 10 and 40 minutes incubation period, and a one-way Anova shows no significant difference between various times. Therefore, it can be concluded that the effect of ACh on food uptake is independent of incubation time. The dose-response curve at 10 minute incubation is barely significant ($p < .05$) and non-significant at 20 and 40 minutes. Visual microscopic observations indicates that B. calyciflorus can survive at a concentration of $5x10^{-3}$ M Ach for days without any noticeable changes in pattern of movement. In another experiment the number of changes of direction per minute was measured on 20 animals per group in the presence of $5x10^{-3}$ M ACh and was compared to the control group. A Student's t-test indicated that there is no difference in the pattern or rate of movement

in the presence of ACh. Therefore, one may assume that the 40% decrease in food uptake might be due to physiological changes other than ciliary movement, responsible for both locomotion and currents to sweep in food particles. Such a change could be a decrease in chemoreception at the pharynx and mastax, resulting in rejection of food, as described by Clément et al., (1980). Should this be the case, an important differentiation in the various phases of feeding (locomotion, creation of feeding currents by coronary cilia, chemosensory effects and mastication) would be possible, and the cholinergic regulation of feeding narrowed down to chemoreceptors. However, at this stage this differentiation is still hypothetical, and only the effect on locomotion and probably feeding currents by ACh is excluded. Thus, as stated in the Introduction (p.4), feeding is not the optimal physiological model for the study of neuronal regulation.

Table 2

Percent food uptake ± s.d. of *B. calyciflorus* after incubation in cholinergic drug solutions.

Drug and Time of incubation \ Molar Conc.	$5x10^{-3}$	$1x10^{-3}$	$1x10^{-4}$	p
AGONIST				
Acetylcholine				
10 min.	59±7	61±7	79±9	.05
20 min.	55±4	50±1	55±2	N.S.
40 min.	62±4	60±8	70±7	N.S.
MUSCARINIC ANTAGONISTS				
Benactyzine				
10 min.	00±0	04±0	28±0	.001
20 min.	00±0	07±0	30±1	.001
40 min.	02±0	44±2	72±2	.001
Homatropine				
10 min.	63±5	87±7	100±4	.001
20 min.	67±3	81±5	81±6	.025
40 min.	84±5	82±3	81±2	N.S.
NICOTINIC GANGLIONIC BLOCKERS				
Pempidine*				
10 min.	41±5	82±16	100±16	.001
20 min.	03±0	58±6	75±14	.001
40 min.	74±3	72±5	90±5	.001
Hexamethonium				
10 min.	51±2	54±2	86±6	.001
20 min.	65±1	64±1	64±1	N.S.
40 min.	31±0	32±1	32±1	N.S.

Drug and Time of incubation \ Molar Conc.	$5x10^{-3}$	$1x10^{-3}$	$1x10^{-4}$	p
NICOTINIC "DEPOLARIZING" -TYPE ANTAGONISTS				
Decamethonium				
10 min.	00±0	08±0	96±4	.001
20 min.	77±4	90±5	90±4	.001
40 min.	27±1	27±1	97±6	.001
NICOTINIC "COMPETITIVE" -TYPE ANTAGONISTS				
d-Tubocurarine				
10 min.	44±1	43±1	87±5	.001
20 min.	19±0	26±0	61±1	.001
40 min.	45±2	54±3	100±3	.001

* $5x10^{-4}$ M is used instead of $1x10^{-4}$ M.

Fig. 5

% Food uptake of B. calyciflorus as a function of acetylcholine concentration at 10, 20 and 40 minutes time.

● 10 min.

▲ 20 min

■ 40 min.

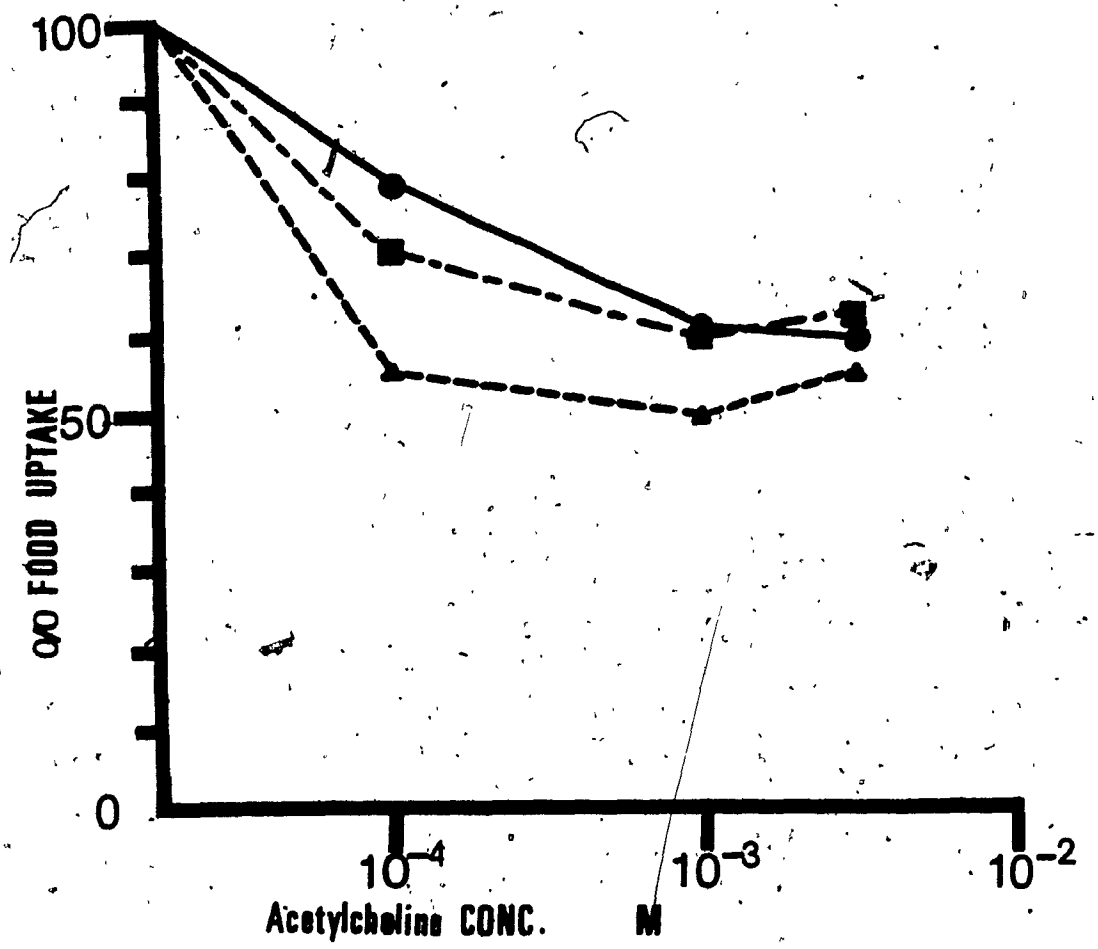

100
50
0
% FOOD UPTAKE
10^{-4}
10^{-3}
10^{-2}
Acetylcholine CONC. M

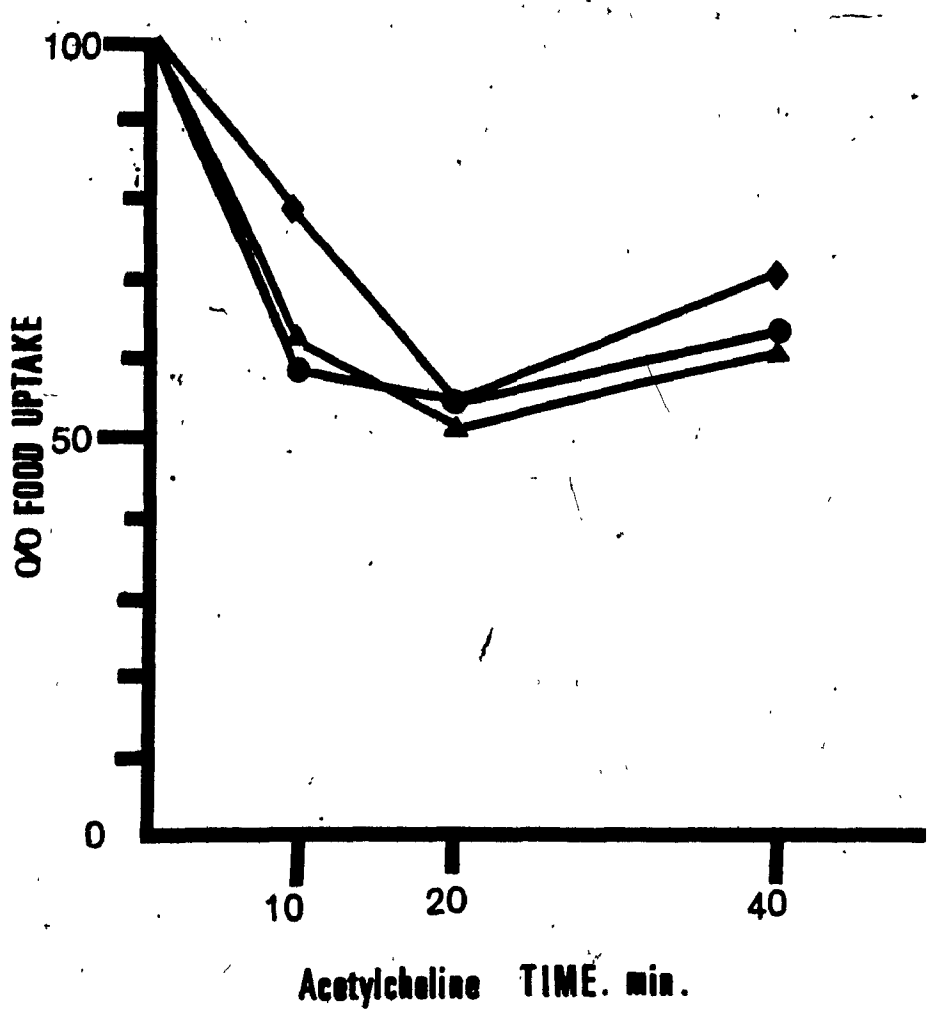
Fig. 6

% Food uptake of B. calyciflorus as a function of 10, 20 and 40 minutes incubation time in the presence of $5x10^{-3}$, $1x10^{-3}$, and $1x10^{-4}$ M concentration of acetylcholine.

