The interactions of pesticides with free-living protozoa

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THE INTERACTIONS OF PESTICIDES WITH FREE-LIVING PROTOZOA.

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SUMMARY

Axenic cultures of the ciliate protozoan Tetrahymena pyriformis and the amoeba Acanthamoeba castellanii were used in investigations of interactions with pesticides.

The sub-acute toxicity of 18 pesticides was assessed using small-scale liquid batch cultures in Repli-dishes and/or microtiter plates. Population growth was monitored by direct microscopic counts and/or Optical Density readings.

In general T.pyriformis was more sensitive than A.castellanii to the inhibitory action of pesticides. The phenylcarbamate herbicides (chlorpropham, propham and barban) were the most inhibitory of the compounds assessed. EC_{50} values for T.pyriformis with these chemicals were 2.9×10^{-6}, 7.5×10^{-5} and 1.2×10^{-5} Moles respectively. None of these chemicals inhibited growth of A.castellanii to the extent of 50%. Chronic toxicity tests (14d) in larger batch cultures (250ml) showed inhibition of population growth to occur at concentrations of these compounds theoretically occuring in the 'field' through normal application rates. The inhibitory effects of the phenylcarbamates on population growth were reversible. Transfer of A.castellanii cells from medium containing propham (70-90 $\mu g ml^{-1}$) to fresh herbicide - free medium induced a tendancy to synchronous division.

Morphological and cytological effects of the phenylcarbamates on T.pyriformis included: cessation of food vacuole formation, cell rounding, de-ciliation, decreased contractile vacuole output and (at 20 μgml^{-1} chlorpropham) the occurrence of 'giant' vacuoles. Similar effects were observed in chlorpropham-treated A.castellanii cells along with the development of an enlarged cell envelope. Chlorpropham (4-20 μgml^{-1}) induced structural changes in oral ciliature, mitochondria and the nucleus of T.pyriformis in under 24h.

Exposure of T.pyriformis to chlorpropham (4-20 μgml^{-1}) for 6h inhibited cell motility, induced periodic ciliary reversals and caused behavioural changes. The phenylcarbamates had no detectable effect on respiration in T.pyriformis but slightly retarded encystment and excystment in A.castellanii. Protozoan transformation of these herbicides to aniline compounds was insignificant.

The effects of the phenylcarbamates on protozoa appear to be consistant with their action as antimitotics and as inhibitors in other cell systems.

CONTENTS

MATERIALS AND METHODS

INTRODUCTION

1.0 Pesticides

Organisms in opposition to the agricultural, industrial, amenity or health requirements of man are controlled by pesticides. Although chemical methods predominate, biological and physical methods are also employed. (Edson, Noakes & Sanderson, 1968).

The world pesticide market is estimated to be worth $12.8 billion (Anon, 1985). Of the 4,400 pesticide products available throughout the world 650 have been approved for use in the UK (Norman, 1985).

Pesticides are grouped according to the target pest they are designed to control. Of the 15 major types of pesticides, herbicides, insecticides and fungicides are the most important, with the sales market being dominated by herbicides.

Early attempts to chemically control pests made use of general poisons, such as copper, mercury and arsenic (Sly, 1977). However, the era of modern synthetic pesticides started just after the second world war. New possibilities were revealed by the discovery of the insecticidal properties of DDT in 1939, the new organophosphorous compounds, and the selective herbicidal properties of 2,4,dichlorophenoxyacetic acid, 2,4-D

(Edson et al.,1968). In the last 45 years a vast chemical arsenal, mainly of organic compounds, have been synthesised, of which some 203 active ingredients have been approved for use in the United Kingdom (Anon, 1984).

1.1 The United Kingdom Pesticide Safety Precaution Scheme

The regulation of the use of pesticides in the UK is in the hands of a non-statutory body, the Pesticide Safety Precaution Scheme, PSPS. It is a formal agreement between trade associations and government departments (PSPS, 1979). Both sides have undertaken to provide for all pesticide products to be cleared by the Ministry of Agriculture, Fisheries and Food (MAFF) before being introduced onto the UK market. The object of the PSPS is to safeguard human beings, livestock, domestic animals, beneficial insects, wildlife (ie non-target flora and fauna) and the environment generally against risks which could arise from the use of pesticides.

A similar body, the Environmental Protection Agency (EPA) exist in the USA. However, unlike the PSPS the EPA is a federal organisation backed by extensive legislation.

Products undergo a 4-stage clearance procedure before they are marketed and only those compounds cleared by the PSPS are placed in the Agricultural Chemicals Approval Scheme,

ACAS. However, this scheme concerns itself with the biological efficiency of the products and not their safety (Temple, 1978).

In their 1979 guidelines, the PSPS suggest that manufacturers should observe the product's effects on the biology of the soil, considering especially its effects on the overall microbiological activity i.e. total respiration, nitrification and the rate of organic matter decomposition.

1.2 Pesticides in the soil

Pesticides enter the soil by direct or indirect routes (Hill & Wright, 1978). The majority of these chemicals present in the soil originate from deliberate applications but include some which miss their targets or fall on the soil as drifts during spraying (Edwards, 1966). Pesticides entering the soil will normally be at, or just below, the recommended field application rate. This is usually in the order of 0.5 - 5kg ai ha^{-1} which, assuming even distribution in the top 10cm of soil, approximates to a concentration level of 0.5 - 5 $\mu g\ g^{-1}$ of soil (Hill & Wright, 1978).

The pesticide formulations can be applied in a variety of ways. As sprays, both emulsifiable and suspension concentrates, wetable powders, dusts, seed dressings,

granules and in enclosed spaces as smoke (Temple, 1978). The chemical formulation of a pesticide affects the localization of the active material, its speed of release, persistence and the duration of its exposure to the target organism(s) (Newman, 1978). Modifications in formulation and application methods have led to more economical use of pesticides by reducing the target dose efficiency ie the smallest quantity of active ingredient reaching and controlling the target organism. Although such economic measures have ecological implications, reducing the amount of chemical available to interact within the environment, they do not totally restrict the biological activity of a pesticide. Thus, once introduced into a soil ecosystem a pesticide can interact with both target and non-target organisms.

2.0 Interaction of pesticides with non-target soil microorganisms

Soil microorganisms are in intimate contact with their environment. Pesticides are not inert in such environments (Pfister, 1972) but have the potential for disturbing microbial processes. The effects of pesticides on non-target microorganisms have been reviewed at length by Anderson (1978). In general pesticides may precipitate shifts in microbial populations, reducing total numbers,

groups or species (Tu & Miles, 1976); inhibit metabolic activity, interrupt nutrient cycles and disrupt ecological associations (Domsch, 1972) and be degraded or accumulated by the organisms themselves (Cairns, 1983). Many chemicals, even at normal field application rates, have some undesirable effects on soil microbiology (Anderson, 1978).

Many reviews on the interaction of pesticides with microorganisms are of a general nature, their extent limited by the chronology of pesticide introduction (eg Wensley, 1953; Fletcher, 1960; Bollen, 1961; Audus, 1970; Pfister, 1972; Tu & Miles, 1976; Hill & Wright, 1978; Ruplal & Saxena, 1980; Ruplal, 1982). Most deal with edaphic locations, although Ware & Roan (1970) were solely concerned with aquatic environments.

Throughout each review the interactions of pesticides with protozoa are omitted or, at best, sparsely documented, eg Edwards, (1978). The interactions of pesticides with free-living protozoa remain one of pesticide microbiology's most neglected areas. Throughout this thesis emphasis is given to the interaction of pesticide with free-living soil protozoa.

3.0 Soil Protozoa

Free-living protozoa occur in soils throughout the world (Sandon, 1927). However, their numbers vary between soil ecosystems (Stout, 1973) and generally decline with depth (Viswaneth & Pillai, 1968),normally being confined to the top 10cm (Sleigh, 1973). In the active state protozoa are well adapted for existing in the soil/water interface of soil colloidal particles (Bamforth, 1973) with some species adapting to periodic adverse conditions by encysting (Sleigh, 1973).

3.1 The role of protozoa in the soil

In 1914 evidence was published that soils contain large numbers of active protozoa repudiating the previously held view that free-living protozoa existed in active forms only in definite accumulations of water in the soil (Martin & Lewis, 1914). Free-living protozoa have now been shown to be associated with virtually all phases of terrestrial ecosystems (Clarholm, 1983) but most commonly in the surface horizons of the soil (Stout, 1973).

The 'Protozoan' theory of soil fertility. The concept of protozoan involvement in soil fertility was proposed by Russel & Hutchinson (1909) to attempt to explain a role for soil protozoa whereby through their consumption of

bacteria, soil protozoa limited bacterial processes within the soil and therefore had a detrimental effect on the growth of field crops. Thus, by partial sterilization, the soil protozoa would be removed and the remaining bacteria allowed to multiply and eventually increase crop yield. The theory was disputed (Goodey, 1914; Russel, 1915) until Skinner (1927) proposed that the protozoan theory was but part of the earlier theory of Stormers (1908). This stated that partial sterilization kills most of the soil fauna and flora, which are subsequently decomposed by bacteria resulting in the release of carbon and nitrogen and subsequent increased soil fertility.

The involvement of protozoa in physiological processes in the soil. By the 1920's other workers were finding evidence of protozoan activity in soils. Nasir (1922) noted that the presence of protozoa stimulated nitrogen fixation by Azotobacter and concluded that protozoa play an important, although indirect, part in this process. This view was upheld by Darbyshire (1972) but the exact mechanism remains unclear.

Protozoa assist in ammonia conversion (Russel, 1927), stimulate the growth of some bacteria (Harvey & Greaves, 1941) and increase soil porosity (Viswanath & Pillai, 1968). Elliot et al. (1979) showed that the presence of protozoa in soils increased CO_2 evolution, ammonia concentrations and the level of inorganic and dissolved

organic phosphorus. The presence of protozoa has a stimulatory effect on the decomposition of organic matter (Stout, 1973; Harrison & Mann, 1975), which may arise from their ability to flocculate and so aid bacterial decomposition through its mechanical activity in a similar manner to earthworms (Bamforth, 1973). Evidence for their preferential selection of bacteria as a food source, thereby influencing both bacterial numbers and population composition, has also accumulated (Singh, 1964; Danso & Alexander, 1975; Habte & Alexander, 1978; Elliot et al., 1979). Protozoa have also been cited as the agents responsible for the disappearance of some plant pathogens (Habte & Alexander, 1975) and enteric bacteria (Stout, 1973) from soils. The role of protozoa in the rhizosphere (Gel'tser, 1975; Darbyshire & Greaves, 1973) and in nutrient cycling and energy flow within soil ecosystems (Stout, 1980) have also been discussed. Microbial interactions involving protozoa, including commensalism, mutualism, parasitism, amensalism and predation, have been reviewed by Curds (1978) although in many cases, the exact relationship between protozoa and other organisms has not been determined experimentally (Curds, 1978).

Clarholm (1981) concluded that protozoa in the rhizosphere accentuate the release of nitrogen and other nutrients from bacterial cells by actively grazing upon them. Her calculations revealed that 60% of the bacterial assimilated nitrogen was excreted as ammonia by the

predatory protozoa and would therefore become available for plant growth. With a standing crop biomass approximately equal to that of earthworms, but with a much shorter turnover time, protozoa have the potential to have a major influence in soil ecosystems (Clarholm, 1983).

3.2 Numbers of protozoa in soils

The majority of soil protozoa vary in size from between 5 and 30 μm (Clarholm, 1981). Direct counting is impossible and their numbers in soils are assessed by indirect methods. Estimates vary from $1x10^3$ - $1x10^4$ g^{-1} soil (Crump, 1920), $1x10^2$ - $1x10^6$ g^{-1} (Viswanath & Pillai, 1968) and $1x10^3$ - $5x10^5$ g^{-1} (Sleigh, 1973). The introduction of improved sampling techniques makes later estimates more reliable eg $2.7x10^4$ g^{-1} (Darbyshire et al., 1974), $1.6x10^4$ (Popovic et al., 1977). Soil moisture may affect numbers, for example Elliot & Coleman (1977) found 2×10^4 protozoa g^{-1} in moist soils but only 1×10^4 g^{-1} in dry prairie soils.

3.3 Population composition

Reports on the species composition of protozoan populations in soil vary. In some soils flagellates are most common (Viswanath & Pillai, 1968; Darbyshire et al. 1974; Popovic et al. 1977), in others both flagellates and small amoebae are reported as being most numerous (Crump, 1920; Macrae & Vinchx, 1973) while in the rhizosphere (Darbyshire & Greaves, 1973) and after rain (Clarholm,

1981) amoebae were found in greatest numbers.

The genera of soil protozoa are similar to aquatic ones though the smaller species tend to be more common in soil. Species common to most soils include Heteromita globsa, Oikomonas termo and Cercomonas spp. (all flagellates) (Nisbet, 1980), Colpoda cucullus and C. steinii (cilates), Naegleria gruberi, Acanthamoeba spp. and Hartmanella hyalina (amoebae) (Campbell, 1977). N. gruberi is possibly the most common small amoeba in soils although Page (1976) lists 23 commonly encountered amoebal species.

4.0 Protozoa as model Eukaryotic cells for research

In a note on the attributes of protozoa as objects of biological research, Corliss (1976) lists their ease of culture, short generation time, ubiquity and adaptability. The role of protozoa as pharmacological research has been reviewed by Hutner (1963) and their role in nutritional studies by Hutner et al. (1973).

Protozoa have been used to study the mode of action and toxicity of a number of pharmacologically active chemicals including adrenergic blocking agents (Iwata, Kuriga & Fujimoto, 1967; Schorr & Boggs, 1973), antibiotics and other drugs (Dryl, 1971; Butzel & Mayer, 1974; Altman et al., 1974; Ricketts & Rappitt, 1975; Bohahtier, 1977;

Rebandel, Gierczak & Karpinska, 1981), sedatives (Tofano, DeBoar & DeBoar, 1975), cigarette smoke (Wang, 1963; Weiss, 1965; Weiss & Weiss, 1966; Gray & Kennedy, 1974), fungal toxins (Hayes, Melton & Smith, 1974; Hayes et al., 1976; Dive, Moreau & Cacan, 1978), vertebrate hormones (Banerjee et al., 1972; Csaba & Nemeth, 1980) and human serum (Nemeth, 1972).

Protozoa, particularly the ciliates, have also been used in a number of laboratory biological assay systems, for example for antitumour and tumour-causing agents (Foley et al. 1958; Epstein et al. 1963; Price et al. 1963), vitamins, reviewed by Baker & Sobotka, (1962) Hill, (1972) and riboflavin analogues (Wallare & Holmund), protein quality (Wang, Miller & Beuchaf, 1980), food anti-oxidants (Surak et al. 1976; Surak, 1977) and proticides, mainly amoebicides (Dobell & Laidlaw, 1926; Pfaffman & Klein, 1966; Gupta et al. 1977; 1978; 1979a; 1979b).

5.0 Protozoa and environmental pollution

Through their ubiquitous nature, their relationship to more advanced metazoans and their rapid response to environmental changes, the protozoa are ideal candidates for pollution and toxicity studies. The impact of a number of xenobiotic chemicals on protozoa at the community, species and cellular level have been reported. Investigations have been on the direct effect of such pollutants as well as the use of protozoa as bioassay and indicator organisms.

5.1 Direct effects of pollutants on protozoa

Among the environmental pollutants which have been investigated for effects on protozoa are: crude oil (Rogerson & Berger, 1981b, 1981c, 1982), oil dispersant chemicals (Rogerson & Berger 1981a), chlorinated effluents (de Jonckheere & Can de Voorde, 1976; Berk & Botts, 1981; 1984), coal conversion by-products (Schultz & Dumont, 1977; Schultz & Allison, 1979; Schultz, Richter & Dumont, 1981), detergents (Cairns et al., 1971; Brujwid-Cwik & Dryl, 1971; Brutkowska, Dryl & Mehr, 1974) heated waste water (Cairns & Lanza, 1972) and heavy metals (Ruthven & Cairns, 1973; Carter & Cameron, 1973; Yamaguchi et al., 1973; Gray & Ventilla, 1973; Berquist, 1974, 1976; Yongue, Berrent & Cairns, 1979; Cairns, Hart & Henerby, 1980).

5.2 Protozoan bioassays of xenobiotic contamination

The assessment of xenobiotic contamination by protozoan bioassays has been investigated on locomotion (Berquist & Bovee, 1974), sedimentation rate (Gittleson, 1975), respiration (Slabbert & Morgan, 1981), the growth rate of populations eg Paramecium sp. (Apostol, 1973), Colpidium sp. (Dive & Leclerc, 1975), Euplotes sp. (Persoone & Uyttersprot, 1975) and Entosiphon sp. (Bringmann & Kuhn, 1980). In addition there have been studies of effects on protozoan associations such as sessile protozoa (Burbank & Spoon, 1967), commensals (Antipa, 1977) and protozoan communities (Cairns, Lanza & Parker, 1972; Henerby & Cairns, 1980).

6.0 A review of the literature on the effects of individual pesticides upon free-living protozoa

(Note: This survey was completed in September 1985)

6.1 The chlorinated hydrocarbon insecticides

DDT (Dichlorodiphenyltrichloroethane). DDT was one of the first insecticides to be investigated for its effects on protozoa and remains the most heavily documented in this respect. Smith & Wenzel (1947) concluded from field and glasshouse trials that the high field application rate of 36kg ha^{-1} of DDT had no detrimental effects upon soil protozoa. Similarly no adverse effects were detected on the growth of laboratory cultures of Paramecium bursaria, P. multimicronucleatum and Euglena gracilis treated with DDT (Gregory, Reed & Priester, 1969). However, when axenic cultures of Tetrahymena pyriformis strain W were grown in the presence of DDT (0.1, 1.0 and 10 $\mu g ml^{-1}$) population growth decreased with increasing concentration of insecticide (Cooley & Keltner, 1970). The authors suggested that T. pyriformis strain W was more sensitive than P. multimicronucleatum or P. bursaria. DDT at concentrations up to 100 $\mu g ml^{-1}$ had no effect on the growth rate or population size of T. vorax (Morgan, 1972). However, at 5 and 50 $\mu g ml^{-1}$ significant reductions in numbers of protozoa in a garden soil were observed

after 1, 2 and 3 months incubation (Macrae & Vinckx, 1973).

Inhibition of growth of cultures of the flagellate Crithidia fasciculata by p,p DDT was observed by French (1974). Initial evidence of transient effects on protein uptake and incorporation in C. fasiculata (French, 1974) were confirmed when French & Roberts (1976) demonstrated inhibitory and then stimulatory effects of p,p DDT on uptake and incorporation of thymidine and uridine in the same organism.

Laboratory cultures of Acanthamoeba castellanii Neff exposed to DDT, and 1, 1, dichloro -2, 2-bis (p-chlorophenyl) ethane (a metabolite of DDT) showed little sensitivity below 10 μgml^{-1} but significant inhibition of growth at concentrations above this value (Prescott & Olson, 1972). Growth of the marine hypotrichous ciliate, Euplotes vannus Muller, was inhibited over 48h by 1-100 μgml^{-1} of DDT and DDD (Persoone & Uyttersprot, 1975). The authors concluded that the residues found in coastal waters (approximately 0.001 μgml^{-1}) were below the sensitivity threshold of the ciliate.

Poorman, (1973) investigating the effects of several pesticides, including DDT, on the photosynthetic Euglena gracilis, also believed that, due to the insolubility of

the compounds, pesticide-induced mortality was unlikely. Using concentrations up to 100 ugml^{-1} Poorman (1973) observed initial inhibitory (48h) and then stimulatory activity (7d) on population growth.

In a systematic study of DDT on a number of ciliate protozoa, Rup Lal and co-workers found DDT (50 and 100 µgml^{-1}) to inhibit population growth and reduce cell size in T. pyriformis (Rup Lal & Saxena, 1979), inhibit cell division in Blepharisma intermedium (Rup Lal, Reddy & Saxena, 1981) and to be readily accumulated and metabolized by Stylonychia notophora (Rup Lal, Saxena & Agarwal, 1981).

Dive, Leclerc & Persoone (1980) investigating the effects of 39 pesticides against Colpidium campylum, found 2,4'-DDD, 4,4'DDD, 4,4'DDE, 2,4'-DDT and 4,4'-DDT to have a minimal toxic activity to this ciliate.

The effect of protozoa on DDT has also been investigated. Euglena gracilis, Paramecium bursaria and P. multimicronucleatum in the presence of 1 µgml^{-1} DDT for 7d concentrated the insecticide 100x, 330x and 1000x, respectively, without adverse effects (Gregory et al. 1969). A. castellanii Neff degraded DDT (5 µgml^{-1}) over 12d into DDE, DDD and DBP (Pollero & de Pollero, 1978). They suggested a protozoan-mediated degradative pathway including dechlorination, hydration, oxidation and

decarboxylation. Rup Lal et al. (1981) found that S. notophora metabolised DDT to DDMV, an intermediate in the conversion to DDD, but not DDD itself. They concluded that S. notophora may have a divergent metabolic pathway from bacteria which generate DDD and DDMU, but noted that the speed of conversion may prevent detection of DDD.

Polychlorinated biphenyls (PCB). The polychlorinated biphenyls are not insecticides, but have physical and chemical properties sufficiently similar to DDT (Morgan, 1972) to warrant inclusion in this review. Commercially-manufactured mixtures of PCB's are called Aroclors. Aroclor is the trade mark of the Monsanto chemical company (Ewald, French & Champ, 1976). They are widespread and persistent pollutants of the environment (Bryan & Olaffson, 1978).

Aroclor 1248, 1254 and 1260 (the last two digits indicate the percentage weight of chlorine) significantly reduced the optical density of axenic cultures of Tetrahymena pyriformis strain W after 96h (Cooley, Keltner & Forrester, 1972; 1973). Inhibition of growth was detectable at 1 $\mu g ml^{-1}$ with all four compounds. However, Aroclor 1242 at concentrations up to 20 $\mu g ml^{-1}$ had no effect on T. vorax (Cooley et al. 1972) and Aroclor 1254, at 10 $\mu g ml^{-1}$ was non-toxic to Euplotes vannus (Persoone & Ulyttersprot, 1975).

Studying 16 pure isomers of PCB and a commercial product, Pyralene 3010, on Colpidium campylum, Dive,Erb & Leclerc (1976) found toxicity to be influenced by both the number and position of the chlorine atoms on the aromatic ring. Ewald et al. (1976) also concluded that toxicity of PCB's to Euglena gracilis was inversely related to the percentage chlorination of the aromatic ring.

Aroclor 1221 reduced carbon fixation, chlorophyl levels and uridine uptake in E. gracilis (Ewald et al., 1976) whilst Aroclor 1242 also inhibited growth in the same organism (Bryan & Olafsson, 1978).

Comparison of the toxicity of DDT and PCB's to C. campylum (Dive et al., 1980) confirmed earlier results (Dive et al., 1976) that PCB's are moderately more toxic that DDT to this species. Like Cooley et al. (1972; 1973), Dive et al. (1976) showed that certain ciliates accumulate PCB's from the culture medium.

Methoxychlor (2,2-bis(p-methoxyphenyl)-1,1,1-thrichloroethane. This compound is chemically related to DDT and its effect on laboratory cultures of Euglena gracilis was identical to that of DDT (Poorman, 1973). After an initial toxic action, lasting 24h, methoxychlor at 10, 50 and 100 $ugml^{-1}$ stimulated growth of E. gracilis over 7d. The insolubility of the compound led Poorman to conclude that methoxychlor's effect on E. gracilis in the environment would be slight.

Methoxychlor was also found to be non-toxic (up to 10 μgml^{-1}) to Colpidium campylum (Dive et al., 1980).

Cyclodienes:- aldrin (1,2,3,4,10,10-hexachloro-1,4,4a,5,6, 8a-hexahydro-exo-1,4-endo-5,8-dimethanon- aphthalene), dieldrin (1,2,3,4,10,10-hexachloro-exo-6,7,-exopoxy-1,4, 4a,5,6,7,8,8a-octahydro-1,4-endo,exo-5,8 dimethanonaphthalene) and endrin (-endo endo isomer).

Aldrin stimulated the growth of Euglena gracilis after an initial toxic action (Poorman, 1973). Concentrations of 10,50 and 100 $ugml^{-1}$ of aldrin stimulated growth between 48 and 81%, as did other chlorinated hydrocarbons, eg DDT and methoxychlor.

Ten $ugml^{-1}$ of both aldrin and dieldrin were, however, found to be lethal to Acanthamoeba castellanii (Prescott, Kubovec & Tryggestad, 1977) but not Colpidium campylum (Dive et al. 1980). DDT, methoxychlor and the cyclodienes (including endrin) had similar inhibition threshold to C. campylum (Dive et al. 1980).

Chlordane (1,2,4,5,6,7,10,10-octachloro-4,7,8,9-tetrahydro -4,7-methyleneindane). The insecticide chlordane at 100 and 500 lb/acre (18-90 $kgha^{-1}$) did not affect soil protozoa, although there was some doubt as to the uniform distribution of the chemical within the soil (Smith & Wenzel, 1947).

Hexachlorocyclohexane (HCH) or Lindane. Often called Benzenehexachloride (BHC), HCH can exist in 8 different isomers, of which only the isomer (lindane) has powerful insecticidal properties (Cremlyn, 1978). Introduced in 1942, HCH was found to have no influence on protozoan populations in the soil as early as 1947 (Smith and Wenzel). However, MacRae & Vinckx (1973) reported that lindane was immediately toxic to soil protozoa at 50 $ugml^{-1}$ and that concentrations as low as 5 $ugml^{-1}$ also affected protozoan numbers over three months. Investigating the effect on lindane on the growth of several aquatic unicellular organisms, Jeanne-Levain (1974) showed that at a high concentration (10 μgml^{-1}) HCH was lethal to Tetrahymena pyriformis, but not Euglena gracilis which withstood concentrations up to 60 μgml^{-1}. Jeanne-Levain observed cytological and behavioural changes in T. pyriformis exposed to lindane (10 μgml^{-1}) for 60h. Cells ceased to form food vacuoles, the cytoplasm became highly granulated and there was a reduction in cell size, with the cells eventually becoming spherical. In contrast, lindane did not induce morphological changes in E. gracilis (Jeanne-Levain, 1974).

Heptachlor (1,4,5,6,7,8,8-heptachloro-3a4,7,7a-tetraphydro -4,7-endo-methanoindene) and Heptachlor epoxide. In a study of the effects of pollutants on cultures of the marine ciliate Euplotes vannus Muller, Persoone & Uyllersprot (1975) found that the insecticide, heptachlor (10 μgml^{-1}) reduced the rate of reproduction by 10%. The same level of heptachlor was also found to reduce the

growth rate of the freshwater ciliate Colpidium campylum although heptachlor expoxide was much more inhibitory, reducing the growth rate at a concentration of 3.16 μgml^{-1} (Dive et al. 1980).

However, the authors did conclude that this species was not a good indicator of the toxicity of a pesticide, due to its comparative lack of sensitivity.

Mirex. Mirex was inhibitory to Tetrahymena pyriformis strain W, reducing the growth rate of the ciliate by 33% with a concentration of 0.009 μgml^{-1} after 96h (Cooley et al., 1972). Population densities were also decreased (12%) and the organism accumulated the insecticide 193-fold.

Toxaphene (chlorinated camphene containing 67 to 69% chlorine). The insecticide toxaphene (at 0.1 μgml^{-1}), used as a pesticide to control undesirable fish populations in Colorado reservoirs, decreased protozoan populations to zero in 2 months (Hoffman & Olive, 1961). Toxaphene was also lethal to laboratory cultures of Euplotes at concentrations below 3 μgml^{1} (Weber, Shea & Berry, 1982).

Isobenzan (1,3,4,5,6,7,8,8-octochloro-1,3,3a,4,7,7a-hexahydro-4,7,methano iso-benzofuran). Field rates of 2.25kg ha^{-1} did not affect numbers of ciliates, flagellates or rhizopods in a

New Zealand soil (Moeed, 1975).

Endosulfan (6,7,8,9,10,10-hexachloro-1,5,5a,6,9,9a, hexahydro-6,9-methano-2,4,3-benzodioxathiepin-3-oxide). Both α and β-isomers of endosulfan were moderately toxic to Colpidium campylum, inhibitory activity was detected at 10 and 10 $ugml^{-1}$ respectively (Dive et al., 1980).

Hexachlorobenzene (HCB). Hexachlorobenzene - a flame retardant, industrial plasticiser and seed dressing (Geike & Parasher, 1976), is commonly found as an impurity in commercially available pentachloronitrobenzene, PCNB (Murphy, Drotar & Fall, 1982). HCB is not synonymous with BHC. Hexachlorobenzene was more inhibitory to the rate of reproduction of Euplotes vannus than Hexachlorcyclohexane (HCH) but the degree of inhibition was low, approx 18% at 10 μgml^{-1} (Persoone & Uyttersprot, 1975).

After a 10d exposure to HCB, cultures of Tetrahymena pyriformis showed a decrease in growth parameters (dry matter, total nitrogen and carbohydrate content) and an increase in the 'porphobilinogen' level in the culture medium (Geike & Parasher, 1976). Porphyria, an hereditary disease of body metabolism producing abdominal pains and mental confusion, can be caused by a number of chlorinated hydrocarbons, DDT, DDE, HCB etc (Geike, 1978). The appearance of porphobilinogen in the culture medium of

T. pyriformis suggested that HCB affected the activity of different enzymes in the ciliate (Geike & Parasher, 1976; Geike, 1978). HCB was believed to act at the level of the mitochondiral cell membrane (Geike, 1978). HCB was much more toxic to Colpidium campylum than hexachlorophene (inhibitory activity was detected at 0.3 and 10 μgml^{-1} respectively (Dive et al., 1980).

6.2 Chlorinated aromatic pesticides

Pentachlorophenol (PCP). The fungicide pentachlorophenol, which is also a degradation product of hexachlorobenzene (HCB) (Geike & Parasher, 1976), was more toxic to Colpidium campylum than were lindane, HCB, DDT and methoxychlor (Dive et al., 1980). Its minimal active dose, the concentration at which inhibitory activity on growth was first detected, was 0.6 μgml^{-1} compared with 10 μgml^{-1} for the parent compound HCB (Dive et al., 1980). Slabbert & Morgan (1982) found PCP (0.5 μgml^{-1}) to continuously increase oxygen uptake in T. pyriformis during 5 min exposure.

Pentachloronitrobenzene (PCNB). PCNB is a widely used soil fungicide and seed disinfectant, which through denitrification and thiomethylation was degraded by Tetrahymena thermophila to its major metabolites, nitrite, pentachlorothioanisole and pentachloraniline (Murphy, Drotar & Fall, 1982). The key enzymes were thought to be

glutathione transferase and thiol 2-methyltransferase. Glutathione-s-transferase is a detoxification enzyme linked in the degradation of a number of electrophillic compounds. Very high levels have been found in T. thermophila and T. pyriformis (Lau et al., 1980).

6.3 Organophosphorus insecticides

Due to their persistence, susceptibility to biomagnification and the development of widespread resistance, the organochlorine insecticides have been banned in many technologically advanced countries (Rup Lal, 1982). The organophosphorus (and carbamate) insecticides have gradually replaced the chlorinated hydrocarbons. These are less persistent, probably due to a higher vapour pressure and increased solubility, and they are more readily oxidized and hydrolozed (Edwards, 1966). However, little is known of the interactions of these chemicals with soil microorganisms (Tu & Miles, 1978). As recently as 1982 Rup Lal in a review of the effects of organophosphorus insecticides on microorganisms, stated that little attention had been paid to the interaction of algae and protozoa (particularly aquatic types) with these chemicals.

Malathion (s-[1,2-Di (ethoxycarbonyl) ethyl] dimethyl phosphorothiolothionate). The rate of respiration of axenic cultures of T. pyriformis exposed to 100 $\mu g ml^{-1}$

100 μgml^{-1} malathion for 7d was reduced by 80% (Duff & Hall, 1970). Lowering the concentration of malathion decreased the inhibitory effect until at 10 μgml^{-1} respiration was stimulated 55% above the control level. Cytological changes in T. pyriformis (increase in cell size and vacuolation) were observed with malathion concentrations between 5 and 100 μgml^{-1}. Vacuolation continued to increase until cell death (Duff & Hall, 1970). Increases in lipid granule accumulation, within 24h, occurred with 5 μgml^{-1} malathion (Duff & Hall, 1971).

As with the chlorinated hydrocarbon insecticides, aldrin, DDT and methoxychlor, malathion had an initial inhibitory effect on growth of cultures of Euglena gracilis (24h) before stimulating the growth of the organism over 168h (Poorman, 1973).

Malathion was slightly inhibitory to Colpidium campylum (minimal active dose 10 μgml^{-1}) (Dive et al., 1980) and to Euplotes spp. (LD50 18 μgml^{-1}) (Weber et al., 1982).

Parathion (0,0-diethyl 0-p-nitrophenyl phosphorotioate). Batch cultures of Euglena gracilis, Paramecium bursaria and P. multimicronucleatum exposed to 1 μgml^{-1} parathion for 7d, did not show any reduction in growth rate or population size (Gregory et al. 1969). However, all of these organisms concentrated parathion from the medium. Poorman (1973), who incubated E. gracilis with 1-100 μgml^{-1} parathion, reported that after 24h the chemical was initially inhibitory but that after 7d it stimulated

growth at the higher levels.

Both ethyl parathion and methyl parathion were slightly inhibitory to Colpidium campylum (Dive et al., 1980).

Some ciliates (Blepharisma seshachari, Spirostomum ambiguum major and S. ambiguum minor) disappeared from hay infusions treated with 1 μgml^{-1} parathion within 7d whilst others (B. intermedium and Frontonia leucas) were still present after 8d (Bai & Dilli, 1974). The chemical was lethal to F. leucas after 30 min (40 μgml^{-1}) and the same concentration lysed both Spirostomum spp. within 24h. Abnormal binary fission, increased macronuclei content and the formation of giant cells were also observed (Bai & Dilli, 1974).

Parathion did not influence the uptake of oxygen in axenic cultures of Tetrahymena pyriformis over 5 min (Slabbert & Morgan, 1982).

Other organophospate insecticides. Fensulfothion and diazinon, at a field application rate of 0.9kg a.i. 0.4 ha^{-1}, had no effect on the composition or density of protozoan populations in New Zealand soils (Moeed, 1975).

Lejczak (1977) found chlorfenvinphos to have only a slight toxic effect to Paramecium caudatum.

Comparative assessment of the toxicity of eight organophosphates to monaxenic cultures of Colpidium campylum revealed azinphosmethyl, bromophos, fenchlorphos and paraxon to be moderately inhibitory(Minimum Active Dose 10 μgml^{-1}) whilst azinphos ethyl, bromophos ethyl, dimenthoate, fenitrothion and trichlorphon were slightly inhibitory (M.A.D. 10 μgml^{-1}) (Dive et al., 1980). The authors commented on the lack of acetylcholinesterase in the ciliate, inhibition of which is the known mode of action of organophosphate insecticides. However, Nistair, Hrusovsky & Benes (1982) showed trichlorphon to be relatively toxic to axenically grown Tetrahymena pyriformis, LD_{50} 4-5 μgml^{-1}, and a similar degree of toxicity was found with dichloros (LD_{50} 5 μgml^{-1}).

6.4 Carbamate herbicides

N-phenylcarbamate herbicides are esters of N-phenyl carbamic acid.

Propham (Isopropyl N-phenylcarbamate). Selected as the most active of a series of arylurethanes, the N-phenylcarbamate herbicide propham was introduced by Templeman & Sexton (1945).

Investigating the regeneration of membranellar bands of oral ciliature, Margulis and Bannerjee (1969) reported that propham inhibited such regeneration on Stentor coeruleus. Further work on the ciliate showed that

propham at 3,4 and 5 x 10^{-4}M (54,72 and 89 μgml^{-1}) prevented 59% of the cells from undergoing regeneration of oral ciliature (Sarras & Burchill, 1975). Interpreting variable results of the effect of propham on the growth of axenic batch cultures of Acanthamoeba castellanii, Prescott & Olson (1972) suggested that 10 μgml^{-1} propham prevented population growth of the organism. These results were confirmed later when the growth of A. castellanii cultures were inhibited 66% by 17.9 μgml^{-1} propham (Prescott et al., 1977).

At 4 x 10^{-4} M (72 μgml^{-1}) propham inhibited both growth and cell division of Euglena gracilis populations (Marcenko, 1980). The lethal concentration for E. gracilis was 3 x 10^{-4} M (54 μgml^{-1}) after 5-7 days growth. Morphological effects were also noted with higher concentrations (5 x 10^{-4}, 89 μgml^{-1}), the most prominent of which was the development of enlarged 'monstrous' cells.

Hydrolysis of propham to isopropyl alcohol and N-phenyl-carbamic acid, followed by decarboxylation of these unstable products to aniline, is thought to account for the disappearance of propham in soils (Newman, DeRose & DeRigo, 1948). Propham is not persistent in soils, its disappearance attributed to microorganisms (Clark & Wright, 1970). Aniline inhibited population growth in Tetrahymena pyriformis at 250 μgml^{-1} after 72h exposure (Schultz & Allison, 1979).

Eptam (s-ethyl dipropylthiocarbamate). Eptam, a thiol carbamate herbicide, inhibited the growth rate of A. castellanii cultures. After 120h exposure to 10 and 100 μgml^{-1}, cell numbers differed significantly ($p=0.01$) from the untreated cultures (Prescott & Olson, 1972).

6.5 Carbamate insecticides

N-methyl carbamate insecticides are esters of N-methyl carbamic acid.

Carbaryl (1-naphthyl N-methylcarbamate). Prescott et al. (1977) reported that 10 $ugml^{-1}$ carbaryl slowed the growth of A. castellanii cultures after 6d, whilst at 7.9 μgml^{-1} carbaryl killed 50% of a Paramecium caudatum culture after 24h. The toxicity of the compound decreased after 48 and 96h (Lezezak, 1977). Dive et al. (1980) found that carbaryl had a minimal active dose (M.A.D.) of 10 μgml^{-1} towards Colpidium campylum, although the ciliate appears to be relatively insensitive to pesticides in general (Dive et al., 1980).

In another investigation, Weber et al. (1982) reported that at 1 μgml^{-1} carbaryl and its derivative 1-naphthol, caused 50% mortality in populations of a Euplotes sp. They concluded that field run-off levels of carbaryl and 1-naphthol were likely to cause significant mortality in protozoan populations and linked the lack of cellulose decomposition in carbaryl-treated soils to the effect of

the insecticide on protozoa.

Propoxur (0, isopropoxyphenyl methylcarbamate). This insecticide also inhibited the population growth of Paramecium caudatum although to a lesser degree than carbaryl, LD_{50} 27.8 μgml^{-1} after 24h (Lezezak, 1977).

6.6 Carbamate fungicides

Benomyl (methyl-1-(butylcarbamoyl)-2-benzimidazol -2-yl carbamate). Benomyl, the active ingredient in Benlate, is the most effective of the benzimidazole fungicides; it is systemic in action (Erwin, 1973).

Benomyl and two of its major hydrolysis products, methyl benzimidazol-2-yl carbamate (MBC) and butylisocyanate (BIC), inhibited the population growth of Tetrahymena pyriformis in a dose-dependent manner whilst a minor degradation product, 2-aminobenzimidazole (2-AB), had no effect on growth (Rankin, Surak & Thompson, 1977). The ED_{50} (effective dose, decreasing cell numbers by 50%) after 12h was 9.1 μgml^{-1} of benomyl. Twenty μgml^{-1} benomyl caused cells to swell, become highly vacuolated and to lose their pyriform shape, whilst MBC and 2-AB at the same concentration had no apparent cytological effect on T. pyriformis (Rankin et al., 1977).

Dithane (ethylene -1,2-bis dithiocarbamate). Dithane at 0.1 - 100 μgml^{-1} was initially toxic to Tetrahymena pyriformis but recovery of cultures treated with 1 and 10 μgml^{-1} was observed after 24h. Cells treated with 100 μgml^{-1} did not recover (Toth & Tomasovicova, 1979).

6.7 Phenylurea herbicides

Monuron (3 - (p-chlorophenyl) -1,1-dimethylurea). Neither monuron, nor the chemically-related linuron (3-(3,4-dichlorophenyl) -1-methoxy-1-methylurea) had any effect on the growth rate of A. castellanii cultures (Prescott & Olson, 1972; Prescott et al., 1977). However, diuron (3 - (3,4-dichlorophenyl) 1-,1-dimethylurea) was toxic towards Hartmannella rhysodes (Singh) and other unspecified species (Gel'tzer and Geptner, 1976). Field trials with monuron, diuron and Cotoran (1,1-dimethyl-3-(3 trifluoromethylphenyl) urea) between the years 1968 and 1973 revealed variable changes in absolute numbers and species composition within protozoan populations (Gel'tzer & Geptner, 1976).

Propanil (3′ 4′ - dichloropropionanilade) or stam F-34. The herbicide propanil, like the phenulurea herbicides is a substituted phenylamide.

The propanil formulation Stam-F-34 reduced the growth rate of Acanthamoeba castellanii populations by 60% after 6d at

a concentration of 10 μgml^{-1} (Prescott & Olson, 1972; Prescott et al., 1977).

6.8 Triazine herbicides

Simazine (2-chloro-4, 6 bis (ethylamino)-s- triazine). Simazine appeared to suppress all physiological functions in Hartmanella rhysodes (synonymous with A. castellanii strain 1534/3) (Gel'tzer, 1967). At 0.4 μgml^{-1} simazine caused changes including; withdrawing of acanthapodia (pseudopodia), cessation of contractile vacuole function and motitity, organelle deformation and cell lysis. Simazine also depressed protozoan populations in soils treated with 1.0kg a.i. ha^{-1} after 1 week, although recovery was evident after 6 weeks (Deshmukh & Shrikhande, 1974).

Atrazine(2-chloro-4-(ethylamino)-6-(isopropylamino)-5-triazine). Atrazine, initially thought to be toxic to A. castellanii, inhibiting growth at a level of 0.1 μgml^{-1} (Prescott & Olson, 1972), was later reported not to significantly inhibit growth at concentrations up to 4 μgml^{-1} (Prescott et al. 1977). Prescott, in an erratum (dated 1972) believed impurities in the atrazine samples to be responsible for the earlier inhibition.

The application of atrazine to soil at 5 and 8kg a.i. ha^{-1} brought a marked reduction in the population of protozoa in $100m^2$ plots (Papovici et al. 1977). The effect was

was dose-related and the most marked action was against the flagellates, mainly Bodo and Monas spp. (75% reduction in numbers).

Atrazine was partially inhibitory to Tetrahymena pyriformis in culture after 24h, but its derivatives MEBT and MBT were much more inhibitory (Toth & Tamasovicova, 1979).

Cyanazine (Cyanazine, 2-(4-chloro-6- (ethylamino)-s-triazin-2-yl) aminol-2-methylpropionitrite. Cyanazine had a similar effect to simazine on the level of protozoan populations in the soil (Deshmukh & Shrikhande, 1974). Five litres a.i. ha^{-1} depressed population levels over 6 weeks but 1 litre a.i. ha^{-1} had no significant effect.

6.9 Phenoxyacetic acid herbicides

The phenoxyacetic acids are systemic herbicides which act like the plant growth hormone indole-3-acetic acid (Cremlyn, 1978).

2,4-D (2,4-dichlorophenoxyacetic acid). The earliest report of the action of 2,4-D on protozoan populations in the soil stated that at concentrations between 1-100 $\mu g ml^{-1}$ no 'injurious' effect was observed although at 500 $\mu g ml^{-1}$, and on a sandy soil, 2,4-D 'seemed to kill some of them' (Smith, Dawson & Wenzel, 1945).

Prescott & Olson (1972) reported that 2,4-D stimulated the growth and reproduction of Acanthamoeba castellanii in laboratory batch culture. Concentrations of 0.1-100 μgml^{-1} stimulated growth, the greatest effect occurring at 0.1 and 10 μgml^{-1}. The authors concluded that either A. castellanii degraded 2,4-D and used it as a carbon/energy source or the herbicide stimulated growth directly.

However, in field trials 2,4-D at rates of 1.6 and 8.0kg a.i. ha^{-1} significantly depressed protozoan populations over a 6 week period (Deshmukh & Shrikhande, 1974). Liberal estimates of the amount of pesticides reaching the soil from such applications would be between 3.5-18 $\mu g \cdot l^{-1}$ according to the conversion factors of Fletcher (1960), Bollen (1961), Sheets & Harris (1965) and Metcalf (1971).

Growth of axenic cultures of Euglena gracilis, exposed to 2,4-D for 24h, was significantly reduced (74% of the control value) at concentrations between 50 and 100 μgml^{-1}. Exposure to the herbicide for a longer period (7d) indicated that the acute toxicity persisted only at the 100 μgml^{-1} level whilst a 7d exposure to 10 μgml^{-1} stimulated population growth by 61% (Poorman, 1973). Observation on all cells revealed that they had been morphologically altered. The majority were 'cyst-like' (rounded) and appeared dead. However, recovery of growth followed removal from 2,4-D solutions was rapid, even after 7d exposure to 100 μgml^{-1}.

2,4,5-T (2,4,5-Trichlorophenoxy acetic acid). Recovery of Tetrahymena pyriformis after inhibition of growth by 2,4,5,-T was also noted (Silberstein & Hooper, 1972). At 4-9 x 10^{-4}M 2,4,5-T, exponential growth of the ciliate was replaced by an inhibition-recovery pattern of growth. 2,4,5-T concentrations above 9 x 10^{-4}M prevented recovery. Cells pre-treated with 2,4,5-T were unaffected by addition of further doses. Silberstein & Hooper (1972) concluded that an inducible recovery system, possibly involving a peroxisomal enzyme system was implicated.

Further studies showed that 2,4,5-T inhibited oxygen utilization and cell division in T. pyriformis (Silberstein & Hooper 1973; 1975). Oxygen utilization by isolated mitochondria was also inhibited by 2,4,5-T and T. pyriformis cells lost their pyriform shape and ceased moving. The herbicide was thought to prevent cell divisin via inhibition of ATP synthesis (Silberstein & Hooper, 1975).

6.10 Insect chemosterilants

The sterilizing effect of Metepa (tris (2-methyl-1-aziridinyl phosphine oxide), an aziridinyl alkylating agent, on insects has been recognised but little is known of its mode of action (Shivaji, Saxena & Pallai, 1975). In a systematic programme Shivaji et al. (1975; 1978a; 1978b; 1978c; 1978d and 1979) attempted to determine the mode of action of metepa using protozoa as model eukaryotic cells.

Marked morphological changes were observed in Stylonychia notophora treated with metepa (Shivaji et al. 1975). Cells became sluggish, highly vacuolated and rounded at high concentrations 2000 - 4000 μgml^{-1}, whilst at 1000 μgml^{-1} the mean generation time was extended. Metepa reduced the synthesis of RNA and DNA in both S. notophora and Blepharisma intermedium (Shivaji et al. 1975; 1978a; 1978b; 1978c; 1978d).

Progressively increasing concentrations of metepa, 2500 - 4000 $ugml^{-1}$ also caused increasing morphological and growth effects with Tetrahymena pyriformis (Shivaji et al., 1979).

6.11 Organomercurial fungicides

Mercury compounds are the by-products of several industrial processes and are widely used in agriculture as an effective bactericide and fungicide (Boules & Wolfson, 1976).

In a study on the impact of mercuric chloride ($HgCl_2$) on a number of sessile ciliates, Burbanck & Spoon (1967) noted that 0.5 μgml^{-1} $HgCl_2$ significantly reduced cell numbers after 4h. Vorticella chlorostigma was the most sensitive organism tested.

Thrasher & Adams (1972), in an effort to determine whether organomercurials affect the ss disulfide formation in microtubular proteins during division, exposed Tetrahymena

pyriformis strain WH14 cultures to four mercury compounds. Concentrations of $HgCl_2$, ethylmercuric acetate increased the generation time of T. pyriformis by between 10 and 100%. Evidence was obtained that methyl mercuric chloride did inhibit disulfide bond formation at the centriole.

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Thrasher & Adams (1972), in an effort to determine whether organomercurials affect the s-s bond formation in microtubular proteins during division, exposed Tetrahymena pyriformis strain WH14 cultures to four mercury compounds. Concentrations of $HgCl_2$, ethylmercuric chloride, methylmercuric chloride and phenylmercuric acetate increased the generation time of T. pyriformis by between 10 and 100%. Evidence was obtained that methyl mercuric chloride did inhibit disulfide bond formation at the centriole of the spindle. Phenyl mercuric acetate was believed to act on the chromosomal-microtubular system of mitosis creating fragmentation of the chromosome. Observations on some dividing cells showed that they remained in cytokinesis and macronuclear division for up to 3h, with no apparent effect on ciliary activity.

Gross morphological and cytological changes were also observed in T. pyriformis strain HSM cells treated with $HgCl_2$ (Tingle, Pavlat & Cameron, 1973).These included swelling of mitochondria, decreased density of the mitochondrial ground matrix, reduction in cell mortality, contractile vacuole activity and changes in cell shape. The threshold of lethal activity after 96h with this strain of T. pyriformis was 4.5 μgml^{-1} (Carter & Cameron, 1973). Mercury ions were also lethal to T. pyriformis at a level of 0.8 μgml^{-1} (Yamaguchi et al., 1973). However, p-chloromercuribenzoate and p-chloromercuri phenyl sulfonate had little influence on T. pyriformis strain GL (Bowles & Wolfson, 1976).

6.12 Miscellaneous pesticides

Rotenone (1,2,12,12a-tetrahydoro-8,9-dimenthoxy-2-(1-methylenyl)-(1) benzopyrano (3,4b) furo (2,3,h) (1)-benzopyran-6(6H)-one). The insecticide rotenone occurs in the roots of Derris elliptica. Some rotenoid compounds have been used as insecticides since 1848 (Cremlyn, 1978). Hoffman & Olive (1961), in an experiment involving three reservoirs, applied derris powder as a piscicide (1 μgml^{-1}) and found it to be inhibitory to protozoan population growth for a period of 9 months.

Previously, Hooper (1948) had attributed similar decreases in protozoan numbers following addition of derris powder

to lakes, to seasonal fluctuations and errors in sampling.

Niclosamide. To determine whether the molluscicidal efficacy of the ethanolamine salt of niclosamide was affected by the composition of the water in which it was used Meredith & Meredith (1972) conducted experiments to investigate its effect on protozoa as a sensitive alternative to time-consuming snail bioassays and inaccurate field chemical methods. Ciliate protozoa of the genera Spirostomum and Euplotes were very sensitive to the chemical and S. ambiguum Ehrenberg var. minor was finally selected as the test organism. The LC_{50} (lethal concentration for 50% of the population) at 10°C was 0.5 $\mu g ml^{-1}$ but was progressively reduced at higher temperatures (20°C and 25°C).

Cartrap (2-(dimethylamino)-1,3-propanediyl dicarbomothioate. Cartrap belongs to a group of insecticides related to nereistoxin, a toxin isolated from species of marine worm. Using genetically homogenous cultures of Paramecium primaureli, Komala (1982) noted a linear relationship between dose of cartrap and lack of motility of the ciliate. Cartrap had a LC_{50} of 2.5 $\mu g ml^{-1}$ and induced morphological abnormalities such as incomplete separation of daughter cells during division which resulted in the formation of 'doublet' cells.

Thiram (tetramethyl thiuram disulphide). Thiram was highly toxic to Colpidium campylum (Dive et al., 1980). The authors noted that all of the compounds which, in this comparative test, were highly toxic were also uncouplers of oxidative phosphorylation.

Ortophaltan (N-trichloro-methylthiophthalmid) and sulikol (a colloidal sulphur preparation). The fungicides Ortophaltan and Sulikol had 24h ED_{50} values of 0.012 and 0.16 $\mu g ml^{-1}$ respectively, and were amongst the most toxic of the compounds against Tetrahymena pyriformis strain T (Toth & Tomasovicova, 1979).

The herbicide 3-amino-1,2,4-triazole inhibited multiplication and pigment synthesis in Euglena gracilis but its mode of action was not established (Aaronson, 1960).

Trifluralin (trifluoro-2-6-dinitro-N,N-dipropyl-p-toluidine). Trifluralin, a dinitroaniline herbicide, delayed oral membranellar band regeneration in the ciliate Stentor coeruleus (Banerjee, Kelleher & Margulis, 1975). Trifluralin was thought to prevent microtubule proteins from polymerization at a site other than the colchicine binding site.

7.0 Objectives of this study

It was firstly proposed, through the use of representative protozoan species, to investigate the influence of some

pesticides on the population growth of free-living protozoa.

One aim was to develop methodology to study such processes and to develop a reliable, reproducible and preferably quick technique for the assessment of the toxicity of pesticides to protozoa, with emphasis on reducing the scale of culture techniques.

Compounds shown to have comparatively high toxicity to the protozoa would be further investigated for their direct effect on; behaviour, feeding activities, respiration, motility, encystment/excystment, morphology and cytology. In accordance with the Environmental Protection Agency (USA) guidelines for environment studies of pesticides, concentrations equivalent to values below and in excess of the estimated field application rate would be considered.

It was also intended to examine, where possible, the ability of the protozoa to absorb and possibly transform certain pesticides.

It was proposed to give particular attention to the effects of some N-phenylcarbamate herbicides (chlorpropham, propham, barban) and the phenylurea diuron as part of a much larger study in this laboratory over a number of years, on the interaction of these chemicals with soil and aquatic micro-organisms. Previous studies have been made with green algae (Wright, 1972; 1975a;

1975b; Wright, Stainthorpe & Downs, 1977; Maule & Wright, 1973; 1984), bacteria (Clark & Wright, 1970a; 1970b; Wright, 1974; Westmacott & Wright, 1975; Quilt, Grossbard & Wright, 1979; 1980; Wright & Maule 1982), cyanobacteria (Maule & Wright, 1983; 1984) and fungi (Wright & Forey, 1972).

8.0 The N-phenylcarbamate herbicides

The N-phenylcarbamates belong to a larger group of chemicals, the phenylamides, which also include the phenylureas (diuron, linuron, monuron etc) and the acylanilides (eg propanil).

The herbicidal properties of propham (Isopropyl N-phenylcarbamate) were first reported by Templeman & Sexton (1945) who found that its toxicity was selective against monocotyledons. Both propham (IPC) and its 3 chloro-derivative chlorpropham (CIPC) are applied to the soil as pre-emergence herbicides to control annual weeds. Chlorpropham is cleared for use amongst annual and perennial vegetables, fruit nursery stock, flowers and in glasshouses, at application rates between 0.84 and 4.5kg a.i.ha^{-1}, whilst propham (2.2 - 6.8kg a.i.ha^{-1}) is cleared only for use amongst annual vegetables (Fryer & Makepeace, 1978). Both compounds are used in conjunction with a number of phenylureas, principally fenuron.

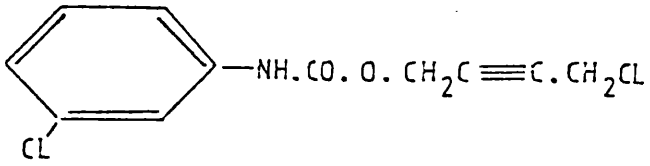
Barban (4-chloro-2-butynyl M-chlorocarbanilate) is a post-emergence herbicide. It is readily absorbed by soil complexes (Dubrovin, 1961) and is therefore applied to the foliage at between 0.53 and 0.70kg a.i.ha^{-1}. It is used to control wild oats amongst annual cereals (wheat and barley) and vegetables (Fryer & Makepeace, 1978).

The chemical structure of propham, chlorpropham and barban is given in Fig. 1.

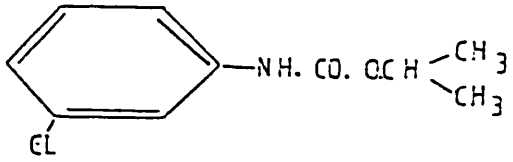
8.1 Mode of action of phenylcarbamates in plants

The N-phenylcarbamate herbicides have been reported to: prevent root and shoot elongation (Ennis, 1948a), reduce inflorescence production (Jones, 1958), inhibit photosynthesis (Moreland & Hill, 1959; Aston et al., 1977; Macheral, Ravanel & Tissat, 1982), retard potato sprouting (Reeve, Forrester & Hendel, 1964) and increase germination in Abutilon theophrasti (Fawcett & Strife, 1975).Cytological and biochemical effects of phenylcarbamates include inhibition of: cell division (Ennis, 1948b; Doxey, 1949; Mann & Storey, 1966; Helper & Jackson, 1969), the Hill reaction in isolated chloroplasts (Moreland & Hill, 1959), polymer synthesis-pectin, hemicellulose and lignin, (Mann, Jorden & Davy, 1965), protein synthesis (Mann, 1967; Aston et al., 1977), amylase production in barley seeds (Mann et al., 1967); they may also uncouple oxidative phosphorylation (Macheral et al., 1982).

BARBAN



CHLORPROPHAM



PROPHAM

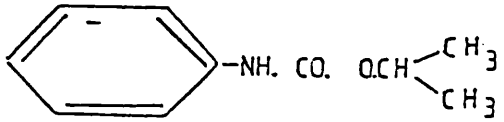


FIG. 1

The structure of some N-phenylcarbamate herbicides.

Observations on phenylcarbamate-treated plant cells undergoing mitosis have shown an arresting of activity at metaphase (Ennis, 1948a,b; Doxey, 1949; Carvin & Friesen, 1959; Mann & Storey, 1966; Helper & Jackson, 1969). Chromosomes were contracted (Mann & Storey, 1966) and failed to separate (Doxey, 1949) or align correctly (Helper & Jackson, 1969),resulting in chromosome aggregation, multipolar spindles, polyploidy and binucleate cells (Ennis, 1948a,b; Moreland & Hill, 1959; Helper & Jackson, 1969).

8.2 Mode of action in other organisms

The mode of action of phenylcarbamates in plants led Helper & Jackson (1969) to believe that mitosis in other systems may also be affected. Subsequent investigations have shown the phenylcarbamates to inhibit mitosis in: human lymphocytes (Timpson, 1970), green algae (Coss & Pickett-Heaps, 1974), the flagellates Ochromonas (Brown & Bouk, 1974) and Euglena (Marcenko, 1980) and mouse oocytes (Magistrini & Szollosi, 1980).

9.0 Representative protozoan species

The protozoan, Tetrahymena pyriformis and the amoeba Acanthamoeba castellanii were selected for the studies to investigate some of the interactions of pesticides with free-living protozoa.

9.1 Acanthamoeba castellanii

This amoeba was originally isolated from a culture of yeast (Castellani, 1930). The name Hartmannella (later Acanthamoeba) castellanii was proposed by Douglas (1930).

Small amoebae of the genera Acanthamoeba, Hartmannella and Mayorella occur widely in soil and aquatic environments and are probably some of the most common protozoa (Page, 1967). They are one of the few groups of protozoa which can be isolated and grown in axenic culture (Griffiths et al., 1978) and rapid growth (14 - 20h mean generation time) in defined media has been reported (Byers et al., 1980). Acanthamoeba castellanii's history in culture has been reviewed by Neff (1957).

The abundance of Acanthamoeba spp. in soil, determined by an overlay technique (Menapace et al., 1975) was as high as 3.2×10^3 amoebae per gram of soil. Wright, Redhead & Maudsley (1981) found Acanthamoeba in all 30 samples examined (taken from fallow and cultivated soil, lake muds, river sediments, canal, lake and pond water).

Acanthamoeba castellanii is morphologically characterised by an oval, elongate or irregular outline (Fig. 2) and is described by Page (1967, 1976). It is approximately 20 um in overall size, though larger specimens have been observed in the trophic, free-living state. It has a

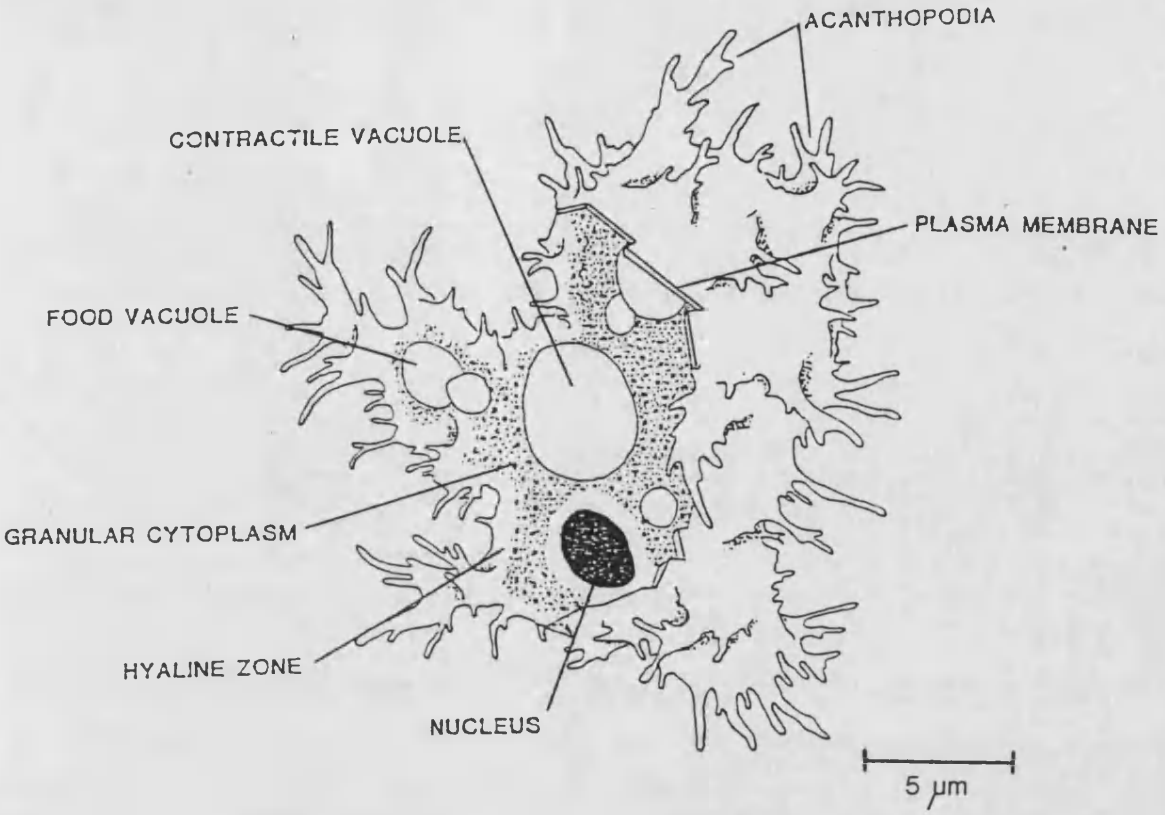


FIG. 2

A schematic diagram of Acanthamoeba castellanii

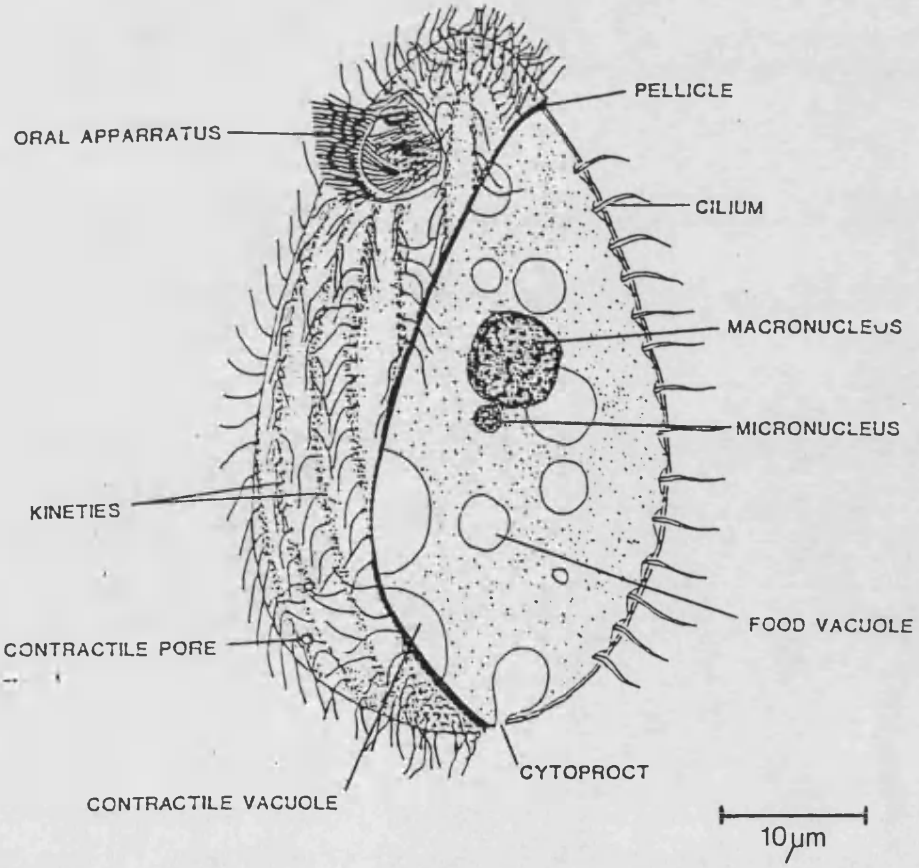


FIG. 3

A schematic diagram of Tetrahymena pyriformis

single vesiculate nucleus and a prominent contractile vacuole. Food vacuoles are clearly visible and numerous. Locomotion is slow and is by the formation of acanthapodia (thin, slender, tapering projections extending from the broad hyaline zone) - Fig.2.

Under unfavourable conditions the trophozoites encyst. Cysts, approximately 14 um diameter, consist of a polygonal or stellate endocyst and a rippled or wrinkled ectocyst. Emergence of the trophozoite from the cyst is through a differentiated structure on the cyst wall, the ostiole (Chambers & Thompson, 1972). In culture the removal of food is the primary stimulus inducing encystment in Acanthamoeba spp. but if cells are starved the process is likely to be variable and asynchronous (Neff et al., 1964). Control of encystment can be achieved by 'replacement' techniques. Cells are harvested at a known growth phase and placed in a cyst-inducing medium. Organisms are obtained which are physiologically similar and the events of encystment can proceed in partial isolation from other physiological functions (eg growth) due to the non-nutrient medium (Band, 1963).

It is recognised that some species of Acanthamoeba, including A. castellanii, can be opportunistic human pathogens causing amoebic meningoencephalitis and non-fatal lung, ear and eye infections (Byers, 1979). They may also be the natural host for Legionella pneumophila (Rowbotham, 1983).

9.2 Tetrahymena pyriformis

The holotrichous ciliate T. pyriformis has been widely used to study the toxic effects of substances as disparate as heavy metals and human sera. It is found ubiquitously in fresh water (Elliot, Addison & Carey, 1962). Its history, life in culture and morphology have been reviewed by Corliss (1952, 1953), the biochemistry and physiology by Hill (1972) and Elliot (1973) and its role in biological research by Corliss (1965, 1976). T. pyriformis can be grown axenically or monoxenically (Everhart, 1972).

It is typically a pear-shaped, highly motile organism approximately 50 x 30 um in size (Fig. 3). It has 16-26 rows of cilia (kinties) which run longitudinally along the pellicle, the extreme plasticity of which permits considerable temporary distortion. The oral apparatus comprises three membranes of fused cilia (tetra-hymena) and one undulating membranes, which direct particles into the oral cavity. Food vacuoles are formed at the base of the oral cavity and undigested components are expelled at the cytoproct. Both the cytoproct and the contractile pore(s) (typically two) have fixed locations on the pellicle (Fig. 3). The contractile vacuole(s) empties through these pores. The macronucleus is typically ovoid to irregular. Small pinocytotic vesicles and autophagic

vacuoles exist and the cytoplasm is granular (Fig.3) (Elliot & Kennedy, 1973).

The life cycle is monomorphic, although Watson (1946) reported cyst formation.

MATERIALS & METHODS

10.0 Culture of Protozoa

10.1 Acanthamoeba castellanii

A. castellanii strain 'G' and 'Neff' (obtained from Dr A Griffiths, University College, Cardiff) were maintained in a proteose-peptone-glucose-yeast extract medium (PGY) (Chagla & Griffiths, 1974) consisting of (gl^{-1}):- Proteose-peptone (Oxoid) 7.5, Glucose (Lab M) 15, yeast-extract (Lab M) 7.5, dissolved in distilled water, pH adjusted to 7.0, and sterilised by autoclaving at 15lb $sq.in^{-1}$ (121°C) for 15 min.

Maintenance cultures of A. castellanii were incubated statically in 10ml PGY medium in Universal bottles for 14d at 30°C. Sub-culturing was done every 4d, by aseptically transferring 1ml of a 14d culture to fresh PGY medium. This regime was perpetuated throughout the study.

Larger volumes of culture were obtained by inoculating 50ml PGY in 250ml Erlenmeyer flasks with 5ml of a 14d maintenance culture, incubating at 30°C and shaking the flasks at 80 cycles min^{-1}. These cultures were transferred every 4d, when the population density was approximately 3.0×10^6 ml^{-1} exponentially growing amoeba (Fig. 4).

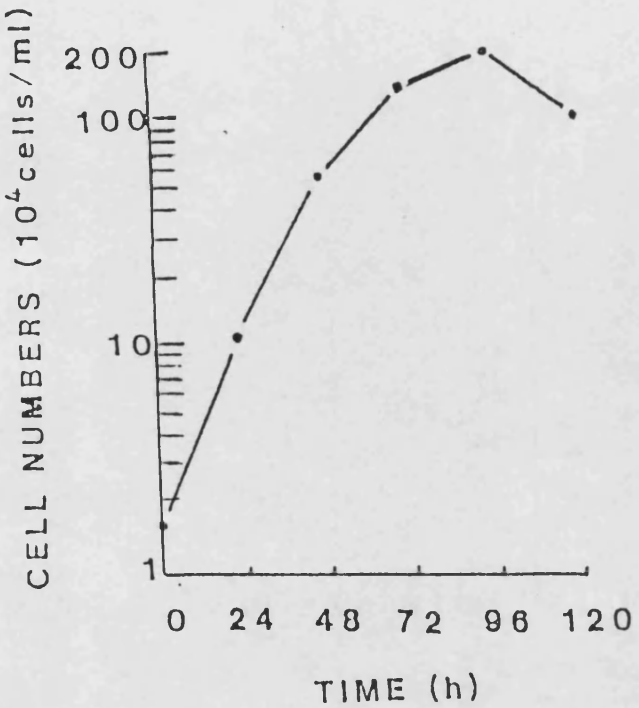


FIG. 4

The growth of A. castellanii in 50 ml of PGY medium in flasks at 30°C.

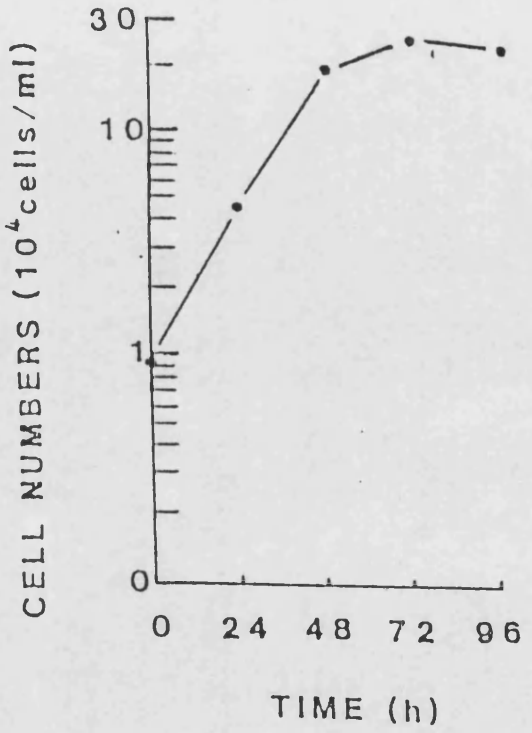


FIG. 5

The growth of T. pyriformis in 50 ml of PY medium in flasks at 20°C.

All transfers of this organism were done aseptically using a Class II safety cabinet (Microflow Pathfinder Flow Laboratories).

10.2 Tetrahymena pyriformis

T. pyriformis strain 1630/1H (CCAP) was maintained on a proteose-peptone-yeast-extract medium (PY) consisting of (gl^{-1}):- Proteose-peptone (Oxoid) 10g and yeast-extract (Lab M) 2.5g dissolved in distilled water, pH adjusted to 6.5, and sterilized by autoclaving at 15lb $sq.in^{-1}$ 121°C for 15 min.

Batch cultures were obtained by inoculating T. pyriformis into 10ml PY medium in Universal bottles and incubating statically at 20°C. Sub-cultures were made every 7 days when 10ml fresh PY medium was inoculated with 0.5ml of T. pyriformis in order to maintain active stock cultures.

Larger culture volumes were achieved by inoculating 50ml PY medium in 250ml Erlenmeyer flasks with 2.5ml of 7d culture and incubating statically at 20°C. These cultures were aseptically sub-cultured every 3d when the cell density was approximately 2.8×10^5 ml^{-1} ciliates (Fig. 5). No significant increase in numbers were obtained by shaking. All transfers of organisms were carried out

aseptically in a Laminar flow cabinet (Microflow).

Cultures of protozoa used in these studies were kept axenic, this being monitored by microscopic examination of wet mounts prior to each experiment.

11.0 Pesticide solutions

A list of pesticides used appears in Table I. All of these were pure compounds, and solutions were made up in either sterile distilled water or in ethanol, before being added to the sterile growth medium. The final concentration of ethanol added to the media never exceeded 0.01% (v/v), a level shown by previous experiments to have no detrimental effects on the protozoa used. Where applicable untreated controls also received this level of ethanol.

11.1 Stock solutions

Stock solutions of pesticides in ethanol were refrigerated and kept no longer than 2 weeks to avoid deterioration or transformation of the chemical. For solutions of pesticides in sterile distilled water, stock solutions

were prepared when required, incubated at 30°C for 2d and then microscopically examined to monitor sterility.

11.2 Dilution of pesticide solutions

The absorbtion of pesticides onto surfaces within the soil is common (Bailey & White, 1970) and the absorbtion of pesticides onto laboratory glassware also occurrs, although the amount lost is difficult to calculate (Wheatley, personal communication). To minimise this possible loss, serial dilution of pesticide solutions were kept to a minimum in this study.

11.3 Pesticide concentrations used

Estimates of the amount of chemical reaching the soil vary, for example, after a field application rate of 1.12 kg active ingredients ha^{-1} estimates ranged from 0.25 - 2.5 $\mu g ml^{-1}$ (Fletcher, 1960; Bollen, 1961; Sheets & Harris, 1965; Metcalf, 1971). A crude estimate of the amount of chemical present in the soil may be obtained by averaging the recommended application rate of each pesticide (the rate varies with soil type and mode of use) and assuming a field application rate of 1.12 kg ha^{-1} is equivalent to 1.5 μg of pesticide in 1 ml of liquid growth media (Table 1).

Table 1

Estimated Field Concentrations (EFC) of pesticides used in this study

Pesticide	Average Recommended application rate ($kgha^{-1}$)	Estimated Field * concentration (μgg^{-1} of soil)
barban	0.5	1
chlorpropham	2.8	4
propham	3.75	5
diuron	2.1	3
fenuron	0.67	1
isoproturon	2.3	3
linuron	0.83	1
terbutryne	0.4	0.5
cyanazine	2.0	2.5
asulam	1.21	2
benomyl	0.6	1
carbaryl	0.6	1
malathion	2.3	3
permethrin	0.2	0.2
pirimicarb	1.0	1

* Estimates are based on conversion factors; see text pp51-52 for explanation

These figures can only realistically serve as nominal indicators of the concentration present, and are possibly only accurate in their order of magnitude. For the purpose of this work, these predicted values have been used as 'Estimated Field Concentrations' (EFC) in order to have a guideline on which to base pesticide concentrations.

EFC's do not reflect actual values in the soil just as axenic cultures of protozoa do not reflect a field habitat.

In the assessment of the inhibitory or stimulatory activity of an unknown compound against a target organism a wide range of concentrations assist correct interpretation of data. Thus, pesticide levels not normally encountered ecologically, from both extremes, may prove useful. However, due to the constituents of PY and PGY media, it could not be assumed that a pesticide would achieve the maximum solubility quoted for water. Therefore no solution exceeding 70% of a compound's stated solubility in distilled water was used.