● $5x10^{-3}$ M

▲ $1x10^{-3}$ M

◆ $1x10^{-4}$ M


100
50
0
% FOOD UPTAKE
10
20
40
Acetylcholine TIME. min.

Benactyzine

This compound showed a strong food uptake inhibition. Fig.8 shows that the effect of benactyzine is maximal and equal at 10 and 20 minutes incubation period. At concentration above $1x10^{-3}$ M, food uptake reaches its minimum before the animals die. Fig. 8 indicates that at 10 and 20 minutes incubation period the effect of benactyzine is maximal, whereas at 40 minutes, the animals seem to recover slightly. Microscopic observations have shown that this habituation period does not last long (often only 1-3 minutes) and the animals start falling down to the bottom of dish again. This pulsatile reaction might continue several times and finally ends in the death of the animals. The identical feeding inhibition at 10 and 20 minutes may be fortuitous, insofar as a minimum point of uptake may have been present in a continuous oscillatory change. Visual observation would not reveal this, since locomotion may be independent of food uptake, as indicated in connection with the ACh effect on page 51.

Fig. 7

% Food uptake of B. calyciflorus as a function of benactyzine concentration at 10, 20 and 40 minutes time.

● 10 min.

▲ 20 min.

■ 40 min.

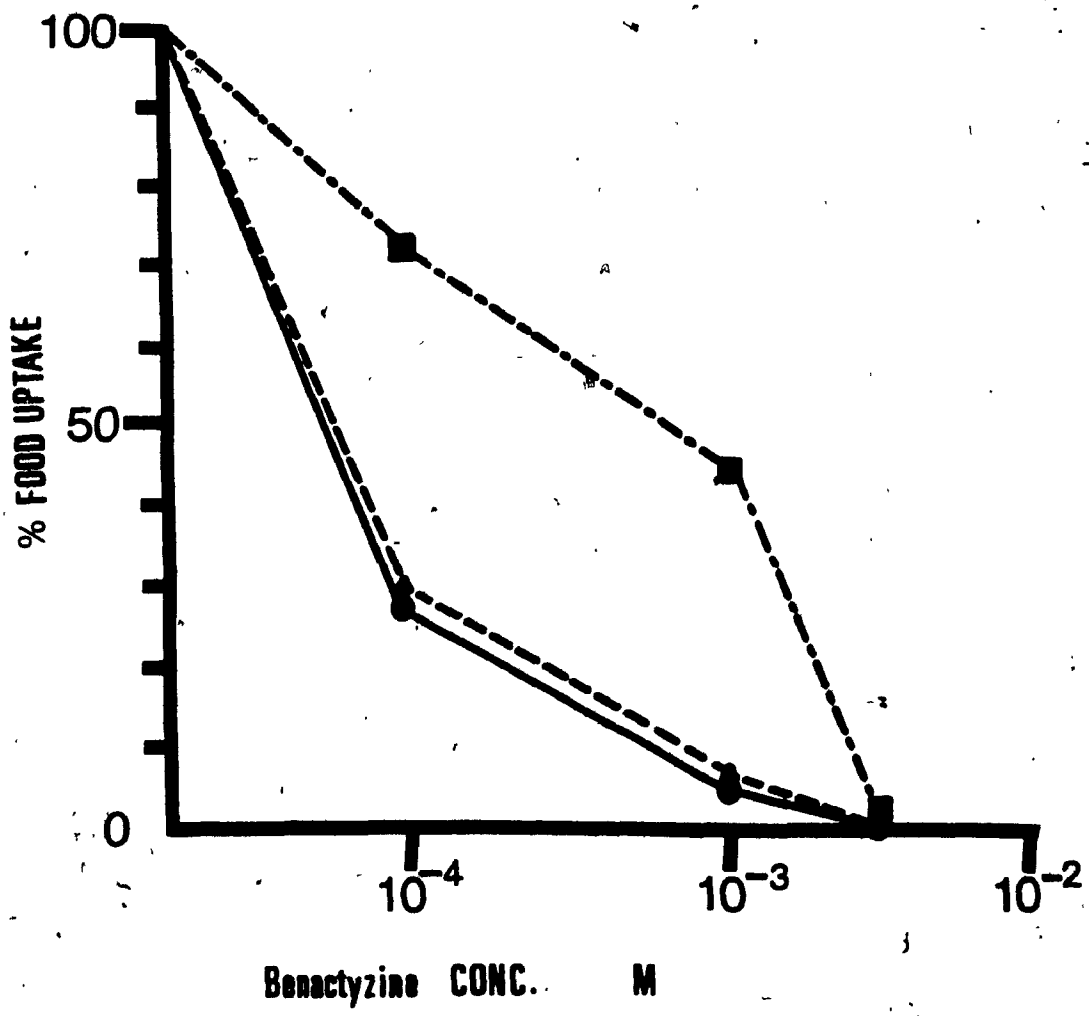

100
50
0
% FOOD UPTAKE
10^{-4}
10^{-3}
10^{-2}
Benactyzine CONC. M

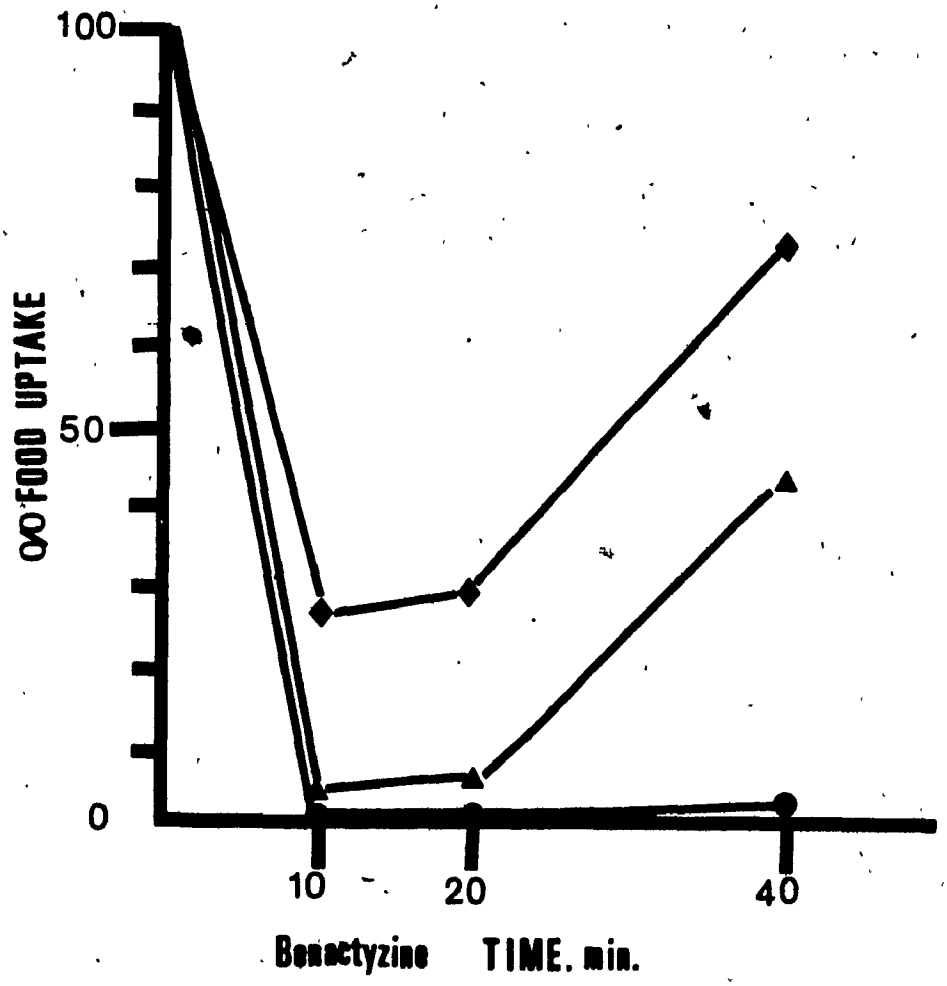
Fig. 8

% Food uptake of B. calyciflorus as a function of 10, 20 and 40 minutes incubation time in the presence of $5x10^{-3}$, $1x10^{-3}$, and $1x10^{-4}$ M concentration of benactyzine.

● $5x10^{-3}$ M

▲ $1x10^{-3}$ M

◆ $1x10^{-4}$ M


100
50
0
% FOOD UPTAKE
10
20
40
Benactyzine
TIME, min.

Homatropine

This muscarinic antagonist appears to have a minimal effect on food uptake. Fig. 9 and 10 shows that the percent inhibition of food uptake is only 10%. Fig.10 illustrates that the effect of homatropine is maximal at 20 minutes period, but does not appear to be different from 10 and 40 minutes periods. Only the dose-response curve measured at 10 minutes is statistically significant ($p < 0.001$). The 20 minutes effect is marginal ($p < 0.025$) and the 40 minutes effect is not statistically significant.

Fig. 9

% Food uptake of B. calyciflorus as a function of homatropine concentration at 10, 20 and 40 minutes time.

● 10 min.

▲ 20 min.

■ 40 min.

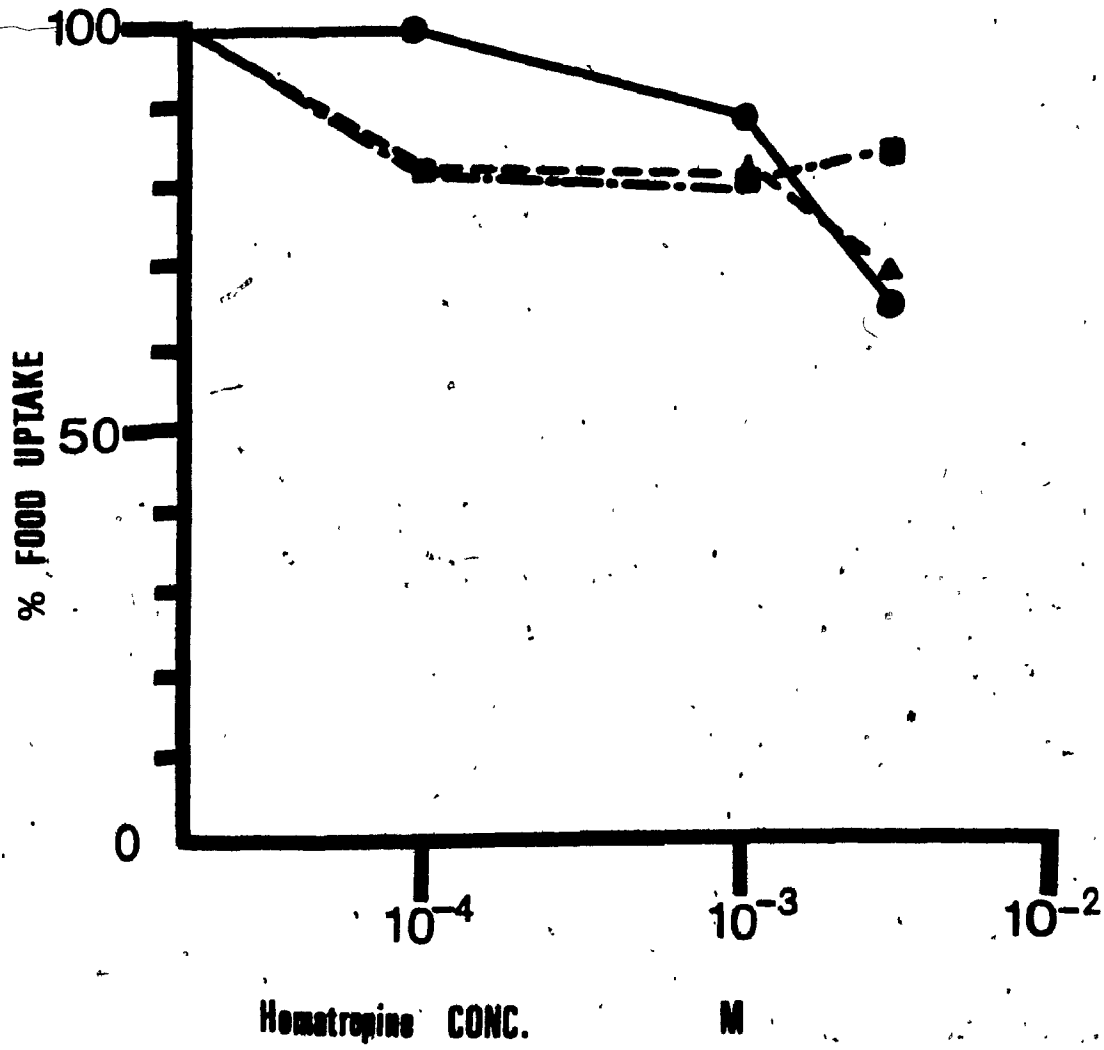

100
50
0
% FOOD UPTAKE
10^{-4}
10^{-3}
10^{-2}
Hematropine CONC. M

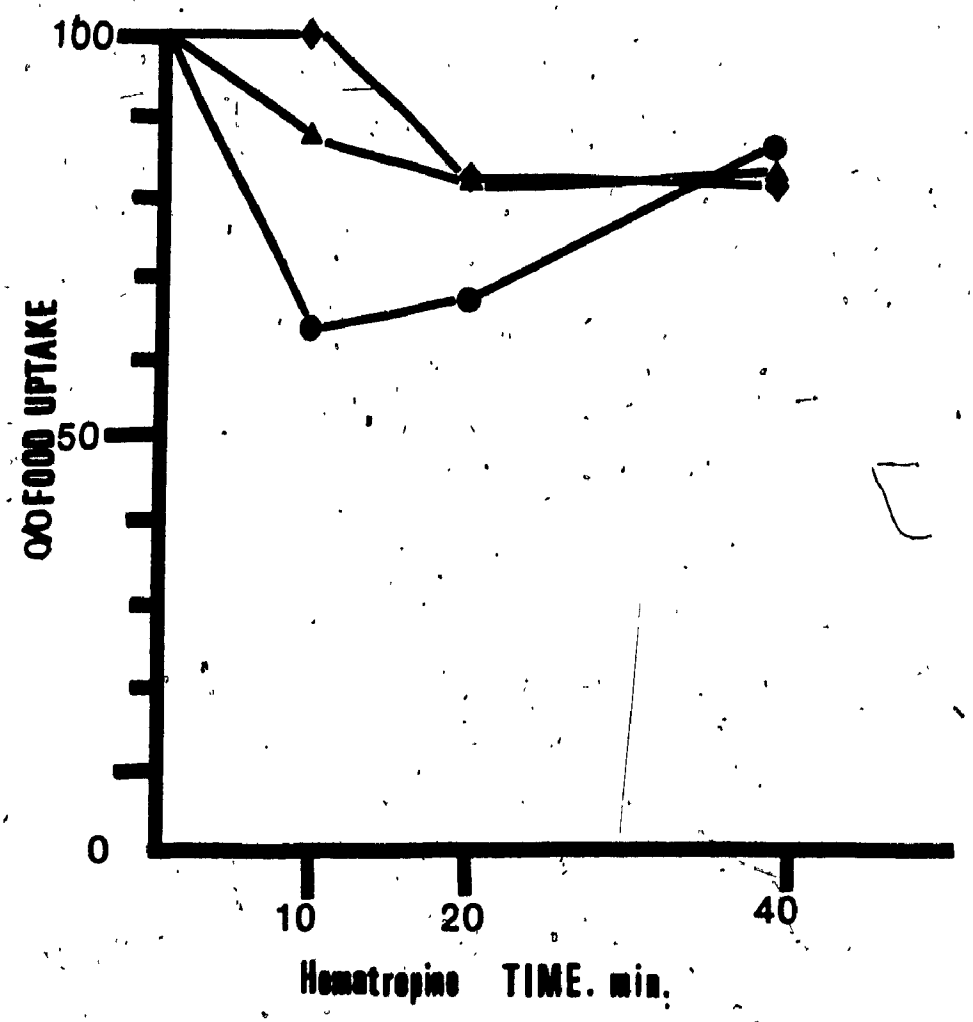
Fig. 10

% Food uptake of B. calyciflorus as a function of 10, 20 and 40 minutes incubation time in the presence of $5x10^{-3}$, $1x10^{-3}$, and $1x10^{-4}$ M concentration of homatropine.

● $5x10^{-3}$ M

▲ $1x10^{-3}$ M

◆ $1x10^{-4}$ M


100
50
0
% FOOD UPTAKE
10
20
40
Hematropine TIME. min.

Pempidine

It appears that this drug shows strong tachyphylaxis since at concentration 5×10^{-3} M and 20 minutes period the percent food uptake is almost zero. This drug has a strong pulsatile effect which will last for a few hours and finally will lead to collapse and death of the animals.

Fig. 11

% Food uptake of B. calyciflorus as a function of pempidine concentration at 10, 20 and 40 minutes time.

● 10 min.

▲ 20 min.

◆ 40 min.