12.0 Definitions for use in Toxicity Testing

The following definitions are based on those found in: The Faber Medical Dictionary (1965); Dorland's Illustrated Medical Dictionary 26th Edition (1981); Butterworth's Medical Dictionary 2nd Edition (1978); Pear Medical Encyclopaedia (1979) and McEwen & Stephenson (1979).

12.1 Acute toxicity tests

Those tests designed to determine over a relatively short period of time, the concentration of a compound that produces symptoms which are: rapid in onset, intense in character, peak sharply and resolve themselves quickly into mortality, chronicity or recovery.

12.2 Sub-acute toxicity tests

Those tests designed to assess the activity of a compound over a range of concentrations, in order to determine those levels which are effective and those that are non-effective against organisms during the formative phase of life.

12.3 Chronic toxicity tests

Those tests designed to expose organisms over their entire life span (or a major part of it) to relevant concentrations of chemicals in order to evaluate their effects. A fuller explanation is given in Appendix 1 (p 226).

13.0 Sub-acute toxicity determinations in Repli-dishes

The toxicity of chlorpropham, propham, barban, 3-chloroaniline, aniline, diuron, asulam, pirimicarb and permethrin to T. pyriformis and A. castellanii were tested.

13.1 Pesticide solutions

With the exception of propham, pesticide stock solutions were freshly prepared in ethanol prior to their aseptic addition to either sterile PY or PGY medium. Pesticide/media solutions were shaken at 180 cycles min^{-1}, on a Griffin flask shaker for 24h at room temperature to monitor sterility.

Propham solutions were prepared by dissolving the compound in distilled water at 50°C whilst stirring continuously. On cooling, the solution was filter-sterilized using a 0.45 um pore membrane filter (Millipore). The pesticide solution was then added aseptically to an equal volume of sterile double strength PY or PGY medium. Membrane filtering of PY medium result in loss of particulate matter and possible feeding distress in Tetrahymena pyriformis (Elliott, 1979).

The wide range of pesticide concentrations employed reflected the objective of the study rather than ecological considerations.

13.2 Culture procedures using Repli-dishes

The pesticide/media solutions were aseptically dispensed in 3ml amounts into each of the 25 wells of sterile Repli-dishes (Sterilin). Eight treatment levels per pesticide were tested with each level, replicated 50 times. Groups of four Repli-dishes were placed on moist paper towels inside pre-warmed 23cm x 23cm plastic antibiotic assay plates (Nunc-bio) as a precaution against evaporative water loss. Before sealing the assay plates, each Repli-dish well was inoculated with 0.15ml from a 72h flask culture of either *Tetrahymena pyriformis* or *Acanthamoeba castellanii* so that the initial number of cells per ml in the wells were 1.0×10^4 and 7.0×10^4 respectively. Previous experiments had determined this to be the most suitable medium volume and inoculum level with each organism. The Repli-dishes were incubated inside the assay plates at 20°C for *T. pyriformis* and 30°C for *A. castellanii* in the dark. The assay plates allowed compact stacking of and easy access to Repli-dishes throughout the experiment.

13.3 Sampling procedures

Individual wells were assigned a number between 1 and 400 and clearly marked. At each successive sampling time (0, 24, 48, 72, 96 and 120h) generated random numbers (Hewett-Packard 'stat-pack') were employed to select 19 replicate wells per treatment per sampling time. The total volume from each well was removed and the protozoa in a sample (1ml) of each replicate were enumerated separately. All sampling procedures were done aseptically using either a Laminar-flow cabinet (T. pyriformis) or a Class II safety cabinet (A. castellanii).

13.4 Enumeration of protozoa

Samples (1 ml) were removed and fixed in a 1.5% v/v glutaraldehyde solution in a 1:1 ration, to a) render T. pyriformis immobile and b) to assist in the handling of A. castellanii. Cells were counted using a modified Fuchs-Rosenthal haemocytometer, Hawksley, Weber BS748, (Prescott & Olson, 1972; Edwards & Lloyd, 1978). The number of cells within the haemocytometer's 9 grid squares (total dimension 0.2 x 3 x 3mm) were counted. Cells were not included in the count if more than 50% of the cell body laid outside the outer 'tramlines' of the grid. Cells undergoing division were classed as individuals. In later experiments the optical density of suspensions of A. castellanii cells were read at 400nm in a sp 6-550 spectrophotometer (Pye Unicam) with an auto-flow-through

system and a 1cm path length curvette. The relationship between cell number (x) and optical density (y) was:

$$x = y - 7.92^{-0.4} - 2.92$$

the correlation being highly significant at P = 0.05.

Reliable results were not obtained using optical density to determine cell numbers in the case of T. pyriformis. The various wavelengths described by other workers; 530nm (Wallace & Holmlund, 1980), 540nm (Schultz & Allison, 1979), 620nm (Surak et al., 1976), 650nm (Iwata et al., 1967) and 660nm (Hayes et al., 1976) did not yield stable and reproducible readings. The use of fixatives, different spectrophotometers, pathlength and sample volume did not alleviate the problem.

However, a strong positive correlation (highly significant at p=0.05) was found between optical density and disrupted cells of T. pyriformis. Sonication of 10ml of a 72h flask culture of cells, approx 2.8 x 10^5m cells ml^{-1} for 30 sec with a MSE 100 watt sonicator (10mm probe) disrupted T. pyriformis cells. Such disrupted cells gave stable optical density readings at 540 or 620 nm in a sp 6-550 spectrophotometer (Pye Unicam) with an auto flow through system and a 1cm path length curvette. This method was not used in any of the experiments for which data are presented.

13.5 Statistical analyses

The size and configuration of the wells within Repli-dishes allowed extensive replication in a compact area. Each well was considered to be separate culture vessel and the data were not pooled.

Initially the data were analysed for the degree of variability within treatments compared to the variability between treatments using one-way analysis of variance. The significance of this variability was subsequently tested using a modified student t-test.

Further analysis of growth rates compared the slopes of linear regression lines and their subsequent significance testing. The significant 't' values for each pesticide were then placed in a matrix, in graphic form, to depict trends which assist in defining the relationship between chemical concentration and growth rate (Appendix 2. p 229).

14.0 Sub-acute toxicity determinations in microtiter plates

41.1 Pesticide solutions

The preparation of pesticide solutions and their incorporation into either PY or PGY media have previously been described. Thirteen pesticides were evaluated using

the qualitative (band-formation) technique. They were chlorpropham, propham, barban, diuron, linuron, isoproturon, asulam, benomyl, pirimicarb, terbutryne, cyanazine, permethrin and MCPA. With the use of a microtiter plate reader (Dynatech MR600) 16 pesticides were assessed for their toxicity to both protozoa. With the exception of asulam and benomyl, all the above compounds were assessed as were fenuron, glyphosate, ethirimol malathion and carbaryl.

14.2 Culture procedures using microtiter plates

14.2.1 Qualitative assessment procedures

A number of simple procedures were devised using microtiter plates to evaluate the toxicity of pesticides to protozoan populations. These qualitative techniques involved visual assessment of the growth of protozoan populations within individual microtiter plate wells. The pesticide/media solutions were dispensed (160 µl) into the 96 'U'-shaped wells of polystyrene microtiter plates (Sterilin or Titertek/Linbro). Each of the wells, with eight replicate wells per treatment, was inoculated with 40 µl of a 72h flask-grown culture (50ml) of either *T. pyriformis* or *A. castellanii* such that the initial number of cells per 200 µl well was 1.45×10^3 and 1.6×10^4, respectively. Strict aseptic conditions were maintained throughout media dispensing and inoculations using

Laminar-flow cabinets (T. pyriformis) or Class II safety cabinets (A. castellanii). Lids were replaced and the plates (x4) were placed on moist paper towels in 23cm square antibiotic assay plates (Nunc-bio). The microtiter plates were incubated at 20°C (T. pyriformis) or 30°C (A. castellanii) in the dark. At 24h intervals the microtiter plates were examined visually and with a dissecting microscope (x 20) to determine the presence or absence of growth. The lowest concentrations to prevent growth or to cause a detectable lowering of growth, as compared to control wells, was recorded. The optimum incubation period to detect these gross effects was 72h.

Enumeration of the protozoan populations within individual wells, overtime, (Fig. 6), showed that at 72h cell numbers of T. pyriformis and A. castellanii were 1.9×10^5 and 4.2×10^5 respectively.

Vital staining. Suspensions of cells (100 µl) of T. pyriformis and A. castellanii were pre-fixed with glutaraldehyde (3% v/v) in a 1:1 ratio before the addition of Steedman's triple stain (Steedman, 1970) to differentiate live and dead cells. The addition of the stain turned live cells pink and dead cells blue but in addition caused gross colourimetric changes, pink-blue, in the wells in which the suspensions were held. A link between intensity of colour change and the number of live cells present was established, but variability curtailed experimentation.

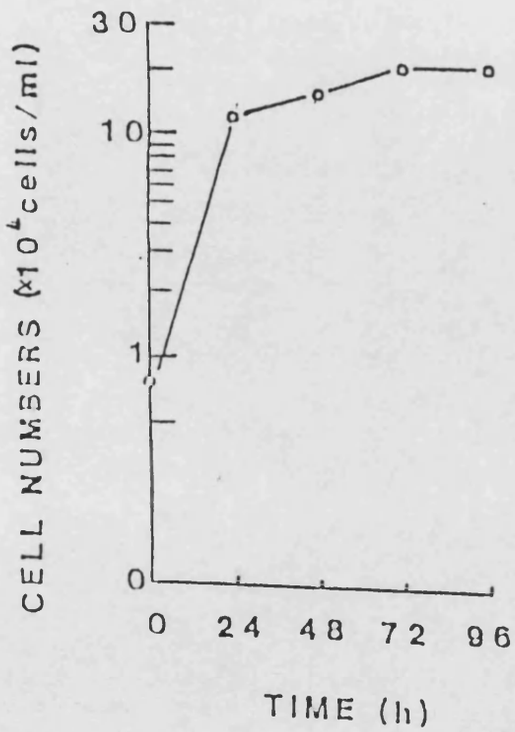


FIG. 6

A The growth of T. pyriformis in 200µl of PY medium in the wells of a microtiter plate at 20°C.

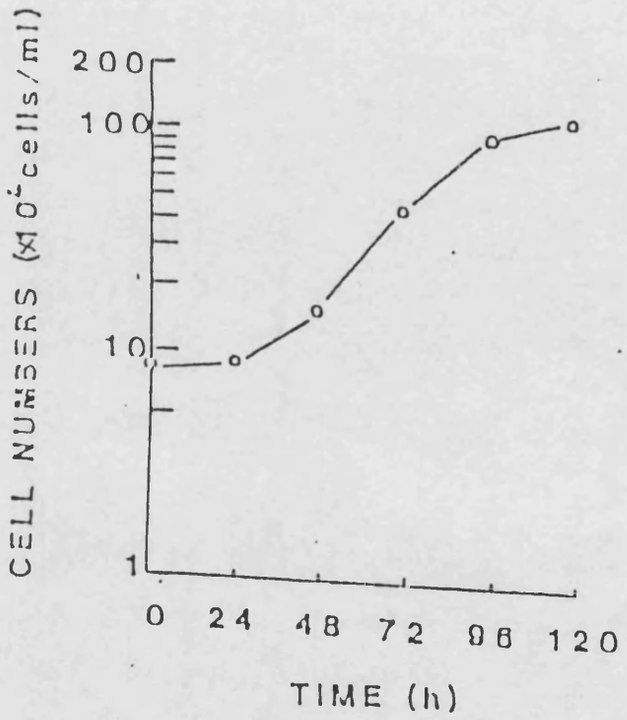


FIG. 6

B The growth of A. castellanii in 200µl of PGY medium in the wells of a microtiter plate at 30°C.

Assessment methods. The 'U' shape of individual wells and the tendency for T. pyriformis cells to settle on the bottom of the wells in response to pesticide treatments, permitted the assessment of the inhibitory effects of pesticides on populations of the ciliate. Direct comparisons with settled cells in untreated wells showed differences in cell mass conformations. Control wells appeared 'cloudy' whereas treated wells had dense cell accumulations on their bases. Despite distinct differences between treated and untreated wells the sensitivity of this system was slight.

A. castellanii cells naturally form aggregates on the base of culture vessels unless they are kept in suspension by agitation. Static incubation and a high initial inoculum (8.2×10^4 cells ml^{-1}) created a distinct circle of sedimented cells in 'U'-shaped wells. No such effects were observed with flat bottomed or 'V'-shaped wells. Successive increases in population size led to differing conformation within the wells (Fig. 7). After 72h a band was clearly visible in untreated wells and the lowest pesticide concentration inhibiting the formation of such a band was termed the 'Minimum Inhibitory Concentration' (MIC) and recorded. Well patterns were preserved by the addition of 50 µl of a 10% (v/v) glutaraldehyde solution. Haemocytometer counts confirmed that gross changes within wells corresponded to increases in population size.

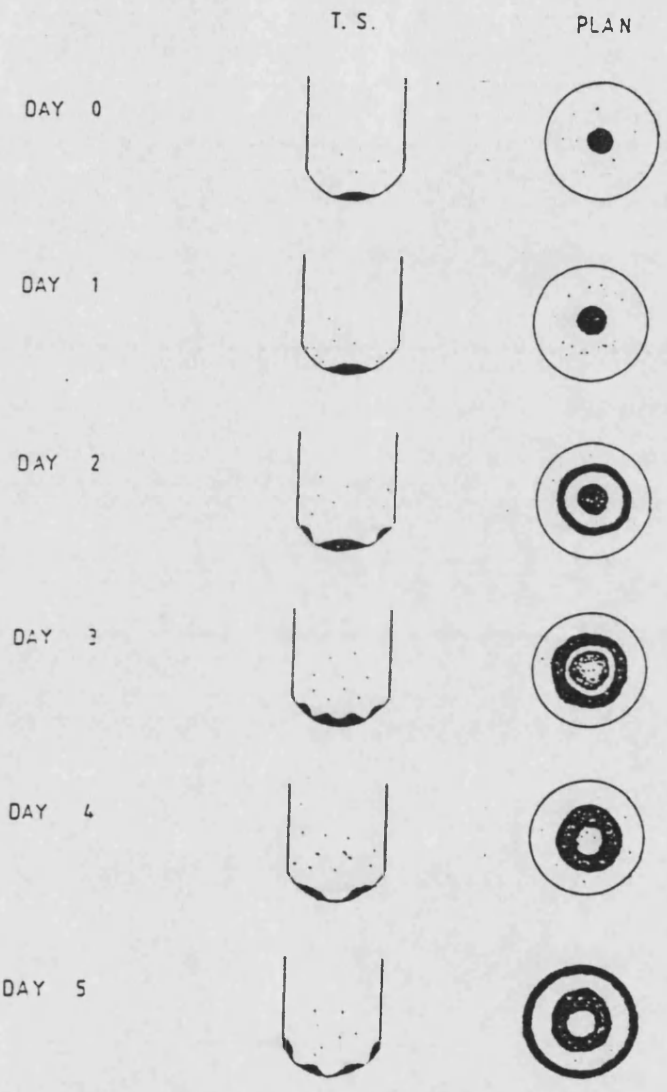


FIG.7

Pattern formation in U-shaped microtitre wells due to population growth of A. castellanii.

No pesticide was added.

14.2.2 Quantitative assessment procedures

The pesticide/media solutions were aseptically dispensed (160 ul) into the 96 'U'-shaped wells of microtiter plates (Sterilin or Titertek/Linbro). The 8 x 12 row well format of the plates allowed extensive replication of all treatments, including reference wells for optical density readings. Each well was inoculated with 40 µl from a 72h exponentially growing culture of either *T. pyriformis* or *A. castellanii* such that initial numbers of cells per ml were approximately 4×10^4 and 3×10^5 respectively. The same volume (200 µl) of sterile medium was dispensed into wells designated for optical density reference. Media dispensing and inoculations were done under strict aseptic conditions utilizing laminar flow and exhaust protective (Class II safety) cabinets where appropriate. Lids were replaced on the plates immediately and they were incubated statically at 20°C (*T. pyriformis*) or 30°C (*A. castellanii*) in the dark. Evaporation loss from the wells was minimised by placing the plates (x4) in 23cm square antibiotic assay plates (Gibco Europe Ltd) lined with moist paper towels.

Sampling procedure. At zero time and subsequently at 24h intervals the protozoan population size was determined according to optical density readings at 490nm (*T. pyriformis*) and 410nm (*A. castellanii*). In some cases a dual wavelength mode (490 or 410 with 630nm) was used to help nullify the effects of light scattering by the microtiter plate lids. The optical density readings were

taken using a Dynatech MR600 microplate reader (Dynatech Laboratories Ltd) linked with a microcomputer (Apple II) to copy and store the generated data. Before readings were taken it was necessary to ensure uniform suspensions of the organisms by shaking (100 cycles min^{-1}) for 30 seconds (Dynatech 4-plate shaker) and to remove condensation from the inside face of the plate lid and moisture from the plate base. Plates were immediately re-incubated after taking optical density readings.

Enumeration of protozoa. For T. pyriformis and A. castellanii a positively correlated relationship was found between growth assessed by optical density and by cell number (Figs. 8 & 9). The linear regression equations (significant at p = 0.01) were:

$x = y + 0.08 / 6.3^{-7}$ (T. pyriformis)

$x = y + 0.65 / 1.3^{-6}$ (A. castellanii)

(x = cell number, y = optical density)

and the relationship is shown in Fig. 10.

Statistical analyses. The 96-well format of the microtiter plates allowed extensive replication, permitting detailed statistical analysis. Each data set was subjected to one-way analysis of variance and a student t-test.

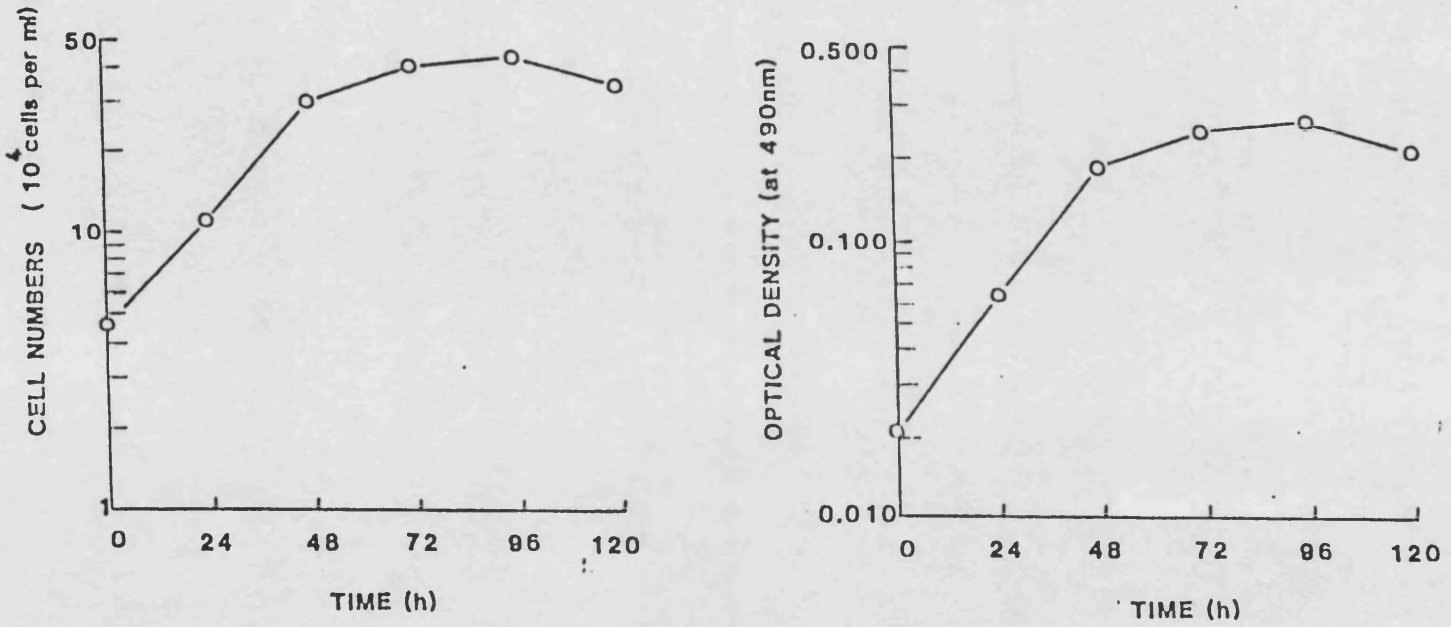


FIG. 8

The growth of T. pyriformis in 200 µl of PY medium in microtiter plates. Growth was assessed by haemocytometer counts and by optical density at 490 nm using a microtiter plate reader.

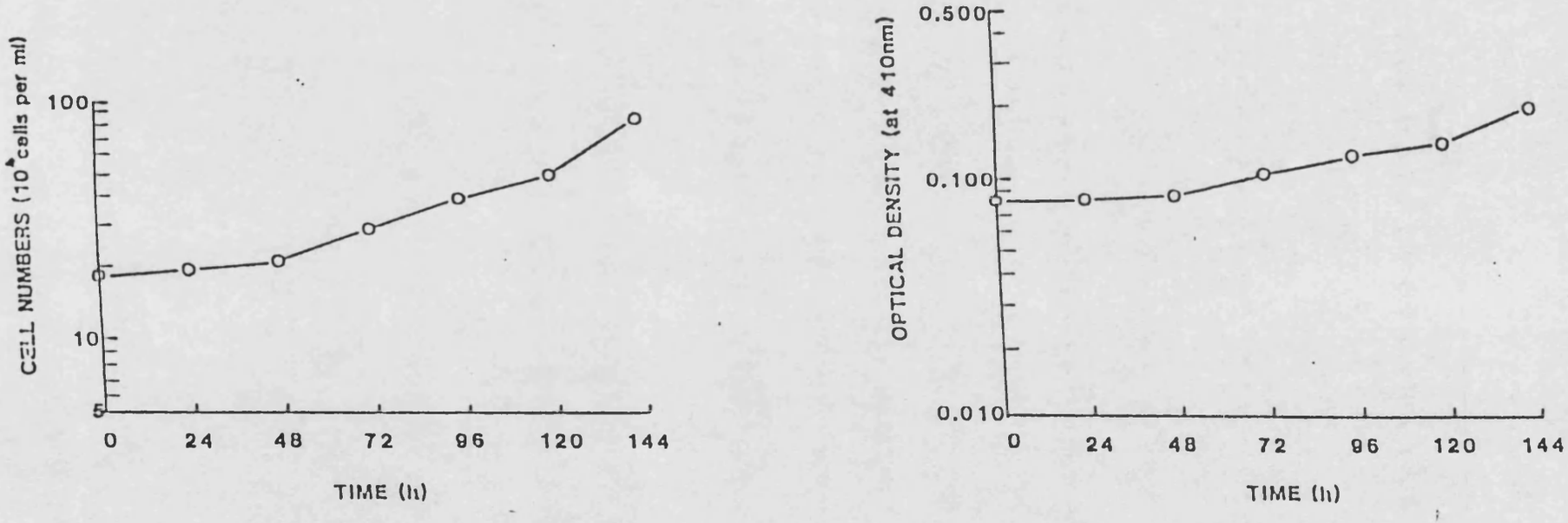


FIG. 9

The growth of A. catellanii in 200 µl of PGY medium in microtiter plates. Growth was assessed by haemocytometer counts and by optical density at 410 nm using a microtiter plate reader.

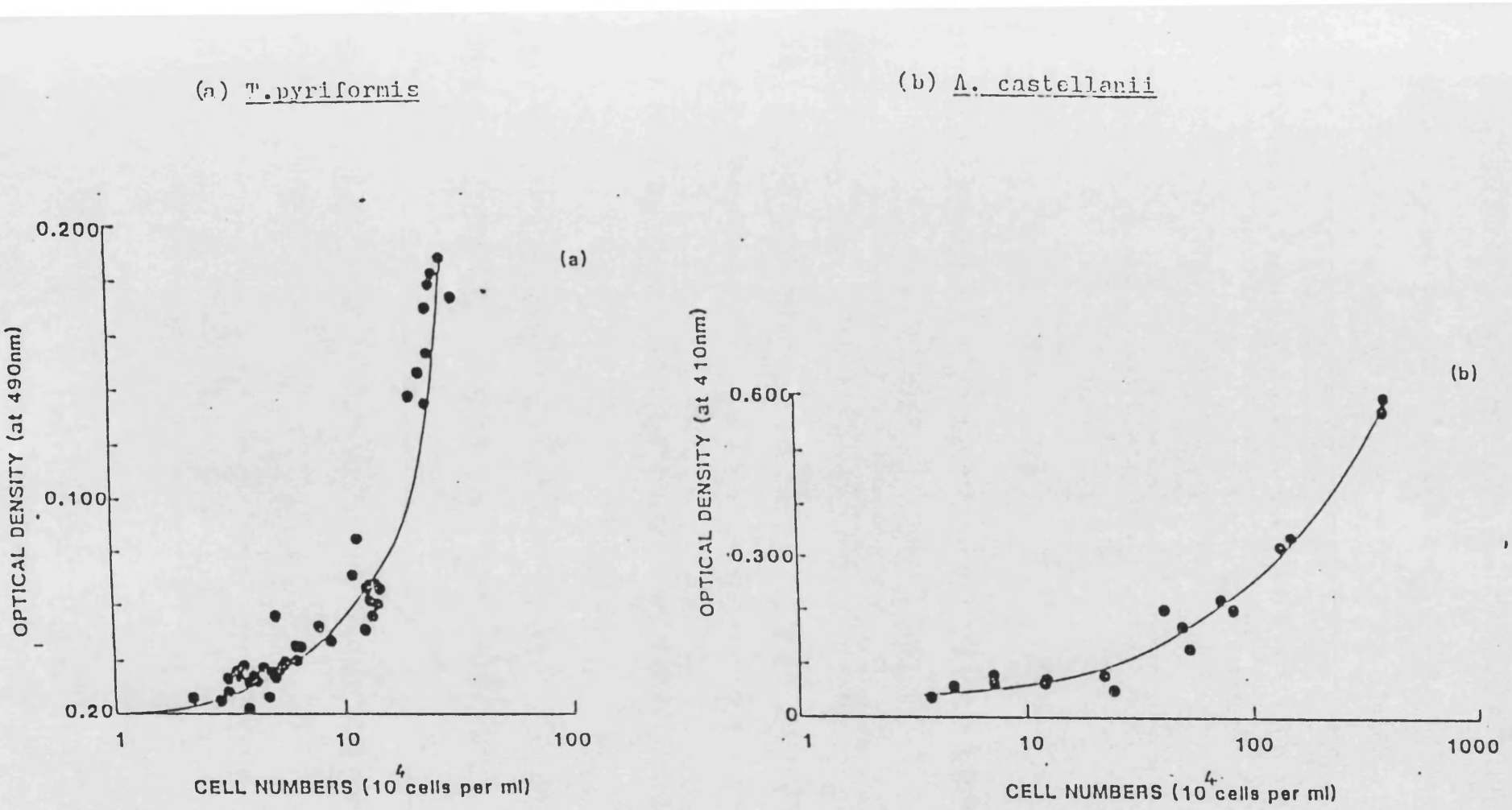


FIG. 10

The relationship between optical density and cell numbers for cultures of T.pyriformis and A.castellanii grown in 200 µl of medium (PY and PGY respectively) in microtiter plates.

15.0 Chronic toxicity determinations in flask cultures

15.1 The effects of some phenylcarbamates on the growth of T. pyriformis and A. castellanii

Stock solutions of chlorpropham, propham and barban were freshly prepared in ethanol. Concentrations of each compound were then made up in either sterile PY or PGY medium. The final concentration of ethanol in the growth media was 0.01% (v/v) and 'control' media without herbicide also had this proportion of ethanol added. The herbicide concentrations used were based on the EFC of each compound, (Table 1), but as this was an initial enquiry both lower and considerably higher levels were also used. Final concentrations used were; chlorpropham, 40,20,4 and 2 μgml^{-1}; propham, 50,25,5 and 2.5 μgml^{-1}, and barban, 10,5,1 and 0.5 μgml^{-1}.

The stock herbicide solutions, made up in either sterile PY or PGY medium were further diluted to the required concentrations (with the appropriate medium) and aseptically transferred (47.5ml) to 250ml Erlenmeyer flasks. The herbicide/medium was inoculated with 2.5ml of liquid cultures of A. castellanii (96h) or T. pyriformis (72h). The final volume in each flask was 50ml. The chronic toxicity of the herbicides was determined for each organism by quadruplicate testing of each herbicide concentration.

Cultures were incubated statically at 20°C (T. pyriformis) or 30°C (A. castellanii) for 10 or 14d, respectively, in the dark.

Sampling procedure; enumeration of protozoa. At 24h intervals 1ml was aseptically withdrawn from each replicate culture and fixed separately in 1.5% (v/v) glutaraldehyde, 1:1 ratio. Previous investigations revealed that this fixative had no deleterious effect on cell number, size or shape with either organism. The number of cells in each replicated treatment was determined and the mean and standard deviation calculated. Cell counts (previously described p 56) were made using a modified Fuchs-Rosenthal haemocytometer (Hawksley, Weber BS748). Sampling was done using aseptic procedure and, where applicable, in a Class II safety cabinet.

In the case of T. pyriformis the cells were further examined for the effect of each herbicide on cell morphology. For each treatment 50 cells were selected (the first 50 cells occurring within the counting area of the haemocytometer starting top right and proceeding to bottom left) and the long and narrow axes of each cell were measured using a calibrated eye piece graticule. The frequency of spherical cells and the occurrence of morphological abnormalities were also recorded and photographed.

All observations on T. pyriformis and A. castellanii were made using a Leitz Dialux research microscope at x250 and x400 magnification. All photographs were taken with a Leitz Orthoplan microscope, x400 magnification with phase contrast optics and using colour slide film (Kodak, ASA 100).

Observations were initially on pre-fixed and fixed cells but no differences between the two were found and subsequently from 48h all observations were on fixed cells. Occasionally pre-fixed cells were examined to help clarify all major observations.

16.0 Morphological and cytological changes caused by some pesticides in Acanthamoeba castellanii and Tetrahymena pyriformis

16.1 Acute effects of some pesticides on Acanthamoeba castellanii

Cells from exponentially growing cultures (48h) of A. castellanii were aseptically transferred to sterile pesticide/medium solutions, gently mixed, and a drop of culture fluid placed in a 0.02mm depth Thoma counting chamber. Sufficient liquid to cover the inner marked circle was applied and covered with a coverslip. Cells were observed after 0,3,5,10,30 and 60 min at room temperature under differential interference contrast

(Nomarski) optics (x250) using a Leitz orthoplan microscope. Photographic records were obtained.

16.2 The influence of some pesticides on cell size in Acanthamoeba castellanii

A. castellanii cells were prepared as for sub-acute toxicity studies in Repli-dishes and grown in the presence of the pesticides for 7d (section 12.0). At 24h intervals 1ml of culture fluid was aseptically removed and fixed in 1% (v/v) glutaraldehyde and the cell size established. Size was defined as the greatest distance between two points on the plasma membrane lying opposite each other in a straight line. Twenty cells were observed per pesticide treatment and the data were analysed by one-way analysis of variance and by t-test. Observation on cells before fixation were also made under phase contrast using a Leitz dialux research microscope (x 400).

16.3 Acute effects of chlorpropham on Tetrahymena pyriformis

T. pyriformis cells (72h exponential culture) were placed in 3ml of fresh PY medium containing chlorpropham (20 $\mu g ml^{-1}$) in Repli-dishes. At intervals, over 8h, 200 μl of culture were removed, placed in a haemocytometer (modified Fuchs-Rosenthal) and the cells observed under phase contrast (x 400 magnification, Leitz dialux research microscope). The morphology, motility and cytology of

cells were noted. Aseptic procedures were maintained throughout. Observations were also made over 24h.

16.4 Electron microscopy

T. pyriformis cells were exposed to chlorpropham (0,2,4 and 20 $\mu g ml^{-1}$) in Erlenmeyer flasks for 24h (cf. chronic toxicity experiments). Samples (50ml) were centrifuged (2000 rpm for 10 min in 50ml Oakridge tubes) and the cells were resuspended in 5ml 3% (v/v) glutaraldehyde in 0.1 M cacodylate buffer (pH 6.8) for 24h. Bulked samples were further centrifuged and resuspended (equal volume) in 1% (v/v) osmium tetroxide for 2h at 4°C.

The fixed cells were passed through a series of acetone solutions (30,50,70 and 100%), 10 min in each concentration, and finally left in 100% acetone for 24h.

16.4.1 Transmission electron microscopy

The cells were suspended in a 1:3 mixture of acetone and Taab embedding resin (Emscope Laboratories) in a 1:1 ratio and left for 12h in a fume cupboard. After further centrifugation the spent resin was decanted and two changes (4h) in fresh resin were given. After a further change the resin was hardened by heating at 60°C for 2d.

Ultra-thin sections were cut with glass knives on a Reichart OM vs ultramicrotome. Sections were selected and mounted on copper grids and stained (10 min) with saturated uranyl

acetate in 95% EtoH. The grids were washed (deionised water) and further stained with Reynold's lead citrate (15 min). The prepared grids were mounted and viewed in a Jeol 100 cx electron microscope (Jeol (UK) Ltd, Colindale, London) at an accelerating voltage of 80 kv.

Further sections were selected, stained with toluene blue (10 min) and observed in the light microscope (x400 magnification) under bright field optics.

16.4.2 Scanning Electron microscopy

Following serial dehydration in 30-100% acetone, the cells were critical-point dried (Polaron Equipment Ltd, Watford, Herts), mounted in a graphite paste on aluminium planchettes, sputter-coated *in vacuo* with a thin layer of gold/paladium (Polaron Equipment Ltd, Watford, Herts) and examined using a J35C scanning electron microscope (Jeol (UK) Ltd, Colindale, London).

17.0 The influence of chlorpropham and diuron on the respiration rate of Tetrahymena pyriformis

17.1 Preparation of cells

Cultures (50ml) of *T. pyriformis* cells (grown statically

at 25°C) in Erlenmeyer flasks for 96h were centrifuged at 1000rpm for 5 min (Centaur 1, bench centrifuge MSE) and resuspended in 10ml of phosphate buffer (0.1M KH_2PO_4 at pH 6.8) in sterile Universal bottles. Cell numbers were determined (Fuchs-Rosenthal haemocytometer) and adjusted to 1 x 10^6 cell ml^{-1} with phosphate buffer.

17.2 Respiration studies

Into the main chamber of small Gilson differential respirometer flasks (20ml) were pipetted 1ml cell suspension, 0.5ml buffer and 0.5ml 0.1M glucose. One ml of pesticide/buffer solution was placed in the side arm and 0.2ml 10% KOH in the central well. Pleated filter paper was placed into the KOH to increase CO_2 absorbing area. The flasks were fitted to a single valve differential respirometer (Gilson Medical Electronics Co) and left to equilibrate for 15 min in the pre-heated (25°C) shaking water bath.

At T=O the pesticide/buffer solutions were tipped into the main chambers. Micrometer readings were taken every 5 min for 200 min. Each treatment had duplicate flasks and each experiment was done twice. In each experiment an endogenous rate control flask was maintained. The treatments were 0,2,4 and 20 μgml^{-1} chlorpropham and 1,1.5,3 and 15 μgml^{-1} diuron. Aseptic techniques were employed throughout.

18.0 Studies on food-vacuole formation in Tetrahymena pyriformis treated with pesticide

18.1 Pesticide solutions

Preparation of pesticide stock solutions, their subsequent dilution and incorporation into PY medium has been previously described (section 11.0). The pesticides used in these studies were chlorpropham, propham, barban, diuron and malathion. The pesticide concentrations used were based on 'estimated field concentrations' (EFC's). The dose levels were 5x, 1x, 0.5x, 0.25x, 0.125x and 0.025x the estimated field concentration. Controls were untreated.

18.2 Culture procedure using Repli-dishes

The pesticide/media solutions were aseptically dispensed (3ml) into the wells of Repli-dishes. The six pesticide treatments plus the untreated level were sufficiently replicated so as to allow duplicate wells per treatment to be removed at each sample point. All wells were inoculated (0.1ml) with a 72h, flask grown culture of *T. pyriformis* such that the initial number of cells per well was 3×10^4. The Repli-dishes were placed on moist tissue paper inside 23cm square plastic trays with lids. Dishes were sealed before being incubated at 20°C in the dark.

18.3 Sampling and microscopic observation procedures

At 3 or 24h intervals samples (3ml) were aseptically withdrawn from each well and 200 µl 3% (v/v) Indian ink solution was added (Rowney's, Kadahar No. 28 black Indian drawing ink). Batches of ink varied in their effects on T. pyriformis. Recently purchased stocks were toxic to the ciliate possibly due to an alkaline anti-flocculating agent (Steedman, personal communication). All feeding studies were carried out with old stocks (2 years) which had been exposed to air for an undetermined period. Cells were exposed to the ink solutions for 15 min before being fixed (1:1) 3% (v/v) glutaraldehyde solution. The optimum exposure time to Indian ink had been previously determined (Plate 13). The contents of each replicate well were treated separately throughout.

Cells were placed in a counting chamber (modified Fuchs-Rosenthal) before observations. A total of 50 cells (25 from each well) per treatment were observed under the microscope (x 250 magnification, Leitz Dialux research microscope) and the number of food vacuoles, readily visualised as black spheres, within each was recorded.

Those pesticide concentrations which inhibited food vacuole formation after 24h were further investigated in repeat experiments sampling every 3h for 24h.

18.4 Statistical analyses

The mean number of food vacuoles formed per cell per pesticide treatment were calculated together with the mode number of food vacuole formed per treatment population. This enabled trends in food vacuole formations within cell populations as well as changes within individual feeding rates to be detected. Cumulative frequency plots, ogives, were also calculated to reveal changes in the frequency distribution of feeding activity within populations.

19.0 Induced encystment in Acanthamoeba castellanii by various replacement techniques

A. castellanii strain G cells from 72h flask cultures (50ml) were harvested by centrifugation in sterile Oakridge tubes (50ml) at 2000rpm for 10 min. The pellet was resuspended in 15ml PGY medium designated suspension a which served as an inoculum source for the following encystment methods.

19.1 Method A. The replacement technique of Neff et al. (1964) for Acanthamoeba sp.

Five ml suspension a (1×10^7) cells ml^{-1}) were added to 45ml encystment medium (EM):- KCl, 0.1M, Amine buffer, 0.02M, $Mg50_4$, 0.008M, $CaCl_2$, 0.004M, and $NaHCO_3$, 0.001M.

The cells were re-spun (2000rpm for 5 min) and resuspended in 50ml EM in 250ml flasks.

19.2 Method B. The replacement technique of Band & Mohrlok (1969) (for Acanthamoeba rhysodes)

Five ml suspension a were added to 45ml filtered (0.22 μm) proteose-peptone-glucose medium, PPGF. The PPGF contained 10 times less glucose than the normal PGY medium and represented a modification of the original method of Band (1963). Flasks were incubated statically at 30°C for 48h. The starved cells were then centrifuged (2000rpm for 5 min) and the pellet resuspended in a high salt medium (HSM):- $NaCl_2$, 0.25M, $MgCl_2.H_2O$, 0.0032M and $CaCl_2$, 0.00036M.

19.3 Method C. The replacement technique of Chagla & Griffiths (1974) (for Acanthamoeba castellanii)

This method was based on the findings of Griffith & Hughes (1968 & 1969) that the essential requirement for encystment in A. castellanii was magnesium ions.

Five ml A. castellanii cell suspension a were washed twice in 0.05M $MgCl_2$ before 5ml (1×10^7 cell ml^{-1}) were added to 45ml of 0.05 and 1.0M solutions of $MgCl_2$ in 250ml flasks.

For all three methods duplicate flasks were incubated either statically or at 80 cycles min^{-1} at 30°C for 72h. At 24h intervals 1ml samples were withdrawn and the cells were examined microscopically. Aseptic procedures were maintained throughout.

A further culture of A. castellanii Neff strain was obtained from Dr A J Griffiths (University College, Cardiff) and induced to encyst by Method C.

20.0 The influence of chlorpropham on encystment in Acanthamoeba castellanii

Flask grown cultures (72h) of A. castellanii Neff strain were centrifuged (2000rpm for 10 min) in sterile Oakridge tubes and the pellet washed in 0.05M of $MgCl_2$. The culture was further centrifuged (2000rpm for 5 min) and the cells resuspended in 50ml chlorpropham/replacement medium solutions containing 0,1,2,4 or 20 $\mu g ml^{-1}$ chlorpropham. Duplicate flasks per treatment were then incubated statically at 30°C. At 24h intervals 1ml samples were removed, fixed (3% (v/v) glutaraldehyde) 1:1 and stained to enhance observations on the morphological appearance with a tri-acid general stain (Steedman, 1970).

After staining, the cells were observed (x 250 x 400 magnification Leitz Dialux research microscope) in a modified Fuchs-Rosenthal haemocytometer. The number of trophozoites and cysts were recorded.

21.0 Excystment of Acanthamoeba castellanii

Removal of cyst suspensions from either replacement media or distilled water and their resuspension in PGY medium results in synchronous excystment in A. castellanii.

22.0 Influence of two herbicides on excystment in Acanthamoeba castellanii

Cysts of A. castellanii Neff strain were obtained using the replacement method of Chagla & Griffiths (1974) Method C. The cysts (1.6×10^4 ml^{-1}) were then stored prior to use at 4°C in distilled water for 1200h. Chambers & Thomson (1973) found both synchrony and extent of excystment to increase with increasing cyst age in A. castellanii. The maximum occurring with cyst between 800 and 1200h old (Chambers & Thomson, 1973).

After 1200h cysts were centrifuged (2000rpm for 10 min) in sterile Oakridge tubes and the pellet re-suspended in PGY medium. The cysts were washed twice before the pellet was re-suspended in pesticide/PGY solutions. The separate pesticide treatments were 0,1,2,4 and 20 $\mu g ml^{-1}$ chlorpropham and 0,0.25,0.5,1 and 5 $\mu g ml^{-1}$ barban. Four replicate flasks per treatment were incubated at 80 cycle

min^{-1} at 30°C. At 24h intervals 1ml culture was removed, fixed and stained as previously described before microscopic observation. The number of cysts and trophozoites per treatment were recorded.

23.0 The influence of chlorpropham on motility in Tetrahymena pyriformis

23.1 Background information on pattern formation in Tetrahymena pyriformis cell cultures

The phenomenon of pattern formation in dense cultures of motile ciliates, including T. pyriformis, was noted by Loef & Melford (1952). As ciliates swim they produce a turbulent vortex ring, due to Newton's third law of motion and despite a low Reynold's number (Jahn & Brown, 1961). Any other ciliate which enters this ring may be hydrodynamically linked to the first organism and tend to follow it (Jahn, Brown & Winet, 1961). The net result in a population of moving cells is a horizontal movement (stream) towards a node, forming a polygonal network with 4 or 5 streams meeting at each node (Hill, 1972). At each node organisms move downwards and then turn horizontally in a radiating pattern to eventually join other streams in a random manner (Jahn et al., 1961). Although individual lines approach an equilibrium, each is actively in a state of dynamic equilibrium and the entire pattern is

relatively fluid, constantly changing shape and size (Loef & Melford, 1952). Under optimal conditions and in a shallow medium the pattern can be formed in less than 10 sec (Hill, 1972). The phenomenon of pattern formation in batch culture of T. pyriformis was found to be predominantly dependant on cell cencentration, with an optimal density of 1.5×10^5 cells ml^{-1}. However, superimposed on this dependancy was an endogenous circadian rhythm, minima 20h, (Willie & Ehret, 1968).

23.2 The inhibition of pattern formation in populations of Tetrahymena pyriformis

A 72h late exponential-phase culture (50ml) of T. pyriformis was centrifuged in sterile 50ml Oakridge tubes at 2000rpm for 10 min. The supernatant was quickly removed by pipette before the cells swam upwards and the cells were resuspended in 20ml of pesticide/medium solution in sterile 250ml flasks. the treatment levels were 0,5,10,30 and 60 $\mu g ml^{-1}$ of chlorpropham with duplicate flasks incubated for 1 or 2h at 20°C. The initial concentration of cells was 6.5×10^4 cells ml^{-1}, previously established as optimal.

After incubation (1,2h), the contents of each flask were agitated to give an even suspension of cells and poured into a 5cm diameter sterile Petri dish (sterilin). All five treatments were poured simultaneously, with

with assistance, and photographed immediately (ASA 125, automatic exposure). Further photographs were taken at 30, 60, 120 and 300 sec. Each experiment was repeated 3 times.

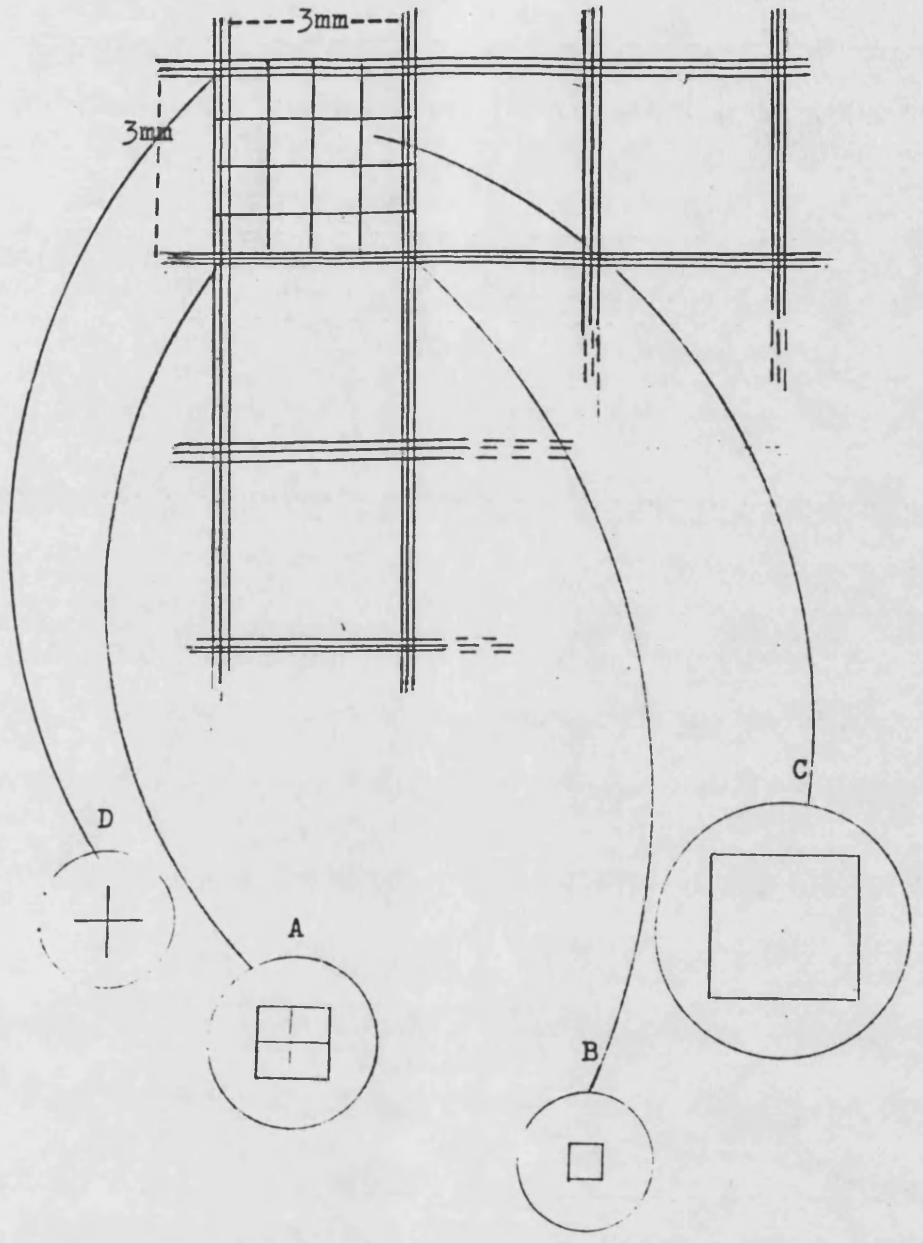
23.3 Quantitative expression of population motility

If a constant number of cells are placed into the known volume of a counting chamber and a fixed location observed, the number of cells entering, touching or crossing that location over a given period of time will provide an indication of the motility of individuals within a population. This index was termed the 'frequency of crossing'.

23.3.1 Determination of constants

The etched rulings of part of a modified Fuchs-Rosenthal haemocytometer appear overleaf.

Schematic diagram of a modified Fuchs-Rosenthal haemocytometer showing the ruled etchings selected for population motility studies with Tetrahymena pyriformis.



A number of fixed locations to measure frequency of crossing were selected. Points A, B and C were boxes of varying size and point D was a 'crosshair' site derived from the marginal tramlines. All locations were evaluated with a range of cell concentrations, 1.5,1.7,2.0,2.3 and 2.8×10^5 cells ml^{-1}, and were observed for various

lengths of time, 30, 60 and 120 sec. The system then adopted was, 1.5 x 10^5 cells ml^{-1} observed for 1 min entering the top left marginal tramline box A. An area approximately 2 x larger than the ciliate.

23.3.2 Method of scoring frequency of crossings

A score of 1 was recorded:

i) if an organism touched or entered any part of the box with any part of the cell body

ii) if an organism entered and remained inside the box even if parts of the cell body left the box

iii) if an organism pivoted outside the box repeatedly touching it but always maintaining contact at some point with the box. If contact was broken each contact was scored separately.

23.3.3 Culture procedures using microtiter plates

Samples (40 µl, containing 7.5 x 10^5 cells ml^{-1}) from a 72h late exponential phase culture of T. pyriformis were used to inoculate 200 µl of pesticide/medium solutions in 96 well microtiter plates (Sterilin). The treatment levels were 40 $\mu g ml^{-1}$ chlorpropham, 10 $\mu g ml^{-1}$ barban, 50 $\mu g ml^{-1}$ propham and 30 $\mu g ml^{-1}$ diuron (approx 5 x the EFC's for each compound) and an untreated control.

Each treatment was replicated 5 times. At staggered intervals for 4h the contents of each of the 5 individual wells were removed from each treatment and placed in a modified Fuchs-Rosenthal haemocytometer and the frequency of crossings recorded.

The effect of lower concentrations of chlorpropham (0,1,2,4 and 20 μgml^{-1}) were further investigated over a 6h period at 1h intervals and the effect of 20 μgml^{-1} of chlorpropham over 1h at 10 min intervals. All cultures were maintained axenic and incubated at room temperature.

23.3.4 Analysis of data

To prevent undue emphasis being placed on the response of individuals to pesticide concentration, the frequency of crossings were expressed as medians and not mean values. Medians being the middle values in a distribution frequency and thus reflect population behaviour better than means or modes which can be unduly influenced by the behaviour of individuals.

24.0 Examination of the ability of protozoa to recover after exposure to some herbicides

The basis of the experiments was to incubate protozoa in liquid culture with the herbicides for 48h, then harvest and wash the cells and examine the 'recovery' growth in

fresh medium lacking the herbicide.

24.1 Acanthamoeba castellanii: propham treatments

Concentrations of propham (0,50,60,70,80,90,100,110, 120,130,140 and 150 $\mu g ml^{-1}$) in sterile PGY medium were obtained as previously described (section 11.0). Fifty ml of each pesticide/medium solution were placed in duplicate 250ml Erlenmeyer flasks and inoculated with cells (5ml) from a 72h late exponential phase growing culture to give a cell concentration of 7.2×10^5 ml^{-1}. Flasks were incubated at 30°C on an orbital shaker (100 cycles min^{-1}) for 48h. At 24 and 48h 1ml samples were aseptically removed and cell numbers counted using a modified Fuchs-Rosenthal haemocytometer.

After 48h the flask contents were centrifuged (2000rpm for 10 min), the supernatant removed and cells washed twice with sterile PGY medium. Erlenmeyer flasks (250ml) containing sterile PGY (50ml) were then inoculated with the washed cells so that the concentration of cells in each flask was 1.6×10^4ml. All propham-treated population were replicated 4 times and incubated at 30°C on an orbital shaker (100 cylces min^{-1}) in the dark.

At 3h intervals 1ml of culture from each flask was aseptically removed, fixed and the cells counted in a modified Fuchs-Rosenthal haemocytometer.

24.2 Tetrahymena pyriformis: chlorpropham, propham, barban and diuron treatments

Herbicide concentrations, based on EFC's, were obtained in sterile PY medium through standard procedures (section 11.0). The concentrations were; chlorpropham 1,2,4,20 and 40 μgml^{-1}, propham 1.75,2.5,5,25 and 50 μgml^{-1}, barban 0.25,0.5,1 and 5 μgml^{-1}, diuron 0.75,1.3,1.5 and 3.0 μgml^{-1}.

Duplicate 50ml portions of each pesticide/medium solution were placed in 250ml Erlenmayer flasks and inoculated with cells from a 72h flask culture of T. pyriformis cells to give 1.4×10^4 cells ml^{-1}. Flasks were incubated statically at 20°C for either 24 or 48h. After 24 or 48h cell density was adjusted to a uniform level with fresh PY medium and centrifuged (2000rpm for 5 min). The loose pellet was removed, washed twice and re-suspended in 50ml sterile PY medium in 250ml Erlenmeyer flasks (final concentration 1×10^3 cells ml^{-1}). The cells were then aseptically dispensed (multi-channel micro-pipette) in 200 µl aliquots into 96 'U'-shaped wells of polystyrene microtiter plates (Sterilin). The 8 x 12 row format allowed 'treatment' replication (x7), including untreated controls, plus a row of PY medium 'blank' reference wells in each plate. The microtiter plates (x4) were placed inside large (23cm square) antibiotic assay plates (Gibco, Europe Ltd) lined with moist paper towels. Each plate

was replicated (x4) giving 28 replicate wells per 'treatment'. All plates were incubated at 20°C in the dark.

At zero time and at 3h intervals the plates were removed and the optical density of each well (at 400 and 600nm, dual mode) was recorded with a Titertek MC microtiter plate recorder (Flow Laboratories). The cells were then fixed, 3% (v/v) glutaraldehyde (1:1) and morphological details recorded using a Leitz Orthoplan microscope (x250 magnification).

Linear regressions of the recovery slopes were compared and the significance tested. Photographic records of cell morphology were obtained.

24.3 Changes in the response of Acanthamoeba castellanii and Tetrahymena pyriformis to phenlycarbamate herbicides with time

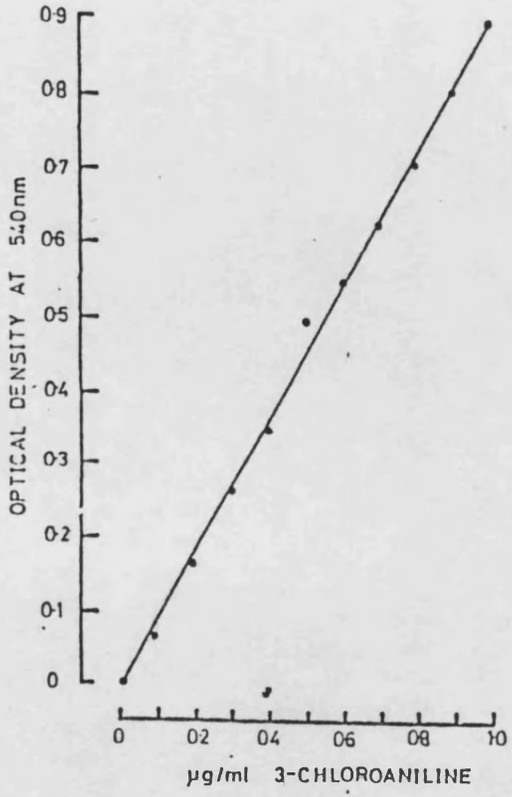
Data obtained from sub-acute (Section 12.0) and chronic (Section 14.0) toxicity determinations on the effect of phenylcarbamates on both organisms were compared and analysed for changes in the dose-response of the protozoa.

25.0 The transformation of some phenylamide herbicides by Tetrahymena pyriformis and Acanthamoeba castellanii

The transformation of chlorpropham and barban to 3-chloroaniline and propham to aniline was investigated.

25.1 3-chloroaniline and aniline assays

Amounts of 3-chloroaniline and aniline in stock solutions were determined using a diazotization-dye-coupling method (Gard & Ferguson, 1964). A range of concentrations (0.1 - 1.0 $\mu g ml^{-1}$) of each compound, dissolved in 1M HCl, was used to obtain separate standard curves (Figs II & 12). To duplicate 25ml volumetric flasks containing 5ml of each of these solutions, 1ml 2% (v/v) aqueous sodium nitrite was added, mixed thoroughly and left to stand for 20 min to allow complete diazotization. One ml of 10% (w/v) aqueous sulphamic acid (sigma) was then added, the mixture shaken and allowed to stand for 15 min to destroy excess nitrite. To the mixture 5ml 2% (w/v) aqueous N-1-naphthylethylenediamine dihydrochloride (sigma) was then added and the solution diluted to 25ml with 1M HCl. After thorough mixing the flasks were left to stand for a further 120 min for the purple colour to develop. The absorbance of the mixtures was read at 540nm (3-chloroaniline) and 555nm (aniline) in 1cm pathlength class cell using a flow-through delivery



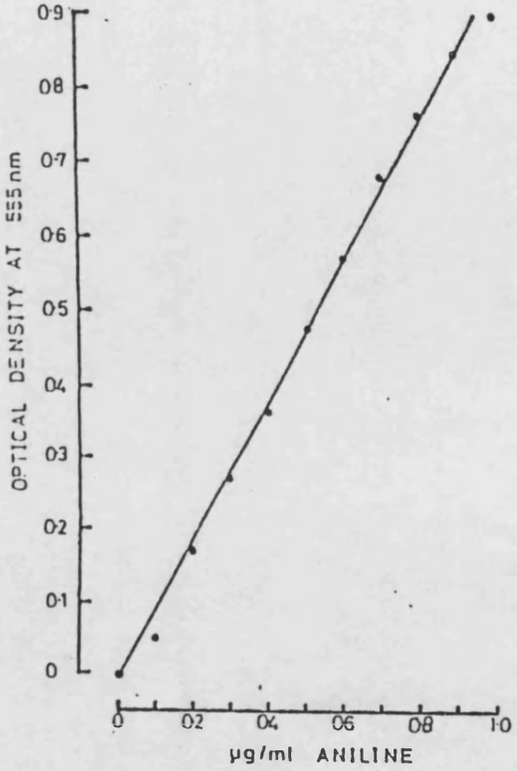


FIG. II and 12

Standard curves for the compounds aniline and 3-chloroaniline estimated by the diazotisation dye coupling method and the absorbance of the diazo compounds measured in 1cm path length glass cell using a Pye Unicam Sp 500 spectrophotometer

system on a Pye Unicam SP500 spectrophotometer. A solution of 1M hydrochloric acid treated with the reagents was used as a reference sample.

All reagents were made up freshly for each assay.

25.2 Experimental procedure

T. pyriformis cell suspensions of different density were obtained by resuspending centrifuged cells (2000rpm for 5 min) in sterile ¼ strength Ringers solution. The cells were washed 3 times before being resuspended in fresh ¼ strength Ringers/herbicide solutions and incubated at 25°C for 4 and 24h. All cell counts were done with a modified Fuchs-Rosenthal haemocytometer. The pesticide concentrations used were; chlorpropham 40 $\mu g ml^{-1}$, propham 50 $\mu g ml^{-1}$, 30 $\mu g ml^{-1}$ of diuron and barban 10 $\mu g ml^{-1}$. Reference solutions, cells in ¼ strength Ringers solution with no herbicide and ¼ strength Ringers/herbicide solution with no cells, were also incubated at 25°C for 4 and 24h.

Duplicate cell suspensions, disrupted by sonication (30 sec) with a MSE 100 watt sonicator with a 10mm probe were also tested.

After 4 or 24h amounts of 3-chloroaniline and aniline in the solutions were measured using the previously

described diazotozation-dye-uncoupling method (Gard & Ferguson, 1964).

A. castellanii cells (96h exponential-culture) were treated similarly.

All treatments were replicated 3x and the data pooled.

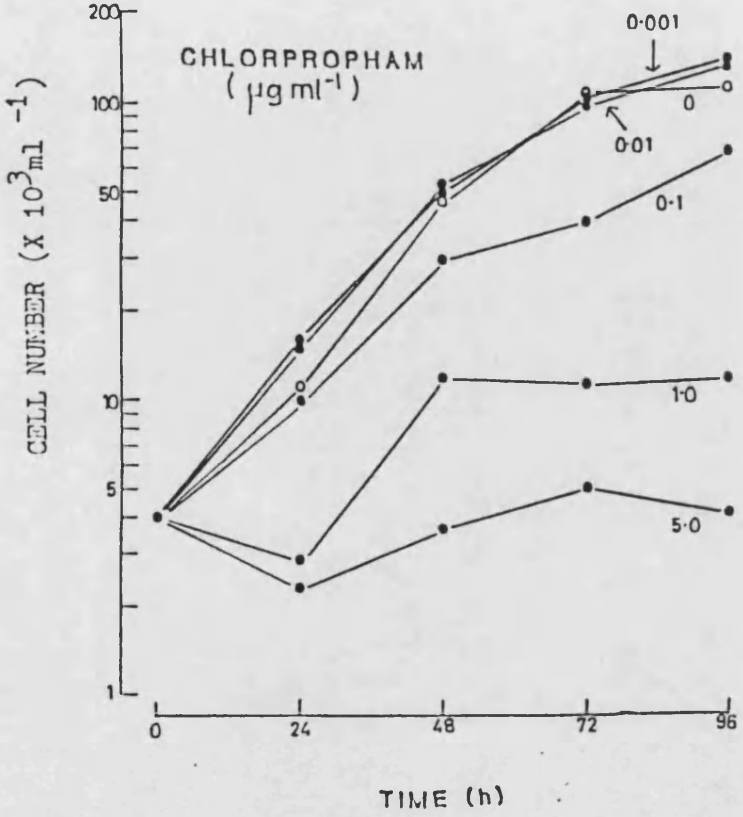
RESULTS

26.0 Evaluation of the sub-acute toxicity of some pesticides and metabolites to Tetrahymena pyriformis using the Repli-dish culture technique

26.1 Growth characteristics of Tetrahymena pyriformis in Repli-dishes

Cultures of T. pyriformis in Repli-dishes did not exhibit a lag-phase. The doubling time for cells varied with initial innoculum size. An initial innoculum level of 2.0 x 10^4 ml^{-1} gave a doubling time of 4h (Fig. 14) whilst with 1.2 x 10^4 ml^{-1} the doubling time was 8h (Fig. 21) and with 0.4 x 10^4 ml^{-1} as the initial innoculum it increased to 12h (Fig. 13). Final yield of cells was also affected by the initial innoculum size and was, after 96h, 1.4 x 10^5, 3.1 x 10^5 and 1.1 x 10^5 cells ml^{-1} respectively. Decreasing the initial innoculum size increased the duration of the log-phase of culture growth eg with an intial innoculum of 0.4 x 10^4 cell ml^{-1} the duration of the log-phase was 4d whilst with 2.0 x 10^4 cells ml^{-1} it was only 1d.

NB: Attention is drawn to the fact that the different initial innoculum levels used in the following experiments preclude all but general comparisons between the effects of different pesticides on T. pyriformis.



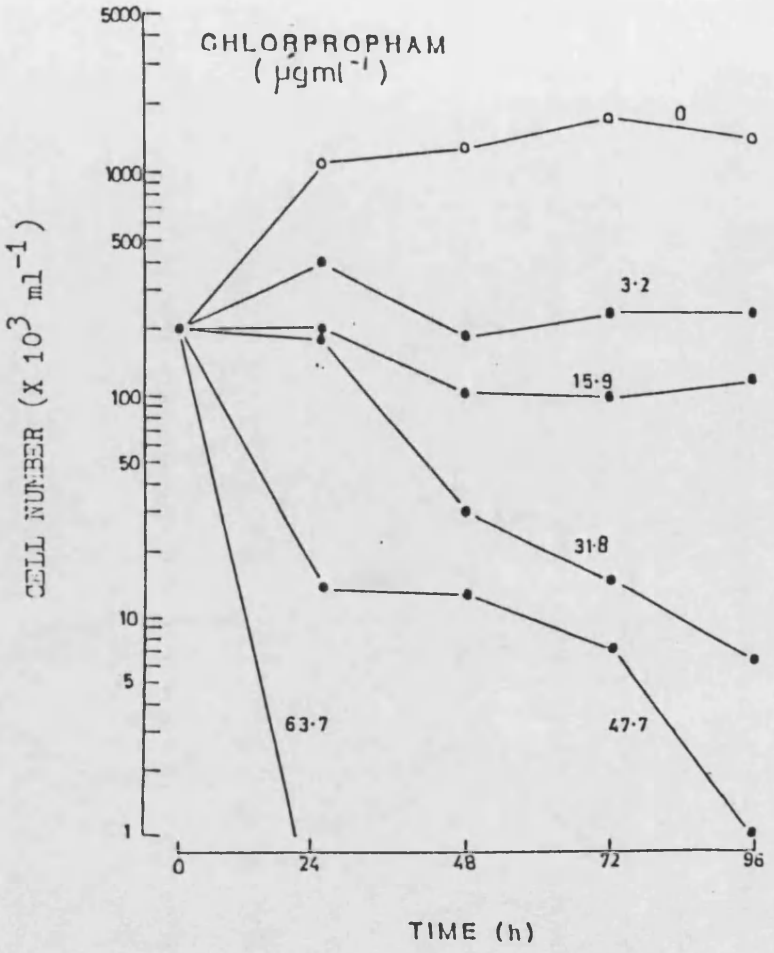


FIG. 13 (Expt. 1)

FIG. 14 (Expt. 2)

The sub-acute effects of chlorpropham on the growth of Tetrahymena pyriformis in Repli-dishes in PY medium at 20°C. Chlorpropham concentration ranges: Expt. 1, 0 - 5 µgml^{-1}. Expt. 2 0 - 63.7 µgml^{-1}.

26.2 Chlorpropham

The effects of chlorpropham on the population growth of T. pyriformis in PY medium are shown in Figs 13 & 14. The analyses of variance for each sampling point are in Appendix 3 and the significance of the differences in cell number caused by chlorpropham concentrations in Table 2.

In both experiments the degree of variation between treatments was significantly greater than the variation within treatments for each sampling point. With the lower concentration range of chlorpropham (Fig. 13) the amount of variation between treatments became more pronounced with time due to the stimulatory action of 0.001 and 0.01 μgml^{-1} and the inhibitory action of 1.0 and 5 μgml^{-1} on cell proliferation. Highly significant inhibition of growth ($p=0.05$) after 48h was caused by 0.1, 1.0 and 5.0 μgml^{-1} chlorpropham (Table 2). Both 1.0 and 5.0 μgml^{-1} had an initial inhibitory action reducing the cell number by 29.5 and 42% respectively. Chlorpropham at 0.1 μgml^{-1} caused no significant difference from the control cell number until 48h when a marked inhibitory effect was seen (significant at $p=0.05$). Concentrations of 0.001 and 0.01 μgml^{-1} chlorpropham caused increases in cell nummber significantly greater than untreated controls, 13 to 14% respectively after 72h and maintained these higher levels until the end of the experiment. Concentrations above 0.1 μgml^{-1} were inhibitory and concentrations above 3.2 μgml^{-1} prevented any increase in cell number (Fig. 14).

Table 2

Cell numbers in Tetrahymena pyriformis cultures treated with chlorpropham, in Repli-dishes. The significances of the differences in cell number of chlorpropham-treated cultures from the control cultures are given

Concentration ($\mu g ml^{-1}$)	Number of cells ml^{-1} Sampling times (h)				
	0	24	48	72	96
63.7	20873	0***	0***	0***	0***
47.7	20873	1433***	1344***	633***	100***
31.8	20873	18178***	3100***	1511***	632***
15.9	20873	20322***	10956***	10000***	12311***
3.2	20873	40878***	18889***	24600***	24833***
0.0	20873	113567	136267	174911	148700
5.0	4000	2333***	3778***	5111***	4222***
1.0	4000	2889***	12333***	11556***	12222***
0.1	4000	10111	30556***	41889***	71556***
0.01	4000	15222	52667	98444	135778***
0.001	4000	16111	51778*	103556	140667***
0.0	4000	11333	47778	111111	117778

Significance testing (t - test)

*** = highly significant (p=0.05)
** = very significant (p=0.1)
* = significant (p=0.5)

All values given represent the mean of 19 replications

Comparison of the regression lines (Appendix 4.1) for growth of T. pyriformis treated with different concentrations of chlorpropham (Fig. 15) confirmed these statements in terms of the effect on growth rates. The growth rates of T. pyriformis cells treated with 1.0 and 5.0 μgml^{-1} were significantly different from the untreated at p=0.1 and p=0.01 respectively. The growth rates with 0.01 and 0.001 μgml^{-1} chlorpropham did not significantly differ from the untreated (Table 3). Further comparisons of each concentration level tested against every other concentration (Figs 17 & 18) gave a matrix which showed that the lines for growth in the presence of 0.001 and 0.01 μgml^{-1} chlorpropham were significantly different from 0.1 μgml^{-1} whereas the untreated control was not. If the growth rate of cultures treated with 0.001 and 0.01 μgml^{-1} were the same as the untreated cultures growth rate then the relationship of all three to the 0.1 μgml^{-1} chlorpropham growth line would be the same. This was not the case, therefore 0.001 and 0.01 μgml^{-1} have a stimulatory effect on the growth rate of T. pyriformis cells. There was no detectable difference between the growth rates of cells treated with 0.01 and 0.001 μgml^{-1} chlorpropham.

The growth rates in the presence of 1.0 and 5.0 μgml^{-1} chlorpropham were markedly different from any other treatment yet did not differ from each other (Table 3) (Fig. 15), thus the inhibitory action of chlorpropham on the growth rate of T. pyriformis occurred at 1.0 μgml^{-1}.

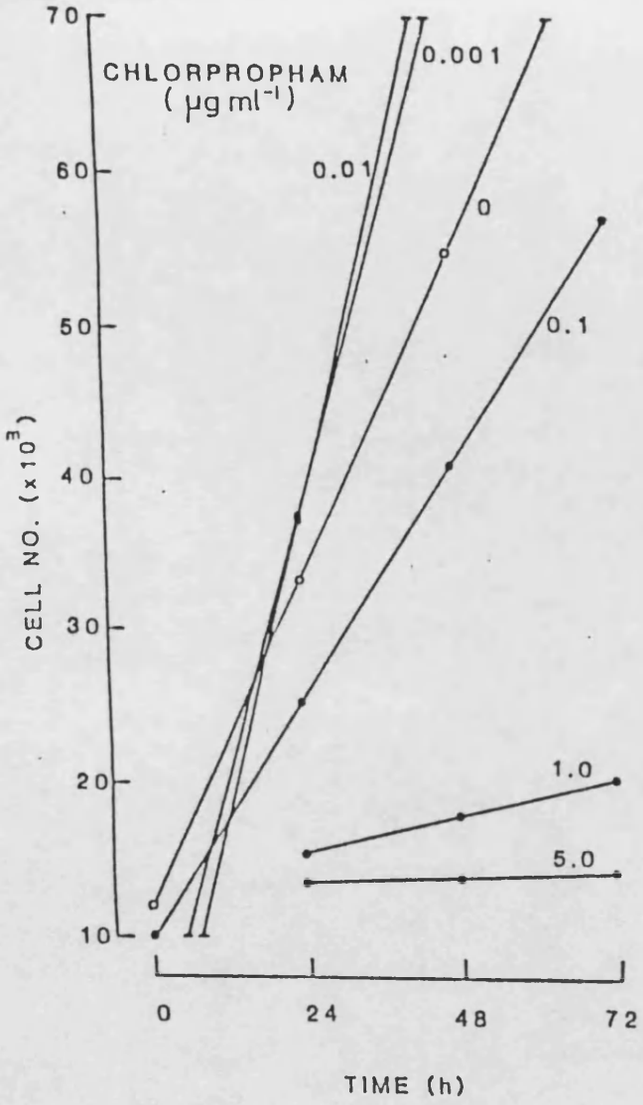


FIG. 15 (Expt 1)

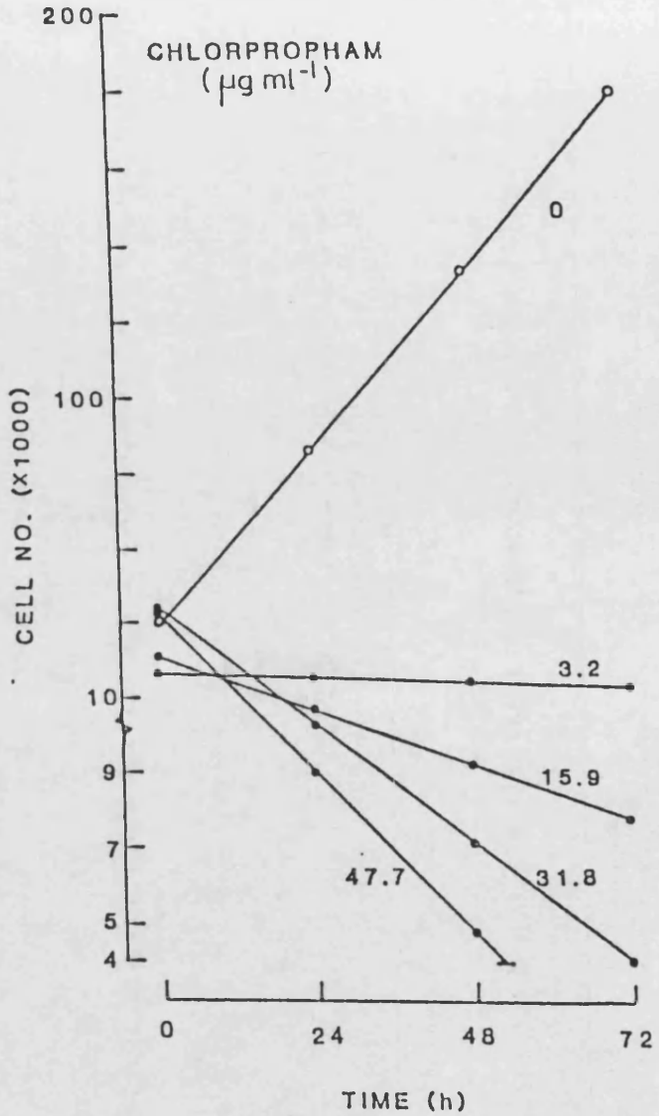


FIG. 16 (Expt. 2)

The effect of chlorpropham on the growth rate of T. pyriformis cells in Repl-dishes. All points were derived from calculated regression lines.

Table 3

Comparison of the growth rates of Tetrahymena pyriformis, treated with chlorpropham, (concentration range 0 to 5.0 $\mu g ml^{-1}$), in Repli-dishes

Treatments			df	t-values	significance
0	vs	0.001	5	-2.02	NS
		0.01	5	-1.92	NS
		0.1	5	1.69	**
		1.0	4	4.97	**
		5.0	5	7.66	***
0.001	vs	0.01	6	0.26	NS
		0.1	6	4.31	*
		1.0	5	6.14	**
		5.0	6	9.04	***
0.01	vs	0.1	6	4.30	*
		1.0	5	6.46	**
		5.0	6	9.59	***
0.1	vs	1.0	5	4.40	**
		5.0	6	7.45	***
1.0	vs	5.0	6	1.82	NS

Confidence limits

NS = not significant
* = significant with 95% confidence
** = significant with 99% confidence
*** = significant with 99.9% confidence
df = degrees of freedom

The growth rates of T. pyriformis cultures were converted to regression lines and compared one with another

FIG 17 (Expt. 1)

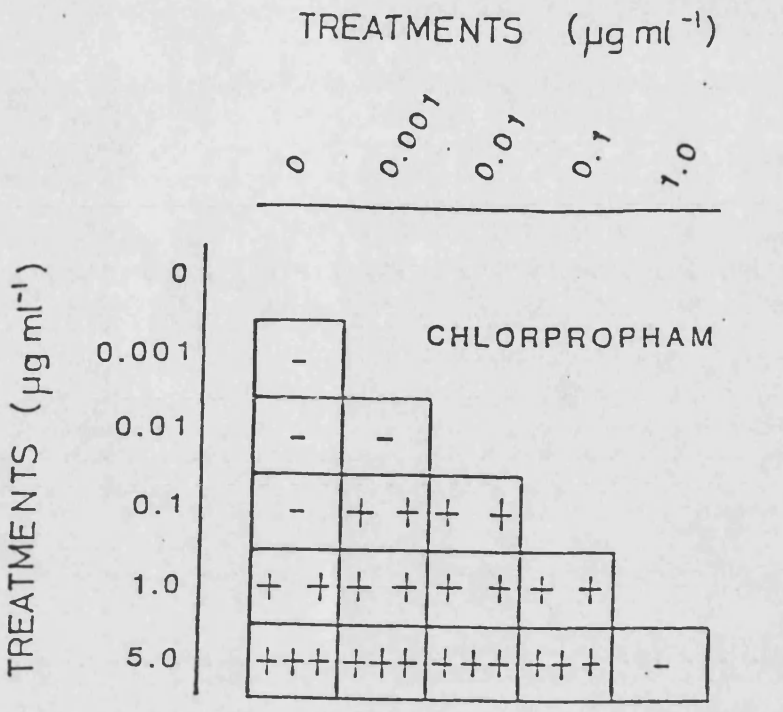


FIG. 18 (Expt. 2)

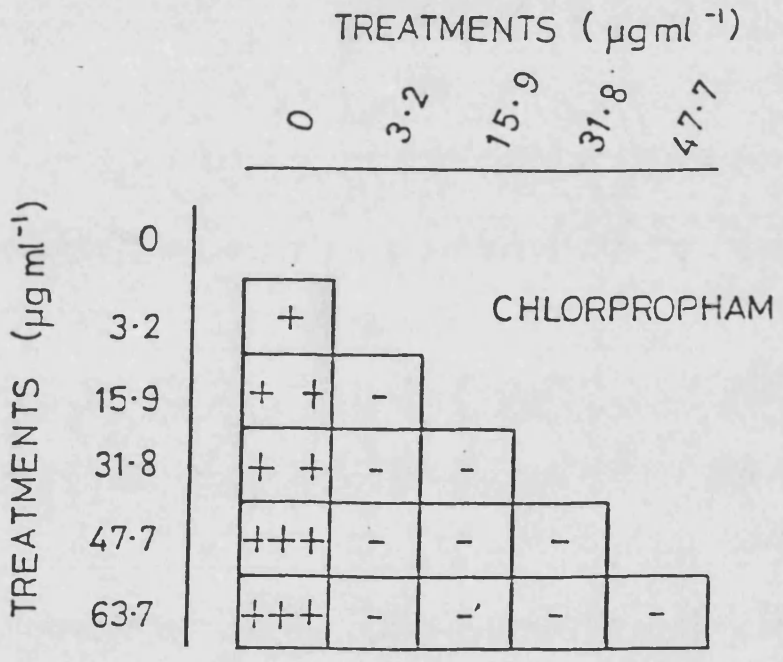


FIG. 17 and 18

A diagramatic plot of significant t-values obtained by comparison of growth rates for Tetrahymena pyriformis in Repli-dishes, treated with chlorpropham (+,++ and +++ indicate significance with 95% , 99% and 99.9% confidence)

Table 4

Comparison of the growth rates of Tetrahymena pyriformis cultures, treated with chlorpropham, (concentration range 0 to 60 $\mu g ml^{-1}$), in Repli-dishes

Treatments			df	t-values	significance
0	vs	3.2	4	4.20	*
		15.9	4	4.99	**
		31.8	4	5.24	**
		47.7	5	6.10	***
		63.7	5	6.02	***
3.2	vs	15.9	4	0.56	NS
		31.8	4	1.09	NS
		47.7	5	0.62	NS
		63.7	5	0.58	NS
15.9	vs	31.8	4	1.41	NS
		47.7	5	-0.02	NS
		63.7	5	-0.07	NS
31.8	vs	47.7	5	-0.95	NS
		63.7	5	-0.89	NS
47.7	vs	63.7	6	-0.02	NS

Confidence limits

NS = not significant
* = significant with 95% confidence
** = significant with 99% confidence
*** = significant with 99.9% confidence
df = degrees of freedom

The growth rates of T. pyriformis cultures were converted to regression lines and compared one with another

The stimulatory action of chlorpropham on the growth rate of T. pyriformis occurred at 0.01 μgml^{-1}.

With the higher concentration range of chlorpropham (Fig. 14 and Table 2), all levels of chlorpropham significantly prevented growth of T. pyriformis ($p=0.05$). Concentrations between 3.2 and 15.9 μgml^{-1} stopped growth whilst concentrations above 15.9 μgml^{-1} caused death. The lethal action of chlorpropham was evident after 48h with 31.8 μgml^{-1} and after 24h with 47.7 and 63.7 μgml. No living cells were seen after 24h with 63.7 μgml^{-1} chlorpropham. A slight decrease in cell numbers was seen with 15.9 μgml^{-1} chlorpropham after 48h.

Growth rates (Fig. 16) derived from regression analysis (Appendix 4) confirm these statements. The comparison of regression lines (Table 4) and the matrix obtained (Figs 17 & 18) showed the inhibitory action on growth rates to occur at 3.2 μgml^{-1}.

26.3 3-chloroaniline

The effect of 3-chloroaniline on the growth rate of T. pyriformis is shown in Fig. 19. The effect on cell numbers is shown in Table 5 and results of the analyses of variance in Appendix 3.3. Graphic depiction of population growth curves have been omitted due to the close proximity of each curve, one to another. Concentrations above 15.9 μgml^{-1} of 3-chloroaniline had an inhibitory effect on

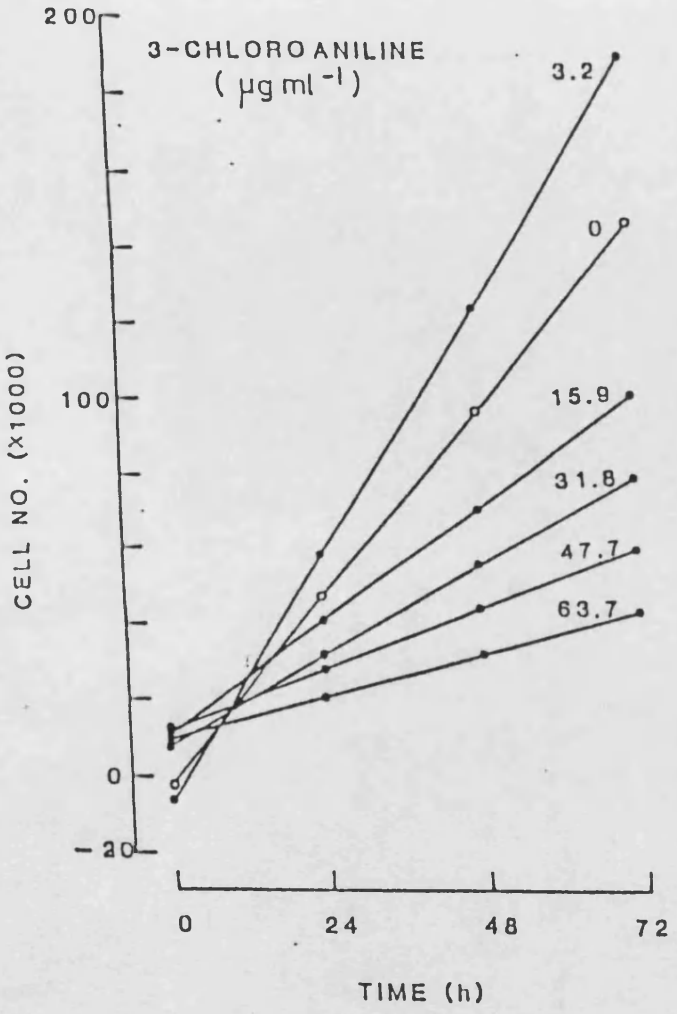


FIG. 19

The effect of 3-chloroaniline on the growth of Tetrahymena pyriformis cells in Repli-dishes. All points derived from regression lines.

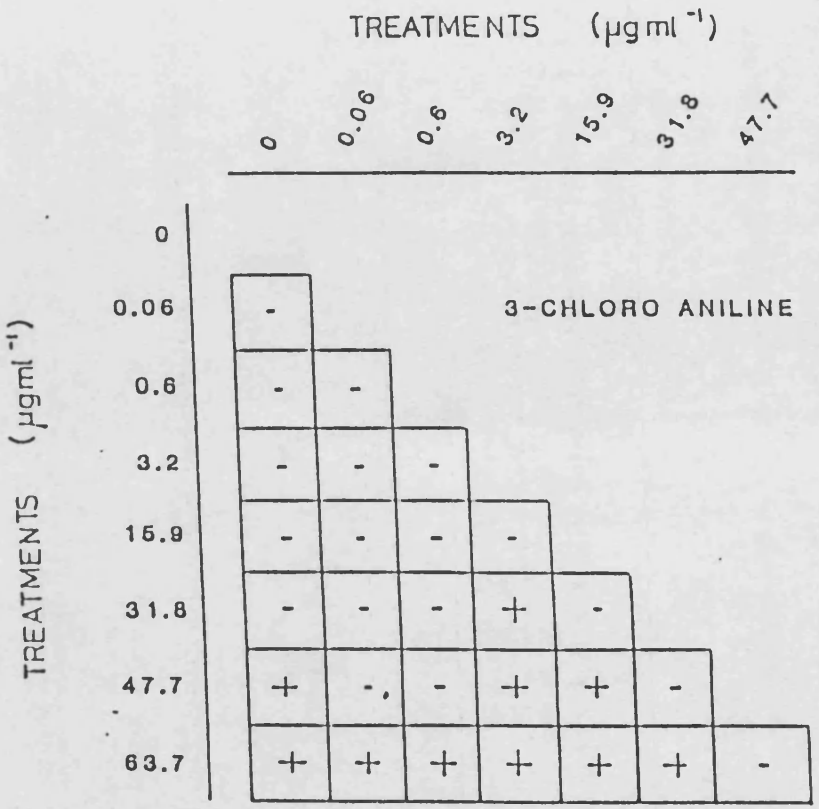


FIG. 20

A diagramatic plot of significant t-values obtained by comparison of growth rates of Tetrahymena pyriformis, in Repli-dishes, treated with 3-chloroaniline. (+ indicates significant with 95% confidence).

Table 5

Cell numbers in Tetrahymena pyriformis cultures treated with 3-chloroaniline, in Repli-dishes. The significance of the differences in cell number of 3-chloroaniline-treated cultures from the control cultures are given

Concentration (µgml-1)	Number of cells ml-1 Sampling times (h)					
	0	24	48	72	-	120
63.7	13888	16222	35111**	42777**	-	96666**
47.7	13888	27555	42000**	62000**	-	136000**
31.8	13888	25111	53777	84888**	-	111555**
15.9	13888	30666	82222	97333**	-	250000
3.2	13888	33555	107111*	211777*	-	205333
0.6	13888	32000	77333	197333	-	222000
0.06	13888	34888	75111	180111	-	254666
0.0	13888	33333	78222	165777	-	252000

Significance testing (t - test)

*** = highly significant (p=0.05)
** = very significant (p=0.1)
* = significant (p=0.5)

All values given represent the mean of 19 replications

Table 6

Comparisons of the growth rates of Tetrahymena pyriformis cultures, treated with 3-chloroaniline, in Repli-dishes

Treatments (µg/ml)			df	t-values	significance
0	vs	0.06	4	-0.22	NS
		0.6	4	-0.48	NS
		3.2	4	-0.96	NS
		15.9	4	1.66	NS
		31.8	4	2.28	NS
		47.7	4	3.14	*
		63.7	4	3.55	*
0.06	vs	0.6	4	-0.27	NS
		3.2	4	-0.67	NS
		15.9	4	1.63	NS
		31.8	4	2.11	NS
		47.7	4	2.77	NS
		63.7	4	3.11	*
0.6		3.2	4	-0.34	NS
		15.9	4	1.71	NS
		31.8	4	2.11	NS
		47.7	4	2.65	NS
		63.7	4	2.94	*
3.2		15.9	4	2.53	NS
		31.8	4	3.05	*
		47.7	4	3.74	*
		63.7	4	4.08	*
15.9		31.8	4	0.99	NS
		47.7	4	2.78	*
		63.7	4	3.53	*
31.8		47.7	4	2.38	NS
		63.7	4	3.37	*
47.7		63.7	4	2.10	NS

Confidence limits

NS = not significant
* = significant with 95% confidence
** = significant with 99% confidence
*** = significant with 99.9% confidence
df = degrees of freedom

The growth rates of T. pyriformis cultures were converted to regression lines and compared one with another

population growth. Concentrations below 0.6 μgml^{-1} had no effect on growth whilst a level of 3.2 μgml^{-1} caused a significant increase in cell number after 48h. A progressive reduction in growth rate occurred with increasing concentrations of 3-chloroaniline. At 47.7 and 63.7 μgml^{-1} 3-chloroaniline significantly reduced cell nuber below the untreated control after 48h. This reduction in cell numbers persisted to the end of the experiment. A similar effect was observed with 31.8 μgml^{-1} 3-chloroaniline after 72h.

Comparison of the growth rates of T. pyriformis cells treated with 3-chloroaniline can be seen in Fig 19 (the regression equations for these curves are shown in Appendix 4). The growth rates, in the presence of 47.7 and 63.7 μgml^{-1} 3-chloroaniline, were significantly reduced from that of the untreated (Table 6). At 3-chloroaniline concentrations below these the growth rates were not statistically different from the untreated. However, further analysis (by comparison of each treatment level with another, Table 6 and Fig. 20) showed that the growth rate of 3.2 μgml^{-1} was significantly different from 31.8, 47.7 and 63.7 μgml^{-1} 3-chloroaniline whereas the untreated control growth rate was significantly different from only 47.7 and 63.7 μgml^{-1}. This indicated that of the concentrations tested, only 3.2 μgml^{-1} stimulated the growth rate of T. pyriformis above the control value. The matrix (Fig. 20) showed the inhibitory effect of 3-chloroaniline to occur at 63.7 μgml^{-1} (all other treatments except 47.7 μgml^{-1}

were significantly different from this concentration). A stepwise inihibition of growth rates was observed with concentrations ranging from 15.9 to 63.7 μgml^{-1}, such that 63.7 was significantly difference from 31.8 μgml^{-1}, 47.4 was significantly different from 15.9 μgml^{-1} and 31.8 was significantly different from 15.9 μgml^{-1}. Comparison on the N-phenylcarbamate herbicides, chlorpropham and propham with their respective metabolites 3-chloroaniline and aniline in terms of the effect on growth of T. pyriformis and A. castellanii occurs in section 28.0.

26.4 Propham

The effect of propham on the growth of T. pyriformis is shown in Fig. 21. The analyses of variance for each sampling point are shown in Appendix 3.4 & 3.5 with the significance of these differences from the untreated in Table 7.

The differences in the response of T. pyriformis to the treatments was significantly greater than the variation within individual treatments for each sampling point. With propham concentrations in the range 0 to 22.4 $\mu gml-1$ the analyses of variance F values (Appendix 3.5) were considerably larger than in the 0 to 5 $\mu gml-1$ range (Appendix 3.4), reflecting the greater differences observed.

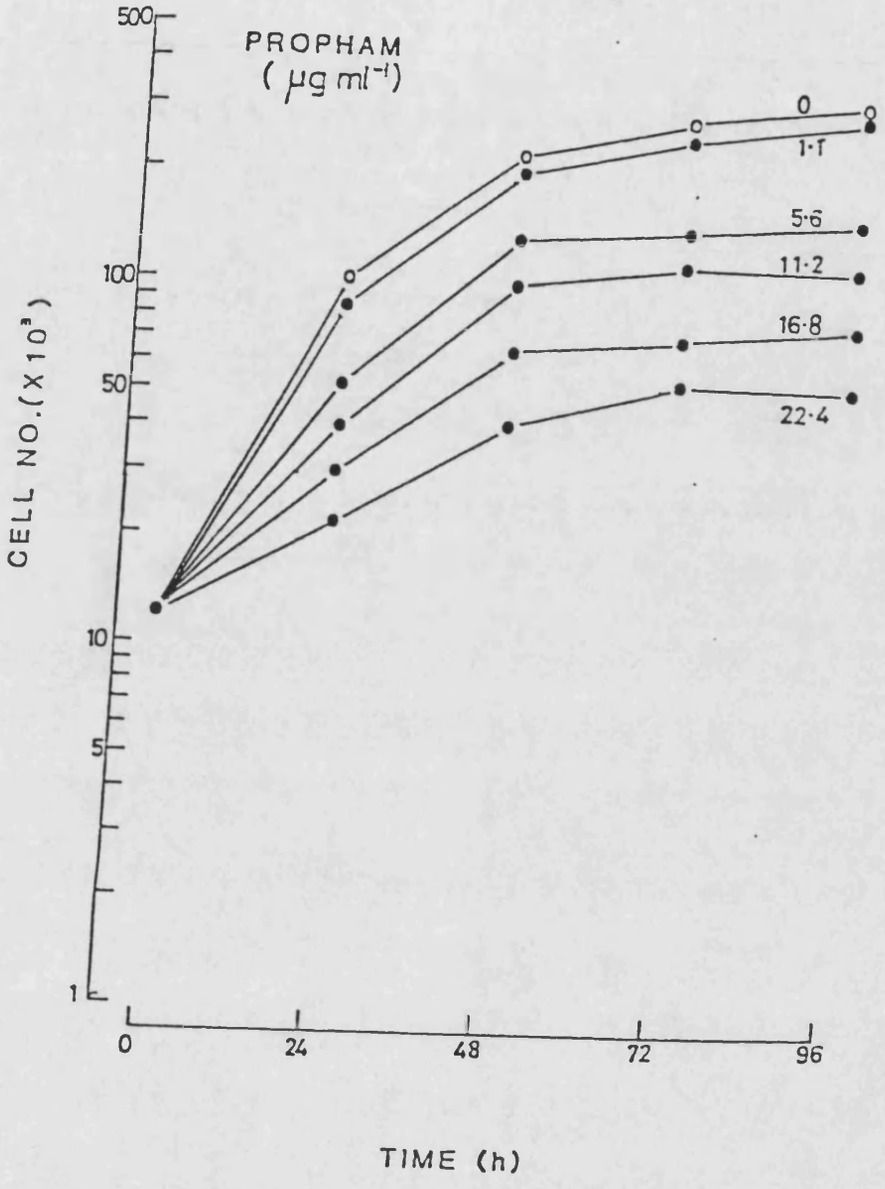


FIG. 21

The sub-acute effect of propham on the growth of Tetrahymena pyriformis in PY medium at 20°C

Table 7

Cell numbers in Tetrahymena pyriformis cultures treated with propham, in Repli-dishes. The significance of the differences in cell number of propham-treated cultures from the control cultures are given.

Concentration ($\mu g ml^{-1}$)	Number of cells ml^{-1} Sampling times (h)				
	0	24	48	72	96
22.4	12333	21889***	40722***	54278***	51444***
16.8	12333	30111***	65444***	71000***	77555***
11.2	12333	40333***	98556***	112889***	110889***
5.6	12333	52333***	130333***	139333***	148556***
1.1	12333	85111*	199444***	253778**	288333
0.0	12333	101333	216444	287111	312889
5.0	4000	12889*	31444***	59222*	91555*
1.1	4000	19222	48222	103333	132444
0.1	4000	16889	52333	116889***	163889***
0.01	4000	17667	69333	102222	133056
0.001	4000	18889	57333	105167***	143889
0	4000	16444	55778	89222	140222

Significance testing (t - test)

*** = highly significant (p=0.05)
** = very significant (p=0.1)
* = significant (p=0.5)

Only those concentrations in the range 0 to 22.4 $ugml^{-1}$ are represented in Fig. 21

All values given represent the mean of 19 replications

In Fig 21 increasing concentrations of propham progressively reduced population growth, after 24h, in a linear manner. All concentrations above 1.1 μgml^{-1} were inhibitory. Statistically significant detection of inhibition of growth was evident after 24h with all concentrations above 1.1 μgml^{-1} and continued to the end of the experiment (Table 7). None of the propham concentrations tested prevented population growth or caused cell death.

Increasing exposure to 0.1 and 0.001 μgml^{-1} propham caused an increase in cell numbers above that of the control.

Different inoculum levels prevent direct comparison of the two experiments shown in Table 7 beyond the observation that concentrations above 5.0 (5.6) μgml^{-1} caused a reduction in cell number.

Comparison of the growth rates of T. pyriformis cells (Table 8) showed no significant differences between treatments. Although 0.001 and 0.1 μgml^{-1} increased cell number above the control, stimulation of the growth rate was not observed. However, these growth rates were obtained from data derived from the exponential phase of the culture (0 to 72h), the increase in cell number with 0.1 μgml^{-1} propham in particular, occurred after 72h and represents an increase in growth over that of control after the exponential phase of culture.

Table 8

Comparison of the growth rates of Tetrahymena pyriformis cultures, treated with propham (concentration range 0 to 5.0 ugml^{-1}), in Repli-dishes

Treatments (µg/ml)			df	t-values	significance
0	vs	0.001	4	-0.70	NS
		0.01	4	-0.79	NS
		0.1	4	-0.87	NS
		1.1	4	-0.24	NS
		5.0	5	1.51	NS
0.001	vs	0.01	4	-0.06	NS
		0.1	4	-0.33	NS
		1.1	4	0.43	NS
		5.0	5	2.18	NS
0.01	vs	0.1	4	-0.29	NS
		1.1	4	0.50	NS
		5.0	5	2.32	NS
0.1	vs	1.1	4	0.67	NS
		5.0	5	2.08	NS
1.1	vs	5.0	5	1.69	NS

Confidence limits

NS = not significant
df = degrees of freedom

The growth rates of T. pyriformis cultures were converted to regression lines and compared one with another

Table 9

Comparison of the growth rates of Tetrahymena pyriformis cultures, treated with propham (concentration range 0 to 22.4 $\mu g ml^{-1}$), in Repli-dishes

Treatments (µg/ml)			df	t-values	significance
0	vs	1.1	4	1.04	NS
		5.6	4	4.49	**
		11.2	4	7.17	***
		16.8	4	10.48	***
		22.4	4	13.48	***
1.1	vs	5.6	4	3.19	*
		11.2	4	4.99	**
		16.8	4	7.24	***
		22.4	4	8.85	***
5.6	vs	11.2	4	0.93	NS
		16.8	4	2.53	NS
		22.4	4	3.46	*
11.2	vs	16.8	4	2.17	NS
		22.4	4	3.71	*
16.8	vs	22.4	4	1.65	NS

Confidence limits

NS = not significant
* = significant with 95% confidence
** = significant with 99% confidence
*** = significant with 99.9% confidence
df = degrees of freedom

The growth rates of T. pyriformis cultures were converted to regression lines and compared one with another

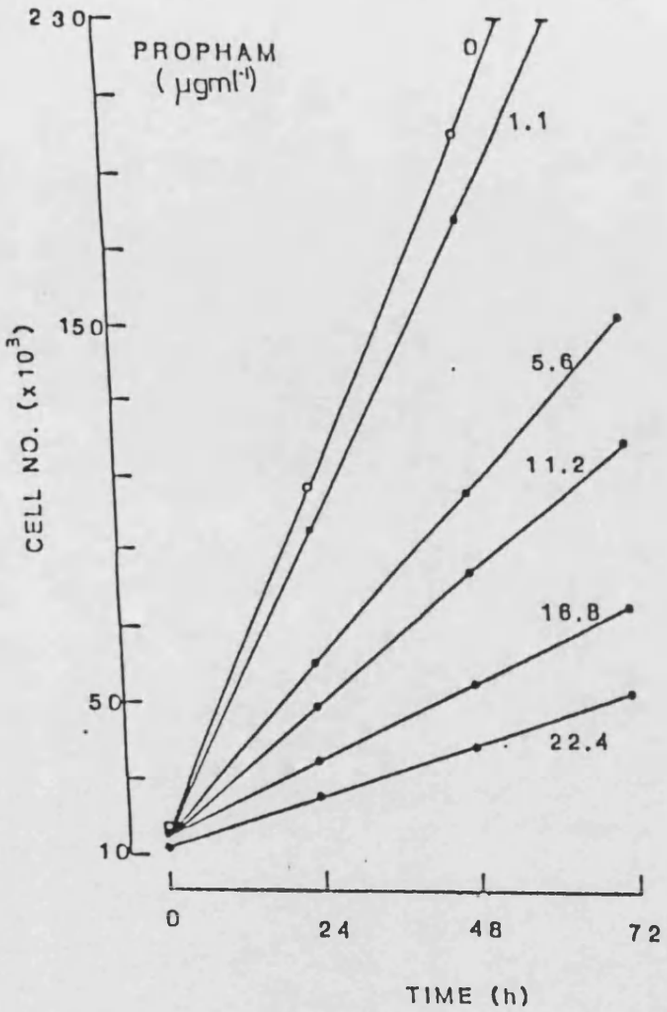


FIG. 22

The effect of propham on the growth of Tetrahymena pyriformis cells, in Repli-dishes. All points are derived from regression lines.

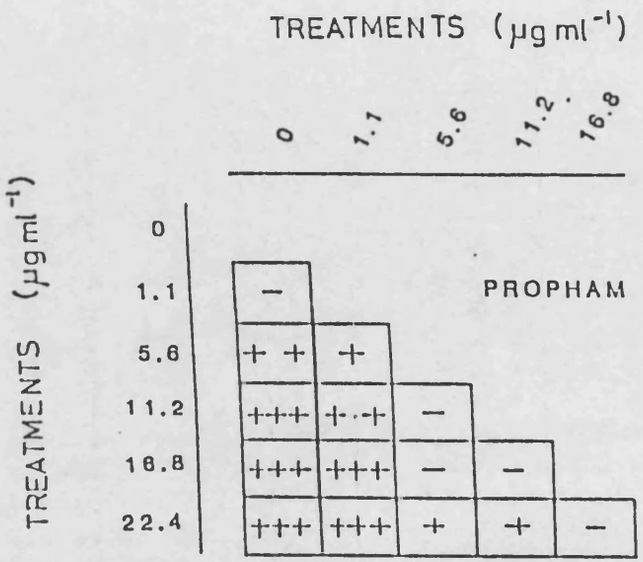


FIG. 23

A diagramatic plot of the significant t-values obtained by comparison of growth rates of Tetrahymena pyriformis , in Repli-dishes, treated with propham (+, ++ and +++ indicate significant with 95%, 99% and 99.9% confidence).

The growth rates (as exponential phase regression lines) of T. pyriformis treated with 0 to 22.4 μgml^{-1} propham, (Fig 22), were derived from the equation in Appendix 4. The slopes of the 5.6 to 22.4 μgml^{-1} treatments differ from those of the untreated control and 1.1 μgml^{-1}. Comparison of the growth rates, one with another (Table 9) showed that concentrations 5.6 to 22.4 μgml^{-1} of propham were significantly different from both the untreated and 1.1 μgml^{-1}. Expressed as a matrix (Fig.23) these significances showed that the lowest concentration which reduced the growth rates was 5.6 μgml^{-1}[1] of propham. No such threshold was observed with 0 to 5.0 μgml^{-1} propham (Table 7).

26.5 Aniline

The affect of aniline on the growth rate of T. pyriformis is shown in Fig. 24 while the effect on cell numbers is shown in Table 10 and the analyses of variance for each sampling point in Appendix 3.6. Population growth curves lie too close to one another to permit clear graphic representation.

The degree of variation between treatments (F values, Appendix 3.6) is significant for all sampling points. The variation was due to the significant inhibitory action of 150 μgml^{-1} and the significant stimulatory action of 37.5 μgml^{-1} of aniline (Table 10). All other concentrations

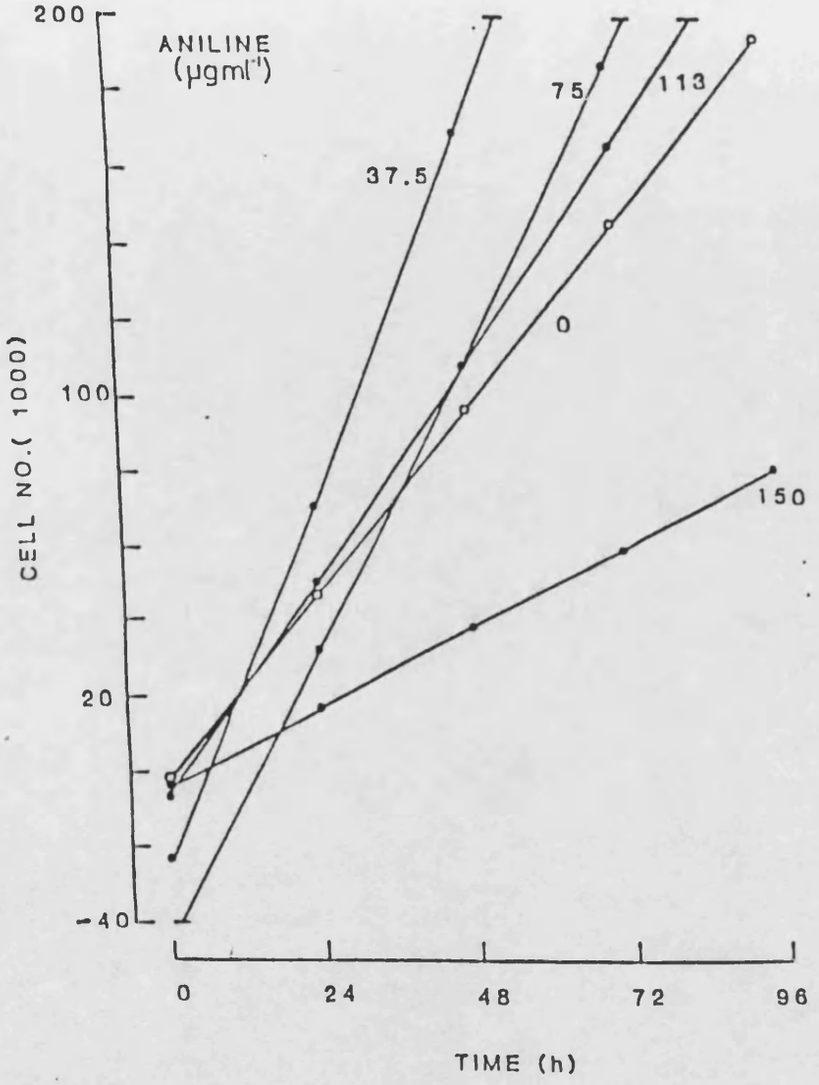


FIG. 24

The effect of aniline on the growth of Tetrahymena pyriformis cells, in Repli-dishes. All points were derived from regression lines.

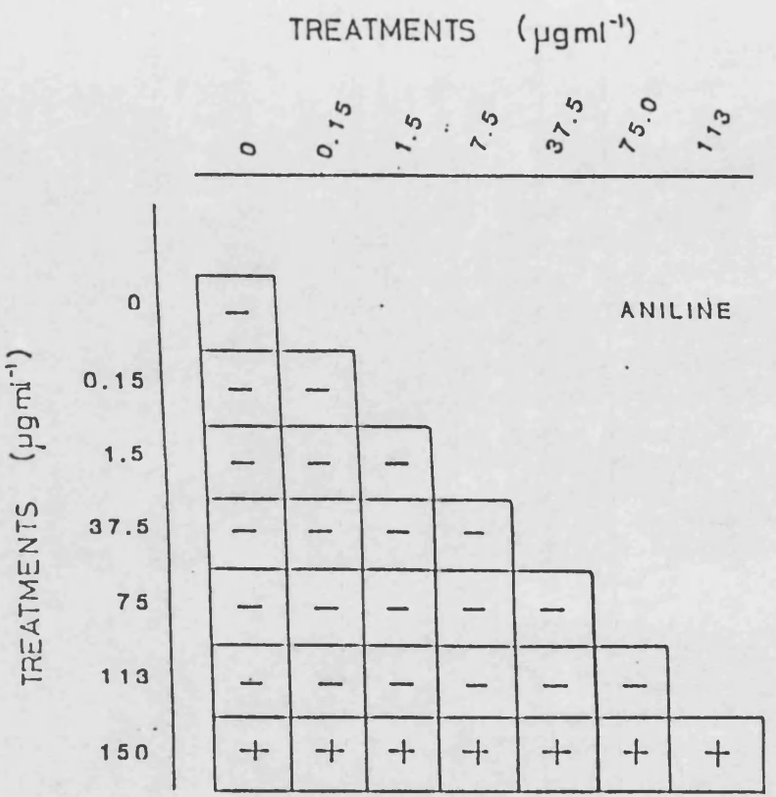


FIG. 24b

A diagramatic plot of the significant t-values obtained by comparison of the growth rates of Tetrahymena pyriformis cells, in Repli-dishes, treated with aniline (+= significant with 95% confidence).

Table 10

Cell numbers in Tetrahymena pyriformis cultures treated with aniline, in Repli-dishes. The significance of the differences in cell number of aniline-treated cultures from the control are given

Concentration ($\mu g ml^{-1}$)	Number of cells ml^{-1} Sampling times (h)					
	0	24	48	72	-	120
150	13888	23111	29555**	61555**	-	136666**
113	13888	34444	82888	190000	-	217333
75	13888	36888	98666	208666	-	247333
37.5	13888	43333	119777**	252000**	-	239333
7.5	13888	38000	80444	155111	-	260000
1.5	13888	36000	81333	160666	-	257333
0.15	13888	36666	68444	158666	-	245333
0	13888	33333	78222	165777	-	252000

All values given represent the mean of 19 replications

Significance testing (t - test)

*** = highly significant (p=0.05)
** = very significant (p=0.1)
* = significant (p=0.5)

Table 11

Comparison of the growth rates of Tetrahymena pyriformis cultures, treated with aniline, in Repli-dishes

Treatments (µg/ml)		df	t-values	significance
0	vs 0.15	4	0.22	NS
	1.5	4	0.11	NS
	7.5	4	0.26	NS
	37.5	4	-1.49	NS
	75.0	4	-0.42	NS
	113.0	4	-0.43	NS
	150.0	4	3.03	*
0.15	vs 1.5	4	-0.14	NS
	7.5	4	-0.00	NS
	37.5	4	-1.64	NS
	75.0	4	-0.61	NS
	113.0	4	-0.62	NS
	150.0	4	2.65	*
1.5	vs 7.5	4	0.26	NS
	37.5	4	-1.63	NS
	75.0	4	-0.54	NS
	113.0	4	-0.55	NS
	150.0	4	3.37	*
7.5	vs 37.5	4	-1.79	NS
	75.0	4	-0.69	NS
	113.0	4	-0.69	NS
	150.0	4	3.50	*
37.5	vs 75.0	4	1.02	NS
	113.0	4	1.00	NS
	150.0	4	3.82	*
75.0	vs 113.0	4	-0.01	NS
	150.0	4	3.00	*
113.0	vs 150.0	4	2.96	*

Confidence limits

NS = not significant
* = significant with 95% confidence
** = significant with 99% confidence
*** = significant with 99.9% confidence
df = degrees of freedom

The growth rates of T. pyriformis cultures were converted to regression lines and compared one with another

of aniline did not differ significantly from the untreated in their effect on growth of T. pyriformis (Table 11).

A reduction in cell number after 24h was evident with 150 μgml^{-1} of aniline but only significant at 48, 72 and 120h. Comparison of cell numbers from this treatment with the untreated control at these points showed a fall of 53, 53 and 46% respectively.

The increase in cell number caused by 37.5 μgml^{-1} was also evident after 24h but only significant at 48 and 72h. The cell number declined to the control level by 120h (Table 10). No concentration of aniline induced stasis in or was lethal to T. pyriformis cells.

The growth rates (Fig. 24) obtained from the regression equations in Appendix 4.4 showed that aniline at 150 μgml^{-1} reduced the growth rate of T. pyriformis . No significant stimulation of the growth rate was detected with 37.5 μgml^{-1} (Table 11). However, Table 11 did show the t-values for this value to be negative. Negative t-values in these comparisons signify growth rates which exceed those of the untreated, indicating stimulation of growth.

Comparison of growth rates (Fig. 24b) showed the inhibitory action on growth rates to occur at 150 μgml^{-1} aniline, the other concentrations having no significant effect.

26.6 Pirimicarb

The effect of pirimicarb on the growth rate of T. pyriformis is shown in Fig. 25. The significance of the effects of pirimicarb on cell numbers are shown in Table 12 and the analyses of variance for each sampling point in Appendix 3.7. The variation between treatments was greater than the variance within treatment for each sampling point tested(F values Appendix 3.7). All concentrations above 50 μgml^{-1} caused a significant reduction in both growth rate and cell numbers. The reduction (in numbers) commenced before 24h and lasted for a period greater than 96h (Table 12). At 50 μgml^{-1} pirimicarb a significant reduction in cell number was detected at 24h but was not detected at 48h. An increase in cell numbers was found with 1.0 and 0.1 μgml^{-1} of pirimicarb but only in the latter part of the experiment (72 to 96h) was this significant. The percentage increase in cell numbers over this period was 23 and 36% for 1.0 μgml^{-1} and 20% for 0.1 μgml^{-1} pirimicarb.

No concentration of pirimicarb was lethal to T. pyriformis over 96h although slight fluctuation in cell number, during the initial stages of exponential growth, were observed with 1000 μgml^{-1}.

Successive concentrations of pirimicarb, above 50 μgml^{-1}, became progressively more inhibitory to the growth rate of

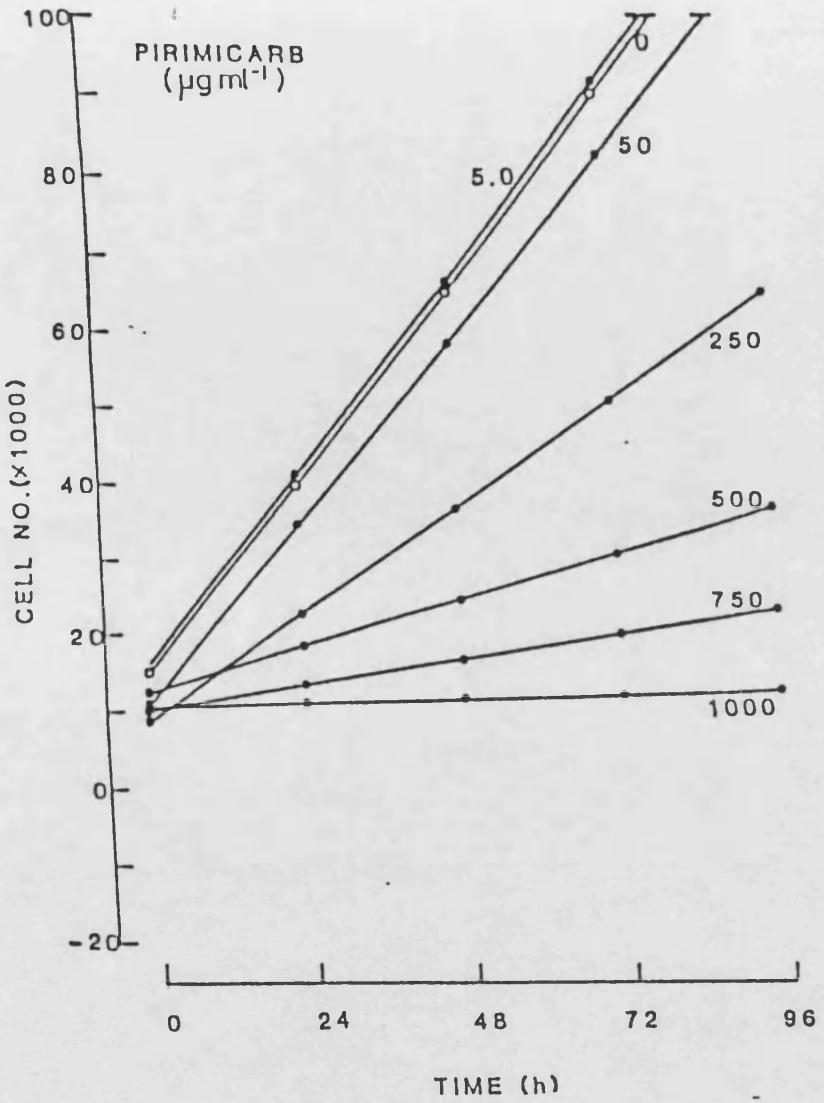


FIG. 25

The effect of pirimicarb on the growth rate of Tetrahymena pyriformis cells, in Repli-dishes. All points were derived from regression lines.

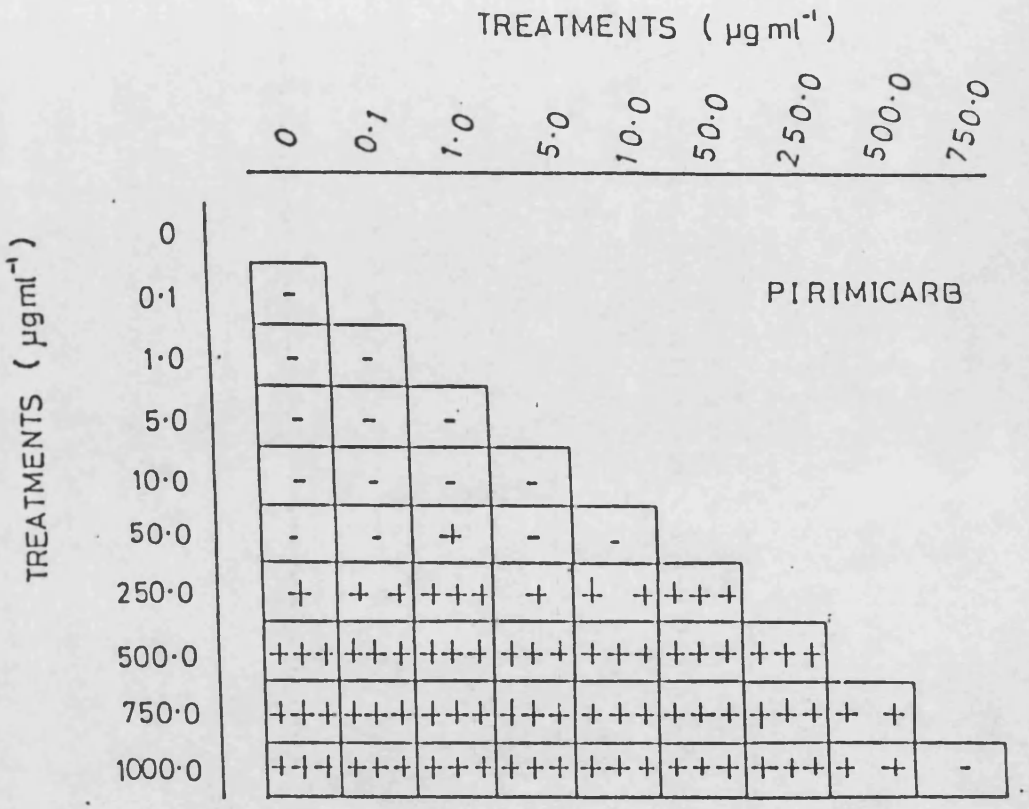


FIG. 26

A diagramatic plot of the significant t-values obtained by comparison of the growth rates of Tetrahymena pyriformis cells, in Repli-dishes, treated with pirimicarb (+,++ and +++ indicate significance with 95%,99% and99.9% confidence).

Table 12

Cell numbers in Tetrahymena pyriformis cultures treated with pirimicarb, in Repli-dishes. The significance of the differences in cell number of pirimicarb-treated cultures from the control cultures are given

Concentration (µgml^{-1})	Number of cells ml^{-1} Sampling times (h)				
	0	24	48	72	96
1000	11020	11444***	9444***	13889***	15333***
750	11020	12444***	17111***	20778***	25000***
500	11020	17667***	24778***	34111***	57889***
250	11020	23444***	36222***	52444***	63333***
50	11020	33222***	61222	80778	106889
10	11020	43333	61000	90444	106444
5	11020	45444	70556	87667	106667
1	11020	52333	74889	109444***	135444***
0.1	11020	48000	66111	97778	120222***
0.01	11020	47333	63444	97444	100333
0.0		47222	64111	88778	99556

Significance testing (t - test)

*** = highly significant (p=0.05)
** = very significant (p=0.1)
* = significant (p=0.5)

All values given represent the mean of 19 replications

Table 13

Comparison of the growth rates of Tetrahymena pyriformis cultures, treated with pirimicarb, in Repli-dishes

Treatments (µg/ml)		df	t-values	significance
0	vs 0.1	4	-0.78	NS
	1.0	4	-1.89	NS
	5.0	4	-0.13	NS
	10.0	4	-0.18	NS
	50.0	4	0.45	NS
	250.0	4	4.16	*
	500.0	4	6.48	**
	750.0	4	8.05	***
	1000.0	4	8.72	***
0.1	vs 1.0	4	-1.15	NS
	5.0	4	0.64	NS
	10.0	4	0.72	NS
	50.0	4	1.54	NS
	250.0	4	5.60	**
	500.0	4	8.14	***
	750.0	4	9.83	***
	1000.0	4	10.45	***
1.0	vs 5.0	4	1.72	NS
	10.0	4	2.00	NS
	50.0	4	3.03	*
	250.0	4	7.21	***
	500.0	4	9.80	***
	750.0	4	11.51	***
	1000.0	4	12.05	***
5.0	10.0	4	0.03	NS
	50.0	4	0.60	NS
	250.0	4	4.20	*
	500.0	4	6.44	**
	750.0	4	7.96	***
	1000.0	4	8.62	***
10.0	50.0	4	0.84	NS
	250.0	4	5.87	***
	500.0	4	9.10	***
	750.0	4	11.22	***
	1000.0	4	11.76	***
50.0	250.0	4	7.93	***
	500.0	4	13.67	***
	750.0	4	17.15	***
	1000.0	4	16 37	***

Continued../

Table 13 (continued../)

Treatments (µg/ml)		df	t-values	significance
250.0	500.0	4	7.80	***
	750.0	4	13.05	***
	1000.0	4	11.88	***
500.0	750.0	4	6.62	**
	1000.0	4	7.00	**
750.0	1000.0	4	2.72	NS

Confidence limits

NS = not significant
* = significant with 95% confidence
** = significant with 99% confidence
*** = significant with 99.9% confidence
df = degrees of freedom

The growth rates of T. pyriformis cultures were converted to regression lines and compared one with another

T. pyriformis. The greatest reduction occurred with 1000 μgml^{-1}, which prevented growth. Cultures grown in the presence of 0.1, 1.0, 5.0 and 10.0 μgml^{-1} of pirimicarb had growth rates higher than those of the control. These were not however significant (Table 13).

The results in Table 13 and Fig. 26 showed that cells treated with 1.0 μgml^{-1} pirimicarb had growth rates significantly different from 50 μgml^{-1} of pirimicarb whereas the untreated value was not. Further comparisons, against other treatments, 250 and 500 μgml^{-1}, showed that the degree of difference was greater for 1.0 μgml^{-1} than for the untreated (Fig. 26). These results show a stimulatory effect on growth rate by 1.0 μgml^{-1} pirimicarb.

Indications that 0.1 and 10 μgml^{-1} may also promote increased growth rates are found in the degree of significance of these treatments against higher levels as compared with the untreated (Table 13, Fig. 26).

The inhibitory action of pirimicarb on the growth rate of T. pyriformis commenced at 250 μgml^{-1}. Dose-response curves for the effects of chlorpropham, propham 3-chloroaniline, aniline and pirimicarb on T. pyriformis are compared in section 28.0.

27.0 Evaluation of the sub-acute toxicity of some pesticides and metabolites to Acanthamoeba castellanii using the Repli-dish culture technique

27.1 Growth characteristics of Acanthamoeba castellanii in Repli-dishes

A. castellanii cultures grown in Repli-dishes exhibit no lag-phase. Population doubling time appears related to initial innoculum size, the higher the initial innoculum the lower the population doubling time, eg with an initial innoculum of 6.0×10^4 cells ml^{-1} the mean doubling time is 30h (Fig. 30) whilst with 7.9×10^4 cells ml^{-1} it is 25.8h (Fig. 33).

Cell yield is variable (Tables 14-25). Mean cell yield (all data) at 120h is 2.9×10^5 cells ml^{-1} from a 6.9×10^4 cell ml^{-1} mean initial innoculum. The range of such values however falls between 2.0×10^5 cell ml^{-1} (6.4×10^4 cell ml^{-1} initial innoculum, Table 22) and 4.0×10^5 cells ml^{-1} (7.0×10^4 cells ml^{-1} initial innoculum, Table 25).

27.2 Chlorpropham

The effect of chlorpropham on the growth of A. castellanii is shown in Fig. 27, the analyses of variance for each sampling point in Appendix 3.8 and the significance of these variances in Table 14.

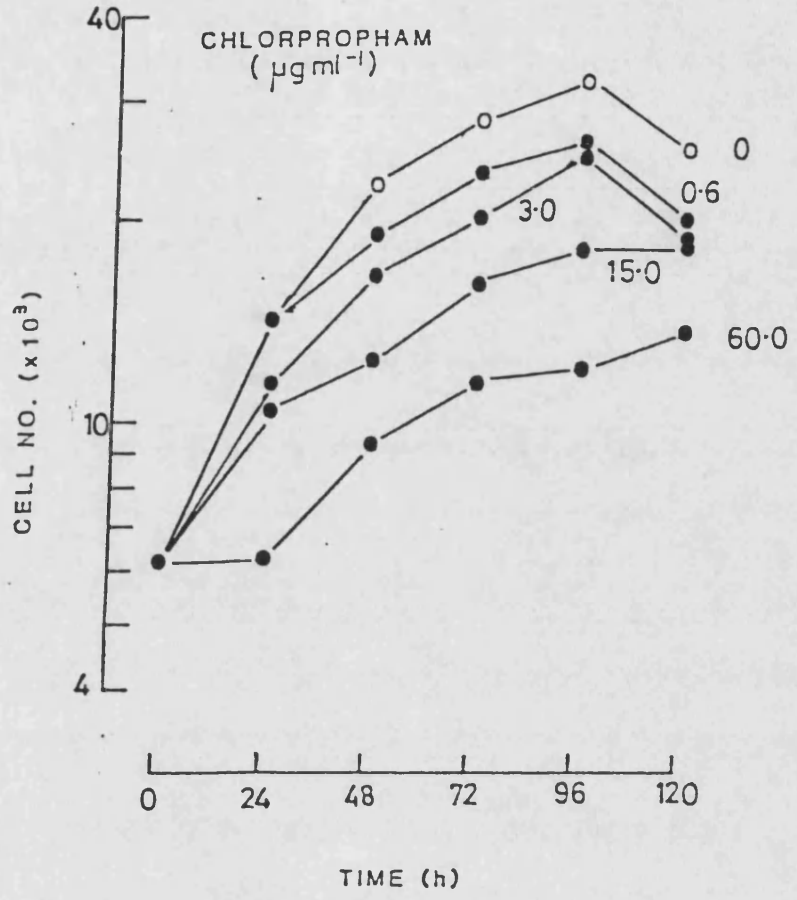


FIG. 27

The sub-acute effect of chlorpropham on the growth of Acanthamoeba castellanii, in Repli-dishes, grown in PGY medium at 30°C

Table 14

Cell numbers in Acanthamoeba castellanii cultures treated with chlorpropham, in Repli-dishes. The significance of the differences in cell number of chlorpropham-treated cultures from the control cultures are given

Concentration ($\mu g ml^{-1}$)	Number of cells ml^{-1} Sampling times (h)					
	0	24	48	72	96	120
60.0	62222	63485***	93030***	114650***	121313***	136212***
45.0	62222	91263***	114495***	123081***	172566***	183182***
30.0	62222	94545***	118283***	150101***	186465***	175859***
15.0	62222	105657***	124848***	161717***	184949***	185707***
3.0	62222	114747*	165505***	202626***	248333***	183434***
0.6	62222	142778	194293*	235455***	259949***	199999***
0.06	62222	160455	200859	234444***	304646	273586
0.0	62222	142778	225859	285202	310202	254646

Significance testing (t - test)

*** = highly significant (p=0.05)
** = very significant (p=0.1)
* = significant (p=0.5)

All values given represent the mean of 19 replications

The degree of variation between treatments was significantly greater than that within treatments at each sampling point over 168h (Appendix 3.8). Concentrations above 0.6 μgml^{-1} of chlorpropham significantly reduced cell numbers over a 120h period (Table 15) this was due to a reduction in growth rate (Fig. 28). Increasing the concentration of chlorpropham progressively depressed population growth (Fig. 27). Chlorpropham (60 μgml^{-1}) caused a 24h delay in the onset of the exponential phase of growth (Fig. 27). No other concentration induced stasis in this way. Population growth was reduced after 24h by all concentrations above 0.6 μgml^{-1} chlorpropham and with 0.6 μgml^{-1} chlorpropham a significant reduction in cell numbers occurred after 48h (Table 14).

No concentration of chlorpropham was lethal to A. castellanii and no concentration increased cell numbers significantly above the levels in untreated cultures.

The effect of chlorpropham on growth rates (Fig. 28), derived from equations of linear regressions for the exponential phase of growth (Appendix 4.6), indicate that no concentrations of chlorpropham stimulated the growth rate of A. castellanii cells. The diagramatic plot of these significances (Fig. 29) shows this inhibition, the threshold of which was 3 μgml^{-1} chlorpropham and highlights the lack of inhibition by 0.06 μgml^{-1} chlorpropham.

Table 15

Comparison of the growth rates of Acanthamoeba castellanii cultures, treated with chlorpropham, in Repli-dishes

Treatments (µg/ml)		df	t-values	significance
0 vs	0.06	4	1.70	NS
	0.6	4	2.43	NS
	3.0	4	6.06	**
	15.0	4	8.76	***
	30.0	4	11.58	***
	45.0	4	10.81	***
	60.0	5	13.08	***
0.06vs	0.6	4	-0.11	NS
	3.0	4	0.76	NS
	15.0	4	2.12	NS
	30.0	4	2.47	NS
	45.0	4	3.10	*
	60.0	5	4.25	*
0.6 vs	3.0	4	1.44	NS
	15.0	4	3.57	*
	30.0	4	4.38	*
	45.0	4	5.10	**
	60.0	5	6.71	**
3.0 vs	15.0	4	3.80	*
	30.0	4	6.51	**
	45.0	4	6.36	**
	60.0	5	7.77	***
15.0 vs	30.0	4	0.90	NS
	45.0	4	2.46	NS
	60.0	5	3.53	*
30.0 vs	45.0	4	2.33	NS
	60.0	5	3.34	*
45.0 vs	60.0	5	0.77	NS

Confidence limits

NS = not significant
* = significant with 95% confidence
** = significant with 99% confidence
*** = significant with 99.9% confidence
df = degrees of freedom

The growth rates of A. castellanii cultures were converted to regression lines and compared one with another

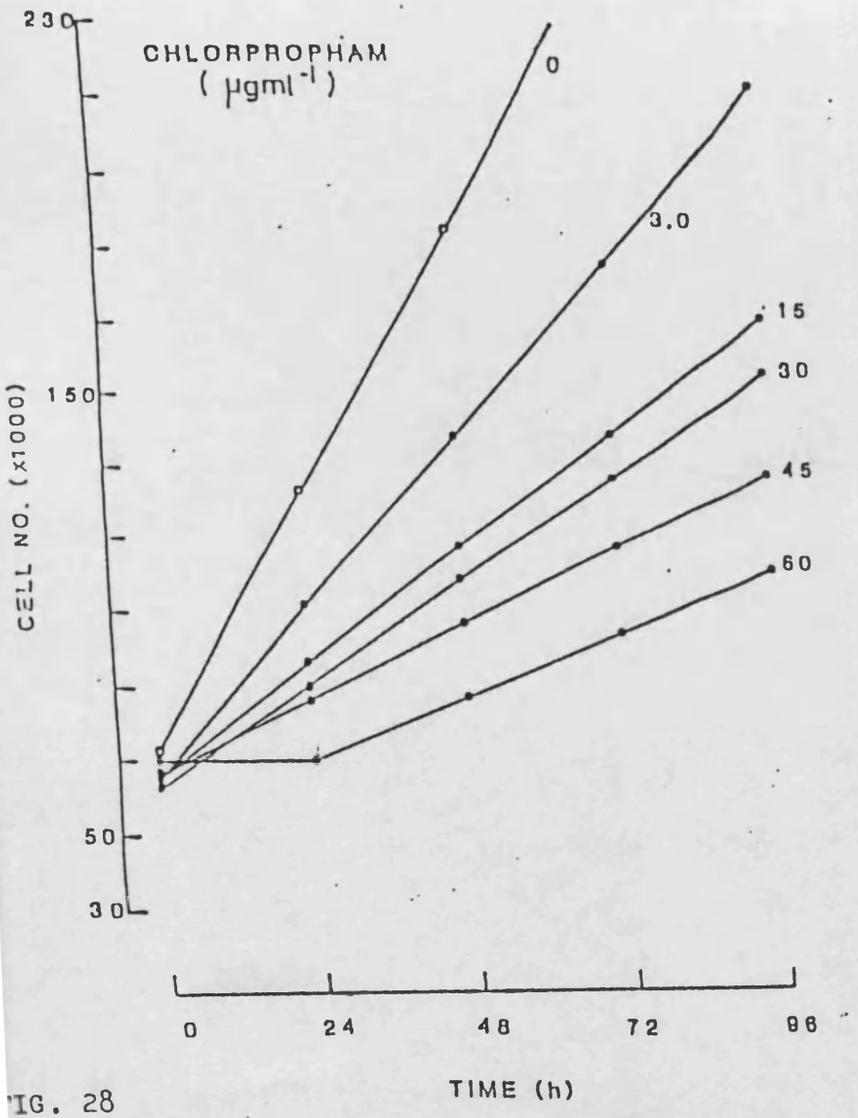


FIG. 28

The effect of chlorpropham on the growth rate of Acanthamoeba castellanii cells, in Repli-dishes. All points derived from regression lines.

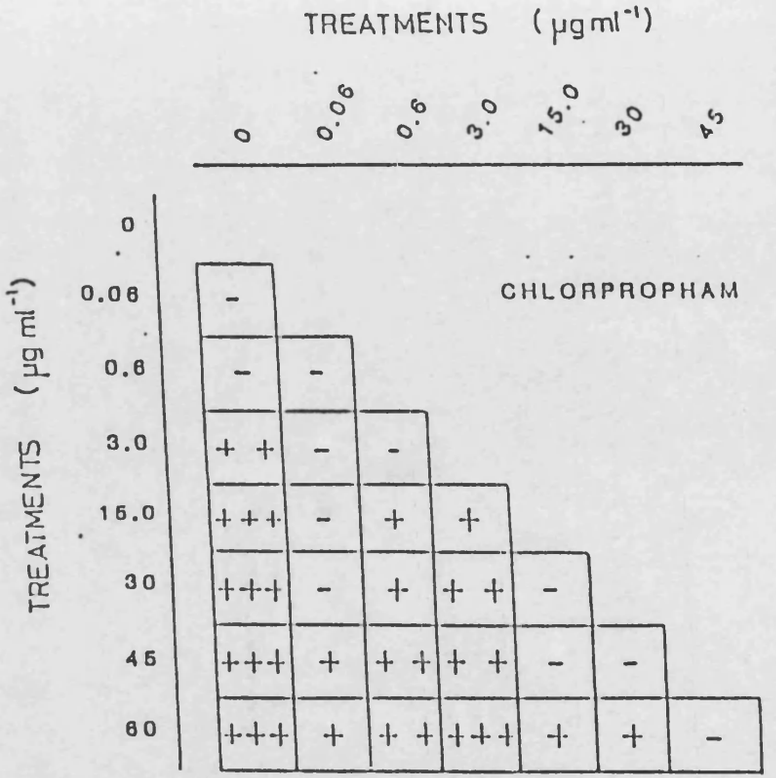


FIG. 29

A diagramatic plot of the significant t-values obtained by comparison of the growth rates of Acanthamoeba castellanii cells, in Repli -dishes treated with chlorpropham (+,++ and+++ indicate significance with 95%,99% and99.9% confidence).

27.3 3-chloroaniline

Growth curves depicting the effect of 3 chloroaniline on A. castellanii have been ommitted due to the confusing pattern that emerged.

The analyses of variance for the effect of 3-chloroaniline on A. castellanii over time (Appendix 3.9) demonstrated that the differences between treatments was greater than that within treatements for all sampling points except 72h. This is reflected in Table 16 (the significance of these differences) where the significant reductions in cell numbers induced by concentrations above 3.2 μgml^{-1} was present at 24, 48 and 120h but not 72h. With 0.6 μgml^{-1} the inhibitory effect was transient, being removed before 48h. The lack of significance of the variance between treatments, as compared with the variance within treatments at the 72h sampling time (F values, Appendix 3.9) accounted for the apparent removal of the inhibitory action of treatments 3.2 to 63.7 μgml^{-1} although the trend was one of diminishing effect. No concentration of 3-chloroaniline induced cell stasis.

Comparison of the growth rates, based on the equations of linear regression (Appendix 4) of A. castellanii treated with 3-chloroaniline, showed that no treatment was significantly different from the untreated control but that 47.7 μgml^{-1} was significantly different from 63.7 μgml^{-1} of 3-chloroaniline to imply a stimulatory action by this treatment on cell growth rates. No threshold of

Table 16

Cell numbers in Acanthamoeba castellanii cultures treated with 3-chloroaniline, in Repli-dishes. The significance of the differences in cell number of 3-chloroaniline-treated cultures from the control cultures is given

Concentration ($\mu g ml^{-1}$)	Number of cells ml^{-1} Sampling times (h)					
	0	24	48	72	-	120
63.7	75754	68178**	112623**	142421	-	152648*
47.7	75754	57067**	142421**	147724	-	159087*
31.8	75754	53532**	110855**	168178	-	193178
15.9	75754	68431**	162370*	151512	-	173485*
3.2	75754	88380**	171714	175754	-	216663
0.6	75754	90906**	200249	192421	-	221714
0.06	75754	123481	111108	180300	-	184845
0.0	75754	161108	183077	207825	-	261108

Significance testing (t - test)

** = very significant (p=0.1)
* = significant (p=0.5)

All values given represent the mean of 19 replications

inhibitory action on cell growth rates was established.

27.4 Propham

Growth of A. castellanii cultures in the presence of propham is depicted in Fig. 30. The analyses of variance for each sampling point (Appendix 3.10) demonstrated significant variations between treatments. The significance of these differences from the untreated (Table 17) shows highly significant differences ($p=0.05$) for all treatments above 1.5 $\mu g ml^{-1}$ propham.

Increasing concentrations of propham progressively inhibited the growth of A. castellanii (Fig. 30). Concentrations above 7.5 $\mu g ml^{-1}$ significantly reduced cell numbers after 24h and continued for 144h. At 1.5 $\mu g ml^{-1}$ propham the inhibitory effect was only first significant after 72h, inhibition persisting for 144h, and with 0.15 $\mu g ml^{-1}$ of propham a significant reduction in cell numbers was seen at 48h but disappeared after 96h (Table 17). No significant increase in cell number over the untreated was seen with any propham treatment, however, with 0.15, 1.5 and 7.5 $\mu g ml^{-1}$ a distinct increase in cell numbers, greater than any preceeding increase, occurred at 96h. (Fig. 30).

At 75 $\mu g ml^{-1}$ propham a distinct lag phase was induced which lasted for 48h before growth commenced. Concentrations of 113 and 150 $\mu g ml^{-1}$ propham were lethal after 24h, no recovery occurred and 150 $\mu g ml^{-1}$ was more toxic than 113 $\mu g ml^{-1}$.

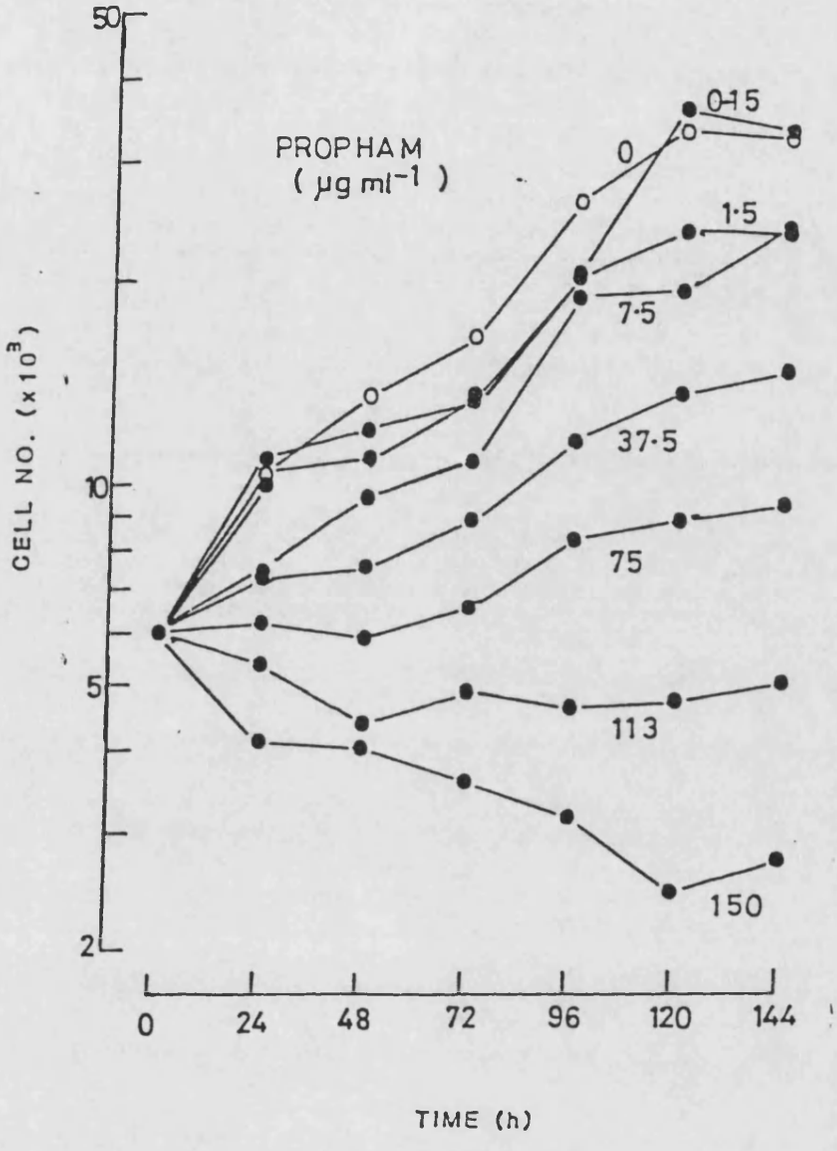


FIG. 30

The sub-acute effect of propham on the growth of Acanthamoeba castellanii, in Repli-dishes, grown in PGY medium at 30°C.

Table 17

Cell numbers in Acanthamoeba castellanii cultures treated with propham, in Repli-dishes. The significance of the differences in cell number of propham-treated cultures from the control cultures are given

Concentration ($\mu g ml^{-1}$)	Number of cells ml^{-1} Sampling times (h)						
	0	24	48	72	96	120	144
150	60000	41000***	40000***	35667***	31667***	24333***	27333***
113	60000	54333***	43889***	48556***	46222***	47000***	49889***
75	60000	61333***	58444***	65444***	82000***	87778***	92778***
37.5	60000	73667***	75000***	88000***	115667***	135333***	146111***
7.5	60000	74000***	95778***	107778***	189444***	19444***	238000***
1.5	60000	108666	122556	134333*	200333*	235667***	232667***
0.15	60000	99222	108111***	128444***	205556*	362222	338667
0.0	60000	103000	136889	166111	261333	333666	324333

Significance testing (t - test)

*** = highly significant (p=0.05)
** = very significant (p=0.1)
* = significant (p=0.5)

All values given represent the mean of 19 replications

Table 18

Comparison of the growth rates of Acanthamoeba castellanii cultures, treated with propham, in Repli-dishes

Treatments (µg/ml)		df	t-values	significance
0.0 vs	0.15	6	0.35	NS
	1.5	6	0.46	NS
	7.5	6	0.68	NS
	37.5	6	2.59	*
	75.0	6	5.53	***
	113.0	8	9.68	***
	150.0	8	10.29	***
0.15vs	1.5	6	0.11	NS
	7.5	6	0.26	NS
	37.5	6	1.91	**
	75.0	6	4.03	***
	113.0	8	7.30	***
	150.0	8	7.83	
1.5 vs	7.5	6	0.12	NS
	37.5	6	1.66	NS
	75.0	6	3.54	*
	113.0	8	6.49	***
	150.0	8	6.98	***
7.5 vs	37.5	6	1.84	NS
	75.0	6	4.48	**
	113.0	8	8.22	***
	150.0	8	8.84	***
37.5 vs	75.0	6	2.34	NS
	113.0	8	5.55	***
	150.0	8	6.26	***
75.0 vs	113.0	8	6.53	***
	150.0	8	8.04	***
113.0vs	150.0	10	2.55	*

Confidence limits

NS = not significant
* = significant with 95% confidence
** = significant with 99% confidence
*** = significant with 99.9% confidence
df = degrees of freedom

The growth rate of A. castellanii cultures were converted to regression lines and compared one with another

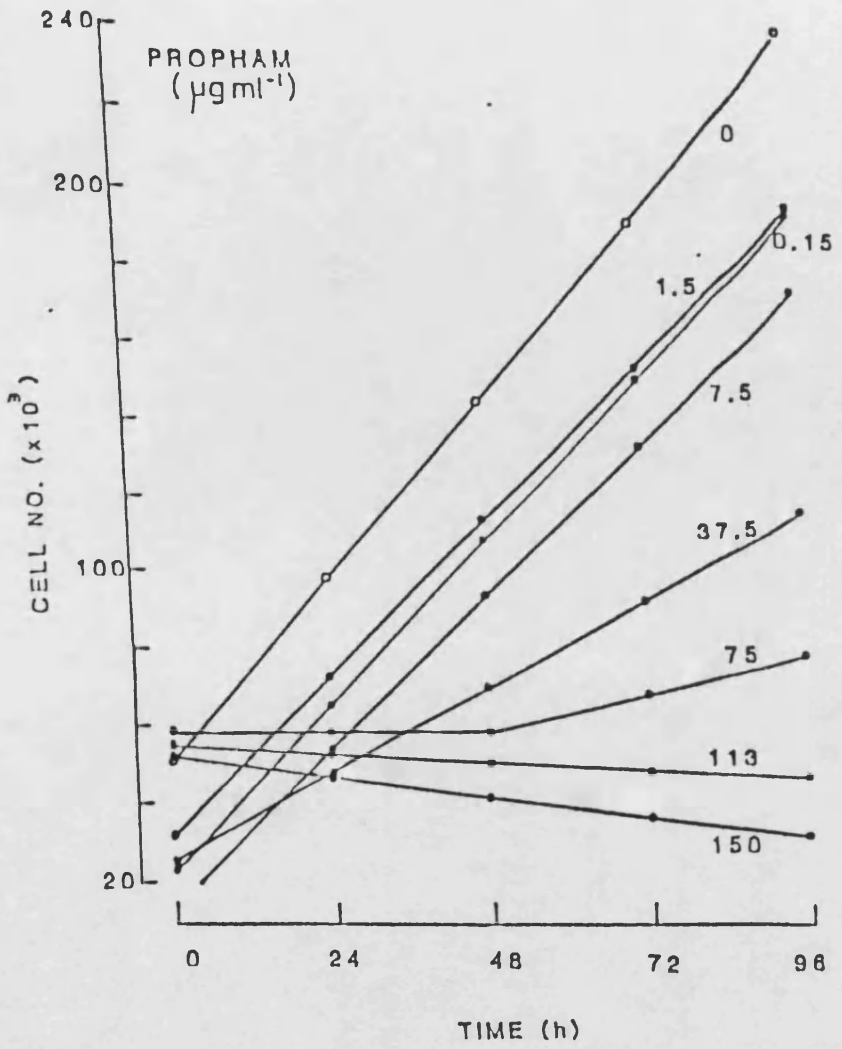


FIG. 31

The effect of propham on the growth rate of Acanthamoeba castellanii cells in Repli-dishes. All points derived from regression lines.

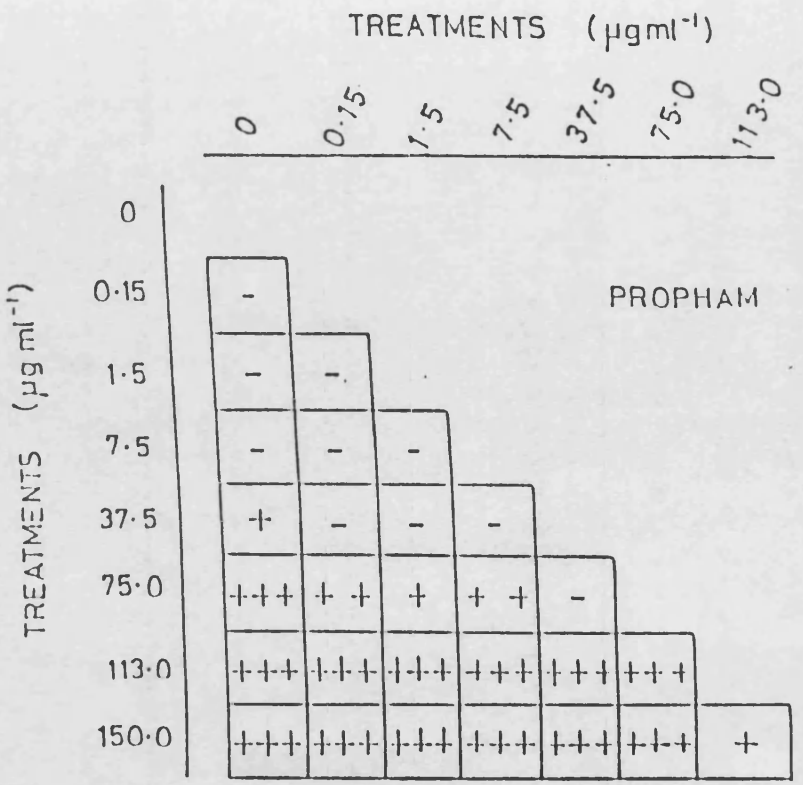


FIG. 32

A diagramatic plot of the significant t-values obtained by comparison of the growth rates of Acanthamoeba castellanii cells, in Repli-dishes, treated with propham (+,++ and +++ indicate significance with 95%, 99% and 99.9% confidence)

The growth rate of A. castellanii cultures treated with propham are shown in Table 18 and Fig. 31. The toxic effect of 113 and 150 μgml^{-1} of propham was clearly seen as negative regression lines in Fig. 31. A reduced rate of growth exists for cultures treated with 75 μgml^{-1} propham. The increase in cell numbers during the later stages of exponential growth with 0.15, 1.5 and 7.5 μgml^{-1} of propham are not in the growth rates for these treatments in Fig. 31.

Comparison of the growth rates one with another showed that there was no stimulation by any propham level (Table 18). Further, treatments of 0.15, 1.5 and 7.5 μgml^{-1} were not significantly different from the control. The comparison of growth rates, expressed as a matrix (Fig. 32) showed the inhibitory action of propham on the growth rate of A. castellanii occurred at 37.7 μgml^{-1}. The growth rates in the presence of 113 and 150 μgml^{-1} of propham were significantly different from all other treatments to suggest that a lethal effect of propham on cells of A. castellanii occurred at this level.

27.5 Aniline

Due to the transient nature of inhibition of growth of A. castellanii in the presence of, growth curves have been omitted.

Table 19

Cell numbers in Acanthamoeba castellanii cultures treated with aniline, in Repli-dishes. The significance of the differences in cell number of aniline-treated cultures from the control cultures are given

Concentration ($\mu g ml^{-1}$)	Number of cells ml^{-1} Sampling times (h)					
	0	24	48	72	-	120
150	75754	100754**	179795**	172724	-	184845
113	75754	120454**	147724**	173986	-	176512*
75	75754	119189**	196209	223734	-	206057
37.5	75754	122219*	152774**	190148	-	236865
7.5	75754	101007**	183077	187875	-	203784
1.5	75754	109340**	147724**	213128	-	252269
0.15	75754	125754*	167673	181057	-	298481
0.0	75754	161108	188077	207825	-	261108

Significance testing (t - test)

** = very significant (p=0.1)
* = significant (p=0.5)

All values given represent the mean of 19 replications

The analyses of variance for the effect of aniline on A. castellanii are in Appendix 3.11, the significance of these differences in Table 19.