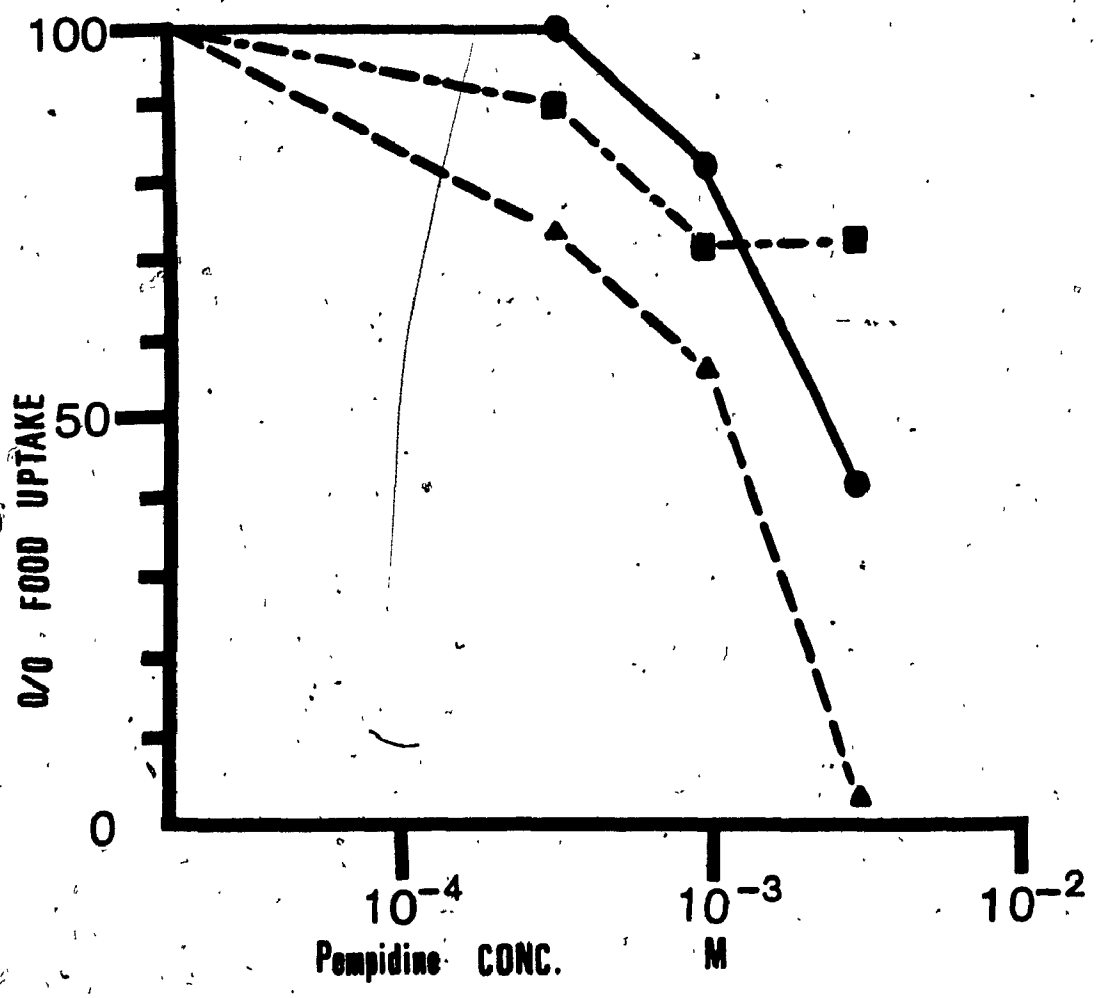

100
50
0
% FOOD UPTAKE
10^{-4}
10^{-3}
10^{-2}
Pempidine CONC.
M

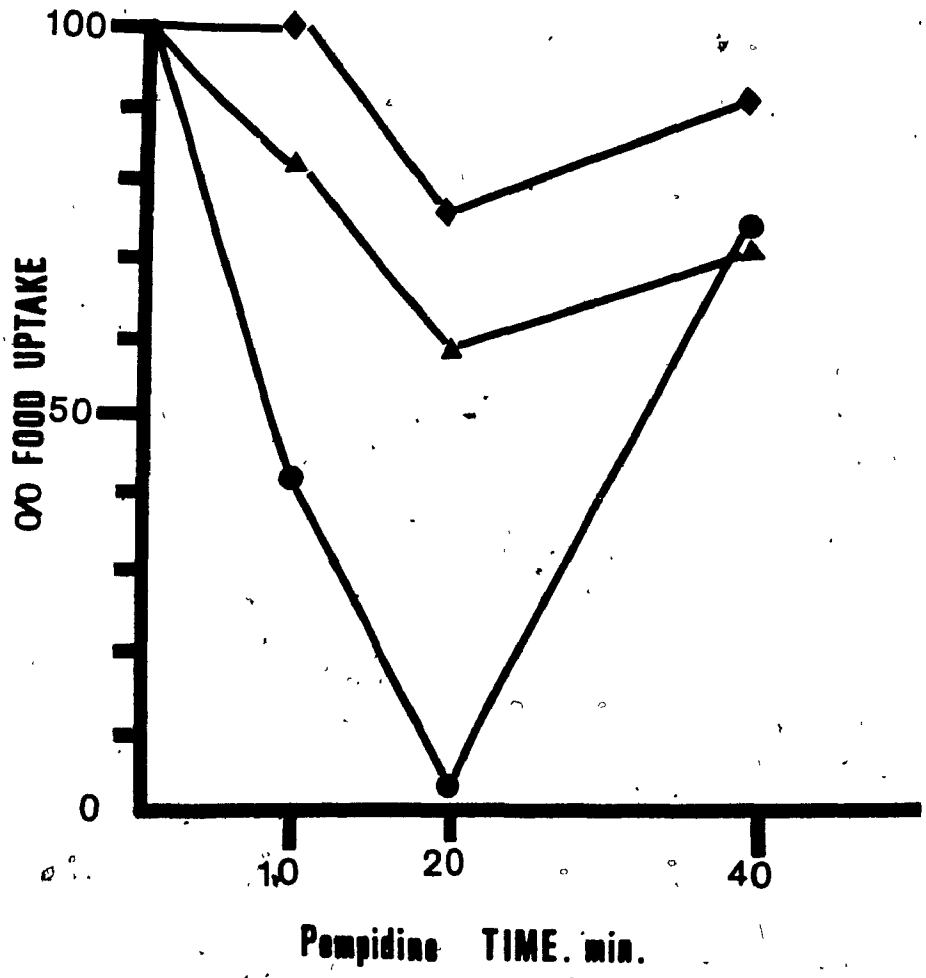
Fig. 12

% Food uptake of B. calyciflorus as a function of 10, 20 and 40 minutes incubation time in the presence of $5x10^{-3}$, $1x10^{-3}$, and $5x10^{-4}$ M concentration of pempidine.

● $5x10^{-3}$ M

▲ $1x10^{-3}$ M

■ $5x10^{-4}$ M


100
50
0
% FOOD UPTAKE
10
20
40
Pempidine TIME. min.

Hexamethonium

As shown on Fig. 14, food uptake decreases in a dose-dependent fashion at 10 minutes. At 20 and 40 minutes respectively, there is an overall decrease, but in a dose-independent way, suggesting a general pharmacological effect, not necessarily restricted to feeding. There seems to be a slight tachyphylaxis at high concentrations at the 20 minute mark, but a linear decrease of food uptake with time at the lowest concentration. This may suggest, that the above-mentioned general effect wears off at lower concentrations, and a more specific, feeding-related activity becomes visible. The available data do not allow further speculation.

Fig. 13

% Food uptake of B. calyciflorus as a function of hexamethonium concentration at 10, 20 and 40 minutes time.

● 10 min.

▲ 20 min.

◆ 40 min.