Aniline had an initial inhibitory action on the growth of A. castellanii at all concentrations tested. The inhibition of growth was transient, but some inhibition persisted after 24h eg 113 $\mu g ml^{-1}$ aniline still significantly inhibited growth at 120h. Inhibition diminished with time suggesting a general recovery (Table 19). No concentration of aniline was lethal or induced cell stasis in A. castellanii.

Inhibition of cell numbers was not recorded for the 72h sample time due to a non-significant F value (Appendix 3).

Analysis of growth rates indicated no significant effect by aniline at any concentration. No threshold of inhibitory action on growth rates was detected.

27.6 Barban

Cultures of A. castellanii grown in the presence of barban (Fig. 33) were analysed for the degree of variance (Appendix 3.12) and the significance of these differences from the untreated (Table 20). Concentrations between 0.4 and 7.7 $\mu g ml^{-1}$ barban were initially lethal to some cells of A. castellanii (Fig. 33, Table 20).

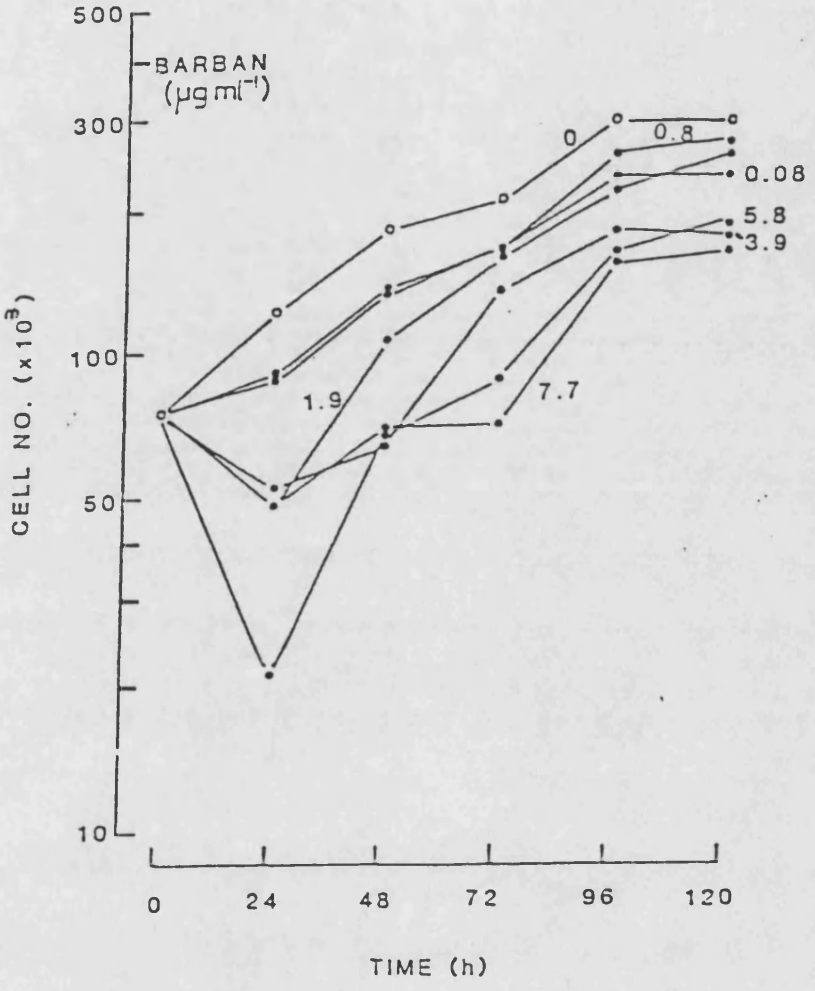


FIG. 33

The sub-acute toxicity of barban on the growth of Acanthamoeba castellanii , in Repli-dishes, grown in PGY medium at 30°C.

Table 20

Cell numbers in Acanthamoeba castellanii cultures treated with barban, in Repli-dishes. The significance of the differences in cell number of barban-treated cultures from the control cultures are given

Concentration ($\mu g ml^{-1}$)	Number of cells ml^{-1} Sampling times (h)					
	0	24	48	72	96	120
7.7	79646	22576***	72828***	74845***	164495***	169798***
5.8	79646	59696***	71566***	91263***	167020***	193535***
3.9	79646	58434***	67020***	138989***	185707***	172323***
1.9	79646	50101***	111970***	165252***	224596***	276616***
0.4	79646	40505***	94798***	178636	315505	336717
0.08	79646	91515***	133687**	163232***	267525***	284192
0.008	79646	92273***	142778*	169293*	245808***	241010
0.0	70646	127274	188232	219040	343939	332677

Significance testing (t - test)

*** = highly significant (p=0.05)
** = very significant (p=0.1)
* = significant (p=0.5)

All values given represent the mean of 19 replications

Cell numbers were reduced by between 55 and 83%. In all cases recovery of growth occurred after 24h. With 7.7 μgml^{-1} barban this recovery was in a distinct step-wise manner. The length of the 'riser' and the 'tread' of each step was 24h in each case (Fig. 33). A concentration of 5.8 μgml^{-1} of barban produced a similar but less distinct growth pattern, no other occurrences were noted.

Concentrations of barban between 3.9 and 7.7 μgml^{-1} caused significant reductions in cell numbers over 120h whilst concentrations between 0.008 and 1.9 μgml^{-1} barban reduced cell number over 96h. No increase in cell number, above the untreated, was observed with any treatment of barban and no concentration induced persistent cell stasis in A. castellanii.

The effect of barban on A. castellanii growth rates (Fig. 34) is complex. At 0.08 and 0.008 μgml^{-1} barban the growth rates were depressed. With barban concentrations ranging from 0.4 to 7.7 μgml^{-1} the cells recovered from an initial inhibitory effect, some with growth rates exceeding that of the untreated control. The significance of these differences (Table 21) show barban (0.08 μgml^{-1}) to significantly depress growth rates while 0.008 μgml^{-1} failed to produce a significant difference.

Barban at 0.4 μgml^{-1} (Fig. 34 but not shown in Fig. 33) caused a significant increase in the growth rate of cells

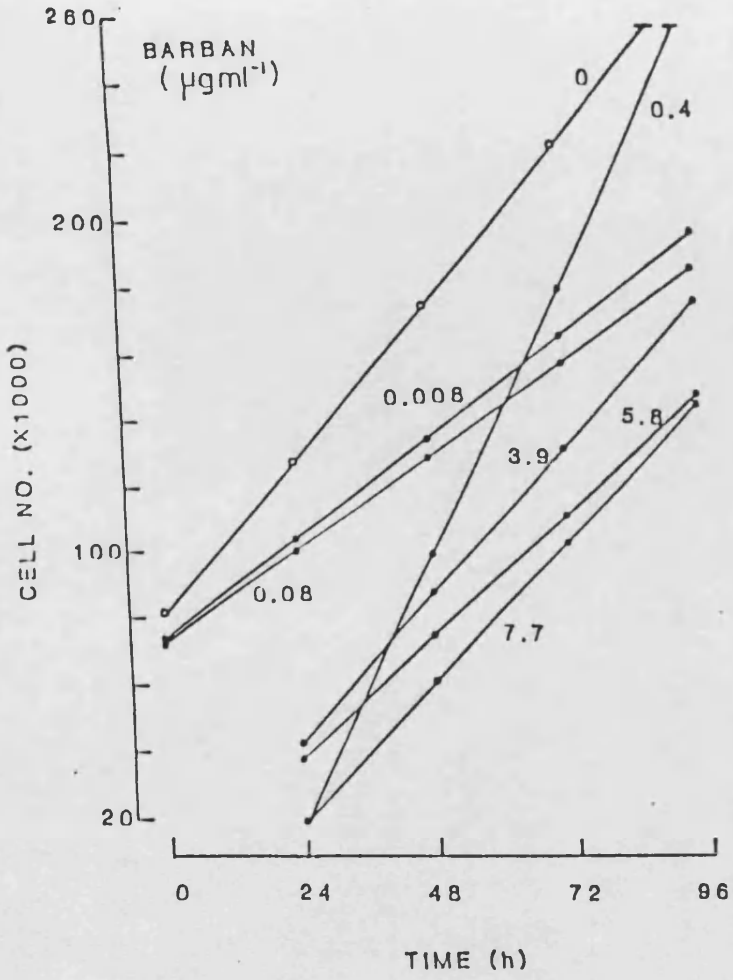


FIG.34

The effect of barban on the growth rates of Acanthamoeba castellanii cells, in Repli-dishes. All points derived from regression lines.

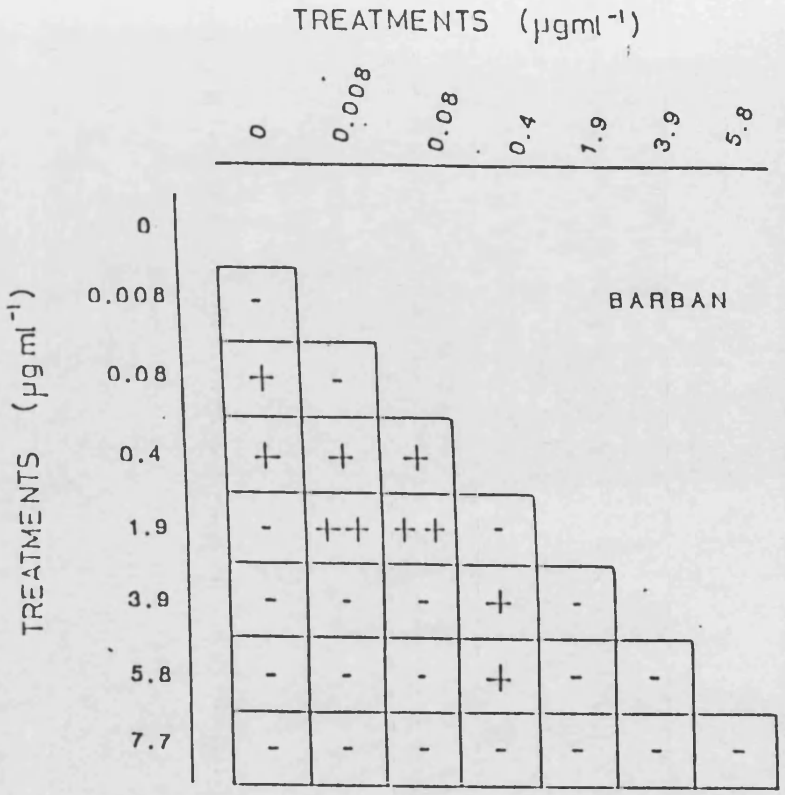


FIG. 35

A diagramatic plot of the significance of t-values obtained by comparison of growth rates of Acanthamoeba castellanii cells, in Repli-dishes, treated with barban (+, ++ and +++ indicate significance with 95%, 99% and 99.9% confidence).

Table 21

Comparison of the growth rates of Acanthamoeba castellanii cultures, treated with barban, in Repli-dishes

Treatments (µglml)			df	t-values	significance
0.0	vs	0.008	4	2.51	NS
		0.08	4	3.22	*
		0.4	4	-3.12	*
		1.9	4	-2.32	NS
		3.9	4	0.26	NS
		5.8	4	1.06	NS
		7.7	4	0.42	NS
0.008	vs	0.08	4	0.41	NS
		0.4	4	-4.20	*
		1.9	4	-5.13	**
		3.9	4	-1.35	NS
		5.8	4	-0.46	NS
		7.7	4	-0.87	NS
0.08	vs	0.4	4	-4.47	*
		1.9	4	-6.64	**
		3.9	4	-1.67	NS
		5.8	4	-0.73	NS
		7.7	4	-1.11	NS
0.4	vs	1.9	4	2.51	NS
		3.9	4	2.88	*
		5.8	4	3.32	*
		7.7	4	2.76	NS
1.9	vs	3.9	4	1.40	NS
		5.8	4	2.16	NS
		7.7	4	1.30	NS
3.9	vs	5.8	4	0.65	NS
		7.7	4	0.18	NS
5.8	vs	7.7	4	-0.39	NS

Confidence limits

NS = not significant
* = significant with 95% confidence
** = significant with 99% confidence
*** = significant with 99.9% confidence
df = degrees of freedom

The growth rate of A. castellanii cultures were converted to regression lines and compared one with another

A. castellanii, 19 μgml^{-1} failed to produce a significant effect. All other treatments were not significantly different from the untreated (Table 21).

In Fig. 35 the growth rates are compared and expressed in a matrix. Comparisons show both significant inhibition (0.08 μgml^{-1}) and stimulation (0.4 μgml^{-1}). Enhanced growth rates were indicated by negative t values with 0.4 and 1.9 μgml^{-1} compared with 0.08 and 0.008 μgml^{-1}. The threshold of stimulatory action on growth rates of A. castellanii was 0.4 μgml^{-1}. The threshold of inhibitory activity on growth rate was 0.08 μgml^{-1}.

27.7 Diuron

Plots of population growth curves have been omitted due to lack of clarity between treatments (Table 22). The analyses of variance for the effect of diuron on the growth of A. castellanii over time are in Appendix 3.13. The significance of these differences, compared with the untreated, are set out in Table 22.

Diuron at all concentrations, 0.03 to 29.4 μgml^{-1}, significantly reduced cell number (A. castellanii) after 24h. All treatments were initially lethal to some cells, the decreases in numbers ranged from 30% with 0.03 μgml^{-1} to 70% with 29.4 μgml^{-1} diuron. With 0.03, 0.3 and 1.5 μgml^{-1} diuron the inhibition was absent after 48h.

Table 22

Cell numbers in Acanthamoeba castellanii cultures treated with diuron, in Repli-dishes. The significance of the differences in cell number of diuron-treated cultures from the control cultures are given

Concentration ($\mu g ml^{-1}$)	Number of cells ml^{-1} Sampling times (h)					
	0	24	48	72	96	120
29.4	64495	25858***	80404***	137980***	156667***	209697
22.1	64495	28131***	76868***	115000***	177626***	210707
14.7	64495	30151***	56919***	129141***	191263***	210328
7.4	64495	52626***	94040*	147828***	192273***	204646
1.5	64495	42778***	112727	168030	245689	250101
0.3	64495	44798***	125606	177879	226111	178636
0.03	64495	59697*	125354	158434*	237475	203383
0.0	64495	84949	134444	202121	258308	203383

Significance testing (t - test)

*** = highly significant (p=0.05)
** = very significant (p=0.1)
* = significant (p=0.5)

All values given represent the mean of 19 replications

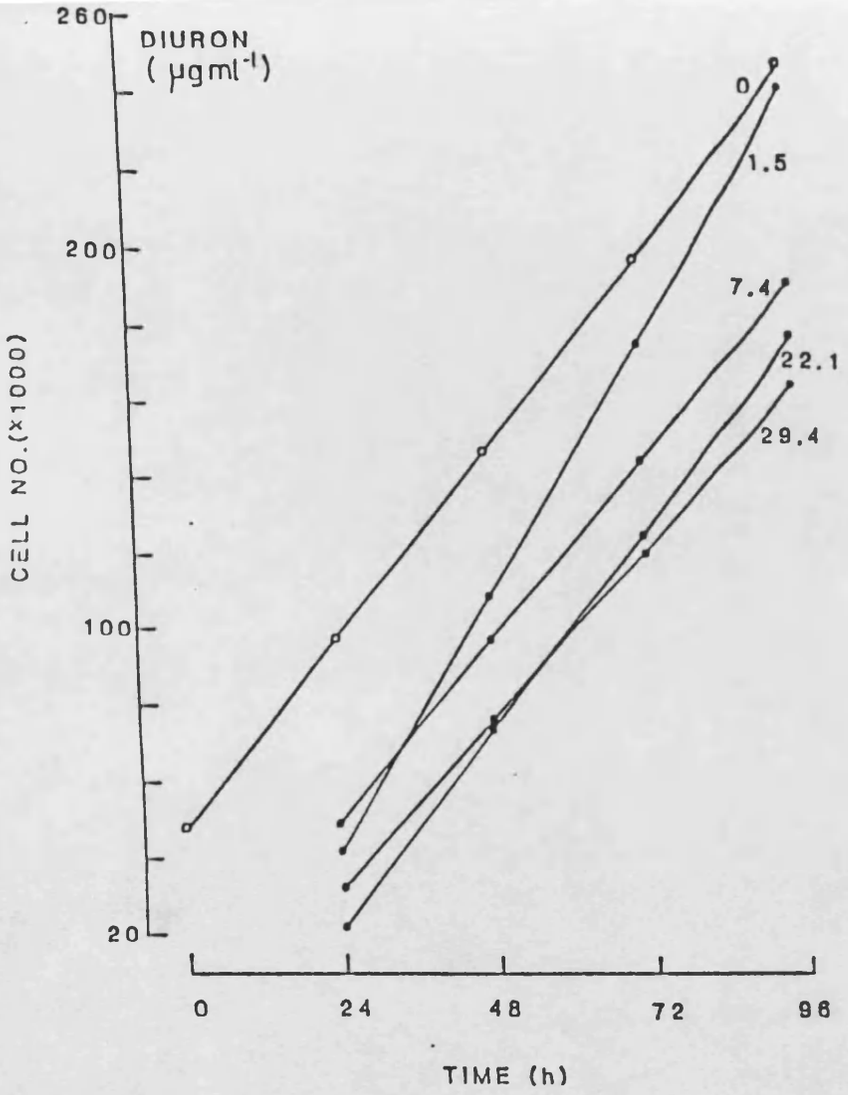


FIG. 36

The effect of diuron on the growth of Acastellanii castellanii cells, in Repli-dishes. All points were derived from regression lines.

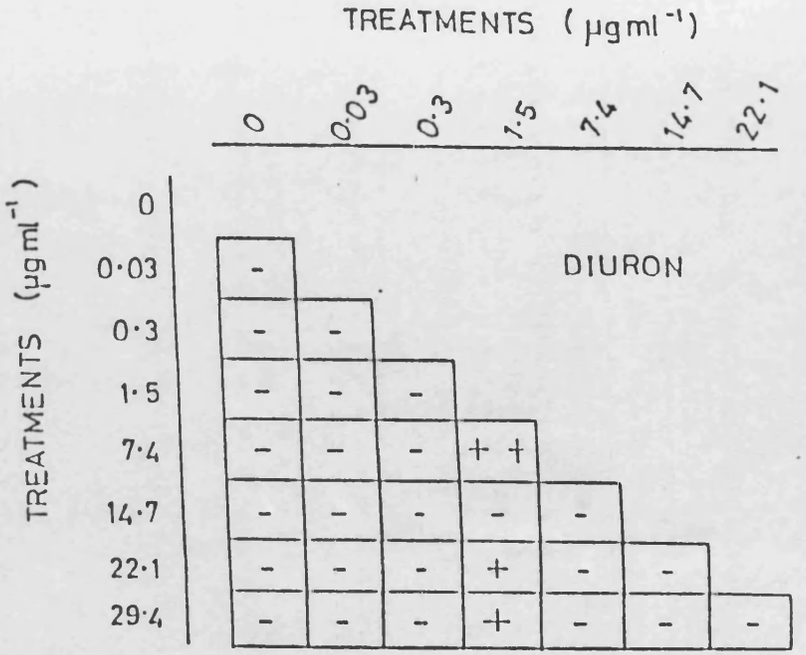


FIG. 37

A diagramatic plot of significant t-values obtained by comparison of growth rates of Acanthamoeba castellanii cells, in Repli-dishes, treated with diuron (+ and ++ indicate significance with 95% and 99% confidence).

Table 23

Comparison of the growth rates of Acanthamoeba castellanii cultures, treated with diuron, in Repli-dishes

Treatments (µg/ml)		df	t-values	significance
0.0	vs 0.03	5	-0.76	NS
	0.3	5	-1.16	NS
	1.5	5	-2.26	NS
	7.4	5	0.47	NS
	14.7	5	-0.59	NS
	22.1	5	0.25	NS
	29.4	5	0.66	NS
0.03	vs 0.3	4	-0.37	NS
	1.5	4	-1.48	NS
	7.4	4	1.52	NS
	14.7	4	0.12	NS
	22.1	4	1.17	NS
	29.4	4	1.33	NS
0.3	vs 1.5	4	-1.10	NS
	7.4	4	2.17	NS
	14.7	4	0.47	NS
	22.1	4	1.72	NS
	29.4	4	1.74	NS
1.5	vs 7.4	4	5.79	**
	14.7	4	1.46	NS
	22.1	4	4.04	*
	29.4	4	3.05	*
7.4	vs 14.7	4	-1.18	NS
	22.1	4	-0.34	NS
	29.4	4	0.34	NS
14.7	vs 22.1	4	0.91	NS
	29.4	4	1.13	NS
22.1	vs 29.4	4	0.51	NS

Confidence limits

NS = not significant
* = significant with 95% confidence
** = significant with 99% confidence
*** = significant with 99.9% confidence
df = degrees of freedom

The growth rates of A. castellanii cultures were converted to regression lines and compared one with another

Concentrations between 7.4 and 29.4 μgml^{-1} of diuron significantly depressed cell numbers for 96h (Table 22). No significant variation between the treatments was found for the 120h sample point (Appendix 3.13), suggesting recovery of cultures from diuron inhibition.

The effect of diuron on the growth rate of cells of A. castellanii (Fig. 36) derived from linear regression equations of the exponential phase of culture (Appendix 4.10) again show the initial lethal actionand subsequent recovery. No recovery growth rate was significantly different from the untreated (Table 23). However, comparison of 1.5 μgml^{-1} treated cultures with other diuron treatments (Fig. 37) indicated that the 1.5 μgml^{-1} treated cultures growth rate was significantly different from 7.4, 22.1 and 29.4 μgml^{-1} treated cultures whereas the untreated control was not. The results suggest a stimulatory effect on the growth rate of A. castellanii treated with 1.5 μgml^{-1} diuron after an initial suppression of growth.

27.8 Permethrin

The analyses of variance for the effect of permethrin on the growth of A. castellanii over 120 appear in Appendix 3.14. The significance of the variation between treatments declined with time. There was no significant difference between treatments after 72h. The significance

Table 24

Cell numbers in Acanthamoeba castellanii cultures treated with permethrin, in Repli-dishes. The significance of the differences in cell number of permethrin-treated cultures from the control cultures are given

Concentration ($\mu g ml^{-1}$)	Number of cells ml^{-1} Sampling times (h)					
	0	24	48	72	96	120
0.14	69545	28131***	97323*	175353	206919	254192
0.11	69545	54141***	96566*	183939	227879	246313
0.07	69545	96067	156667	168535*	219545	269040
0.03	69545	62980*	112980	208939	214495	255909
0.007	69545	79141	135960	149090***	226869	233939
0.001	69545	101869	115253	198081	271568	286717
0.0001	69545	78636	137727	193030	219798	274596
0.0	69545	86212	137222	208939	245303	289495

Significance testing (t - test)

*** = highly significant (p=0.05)
** = very significant (p=0.1)
* = significant (p=0.5)

All values given represent the mean of 19 replications

of the earlier differences (Table 24) show concentrations of 0.14 and 0.11 μgml^{-1} permethrin had an initial lethal effect on some cells. The lethal action was removed after 24h but depression of cell numbers remained until after 48h. Isolated inhibition of cell numbers occurred with 0.07, 0.03 and 0.007 μgml^{-1} of permethrin.

Permethrin had no significant effect on the growth rate of cells of A. castellanii.

27.9 Asulam

The analyses of variance for the effect of asulam on the growth of A. castellanii over 120h reveal significant variations between treatments to occur at 24 and 120h only (Appendix 3.15). The significance of these differences (Table 25) show no toxic effect of asulam on cell numbers. Stimulation of cell numbers above the untreated number was detected after 24h with 0.38 μgml^{-1} asulam, the increase was 128%. Further significant increases in cell numbers were detected with 37.5, 28.1 and 3.8 μgml^{-1} (129h), the increases ranged from 13 to 25%.

Asulam had no significant effect upon the growth rate of A. castellanii cells.

Table 25

Cell numbers in Acanthamoeba castellanii cultures treated with asulam, in Repli-dishes. The significance of the differences in cell number of asulam-treated cultures from the control cultures are given

Concentration ($\mu g ml^{-1}$)	Number of cells ml^{-1} Sampling times (h)					
	0	24	48	72	96	120
375.0	70704	84340	178784	243683	362118	465401**
281.0	70704	98734	185098	223734	327017	458077*
187.5	70704	76764	152774	202269	338633	389391
93.8	70704	75754	178532	229037	340401	382067
18.8	70704	82067	157825	242673	352774	420451
3.8	70704	98986	163380	242168	354163	507320***
0.38	70704	203279***	195956	236360	334593	438759
0.0	70704	89138	150249	213885	327269	405300

Significance testing (t - test)

*** = highly significant (p=0.05)
** = very significant (p=0.1)
* = significant (p=0.5)

All values given represent the mean of 19 replications

28.0 Comparison of the sub-acute toxicity of some pesticides to T. pyriformis and A. castellanii in Repli-dish culture

28.1 The characteristics of dose-response curves

Dose-response curves for T. pyriformis (72h) and A. castellanii (96h) were produced from data given in Section 26 and 27.

Tetrahymena pyriformis: Increasing concentrations of pesticides created dose-response curves which in most cases were typically curvilinear in shape for the majority of chemicals evaluated with T. pyriformis (Fig. 38 and 39). The initial part of the curve was generally shallow, low levels of pesticides having little effect. The middle section was steep, small increases in concentration producing large differences in inhibitory activity. A final section (seen only with chlorpropham) was a 'tailing off' of effects, a shallow curve where large increases in concentration did not influence inhibition.

The evidence that such dose-response curves are sigmoidal is discussed in Section 33.0. Several curves indicated growth stimulation by very low levels of pesticide.

Acanthamoeba castellanii: Less dramatic than T. pyriformis response, the dose-response curves with

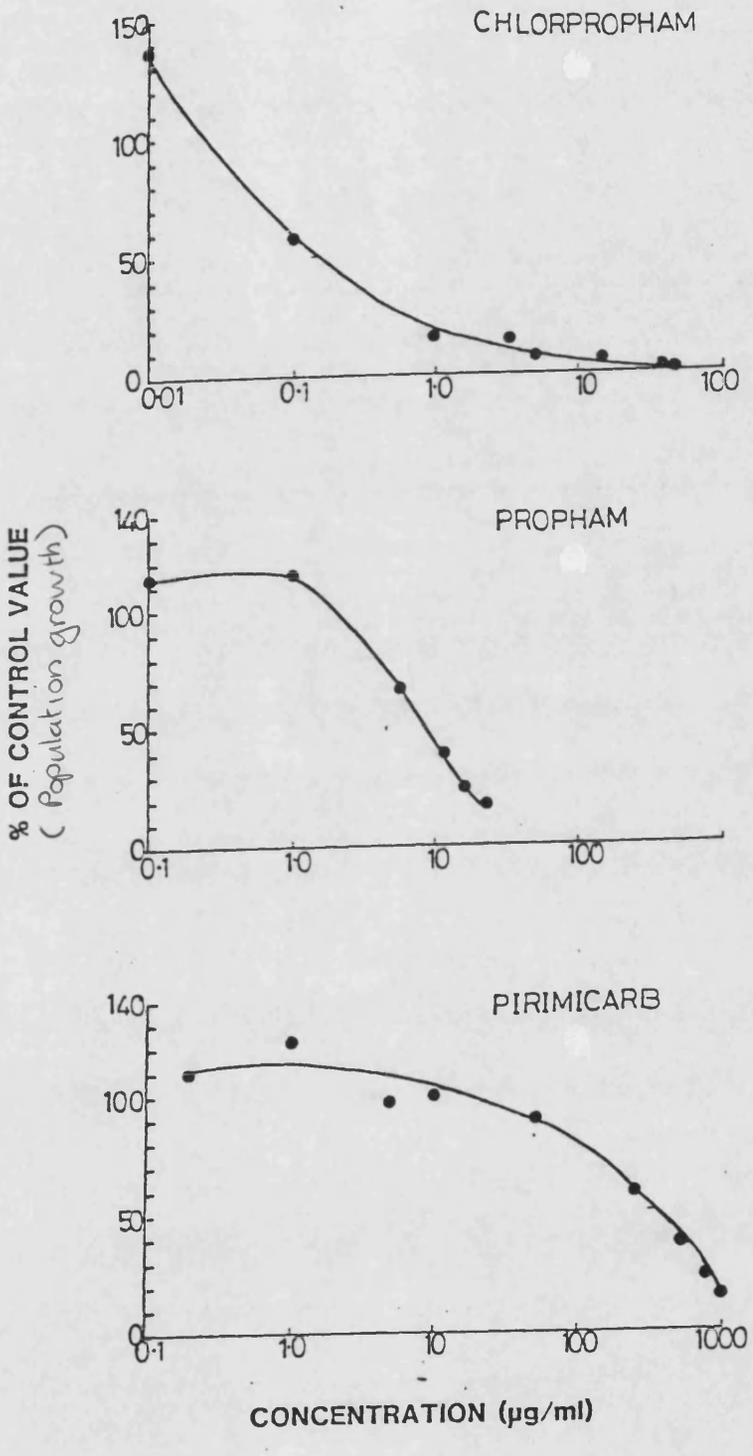


FIG.38

The dose-response curves for Tetrahymena pyriformis (72h) treated with chlorpropham propham and pirimicarb.

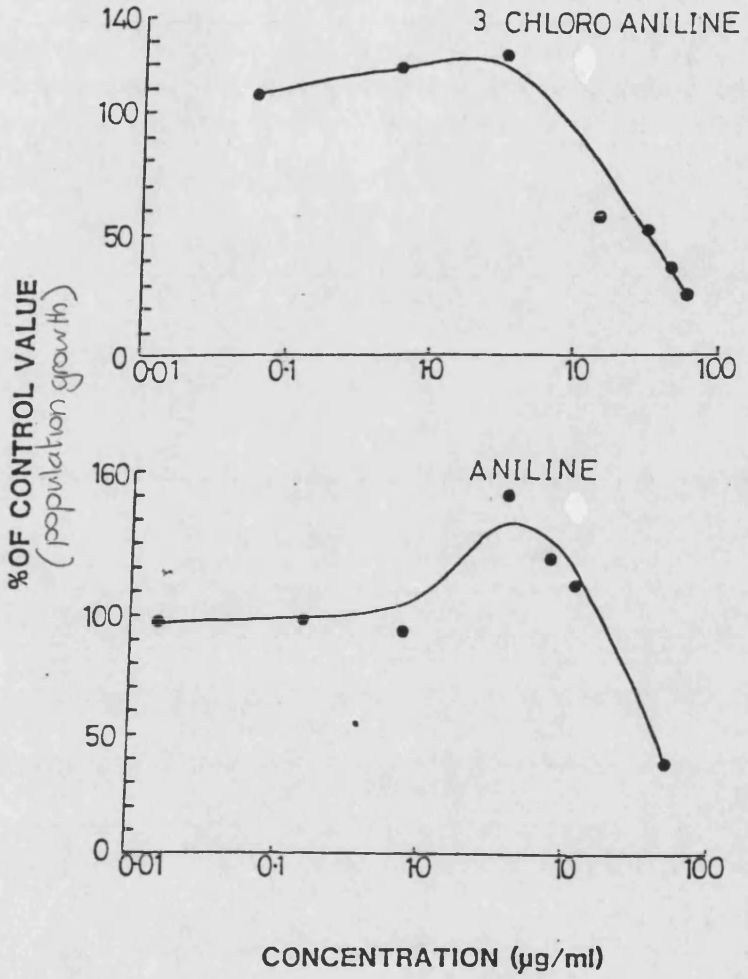


FIG. 39

The dose-response curves for Tetrahymena pyriformis (72h) treated with 3-chloro-aniline and aniline.

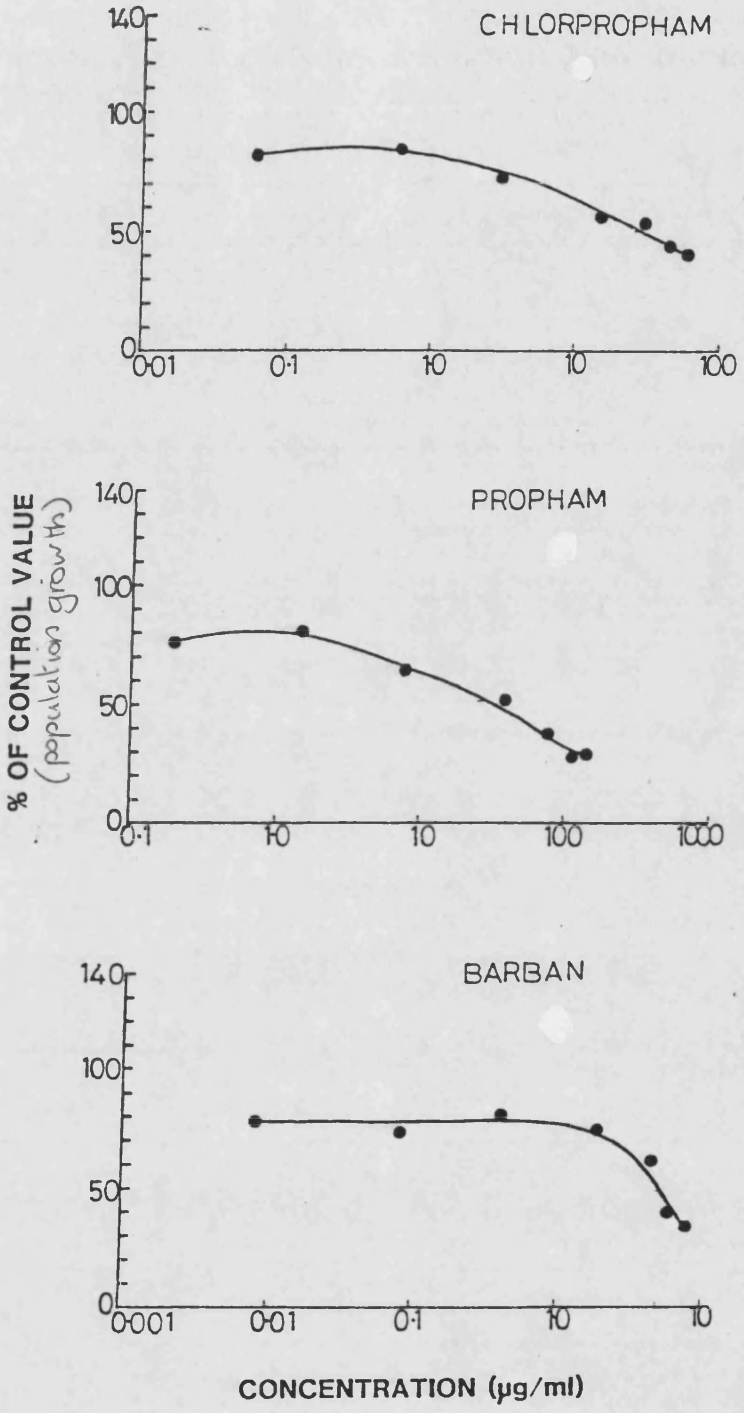


FIG. 40

The dose-response curves for Acanthamoeba castellanii (96 h) treated with chloropropham propham and barban.

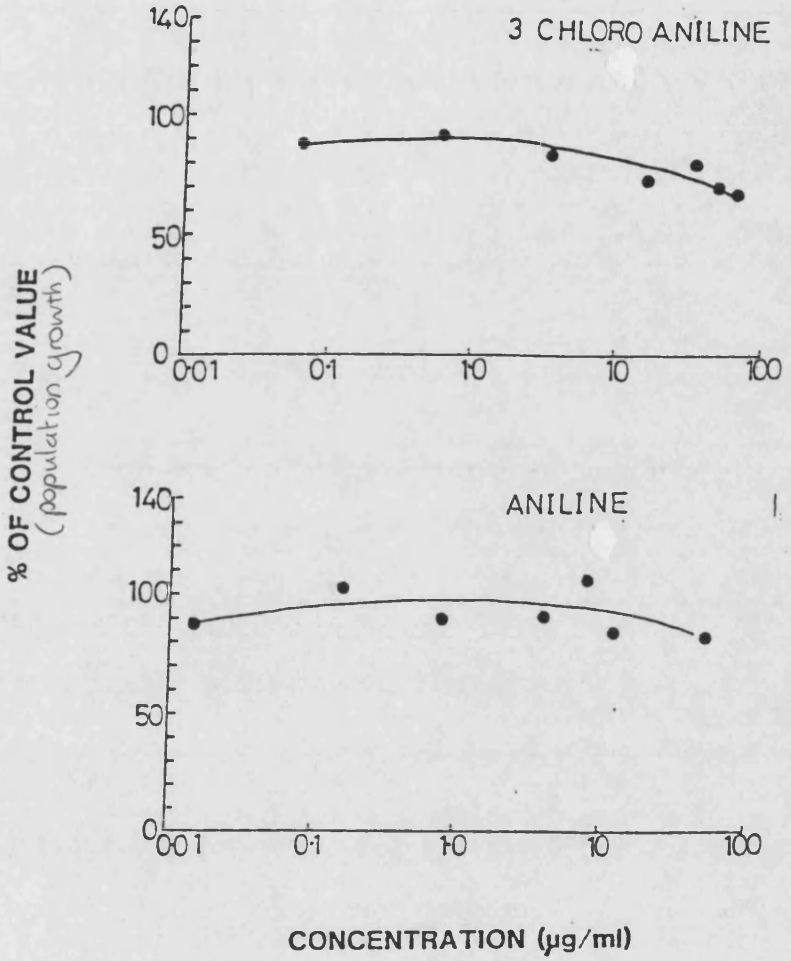


FIG. 41

The dose-response curves for Acanthamoeba castellanii (96h) treated with 3-chloro-aniline and aniline.

A. castellanii were generally shallower and more linear in shape (Fig. 40 and 41).

28.2 The comparative sensitivity of Tetrahymena pyriformis and Acanthamoeba castellanii to phenylcarbamate herbicides and metabolites

T. pyriformis was more sensitive to chlorpropham, propham, 3-chloroaniline and aniline than A. castellanii. The EC_{50} values, the concentration of a pesticide which reduces cell number to 50% of the untreated control, for chlorpropham against T. pyriformis (72h) and A. castellanii (96h) was 0.2 and 30 $\mu g ml^{-1}$ respectively. With propham EC_{50} values were 9 $\mu g ml^{-1}$ (T. pyriformis) and 30 $\mu g ml^{-1}$ (A. castellanii). The metabolites 3-chloroaniline (of chlorpropham) and aniline (of propham) were less toxic to either organism than the parent compounds. The EC_{50} values (72h) for T. pyriformis were 30 $\mu g ml^{-1}$ (3-chloroaniline) and 60 $\mu g ml^{-1}$ (aniline) while for A. castellanii (96h) neither compound caused cell number to fall to a value of 50% of the untreated control.

The order for inhibitory activity, based on EC_{50} values (72h) towards T. pyriformis was chlorpropham (0.2 $\mu g ml^{-1}$) > propham (9 $\mu g ml^{-1}$) > 3-chloroaniline (30 $\mu g ml^{-1}$) > aniline (60 $\mu g ml^{-1}$). With A. castellanii (96h) it was (barban 4.7 $\mu g ml^{-1}$)>chlorpropham and propham (30 $\mu g ml^{-1}$)> both 3-chloroaniline and aniline (no value).

With T. pyriformis low concentrations of chlorpropham, propham, 3-chloroaniline, aniline and pirimicarb increased cell numbers above the control value whilst no stimulation of cell population numbers was indicated in the dose-response curves of A. castellanii. Maximum stimulation of cell population numbers with 3-chloroaniline and aniline occurred over the range 0.1 - 5.0 (3-chloroaniline) and 1.0 - 50 μgml^{-1} (aniline) while with propham and chlorpropham stimulation, to a similar degree, occurred over the concentration range 0.1 - 1.0 μgml^{-1} (propham) and 0.01 to 0.03 μgml^{-1} (chlorpropham). Both organisms were more sensitive to stimulation by the parent compounds than by the metabolites.

The phenylcarbamate barban had a dose-response curve with A. castellanii which was steeper in the latter stages than either chlorpropham or propham. The 96h EC_{50} value was 4.7 μgml^{-1}, considerably lower than either chlorpropham or propham. No stimulation of population numbers above the control value was detected.

28.3 Pirimicarb

The 72h EC_{50} value for pirimicarb towards T. pyriformis was 400 μgml^{-1} (Fig. 38). The dose-response curves, typically shows both stimulation and considerable inhibition of cell numbers.

29.0 Growth characteristics of Acanthamoeba castellanii in microtiter plate wells

The population growth of A. castellanii in microtiter plate wells (200 µl) is shown in Fig 6 & p 60. Cell yield after 120h (1.2 x 10 cells ml^{-1}) was comparable with those obtained in Repli-dish wells (Section 27.1) given that in microtiter plates the initial innoculum level was larger (9.0×10^4 cell ml^{-1}). The high initial innoculum size also reduced the doubling time (15.2h) to below that found in Repli-dish cultures.

30.0 Qualitative evaluation of the sub-acute toxicity of some pesticides to Acanthamoeba castellanii in microtiter plate wells

The results of the qualitative (macroscopic) assessment of the effect of some pesticides on growth of A. castellanii in microtiter wells are given in Table 26. The pesticides were ranked according to the lowest concentrations, in moles, that inhibited 'band formation' in the test system, this was the Minimum Inhibitory Concentration (MIC value).

On this assessment the most toxic pesticide was propham. This was followed by barban, diuron, benomyl and chlorpropham. The other carbamates, the insecticide

Table 26

Comparative inhibition by some pesticides of 'band formation' in microtiter wells inoculated with Acanthamoeba castellanii

Pesticide	Minimum Inhibitory Concentration (M)
propham	5.5×10^{-6}
barban	1.5×10^{-5}
diuron	6.4×10^{-5}
benomyl	6.8×10^{-5}
chlorpropham	7.8×10^{-5}
terbutryne	1.2×10^{-4}
MCPA	4.9×10^{4}
pirimicarb	1.7×10^{-3}
asulam	1.7×10^{-3}
isoproturon	> compound's solubility
permethrin	> compound's solubility
linuron	> compound's solubility
cyanazine	> compound's solubility

Band formation (see p 61) was assessed after 72h incubation at 30°C

pirimicarb and asulam (a herbicide) were both similar in their toxicity effect but were 2 to 3 orders of magnitude less toxic than the above.

The triazines, terbutryne and cyanazine differed in their inhibitory activity. Cyanazine had no toxic effect on A. castellanii in this comparative test, whilst terbutryne was relatively inhibitory to 'band formation'.

The urea herbicides, isoproturon and linuron were not toxic to A. castellanii in contrast to the action of diuron. The pyrethroid insecticide, permethrin was also non-toxic. Both representative insecticides, permethrin and pirimicarb, were much less toxic than the majority of herbicides tested in this system.

Comparison of the phenylcarbamate herbicides showed the order of inhibition to be propham > barban > chlorpropham, (based on absolute quantity, $\mu g ml^{-1}$)

Comparison of these MIC values with the 96h EC_{50} values obtained with A. castellanii in sub-acute toxicity evaluations in Repli-dishes (Section 28.2) showed the order of inhibition to be altered. In the latter cases EC_{50} values gave the order of toxicity to be barban ($2.7 \times 10^{-5}M$) > chlorpropham ($1.4 \times 10^{-4}M$) > propham ($1.6 \times 10^{-4}M$).

31.0 Quantitative evaluation of the sub-acute toxicity of some pesticides to Tetrahymena pyriformis and Acanthamoeba castellanii using the microtiter plate culture technique

31.1 Growth characteristics of Tetrahymena pyriformis and Acanthamoeba castellanii in microtiter plates

Comparison of the population growth of T. pyriformis measured by haemocytometer and by optical density (Fig. 8 & p. 63) showed a high correlation throughout culture growth (120h). The population doubling time was 16.8h and the yield of cells was 4.5×10^5 cells ml^{-1} after 96h. The doubling time for cell populations in this culture procedure was 3 x longer than for those cells grown for the qualitative assessment in microtiter plates. However, the initial inoculum was 5 x larger to accommodate the detection threshold of the microplate readers (approximately 4×10^4 cells ml^{-1} (Fig. 10). No lag phase was detected in these cultures.

Population growth of A. castellanii measured by haemocytometer and optical density was also correlated throughout 144h of growth in microtiter plates (Fig. 9 & p 63). A. castellanii cells grown for quantitative studies in microtiter plates had a slower growth rate (doubling time 21.6h) and an extended lag phase (48h) unlike those

grown for qualitative studies in microtiter plates (Fig. 6). However, in the quantitative studies the initial inoculum was 2.5 x higher than previous to allow detection by the microplate reader, threshold approximately 7 x 10^4 cells ml^{-1} (Fig. 10). Cell yields were also below those of cells grown for the qualitative studies. Final cell yields (144h) were 8.2 x 10^5 cells ml^{-1}.

31.2 Analyses of data

All subsequent tables of significant t-value differences between cells grown in the presence of pesticides and untreated control cells had significant (p=0.01) analysis of variance F-values for all sample points. The F-value tables are not presented.

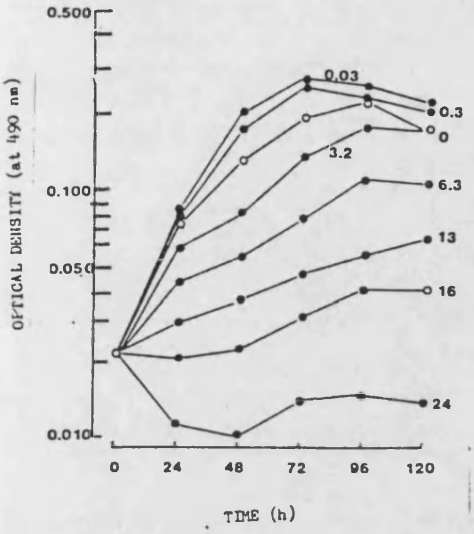
Comparison of the dose-response curves generated from the t-value tables are in section 32.0.

31.3 Barban

The effects of barban on the population growth of *T. pyriformis* and *A. castellanii* are shown in Fig. 42, with the significance of these effects in Tables 27 and 28 respectively. Concentrations between 1.0 and 8.0 $\mu g ml^{-1}$ were inhibitory to *T. pyriformis*. Barban at 4 and 3 $\mu g ml^{-1}$ caused an initial decline in optical density values of *T. pyriformis* but the effect did not persist after 24h.

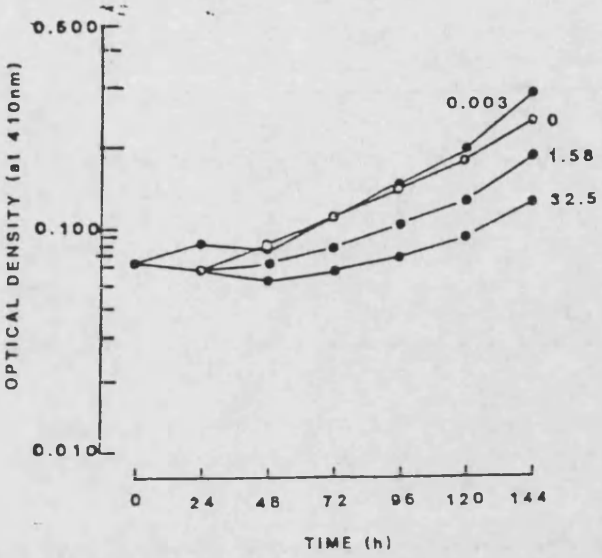
T. PYRIFORMIS (a)

DIURON



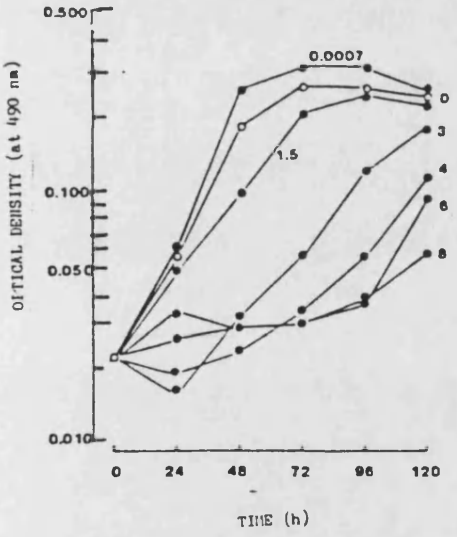
A. CASTELLANII (b)

DIURON



T. PYRIFORMIS (a)

BARBAN



A. CASTELLANII (b)

BARBAN

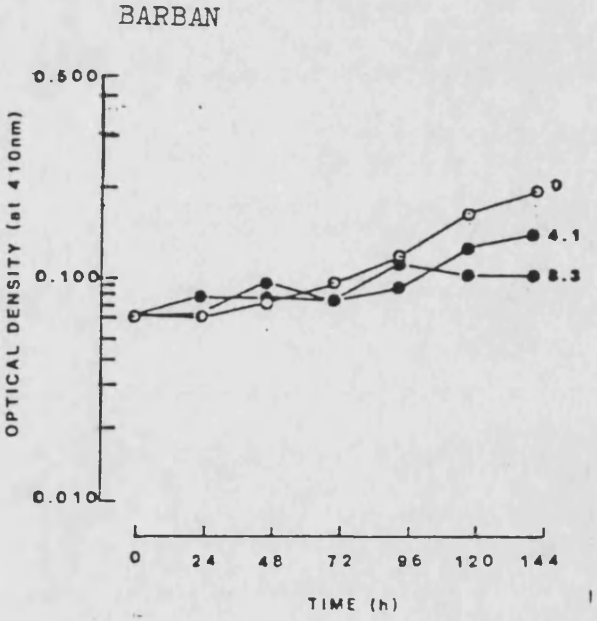


FIG. 42

The sub-acute effects of barban and diuron on the growth of Tetrahymena pyriformis (a) and Acanthamoeba castellanii (b) in microtiter plates. Numbers on the figures refer to herbicide concentration. Not all concentrations tested are shown on the graphs.

Table 27

Optical density values at 490nm of Tetrahymena pyriformis cultures treated with barban, in microtiter plates. The significance of the difference in optical density values of barban-treated cultures from control cultures are given

	Optical density values					
Concentration (μgml^{-1})	Sampling times (h)					
	0	24	48	72	96	120
8.0	0.023	0.027**	0.030**	0.031**	0.039**	0.058**
6.0	0.023	0.035**	0.031**	0.031**	0.037**	0.095**
4.0	0.023	0.020**	0.024**	0.034**	0.057**	0.115**
3.1	0.023	0.017**	0.033**	0.057**	0.122**	0.182
2.3	0.023	0.035	0.074**	0.169**	0.276	0.212
1.5	0.023	0.050	0.101**	0.202*	0.238	0.219
1.0	0.023	0.036	0.130**	0.224	0.213	0.195
0.4	0.023	0.040	0.150	0.283	0.233	0.207
0.07	0.023	0.070	0.179	0.251	0.221	0.204
0.007	0.023	0.054	0.204**	0.285	0.257	0.242
0.0007	0.023	0.060	0.248**	0.304	0.305	0.345
0.0	0.023	0.156	0.180	0.255	0.253	0.235

Confidence limits

* = significant with 95% confidence
** = significant with 99% confidence

All values given represent the mean of 14 values

Table 28

Optical density values at 410nm of Acanthamoeba castellanii cultures treated with barban in microtiter plates. The significance of the difference in optical density values of barban-treated cultures from control cultures are given

	Optical density values						
Concentration ($\mu g ml^{-1}$)	Sampling times (h)						
	0	24	48	72	96	120	144
8.3	0.082	0.082	0.097	0.087	0.107	0.101**	0.101**
6.2	0.082	0.073	-	-	-	0.102**	0.102**
4.1	0.082	0.091	0.090	-	0.094	0.117	0.128**
3.3	0.082	0.071	0.074	0.097	0.094	0.127	0.119**
2.5	0.082	0.066	0.077	0.093	0.094	0.120	0.119**
1.65	0.082	0.062	0.070	0.12	0.091	0.128	0.138
0.83	0.082	0.073	-	0.109	0.109	0.151	0.137
0.41	0.082	0.063	0.074	0.105	0.085**	0.123	0.131**
0.08	0.082	0.062	0.075	0.106	0.095	0.133	0.128**
0.008	0.082	0.079	0.083	0.105	0.109	0.146	0.141
0.0008	0.082	0.068	0.074	0.120	0.100	0.136	0.149
0.0	0.082	0.081	0.088	0.096	0.113	0.143	0.160

Confidence limits

* = significant with 95% confidence
** = significant with 99% confidence

All values given represent the mean of 14 values

No concentration of barban induced stasis, although 4, 6 and 8 μgml^{-1} depressed population growth for 72h after which rapid increases in population size were observed. Population growth of T. pyriformis was unaffected by concentrations between 0.07 and 0.4 μgml^{-1}. At 0.007 and 0.0007 $\mu gm\ l^{-1}$ barban stimulated population growth, significant at 48h only.

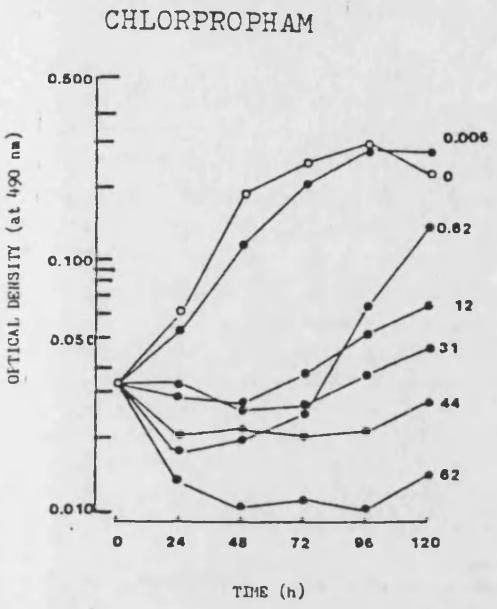
Concentrations between 0.08 and 8.3 μgml^{-1} barban, with the exception of 0.83 and 1.65 μgml^{-1}, significantly reduced optical density values of A. castellanii after 144h. Barban at 6.2 and 8.3 μgml^{-1} significantly inhibited growth at 120h. Barban was not lethal or stimulatory to A. castellanii.

T. pyriformis was more susceptible than A. castellanii to the inhibitory action of barban.

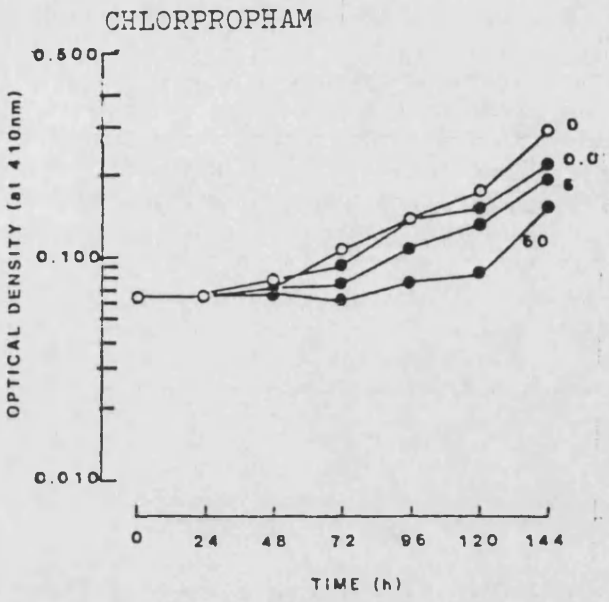
31.4 Chlorpropham

Chlorpropham at 44 μgml^{-1} and above was toxic to T. pyriformis throughout 120h (Table 29) although slight recovery was evident after 96h (Fig. 43). An initial suppression of optical density values in T. pyriformis cultures was exhibited by concentration of chlorpropham at or above 0.62 μgml^{-1}. No similar effect was evident in populations of A. castellanii (Table 30). Cell stasis was induced in T. pyriformis by 31 μgml^{-1} for 72h and by 19 μgml^{-1} for 24h (Table 29). Growth was not inhibited by 0.06 and 0.006 μgml^{-1} except after 48h.

T. PYRIFORMIS (a)



A. CASTELLANII(b)



T. PYRIFORMIS (a)

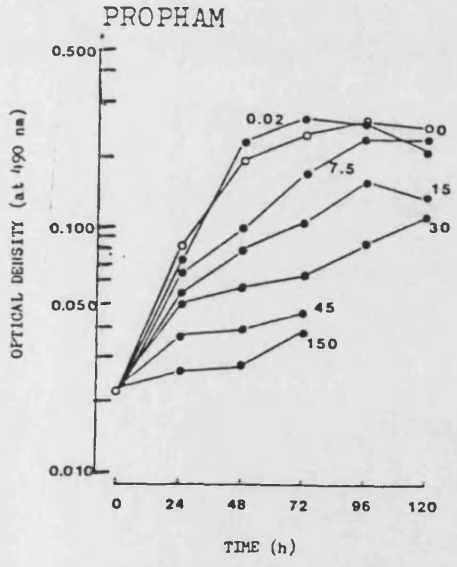


FIG 43.

The sub-acute effects of chlorpropham and propham on the population growth of Tetrahymena pyriformis (a) and Acanthamoeba castellanii (b) in microtiter plates. Numbers on the figures refer to herbicide concentration. To avoid overcrowding in figures not all herbicide treatments are shown.

Table 29

Optical density values at 490nm of Tetrahymena pyriformis cultures treated with chlorpropham in microtiter plates. The significance of the difference in optical density values of chlorpropham-treated cultures from control cultures are given

Concentration ($\mu g ml^{-1}$)	Optical density values Sampling times (h)					
	0	24	48	72	96	120
62	0.033	0.014**	0.011**	0.012**	0.011**	0.015**
44	0.033	0.021**	0.022**	0.021**	0.020**	0.029**
31	0.033	0.033**	0.026**	0.027**	0.036**	0.045**
25	0.033	0.030**	0.034**	0.034**	0.029**	-
19	0.033	0.034**	0.039**	0.040**	0.067**	-
12	0.033	0.029**	0.028**	0.036**	0.051**	0.066**
6.2	0.033	0.022**	0.033**	0.028**	0.056**	-
3.1	0.033	0.019**	0.033**	0.036**	0.050**	-
0.62	0.033	0.018**	0.020**	0.025**	0.062**	0.132*
0.06	0.033	0.048	0.126**	0.219	0.268	0.291
0.006	0.033	0.052	0.111**	0.190	0.258	0.253
0.0	0.033	0.060	0.172	0.227	0.280	0.208

Confidence limits

* = significant with 95% confidence
** = significant with 99% confidence

All values given represent the mean of 14 values

Table 30

Optical density values at 410nm of Acanthamoeba castellanii cultures treated with chlorpropham in microtiter plates. The significance of the difference in optical density values of chlorpropham-treated cultures from control cultures are given

Concentration ($\mu g ml^{-1}$)	Optical density values Sampling times (h)						
	0	24	48	72	96	120	144
60	0.082	0.083	0.083	0.080**	0.088**	0.091**	0.133**
45	0.082	0.082	0.083	0.084**	0.090**	0.099**	0.128**
30	0.082	0.083	0.084	0.083**	0.098**	0.111**	0.145**
24	0.082	0.086	0.069	0.074**	0.086**	0.101**	0.136**
18	0.082	0.080	0.084	0.087**	0.104	0.119*	0.152**
6	0.082	0.082	0.085	0.087**	0.105	0.119	0.152**
3	0.082	0.082	0.088	0.095**	0.113	0.128	0.162
0.6	0.082	0.082	0.086	0.094	0.119	0.127	0.165
0.06	0.082	0.081	0.087	0.095	0.121	0.131	0.164
0	0.082	0.082	0.085	0.104	0.124	0.142	0.198

Confidence limits

* = significant with 95% confidence
** = significant with 99% confidence

All values given represent the mean of 14 values

Concentrations between 24 and 60 ugml^{-1} prolonged the lag-phase, by 24h, in A. castellanii and significantly inhibited growth thereafter. Chlorpropham at 0.6 and 0.06 ugml^{-1} had no effect on A. castellanii whilst concentrations between 3 and 18 µgml^{-1} were inhibitory after 72h (Table 30). No stimulation of growth of either organism was detected with chlorpropham. Representative growth curves for the effect of chlorpropham on A. castellanii are given (Fig. 43). For clarity not all growth curves are given.

31.5 Propham

The growth of T. pyriformis cultures treated with propham was significantly affected by concentrations above 7.5 µgml^{-1} (Table 31). The inhibitory action of propham was dose-related, increasing concentrations progressively inhibiting growth (Fig. 43). At 0.02 and 0.15 µgml^{-1} propham the O.D. values were increased above those of the untreated. The stimulatory action was significant at the 48h sample point only (Table 31).

Propham was markedly less inhibitory to A. castellanii (Table 32). A significant inhibitory action was detected after 144h with concentrations of 75 and 150 µgml^{-1} and after 120h with 112.5 µgml^{-1} propham. There was no significant evidence of a lethal, stimulatory or static effect of propham on populations of A. castellanii (Table 32). The growth curve depicting the effect of propham on A. castellanii are confusing and have therefore been omitted.

Table 31

Optical density values at 490nm of Tetrahymena pyriformis cultures treated with propham in microtiter plates. The significance of the difference in optical density values of propham-treated cultures from control cultures are given

	Optical density values					
Concentration ($\mu g ml^{-1}$)	Sampling times (h)					
	0	24	48	72	96	120
150	0.023	0.028**	0.029**	0.040	-	-
113	0.023	0.030**	0.030**	0.049**	-	-
75	0.023	0.030**	0.030**	0.041**	-	-
60	0.023	0.041**	0.031**	0.042**	-	-
45	0.023	0.038**	0.040**	0.047**	-	-
30	0.023	0.051**	0.059**	0.066**	0.087**	0.112**
15	0.023	0.056**	0.082**	0.108**	0.152**	0.131
7.5	0.023	0.067	0.100**	0.165**	0.228	0.225
1.5	0.023	0.071	0.153	0.255	0.248	0.212
0.15	0.023	0.070	0.217**	0.228	0.233	0.193
0.002	0.023	0.076	0.217**	0.270	0.258	0.203
0.0	0.023	0.085	0.183	0.231	0.261	0.250

Confidence limits

* = significant with 95% confidence
** = significant with 99% confidence

All values given represent the mean of 14 values

Table 32

Optical density values at 410nm of Acanthamoeba castellanii cultures treated with propham in microtiter plates. The significance of the difference in optical density values of propham-treated cultures from control cultures are given

	Optical density values						
Concentration ($\mu g ml^{-1}$)	Sampling times (h)						
	0	24	48	72	96	120	144
150	0.147	0.158	-	0.169	0.152	0.176	0.190**
112.5	0.147	0.132	-	0.139	0.141	0.150**	0.168**
75	0.147	0.133	-	0.149	0.147	0.174	0.197*
60	0.147	0.163	-	0.174	0.178	0.192	0.216
45	0.147	0.158	-	0.168	0.170	0.192	0.231
30	0.147	0.148	-	0.163	0.169	0.184	0.210
15	0.147	0.152	-	0.150	0.170	0.189	0.214
7.5	0.147	0.143	-	0.160	0.162	0.180	0.200
1.5	0.147	0.150	-	0.172	0.194	0.203	0.232
0.15	0.147	0.147	-	0.166	0.186	0.200	0.237
0.015	0.147	0.137	-	0 .183	0.158	0.182	0.223
0.0	0.147	0.147	-	0.173	0.193	0.212	0.254

Confidence limits

* = significant with 95% confidence
** = significant with 99% confidence

All values given represent the mean of 14 values

31.6 Diuron

Population growth of T. pyriformis treated with diuron showed a dose-dependent response (Fig. 42). Increasing concentrations of the chemical become progressively and significantly more inhibitory(Table 33). Concentrations above 24 μgml^{-1} were highly toxic to T. pyriformis cells, decreasing O.D. values for 120h. At 16 μgml^{-1} diuron, induced a lag-phase of 48h. Recovery occurred but the growth rate was greatly reduced compared with the control. Concentrations between 3.2 and 13 μgml^{-1} also reduced the growth rate, inhibition increasing with concentration. Growth of T. pyriformis was not significantly affected by 1.6 μgml^{-1} diuron, but at 0.003, 0.03 and 0.3 μgml^{-1} the herbicide had a significant stimulatory action on O.D. values. The maximum stimulatory effect occurred at 0.03 μgml^{-1}.

With A. castellanii (Fig 42., Table 34) a dose-dependent inhibitory action of diuron on growth was observed. Diuron at concentrations between 1.5 and 3.2 μgml^{-1} significantly inhibited population growth, and higher concentrations progressively depressed growth. Diuron did not depress O.D. values in A. castellanii cultures but 32 μgml^{-1} prolonged the lag-phase of the culture by 24h. Significant increases in O.D. values were observed after 144h with 0.003 μgml^{-1} but 0.3 and 0.03 μgml^{-1} of diuron had no effect on O.D. values. In general, diuron had a

Table 33

Optical density values at 490nm of Tetrahymena pyriformis cultures treated with diuron in microtiter plates. The significance of the difference in optical density values of diuron-treated cultures from control cultures are given

	Optical density values					
Concentration ($\mu g ml^{-1}$)	Sampling times (h)					
	0	24	48	72	96	120
32	0.023	0.005**	0.002**	0.001	0.001**	0.001**
24	0.023	0.012**	0.011**	0.015**	0.016**	0.015**
16	0.023	0.022**	0.024**	0.032**	0.041**	0.041**
13	0.023	0.030**	0.037**	0.047**	0.056**	0.065**
9.5	0.023	0.037**	0.046**	0.055**	0.087**	0.086**
6.3	0.023	0.043*	0.054**	0.076**	0.111**	0.107**
3.2	0.023	0.059	0.080**	0.133**	0.176**	0.173
1.6	0.023	0.069	0.100	0.182	0.194	0.189
0.3	0.023	0.077	0.170**	0.251	0.225	0.204
0.03	0.023	0.080	0.198**	0.272**	0.256	0.220
0.003	0.023	0.082	0.166**	0.244*	0.234	0.215
0.0	0.023	0.072	0.127	0.191	0.220	0.180

Confidence limits

* = significant with 95% confidence
** = significant with 99% confidence

All values given represent the mean of 14 values

Table 34

Optical density values at 410nm of Acanthamoeba castellanii cultures treated with diuron in microtiter plates. The signficiance of the difference in optical density values of diuron-treated cultures from control cultures are given

Concentration ($\mu g ml^{-1}$)	Optical density values Sampling times (h)						
	0	24	48	72	96	120	144
32	0.082	0.077	0.075	0.079**	0.085**	0.094**	0.114**
24	0.082	0.082	0.077	0.089	0.096**	0.107**	0.131**
15	0.082	0.080	0.081	0.083**	0.095**	0.112**	-
13	0.082	0.082	0.082	0.090	0.097**	0.113**	0.145**
9.5	0.082	0.085	0.081	0.087	0.095**	0.109**	0.146*
6.3	0.082	0.080	0.076	0.081**	0.090**	0.104**	0.142**
3.2	0.082	0.077	0.077	0.080**	0.091**	0.103**	0.137**
1.5	0.082	0.084	0.081	0.089	0.100*	0.115**	0.148*
0.3	0.082	0.083	0.083	0.093	0.108	0.123	0.176
0.03	0.082	0.086	0.084	0.100	0.117	0.139	0.178
0.003	0.082	0.092	0.089	0.105	0.125	0.152	0.207*
0.0	0.082	0.077	0.090	0.105	0.121	0.143	0.177

Confidence limits

* = significant with 95% confidence
** = significant with 99% confidence

All values given represent the mean of 14 values

more pronounced effect on T. pyriformis than A. castellanii, for example, the degree of inhibition after 96h with 16 μgml^{-1} was 81% and 22% respectively. However, A. castellanii was more sensitive than T. pyriformis to the inhibitory action of low levels of diuron. Significant inhibition occurred at 1.6 μgml^{-1} with A. castellanii and 3.2 μgml^{-1} with T. pyriformis (Tables 34 and 33).

31.7 Fenuron

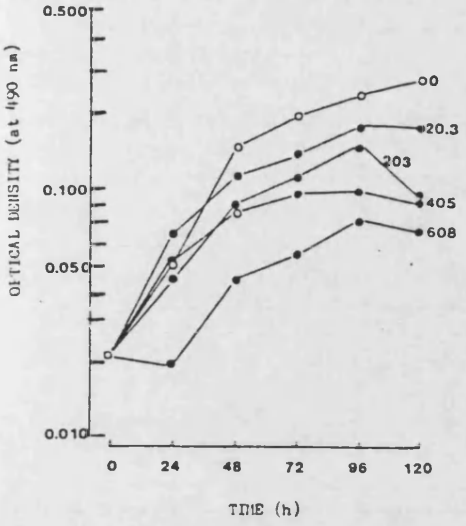
Fenuron had a dose-dependent action on T. pyriformis (Fig. 44). Concentrations between 270 and 2695 μgml^{-1} significantly affected the growth rate of T. pyriformis cultures (Table 35). Fenuron, at 1348 and 1887 μgml^{-1}, initially decreased O.D. values in T. pyriformis cultures. At 2695 μgml^{-1}, fenuron induced a 24h lag-phase which was followed by slight growth before a decrease in O.D. values was seen (Table 35).

In the concentration range 0.27 to 135 μgml^{-1}, fenuron had no effect on T. pyriformis cells. Evidence of a stimulatory action at 2.7 and 27 μg fenuron was not found to be significant (Table 35).

Fenuron was not tested with A. castellanii cultures.

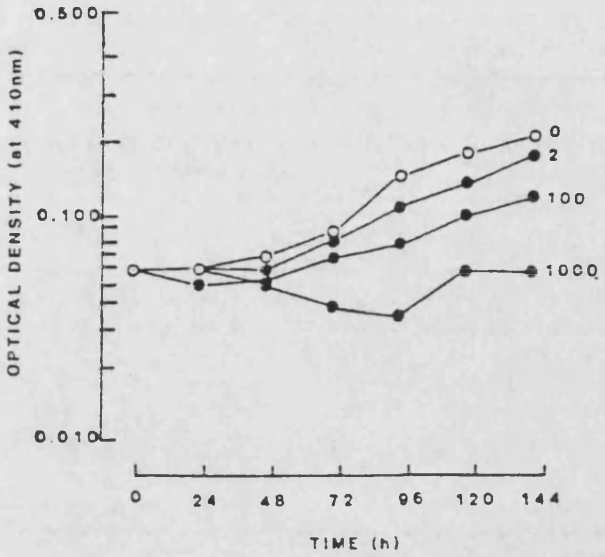
T. PYRIFORMIS (a)

PIRIMICARB



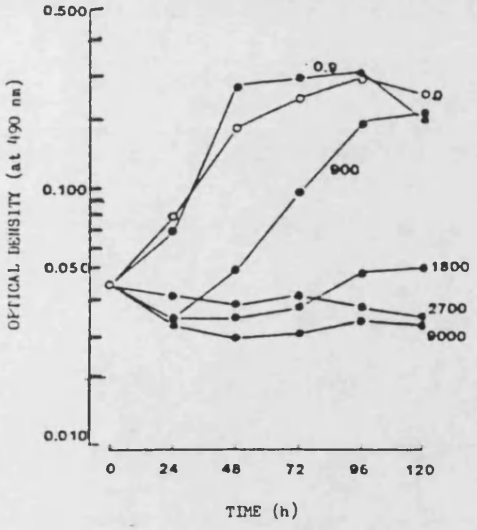
A. CASTELLANII (b)

PIRIMICARB



T. PYRIFORMIS (a)

GLYPHOSATE



T. PYRIFORMIS(a)

FENURON

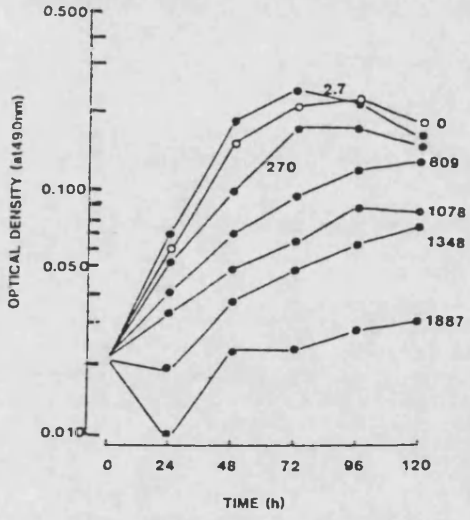


FIG 44

The sub-acute effects of pirimicarb, glyphosate and fenuron on the population growth of Tetrahymena pyriformis (a) and Acanthamoeba castellanii (b) in microtiter plates. Numbers on the figures refer to pesticide concentration. Graphs show only a selection of pesticide concentrations.

Table 35

Optical density values at 490nm of Tetrahymena pyriformis cultures treated with fenuron in microtiter plates. The significance of the difference in optical density values of fenuron-treated cultures from control cultures are given

	Optical density values					
Concentration ($\mu g ml^{-1}$)	Sampling times (h)					
	0	24	48	72	96	120
2695	0.022	0.022**	0.040**	0.041**	0.035**	0.028**
1887	0.022	0.011**	0.024**	0.024**	0.029**	0.032**
1348	0.022	0.020**	0.037**	0.049**	0.062**	0.073
1078	0.022	0.033**	0.049**	0.063**	0.088**	0.083**
809	0.022	0.040**	0.068**	0.094**	0.123**	0.130**
539	0.022	0.048	0.073**	0.112**	0.141**	0.129**
270	0.022	0.054	0.098**	0.177	0.172**	0.150
135	0.022	0.058	0.130	0.215	0.199	0.169
27	0.022	0.071	0.170	0.233	0.247	0.200
2.7	0.022	0.067	0.183	0.250	0.219	0.164
0.27	0.022	0.070	0.182	0.222	0.235	0.198
0.0	0.022	0.059	0.152	0.214	0.231	0.184

Confidence limits

* = significant with 95% confidence
** = significant with 99% confidence

All values given represent the mean of 14 values

31.8 Isoproturon

Significant inhibition of population growth (p=0.01) occurred with 52 μgml^{-1} of isoproturon after 24h and continued for a further 72h. No other concentration within the range 0.005 - 52 μgml^{-1} of the herbicide significantly inhibited *T. pyriformis*. At 0.52 μgml^{-1} isoproturon had a stimulatory effect on *T. pyriformis* after 48h but this was significant only after 72h. Isoproturon did not induce stasis and its inhibitory activity was not dose-dependant over the concentration range tested.

Isoproturon was not tested against *A. castellanii*.