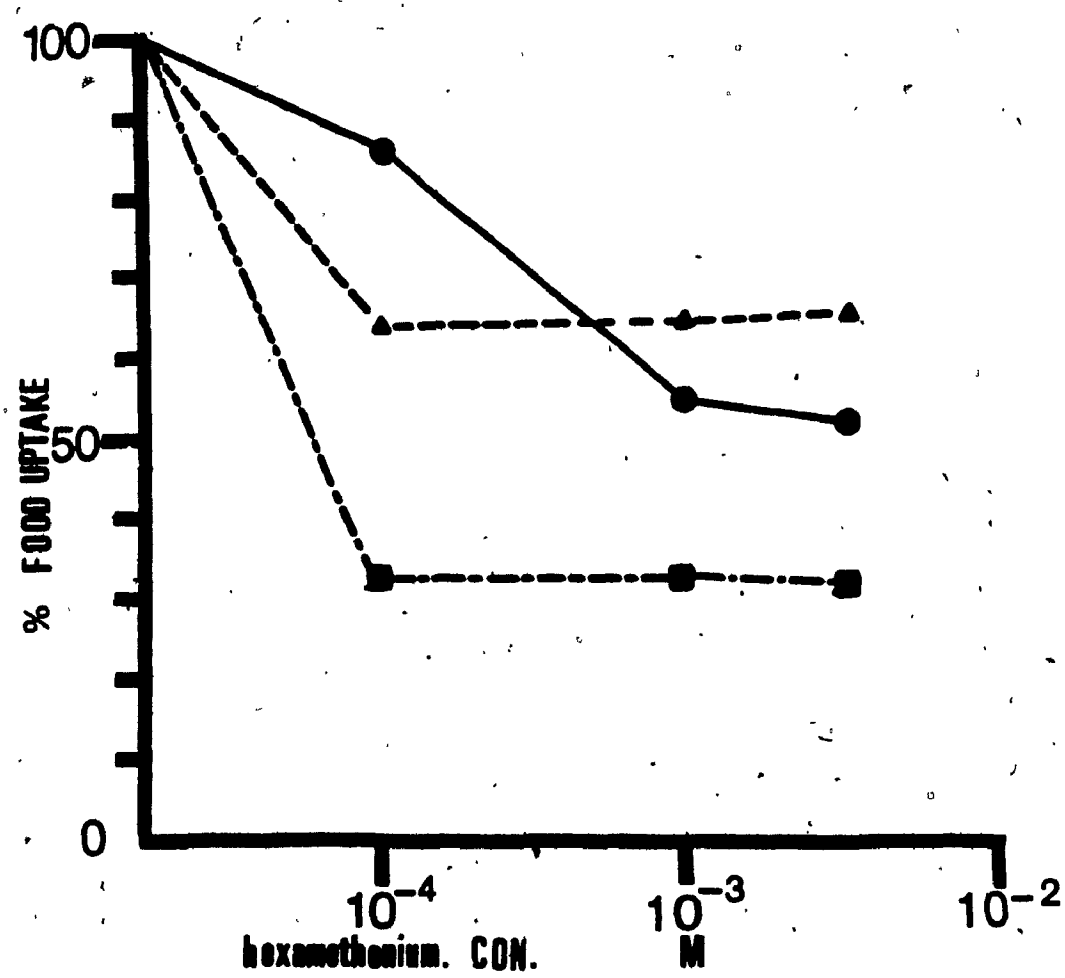

100
50
0
% FOOD UPTAKE
10^{-4}
10^{-3}
10^{-2}
hexamethonium. CON.
M

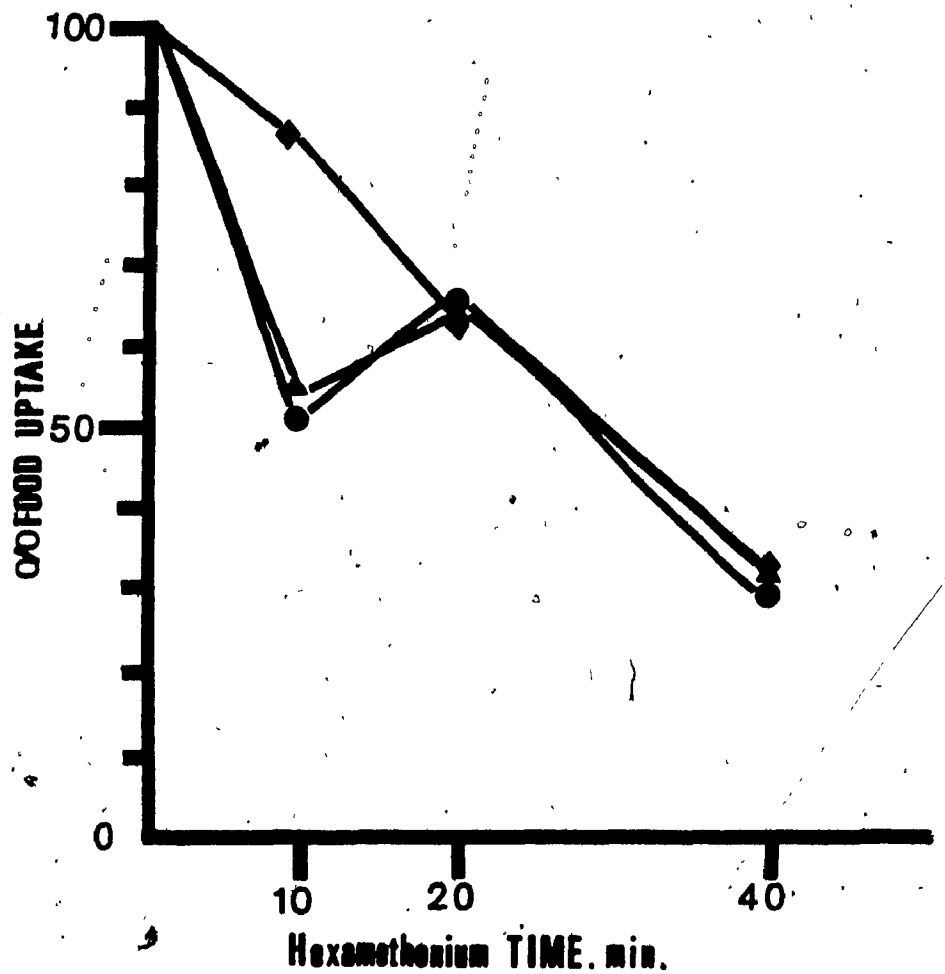
Fig. 14

% Food uptake of B. calyciflorus as a function of 10, 20 and 40 minutes incubation time in the presence of $5x10^{-3}$, $1x10^{-3}$, and $1x10^{-4}$ M concentration of hexamethonium.

● $5x10^{-3}$ M

▲ $1x10^{-3}$ M

■ $1x10^{-4}$ M


100
50
0
O/O FOOD UPTAKE
10
20
40
Hexamethonium TIME. min.

Decamethonium

This drug shows a different pattern from normal at 20 minutes (Fig.16). One reason for this might be that the first recovery period is at 20 minutes, therefore, the minimum effect seen is dose-independent. One may notice that this drug has a very immediate activity which paralyses the animals in the first minutes. Therefore, the maximal effect occurs at 10 minutes. This time-course of the drug effect is different from that shown by the other drugs. At $1x10^{-4}$ M, the drug is not active anymore, regardless of the incubation time.

Fig. 15

% Food uptake of B. calyciflorus as a function of decamethonium concentration at 10, 20 and 40 minutes' time.

● 10 min.

▲ 20 min.

■ 40 min.

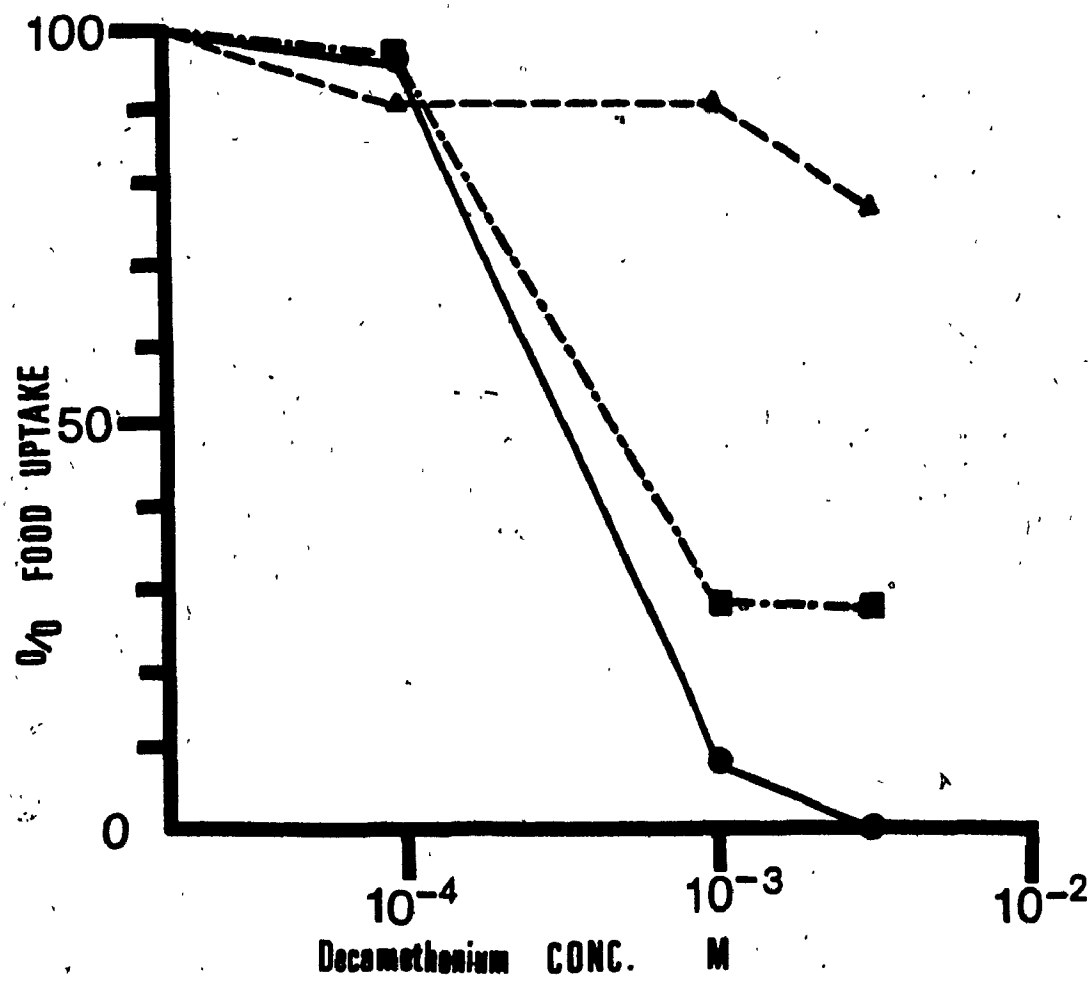

100
50
0
% FOOD UPTAKE
10^{-4}
10^{-3}
10^{-2}
Decamethonium CONC. M

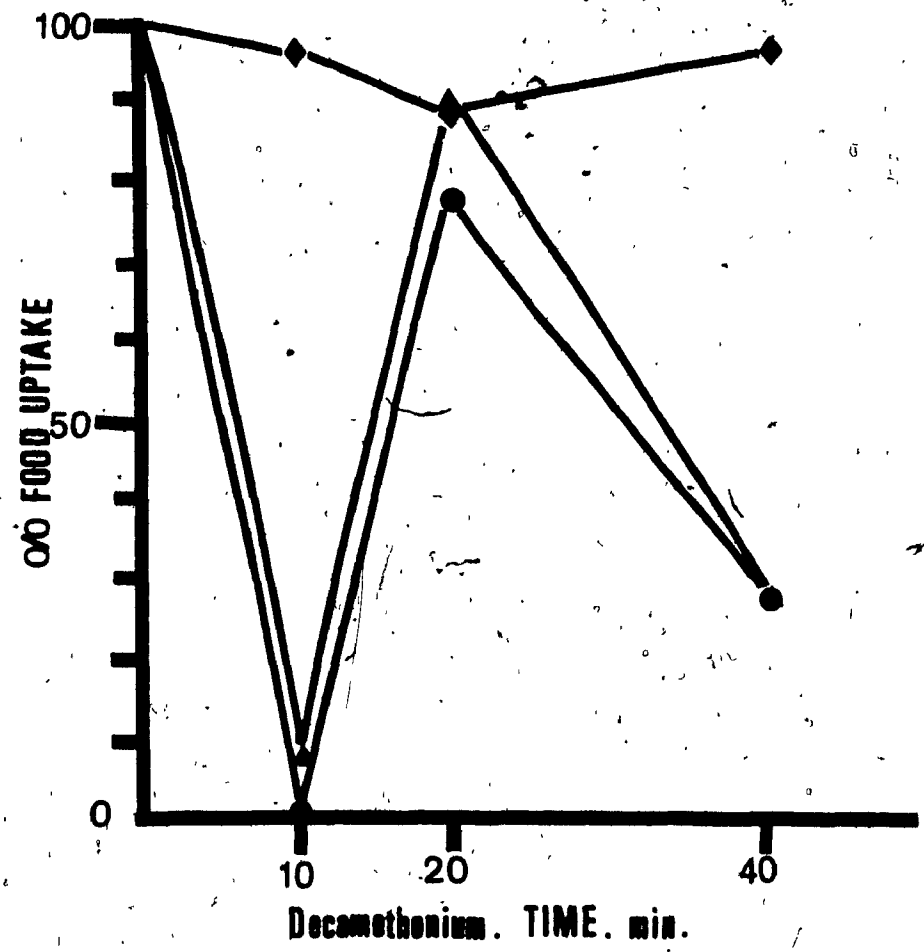
Fig. 16

% Food uptake of B. calyciflorus as a function of 10, 20 and 40 minutes incubation time in the presence of $5x10^{-3}$, $1x10^{-3}$, and $1x10^{-4}$ M concentration of decamethonium.

● $5x10^{-3}$ M

▲ $1x10^{-3}$ M

◆ $1x10^{-4}$M


100
50
0
% FOOD UPTAKE
10
20
40
Decamethonium. TIME. min.

Tubocurarine

Tubocurarine is a nicotinic "competitive" type neuromuscular antagonist. Fig. 18 illustrates that at 20 minutes period the drug will reach its maximal effect, in keeping with the general pattern seen. Considering the protracted effect of tubocurarine in mammalian systems, the rapid recovery period in *B. calyciflorus* is remarkable, and may indicate that inhibition of food uptake is not based on a nicotinic (persumably neuromuscular) effect but perhaps depends more on muscarinic receptors.

Fig. 17

% Food uptake of B. calyciflorus as a function of d-tubocurarine concentration at 10, 20 and 40 minutes time.

● 10 min.

▲ 20 min.

■ 40 min.