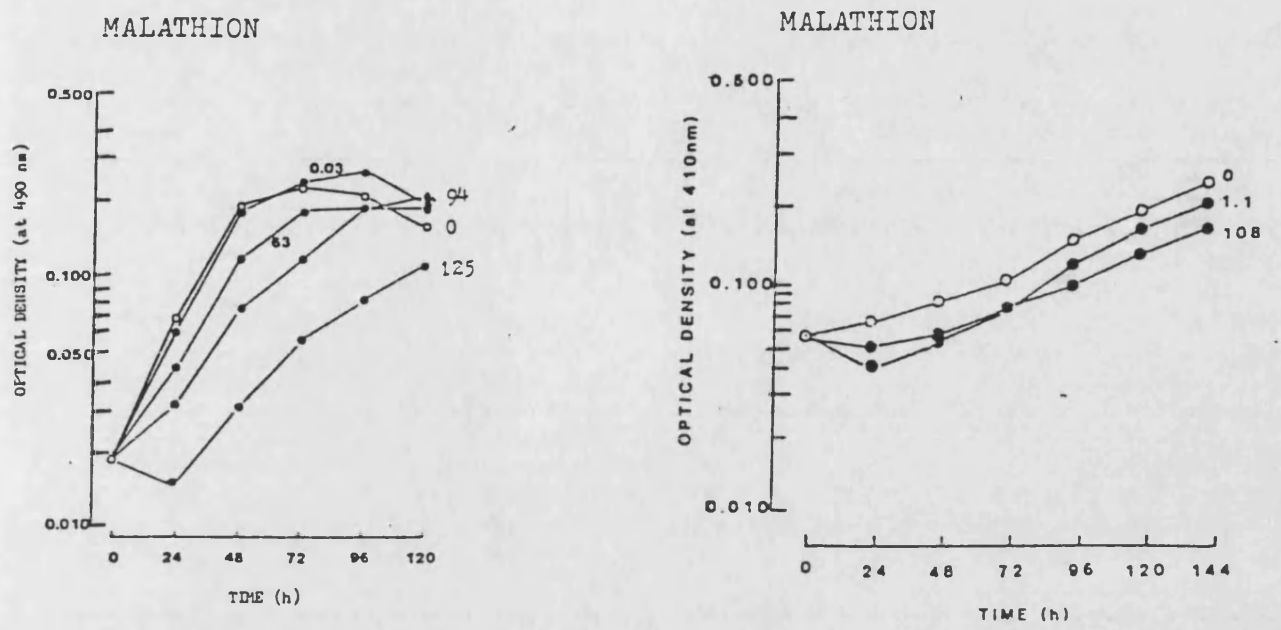
31.9 Linuron

Linuron, like diuron and fenuron, had a dose-dependent inhibitory action on growth of *T. pyriformis* (Fig. 45). Concentrations between 11.2 and 56 μgml^{-1} significantly reduced population numbers over 120h (Table 36). At 5.6 μgml^{-1} linuron was inhibitory between 48 and 96h. Stasis was induced by 56,42 and 22.4 μgml^{-1} for 120h (Table 36). Linuron at 28 μgml^{-1} decreased O.D values in *T. pyriformis* cultures after 72h, having initially prolonged the lag-phase of the culture. This result was not consistent with the dose-dependent action of linuron.

Population growth of *T. pyriformis* was not affected by linuron at 0.56 and 2.8 μgml^{-1} but 0.06 μgml^{-1} linuron did

T. PYRIFORMIS (a)

A. CASTELLANII (b)



T. PYRIFORMIS (a)

A. CASTELLANII (b)

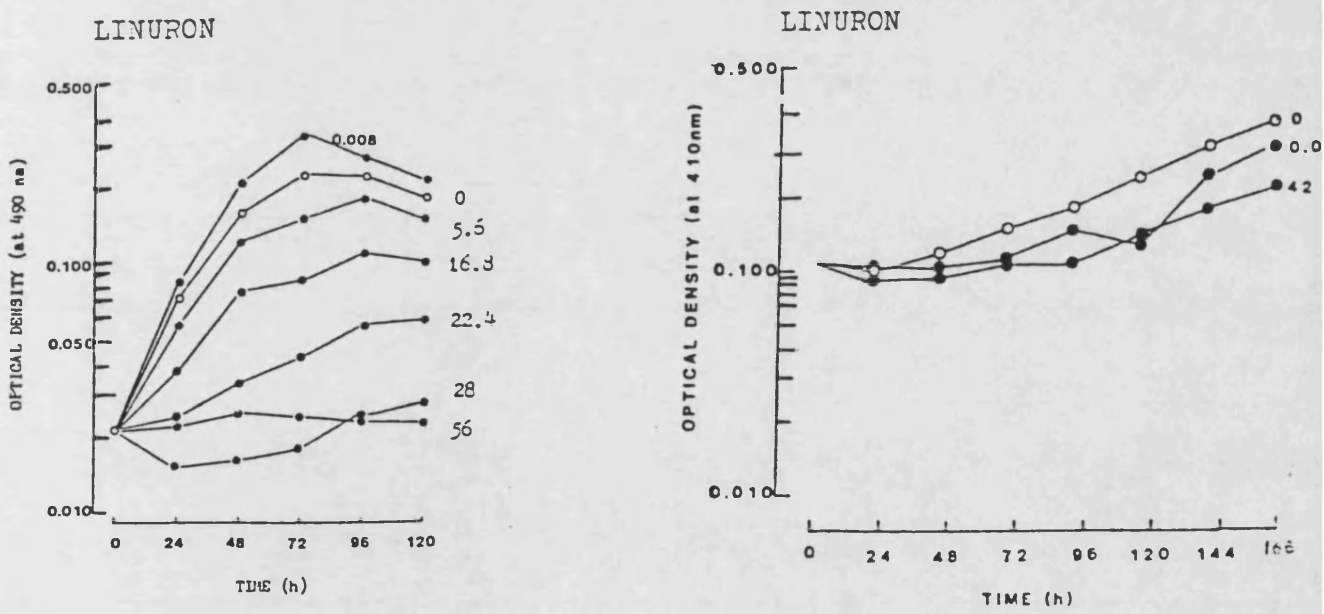


FIG. 45

The sub-acute effects of malathion and linuron on the population growth of Tetrahymena pyriformis (a) and Acanthamoeba castellanii (b) in microtiter plates. Numbers on figures refer to pesticide concentration. Graphs show only a selection of pesticide concentrations.

Table 36

Optical density values at 490nm of Tetrahymena pyriformis cultures treated with linuron in microtiter plates. The significance of the difference in optical density values of linuron-treated cultures from control cultures are given

	Optical density values					
Concentration ($\mu g ml^{-1}$)	Sampling times (h)					
	0	24	48	72	96	120
56	0.022	0.023**	0.026**	0.025**	0.024**	0.024**
42	0.022	0.025**	0.023**	0.026**	0.025**	0.023**
28	0.022	0.022**	0.020**	0.018**	0.013**	0.000**
22.4	0.022	0.016**	0.017**	0.019**	0.025**	0.029**
16.8	0.022	0.025**	0.034**	0.043**	0.058**	0.060**
11.2	0.022	0.038**	0.077**	0.085**	0.110**	0.103**
5.6	0.022	0.057	0.121*	0.148**	0.179**	0.149
2.8	0.022	0.063	0.118	0.195	0.192	0.154
0.56	0.022	0.071	0.156	0.240	0.223	0.190
0.06	0.022	0.068	0.171	0.264*	0.239*	0.204
0.006	0.022	0.084	0.201**	0.320**	0.257**	0.208
0.0	0.022	0.073	0.154	0.220	0.218	0.177

Confidence limits

* = significant with 95% confidence
** = significant with 99% confidence

All values given represent the mean of 14 values

Table 37

Optical density values at 410nm of Acanthamoeba castellanii cultures treated with linuron in microtiter plates. The significance of the difference in optical density values of linuron-treated cultures from control cultures are given

Concentration ($\mu g ml^{-1}$)	Optical density values Sampling times (h)							
	0	24	48	72	96	120	144	168
56	0.108	0.106	0.117	0.115	0.113**	0.133**	0.148**	0.170**
42	0.108	0.094	0.095	0.102**	0.101**	0.120**	0.138**	0.155**
28	0.108	0.100	0.099	0.108	0.117*	0.137**	0.158**	0.173**
22.4	0.108	0.094	0.089	0.097**	0.106**	0.125**	0.149**	0.163**
16.8	0.108	0.110	0.100	0.110	0.123	0.143**	0.165**	0.180**
11.2	0.108	0.92	0.077	0.091**	0.104**	0.126**	0.151**	0.167**
5.6	0.108	0.112	0.103	0.114	0.130	0.152	0.181	0.195**
2.8	0.108	0.115	0.108	0.119	0.134	0.159	0.193	0.218
0.56	0.108	0.104	0.101	0.111	0.127	0.151	0.185	0.215
0.06	0.108	0.103	0.102	0.106	0.127	0.115**	0.169**	0.197**
0.006	0.108	-	-	-	0.095**	0.117**	0.147**	0.176**
0.0	0.108	0.100	0.108	0.124	0.139	0.165	0.197	0.226

Confidence limits

* = significant with 95% confidence
** = significant with 99% confidence

All values given represent the mean of 14 values

increase population O.D. values after 72h and 0.006 μgml^{-1} linuron after 48h. The stimulatory action persisted for 24 and 48h, respectively.

Linuron did not reduce or increase O.D. values in A. castellanii cultures (Table 37). Concentrations between 11.2 and 56 μgml^{-1} depressed the population growth of the amoeba and caused an extension of the lag-phase of the culture by 24h.

No effect on population size was detected with linuron at 2.8 and 0.56 μgml^{-1} and with 56 μgml^{-1} a significant inhibitory effect was seen only after 168h treatment.

Low concentrations of the urea herbicides in general had little inhibitory action on A. castellanii although uncharacteristically linuron was inhibitory at 0.006 and 0.06 μgml^{-1}. Effects of some concentrations of linuron on the population growth of A. castellanii are shown (Fig. 45).

T. pyriformis was more susceptible than A. castellanii to the full range of linuron concentrations (both stimulatory and inhibitory).

31.10 Malathion

Concentrations between 38 and 125 μgml^{-1} of malathion inhibited the population growth of T. pyriformis (Table 38). The inhibitory effect increased with higher

Table 38

Optical density values at 490nm of Tetrahymena pyriformis cultures treated with malathion in microtiter plates. The significance of the difference in optical density values of malathion-treated cultures from control cultures are given

Concentration ($\mu g ml^{-1}$)	Optical density values Sampling times (h)					
	0	24	48	72	96	120
125	0.020	0.016**	0.032**	0.057**	0.081**	0.113
94	0.020	0.034**	0.075**	0.116	0.181	0.202
63	0.020	0.045**	0.116**	0.181	0.184	0.186
50	0.020	0.052**	0.135	0.214	0.196	0.187
38	0.020	0.051*	0.161	0.217	0.211	0.197
25	0.020	0.056	0.157	0.226	0.232	0.207
13	0.020	0.056	0.183	0.226	0.231	0.188
6.3	0.020	0.077	0.155	0.170	0.159	0.131
1.25	0.020	0.067	0.188	0.226	0.237	0.209
0.13	0.020	0.060	0.179	0.222	0.252	0.192
0.01	0.020	0.053	0.182	0.208	0.217	0.167
0.0	0.020	0.069	0.184	0.216	0.206	0.155

Confidence limits

* = significant with 95% confidence
** = significant with 99% confidence

All values given represent the mean of 14 values

Table 39

Optical density values at 410nm of Acanthamoeba castellanii cultures treated with malathion in microtiter plates. The significance of the difference in optical density values of malathion-treated cultures from control cultures are given

Concentration ($\mu g ml^{-1}$)	Optical density values Sampling times (h)						
	0	24	48	72	96	120	144
108	0.075	0.071	0.077	0.087	0.099**	0.118*	0.136**
81	0.075	0.088	0.085	0.095	0.106**	0.123**	0.142**
54	0.075	0.069	0.078	0.087	0.104**	0.122**	0.145**
43.2	0.075	0.061**	0.071*	0.076**	0.098**	0.119**	0.142**
32.4	0.075	0.061**	0.070*	0.078**	0.101**	0.122**	0.145**
10.8	0.075	0.071	0.081	0.100	0.112	0.135	0.158
5.4	0.075	0.073	0.080	0.079**	0.112	0.131*	0.152*
1.1	0.075	0.062*	0.074	0.082**	0.105**	0.128**	0.149**
0.1	0.075	0.056**	0.064**	0.074**	0.098**	0.122**	0.141**
0.01	0.075	0.064	0.074	0.086	0.110	0.135	0.155
0.0	0.075	0.081	0.091	0.104	0.127	0.149	0.172

Confidence limits

* = significant with 95% confidence
** = significant with 99% confidence

All values given represent the mean of 14 values

insecticide concentrations and it persisted for 24h with 38 and 50 μgml^{-1}, 48h with 63 and 94 μgml^{-1} and 96h with 125 μgml^{-1} (Table 38). At 125 μgml^{-1} malathion was initially toxic but recovery was evident after 24h (Fig. 45).

A. castellanii was significantly inhibited by concentrations of malathion ranging from 0.1 to 108 μgml^{-1} (Table 39). Throughout this range some concentrations caused an initial decline in O.D. values, for example 1.1 μgml^{-1} (Fig. 45). A. castellanii was more sensitive to the inhibitory action of malathion than T. pyriformis. Significant inhibitory effects on population growth in A. castellanii were detected at 0.1 μgml^{-1} whereas in T. pyriformis cultures the lower limit for significant inhibitory effects was 38 μgml^{-1}.

31.11 Pirimicarb

Pirimicarb was inhibitory to T. pyriformis growth after 72h at a concentration range of 0.2 to 608 μgml^{-1} (Table 40). At 608 μgml^{-1} pirimicarb caused a lag-phase of 24h (Fig. 44). No concentration of pirimicarb caused a decline in O.D. values in T. pyriformis cultures. Only 405 and 608 μgml^{-1} pirimicarb significantly depressed population growth at 48h, all other concentrations significantly inhibited cultures at 72 and 96h (Table 40). No significant stimulation of T. pyriformis occurred with pirimicarb, although at 2.0 and 20.3 μgml^{-1} the

Table 40

Optical density values at 490nm of Tetrahymena pyriformis cultures treated with pirimicarb in microtiter plates. The significance of the difference in optical density values of pirimicarb-treated cultures from control cultures are given

	Optical density values					
Concentration ($\mu g ml^{-1}$)	Sampling times (h)					
	0	24	48	72	96	120
608	0.023	0.021	0.045**	0.057**	0.078**	0.071**
405	0.023	0.054	0.083**	0.099**	0.103**	0.090**
203	0.023	0.045	0.088	0.115**	0.156**	0.096**
101	0.023	0.066	0.084	0.097**	0.157	0.134**
20.3	0.023	0.068	0.114	0.141**	0.182	0.180**
2.0	0.023	0.068	0.105	0.169**	0.186	0.178**
0.2	0.023	0.062	0.115	0.167**	0.219	0.179**
0.0	0.023	0.052	0.149	0.194	0.239	0.276

Confidence limits

* = significant with 95% confidence
** = significant with 99% confidence

All values given represent the mean of 14 values

Table 41

Optical density values at 410nm of Acanthamoeba castellanii cultures treated with pirimicarb in microtiter plates. The significance of the difference in optical density values of pirimicarb-treated cultures from control cultures are given

Concentration ($\mu g ml^{-1}$)	Optical density values Sampling times (h)						
	0	24	48	72	96	120	144
2000	0.075	0.067	0.073	0.061**	0.072**	0.071**	0.074**
1500	0.075	0.069	0.065	0.065**	0.068**	0.071**	0.073**
1000	0.075	0.074	0.069	0.062**	0.059**	0.075**	0.076**
800	0.075	0.102**	0.093	0.099	0.103**	0.098**	0.105**
600	0.075	0.073	0.079	0.069**	0.075**	0.082**	0.081**
400	0.075	0.078	0.087	0.055**	0.078**	0.097**	0.095**
200	0.075	0.079	0.090	0.084	0.091**	0.102**	0.118*
100	0.075	0.070	0.072	0.082	0.087**	0.106**	0.117**
2	0.075	0.076	0.076	0.089	0.108	0.122	0.141
0.2	0.075	0.083	0.85	0.102	0.131	0.147	0.171
0.0	0.075	0.074	0.080	0.092	0.126	0.144	0.157

Confidence limits

* = significant with 95% confidence
** = significant with 99% confidence

All values given represent the mean of 14 values

optical density values at 24h were 30% greater than for the untreated cultures.

Growth of A. castellanii was significantly inhibited after 96h by pirimicarb concentrations ranging from 100-2000 μgml^{-1} (Table 41). In each case depression of population growth persisted for a further 48h. Concentrations of between 400 and 2000 μgml^{-1} were inhibitory to A. castellanii after 72h. An uncharacteristic stimulatory effect (significant at p=0.01) occurred after 24h with 800 μgml^{-1} pirimicarb. This was not in unity with adjacent values.

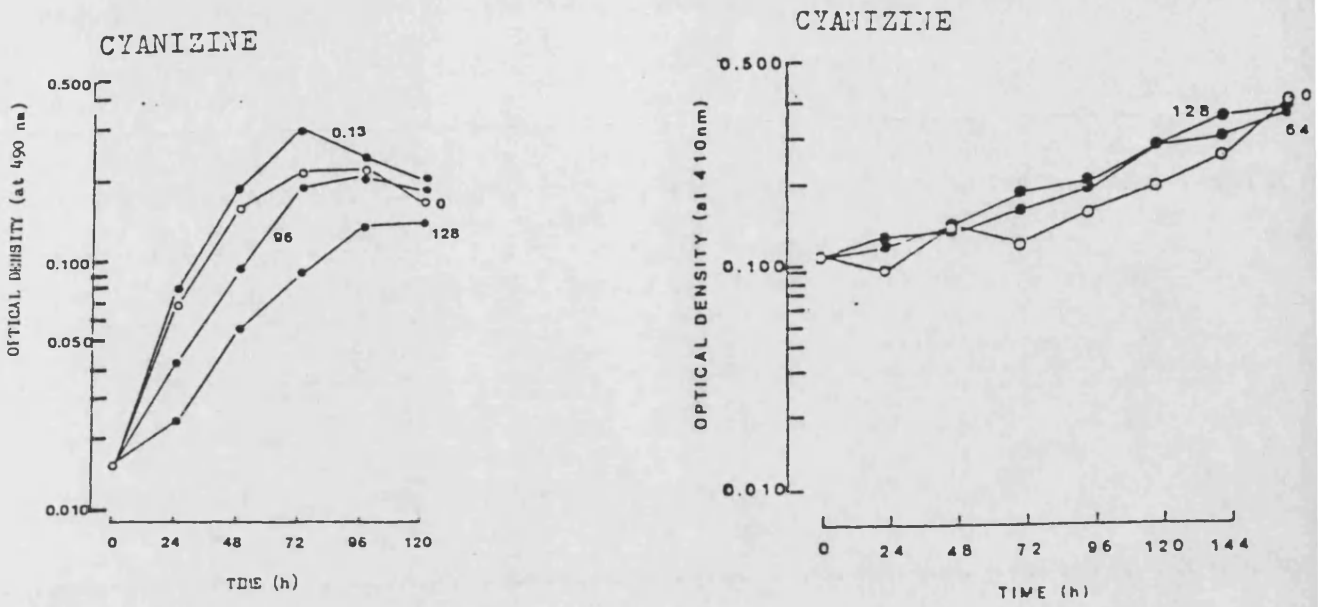
A prolonged lag-phase in the culture (72h) was observed with 400 and 600 μgml^{-1} whilst 1000, 1500 and 2000 μgml^{-1} prevented increases in O.D. values for 144h (Table 41). Pirimicarb at 0.2 and 2.0 μgml^{-1} had no significant effect on A. castellanii and no concentration of pirimicarb was stimulatory to A. castellanii (Fig. 44).

31.12 Cyanazine

Cyanazine did not decrease O.D. values in either T. pyriformis or A. castellanii cultures (Fig. 46). Concentrations of 64, 96 and 128 μgml^{-1} significantly depressed the population growth of T. pyriformis (p=0.01) for 24, 48 and 96h, respectively. No other concentration of cyanazine (0.01 - 51.2 μgml^{-1}), except 0.13 μgml^{-1}

T. PYRIFORMIS (a)

A. CASTELLANII (b)



T. PYRIFORMIS (a)

A. CASTELLANII (b)

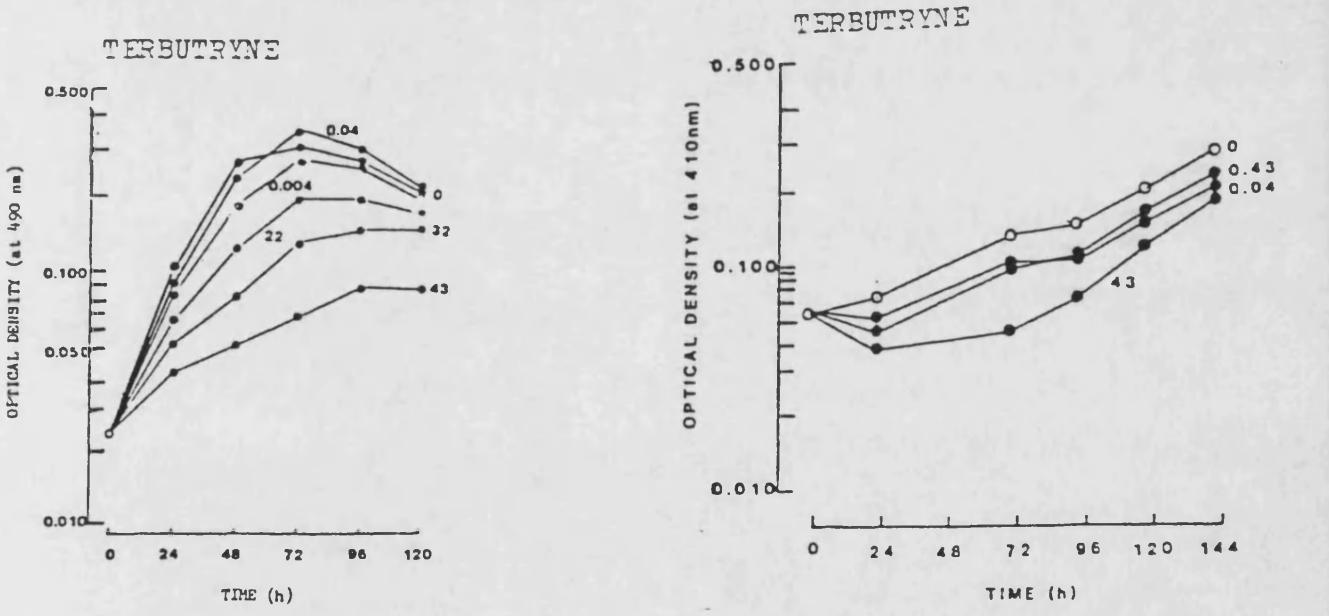


FIG. 46

The sub-acute effects of cyanzine and terbutryne on the population growth of Tetrahymena pyriformis (a) and Acanthamoeba castellanii (b) in microtiter plates. Numbers on figures refer to herbicide concentrations. Graphs show only a selection of pesticide concentrations.

which was stimulatory, had an effect on T. pyriformis, the data is therefore not presented.

Cyanazine stimulated the growth of A. castellanii. The majority of cyanazine-treated cultures (0.13 to 128 μgml^{-1}) had optical density values greater than those of the untreated, although this action was only significant ($p=0.01$) with 6.4 and 128 μgml^{-1}. This data is therefore not presented.

31.13 Terbutryne

Terbutryne and did not prevent growth of T. pyriformis populations, but increasing concentrations became progressively more inhibitory (Fig. 46). At 32.3 and 43 μgml^{-1}m significant inhibition of population growth occurred for 96 and 120h respectively (Table 42). Concentrations of 12.9, 17.2 and 21.5 also depressed growth but were significant at the 48h sample point only. Stimulation of population growth occurred with 2.15, 0.04 and 0.004 μgml^{-1}.

Terbutryne was inhibitory to A. castellanii at all the concentrations evaluated (Table 43). Population growth was significantly inhibited after 72h with concentrations from 2.15 to 43 μgml^{-1} and after 96h with 0.04 and 0.43 μgml^{-1} also. Initial toxicity was evident with all treatments (Table 43) except 0.004 μgml^{-1}, but was

Table 42

Optical density values at 490nm of Tetrahymena pyriformis cultures treated with terbutryne in microtiter plates. The significance of the difference in optical density values of terbutryne-treated cultures from control cultures are given

Concentration ($\mu g ml^{-1}$)	Optical density values Sampling times (h)					
	0	24	48	72	96	120
43	0.024	0.040**	0.051**	0.066**	0.085**	0.083**
32.3	0.024	0.053**	0.078**	0.126**	0.140**	0.141
21.5	0.024	0.064	0.120**	0.189	0.186	0.163
17.2	0.024	0.075	0.132**	0.191	0.184	0.155
12.9	0.024	0.080	0.145**	0.196	0.185	0.148
8.6	0.024	0.084	0.161	0.219	0.196	0.153
4.3	0.024	0.087	0.187	0.257	0.197	0.137
2.15	0.024	0.098*	0.188	0.251	0.218	0.161
0.43	0.024	0.090	0.191	0.278	0.232	0.174
0.04	0.024	0.288	0.223**	0.346	0.288	0.201
0.004	0.024	0.100**	0.258**	0.291	0.255	0.297
0.0	0.024	0.080	0.177	0.260	0.241	0.182

Confidence limits

* = significant with 95% confidence
** = significant with 99% confidence

All values given represent the mean of 14 values

Table 43

Optical density values at 410nm of Acanthamoeba castellanii cultures treated with terbutryne in microtitre plates. The significance of the difference in optical density values of terbutryne-treated cultures from control cultures are given

Concentration ($\mu g ml^{-1}$)	Optical density values Sampling times (h)						
	0	24	48	72	96	120	144
43	0.082	0.066	-	0.074**	0.090**	0.117**	0.152**
32.3	0.082	0.078	-	0.090**	0.104**	0.124**	0.158**
21.5	0.082	0.088	-	0.087**	0.097**	0.117**	0.150**
17.2	0.082	0.072	-	0.089**	0.108**	0.126**	0.157**
12.9	0.082	0.068	-	0.085**	0.095**	0.118**	0.159**
8.6	0.082	0.055**	-	0.093**	0.114**	0.132**	0.177
4.3	0.082	0.065*	-	0.081**	0.095**	0.122**	0.153**
2.15	0.082	0.067	-	0.092**	0.114**	0.138**	0.174*
0.43	0.082	0.073	-	0.103	0.113**	0.142*	0.177
0.04	0.082	0.79	-	0.107	0.108**	0.135**	0.163**
0.004	0.082	0.97	-	0.090**	0.123	0.134**	0.173**
0.0	0.082	0.87	-	0.127	0.134	0.164	0.200

Confidence limits

* = significant with 95% confidence
** = significant with 99% confidence

All values given represent the mean of 14 values

significant with only 4.3 and 8.6 μgml^{-1} of terbutryne. No concentration of terbutryne stimulated A. castellanii population growth.

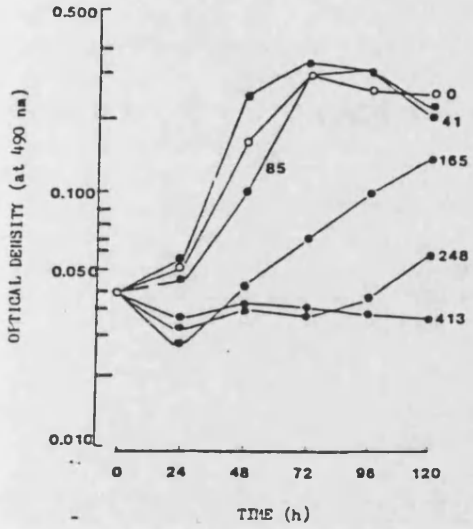
A. castellanii was more sensitive than T. pyriformis to the inhibitory action of terbutryne, significant inhibition occurred with 0.004 μgml^{-1} with A. castellanii and 12.9 μgml^{-1} with T. pyriformis. However, the ciliate was more sensitive to the stimulatory influence of terbutryne.

31.14 Carbaryl

The insecticide carbaryl inhibited population growth of T. pyriformis at concentrations between 9 and 30 μgml^{-1} (Table 44). At 225, 15 and 30 μgml^{-1} carbaryl caused significant inhibition after 24h but only persisted for 24, 72 and 96h respectively. The inhibitory concentrations of 9 and 12 μgml^{-1} were significant only at the 96h sample point. T. pyriformis was not significantly affected by 0.3 to 6 μgml^{-1} of carbaryl but population growth was stimulated by 0.003 and 0.03 μgml^{-1} after 48h (significant at p=0.01). Carbaryl did not prevent O.D. values from increasing with time in T. pyriformis cultures (Fig. 47). No significant effect of carbaryl was observed with A. castellanii.

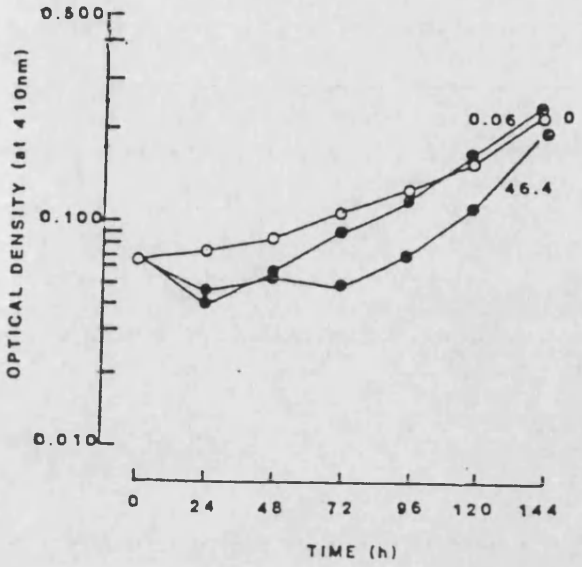
T. PYRIFORMIS (a)

MCPA



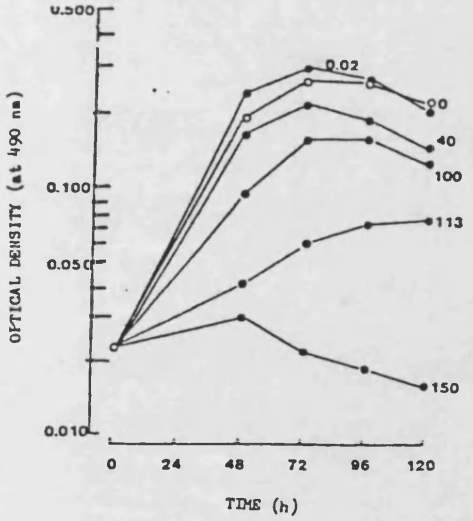
A. CASTELLANII (b)

MCPA



T. PYRIFORMIS (a)

ETHIRIMOL



T. PYRIFORMIS (a)

CARBARYL

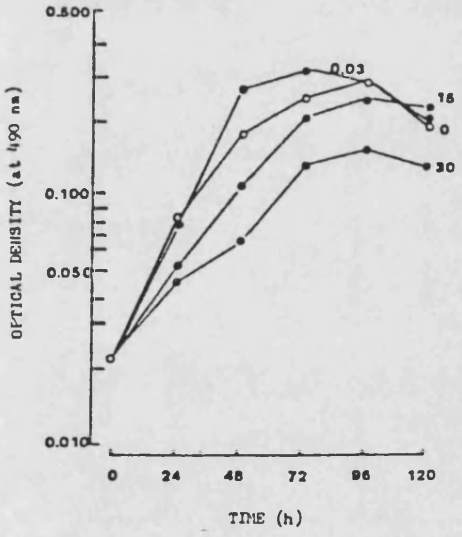


FIG. 47

The sub-acute effects of MCPA, ethirimol and carbaryl on the population growth of Tetrahymena pyriformis (a) and Acanthamoeba castellanii (b) in microtiter plates. Numbers on figures refer to pesticide concentration. Graphs show only a selection of pesticide concentrations.

Table 44

Optical density values at 490nm of Tetrahymena pyriformis cultures treated with carbaryl in microtiter plates. The significance of the difference in optical density values of carbaryl-treated cultures from control cultures are given

	Optical density values					
Concentration ($\mu g ml^{-1}$)	Sampling times (h)					
	0	24	48	72	96	120
30	0.023	0.045**	0.065**	0.128**	0.148**	0.128**
22.5	0.023	0.041**	0.076**	0.149**	0.188**	0.196
15	0.023	0.052**	0.106**	0.193	0.231	0.215
12	0.023	0.062	0.128	0.234	0.219**	0.189
9	0.023	0.060	0.123	0.215	0.223**	0.194
6	0.023	0.080	0.179	0.251	0.237	0.195
3	0.023	0.077	0.224	0.275	0.256	0.183
1.5	0.023	0.069	0.180	0.230	0.229	0.184
0.3	0.023	0.064	0.199	0.262	0.240	0.177
0.03	0.023	0.076	0.248**	0.299	0.268	0.188
0.003	0.023	0.169	0.207**	0.261	0.246	0.185
0.0	0.023	0.078	0.166	0.227	0.269	0.178

Confidence limits

* = significant with 95% confidence
** = significant with 99% confidence

All values given represent the mean of 14 values

No significant effect of carbaryl was observed with A. castellanii.

31.15 Ethirimol

Ethirimol stimulated population growth of T. pyriformis at 0.02, 0.15 and 1.5 μgml^{-1} but was significant only at 48h. Concentration of 0.15 and 1.5 μgml^{-1} had the distinction of being both initially stimulatory and subsequently inhibitory T. pyriformis growth. Significance for the inhibitory action of 0.15 μgml^{-1} was not upheld (Table 45).

At 150 μgml^{-1} the fungicide was toxic to T. pyriformis after 48h (Fig. 47). Concentrations between 40 μgml^{-1} and 112.5 μgml^{-1} caused significant inhibition of population growth after 48h whilst concentrations between 7.5 and 15 μgml^{-1} were inhibitory (significant at $p=0.01$) at the 120h sample time only (Table 45).

Ethirimol had little effect on A. castellanii except to inhibit population growth by 14-18% with 112.5 and 150 μgml^{-1}. This inhibition was significant ($p=0.05$) at 144 and 168h respectively. No other concentrations affected A. castellanii.

Table 45

Optical density values at 490nm of Tetrahymena pyriformis cultures treated with ethirimol in microtiter plates. The significance of the difference in optical density values of ethirimol-treated cultures from control cultures are given

Concentration ($\mu g ml^{-1}$)	Optical density values Sampling times (h)					
	0	24	48	72	96	120
150	0.024	-	0.032**	0.023**	0.020**	0.017**
112.5	0.024	-	0.042**	0.061**	0.073**	0.076**
75	0.024	-	0.095**	0.155**	0.156**	0.123**
60	0.024	-	0.120**	0.165**	0.154**	0.120**
40	0.024	-	0.137**	0.182**	0.162**	0.124**
30	0.024	-	0.164	0.212	0.183**	0.142**
15	0.024	-	0.192	0.186	0.182	0.140**
7.5	0.024	-	0.204	0.202	0.192	0.111**
1.5	0.024	-	0.236**	0.254	0.234	0.172**
0.15	0.024	-	0.255**	0.268	0.250	0.179
0.02	0.024	-	0.237*	0.293	0.266	0.204
0.0	0.024	-	0.199	0.265	0.258	0.214

Confidence limits

* = significant with 95% confidence
** = significant with 99% confidence

All values given represent the mean of 14 values

31.16 MCPA

High levels (330-825 μgml^{-1}) MCPA prevented growth of *T. pyriformis*. At 248 μgml^{-1} MCPA an extended lag-phase occurred (96h) and at 165 μgml^{-1}, population growth was significantly inhibited (Table 46).

The population growth of *A. castellanii* was significantly inhibited ($p=0.01$) after 72h with 46.6 μgml^{-1} MCPA. This concentration also extended the lag-phase of *A. castellanii* cells by 24h (Fig. 47). No other pesticide level caused significant inhibitory effects on *A. castellanii* and MCPA was not stimulatory to *A. castellanii* or *T. pyriformis*.

31.17 Glyphosate

High levels (1800 - 9000 μgml^{-1}) glyphosate prevented growth of *T. pyriformis* (Fig. 44). Some concentrations (eg 6750 and 9000 μgml^{-1}) were initially lethal (Table 47).

The growth of *T. pyriformis* was significantly depressed by 900 μgml^{-1} although there was recovery from the initial toxic action of this dose. Some stimulation of the population growth occurred with 90 μgml^{-1} glyphosate.

Glyphosate was not tested against *A. castellanii*.

Table 46

Optical density values at 490nm of Tetrahymena pyriformis cultures treated with MCPA in microtiter plates. The significance of the difference in optical density values of MCPA-treated cultures from control cultures are given

	Optical density values					
Concentration (μgml^{-1})	Sampling times (h)					
	0	24	48	72	96	120
825	0.040	0.039	0.041**	0.044**	0.040**	0.035**
619	0.040	0.041	0.056**	0.068**	0.068**	0.057**
413	0.040	0.032	0.037**	0.036**	0.034**	0.033**
330	0.040	0.028	0.036**	0.036**	0.034**	0.030**
248	0.040	0.029	0.035**	0.033**	0.039**	0.059**
165	0.040	0.025**	0.043**	0.063**	0.099**	0.137**
83	0.040	0.050	0.154	0.272	0.295	0.204
41	0.040	0.054	0.231	0.314	0.293	0.193
8	0.040	0.045	0.177	0.281	0.246	0.208
0.8	0.040	0.060	0.205	0.309	0.267	0.232
0.08	0.040	0.046	0.175	0.286	0.259	0.214
0	0.040	0.044	0.191	0.280	0.247	0.239

Confidence limits

* = significant with 95% confidence
** = significant with 99% confidence

All values given represent the mean of 14 values

Table 47

Optical density values at 490nm of Tetrahymena pyriformis cultures treated with glyphosate in microtiter plates. The significance of the difference in optical density values of glyphosate-treated cultures from control cultures are given

Concentration ($\mu g ml^{-1}$)	Optical density values Sampling times (h)					
	0	24	48	72	96	120
9000	0.043	0.030**	0.027**	0.028**	0.031**	0.030**
6750	0.043	0.030**	0.024**	0.032**	0.029**	0.026**
4500	0.043	0.039**	0.034**	0.029**	0.033**	0.033**
3600	0.043	0.035**	0.034**	0.037**	0.039**	0.033**
2700	0.043	0.039**	0.036**	0.039**	0.035**	0.032**
1800	0.043	0.032**	0.032**	0.035**	0.049**	0.050**
900	0.043	0.032**	0.049**	0.098**	0.182**	0.198
450	0.043	0.059	0.125**	0.222	0.244	0.239
90	0.043	0.063	0.200	0.231	0.256	0.191
9	0.043	0.059	0.189	0.296	0.308	0.210
0.9	0.043	0.069	0.252**	0.269	0.286	0.188
0.08	0.040	0.046	0.175	0.286	0.259	0.214
0	0.043	0.077	0.172	0.228	0.276	0.234

Confidence limits

* = significant with 95% confidence
** = significant with 99% confidence

All values given represent the mean of 14 values

31.18 Permethrin

Permethrin had no significant effect on T. pyriformis and was not tested against A. castellanii.

32.0 Comparison of the sub-acute toxicity of some pesticides to Tetrahymena pyriformis and Acanthamoeba castellanii

Dose-response curves were produced from the growth curve data presented in Section 31. these responses were measured at 72h and 120h for T. pyriformis and A. castellanii respectively. These times were selected in order to ensure high population densities.

32.1 The characteristics of dose-response curves

32.1.1 Tetrahymena pyriformis (72h)

Increasing concentrations of pesticides created dose-response curves which in most cases were typically sigmoidal in shape for the majority of chemicals evaluated with T. pyriformis (Fig. 48a-52a).

The dose-response curves comprised three sections. The initial part of the curve was generally shallow as a

result of increasing pesticide concentrations having little inhibitory effect. With malathion (Fig. 50a) this part of the curve was almost flat but with other compounds eg diuron, carbaryl and terbutryne (Fig. 49a, 50a and 52a) the curve was steeper. The severity of the curve was a result of the compounds, decreasing stimulatory activity and their progressive (low dose) inhibitory action (Tables 33, 44,42).

The middle part of the curve was the steepest. Generally, small increases in concentration produced large differences in inhibitory activity. With pirimicarb (Fig. 50a) initial and middle section were indistinguishable, but with the majority of chemicals evaluated the middle section was clearly evident eg barban and propham (Fig. 48a).

The final section was a tail or shallow curve at the nadir of a compound's inhibitory action. Further increases in pesticide concentration produced little (eg chlorporpham Fig. 48a) or no further increases (eg glyphosate Fig. 52a). This section was not always present (eg carbaryl and malathion Fig. 50a).

32.1.2 Acanthamoeba castellanii (120h)

The response of A. castellanii to increasing concentrations of most pesticides evaluated was less

Comparison of the sub-acute toxicity of some pesticides to Tetrahymena pyriformis (a) and Acanthamoeba castellanii (b) in microtiter plates.

FIG. 48

The dose-response curves of barban , chlorpropham and propham.

FIG. 49

The dose-response curves of diuron , fenuron , isoproturon and linuron.

FIG. 50

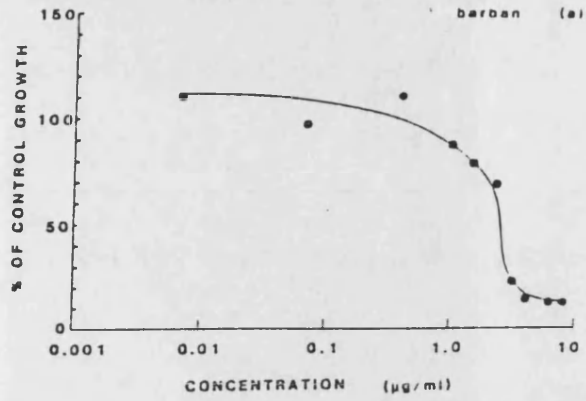
The dose-response curves of carbaryl , malathion and pirimicarb.

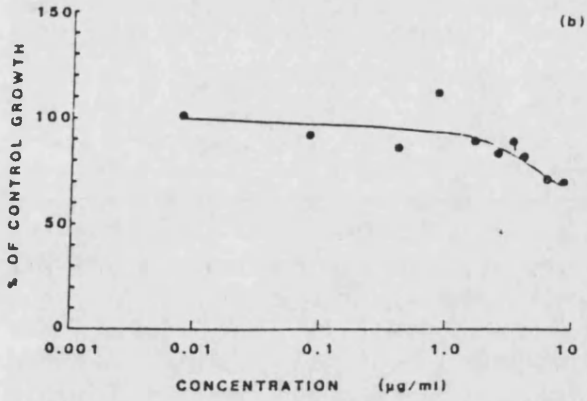
FIG. 51

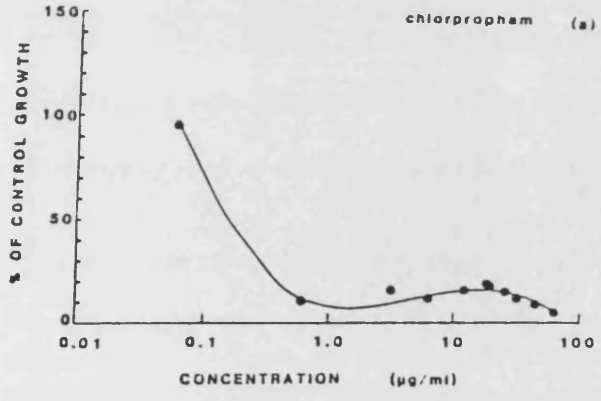
The dose-response curves of MCPA , ethirimol and cyanazine.

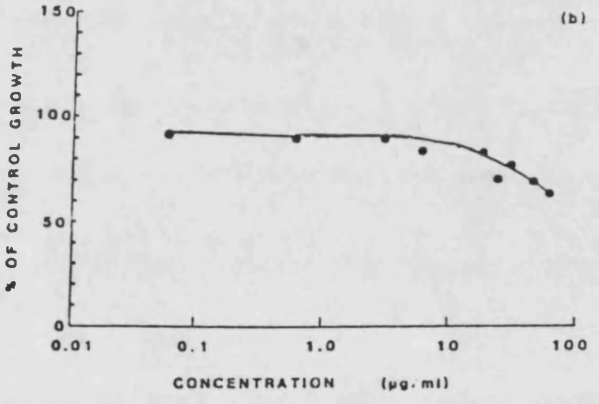
FIG. 52

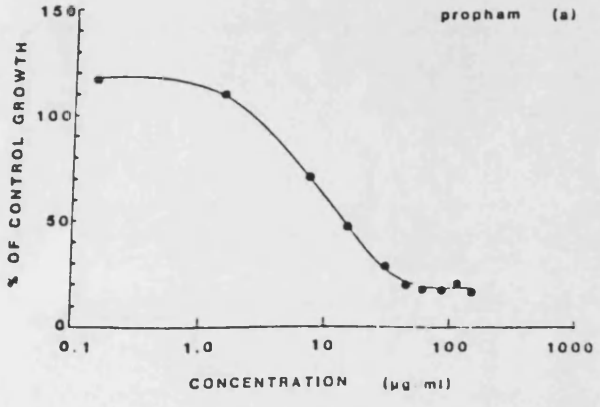
The dose-response curves of terbutryne , permethrin and glyphosate.


barban (a)
% OF CONTROL GROWTH
150
100
50
0
0.001
0.01
0.1
1.0
10
CONCENTRATION (µg/ml)


(b)
% OF CONTROL GROWTH
150
100
50
0
0.001
0.01
0.1
1.0
10
CONCENTRATION (µg/ml)


chlorpropham (a)
% OF CONTROL GROWTH
150
100
50
0
0.01
0.1
1.0
10
100
CONCENTRATION (µg/ml)


(b)
% OF CONTROL GROWTH
150
100
50
0
0.01
0.1
1.0
10
100
CONCENTRATION (µg/ml)


propham (a)
% OF CONTROL GROWTH
150
100
50
0
0.1
1.0
10
100
1000
CONCENTRATION (µg/ml)

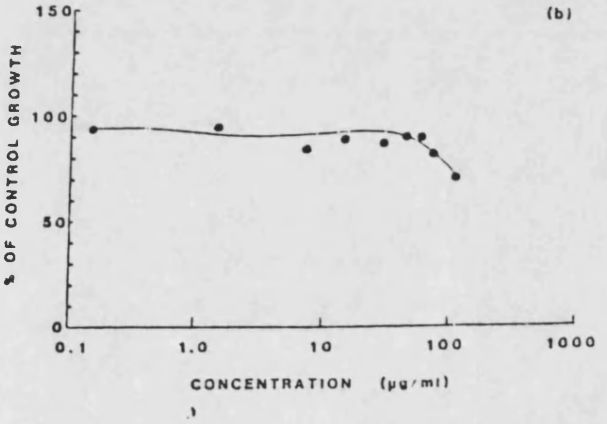
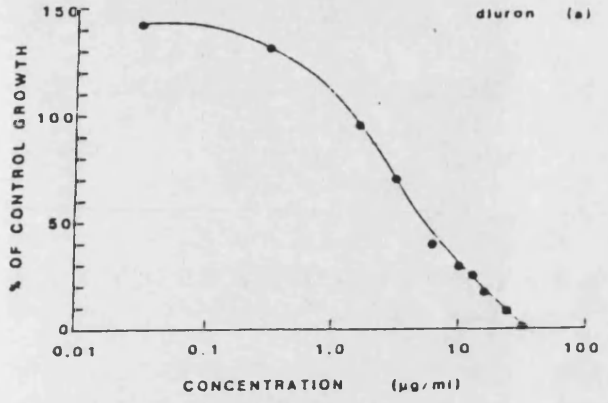
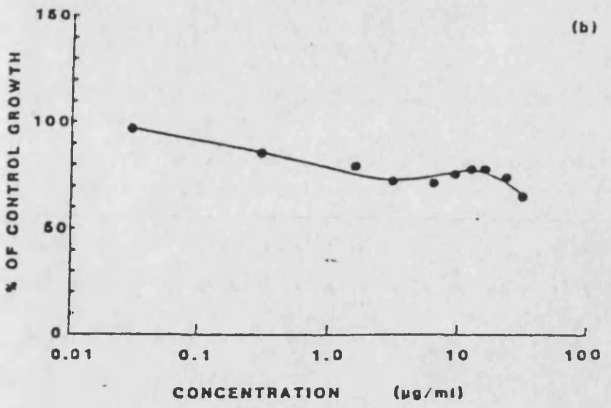
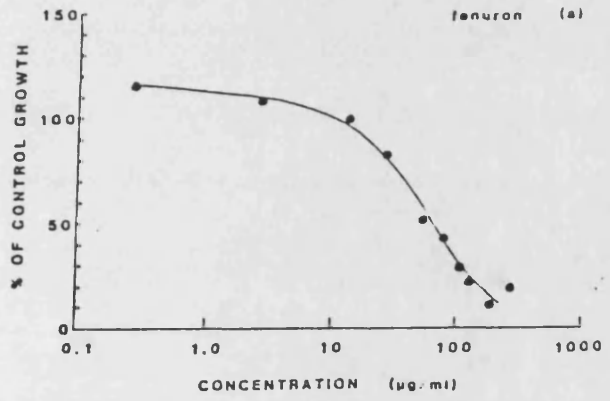
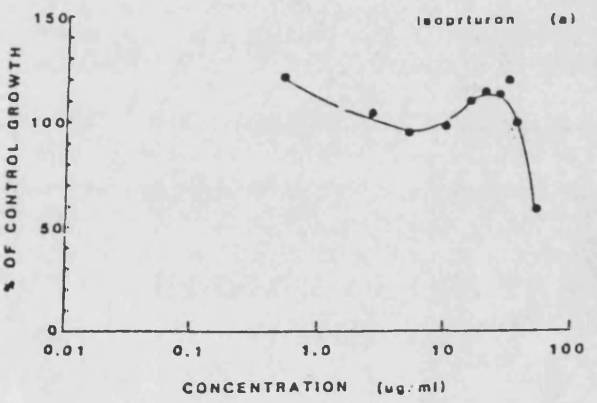

(b)
% OF CONTROL GROWTH
150
100
50
0
0.1
1.0
10
100
1000
CONCENTRATION (µg/ml)

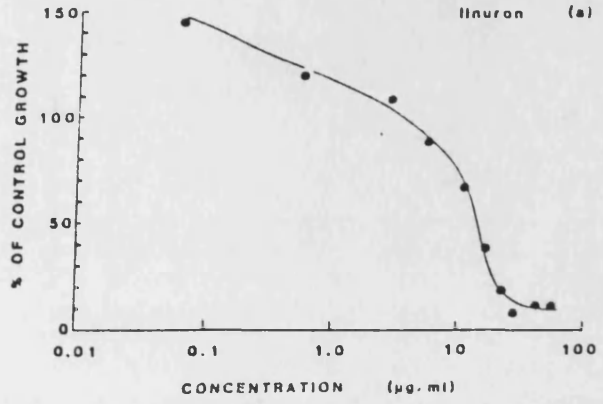
FIG. 48


diuron (a)
% OF CONTROL GROWTH
150
100
50
0
0.01
0.1
1.0
10
100
CONCENTRATION (µg/ml)


(b)
% OF CONTROL GROWTH
150
100
50
0
0.01
0.1
1.0
10
100
CONCENTRATION (µg/ml)


fenuron (a)
% OF CONTROL GROWTH
150
100
50
0
0.1
1.0
10
100
1000
CONCENTRATION (µg/ml)


isoprturon (a)
% OF CONTROL GROWTH
150
100
50
0
0.01
0.1
1.0
10
100
CONCENTRATION (µg/ml)


linuron (a)
% OF CONTROL GROWTH
150
100
50
0
0.01
0.1
1.0
10
100
CONCENTRATION (µg/ml)

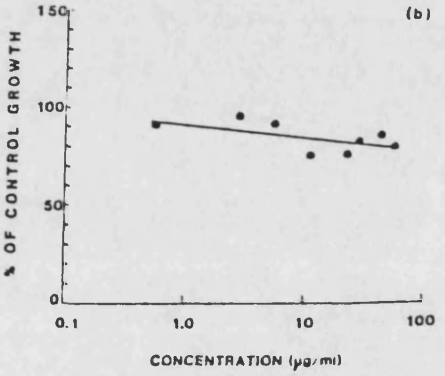
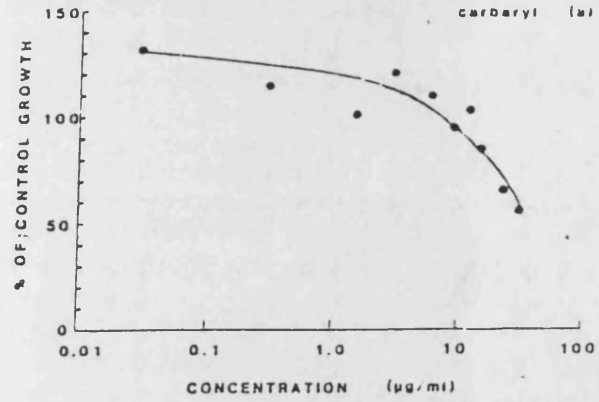
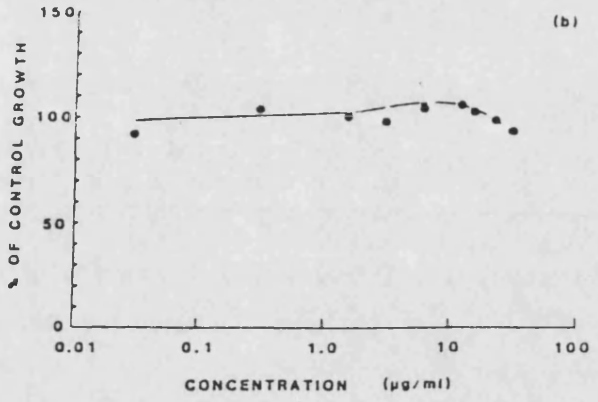
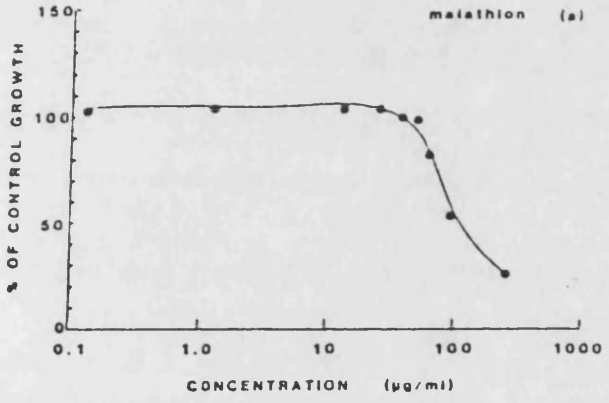
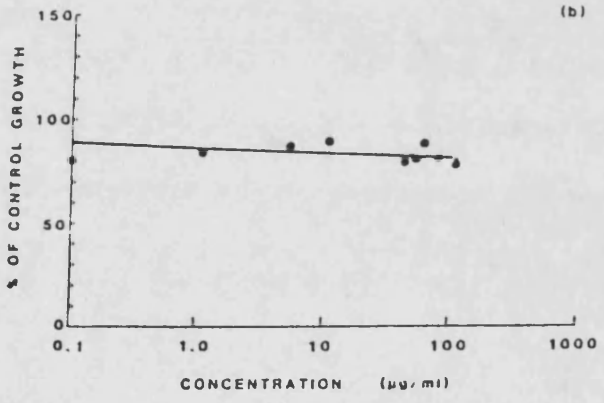

(b)
% OF CONTROL GROWTH
150
100
50
0
0.1
1.0
10
100
CONCENTRATION (µg/ml)

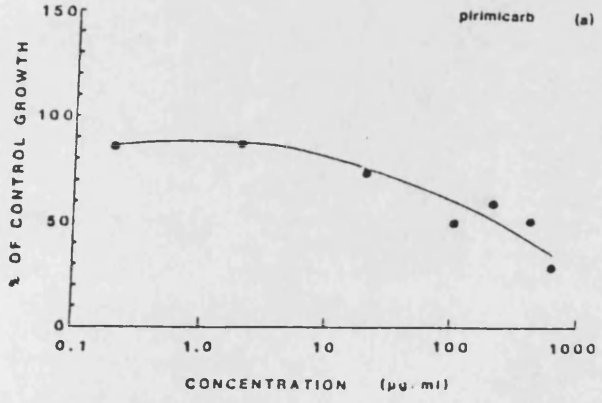
FIG. 49


carbaryl (a)
% OF CONTROL GROWTH
150
100
50
0
0.01
0.1
1.0
10
100
CONCENTRATION (µg/ml)


(b)
% OF CONTROL GROWTH
150
100
50
0
0.01
0.1
1.0
10
100
CONCENTRATION (µg/ml)


malathion (a)
% OF CONTROL GROWTH
150
100
50
0
0.1
1.0
10
100
1000
CONCENTRATION (µg/ml)


(b)
% OF CONTROL GROWTH
150
100
50
0
0.1
1.0
10
100
1000
CONCENTRATION (µg/ml)


pirimicarb (a)
% OF CONTROL GROWTH
150
100
50
0
0.1
1.0
10
100
1000
CONCENTRATION (µg/ml)

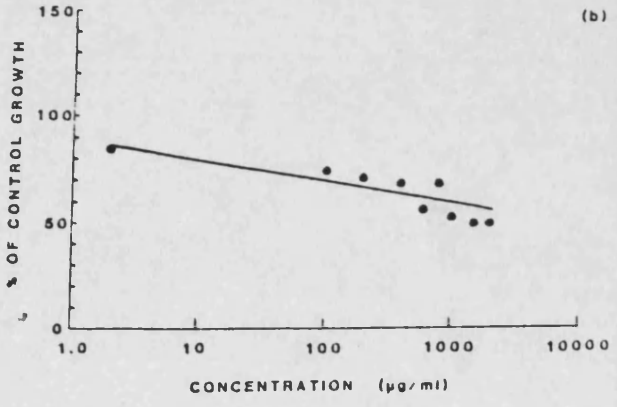

(b)
% OF CONTROL GROWTH
150
100
50
0
1.0
10
100
1000
10000
CONCENTRATION (µg/ml)

FIG. 50

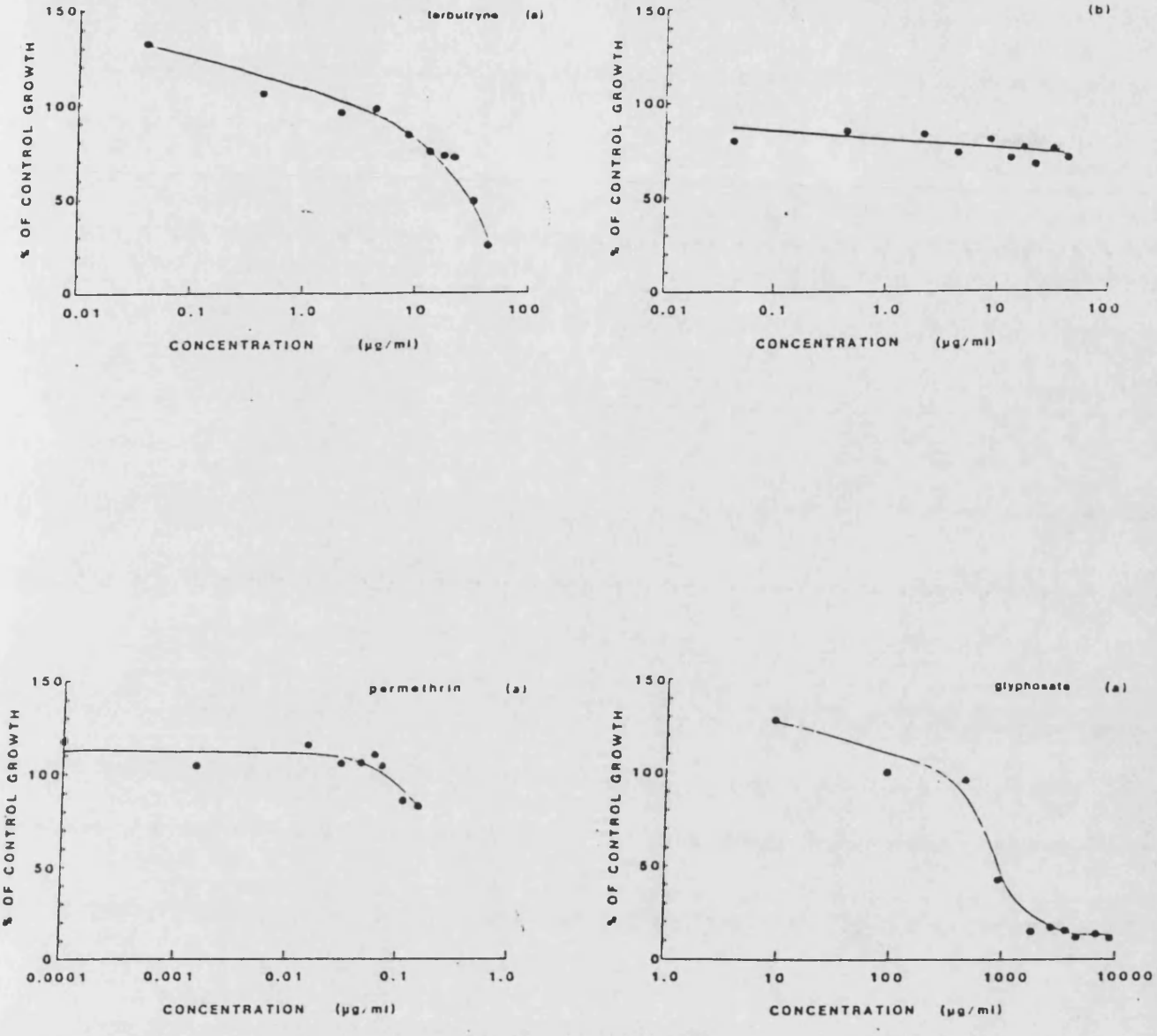

terbutryne (a)
% OF CONTROL GROWTH
150
100
50
0
0.01
0.1
1.0
10
100
CONCENTRATION (µg/ml)
(b)
% OF CONTROL GROWTH
150
100
50
0
0.01
0.1
1.0
10
100
CONCENTRATION (µg/ml)
permethrin (a)
% OF CONTROL GROWTH
150
100
50
0
0.0001
0.001
0.01
0.1
1.0
CONCENTRATION (µg/ml)
glyphosate (a)
% OF CONTROL GROWTH
150
100
50
0
1.0
10
100
1000
10000
CONCENTRATION (µg/ml)

FIG. 51

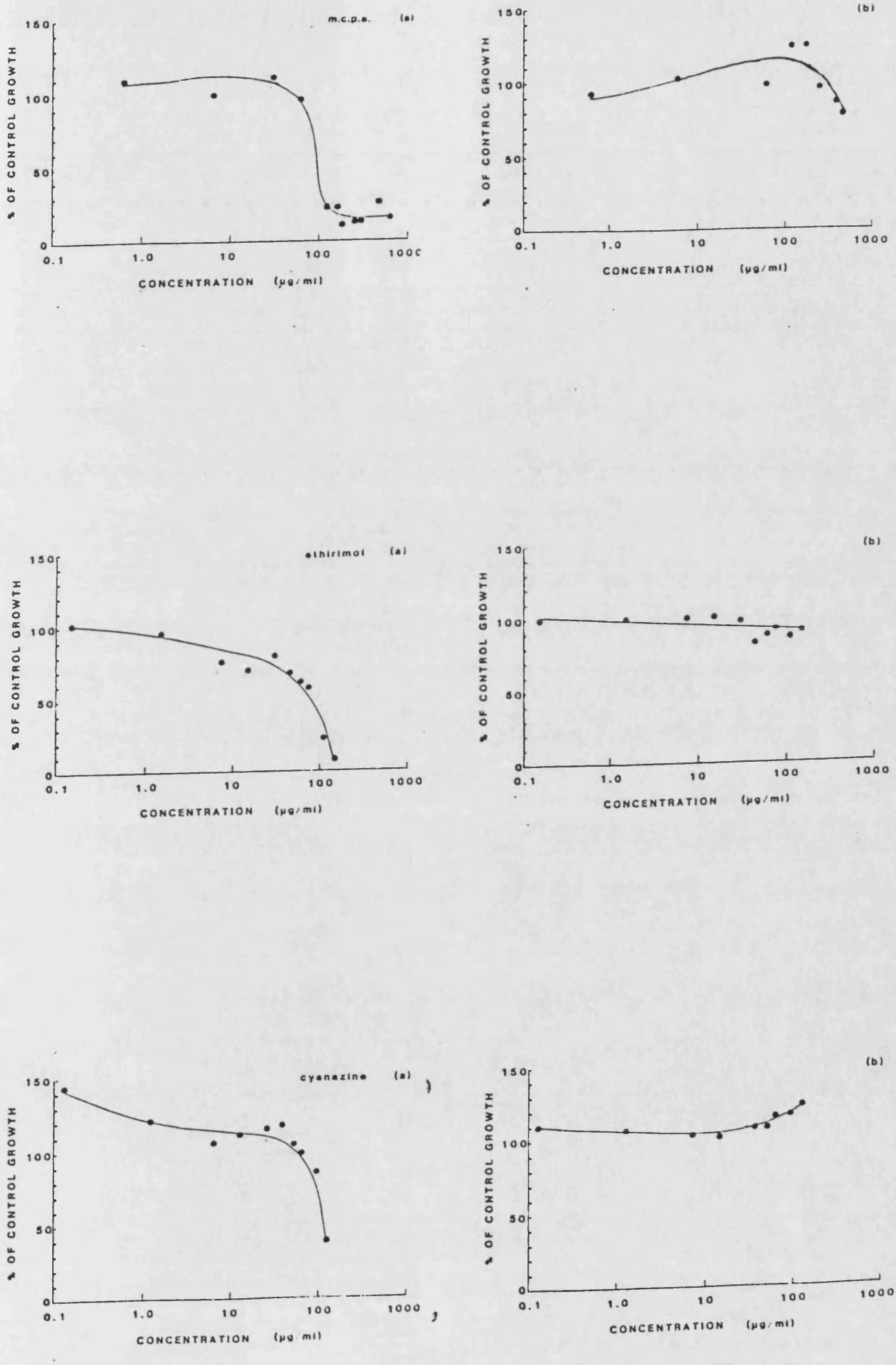

m.c.p.a. (a)
(b)
ethirimol (a)
(b)
cyanazine (a)
(b)
% OF CONTROL GROWTH
CONCENTRATION (µg/ml)
0.1
1.0
10
100
1000
0
50
100
150

FIG. 52

dramatic than T. pyriformis. The flat uncharacteristic growth curves of A. castellanii suggest the culture system was not optimal for the organism. The dose-response curves were therefore drawn from the 120h sample point data in order to capitalise on the maximum effects. Recovery of cultures from initial inhibitory activity may limit the usefulness of this sample time.

The dose-response curves (Fig. 48b - 52b) were generally curvilinear, comprising two parts, the final section of the curve (as defined in 32.1.1) was absent.

The initial section was proportionally longer than the middle section. It was a shallow curve, linear with malathion, terbutryne, pirimicarb, ethirimol, diuron and linuron (Fig. 49b - 52b) over which large increases in pesticide concentration had little inhibitory effect. The middle section was not evident in the above cases but where present it indicated a steepening of the curve. An exception to this was cyanazine (Fig. 51b) which was progressively more stimulatory to A. castellanii at increasing concentrations.

32.2 The phenylcarbamate herbicides

T. pyriformis was inhibited to a greater degree than A. castellanii by barban, chlorpropham and propham and was also more sensitive to the stimulatory effects of barban

and propham (Fig. 48a and b). A. castellanii detected the inhibitory effects of propham at a much lower level than T. pyriformis (Table 48).

The comparative order of the inhibitory activity of the phenylcarbamates was chlorpropham > barban > propham (T. pyriformis 72h EC_{50} values) and chlorpropham > barban and propham (A. castellanii 120h EC_{50} values). Chlorpropham was the most inhibitory pesticide to T. pyriformis (Table 48).

32.3 The phenylurea herbicides

Diuron and linuron were more inhibitory to T. pyriformis than A. castellanii (Fig. 49), although population growth of A. castellanii was inhibited at lower concentration (120h EC_{10} values, Table 48). The comparative order of inhibitory action of the phenylureas was diuron > linuron > fenuron > isoproturon (76h EC_{50} values T. pyriformis) and diuron > linuron with A. castellanii (120h EC_{50} values). All the phenylureas had a stimulatory action on the growth of T. pyriformis at low doses (Fig. 49).

32.4 The triazine herbicides

Cyanazine (Fig. 51) and terbutryne (Fig. 52) stimulated T. pyriformis growth at low concentrations, but only cyanazine was stimulatory to A. castellanii over the whole concentration range evaluated. The maximum stimulatory

effect for each organism was 0.1 μgml^{-1} (T. pyriformis) and 100 μgml^{-1} (A. castellanii). Both triazines were more inhibitory to T. pyriformis than A. castellanii, however, A. castellanii was considerably more sensitive to terbutryne than the ciliate (based on EC_{10} values, Table 48).

32.5 Miscellaneous insectides

The dose-response curves for carbaryl, malathion and pirimicarb (Fig. 50) showed that each was more inhibitory to T. pyriformis than A. castellanii. Carbaryl stimulated both organisms, the maximum effect was at 0.05 and 10 μgml^{-1} respectively.

The pyrethroid insecticide permethrin was the only compound not to inhibit T. pyriformis by 50% of the untreated population growth, consequently no EC_{50} value was obtained (Table 48). This lack of toxicity was depicted by the curvilinear profile of its dose-response curve (Fig. 52).

32.6 Miscellaneous pesticides

The herbicide MCPA and the fungicide ethirimol were more inhibitory to T. pyriformis than A. castellanii (Table 48). The EC_{10} values however suggest that A. castellanii was more sensitive to inhibition by low levels of both compounds than T. pyriformis. Stimulation of A. castellanii occurred at approximately 100 μgml^{-1} MCPA,

Table 48

Comparison of the concentrations of pesticides which inhibited population growth of *Acanthamoeba castellanii* and *Tetrahymena pyriformis* by 10% and 50%

Pesticide	Effective concentration which inhibited population growth, in Moles.			
	A. castellanii (120h)		*T. pyriformis* (72h)	
	EC_{10}	EC_{50}	EC_{10}	EC_{50}
chlorpropham	2.8×10^{-6}		3.2×10^{-7}	2.8×10^{-7} to 2.9×10^{-6}
barban	6.0×10^{-6}		2.9×10^{-6}	8.9×10^{-6} to 1.2×10^{-5}
diuron	6.0×10^{-7}		8.2×10^{-6}	2.1×10^{-5}
linuron	4.8×10^{-6}		2.2×10^{-5}	6.4×10^{-5}
propham	2.8×10^{-6}		2.1×10^{-5}	7.5×10^{-5}
terbutryne	4.2×10^{-8}		2.4×10^{-5}	1.2×10^{-4}
carbaryl	stimulatory		5.7×10^{-5}	1.9×10^{-4}
ethirimol	7.6×10^{-4}		1.3×10^{-5}	3.1×10^{-4}
malathion	3.0×10^{-7}		1.7×10^{-4}	3.3×10^{-4}
cyanazine	stimulatory		3.3×10^{-4}	5.0×10^{-4}
MCPA	3.0×10^{-6}		3.4×10^{-2}	5.0×10^{-4}
pirimicarb	7.1×10^{-7}		2.9×10^{-4}	6.7×10^{-4}
fenuron	NA	NA	0.1×10^{-2}	2.7×10^{-2} - 4.0×10^{2}
isoproturon	NA	NA	1.9×10^{-4}	>
permethrin	NA	NA	2.8×10^{-7}	>

> = greater than compound's solubility
NA = no data available

occurred at approximately 100 μgml^{-1} MCPA, similar doses caused 80-90% inhibition of T. pyriformis (Fig. 51).

The herbicide glyphosate also stimulated T. pyriformis at low doses (Fig. 52).

Direct comparisons of the EC_{50} values (effective concentration which inhibited growth by 50%) showed that T. pyriformis was more susceptible than A. castellanii to all 14 chemicals evaluated. Comparison of EC_{10} values showed A. castellanii to be more sensitive than T. pyriformis in 6 out of 12 cases (Table 48).

33.0 Evaluation of the chronic toxicity of some phenylcarbamate herbicides to Tetrahymena pyriformis in larger scale batch culture in Erlenmeyer flasks

Further investigations on the chronic toxicity of pesticides to T. pyriformis were restricted to the phenylcarbamate herbicides, chlorpropham, propham and barban, on the basis of their sub-acute toxicity in Repli-dishes and microtiter plates. Concentrations of each herbicide were confined to the values with the range 0.5 to 10x EFC of each chemical.

33.1 Population growth

Dose-response curves for the effect of chlorpropham, propham and barban on *T. pyriformis* appear in Section 43.0 (p 170) as they clearly demonstrate changes in the response of *T. pyriformis* to some herbicide concentrations.

Chlorpropham: Chlorpropham (2,4,20 and 40 $\mu g ml^{-1}$) significantly inhibited the population growth of *T. pyriformis* throughout 10d (Fig. 53). At 2 and 4 $\mu g ml^{-1}$ growth of the ciliate was prevented. A lethal action of these concentrations was evident after 4d. With 20 and 40 $\mu g ml^{-1}$ cell death was apparent before 1d. Continued exposure to the chemical resulted in a progressive decline in cell number which proceeded linearly until 5d (40 $\mu g ml^{-1}$) and 6d (20 $\mu g ml^{-1}$) when cell numbers declined below the limit of detection. Increasing concentrations of chlorpropham were progressively more toxic to this organism. Toxicity increased with dose and length of exposure to the compound.

Propham: High levels (25 and 50 $\mu g ml^{-1}$) propham prevented cell division over 10d after an initial lethal action lasting 1d (Fig. 54). With 25 $\mu g ml^{-1}$ a suggestion of recovery of cell division occurred in the later stages of the experiment. Twenty five $\mu g ml^{-1}$ propham was not as lethal as 50 $\mu g ml^{-1}$ to *T. pyriformis*.

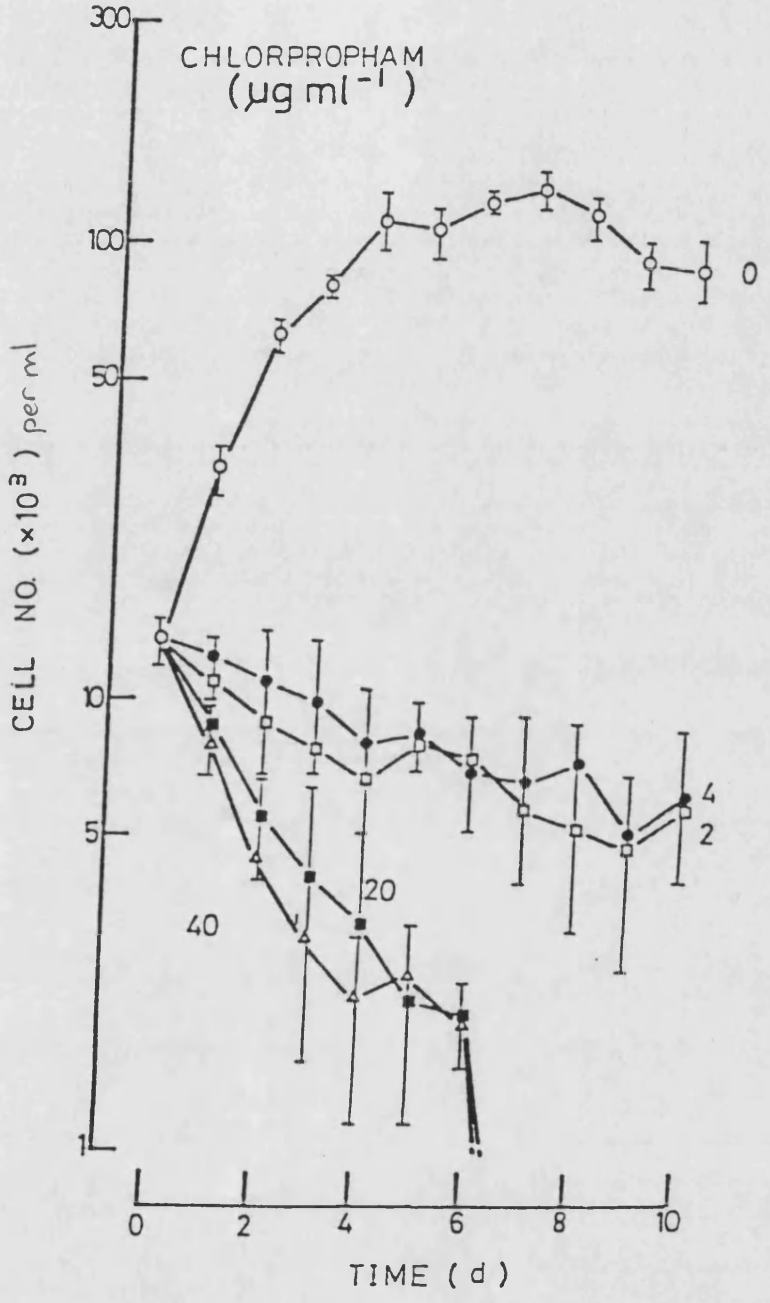


FIG. 53

The effect of chlorpropham on the growth of Tetrahymena pyriformis, in 50 ml of PY medium in Erlenmeyer flasks, at 20°C. (mean values +/- I standard deviation).

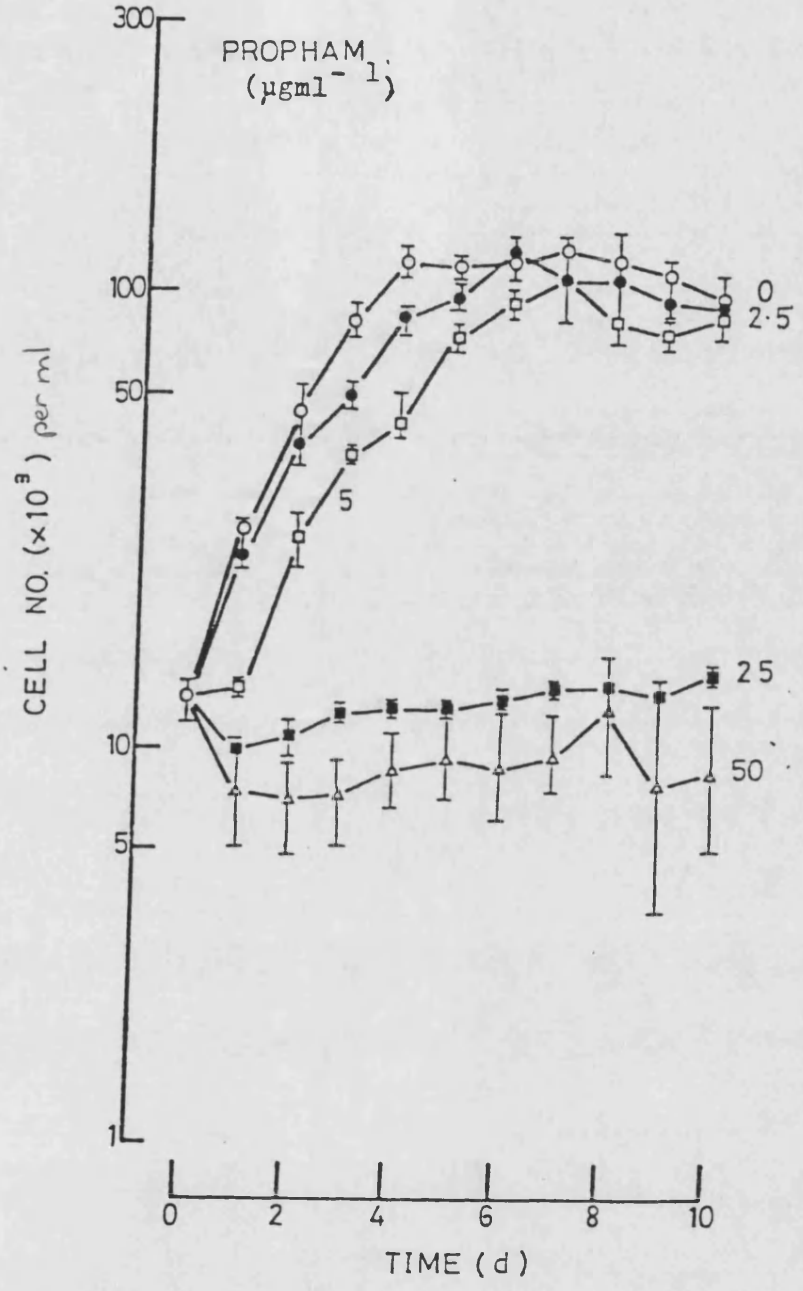


FIG. 54

The effect of propham on the growth of Tetrahymena pyriformis, in 50 ml of PY medium in Erlenmeyer flasks, at 20°C . (mean values +/- 1 standard deviation).

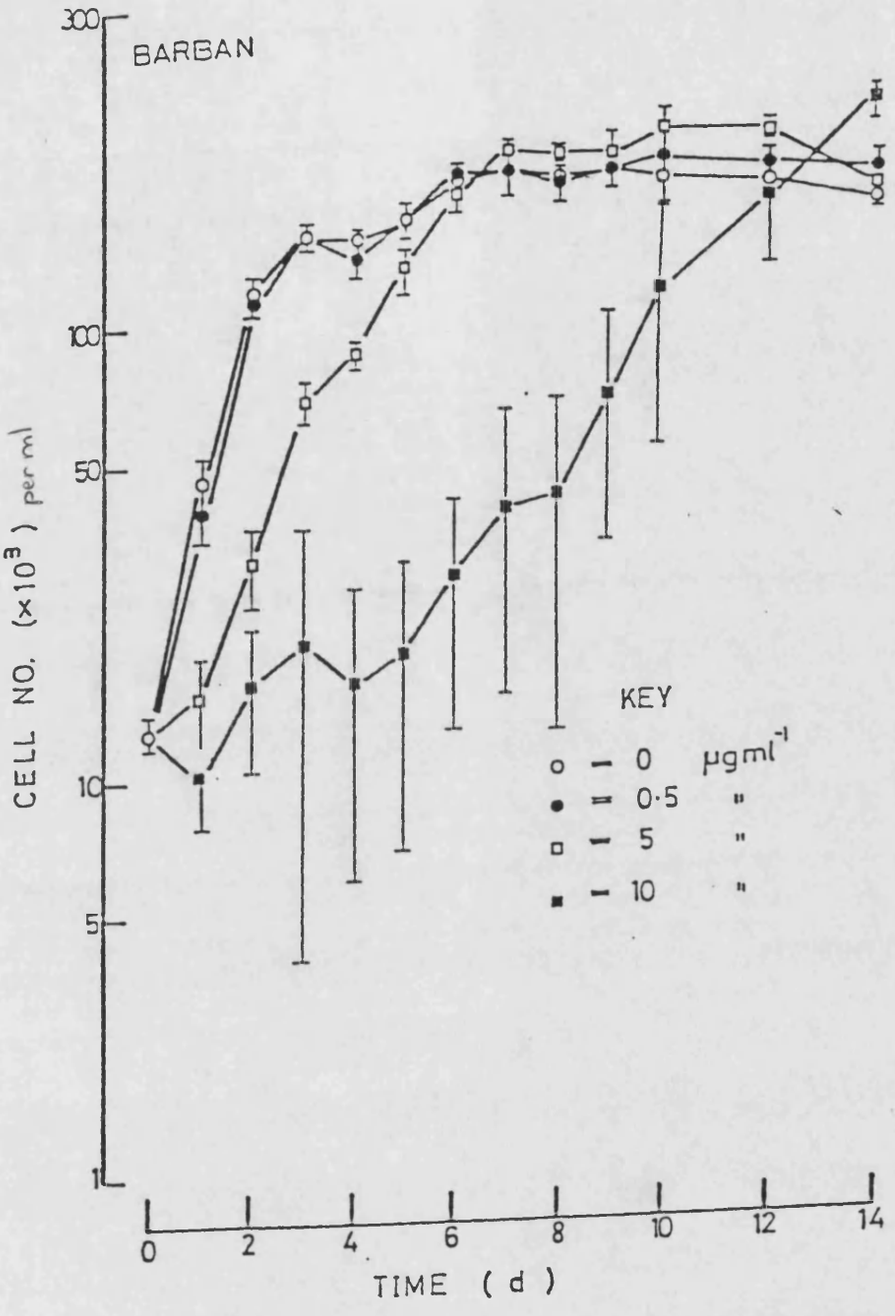


FIG. 55

The effect of barban on the growth of Tetrahymena pyriformis, in 50 ml of PY medium in Erlenmeyer flasks, at 20°C (mean values +/- 1 standard deviation).

Suppression of growth also occurred with 5 μgml^{-1} propham after an initial lag phase (24h) occurred. There were no difference between the exponential phase growth rates of 0, 2.5 and 5 μgml^{-1} of propham. After 7d differences between control (untreated) and 2.5 μgml^{-1} propham-treated cultures were not significant.

Barban: Barban initially decreased cell numbers of T. pyriformis at 10 μgml^{-1}, the effect lasting 1d only (Fig. 55). Cell numbers recovered and population size increased at a reduced rate throughout the experimental period such that at the end of the experiment cell number, in this treatment, was 48% higher than the untreated control. At 5 μgml^{-1} barban slowed the growth of the ciliate throughout the exponential phase of the culture (4d) but after 6d there was no significant reduction in cell number.

Barban, at 1.0 and 0.5 μgml^{-1} had no inhibitory effect on T. pyriformis over the entire experiment.

33.2 Cell size

Chlorpropham: At all concentrations of chlorpropham cell length was decreased dramatically within 24h by approximately 40% (Fig. 56). At 2 μgml^{-1} and above, chlorpropham caused a marked decrease in cell size throughout 9d. With 2 μgml^{-1} chlorpropham cell size was reduced throughout 9d, however at 7d the standard

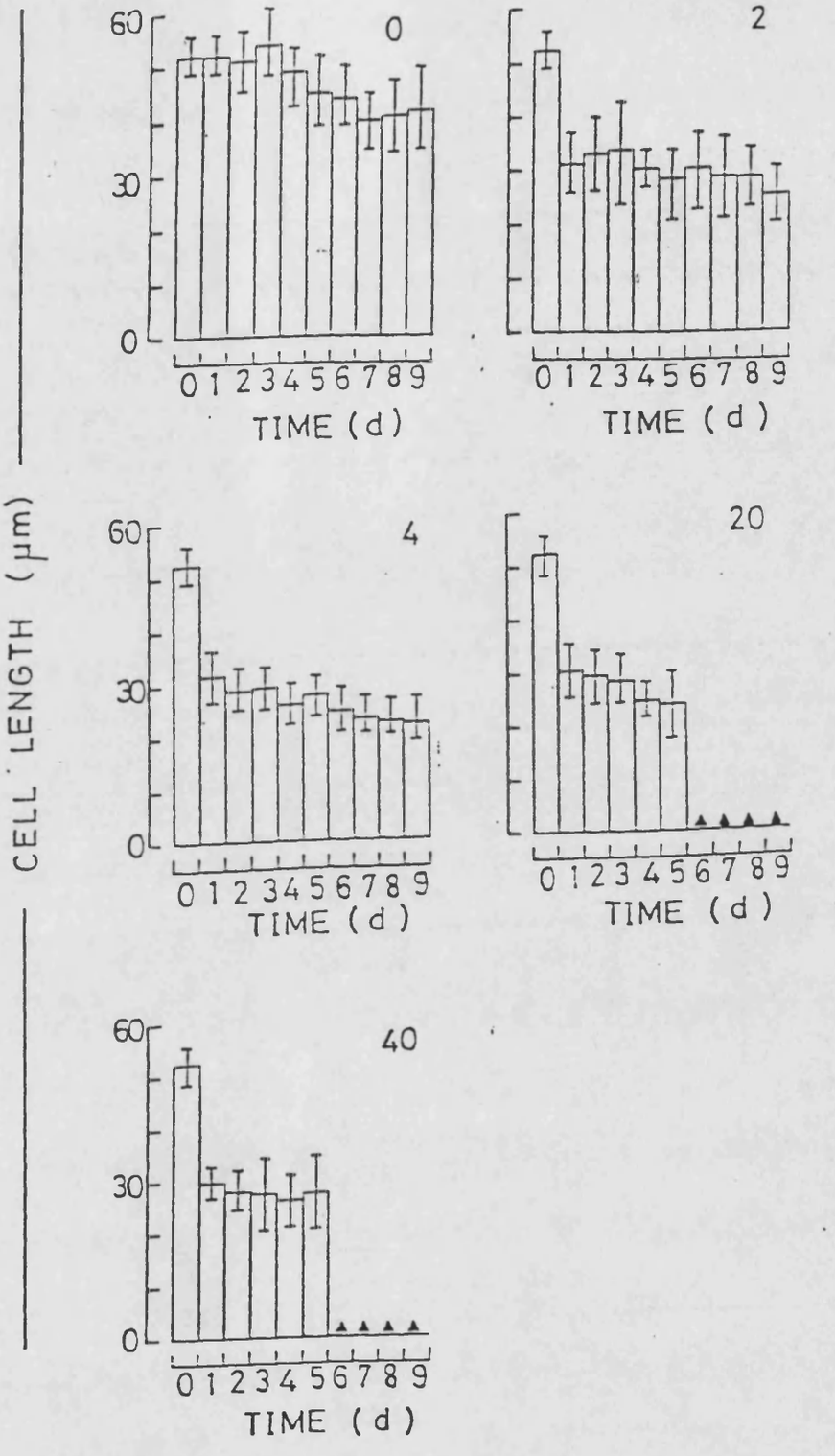


FIG. 56

The influence of chlorpropham on the length of Tetrahymena pyriformis cells (▲ indicates no cells present in sample). Numbers on the digrams refer to the herbicide concentrations

deviation of this treatment and that of the control overlap.

Readings were not taken after 5d with 20 and 40 μgml^{-1} chlorpropham due to the number of cells available for measurement declining below any reasonable detection level (Fig. 56).

Propham: Concentrations of propham below 5 μgml^{-1} had no effect on cell size (Fig. 57). Cell length of 50 μm (0d), declined to 40 μm after 9d in these treatments as well as in the control. With 25 μgml^{-1} propham there was an initial decline in cell length followed by a slight recovery (6d). Differences between the control and this treatment were present at 2, 3 and 4d. Cell length declined by at least 10 μm on 1d. Comparison with Fig. 54 shows that 25 μgml^{-1} propham also reduced the number of cells up until 3d and then caused stasis, with possible recovery at a later stage. The initial toxic action of propham may be reflected in this initial decrease in cell length.

Propham (50 μgml^{-1}) reduced cell length over the entire 9d, this reduction appears to increase with time. The increase in cell numbers at 8d (Fig. 54) and then sudden decline is not depicted in these findings. It may represent a sampling error.

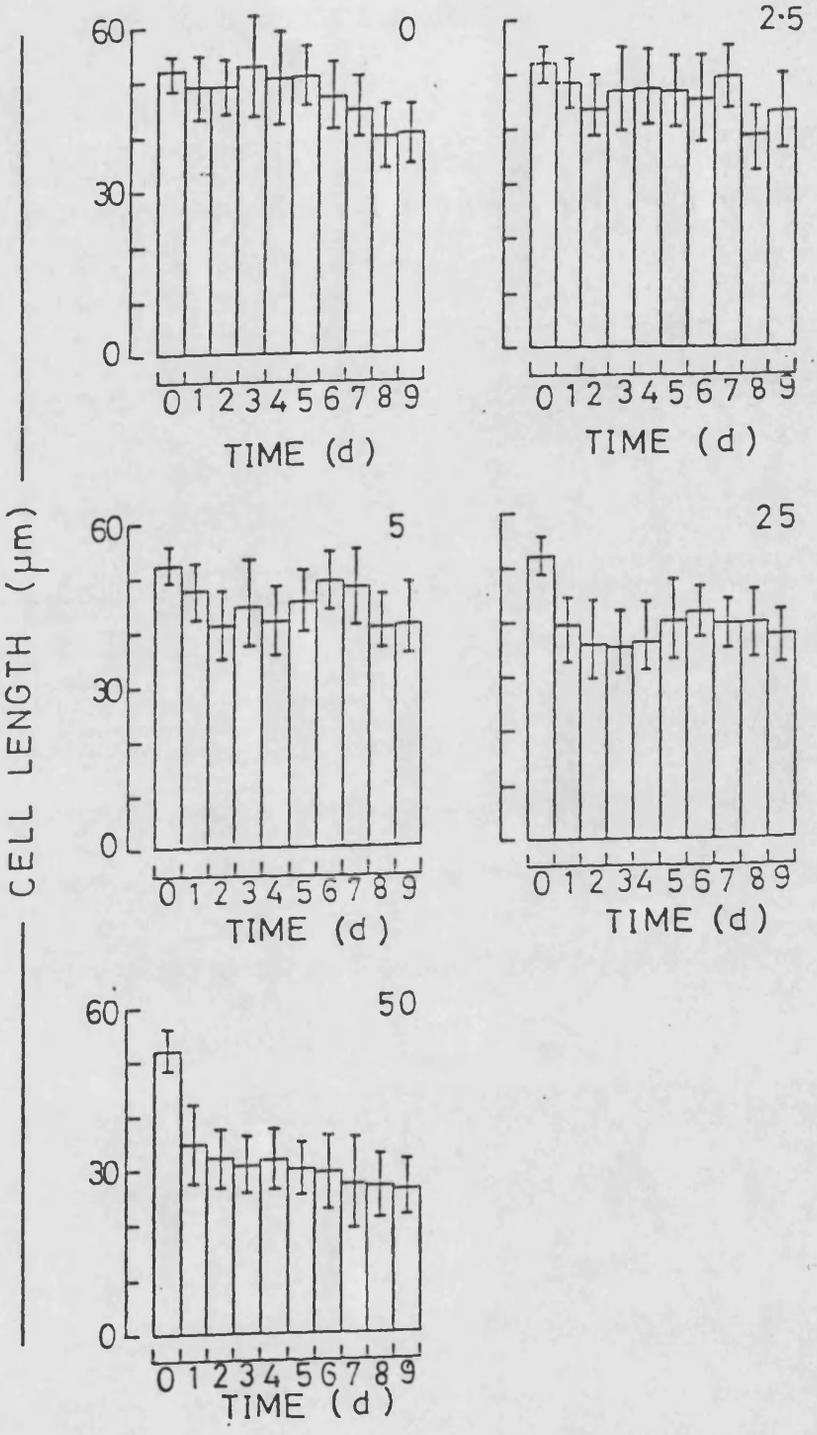


FIG. 57

The influence of propham on the length of Tetrahymena pyriformis cells (figures on diagrams refer to herbicide concentrations)

Comparisons with chlorpropham show that 50 μgml^{-1} propham causes the same decrease in cell length as approximately 2-4 μgml^{-1} chlorpropham.

The effect of propham on cell length appears to be dose-dependent. As with chlorpropham, increasing concentrations of the compound progressively decreased cell length, but to a lesser degree.

Barban: Low levels of barban (0.5, 1 and 5 μgml^{-1}) did not influence cell length in any significant manner (Fig. 58). However, with 5 μgml^{-1} barban a decrease in cell length, of nearly 9 μm (x) occurred at 1d. This decrease was not evident with 0.5 and 1 μgml^{-1} barban but was present with 10 μgml^{-1}. Barban, at 10 μgml^{-1} (Fig. 55) also caused an initial decrease in cell number followed by a progressive recovery, though at a much lower growth rate than the untreated. This is paralleled in the effect of this level of barban on cell size, an initial decrease followed by a recovery to normal size. Although the recovery of cell length was erratic it was parallel with the recovery of cell numbers (Fig. 55).

With all three phenylcarbamates tested cells did not decrease in cell width. Thus, as cell length declined the cells changed from being pyriform, through ovoid, to spherical. The frequency of spherical cells is discussed in Section 35.4.

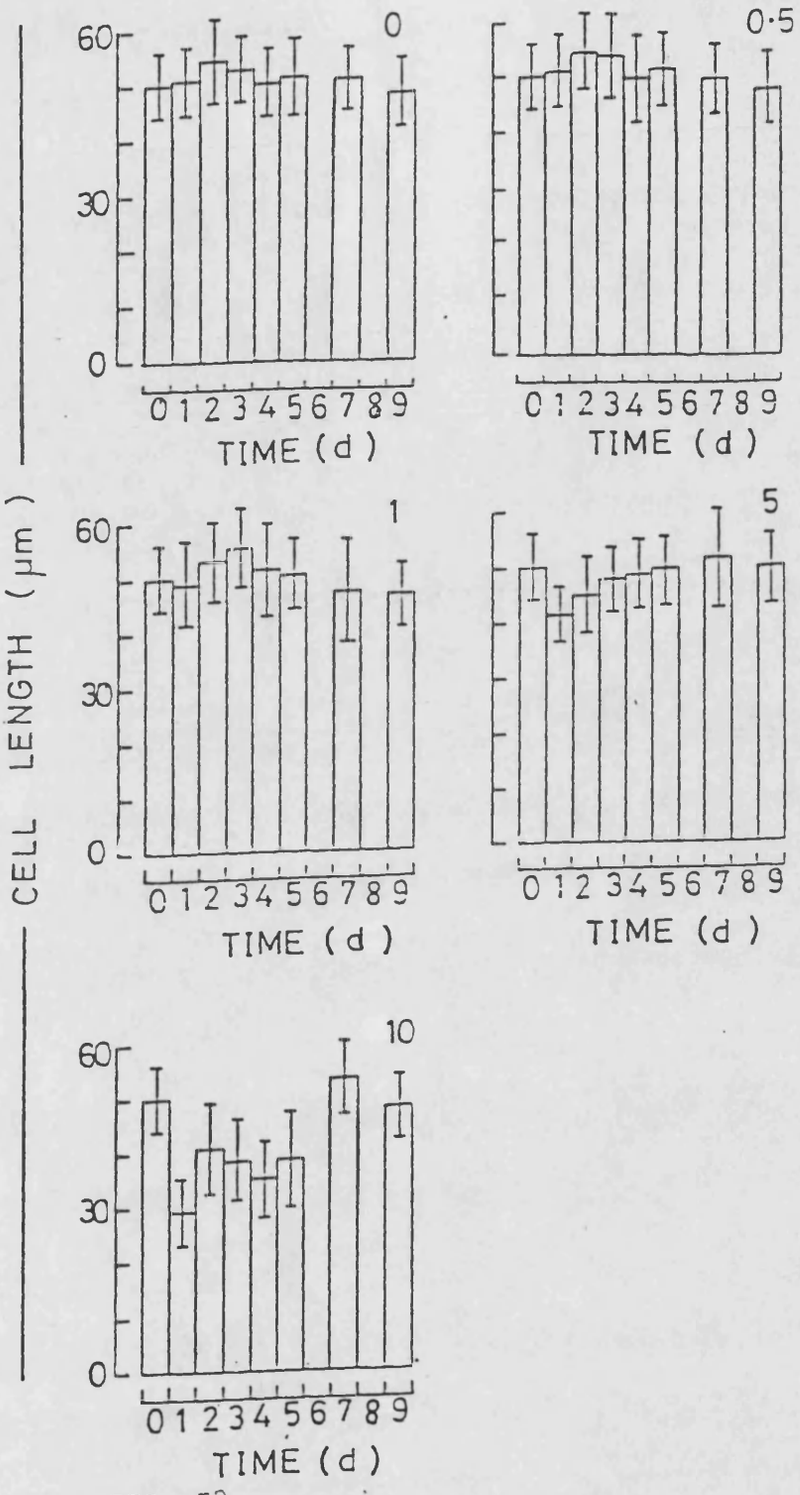


FIG. 58

The influence of barban on the length of Tetrahymena pyriformis cells. Numbers on the diagrams refer to herbicide concentration.

Any drop in cell numbers, caused by the herbicides, was to a lesser extent paralleled in a decrease in cell length. Interestingly, with propham and chlorpropham, their effects on cell size and on cell numbers were dose-dependent, the effects increasing with concentration and length of exposure (dose = concentration x exposure). However, only barban appeared inhibitory in terms of concentration. Cells continuously exposed to barban appeared to recover from its initial effect. With cell numbers this recovery is proportional to the length of exposure.

34.0 Evaluation of the chronic toxicity of some phenylcarbamate herbicides to Acanthamoeba castellanii in Erlenmeyer flasks

In investigating the chronic toxicity of pesticides to A. castellanii choice of compounds was restricted to the phenylcarbamate herbicides, chlorpropham, propham and barban, which had already demonstrated their sub-acute toxicity to this organism in Repli-dishes and microtiter plates. Herbicide concentrations were again confined to levels within 0.5 to 10x EFC for each chemical.

34.1 Population growth

Chlorpropham: Normal growth of untreated cell populations occurs up until 7d after which the cell number gradually

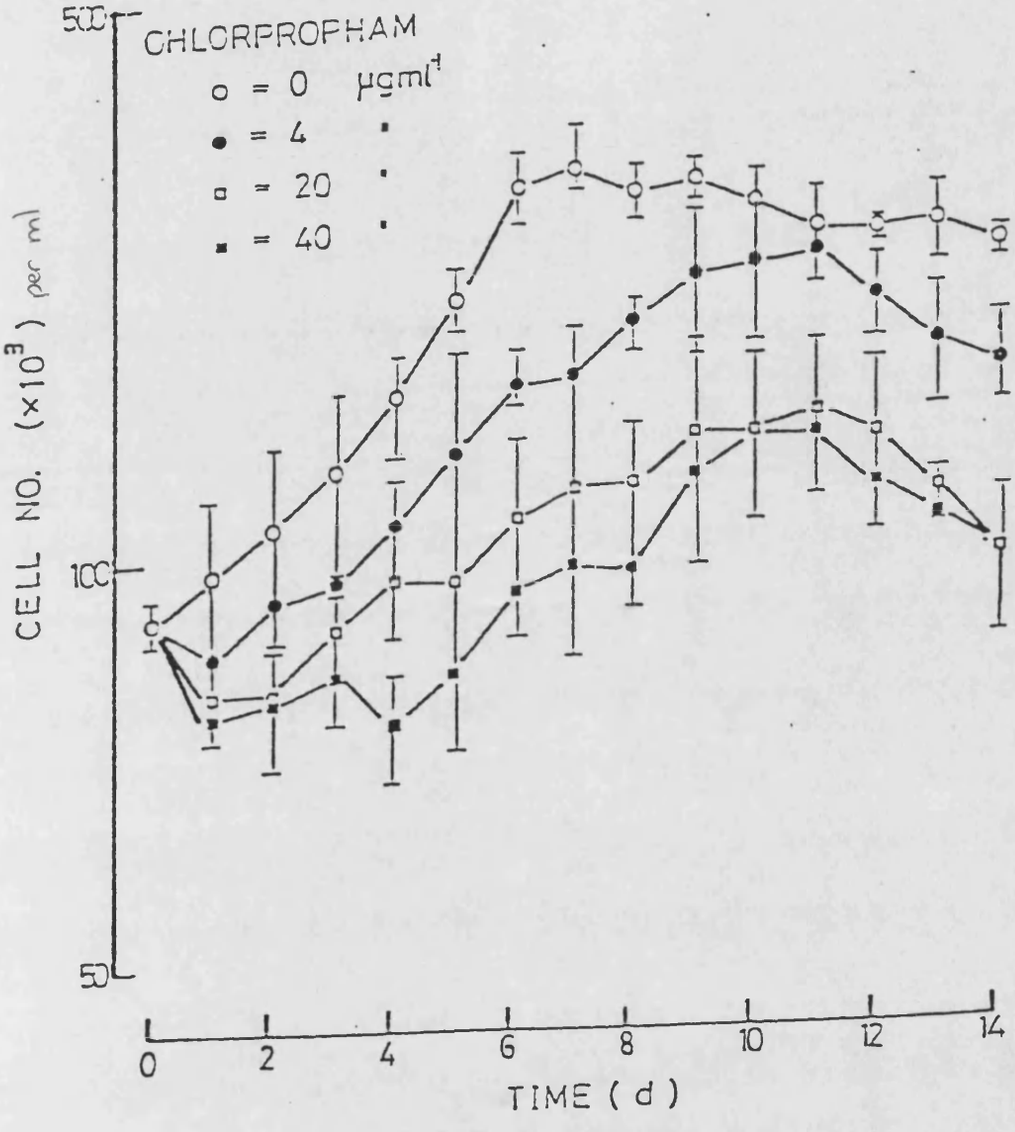


FIG. 59

The effect of chlorpropham on the growth of Acanthamoeba castellanii, in 50 ml of PGY medium in Erlenmeyer flasks, at 30°C (mean values +/- 1 standard deviation).

declines (stationary phase). Treatment with all levels of chlorpropham gave similar shaped curves but of differing growth rates. Chlorpropham at 4, 20 and 40 μgml^{-1} gave growth rates which were significantly different ($p=0.01$) from the untreated, but not from each other.

The high variability of the data within the untreated cell numbers prevented detailed discussion of initial inhibitory effects of chlorpropham. However, Fig. 59 indicates that all concentrations of chlorpropham had an initial lethal action on some A. castellanii cells. With 40 and 20 μgml^{-1} there appears to be an initial lag-phase of 4 and 2d respectively.

The inhibition of A. castellanii became more pronounced with time with all chlorpropham concentrations. After 10d the cell number in both control and 4 μgml^{-1} (chlorpropham) populations coincided. However, at this point the cell number of the untreated had been declining for 4d whereas cells in the chlorpropham (4 μgml^{-1}) treatment were still rising. The two cultures were at different physiological ages.

With 20 μgml^{-1} chlorpropham the lag-phase follows an initial lethal action on a proportion of the population in a similar manner to that with 40 μgml^{-1} of chlorpropham although to a lesser degree. Both populations of cells had growth rates significantly different from the control ($p=0.01$) but not from each other. Cell number declined at

9d (20 μgml^{-1}) and 11d (40 μgml^{-1}) compared with 6d for the untreated control.

The effect of chlorpropham on A. castellanii was dose-dependent; the inhibition becoming more pronounced with time. No stimulatory activity was detected over the concentration range tested.

A. castellanii was less sensitive than T. pyriformis to the inhibitory effects of chlorpropham.

Chlorpropham (40 μgml^{-1}) prevented division in A. castellanii for 4 d whilst a 2 μgml^{-1} chlorpropham was lethal to T. pyriformis after 4d.

Propham: Increasing concentrations of propham progressively inhibited the growth of A. castellanii (Fig. 60). Inhibition of the amoeba is further enhanced with time (dose being dependent on concentration and exposure). Unlike the untreated growth curve shown with chlorpropham (Fig. 59) the experimental control with propham does not exhibit any great variation at the beginning of the experiment. All concentrations of propham (2.5 - 50 μgml^{-1}) caused an initial decline in numbers followed by resumed division and normal growth rates after 2d. All cultures entered a stationary phase after 7d whereas the control entered this phase after 6d. Propham (5 - 50 μgml^{-1}) significantly inhibited growth

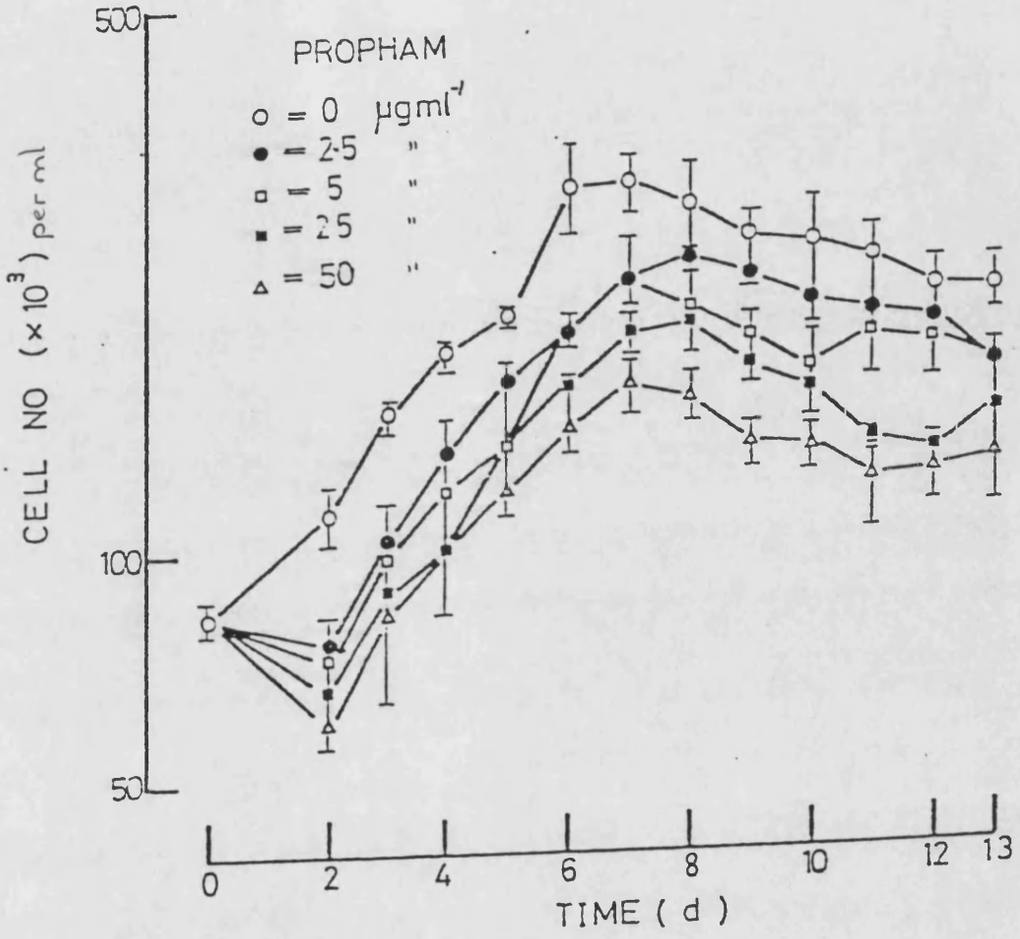


FIG. 60

The effect of propham on the growth of Acanthamoeba castellanii, in 50 ml of PGY medium in Erlenmeyer flasks, at 30°C (mean values +/- 1 standard deviation).

yield (13d) in a constant dose-related manner. With 2.5 μgml^{-1} propham after 8d no significant inhibition was observed.

The amoeba A. castellanii was not as sensitive to inhibition by 25 and 50 μgml^{-1} propham as T. pyriformis but both organisms had similar responses to 2.5 and 5 μgml^{-1}.

Barban: Dose-response curves for the inhibition of A. castellanii by barban appear in section 44.0 (p 171) as they clearly demonstrate changes in the response of A. castellanii to the herbicide.

The initial toxicity of the phenylcarbamates to A. castellanii was most clearly demonstrated by barban (Fig. 61). Concentrations 0.5 - 1.0 μgml^{-1} caused the cell population density to fall to nearly half of its original value over 24h. However, within 2d full recovery of cell number had occurred. This initial and severe reduction in cell numbers and subsequent recovery at a significantly reduced growth rate ($p=0.01$) as compared with the control is surprising. A collapse of the culture regime in all 16 treated flasks is unlikely and would have affected the 4 control flasks had it originated from any source other than from the herbicide.

Barban reduced cell numbers at all concentrations tested

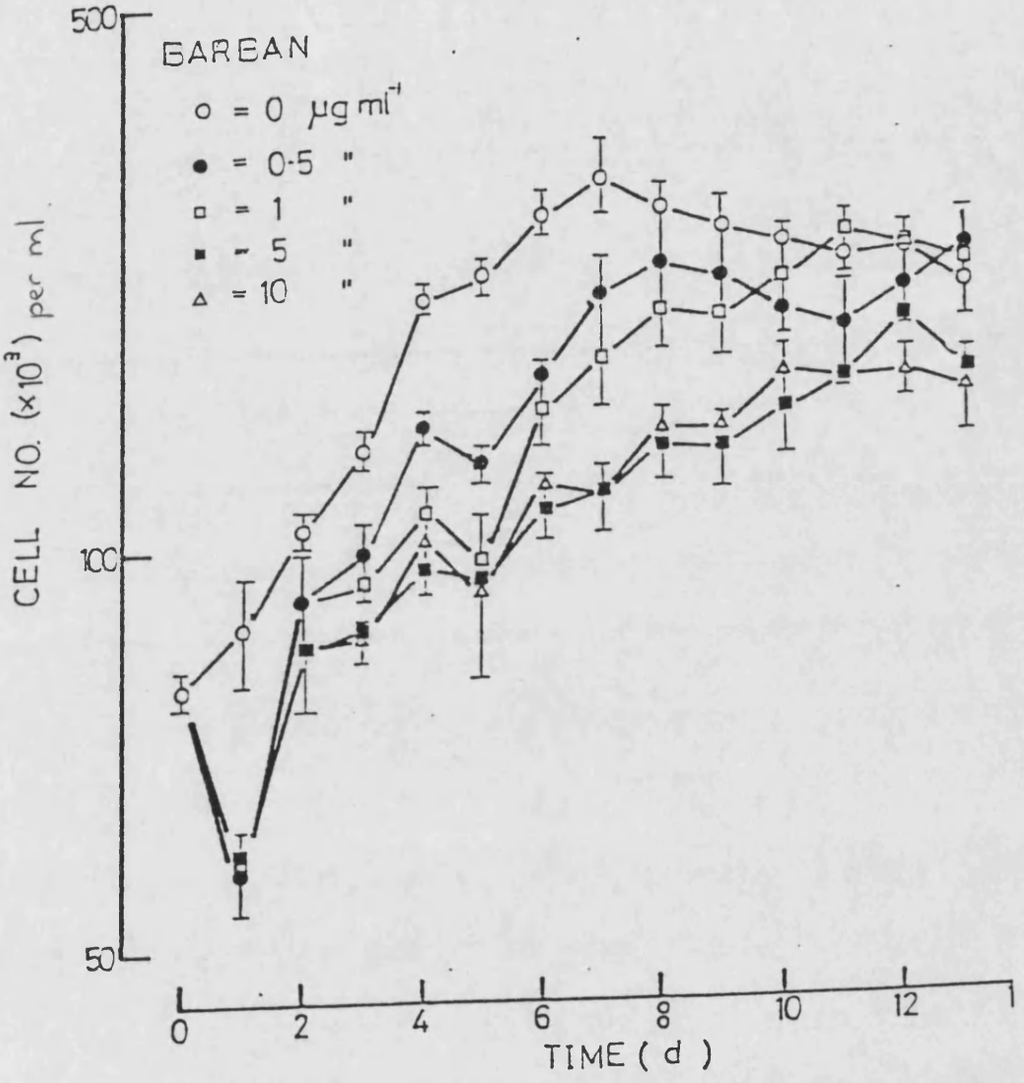


FIG. 61

The effect of barban on the growth of Acanthamoeba castellanii, in 50 ml of PGY medium in Erlenmeyer flasks, at 30 C (mean values +/- 1 standard deviation).

in a dose-dependent manner. The length of exposure to the chemical increased its inhibitory action. With all levels the length of the log-phase was increased; 8d (0.5 μgml^{-1}), 11d (1 μgml^{-1}), 12d (5 μgml^{-1}) and 10d (10 μgml^{-1}) as opposed to 7d for the untreated control. No stimulation of A. castellanii growth occurred with barban unlike its activity with T. pyriformis. Interestingly the growth curves of A. castellanii treated with barban tended to be step-wise in nature. This trend breaks down after 5d (0.5 μgml^{-1}) and 6d (1,5,10 μgml^{-1}) barban, though in the latter there is qualitative evidence of small undulations extending up until the 9th day. These steps can also be seen in Fig. 55 (10 μgml^{-1} barban) with T. pyriformis although the variability of the data prevents further comment.

Unlike propham and chlorpropham, barban appears to be more inhibitory to A. castellanii than T. pyriformis. However in both cases recovery is suggested from the lower doses (0.5 and 1 μgml^{-1}) but not the higher doses (5 and 10 μgml^{-1}).

35.0 Morphological and cytological effects of some pesticides on Acanthamoeba castellanii and Tetrahymena pyriformis

Being observations on cells gathered from both sub-acute and chronic toxicity experiments.

35.1 The acute effects of some pesticides on the morphology and cytology of Acanthamoeba castellanii

From cultures initially set up for quantitative evaluation of the sub-acute toxicity of a number of chemicals on A. castellanii in Repli-dishes (Section 13.0) samples were withdrawn and observation on acute effects on morphology and cytology were made.

Barban: At 10 μgml^{-1} barban caused rounding of some A. castellanii cells within 5 min. Many cells had stopped moving after 10 min and acanthapodia were withdrawn. All cells had become round by 60 min.

There were significant reductions (27.4%) in cell sizes after 48h caused by 5 μgml^{-1} and after 120h (20%) with 1 μgml^{-1} of barban. All values were significant at p=0.5.

Chlorpropham: Chlorpropham (60 μgml^{-1}) produced variable results in three trials. In general the cells were seen to withdraw their acanthapodia and become rounded (Plate 1) within 60 min (in one cell within 10 min). After 2h cell movement had ceased, the cytoplasm became dense and no food vacuoles were seen. The hyaline zone was not detected and cells had the general appearance of smooth spheres. The nucleus was not observed.

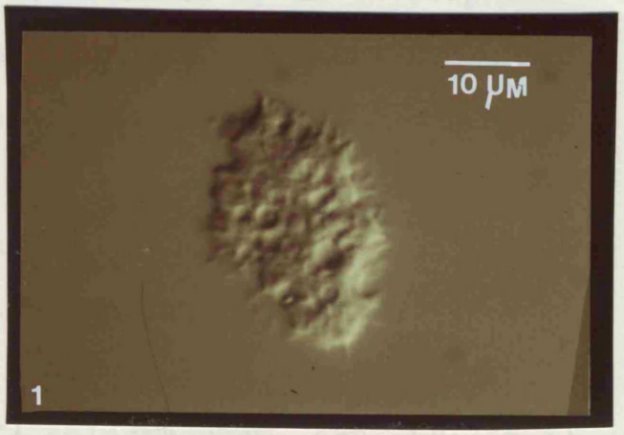
Cell size was reduced, by 39.7%, after 24h treatment with

PLATE I.

The appearance of Acanthamoeba castellanii cells after 48h exposure to chlorpropham solutions in PCY medium (x400 magnification)

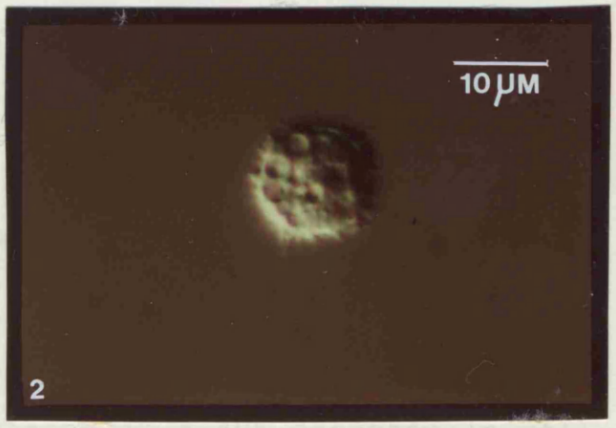
PHOTOGRAPH I.

Untreated A.castellanii cell showing acanthapodia, vacuoles and irregular cell shape.



PHOTOGRAPH 2.

Cell treated with 45µgml^{-I} of chlorpropham. Small acanthapodia and vacuoles present but the cell has rounded up.



PHOTOGRAPH 3.

Cell treated with 60 µgml^{-I} of chlorpropham. Cytoplasm confined to the centre of the cell. No cytoplasmic differentiation and the cell is surrounded by a large open structured envelope.

60 μgml^{-1} chlorpropham and by 29.9% with 45 μgml^{-1} after 72h (both were significantly different from controls at p=0.5). After 48h with 60 μgml^{-1} of chlorpropham (Plate 1) the cells appeared to be surrounded by an envelope. The envelope varied in size, but was always distinct from the cytoplasm which was confined to the centre of the cell. The cytoplasm had a granular appearance but no vacuoles. Occasional 'granules' were observed within the envelope space although this was generally clear.

Cells treated with 45 μgml^{-1} chlorpropham, 48h, (Plate 1) did not possess an envelope but were generally round. Food vacuoles were observed within the cytoplasm but little acanthapodial activity was detected.

Propham and benomyl: Neither of these compounds affected the morphology of A. castellanii over 60 min. However, some rounding-up of the cells occurred after 72h exposure to 150 μgml^{-1} propham, resulting in a 40% decrease in cell size.

Asulam: Variable results were obtained with cells exposed to 4 μgml^{-1} asulam. In one experiment all cells rounded up after 30 min, in a second no effect was detected, whilst in a third cytoplasmic 'blips' occurred at the membrane edge. Asulam had no significant effect on the size of A. castellanii cells throughout 120h.

Pirimicarb: After 10 min exposure to pirimicarb (1800 μgm^{-1}) A. castellanii cells ceased moving and withdrew their acanthapodia. After 30 min rounding-up of some cells began and all cells were round after 60 min. Food vacuoles were observed in all cells.

35.2 Observations on the acute effects of chlorpropham on the morphology, cytology, motility and behaviour of Tetrahymena pyriformis

The observations were made on cells from Repli-dish cultures initially set up to investigate chlorprophams sub-actute toxicity.

Acute effects of 20 μgml^{-1} chlorpropham on the morphology, cytology and motility of the ciliate included the disappearance of food vacuoles, the appearance of large (contractile) vacuoles, cilia reversals and a gradual rounding of cells (Table 49). After 5 min the treated cells showed abnormal movement patterns, cilia tended to reverse direction and cells showed characteristic responses to adverse conditions (swimming backwards, repeatedly changing forward direction, and pivoting around a fixed point). After 20 min cell movement gradually slowed although population 'swarming' was seen after 40 min. Some cells ceased moving after 240 min, these cells were irregularly shaped and stained blue with Steedmans triple stain, cells alive prior to staining stain red, cells which are dead stain blue, others underwent

prolonged periods of swimming backwards. Directional movement ceased after 300 min and was replaced by sporadic but rhythmic jerking action. This activity also declined leaving cells oscillating (360 min). De-ciliation occurred, the tendency increased with time. Ciliary rows and the oral ciliature were difficult to see, whilst in untreated cells both oral and body ciliature remained visible and intact.

Cell shape altered from the normal pyriform through blunt-ended pyriform (40 min) then to rounded forms (60 min) and finally to irregular shapes (240 min). Both the irregular and round cells had areas within the cell which were devoid of any contents. The irregular shaped cells stained blue with Steedman's triple stain.

The number of food vacuoles declined in treated cells, to the extent that after 60 min none were observed in the majority of cells. Contractile vacuoles were visible throughout the experiment (480 min). Both the size of the vacuole and the duration of the systole/diastole function increased with length of exposure to 20 $\mu g ml^{-1}$ chlorpropham (Table 49 and 50). The time taken for systole/diastole function was twice that for the control cells after 120 min, 3 x after 300 min and over 5 x after 480 min. The size of contractile vacuoles, at the same sample points increased, 1.5 - 2.0x, 2.0 - 3.0x and 3.0 - 4.0x, respectively, with some cells possessing such

Table 49 The acute effect of chlorpropham (20 $\mu g ml^{-1}$) on the morphology and cytology of Tetrahymena pyriformis

Time (min)	Cell movement	Cilia movement	Cell shape: normal	Cell shape: round	Cell shape: irregular	Food vacuole appearance	Contractile vacuole appearance	Cytoplasm appearance	% staining blue with Steedman's triple stain *
5	slow	some periodic ciliary reversals (PCR's)	*			normal	normal	denser than normal	0
20	very slow	"	*			fewer than normal	"	"	0
40	active	"	*			"	1.5x larger than normal	"	0
60	restricted	cilia operating: no PCR's	*	*		not visible	"	"	0
120	very restricted	"	*	*		"		"	0
180	extremeley restricted	"	*	*	*	"	2 x larger than normal	"	30
240	some ceased moving	extended reversals	*	*	*	"	2-3x larger than normal	"	50
300	jerking action	cilia operating		*	*	present but fewer than normal	"	"	50
360	oscillation only	cilia operating; some cilia loose in media		*	*	"	"	cytoplasm 'patchy'	50
420	"	cilia rows difficult to see		*	*	not visible	3-4 x larger than normal	areas within cytoplasm appear empty	50
480	"	cilia operating; oral ciliature diff. to see		*	*	"	(1 cell 15x larger)	"	60

* Dead or dying cells of T. pyriformis stain blue with Steedman's triple stain

Table 50

The effect of chlorpropham on the duration of contractile vacuole activity in Tetrahymena pyriformis over time

	Mean duration of systole and diastole (sec)	
Time (min)	Untreated cells	Treated cells (20 $\mu g ml^{-1}$ chlorpropham)
5	25	25
20	20	21
40	-	25
60	15	28
120	20	40
180	20	35
240	-	58
300	18	55
360	34	66
420	34	102
480	34	193

vacuoles 15.0x larger. Cells with large contractile vacuoles visibly swelled outwards during systole. All contractile vacuoles underwent systole and diastole up to 480 min into chlorpropham treatments. After this time such large vacuoles were not observed to contract.

Cells exposed to 20 μgml^{-1} chlorpropham for 10h had ceased moving but still possessed cilia, although these were fewer in number. All cells that contained large vacuoles were round. After 21h many cells had lysed. The contractile vacuole occupying 20 - 30% of the cell volume (estimated from photographs) were not observed to undergo disystole. Cells exposed for 24h had up to four large vacuoles. These vacuoles collectively occupied 30 to 90% of the total cell volume. They had the appearance of other large contractile vacuoles.

Cells treated with 10 μgml^{-1} chlorpropham for 24h showed similar morphological and cytological effects to those cells treated with 20 μgml^{-1}. Cells exposed over the same period to 2 and 4 μgml^{-1} chlorpropham showed similar, but less severe, symptoms. At a concentration of 2 μgml^{-1} chlorpropham (24h) the contractile vacuoles, although 4x larger than those in untreated cells, were still able to contact. Such vacuoles were not as large as those observed with 20 μgml^{-1}. However, with 4 μgml^{-1} similar large-sized vacuoles were not observed to undergo diastole, such cells were observed for over 25 min.

The development of large contractile vacuoles with time is shown in Plate 2. Photographs, taken at T= 0,5,10,21 and 24h of cells treated with 20 μgml^{-1} chlorpropham show first the change from pyriform to round cell shape, after 5h, the development of a large contractile vacuole (10h) and effects on cell integrity after 24h. Other photographs (Plate 2) show a normal pyriform shaped cell with a large contractile vacuole (4 μgml^{-1} after 24h) and (with 40 μgml^{-1} after 24h) a many-vacuolated lysed cell.

35.3 The effect of chlorpropham on the morphology and cytology of Tetrahymena pyriformis observed under high resolution microscopy

Flask cultures of T. pyriformis cells were treated with chlorpropham (24h) prior to their preparation for the electron microscope.

Microtome section micrographs: Sections of T. pyriformis cells which had been treated for 24h with chlorpropham revealed cytological changes similar to those seen in whole cells (Plate 2). The untreated cells were pyriform and contained food vacuoles and nuclei and body and oral ciliature were present (Plate 3). Fewer of the cells treated with 2 μgml^{-1} chlorpropham were pyriform and many were circular or barrel-shaped. Many cells lacked cytoplasmic inclusions although a few did possess food vacuoles. Abnormally large vacuoles were observed in a high proportion of the chlorpropham-treated cells (Plate 3).

PLATE 2.

Acute effects of chlopropham on the morphology and cytology of Tetrahymena pyriformis (x 250 magnification).

PHOTOGRAPH I.

Cell at the beginning of the experiment showing normal pyriform shape and cilia.

PHOTOGRAPH 2.

Appearance of cell after 5h exposure to 20μgml^{-I} of chlorpropham. Cell rounded up but cilia still present. Areas within the cytoplasm have irregular edged clearings. Contractile vacuoles (not shown) are over large.

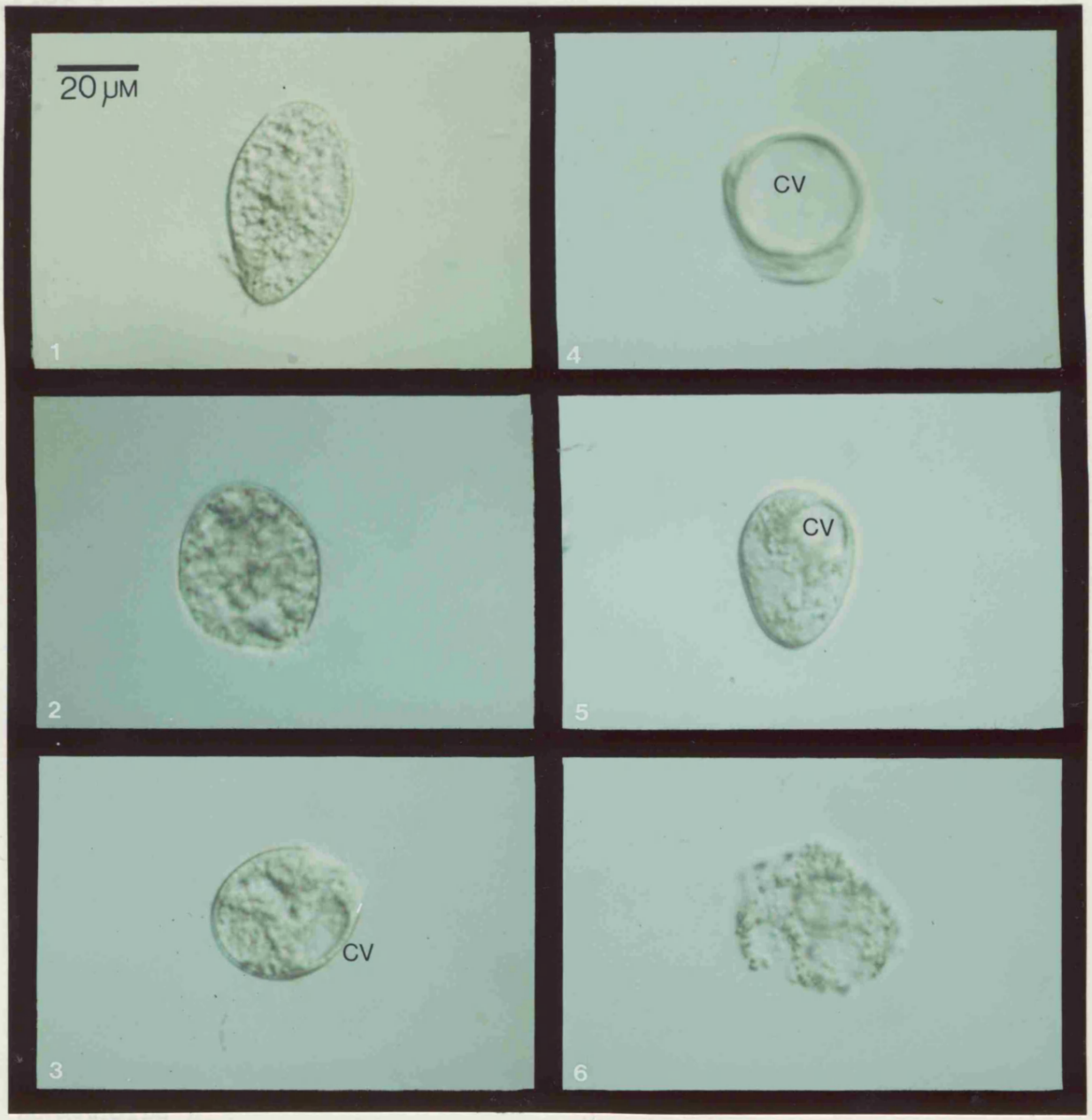
PHOTOGRAPH 3.

Cell after IOh exposure to 20 μgml^{-I} chlorpropham large vacuole (cv) present and some cilia remain visible.

PHOTOGRAPH 4.

After 24h exposure to 20 μgml^{-I} to chlorpropham the contractile vacuole (cv) occupies nearly all of the cell.

Scale marker in photograph I refers to all photographs in the plate.



PHOTOGRAPH 5.

A cell exposed to chlorpropham ($4\mu gml^{-1}$) for 24h.
Cell retains pyriform shape but has an enlarged
contractile vacuole (cv).

PHOTOGRAPH 6.

Cell after 24h exposure to 40 μgml^{-1} chlorpropham.
Cell lysed.

Scale marker in photograph I refers to all photographs.

PLATE 3.

The cytological appearance of Tetrahymena pyriformis cells exposed to chlorpropham for 24h: Thin section light micrographs stained with methylene blue.

PHOTOGRAPH I.

Untreated T.pyriformis cell (x400) showing characteristic pyriform shape. Food vacuoles (FV) are present along with the dense staining nuclei (N) and somatic and oral ciliature.

PHOTOGRAPH 2.

Cells treated with 2 μgml^{-I} chlorpropham (x 400). Fewer pyriform shaped cells. Cytoplasm appears dense with few inclusions although some cells still posses food vacuoles (FV).

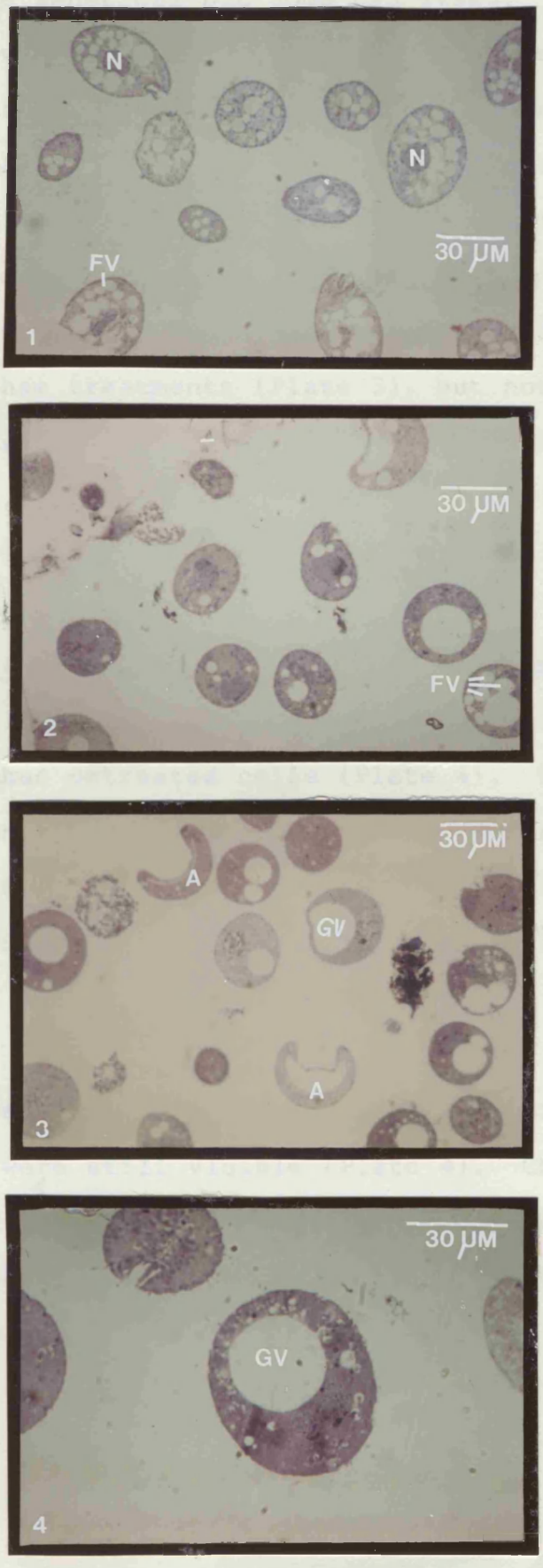
PHOTOGRAPH 3.

Cells treated with 4 μgml^{-I} chlorpropham (x 400). Some cells have lysed and 40% have 'giant' vacuoles (GV). Nuclei are present but appear less dense than in untreated cells. The crescent shaped cells(A) were thought to be artefacts of the fixation process.

PHOTOGRAPH 4.

Single cell (x I000) treated with 4 μgml^{-I} chlorpropham showing a 'giant' vacuole (GV) surrounded by dense staining cytoplasm. No food vacuoles were seen.

PLATE 3.



A higher level of chlorpropham (4 μgml^{-1}) led to a larger proportion of cells containing a 'giant' vacuole. Most cells were spherical but some had lysed. Oral ciliature was observed in these cells but at a lower frequency than in untreated populations. Chlorpropham-treated cells (4 μgml^{-1}) had less dense cytoplasm and nuclei were observed. Nuclei of treated (4 μgml^{-1}) cells appeared less dense and more granular than those in untreated cells. The crescent-shaped cells observed in all chlorpropham treatments (Plate 3), but not in untreated samples, were possibly artefacts of the preparation technique, the procedure possibly collapsing the giant vacuole (Steedman, personal communication).

Scanning Electron micrographs: T. pyriformis cells treated with chlorpropham (2 and 4 μgml^{-1}) appeared more rounded than untreated cells (Plate 4). With 4 μgml^{-1} chlorpropham the majority of cells had irregular indentations and such cells were often curved or crescent shaped. Such structures were thought to be fixation artefacts (see Plate 3).

Treated cells generally possessed fewer cilia although kineties were still visible (Plate 4). Changes in the outer surface of the pellicle were apparent with 4 μgml^{-1} chlorpropham (Plate 5). The surface was convoluted and more deeply etched than with untreated cell surfaces. Contractile vacuole pores, clearly visible in untreated cells, were present but distorted in treated cells (4 μgml^{-1}) (Plate 5).

The oral ciliature of cells was also affected by chlorpropham (Plate 6). Chlorpropham (4 μgml^{-1}) reduced the number of cilia around the buccal cavity and distorted the buccal cavity. Cilia appeared short and blunt-ended. No adoral zone membrane was detected.

Transmission electron micrographs: Cell size was reduced (2-4 μgml^{-1}) in cells exposed to chlorpropham for 24h. Chlorpropham induced the formation of a large single vacuole and the disappearance of small vacuoles (Plate 7), the frequency of occurrance of the large vacuoles increased with concentration. At 20 μgml^{-1} chlorpropham caused cells to lose this vacuole but increased the incidence of numerous small vesicles. Such vesicles, believed to be autophagic vacuoles, were seen in other chlorpropham-treated cells (2-4 μgml^{-1}) but at a reduced frequency. With 4 and 20 μgml^{-1}, chlorpropham areas of the pellicle appeared to be discontinuous with the cytoplasm forming undulating bulges. Mitochondria, endoplasmic reticulum and kinetodesmal fibres were observed in untreated and 2 μgml^{-1}-treated cells.

Chlorpropham at 4 and 20 μgml^{-1} decreased the oral ciliature of the cells (Plate 8).

The mitochondria in cells treated with chlorpropham appeared rounded with slight bulges (2 μgml^{-1}) and devoid of an internal structure (4 μgml^{-1}) (Plate 9). In

PLATES 4 - 6.

The appearance of Tetrahymena pyriformis cells after 24h exposure to chlorpropham: Scanning Electron Micrographs.

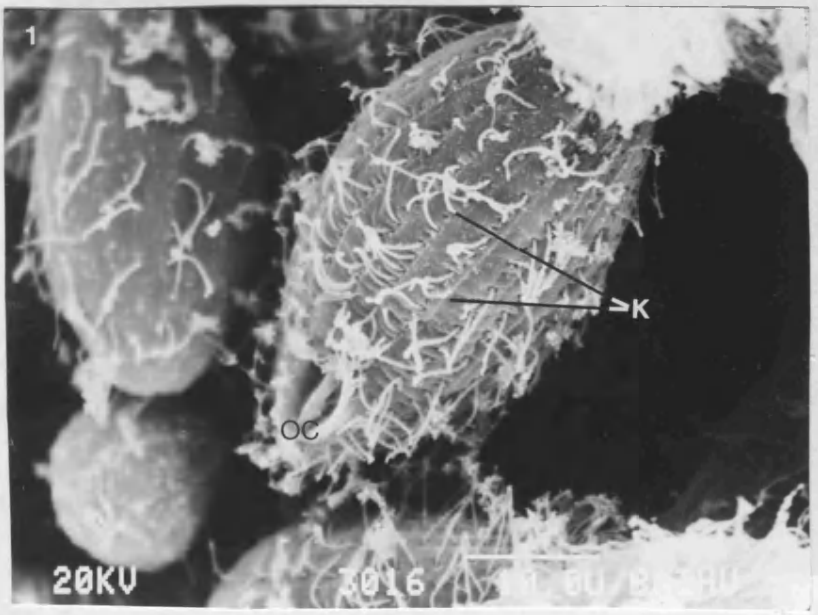
PLATE 4.

The appearance of the cilia.

PHOTOGRAPH I. Untreated cells showing oral cavity (OR) with associated ciliature with kineties (K).

PHOTOGRAPH 2. Cells exposed to 2 μgml^{-I} chlorpropham. Cilia (C) appear blunt ended and fewer in number.

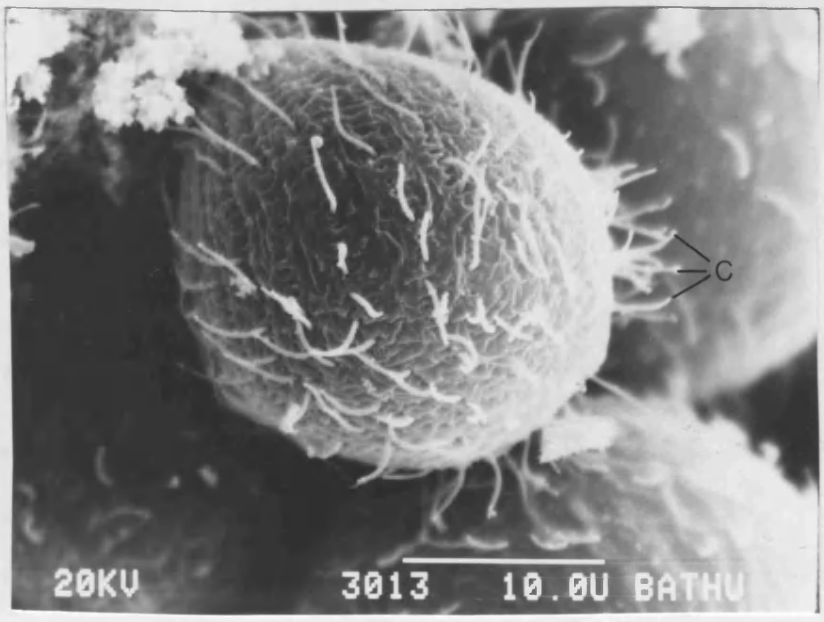
PHOTOGRAPH 3. Cells exposed to 4 μgml^{-I} chlorpropham. Cilia appear less numerous. The large cavity (A) was believed to be an artefact of the fixation process (cf Plate 3)



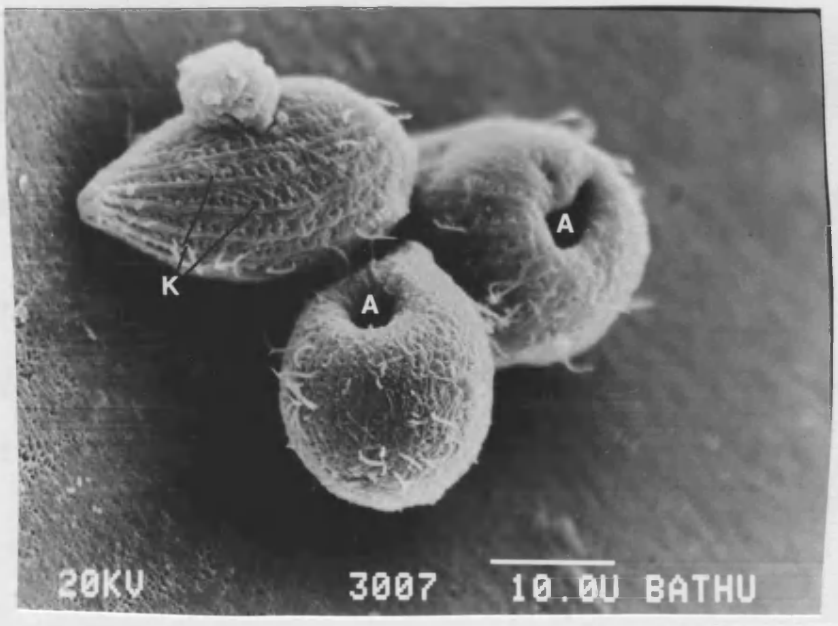
PHOTOGRAPH I.

PLATE 4.

The appearance of the cilia.



PHOTOGRAPH 2.



PHOTOGRAPH 3.

PLATE 5.

The appearance of the pellicle

PHOTOGRAPH I.

Untreated cell showing the posterior end and detail of the contractile vacuole (CV). Pellicle(P) surface appears smooth and individual cilia (C) clearly visible.

PHOTOGRAPH 2.

Cell treated 24h with 4 $\mu g ml^{-I}$ chlorpropham, detail of posterior end. Pellicle (P) surface uneven with large depressions (2) these possibly represent artefacts induced by the fixation process or may be the remains of the contractile vacuole pores (CVP).

PLATE 6.

The appearance of oral ciliature.

PHOTOGRAPH I.

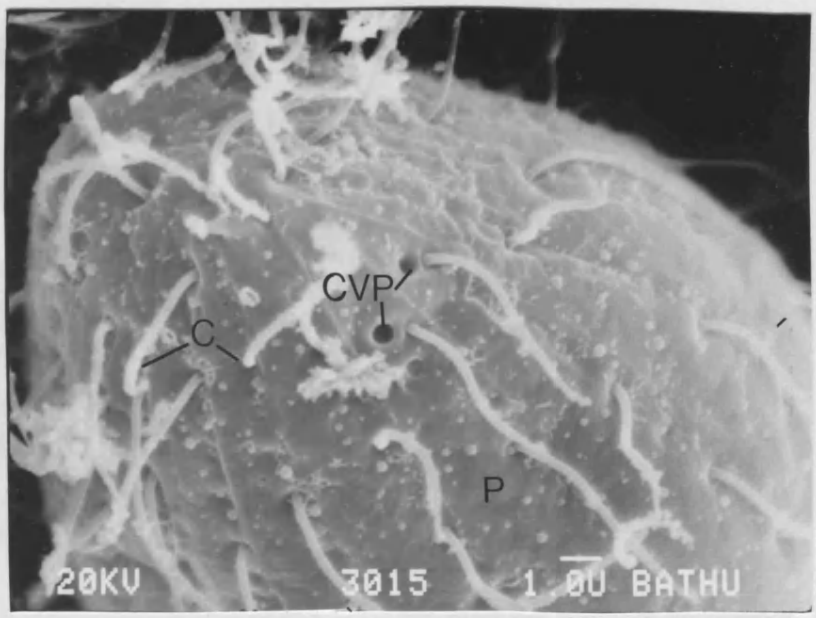
Untreated cell showing details of the oral ciliature. Individual ciliature (C) visible as well as the buccal cavity (BC) and undulating membranes (UM) (top of photograph).

PHOTOGRAPH 2.

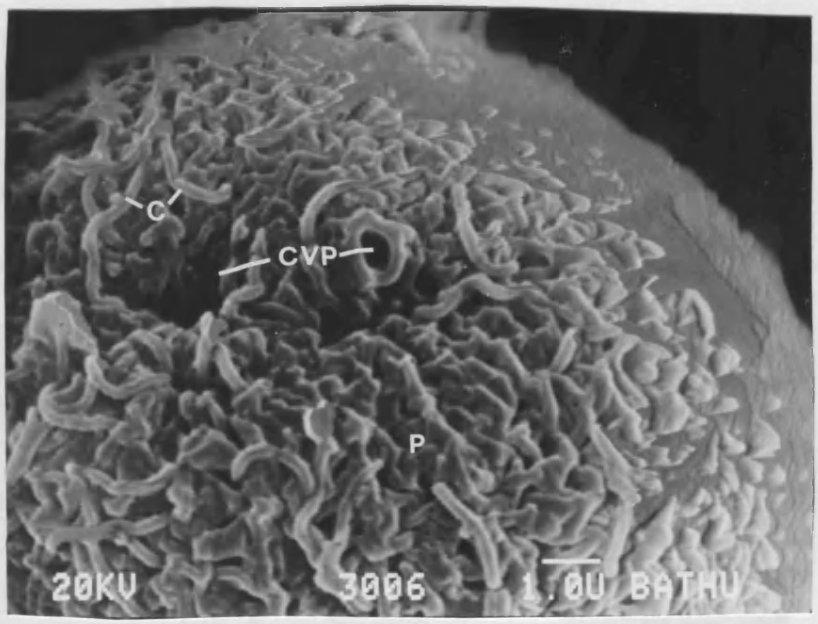
Cell treated with $4 \mu g ml^{-I}$ chlorpropham. Cilia (C) of the buccal cavity (BC) appear indistinct, short and thickened. No detail of the structure of the oral zone can be seen.

PLATE 5.

The appearance of the pellicle.



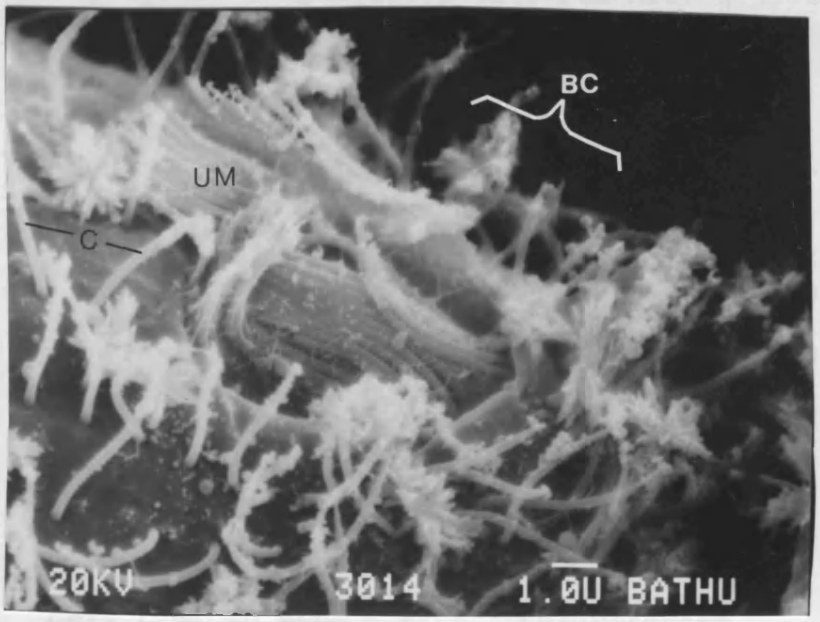
PHOTOGRAPH I.



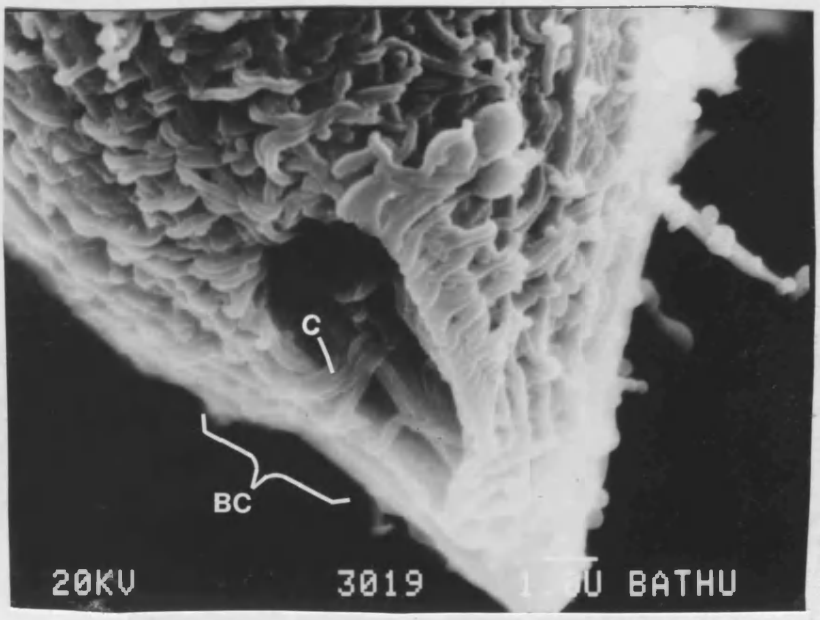
PHOTOGRAPH 2.

PLATE 6.

The appearance of the oral ciliature.



PHOTOGRAPH I.



PHOTOGRAPH 2.

PLATES 7 - IO.