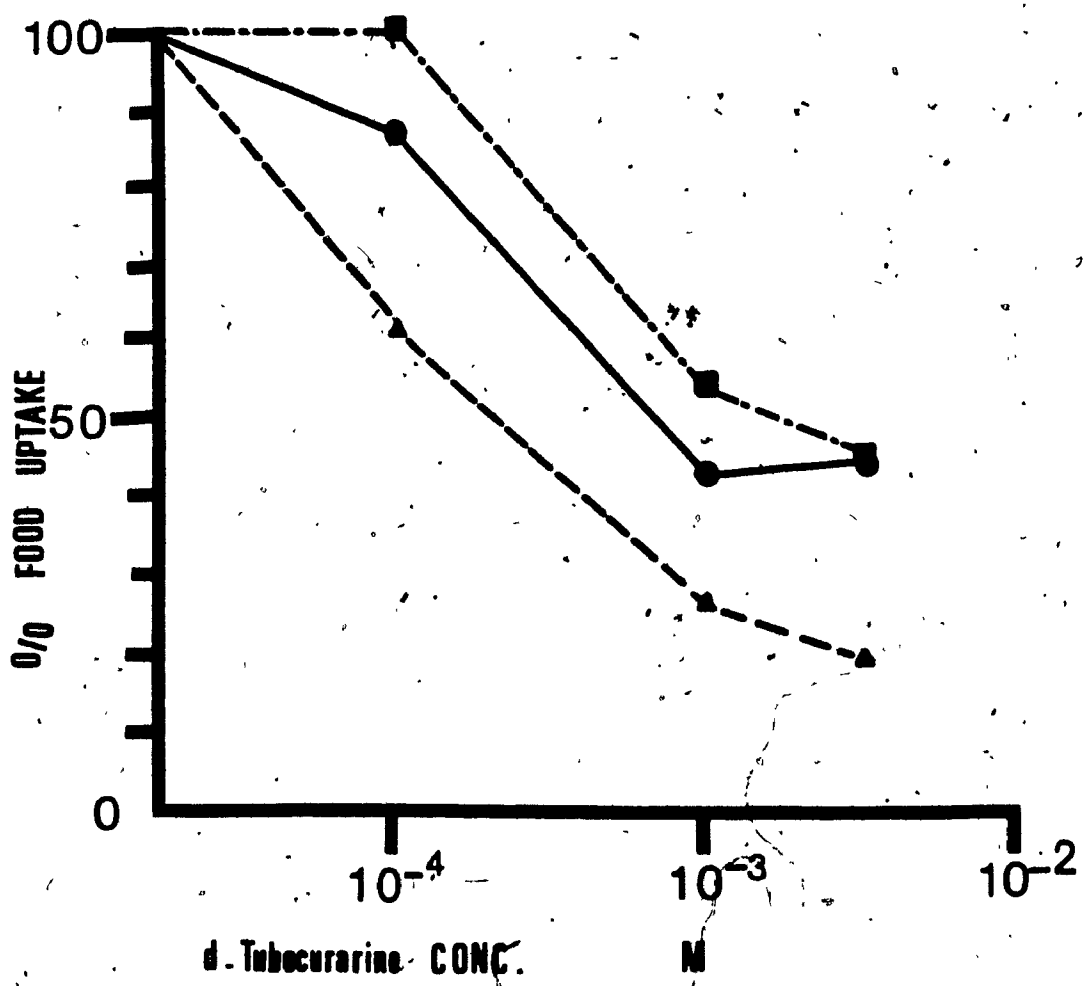

100
50
0
% FOOD UPTAKE
10^{-4}
10^{-3}
10^{-2}
d-Tubocurarine CONC. M

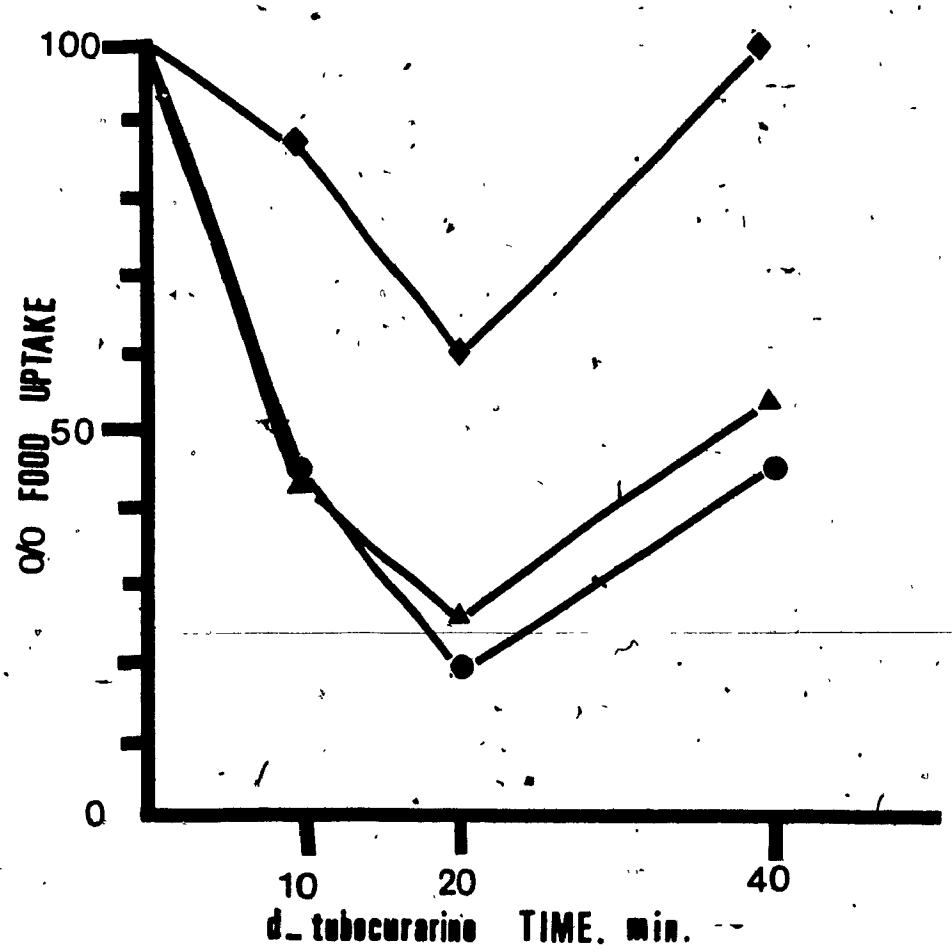
Fig. 18

% Food uptake of B. calyciflorus as a function of 10, 20 and 40 minutes incubation time in the presence of $5x10^{-3}$, $1x10^{-3}$, and $1x10^{-4}$ M concentration of d-tubocurarine.

● $5x10^{-3}$ M

▲ $1x10^{-3}$ M

◆ $1x10^{-4}$ M


100
50
0
% FOOD UPTAKE
10
20
40
d_ tubocurarine TIME. min.

Agonist-antagonist interactions

I investigated the quantitative interaction of two agonists with four antagonists, to see if antagonism, as seen in vertebrate systems, exists in lower invertebrates. The present as well as other investigations in this laboratory, and data shown by Leake and Walker (1980) seem to suggest, that this is not the case. We shall return to this topic in the Discussion.

Acetylcholine / homatropine

The presence of ACh at the highest concentration increased food uptake as compared to values without ACh. However, the ACh effect is not dose related. The data may also be interpreted as a homatropin antagonism of food uptake inhibition caused by ACh alone. However, in the absence of statistical significance, such speculations cannot be supported.

Acetylcholine / hexamethonium

Acetylcholine antagonizes the hexamethonium induced food uptake inhibition in a statistically highly significant fashion. What makes these data suspect is the fact, that food uptake at the lowest ACh concentration is only 17%, while in other series it was 51% in the absence of

ACh. Since reproducibility between replicate series was normally good, this discrepancy cannot be explained, because variance within data points was low.

Carbachol / pempidine and carbachol / benactyzine

I wished to investigate if other cholinergic agonists were capable of antagonizing the effect of anticholinergic drugs. Since carbachol is known as a primarily muscarinic agonist, its interaction with benactyzine, a muscarinic blocker, was of special interest. However, the result was another surprize in this unconventional field of rotifer pharmacology. While both carbachol and the antagonists proved to be nontoxic in the concentrations used when investigated during our qualitative experiments, in combination they killed the animals at all concentrations. Thus, instead of antagonism, these drug combinations showed a synergistic increase in toxicity.

Table 3

Percent food uptake ± s.d. of *R. calyciflorus* after simultaneous incubation in a mixture of 5×10^{-3} M antagonist and varying agonist concentration, for 10 minutes.