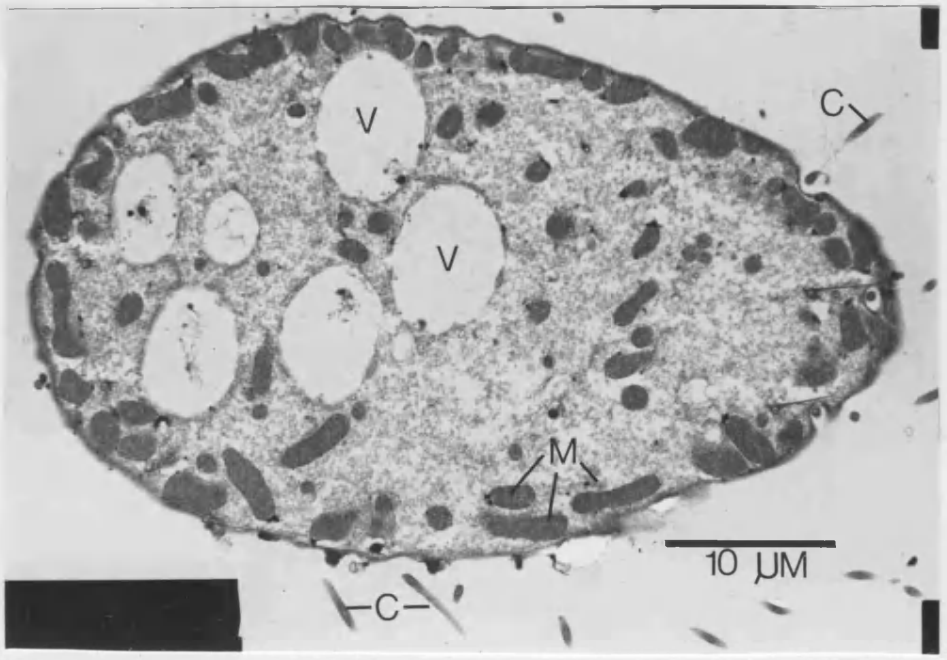
The appearance of Tetrahymena pyriformis cells after 24h exposure to chlorpropham: Transmission Electron Micrographs.

PLATE 7. The appearance of whole cells.

PHOTOGRAPH I. Untreated cell showing somatic cilia (C), mitochondria (M) and vacuoles (V).

PHOTOGRAPH 2. Cell treated with 2 $\mu g ml^{-I}$ chlorpropham, the normal pyriform shape is absent and a 'giant' vacuole (GV) is present. Cilia, both oral (OC) and somatic (C), and mitochondria (M) are visible. Small vacuoles not present.

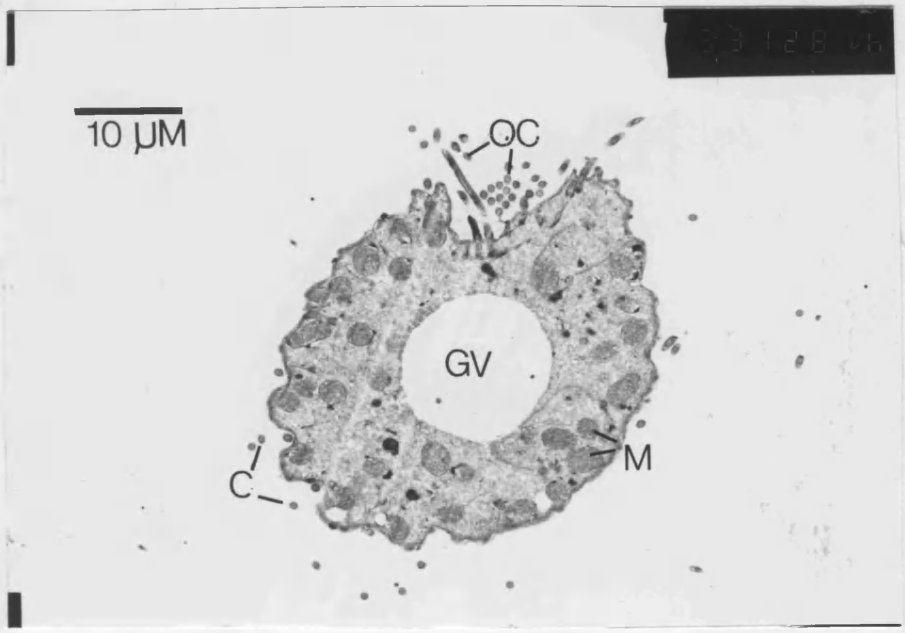
PHOTOGRAPH 3. Cell treated with 4 $\mu g ml^{-I}$ chlorpropham. Cytoplasm appears less dense with distinct clear zones (CZ). Mitochondria are present but are again less dense than the control cells. Small vessicles (SV) were also observed.



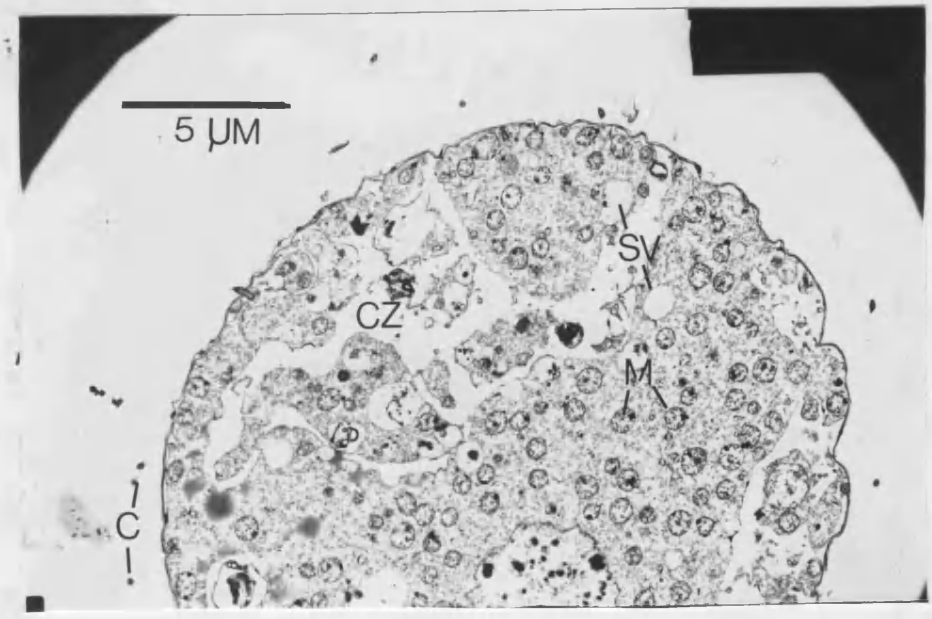
PHOTOGRAPH I.

PLATE 7.

The appearance of whole cells.



PHOTOGRAPH 2.



PHOTOGRAPH 3.

PLATE 8.

The appearance of the oral ciliature.

PHOTOGRAPH I.

Untreated cell showing cross section of the oral cavity cilia (C), arranged in rows, which are clearly visible. A singular distinct row (possibly the undulating membrane, UM) can be seen. A mass of cilia at the bottom of the photograph may represent the adoral zone of membranes (AZ).

PHOTOGRAPH 2.

Cell treated (24h) with 4 μgml^{-I} chlorpropham showing detail of the oral cavity. Cilia (C) are fewer in number and their organization into the various membranes is not apparent.

PLATE 9.

The appearance of the mitochondria.

PHOTOGRAPH I.

Untreated cell showing the mitochondria (M) aligned around the cell perifery (P).

PHOTOGRAPH 2.

Cell exposed to 2 μgml^{-I} chlorpropham (24h). Mitochondria (M) are rounded and damaged. The internal structure is evident.

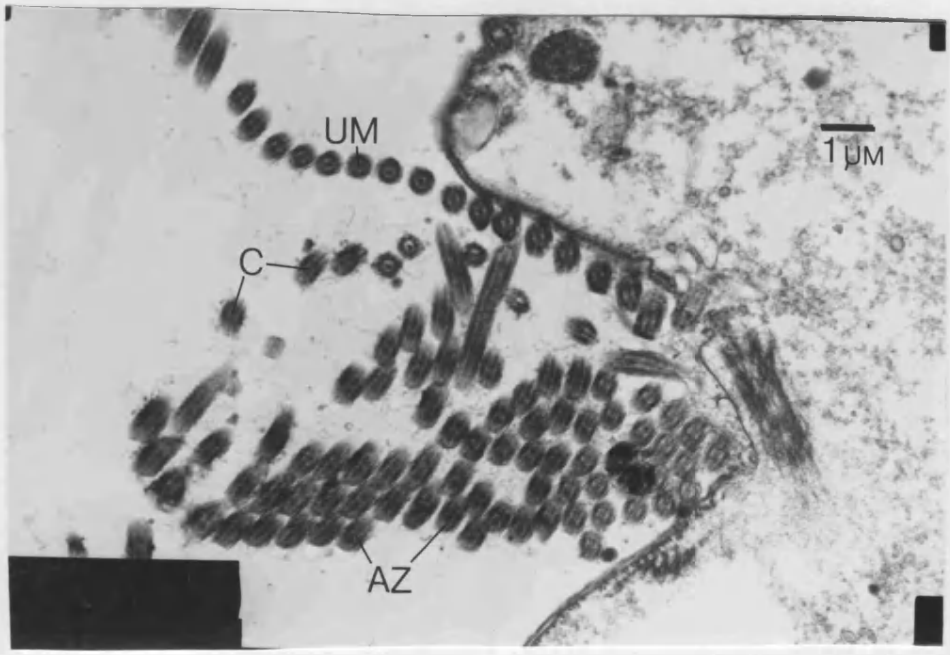
PHOTOGRAPH 3.

A cell treated with 4 μgml^{-I} chlorpropham (24h). Mitochondria (M) are situated throughout the cytoplasm. The mitochondria are round and are internally disrupted.

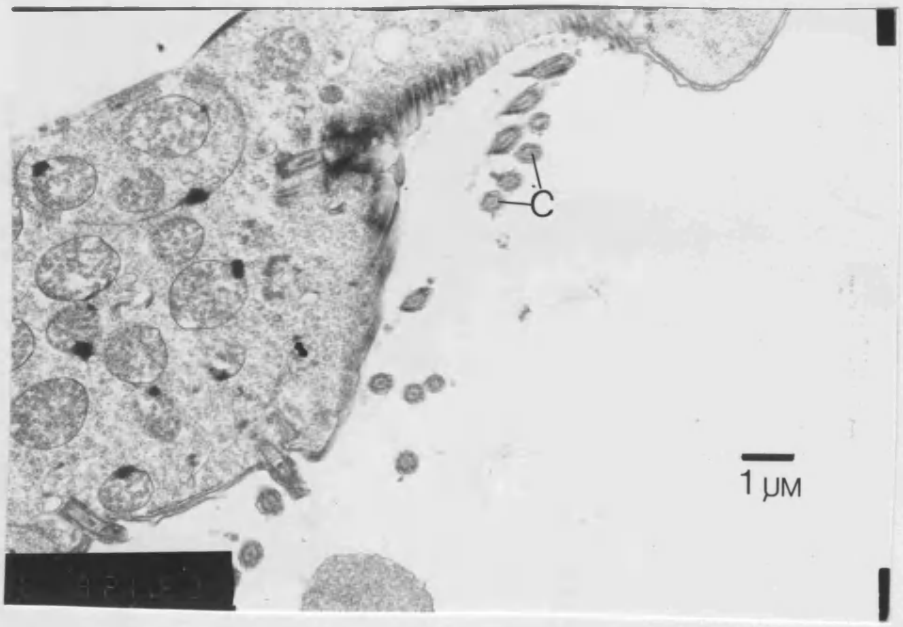
Scale marker in photograph I refers to all photographs in the plate.

PLATE 8.

The appearance of the oral ciliature.



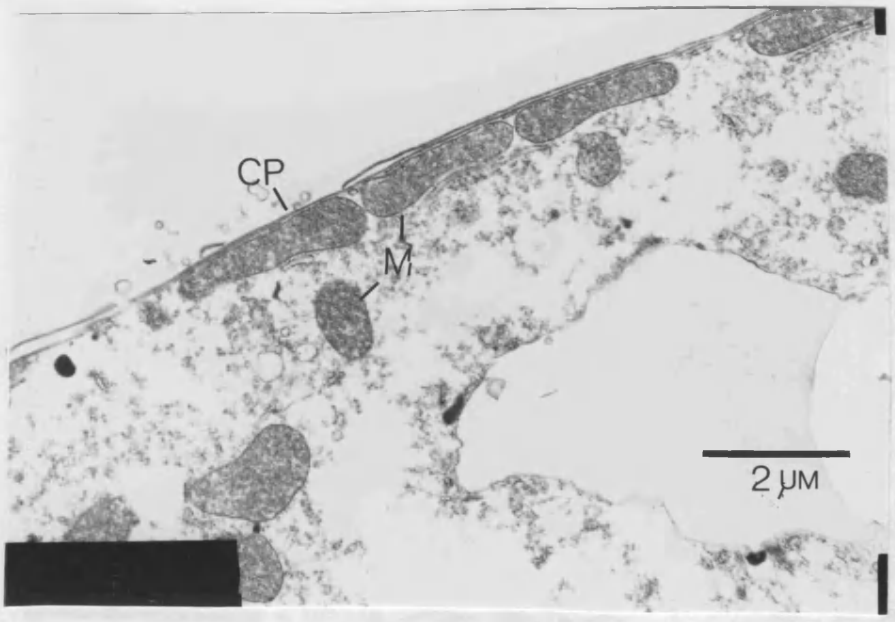
PHOTOGRAPH I.



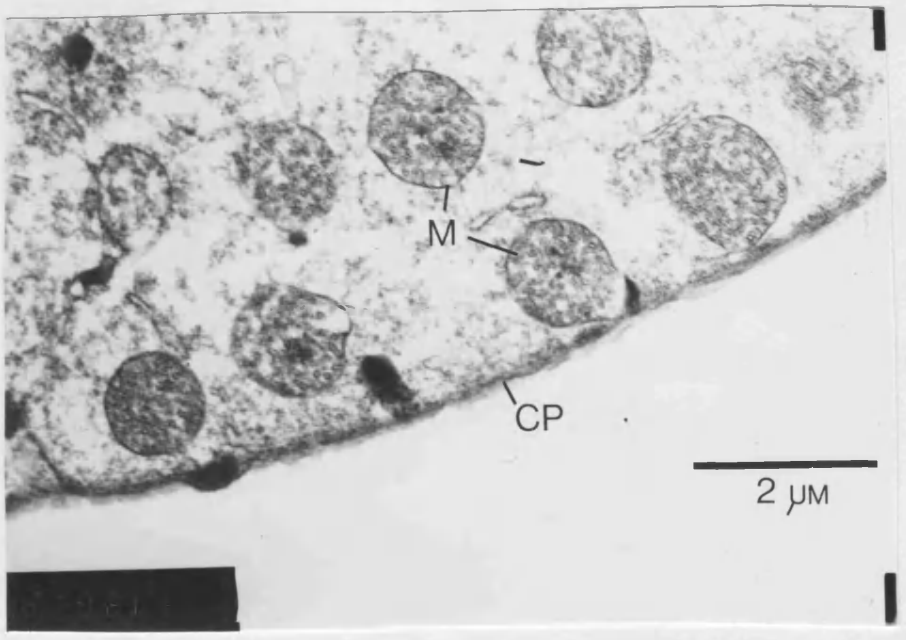
PHOTOGRAPH 2.

PLATE 9.

The appearance of the mitochondria.



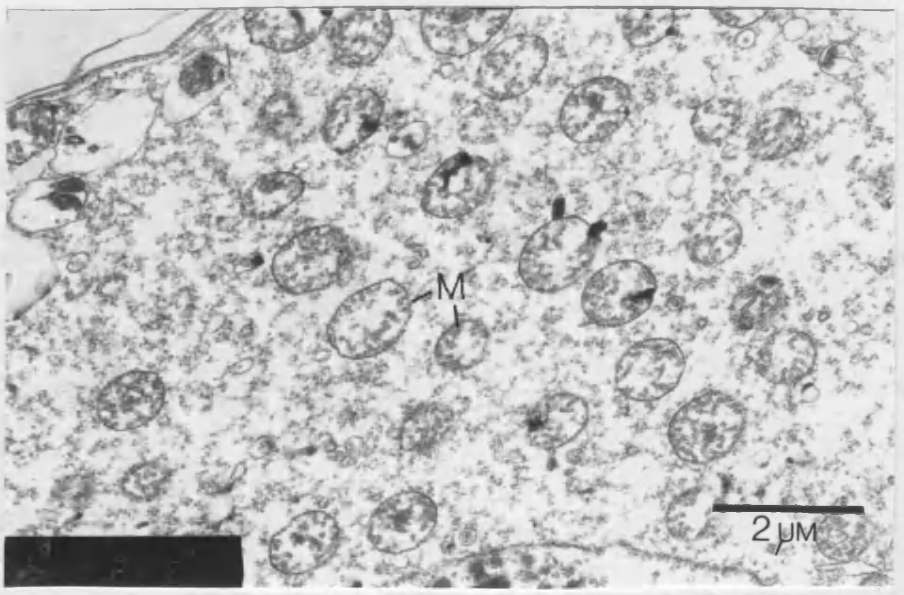
PHOTOGRAPH I.



PHOTOGRAPH 2.

PLATE 9 CONT/....

The appearance of the mitochondria.



PHOTOGRAPH 3.

PLATE IO.

The appearance of the nucleus.

PHOTOGRAPH I.

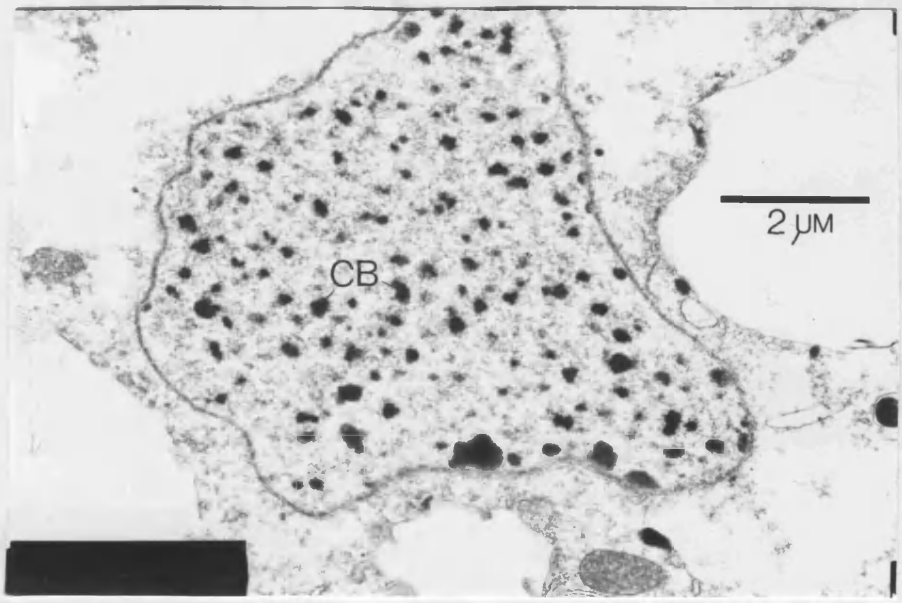
The nucleus of an untreated cell. The numerous electron dense areas are believed to be chromatin bodies (CB).

PHOTOGRAPH 2.

The nucleus of a cell treated (24h) with 2 μgml^{-I} chlorpropham. Large electron dense bodies (CB) appear concentrated to one side of the nucleus, this was not thought to be attributable to centifugation.

PHOTOGRAPH 3.

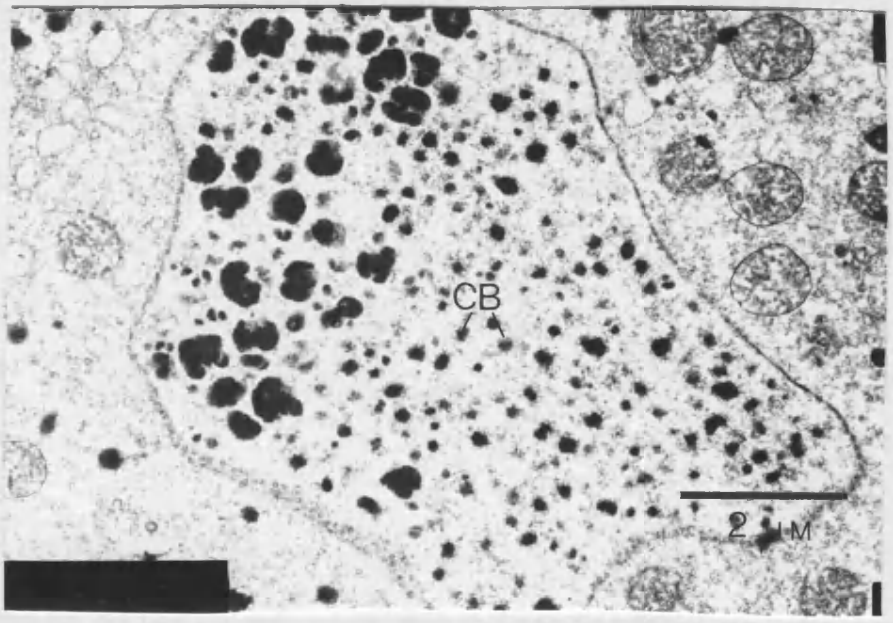
The nucleus of a cell treated (24h) with 4 μgml^{-I} chlorpropham. The nucleus appears round and less dense than the control. Electron dense bodies (CB) are present but fewer in number than in either the control of 2 μgml^{-I} chlorpropham - treated cells.



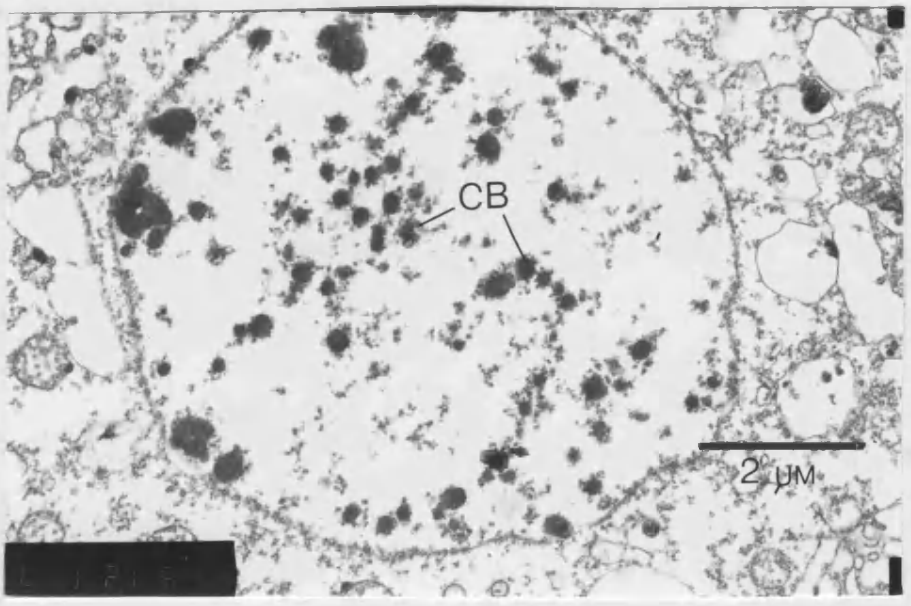
PHOTOGRAPH I.

PLATE IO.

The appearance of the nucleus.



PHOTOGRAPH 2.



PHOTOGRAPH 3.

untreated cells round endoplasmic reticulum appeared adjacent to the mitochondria. This was not observed in treated cells. The cytoplasm became progressively less dense with increasing chlorpropham concentrations.

Chlorpropham at μgml^{-1} caused rounding of the nucleus and a loss of spacial continuity of chromatin bodies (Plate 10). Discrete areas of electron dense material accumulated at one side of the nucleus but no change in shape was observed with 2 μgml^{-1}. In untreated cells no such changes were observed (Plate 10).

35.4 Chronic effects of some phenylcarbamates on the morphology and cytology of Tetrahymena pyriformis

Chlorpropham: The frequency of occurrence of spherical cells in cultures grown in the presence of chlorpropham is given in Fig. 62. In untreated cells there was a tendency for a small percentage to become spherical towards the end of the experiment. Those cells were in the stationary phase of the growth curve (Fig. 53). However, with chlorpropham (4, 20 and 40 μgml^{-1}) spherical cells were observed after 1d, whilst with 2 μgml^{-1} chlorpropham such cells were seen after 2d. As with cell length the frequency of spherical cells appears dose-dependent; increasing concentrations progressively reducing the ratio of cell length to width.

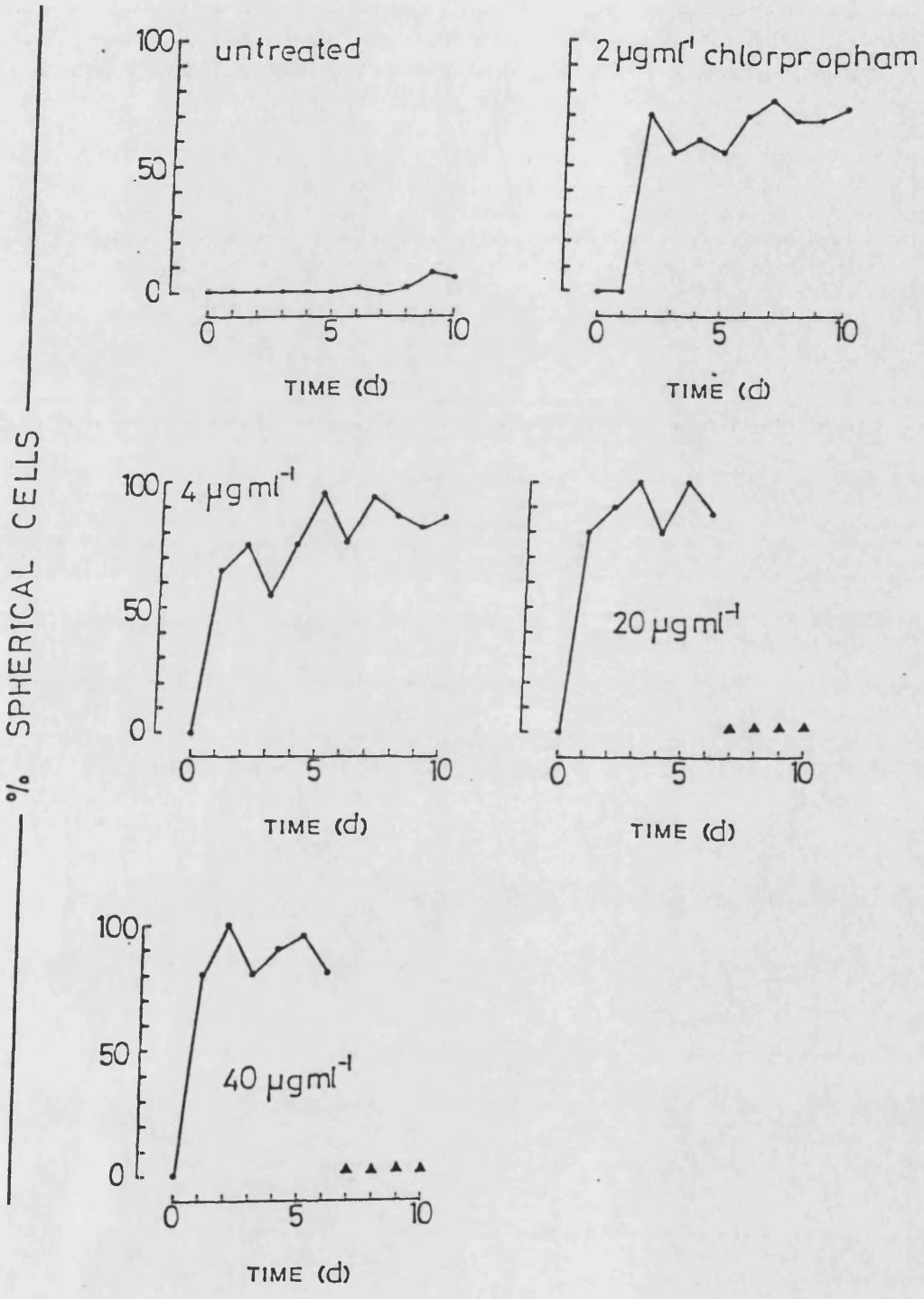


FIG. 62

The proportion of spherical cells in cultures of Tetrahymena pyriformis treated with chlorpropham. Cultures were grown in the presence of the herbicide for up to 10d in 50ml of PY medium in Erlenmeyer flasks at 20°C.

There was a marked difference between cells that were spherical in the control and those found in chlorpropham treatments. The control cells retained their cilia, which were evenly spaced around the circumference, and were of uniform appearance. With chlorpropham treated cells, the cilia appeared more sparse and unevenly distributed around the cell. Chlorpropham (20 μgml^{-1}) treated cells were de-ciliated and a large vacuole (possibly contractile) was visible in each cell (Plate 11). With 2 and 4 μgml^{-1} chlorpropham the incidence of these 'giant' vacuoles in the population was low (5 and 12% respectively after 48h, Fig. 63). With 20 μgml^{-1} there was a sharp increase in the number of such cells in the population after 24h, reaching a maximum after 96h when 75% of the population were vacuolated in this way. This declined to 44% after 144h and paralleled the decline in total cell numbers with this concentration (Fig. 53). With 40 μgml^{-1} chlorpropham no cells were observed to contain a 'giant' vacuole. This finding coincides with the observation that after 24h, cells treated with 40 μgml^{-1} chlorpropham showed no internal differentiation of cilia (Plate 11) and had lysed (Fig. 53).

In untreated cells 'giant' vacuoles were rarely observed. Such cells (late stationary phase) were spherical and possessed uniformly distributed cilia unlike chlorpropham treated cells (Plate 11).

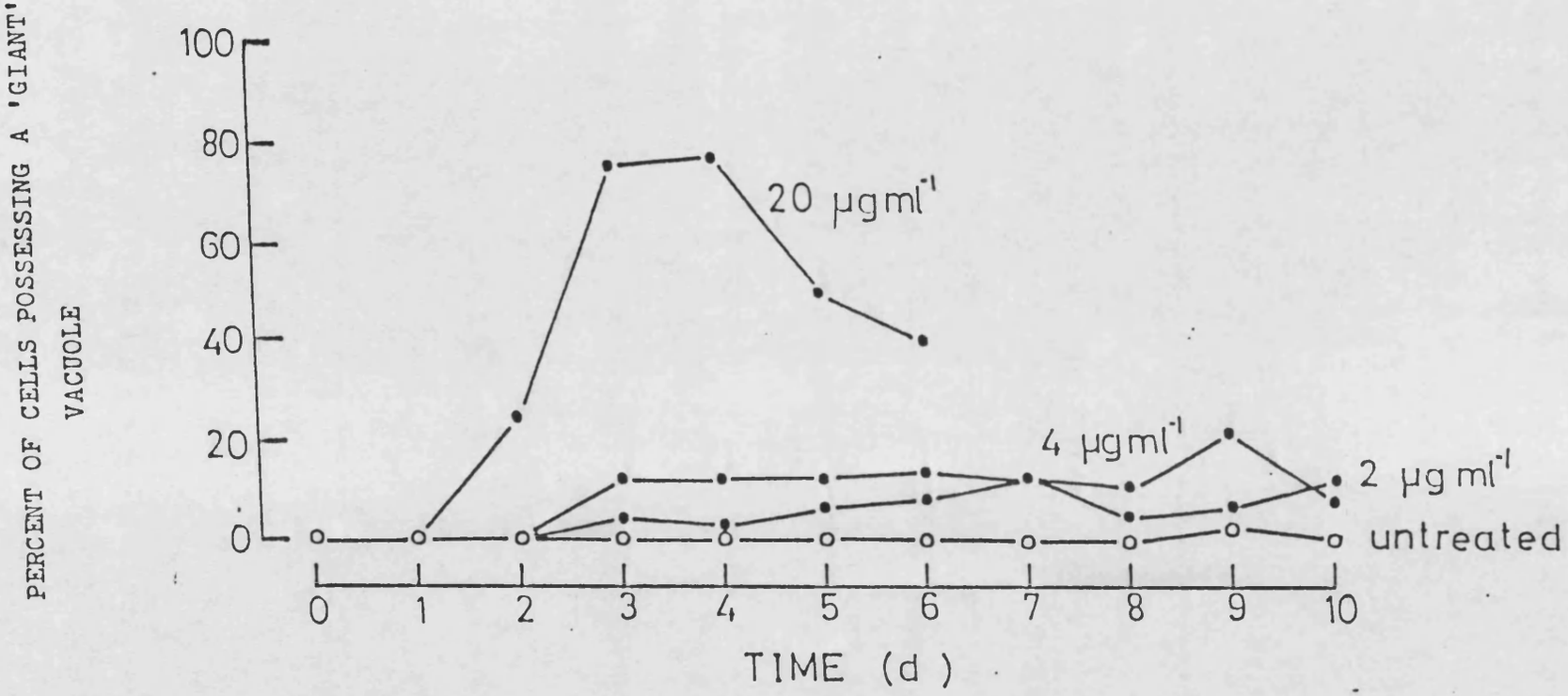


FIG. 63

The influence of chlorpropham on the occurrence of 'giant' vacuoles within cells of Tetrahymena pyriformis .

PLATE II.

The morphology of Tetrahymena pyriformis cells grown in the presence of chlorpropham in PY medium in Erlenmeyer flasks (x400 magnification, phase contrast microscopy). Pictures taken after 72h exposure to chlorpropham.

PHOTOGRAPH I. Cells grown in the absence of chlorpropham (untreated control) showing division.

PHOTOGRAPH 2. Cell grown in the absence of chlorpropham (untreated control).

PHOTOGRAPH 3. Cell grown in the presence of 4µg/ml chlorpropham. Cell rounded in appearance. cilia present but little cell movement detected.

PHOTOGRAPH 4. Cell grown in the presence of 4µg/ml chlorpropham. Cytoplasm concentrated towards cell centre. Cilia present but no oral apparatus detected.

PHOTOGRAPH 5. Cell grown in the presence of 20µg/ml chlorpropham. No cilia observed, no movement detected. Large vacuole (GV) and cytoplasm concentrated around the periphery. Small vacuoles observed (not shown).

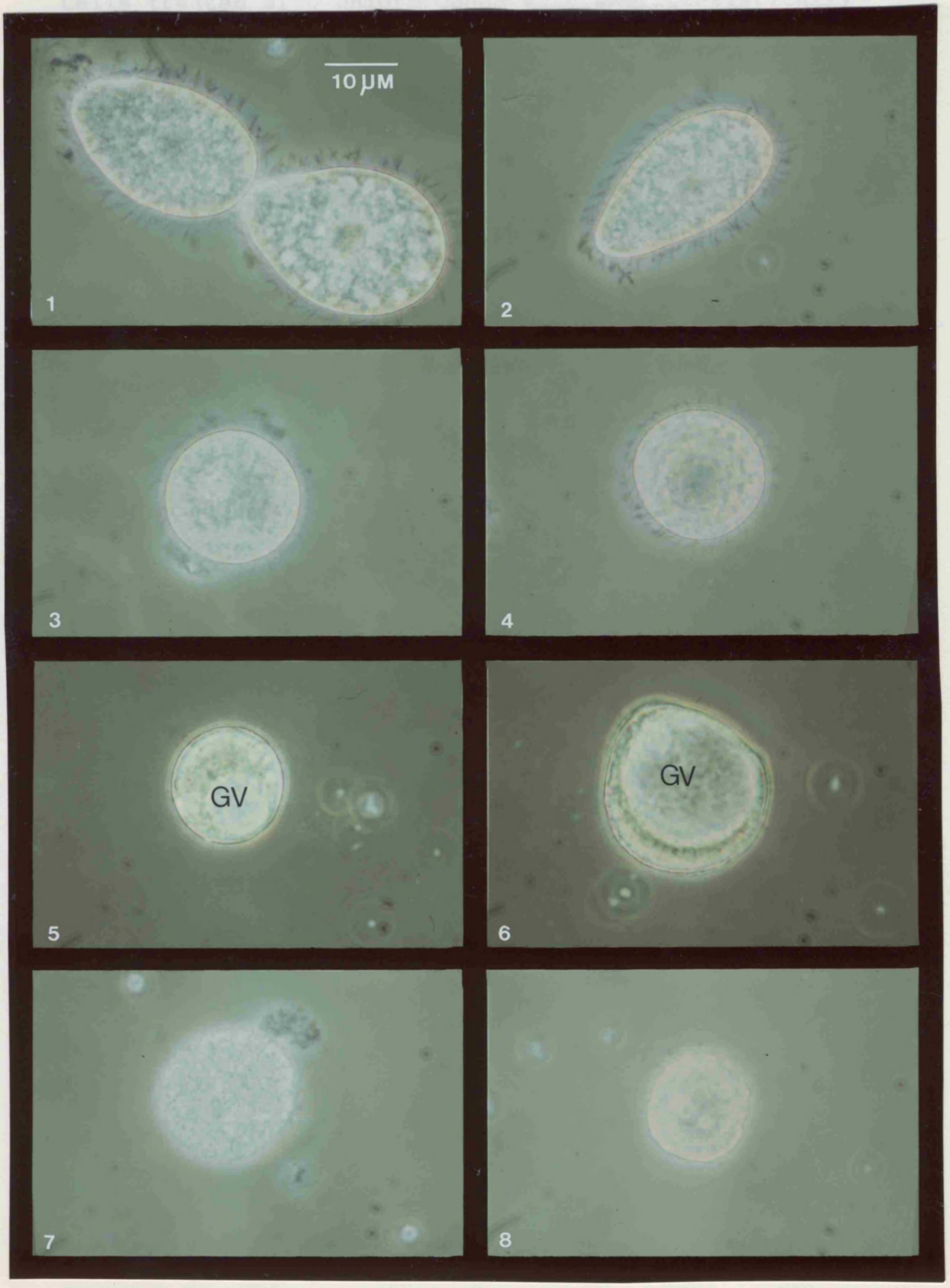
PHOTOGRAPH 6. Cell grown in the presence of 20µg/ml chlorpropham. Cilia absent, large vacuole (GV) present nearly filling the whole cell. Smaller vacuoles not observed.

PHOTOGRAPH 7. Cell grown in the presence of 40µg/ml chlorpropham. Cilia absent, cell integrity lost. Cell shape wrinkled, no large vacuole.

PHOTOGRAPH 8. Cell lysing(40 µg/ml chlorpropham).

Scale marker in photograph I refers to all phorographs in the plate.

PLATE 11



Cells treated with chlorpropham were characteristically poorly differentiated from the background. The appearance of fixed and unfixed cells was identical. The nucleus was not apparent in chlorpropham-treated cells.

Morphological changes induced by chlorpropham occurred progressively. After 48h T. pyriformis cells treated with 4 $\mu g ml^{-1}$ chlorpropham were spherical and dense, some with cilia, others without. No enlarged vacuole was observed and no food vacuoles were seen. Oral ciliature could not be detected. By 72h 12% of the cells were vacuolated and some had mis-shapen areas around the pellicle. Such morphological changes were also dose-dependent. For instance, with 20 $\mu g ml^{-1}$ chlorpropham after 48h, 25% of the population possessed enlarged vacuoles, all cells were rounded and no ciliated cells were observed.

After 48h exposure to chlorpropham (20 $\mu g ml^{-1}$) some cells contained more than one 'giant' vacuole (maximum 4). No 'giant' vacuoles were observed to contract. Untreated cells had normal size contractile vacuoles which underwent dysystolic activity.

An artefact of glutaraldehyde fixation (1.5% v/v 1:1) was the disappearance of contractile vacuoles in untreated cells. The enlarged vacuoles of chlorpropham-treated cells did not disappear even after prolonged fixation (14d).

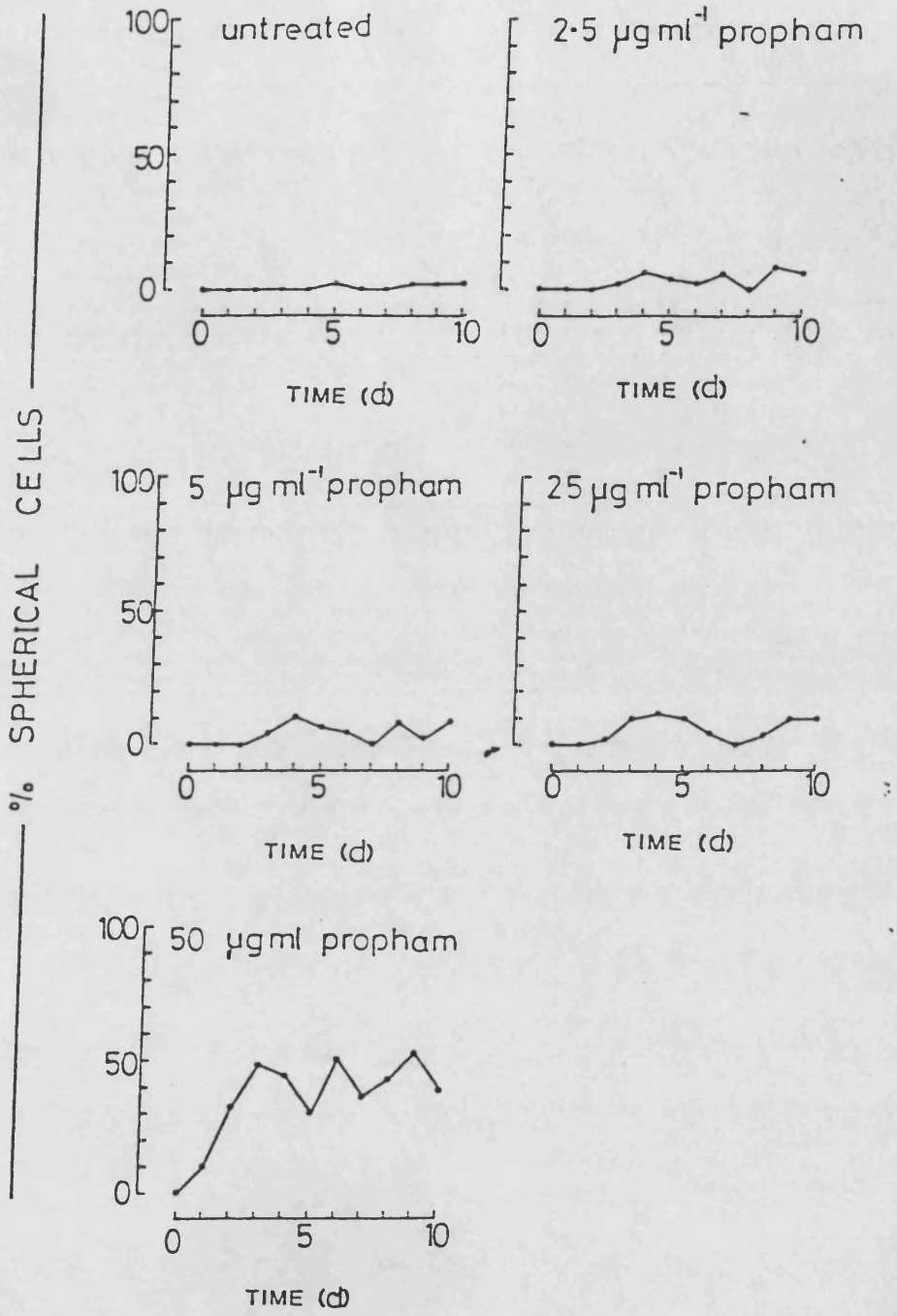


FIG. 64

The induction of spherical cells in cultures of Tetrahymena pyriformis cells treated with propham. Cells were grown in the presence of the herbicide for up to 10d in 50 ml of PY medium in flasks at 20°C.

Propham: Propham-treated cells tended at the higher doses to form spherical cells (Fig. 64). However, at the highest level tested (50 μgml^{-1}) propham induced only 50% of the population to become spherical compared with 70% with 2 μgml^{-1} chlorpropham (Fig. 62). The onset of this effect also differs between the two herbicides. With propham the onset of rounding-up was 3d for 2.5 and 5 μgml^{-1}, 2d for 25 μgml^{-1} and 1 d for 50 μgml^{-1}. With chlorpropham the delay is shortened with 2 μgml^{-1} taking 2d to exhibit rounding-up and all other doses (4, 20 and 40 μgml^{-1}) 1d. The exact times for this commencement of rounding-up cannot be determined from this experiment due to the sample interval being set at 24h. The commencement of rounding-up in untreated cells occurred at 5d (Fig. 64).

Enlarged vacuoles similar to those encountered in T. pyriformis cells treated with chlorpropham also occured in propham-treated cells. However, the incidence was considerably lower (4% of the population at its maximum) and such cytological changes were only seen with 25 and 50 μgml^{-1} propham.

Broadly speaking, the effects of propham on the morphology of T. pyriformis were similar to those of chlorproham, differing only in the degree of severity. For instance, with chlorpropham, cells in the later stages of division were never observed in treatments greater than 2 μgml^{-1}.

At 2 μgml^{-1} dividing cells did not appear normal, being stunted and occasionally oddly shaped. However, with propham, division in the stages prior to separation of the daughter cell have been observed in concentrations up to 25 μgml^{-1}. These observations correlated with the effects of both herbicides on population growth (Fig. 53,54). At 50 μgml^{-1} proham cells appeared round, some areas of cytoplasm were empty, while others were dense and the nucleus was visible.

A feature of cells treated with both chlorpropham and propham was the apparent lack of oral ciliature or discernible oral apparatus. With chlorpropham these were not evident at concentrations greater than 4 μgml^{-1} whilst with propham such structures were not observed in 50 μgml^{-1} treated cells only.

Barban: Barban did not cause the formation of any spherical cells or cells with abnormal shapes. Enlarged vacuoles were not present in any untreated cells and all cells had nuclei, cilia, oral apparatus and were observed to undergo division.

In Fig. 65 the ratios of cell length to cell width have been plotted to compare the effect of certain concentrations of some phenylcarbamates. As the ratio tends to 1:1 so T. pyriformis cells tend towards a spherical shape. With chlorpropham, even at the lowest

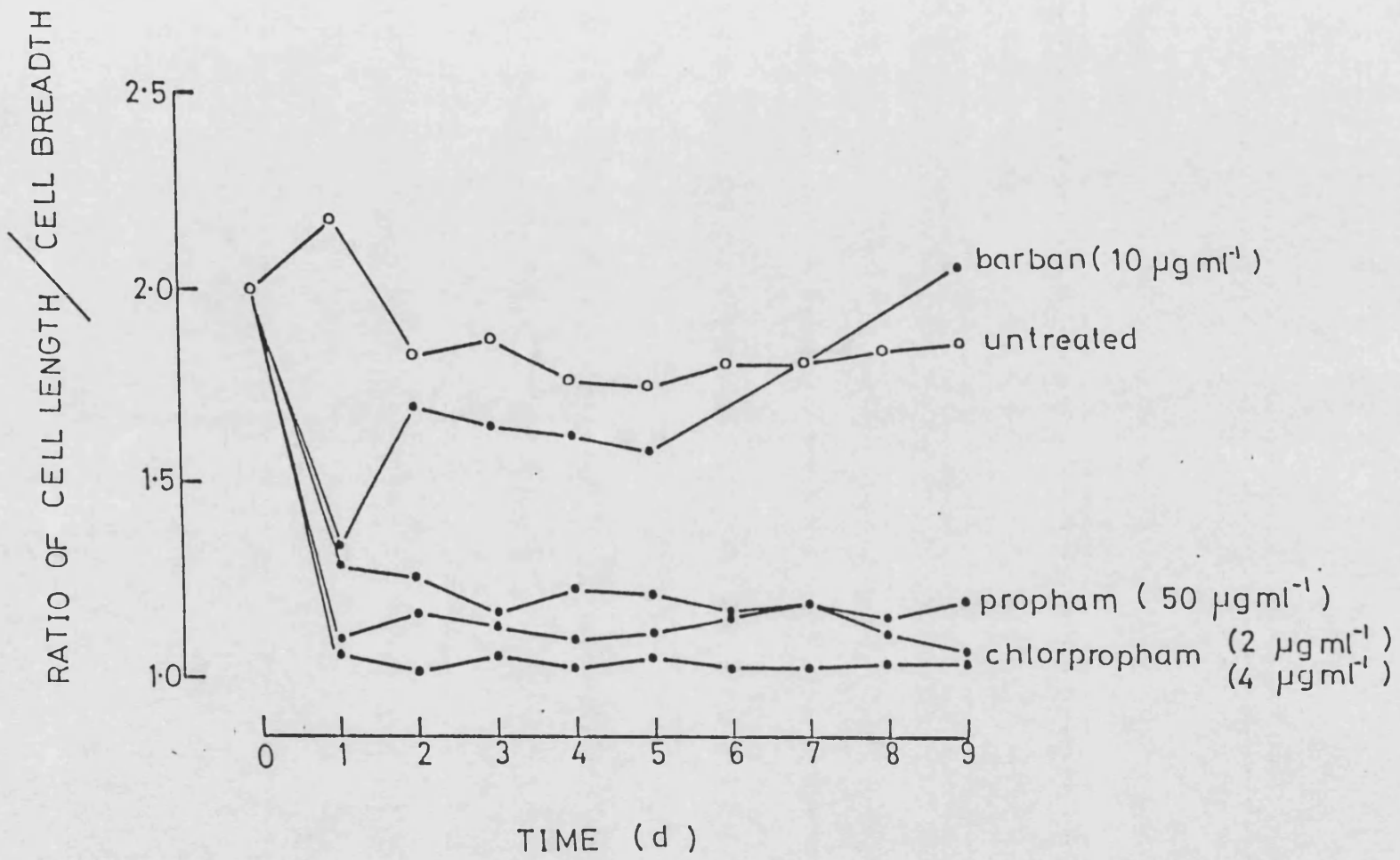


FIG. 65

The effect of some phenylcarbamate herbicides on the morphology of Tetrahymena pyriformis. Cells were grown in the presence of the herbicide for up to 9d in 50 ml of PY medium in flasks at 20°C.

concentration tested, (2 μgml^{-1}) dimension ratios approached 1:1 after 1d. With propham the ratios approached 1:1 in cells treated with the highest concentration only (50 μgml^{-1}). The initial effect of barban on reducing cell length is clearly shown though even at its nadir the ratio is sufficiently higher than 1:1 to give blunt-ended, ovoid cells and not spherical ones. Recovery of cell length in barban treated cultures is clearly shown along with what appears to be cells of a greater length than untreated ones (9d).

Based on EFC values (estimated field concentrations) the order of inhibitory activity on cell numbers, cell size, morphology and cytology was chlorpropham > propham > barban.

36.0 The effect of chlorpropham and diuron on the respiration rate of Tetrahymena pyriformis cells

Chlorpropham (2,4 and 20 μgml^{-1}) and diuron (1.5,3 and 20 μgml^{-1}) had no effect on the respiration rate of *T. pyriformis* cells.

37.0 The influence of some pesticides on the ability of Tetrahymena pyriformis cells to form food vacuoles

37.1 A note on food vacuole formation in T. pyriformis shape and enumeration

Food vacuoles are formed at the base of the cytopharynx and once formed they move about in the cytoplasm until digestion of the contents is complete. The contents of a spent vacuole are ejected into the environment through a fixed pore in the pellicle, the cytoproct (Jones, 1974). Examples of the variation in food vacuole size and shape are shown in Plate 12. Changes in food vacuoles during their passage through the cytoplasm may account for differences in size and density of India ink particles. The line-like appearance of ink particles (photograph 3, Plate 13) was found in a number of cells in anterior positions and, when visible, extending towards the oral cavity. This was assumed to be a newly-forming food vacuole at the base of the cytopharynx and was included in total food vacuole counts.

The use of phase contrast or differential interferance contrast (Nomarski) optics or the use of any objective other than x25 did not facilitate observation of vacuoles, although varying the focal plane greatly assisted food vacuole enumeration.

Tetrahymena pyriformis forms food vacuoles continuously except during the later stages of division (Chapman-Anderssen & Nilsson, 1969).

Observations on the ability of the cells to form food vacuoles, after exposure to herbicides for different

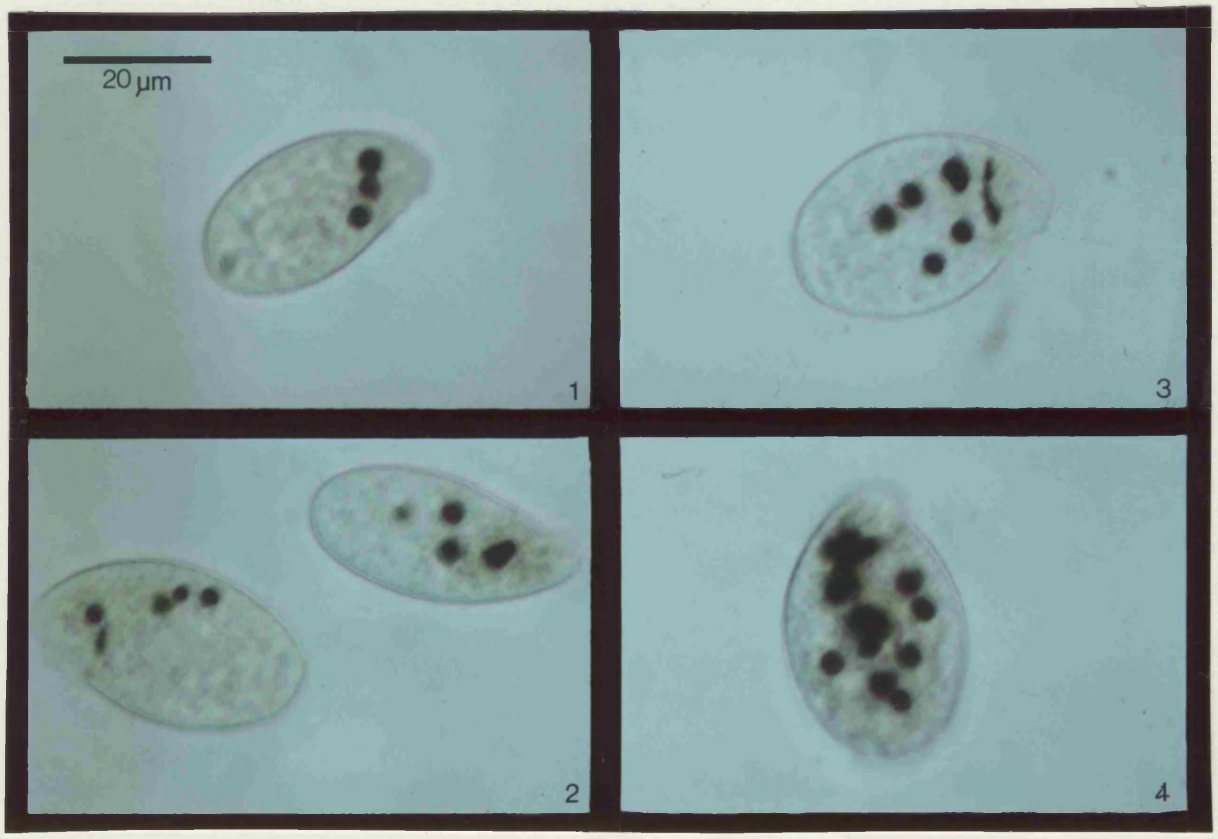


PLATE I2.

Food vacuole formation in Tetrahymena pyriformis: influence of exposure time on the uptake of India ink particles from the culture fluid.

PHOTOGRAPH I. Exposure to India ink solutions for 5 min before fixation. Three vacuoles formed.

PHOTOGRAPH 2. Exposure to India ink solutions for I0 min before fixation. Approx. 4 vacuoles formed.

PHOTOGRAPH 3. Exposure to India ink solutions for I5 min before fixation. Approx. 6 vacuoles formed.

PHOTOGRAPH 4. Exposure to India ink solutions for 20 min before fixation. Numerous vacuoles formed.

Fixative, 3% (V/V) glutaraldehyde solution (I:I ratio).

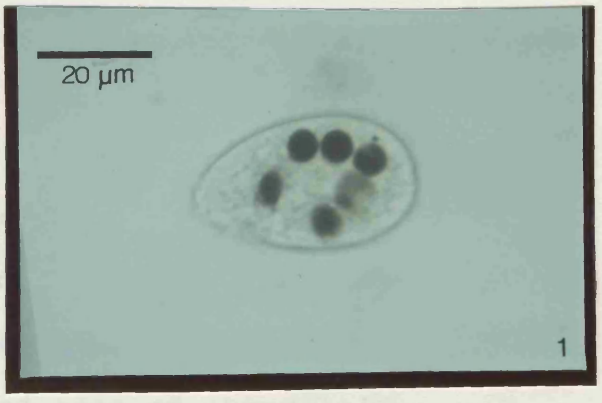
Scale marker in photograph I refers to all photographs in the plate.

PLATE I3.

Food vacuole formation in Tetrahymena pyriformis: appearance of food vacuoles after I5 min exposure to India ink particles (x 250 magnification).

PHOTOGRAPH I.

Vacuoles appear either as dense black circles (3) or as less dense grey patches of differing sizes (3).



PHOTOGRAPH 2.

Vacuoles appear similar to those in Photo. I. and also as a line of particles situated anterioraly.

PHOTOGRAPH 3.

One vacuole appears as a 'hollow' circle. Areas within the cytoplasm show signs of India ink inclusion but no distinct vacuoles can be seen.

times, were made on cells incubated in a suspension of India ink for 15 min.

Preliminary experiments, on India ink uptake in untreated cells (Plate 13), showed a linear relationship between length of exposure to India ink and number of food vacuoles formed, up to 15 min.

Exposure of the cells to India ink for 15 min gave an average of 8 detectable food vacuoles per cell. This was selected as the optimum exposure time with respect to number of vacuoles formed and ease of observation.

37.2 Chlorpropham

Chlorpropham at 0.5,1,2,4 and 20 μgml^{-1} significantly inhibited the formation of food vacuoles in T. pyriformis over 144h (Fig. 66). The ability to form food vacuoles diminished as culture age progressed. The rate of decline in food vacuole number was related to increasing chlorpropham concentrations. At 20 μgml^{-1} no food vacuoles were formed after 24h and with 4 and 2 μgml^{-1} no food vacuoles were formed after 48h and 72h respectively. Mode values (Fig. 67) suggested a decline in frequency of food vacuoles formed by individuals with all chlorpropham treatments. With 0.5,1,2,4 and 20 μgml^{-1} the most common number of food vacuoles per cell (mode) fell to 0 after 24h. The equivalent number of food vacuoles in untreated cells was 9. Chlorpropham at 0.1 μgml^{-1} caused a marked decline in mode values over the corresponding period.

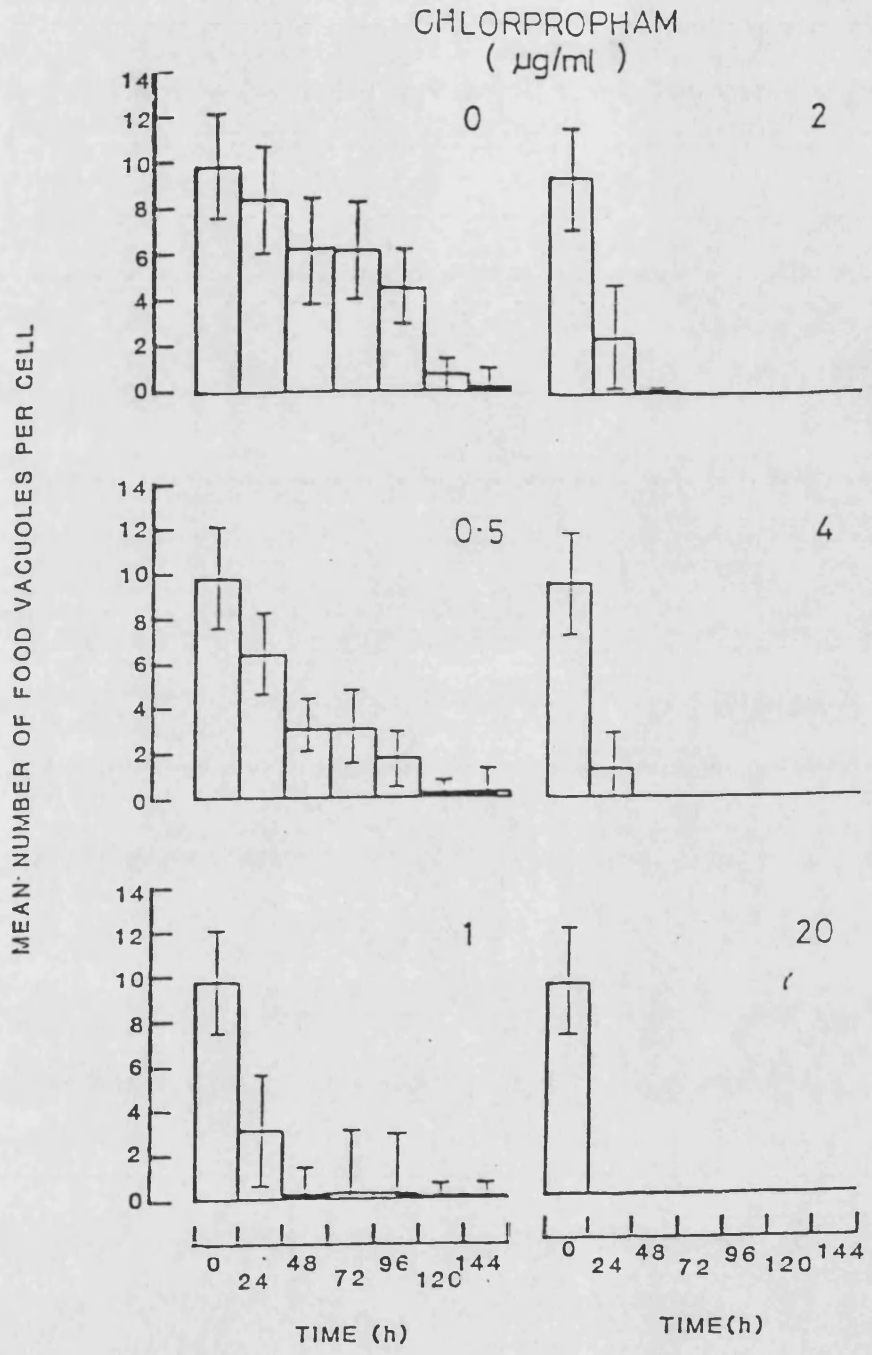


FIG. 66

The influence of chlorpropham on food vacuole formation in Tetrahymena pyriformis cells: The effect on the mean number of food vacuoles formed per cell. All cells were exposed to India ink (15min) after herbicide treatment, for different times.

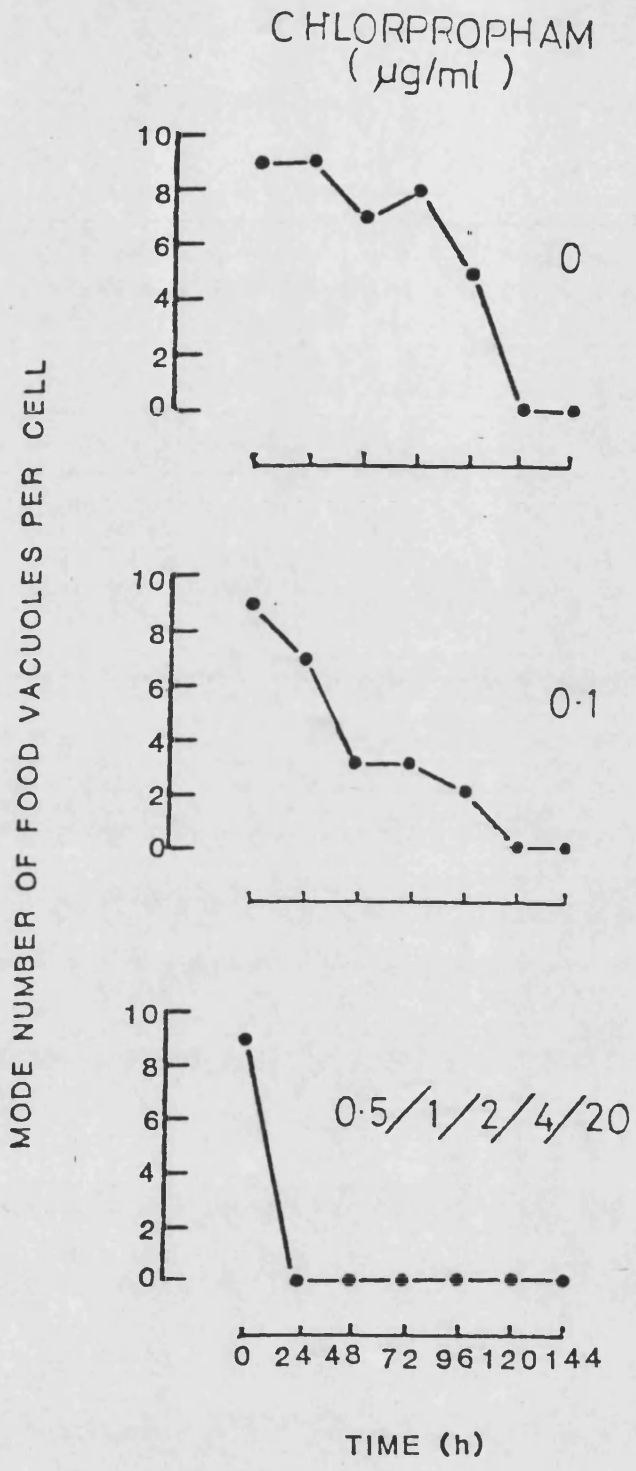


FIG. 67

The influence of chlorpropham on food vacuole formation in Tetrahymena pyriformis cells: the effect on the mode number of food vacuoles formed per cell. All cells were exposed to India ink (15min) after herbicide treatment (for up to 144h).

The effect of chlorpropham on mean food vacuoles over 24h is shown in Fig. 68. The mean number per cell declined with all chlorpropham treatments.

Generally, significant differences were observed after 18h, but with 20 μgml^{-1} food vacuole formation ceased after 3h. Chlorpropham reduced the frequency of food vacuole formation in individuals at all concentrations tested (Fig. 69). The mode declined rapidly after 9h with 0.5,1 and 4 μgml^{-1} and after 3h with 20 μgml^{-1} of chlorpropham. A more gradual decline in such values was observied with 2 μgml^{-1} chlorpropham.

The number of cells in which no food vacuoles were observed increased with exposure and concentration of chlorpropham used (Fig. 70). After 6h the percentage of cells without food vacuoles for 20, 4 and 0.5 μgml^{-1} were 100, 10 and 0%. After 24h the respective percentages were 100, 62 and 54%. The corresponding untreated values were 2% (6h) and 6% (24h). In each case cells in the later stages of division were not considered.

The effect of chlorpropham on food vacuole formation after 24h in T. pyriformis cells is shown in Plate 14. As the herbicide concentration increased, the number of food vacuoles formed decreased and the cells became spherical. Above 1 $ugml^{-1}$ distinct cell rounding occurred (photographs 5-7, Plate 14). At 20 μgml^{-1} (photograph 7) no food vacuoles were seen, the cells were spherical,

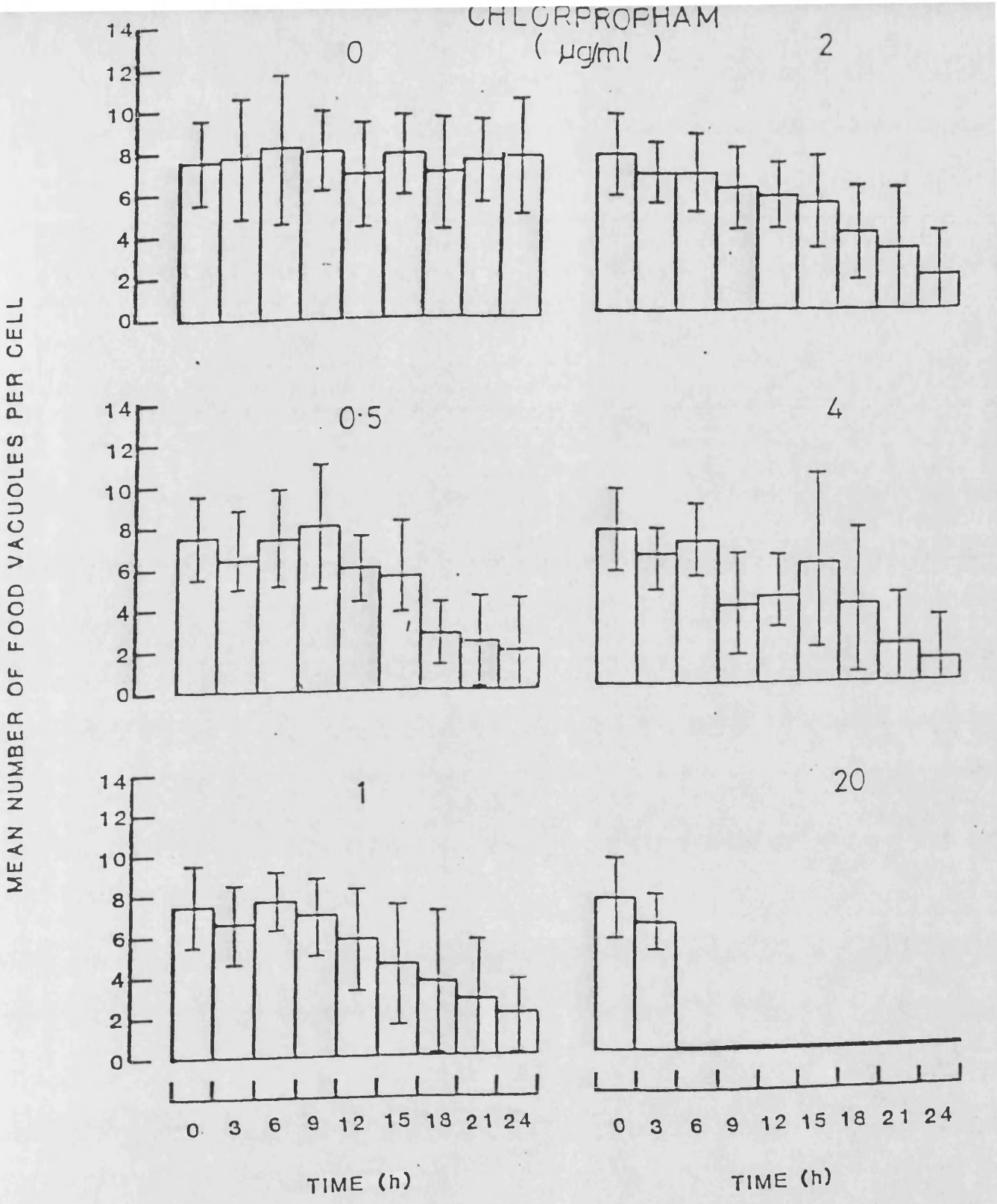


FIG. 68

The influence of chlorpropham (24h exposure) on food vacuole formation in Tetrahymena pyriformis cells: the effect on the mean number of food vacuoles formed over 24 h herbicide treatment. All cells were exposed to India ink (15 min) after herbicide treatment.

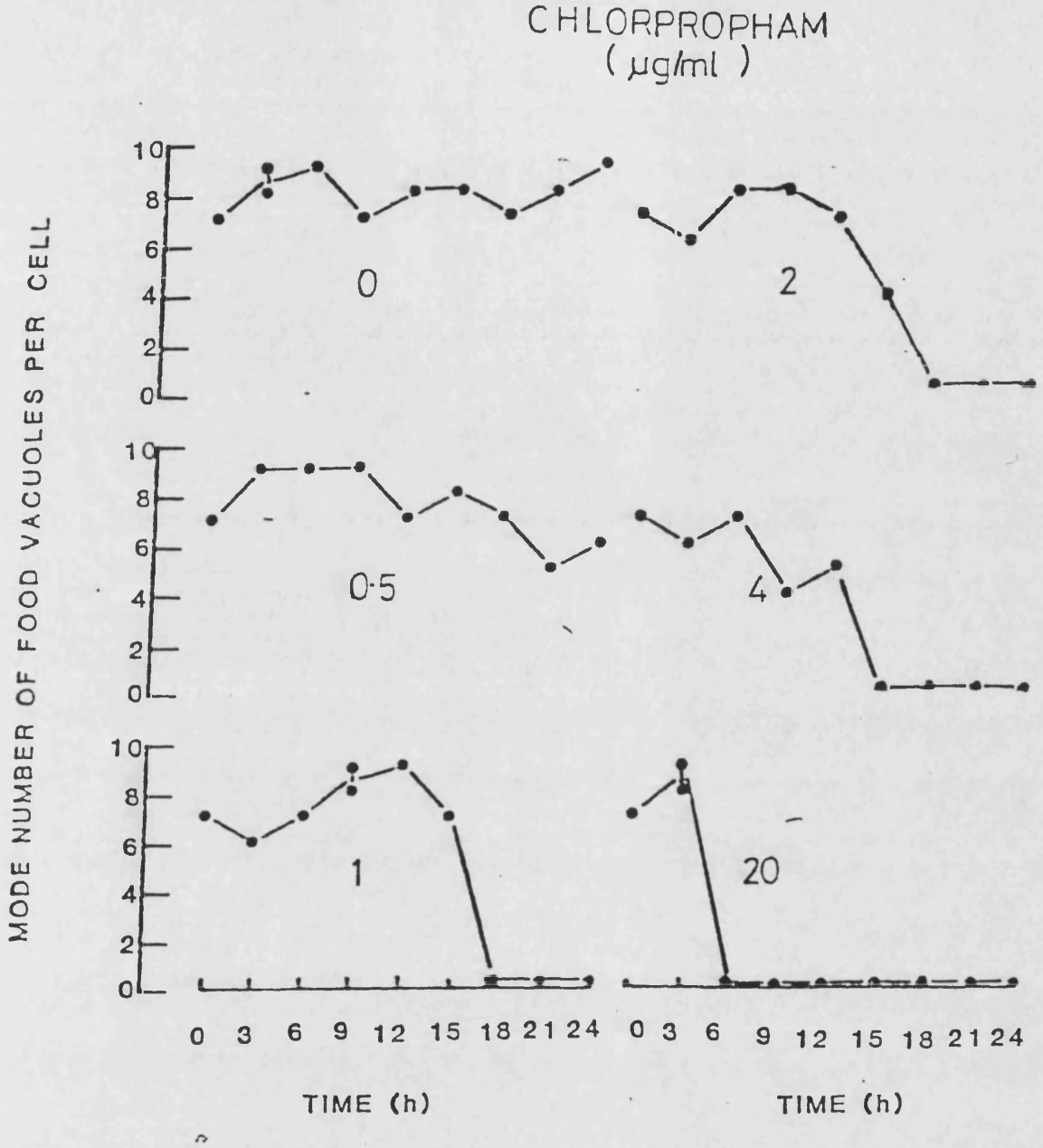


FIG. 69

The influence of chlorpropham on food vacuole formation in Tetrahymena pyriformis cells: the effect on the mode number of food vacuoles formed over 24 h exposure to herbicide. All cells were exposed to India ink (15 min) after herbicide concentration.

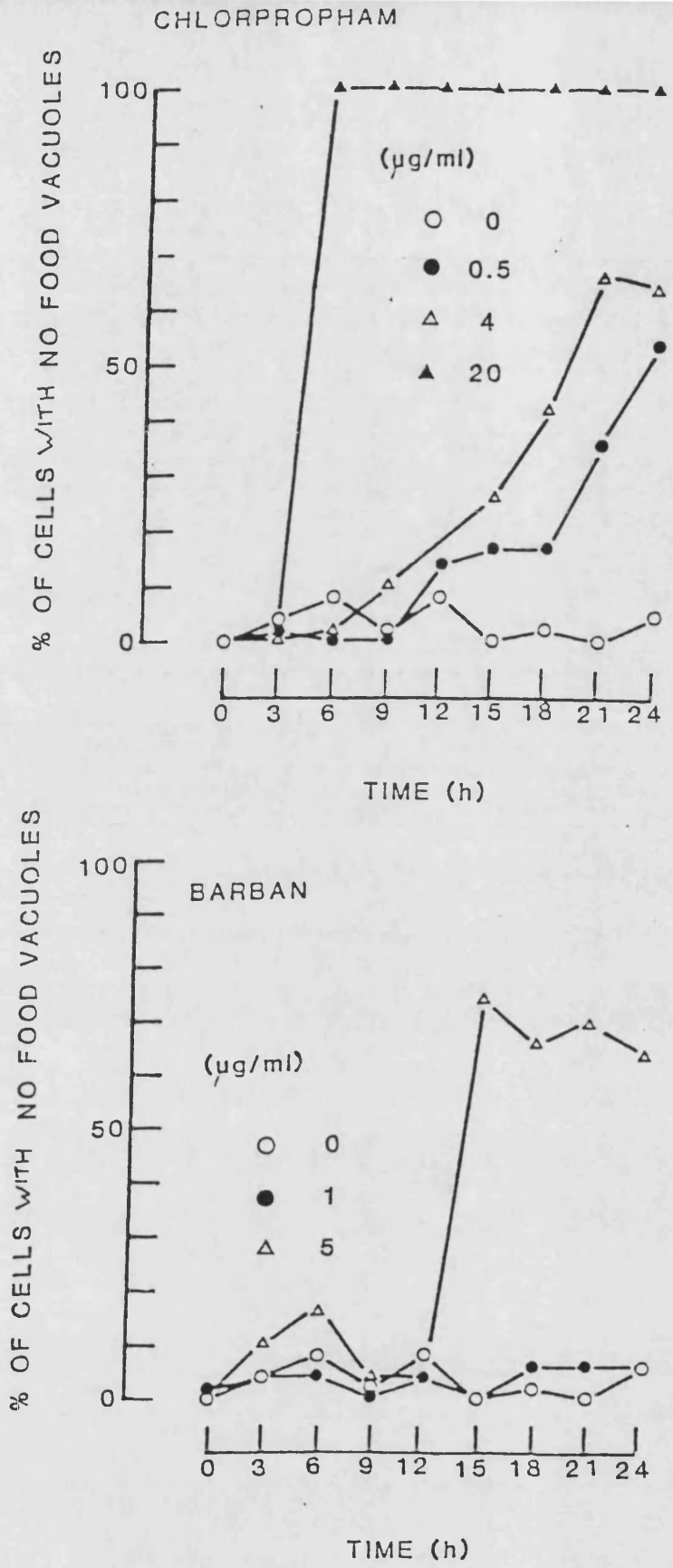


FIG. 70

The effect of chlorpropham and barban on food vacuole formation in Tetrahymena pyriformis cells. All cells were exposed to India ink (15min) after herbicide treatment up to 24 h.