Molar Conc. of agonist / Agonist - 5×10^{-3}M antagonist	5×10^{-3}	1×10^{-3}	1×10^{-4}	p
Acetylcholine / Homatropine	93±4	91±4	88±7	N.S.
Acetylcholine / Hexamethonium	46±3	18±1	17±0	.001
Carbachol / Pempidine	00±0	03±0	03±0	-
Carbachol / Benactyzine	00±0	00±0	03±0	-

Fig. 19

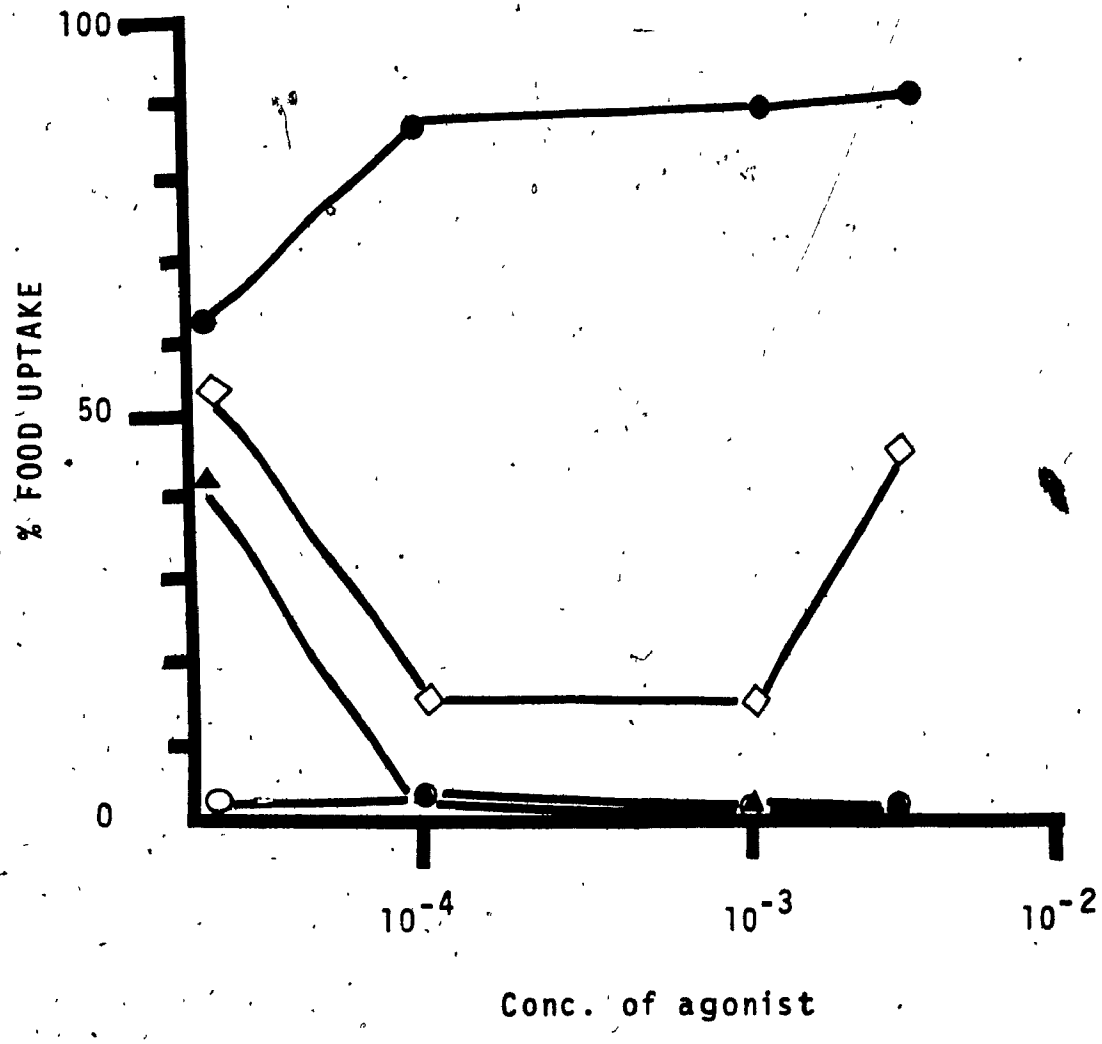
% Food uptake of B. calyciflorus in the simultaneous presence of agonist/antagonist after 10 minutes incubation. The concentration of antagonist was kept at 5×10^{-3} M while agonist concentration was varied.

● Acetylcholine / Homatropine

◇ Acetylcholine / Hexamethonium

▲ Carbachol / Pempidine

○ Carbachol / Benactyzine


100
50
0
% FOOD UPTAKE
10^{-4}
10^{-3}
10^{-2}
Conc. of agonist

DISCUSSION and SUMMARY

As stated in the introduction, the purpose of this investigation was to contribute to answers to the following questions:

1. Is the response of rotifers to cholinergic drugs similar to the effects known from classical vertebrate or invertebrate pharmacology?

2. Using food uptake as a quantitative model, how do cholinergic drugs influence feeding?

3. Are there any other physiological or behavioral changes observable upon exposure to these drugs?

The answer to the first question seems to be the rather unsurprising fact, that vertebrate pharmacology is not applicable in lower invertebrates. Since feeding as a model was not previously used in invertebrate neuropharmacology, comparison with other invertebrate phyla is not possible either at this time. Similarly, since the present investigation is the first of its kind, it cannot even attempt to answer any questions regarding details on rotifer neurophysiology.

The second question could be answered more satisforily. The results, that form the principal body of my Thesis, are

summarized in the subsequent paragraphs, and in a concise form in the Sumary as well.

The third question pertained to other, unforseeable and specific pharmacological effects that emerged during my observatiions. Several novel phenomena were observed: an oscillating tachyphylaxis (drug habituation) and a foot paralysis caused by muscarinic blockers.

The general inhibitory effect of chp;omergoc drugs on feeding and the above mentioned unusual and physiologically unfavourable drug effects intimate, that any environmental pollutant acting through a cholinergic mechanism (e.g. an insecticide like malathion) could have a serious effect on the rotifer population in a natural environment, and unbalance the aquatic food chain of which rotifers are an integral part. Thus the ecotoxicological implications of the present work warrant further investigation (Nogrady and Keshmirian, 1986).

The paucity of any previous information on rotifer pharmacology was a definite drawback in our investigations. The two previous papers in the literature (Marriott et al., 1948; Lindner and Goldman, 1964) were either obsolete or restricted to specific phenomena (oviposition) and non-

systematic. Thus, our first task was to develop the methodology suitable to follow rotifer pharmacology in some way. Such a problem does not arise in vertebrate pharmacology, where an infinite wealth of sophisticated, specific test methods exists, and the difficulty lies in the choice of the most appropriate one. Our problem was compounded by scant information on rotifer biochemistry and systems physiology as well. Eventually, we utilized the investigations of Gilbert, Starkweather and their collaborators, who thoroughly and systematically explored the feeding biology of a common rotifer, Brachionus calyciflorus. These authors used radioactively labelled algae and yeast cells to follow food uptake in B. calyciflorus. We modified their method extensively to suit our purpose, and eventually developed it into a highly reproducible test with a low variance. Once this time-consuming developmental work was accomplished, we started to follow food uptake in B. calyciflours under the influence of a systematically chosen series of cholinergic drugs.

One might argue that feeding is too complex a phenomenon to serve as a pharmacological "test", and we agree with that evaluation. Feeding in rotifers is a composite result of locomotion, creation of feeding currents, chemoreception with resultant acceptance or rejection of food particles, and mastication. It would

have been impractical, however, to begin developing an entirely new test system, when nothing was known about rotifer behavioural pharmacology. Nevertheless, we searched constantly for new leads on useful systems, and explored qualitatively effects, that eventually could be evaluated quantitatively, and found some interesting and unusual phenomena. Some of these are now under investigation in this Laboratory.

There are two novel findings that have been uncovered during these investigations, unconnected to the central problem of food uptake regulation. Both are without precendent in pharmacology. The first one is a peculiar selective paralysis of the foot in B. calyciflorus.

Foot paralysis was shown by exposure of the animals to benactyzine, a muscarinic blocker. Subsequent investigations have shown (Nogrady and Keshmirian, in preparation), that other muscarinic cholinergic blockers like atropine (but not the short-acting homatropine) and even beta-adrenergic blockers like propranolol also show the same phenomenon. Acetylcholine antagonism (or lack of it) is just as peculiar as the phenomenon itself, and is discussed below.

The second unprecedented pharmacological phenomenon uncovered was an oscillating tachyphylaxis. Tachyphylaxis, rapid drug habituation, is usually explained by the uncoupling of the chemorecognitive part of a receptor from the effector, e.g. an ionophore. It may also be the result of neurotransmitter depletion of a synapse, and consequent inability to respond to receptor activation. There are many examples for both mechanisms in the literature (Nogrady, 1985; Goth, 1984). Nowhere, however, have we found a report on rapid recovery from tachyphylaxis, followed by several more tachyphylactic episodes, eventually leading to death caused by the acute toxicity of the drug. Such oscillating tachyphylaxis was shown by the muscarinic blocker benactyzine, by the ganglionic blocker pempidine, and by the neuromuscular blocker decamethonium. At appropriate concentrations, they will immobilize the test animals, followed by recovery, and possibly another two or three episodes of paralysis, and finally death, all within 60-90 minutes only. For unknown reasons, other similar drugs (e.g. the ganglionic blocker hexamethonium) shows no such effect. We are at a loss to explain this phenomenon.

The quantitative investigations on food uptake in B. calyciflours also led to some interesting results. The effect of the agonist and neurotransmitter acetylcholine (ACh) itself was rather remarkable. ACh is an excitatory

neurotransmitter, and penetrates the neuronal membranes of rotifers easily, even though it is a quaternary compound, that would not reach the CNS of vertebrates from an external solution. However, rotifers do not possess a "blood-brain barrier", as first shown by Lindner and Goldman (1964) and proven by many experiments in our Laboratory. Therefore, one might have expected an excitatory effect, manifesting itself either in convulsions or at least in an increase in feeding. However, surprisingly, a nonspecific decrease in feeding was seen. This effect is considerable (about a 40% decrease), but is dose-independent, and does not change during 40 minutes, after its onset in the first 10 minutes of exposure. There are no other discernible effects, and measurements of klinokinesis (change in rate of movement) showed no difference between animals treated with unphysiologically high ($5x10^{-3}$M) doses of ACh, and control. In other chronic experiments it has been shown, that B. calyciflorus stays alive in the presence of $1x10^{-3}$M ACh for three days or more. Thus one might speculate, that cholinergic effects of feeding are not due to changes in locomotion or generation of feeding currents, but to affecting chemoreception or mastication. If this could be proven directly by another method, the neuro-pharmacology of rotifer feeding could be pinpointed more precisely. However, we made no further progress on this important finding.

Instead, we concentrated on the effect of individual anticholinergic drugs on food uptake. As shown on Table 2, all six anticholinergic drugs decreased feeding, most of them in a statistically highly significant dose dependence ($p < .001$). Homatropine and hexamethonium were the exceptions, and acted weakly. It is remarkable that other drugs belonging to the same groups (antimuscarinics and ganglionic blockers) are highly active. Thus, it is not possible to decide that "feeding" is regulated by a particular neuronal pathway or receptor group. Considering the complexity of feeding - even if we discount ciliary involvement - this is not surprising, and a drawback of our model.

If one also considers that the agonist ACh, as well as <u>all</u> antagonists act as inhibitors, one has to conclude that the neuronal system of rotifers is so primitive, that a distinction between agonists and antagonists, or muscarinic and nicotinic receptors is not possible. Such a hypothesis was proposed by Leake and Walker (1980) and corroborated by an investigation in this Laboratory (Nogrady and Keshmirian, in press) in connection with inhibition of oviposition by ACh in an other species of rotifer, <u>Philodina acuticornis</u>.

The phenomenon of tachyphylaxis was also observable in the feeding experiments. All the compounds that showed oscillating tachyphylaxis in the qualitative experiments also showed tachyphylaxis in feeding. This manifested itself in minimum food uptake values at 10 or 20 minutes exposure, and partial recovery at 40 minutes. Since at longer, or in-between times, food uptake was not measured, oscillation was not observable.

In any pharmacological investigation agonist - antagonist interactions are of great interest in exploring neurophysiological correlations. Thus we tried some experiments along that line, even though the previously outlined primitive recognition capabilities of the rotifer neuronal system were likely to interfere. Indeed, the results were not straightforward. An ACh - homatropine antagonism, while present, was not dose related; but it has to be recalled that the homatropine feeding inhibition was barely significant in itself. The ACh - hexamethonium antagonism was highly significant, but the absolute values were somewhat lower than expected on the basis of earlier experiments. In the other two antagonism experiments the agonist, carbachol, showed a synergistic toxic effect instead of antagonism.

In the qualitative ACh - benactyzine antagonism, ACh did not show true antagonism when administered simultaneously. However, it was capable of overcoming the benactyzine caused "anesthesia" and foot paralysis more rapidly than mere transfer into pure medium, if administered consecutively. Such an effect is, however not a classical antagonism, but only a flip-flop of an ionophore, depending on the prevailing drug. Results like these further strengthen the hypothesis proposed by Leake and Walker (1980) and Nogrady and Keshmirian (1986), that the cholinoceptor is merely a "lock" on the ionophore, and is capable of mediating effects in rotifers nonspecifically only.

SUMMARY

1. The effect of cholinergic drugs was investigated on the rotifer Branchionus calyciflorus in qualitative studies, and in quantitative feeding experiments. The food source was the yeast Rhodotorula glutinis, labelled with ^{3}H-glucose. Food uptake was measured by scintillation counting of 20 rotifers per group in triplicate.

2. The qualitative investigations uncovered two novel phenomena. One was a specific foot paralysis shown by some muscarinic blockers. The other effect was an oscillating tachyphylaxis, alternate "anesthesia" and recovery caused by some of the anticholinergics used. Several oscillations occurred within 60-90 minutes, ending in the death of the animals. No explanation for these phenomena is offered at this time.

3. Acetylcholine in itself shows a dose- and time-independent inhibition of food uptake up to 40%. No other physiological phenomenon is influenced, and it is assumed that ACh acts through chemoreceptors or the mastax ganglion.

4. Two each of muscarinic antagonists, ganglionic blockers, and neuromuscular blockers were explored in food uptake inhibition. All compounds acted as inhibitors to approximately the same extent, no distinction between them is possible.

5. The same antagonists that showed oscillating tachyphylaxis in the qualitative experiments, also showed tachyphylaxis in the quantitative feeding experiments. This was manifested in the increase after 40 minutes in % food uptake after a minimum uptake shown at 10 or 20 minutes exposure.

6. Some of the antagonist effects could be eliminated by Ach, but the experiments are not conclusive. Carbachol, a muscarinic agonist, showed a synergistic toxicity if co-administered with some anticholinergics.

7. It is concluded, that the cholinoceptors of rotifers are nonspecific and primitive, and act only as a lock on the ionophore.

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APPENDIX

Raw data of feeding experiments.

Numbers shown are dpm of ^{3}H.

Time (min.) / Conc. (M)	10 min.				20 min.				40 min.			
Acetylcholine												
$5x10^{-3}$	364	274	371	295	330	301	340	283	339	-	368	343
$1x10^{-3}$	317	297	323	399	298	291	295	277	391	285	298	398
$1x10^{-4}$	405	402	395	438	323	327	298	301	433	369	328	410
MBL	480	508			510	473			505	519		
Control	83				92				88			
Benactyzine												
$5x10^{-3}$	102	97	79	98	81	99	99	101	89	105	89	101
$1x10^{-3}$	154	140	-	109	-	195	175	179	319	347	312	358
$1x10^{-4}$	524	487	598	512	510	403	464	431	485	-	511	456
MBL	1534	-			1252	1301			642	637		
Control	98				91				84			
Homatropine												
$5x10^{-3}$	-	1099	1044	949	1238	1132	1252	1079	1357	1213	1307	1427
$1x10^{-3}$	1395	1532	1347	1447	1377	1458	1279	1502	1274	1345	1237	1325
$1x10^{-4}$	1493	1623	1535	1540	1453	1244	1374	1529	1238	1234	1259	1323
MBL	1500	1496			1700	-			1520	1601		
Control	108				90				94			

Time (min.) / Conc. (M)	10 min.				20 min.				40 min.			
Pempidine												
$5x10^{-3}$	566	486	365	343	224	180	148	-	748	701	782	693
$1x10^{-3}$	871	886	540	-	387	395	466	382	748	673	771	688
$5x10^{-4}$	867	1079	769	-	507	548	466	372	-	898	793	902
MBL	902	-			663	481			950	-		
Control	115				173				120			
Hexamethonium												
$5x10^{-3}$	440	399	410	447	428	420	413	399	759	698	707	731
$1x10^{-3}$	423	462	416	459	402	423	399	416	756	680	698	822
$1x10^{-4}$	614	652	701	609	413	399	406	420	765	777	688	758
MBL	791	677			575	599			2150	2024		
Control	94				93				114			
Decamethonium												
$5x10^{-3}$	95	110	93	107	204	202	222	-	552	502	612	578
$1x10^{-3}$	158	142	139	148	245	225	234	220	609	531	502	582
$1x10^{-4}$	598	663	614	645	235	230	238	221	1714	1584	1531	1767
MBL	673	631			254	240			1688	-		
Control	99				77				135			

Time (min.) / Conc. (M)	10 min.				20 min.				40 min.			
d-tubocurarine												
$5x10^{-3}$	-	182	184	179	272	229	281	-	367	363	366	424
$1x10^{-3}$	188	181	175	179	341	329	321	334	461	457	-	393
$1x10^{-4}$	260	270	248	272	691	683	698	673	737	756	709	730
MBL	290	284			1069	1114			711	739		
Control	97				63				99			

	Acetylcholine/Homatropine				Acetylcholine/Hexamethonium			
$5x10^{-3}$	1385	1503	1478	1398	1019	907	807	1119
$1x10^{-3}$	1245	1459	1398	1512	523	396	395	442
$1x10^{-4}$	1295	1512	1265	1384	423	399	429	-
MBL	1602	1466			1984	-		
Control	109				85			
	Carbachol/Pempidine				Carbachol/Benactyzine			
$5x10^{-3}$	108	-	101	92	95	79	83	91
$1x10^{-3}$	135	112	115	130	61	107	65	107
$1x10^{-4}$	120	135	101	124	109	111	101	123
MBL	1082	1022			1096	986		
Control	90				83			

SUMMARY OF DRUGS USED

Acetylcholine: Acetylcholine is a neurotransmitter in all ganglia, the neuromuscular junction and the postganglionic synapses of the cholinergic (parasympathetic) nervous system. Both nicotinic and muscarinic receptors bind acetylcholine. Acetylcholine is normally an excitatory neurotransmitter, although it can occasionally show an inhibitory action in cardiac muscle.

Carbachol: A very potent choline carbamate having both muscarinic and nicotinic effects. Its only use at present is in the treatment of glaucoma. The antidote to carbachol is atropine.

Hexamethonium: The ganglionic blocking drug which is poorly absorbed in man. It is a bisquaternary compound with six methylene groups separating the two cationic groups.

Pempidine: A ganglionic blocking drug which is well absorbed from the gasterointestinal tract In large doses it may cause central nervous system stimulation and neromuscular blockade.

Benactyzine: Basically an anticholinergic drug with many central nervous system actions. It produces mydriasis and inhibition of

salivation. Its effects in humans are characterized by drowsiness and inability to concentrate.

Homatropine: In large doses, all of these anticholinergic agents have central excitatory and hallucinogenic effects. It differs from atropine only in the fact that it is an ester of mandelic rather than of tropic acid. It produces mydriasis fairly rapidly and commonly used in opthaemology.

Decomethonium: This drug binds normally to the Ach R and triggers the same response as Ach, a brief contraction of the muscle which is followed by a prolonged period of transmission blockage accompanied by muscular paralysis.

d-Tubocurarine: It is important neuromuscular blocking agent, about 1/3 administered is excreted unchanged in urine, whereas the rest is metabolically altered.

FEEDING EXPERIMENT

20 rotifers per tube starved for 30 minutes in MBL medium

↓

Incubated in drug solution for 10, 20, or 40 minutes

↓

Drug solution drained, leaving 250 µl residue

↓ 25 µl labelled yeast suspension added containing >10 cells

Incubation in duplicate for 20 minutes
controls: No drug + yeast (normal feeding)
No drug, no rotifer, + yeast (to test efficiency of washing)

↓

Rinse all tubes with 3×5 ml medium

↓

Kill with ethanol
Digest with protosol
Count ^3H

↓

All experiments were done in triplicate

FLOW CHART FOR LABELLING YEAST

Yeast cultured on agar plate

washed and centrifuged 5000RPM

Supernatant discarded

Yeast cells

+ 1ml dist. H_2O counted

Yeast cell/ml

+ 2 cpm of 3H glucose added/yeast cell

(i.e. 1 ul of ^3H glucose contain 2.2×10 dpm)

+ incubated at 37 +shaken for 14-16 hrs

Labelled yeast

5× washed in 2.5ml H_2O

centrifuged 5000RPM

Supernatant (discarded)

Supernatant (discarded)

labelled yeast

add 1ml H_2O

Yeast cell/ml

stored at 0°C.