PLATE I4.

The appearance of the food vacuoles of Tetrahymena pyriformis cells grown in the presence of chlorpropham for 24h. All cells were exposed to India ink solutions for I5 min after herbicide treatment.

PHOTOGRAPH I. Untreated cell showing normal food vacuole (FV) formation.

PHOTOGRAPH 2. Cells exposed to chlorpropham (0.Iμgml^{-I}) showing reduced food vacuole formation (FV).

PHOTOGRAPH 3. Cell exposed to chlorpropham (0.5 μgml^{-I}) showing a further reduction in food vacuole (FV) number.

PHOTOGRAPH 4. Two cells exposed to I μgml^{-I} chlorpropham showing alteration in cell shape and a reduction in food vacuoles (FV).

PHOTOGRAPH 5. Cells treated with 2 μgml^{-I} chlorpropham cell rounding evident but food vacuole (FV) still present.

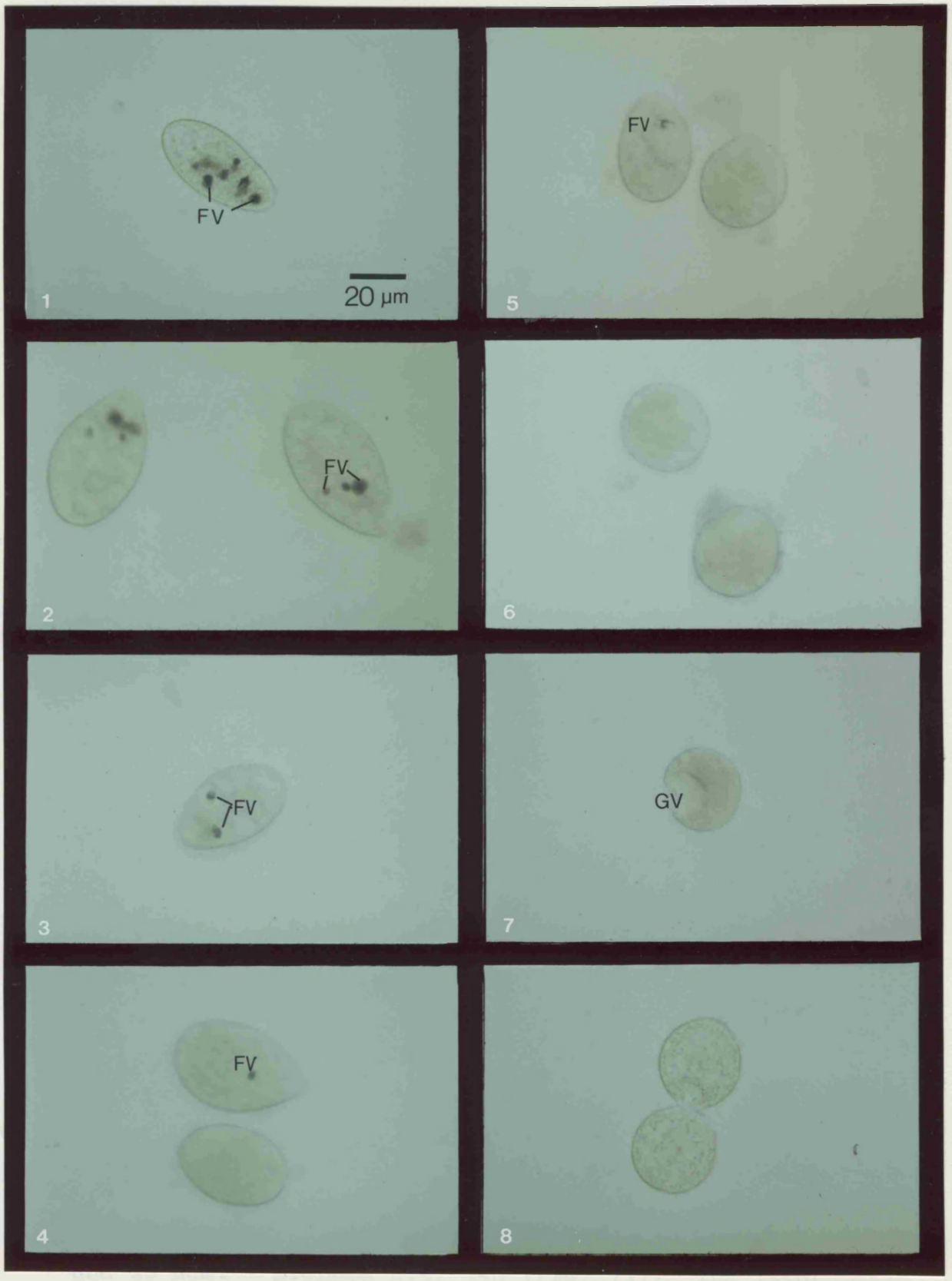
PHOTOGRAPH 6. Cells exposed to 4 μgml^{-I} chlorpropham. Food vacuole (FV) formation has ceased and cells have become spherical.

PHOTOGRAPH 7. Cell treated with 20 μgml^{-I} chlorpropham. No food vacuoles present and cells are spherical with a 'giant' vacuole (GV), possibly a contractile.

PHOTOGRAPH 8. An untreated dividing cell showing the ceasation of food vacuole formation during cell division.

Scale marker in photograph I refers to all photographs in the plate.

PLATE I4.



smaller than control cells and possessed a large vacuole, possibly a contractile vacuole, which did not contain India ink particles. The rounding of cells treated with 20 μgml^{-1} chlorpropham occurred after 9h and, after 12h, distinct regions of 'clear' cytoplasm were observed. After 15h such cells appeared dead. In cells treated with lower concentrations (photographs 2-4, Plate 14) the food vacuoles appeared smaller than those in untreated cells. The final photograph (8, Plate 14) shows an untreated cell in the late stages of division. No food vacuoles were formed in such cells.

37.3 Propham

Depression of food vacuole formation occurred with cells which had been treated with 5 and 25 μgml^{-1} propham. The mean number of food vacuoles formed was, however, significantly different from the untreated only at the 96h sample point (Fig. 71). The mode number of vacuoles formed per individual fell to 0 after 24h with both 5 and 25 μgml^{-1} (Fig. 71) but 0.125, 2.5 μgml^{-1} propham had no inhibitory effect on the frequency of vacuole formation over 144h. However, an increase in the mode number of food vacuoles formed was seen at 0.125 μgml^{-1} after 24h (Fig. 71).

No inhibition of vacuole formation occurred with 0.63, 2.5 and 25 μgml^{-1} propham over 24h (Fig. 72). The later

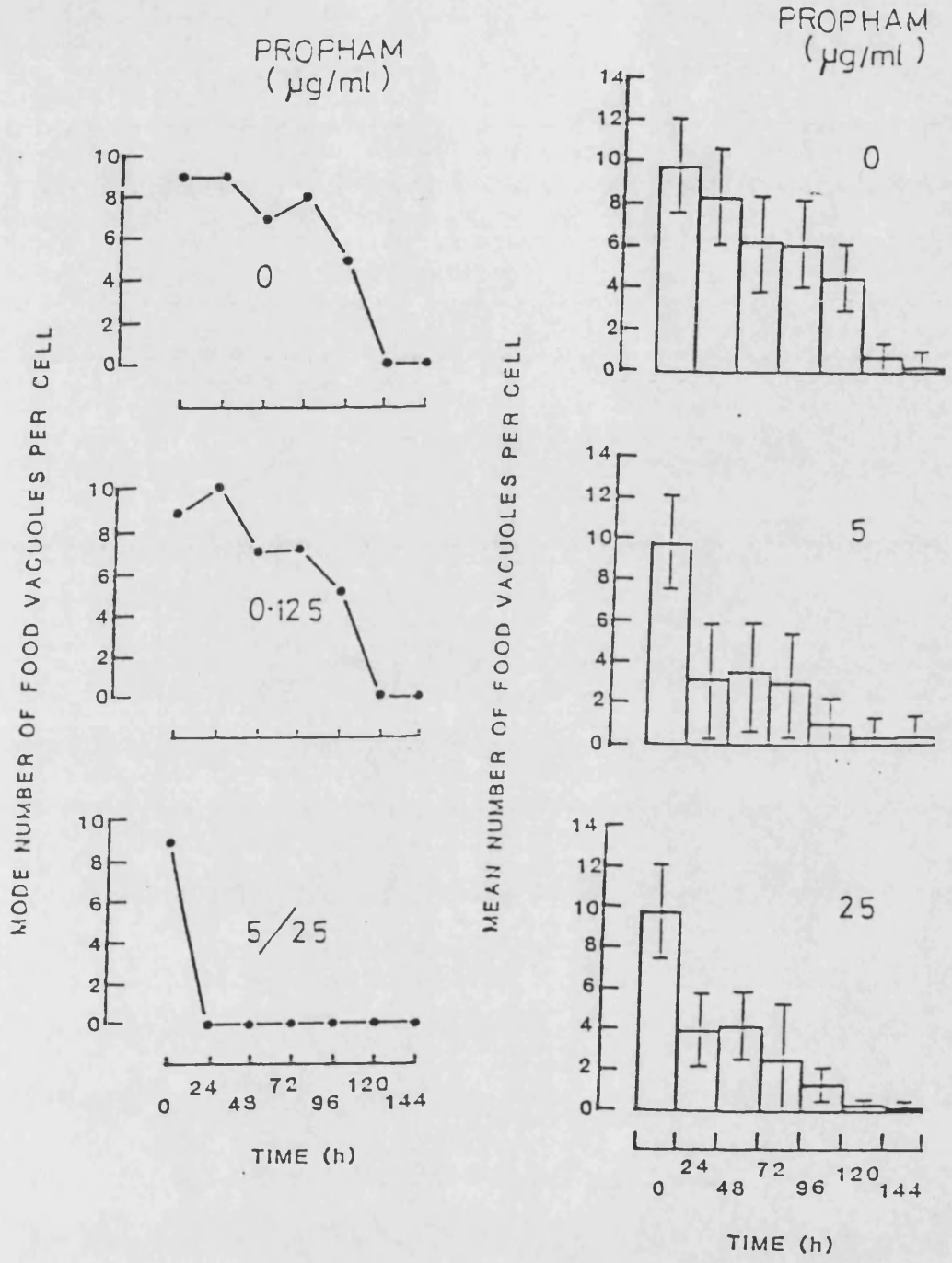


FIG. 71

The influence of propham on food vacuole formation in Tetrahymena pyriformis cells; the effect on the mode and mean number of food vacuoles formed per cell. All cells were exposed to India ink (15 min) after herbicide treatment for different times up to 144 h.

observation that 25 μgml^{-1} propham did not affect vacuole numbers conflicts with the results shown in Fig. 71. Large increases in the number of vacuoles formed per cell over 24h were observed with 0.63 and 2.5 μgml^{-1} (Fig. 72). After 3h the mean number of food vacuoles formed per cell was 40 and 20% higher than untreated values with 0.63 and 2.5 μgml^{-1} respectively. After 6h stimulation of vacuole formation declined to 18% with 0.63 μgml^{-1} but rose substantially with 2.5 μgml^{-1}. Stimulation of food vacuole formation was so pronounced that cells treated with 2.5 μgml^{-1} after 6h and 1.25 μgml^{-1} after 3h appeared black and the number of food vacuoles contained could not be determined although some discrete vacuoles could be seen at the cell periphery. Where possible, the corresponding increases in mode values are shown in Fig. 72. No difference over 24h was observed in the modal number of vacuoles formed in cells treated with 25 μgml^{-1} propham, again conflicting with the previous result (Fig. 71).

Cells exposed to propham at 25 and 2.5 μgml^{-1} for 3h showed an increase in the number of cells in the later stages of division (5 fold). With 2.5 μgml^{-1} the increase in the number of dividing cells was correlated with an increase in food vacuole formation in non-dividing cells in the same population.

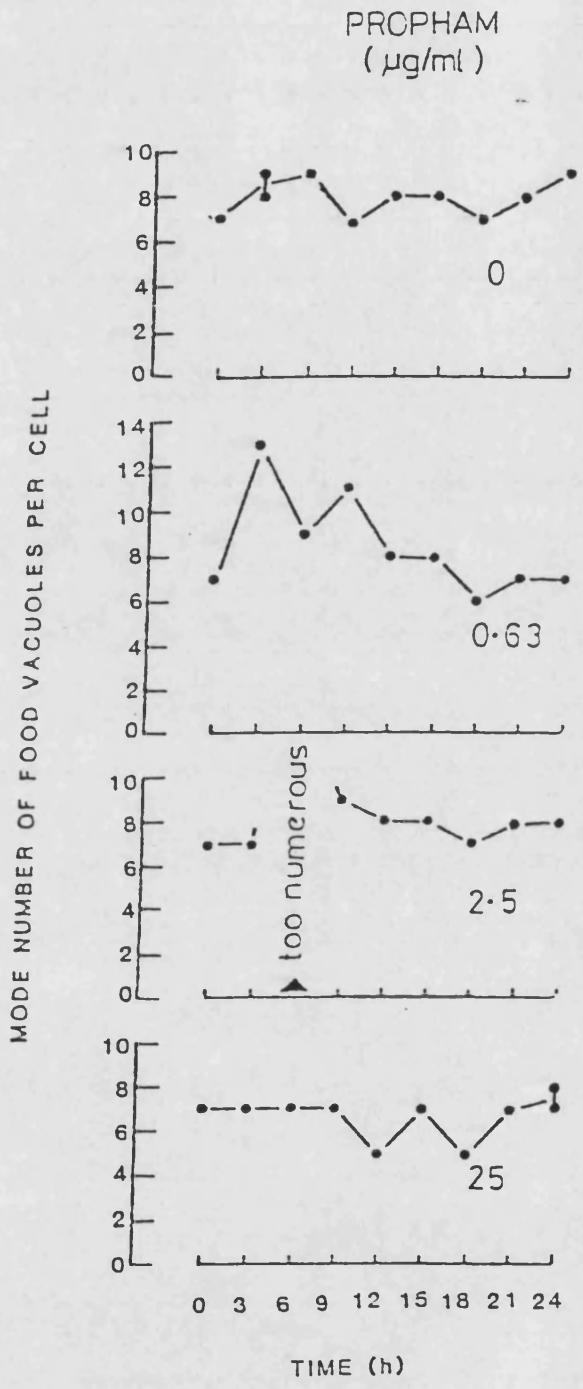


FIG. 72

The influence of propham on food vacuole formation in Tetrahymena pyriformis cells: the effect of propham on the mode number of food vacuoles formed over 24h. All cells were exposed to India ink (15 min) after herbicide treatment.

37.4 Barban

Barban did not signficantly affect food vacuole formation, except at 5 μgml^{-1} (Fig. 73) where the mean number of food vacuoles fell from 9.68 to under one in 24h. The corresponding decrease in modal numbers showed that although no differences in mean number of vacuoles formed were detected at 0.03 - 0.5 μgml^{-1} barban, 0.03 μgml^{-1} barban caused a decline in the number of individuals possessing food vacuoles after 24h (Fig. 73). Examination of these individuals showed that 81% of those which possessed no food vacuoles were in the later stages of division. The incidence of dividing cells was 5 x greater in populations treated with 0.3 μgml^{-1} than that found in the untreated. Barban appeared to have a stimulatory effect on division with the concomittant reduction in modal numbers of food vacuoles.

Over 24h, barban at 1 μgml^{-1} had a stimulatory effect on both the mean and modal number of food vacuoles formed (Fig. 74). The effect was greatest at 3h and declined rapidly afterwards. At 5 μgml^{-1} barban reduced the mean number of food vacuoles formed after 12h and reduced the modal to 0 at 15h.

In Fig. 70 the percentage increase in cells having no food vacuoles (corrected by not including visibly dividing cells) showed that only 5 μgml^{-1} barban caused an increase

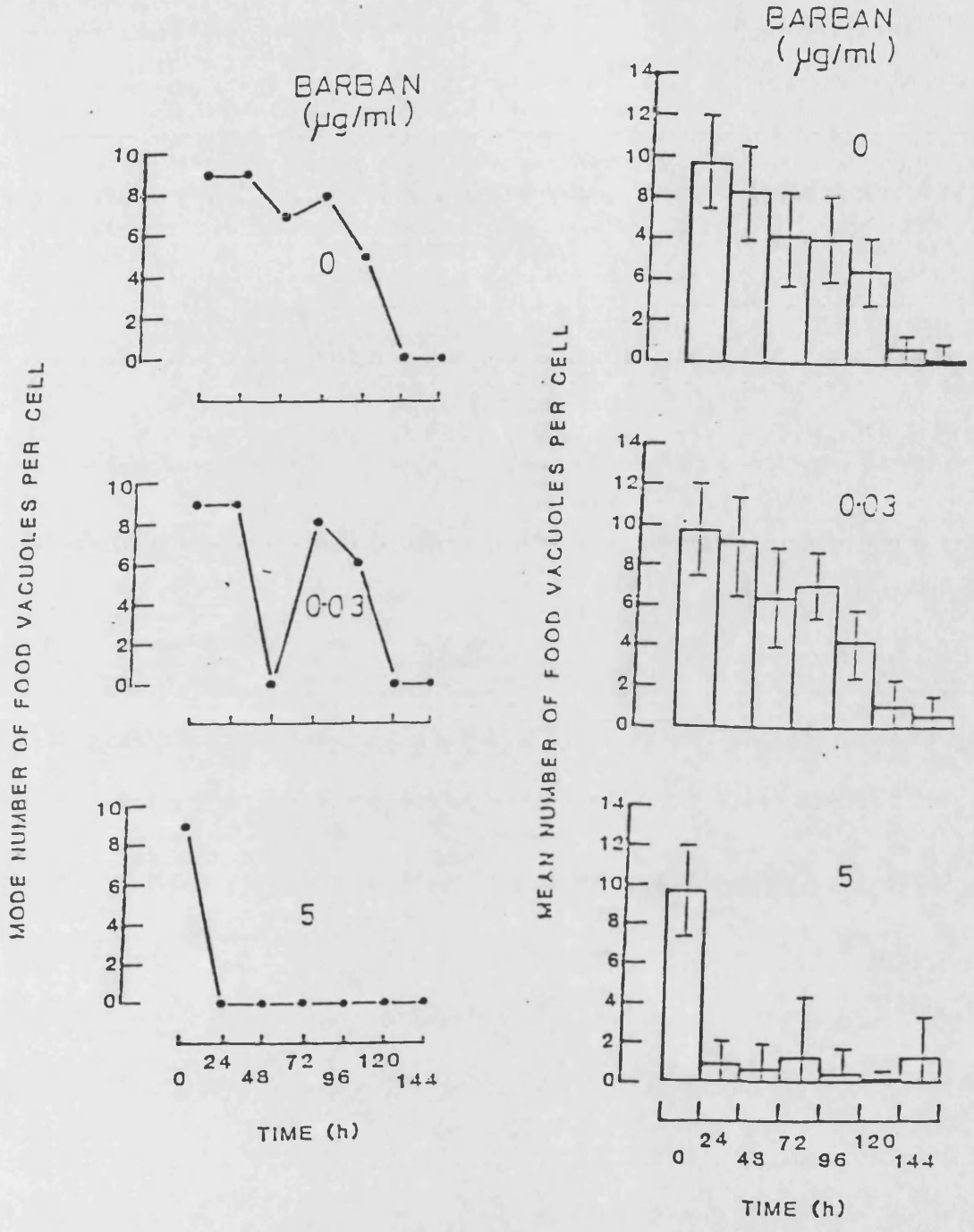


FIG. 73

The influence of barban on food vacuole formation in Tetrahymena pyriformis cells: the effect on the mode and mean number of food vacuoles formed per cell. all cells were exposed to India ink (15 min) after herbicide treatment for different times up to 144h.

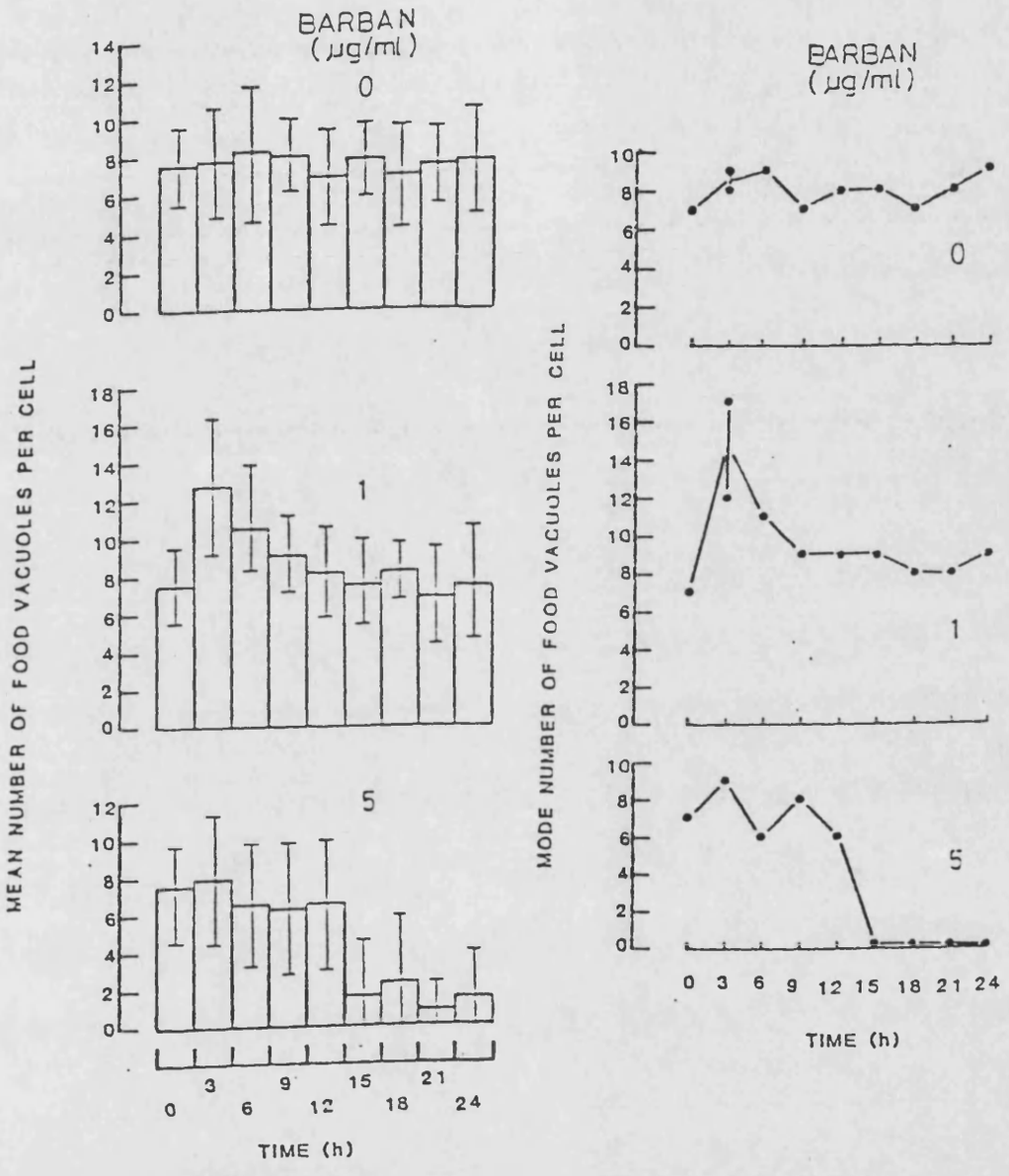


FIG. 74

The influence of barban on food vacuole formation in Tetrahymena pyriformis cells: the effect on the mean and mode number of food vacuoles formed per cell. All cells were exposed to India ink (15 min) after herbicide treatment for different times up to 24h.

in the number of cells containing no food vacuoles. Seventy four % of cells exposed to this level of barban did not form food vacuoles after 12h. This value decreased slightly over the remaining 10h.

37.5 Diuron

Diuron at 0.08 - 1.5 μgml^{-1} had no effet on the mean number of food particles formed in T. pyriformis cells. However, at 3 and 15 μgml^{-1} some inhibitory action was seen (Fig. 75). Food vacuole numbers were reduced from 8 to 3 after 24h and, after 48h, to 1 vacuole per cell with 15 μgml^{-1} of diuron. The number of vacuoles formed by untreated cells remained at 8 over this period. Significant inhibition of mean vacuole number occurred after 72h with 15 μgml^{-1}. After 24h 15 μgml^{-1} diuron decreased the modal value to 0 whilst with 3 μgml^{-1} the decline was less marked (Fig. 75).

No change in cell morphology occurred with any diuron treatment.

37.6 Malathion

Malathion at all concentrations tested (0.08 - 15 μgml^{-1}) did not affect mean food vacuole formation. No differences were observed in cell shape or in the frequency of food vacuole formation per individual.

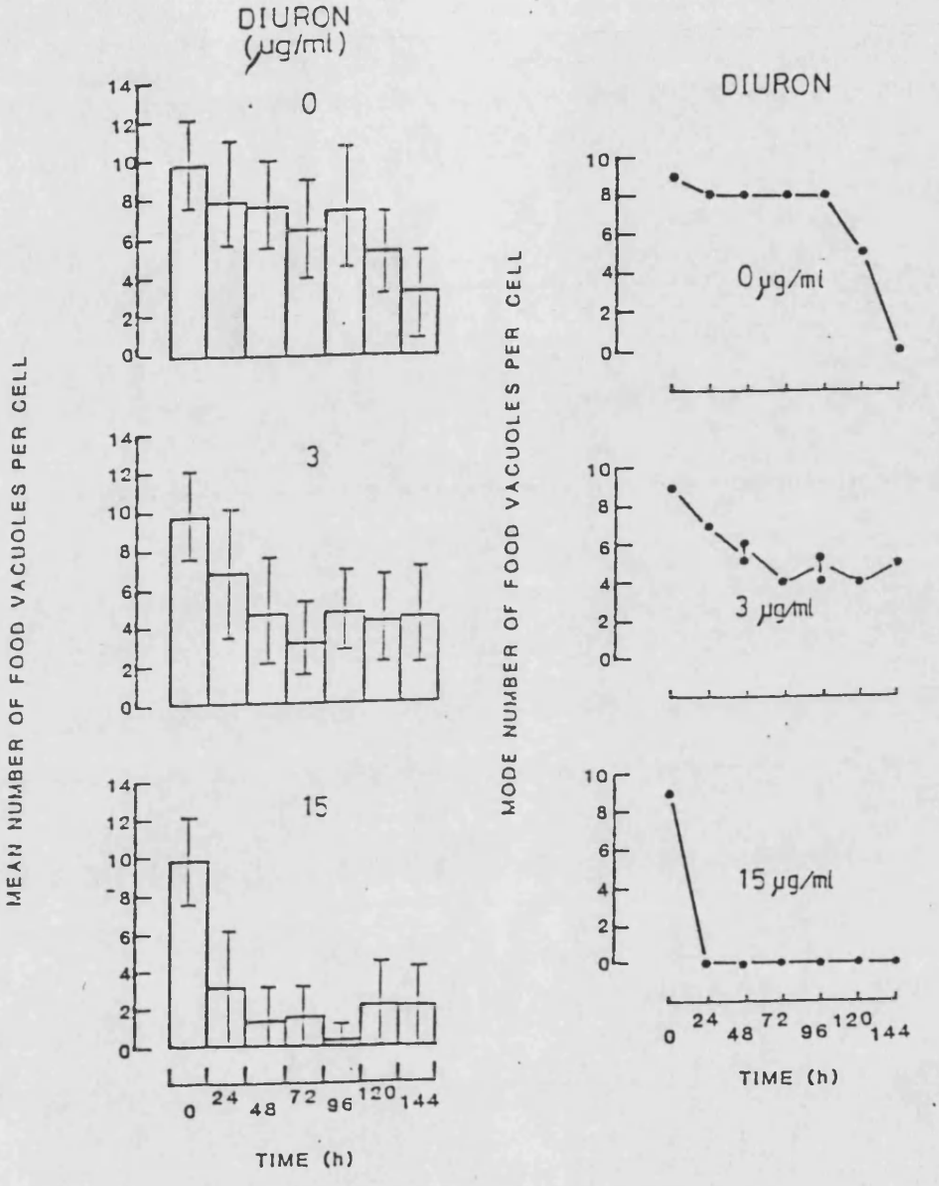


FIG. 75

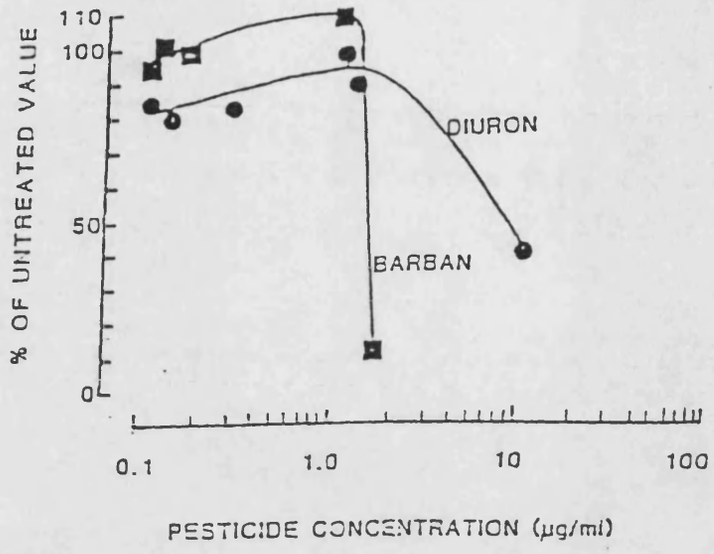
The influence of diuron on food vacuole formation in Tetrahymena pyriformis cells: the effect on the mean and mode number of food vacuoles formed per cell. All cells were exposed to India ink (15 min) after herbicide treatment.

37.7 Comparison of the action of some pesticides on food vacuole formation in Tetrahymena pyriformis

The dose response curves (24h) for the inhibition of food vacuole formation in T. pyriformis are shown in Fig. 76. The concentration of each pesticide which reduced food vacuole formation to 50% of the control value (EC_{50}) are shown in Table 51.

Chlorpropham was the most inhibitory chemical tested whilst malathion was the least inhibitory. Both chlorpropham and propham had similar shaped dose-response curves. Increasing concentrations of each pesticide were progressively more inhibitory to food vacuole formation. With barban and diuron increasing concentrations were not progressively inhibitory and, with barban, stimulation of food vacuole formation occurred at low doses. At 5 μgml^{-1} barban was very inhibitory to food vacuole formation contrasting dramatically with its stimulation at low concentrations. The maximum point of stimulation and the threshold of the compound's inhibitory action was at approx 1 μgml^{-1}. With diuron a similar threshold was found although no stimulation of food vacuole formation occurred and the inhibitory activity was less intense.

Chlorpropham (20 μgml^{-1}) was the only compound to prevent food vacuole formation after 24h.



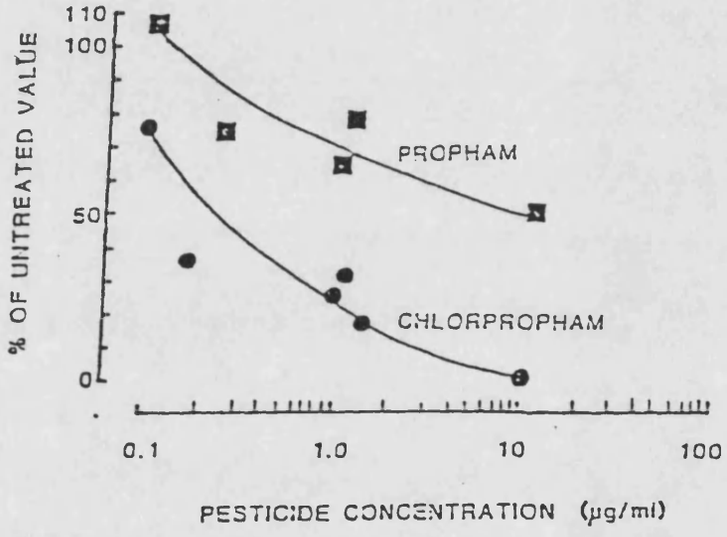


FIG. 76

Comparison of the dose-response curves for the effect of chlorpropham, propham, barban and diuron on the mean number of food vacuoles formed per Tetrahymena pyriformis cell after herbicide treatment for 24 h.

Table 51

Comparison of the herbicide concentrations which reduced the mean number of food vacuoles formed per Tetrahymena pyriformis cell to 50% of the control value (EC_{50}) after 24h

Pesticide	EC_{50} ($\mu g ml^{-1}$)
chlorpropham	0.65
barban	4.4
diuron	9.8
propham	9.8
malathion	not found

Table 52

The percentage inhibition of cyst formation in Acanthamoeba castellanii by chlorpropham

	chlorpropham ($\mu g ml^{-1}$)			
Time (h)	1	2	4	20
24	1	11	7	12
48	7	2	0	14
72	10	3	0	11
96	+2	0	+2	11
120	4	3	+1	13
144	2	2	4	10

The effect of 4 pesticides on the distribution of food vacuole numbers within populations was obtained by cumulative frequency plots, ogives, (Fig. 77). The data for the 'control', untreated populations, were spread over a wide range with the highest number of individuals in the 9-10 food vacuole class. This gave a classical 'S'-shaped curve. The flat extremes of the curve derived from the low incidence of cells possessing a very high or very low number of food vacuoles. In all cases increasing concentrations of pesticides gave rise to successive reductions in the spread of data as the number of food vacuoles per cell declined.

Chlorpropham at 20 μgml^{-1} (Fig. 77) completely inhibited food vacuole formation and produced a horizontal plot. Lower levels of chlorpropham (1 - 4 μgml^{-1}) gradually flattened the S-shape of the plot due to a higher proportion of cells having fewer vacuoles per cell.

Barban at 5 μgml^{-1} also produced a shallow plot, as the proportion of individuals containing low numbers of food vacuoles was large (Fig. 77). However, lower concentrations of barban (0.03 - 1 μgml^{-1}) had no effect on the data spread.

Diuron (15 μgml^{-1}) and propham (24 μgml^{-1}) did alter the data spread within the plots but to a lesser degree than either barban or chlorpropham. Both diuron and propham

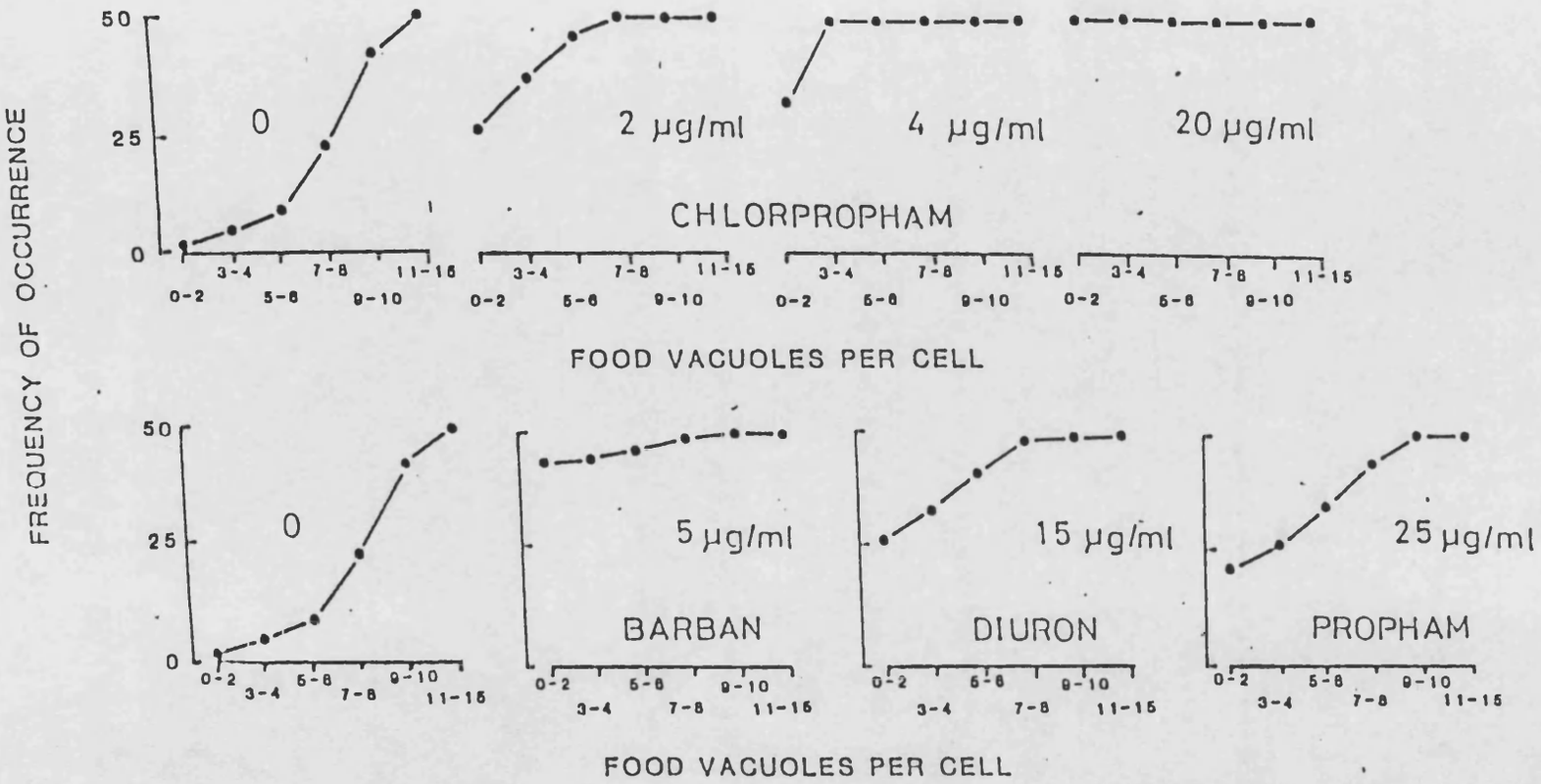


FIG. 77

The effect of chlorpropham barban and diuron on food vacuole formation in Tetrahymena pyriformis : cumulative frequency plots (ogives).

produced curvilinear plots due to over half the cells within the untreated populations possessing only 0-2 or 3-4 food vacuoles per cell. No other concentrations of either compound showed any marked effect upon frequency distribution.

The order of inhibitory activity of pesticides on food vacuole formation in T. pyriformis cells was chlorpropham $>$barban $>$ diuron $>$ propham; with malathion having no effect.

38.0 Observations on Acanthamoeba castellanii induced to encyst by various replacement techniques

The use of HSM, EM and $MgCl_2$ replacement methods did not produce true cysts in A. castellanii Neff strain in this laboratory. In each case cells became rounded and ceased forming acanthapodia within hours. With some methods, notably the EM method, the rounded forms became highly vacuolated and had distinct peripheral thickening. An absence of cytoplasmic components was also noted. With IM $MgCl_2$ the cells appeared damaged after 24h. No cysts were formed over 72h with any treatment.

With the A. castellanii culture (Neff strain) obtained from Dr A J Griffiths the $MgCl_2$ replacement method induced encystment after 24h. This method and culture of A. castellanii was used subsequently in encystment studies.

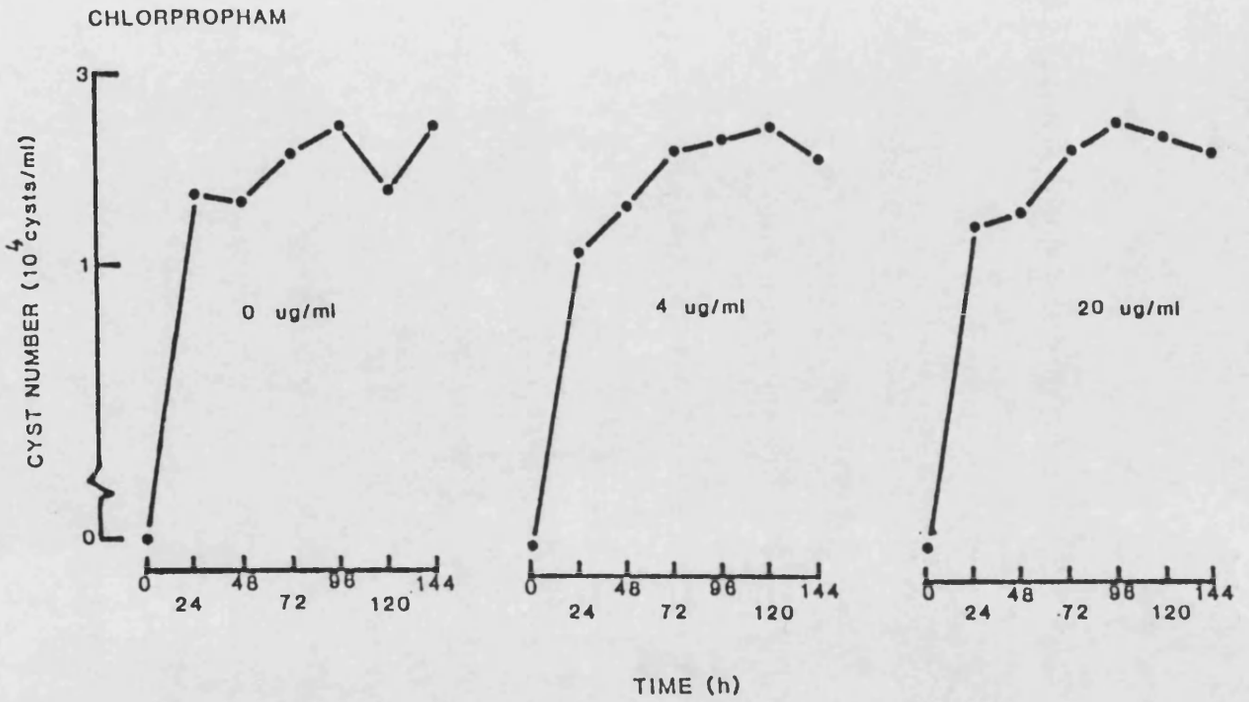


FIG. 78

The effect of chlorpropham on encystment in Acanthamoeba castellanii
All cells were induced to encyst by the replacement technique of
Chagla & Griffiths (1974).

39.0 The effects of chlorpropham on encystment in Acanthamoeba castellanii

Chlorpropham: Slight inhibition of encystment of A. castellanii was found with 20 μgml^{-1} chlorpropham (Fig. 78 and Table 52).

In all treatments rapid encystment occurred within 24h and then slowed (Fig. 78). After 24h 90% of the untreated cells had encysted and this figure rose to 96% after 144h. In the presence of 20 μgml^{-1} chlorpropham, the respective percentages were 79 and 86%. No stimulation of encystment occurred.

40.0 The effects of some phenylcarbamates on excystment of Acanthamoeba castellanii

Plate 15 shows the early emergence of A. castellanii trophozoites from cysts in untreated cultures.

Chlorpropham: Chlorpropham did not affect the low incidence of excystment but did alter the growth rate of the emergent trophozoites (Fig. 79). The doubling time (16h in untreated cells) was increased to 32h with 20 μgml^{-1}. The onset of excystment was not delayed by any treatment and occurred after 24h.

Barban: The onset of excystment was delayed by 24h with 1 and 5 μgml^{-1} barban (Fig. 79). At 1 μgml^{-1} the initial delay in excystment was followed by a stimulatory effect

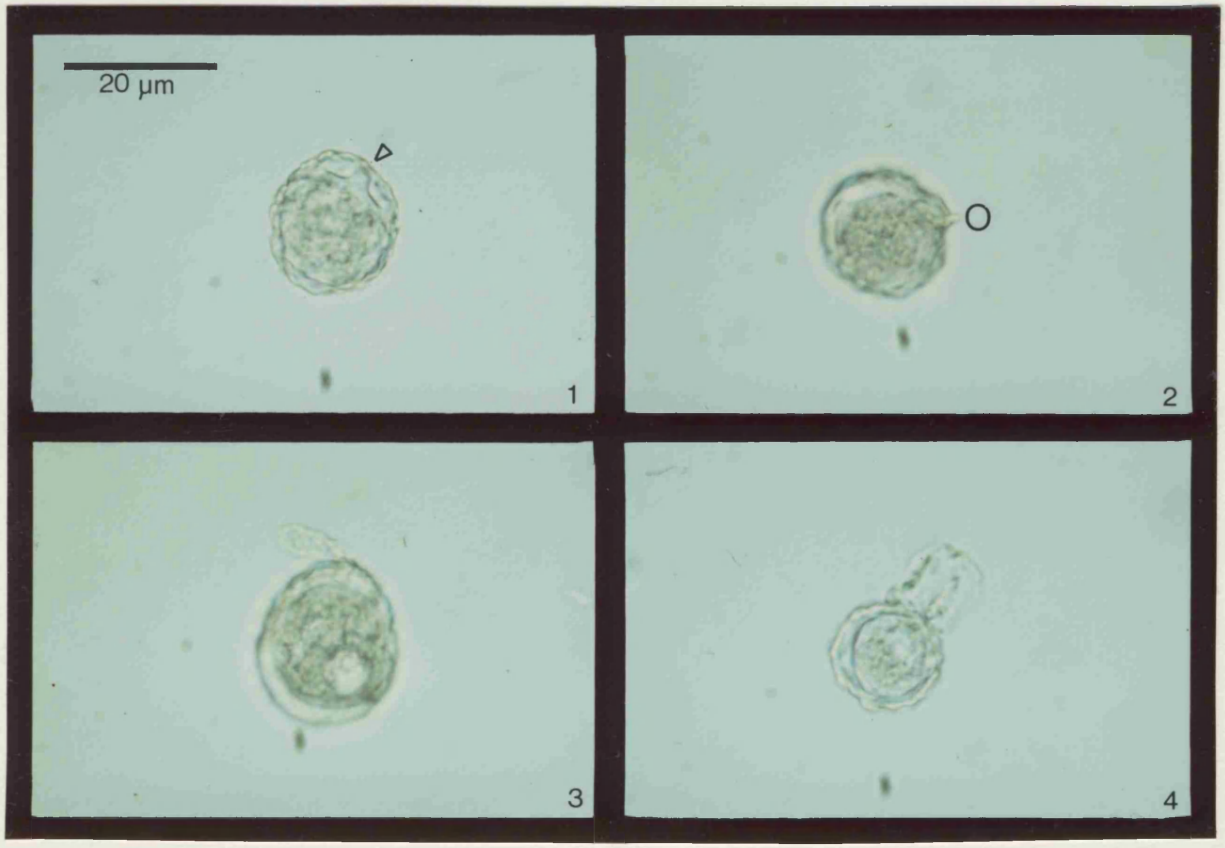


PLATE I5.

Excystment in Acanthamoeba castellanii: emergence of trophozoite from a cyst.

PHOTOGRAPH I. Cyst, showing the internal endocyst making contact with the external exocyst at arrow.

PHOTOGRAPH 2. Cyst showing the projection of cytoplasm emergeing at the operculum (O).

PHOTOGRAPH 3. Trophozoite emerging from the cyst, early stages.

PHOTOGRAPH 4. Trophozoite emerging from the cyst, late stages.

Scale marker in photograph I refers to all photographs in the plate.

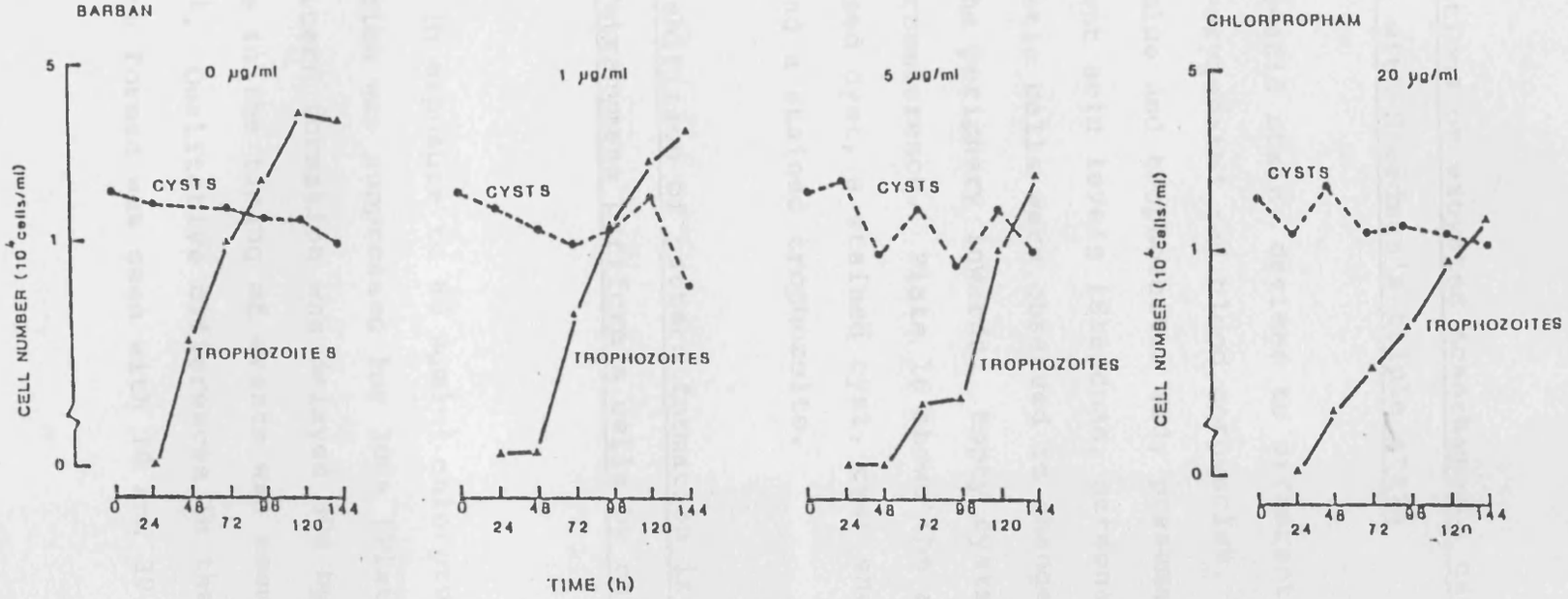


FIG. 79

The effect of barban and chlorpropham on excystment in Acanthamoeba castellanii .

on the growth rate (doubling time 112h). Cells treated with 5 μgml^{-1} of barban had a reduced level of growth until 96h when a similar stimulatory effect was seen (11h).

40.1 Observations on excysted Acanthamoeba castellanii cells stained with Steedman's triple stain

The tri-acid stain, devised to differentiate oxygenated and de-oxygenated red blood corpuscles, was found to stain cysts blue and trophozoites red, presumably in response to different acid levels (Steedman, personal communication). Pre-cystic cells were observed to change from red to blue from the periphery inwards. Empty cysts stained only at the circumference. Plate 16 shows the appearance of an unstained cyst, a stained cyst, cyst shell and precystic cell and a stained trophozoite.

41.0 The inhibition of pattern formation in populations of Tetrahymena pyriformis cells by chlorpropham

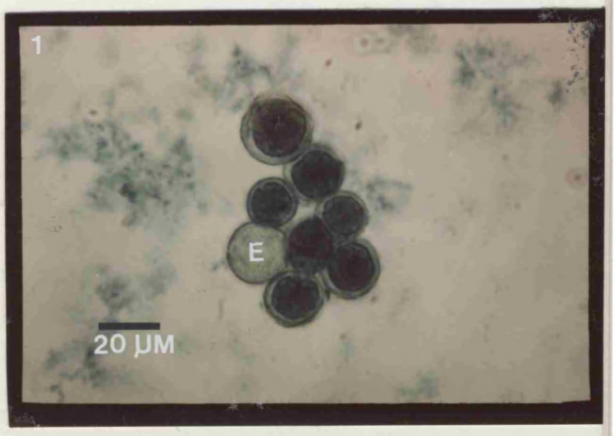
After 1h exposure to 60 μgml^{-1} chlorpropham pattern formation was suppressed for 300s (Plate 17). The onset of pattern formation was delayed 30s by 30 μgml^{-1} but no change in the timing of events was seen with 10 and 5 μgml^{-1}. Qualitative differences in the intensity of the pattern formed was seen with 10 and 30 μgml^{-1}. In the

PLATE I6.

The appearance of encysted Acanthamoeba castellanii after staining with a tri-acid triple stain (Steedman, I970).

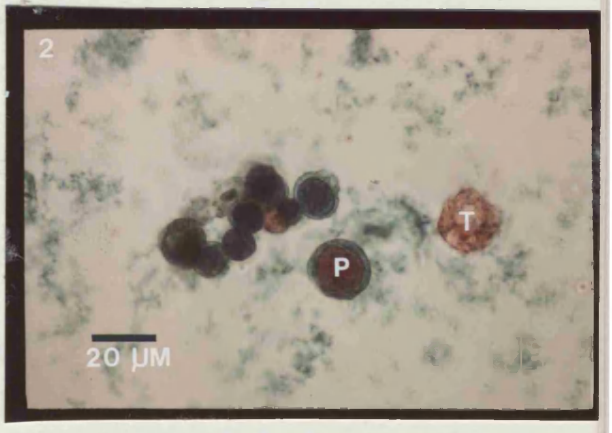
PHOTOGRAPH I.

Cysts of A. castellanii (blue) after staining with the one solution triple stain. Exocyst of empty cyst (E) still remains stained after emergence of trophozoite.



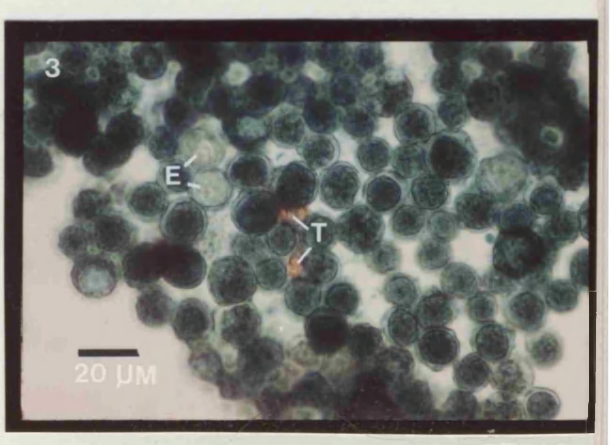
PHOTOGRAPH 2.

Fully formed cysts of A. castellanii (blue) compared with a stained pre-cystic cell (P) with a red centre and blue perifery and a red stained trophozoite (T).



PHOTOGRAPH 3.

Group of encysted A. castellanii cells and two stained empty cysts (E) with two red stained trophozoites (T) clearly visible in the centre of the picture.



PHOTOGRAPH 4.

An encysting cell of A. castellanii unstained.

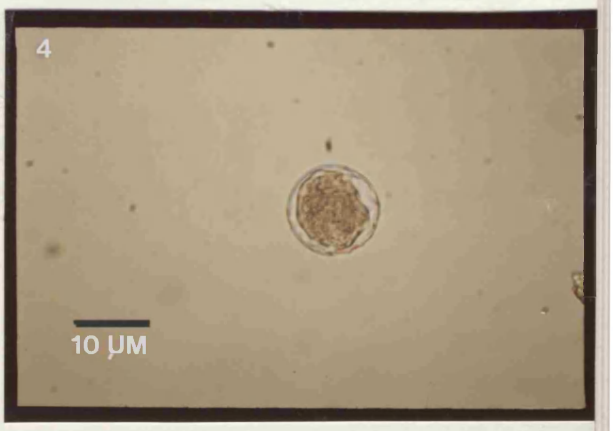


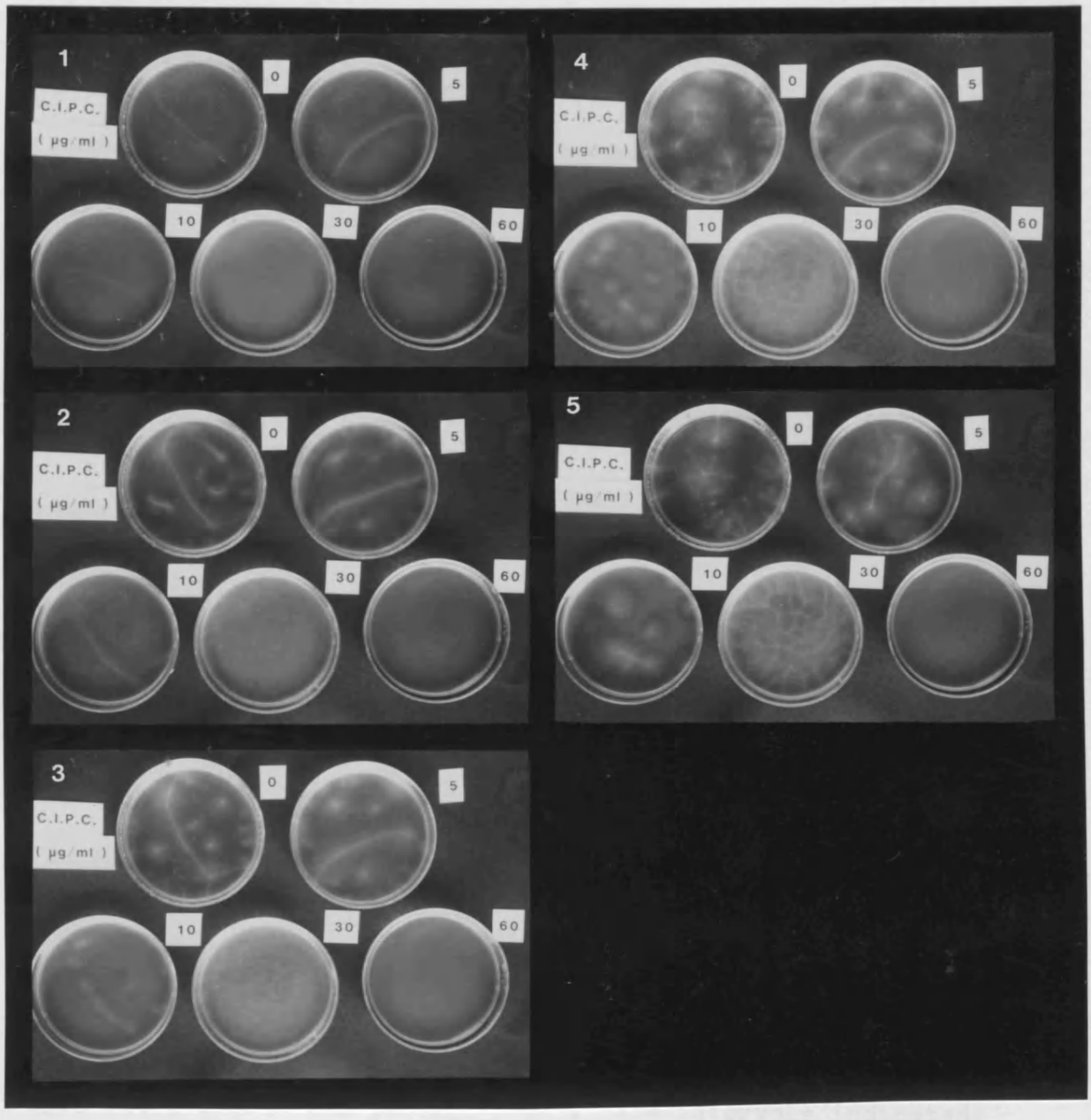
PLATE 17

The inhibition of pattern formation in suspensions of Tetrahymena pyriformis after 1 h exposure to chlorpropham (C.I.P.C.).

PHOTOGRAPH 1.	T=0	Immediate pattern formation in 0,5 and 10 $\mu g ml^{-1}$. No pattern formation with 30 and 60 $\mu g ml^{-1}$.
PHOTOGRAPH 2.	T=30s	Advancing pattern formation in 0 and 5 $\mu g ml^{-1}$, slower development with 10 $\mu g ml^{-1}$. No clear pattern with 30 and 60 $\mu g ml^{-1}$.
PHOTOGRAPH 3.	T=60s	Pattern formation still slower with 10 $\mu g ml^{-1}$, weak pattern formation with 30 $\mu g ml^{-1}$ and still no pattern with 60 $\mu g ml^{-1}$.
PHOTOGRAPH 4.	T=120s	Intensity of pattern formation visibly different with 30 $\mu g ml^{-1}$, increased number of nodes and streams. No pattern formation with 60 $\mu g ml^{-1}$.
PHOTOGRAPH 5.	T=300s	Increased number of nodes and streams still present with 30 $\mu g ml^{-1}$. No pattern formation with 60 $\mu g ml^{-1}$.

Cultures incubated with chlorpropham for 1 h, then tipped into Petri-dish and photographed at intervals indicated.

PLATE 17



The inhibition of pattern formation in suspensions of Tetrahymena pyriformis after 1 h exposure to chlorpropham (C.I.P.C.).

PLATE 18

The inhibition of pattern formation in suspensions of Tetrahymena pyriformis after 2 h exposure to chlorpropham (C.I.P.C.).

PHOTOGRAPH 1. T=0 Vague pattern formation with 0,5 and 10 μgml^{-1}.

PHOTOGRAPH 2. T=30 Distinct pattern formation with 5 μgml^{-1}, no pattern formation with 30 or 60 μgml^{-1}.

PHOTOGRAPH 3. T=60 Pattern formation in 0 and 10 μgml^{-1} Petri-dishes but a greater intensity of activity with 5 μgml^{-1}. No pattern formation with 30 or 60 μgml^{-1}.

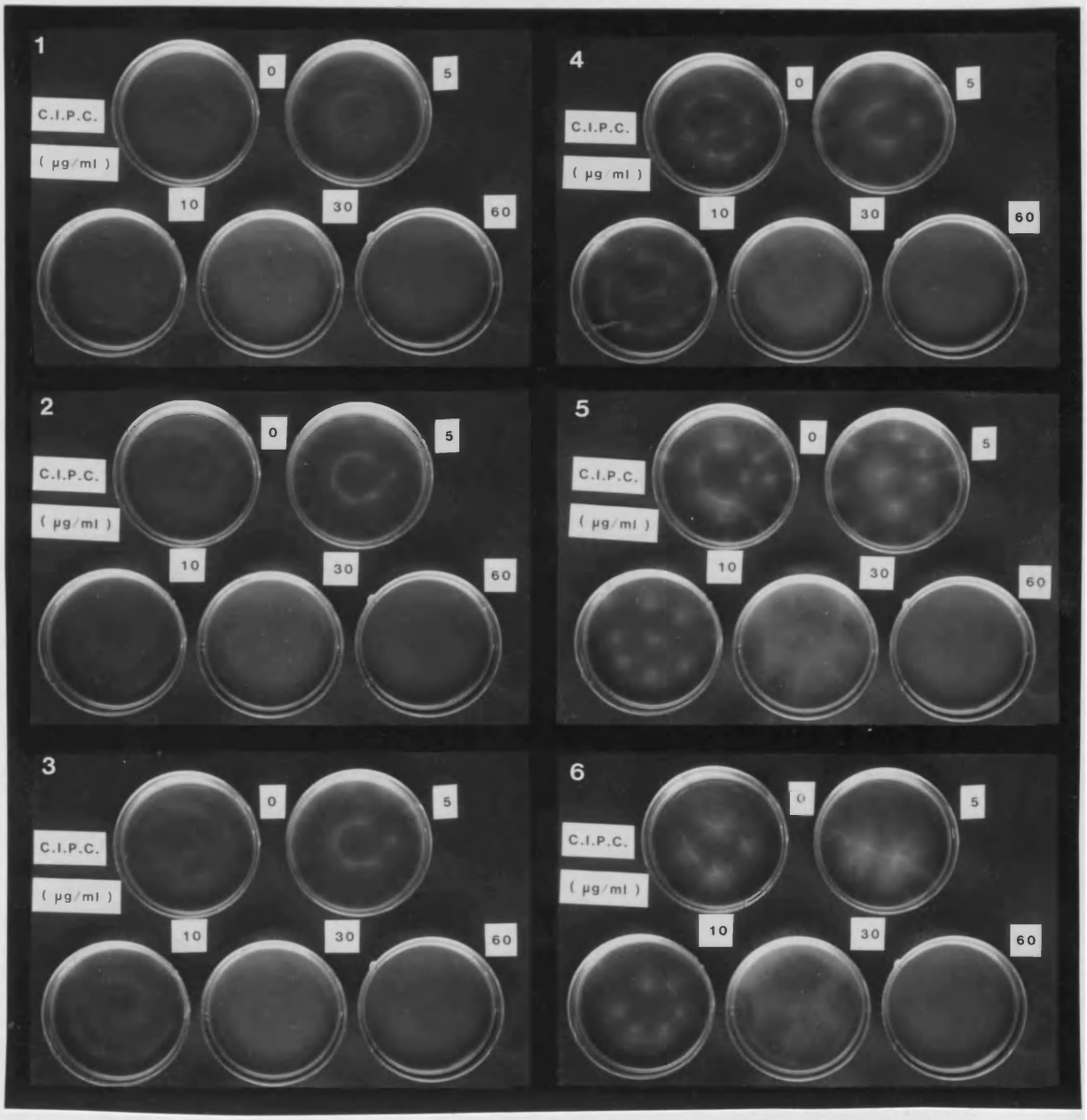
PHOTOGRAPH 4. T=120 No definate difference between pattern formation in 0, 5 and 10 μgml^{-1}. No pattern formation with 30 and 60 μgml^{-1}.

PHOTOGRAPH 5. T=300 No difference in pattern formation between 0, 5 and 10 μgml^{-1}. Vague pattern formation with 30 μgml^{-1} but but no pattern formed with 60 μgml^{-1}.

PHOTOGRAPH 6. T=600 No significant differences in pattern formation between 0, 5 and 10 μgml^{-1}. Coarse pattern formed with 30 μgml^{-1} although no clear nodes and still no pattern formed with 30 and 60 μgml^{-1}.

Cultures incubated with chlorpropham for 2 h, then tipped into Petri-dishes and photographed at the intervals indicated.

PLATE 18



The inhibition of pattern formation in suspensions of Tetrahymena pyriformis after 2 h exposure to chlorpropham (C.P.I.C.).

former, pattern formation advanced slightly slower compared with the control whilst in the latter a significant increase in the number of nodes was observed after 60s. Both conditions persisted until the end of the experiment.

Exposure to 30 μgml^{-1} chlorpropham for 2h delayed the onset of pattern formation further (300s) and after 600s the pattern observed was much coarser than with lower dose treatments and had no clear nodal formation (Plate 18). A shortening of the onset time for pattern formation was observed with 5 μgml^{-1}. Formation occurred after 30s and in advance of the untreated and 10 μgml^{-1} treatments until 120s. There was no difference between the patterns for untreated and 10 μgml^{-1} treated suspensions.

42.0 The effect of some herbicides on cell motility in Tetrahymena pyriformis

All four pesticides decreased the motility of *T. pyriformis* over 4h (Fig. 80). The order of inhibitory action was chlorpropham > barban > propham > diuron. Chlorpropham was the only compound to completely inhibit cell motility. Total inhibition in this case occurred after 1h exposure to 20 μgml^{-1}. Diuron was the only chemical to stimulate (+53% after 1h) before inhibiting motility (-40% after 2h).

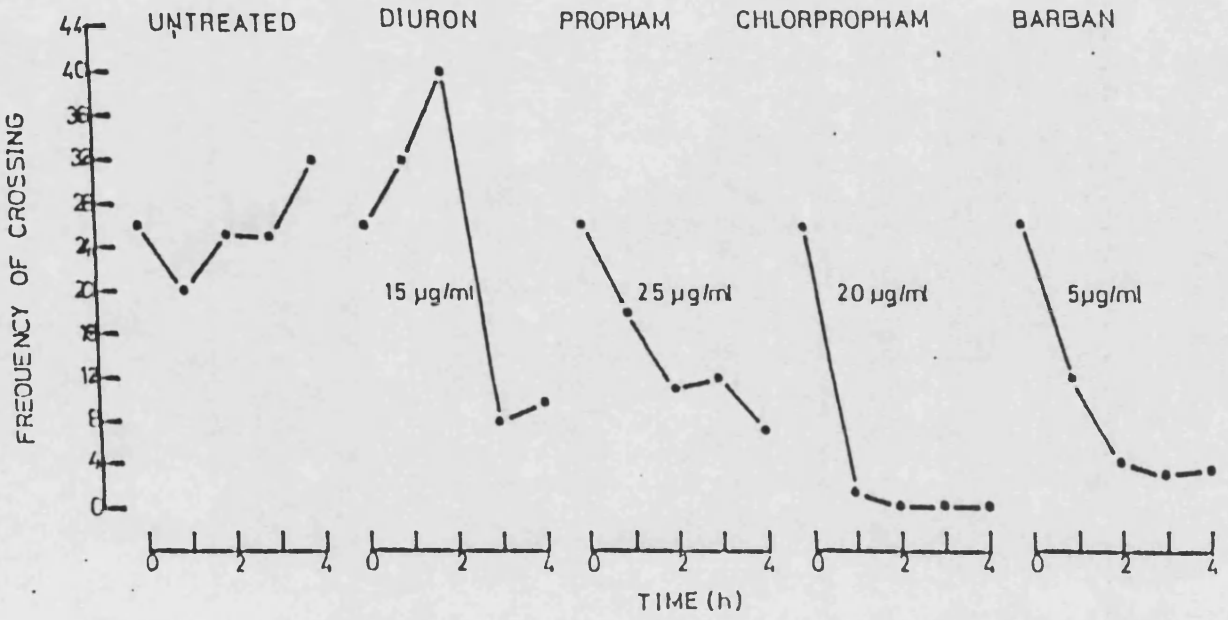


FIG. 80

The effect of diuron, propham, chlorpropham and barban on motility in Tetrahymena pyriformis grown in the presence of the herbicides for 4 h.

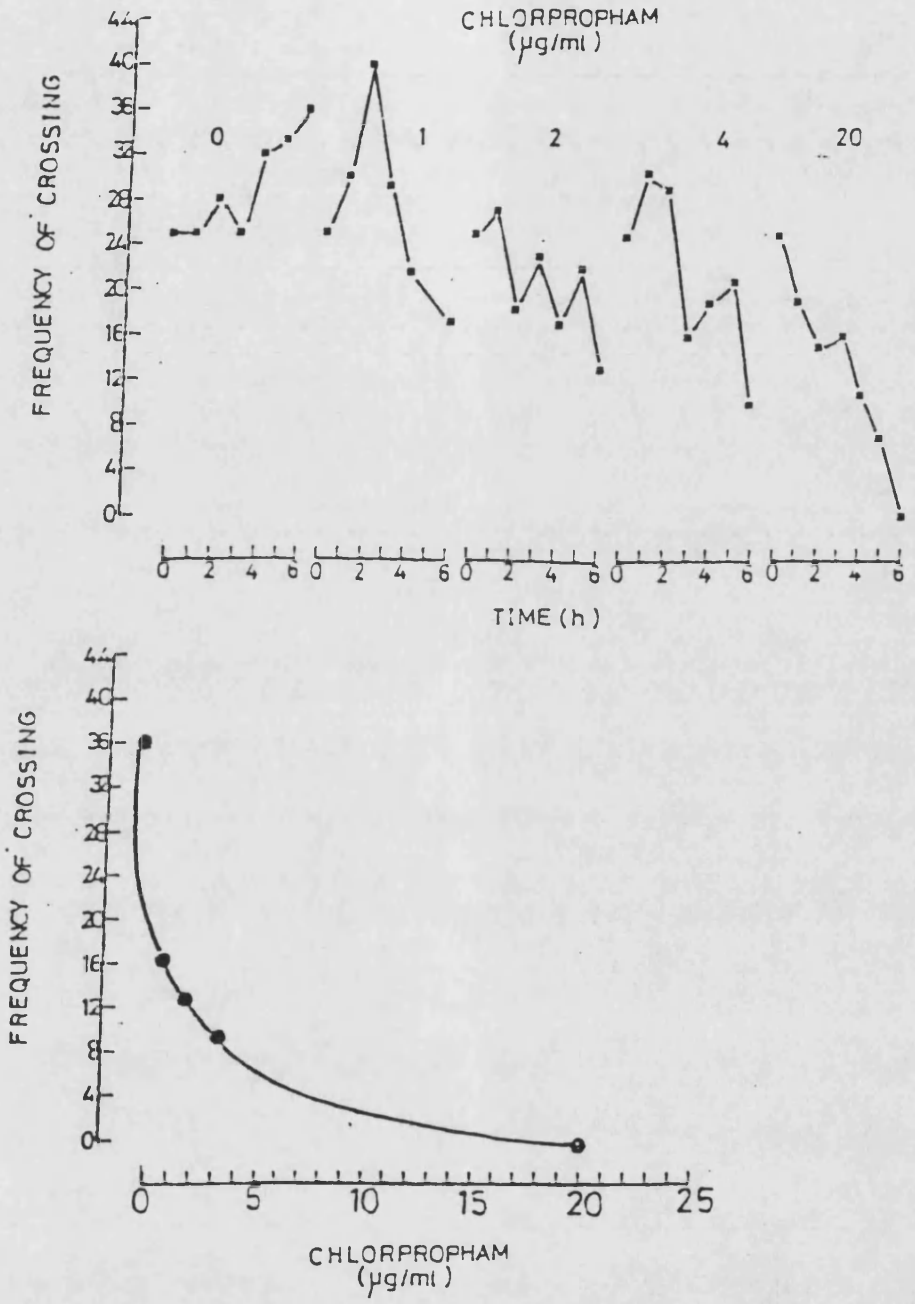


FIG. 81

The effect of chlorpropham on motility in Tetrahymena pyriformis over 6h.

TOP - The dose-dependent inhibition of motility by chlorpropham over 6 h.

BOTTOM - The dose-response curve for the inhibition of motility by chlorpropham (based on data from the 6h sampling point).

Chlorpropham (Fig. 81) inhibited cell motility in a dose-dependent manner. After 6h, 20 μgml^{-1} completely inhibited motility, whilst 4,2 and 1 μgml^{-1} reduced it by 75, 64 and 45% respectively. Chlorpropham at 1 μgml^{-1} stimulated motility over the initial 2h before exerting its inhibitory effect.

Chlorpropham at 20 μgml^{-1} reduced motility by 30% after 10 min, 40% after 30 min and 66% after 50 min. Observations on these cells showed T. pyriformis to exhibit characteristic avoidance behaviour with 20 μgml^{-1}. Cells underwent ciliary reversals whilst others pivoted about a fixed location. Some remained stationary.

43.0 Changes in the response of Tetrahymena pyriformis to some phenylcarbamate herbicides

Dose-response curves calculated at 24, 72, 144 and 240h from flask cultures (chronic toxicity evaluations, section 31) showed changes in the response of T. pyriformis cells to barban, propham and chlorpropham (Fig. 82 and 83).

Chlorpropham: The nature of the inhibition of growth of T. pyriformis by chlorpropham did not change but the degree of inhibition increased with time (eg 2 μgml^{-1} caused 60% inhibition after 24h but 83% after 24h). Little change occurred after 48h.

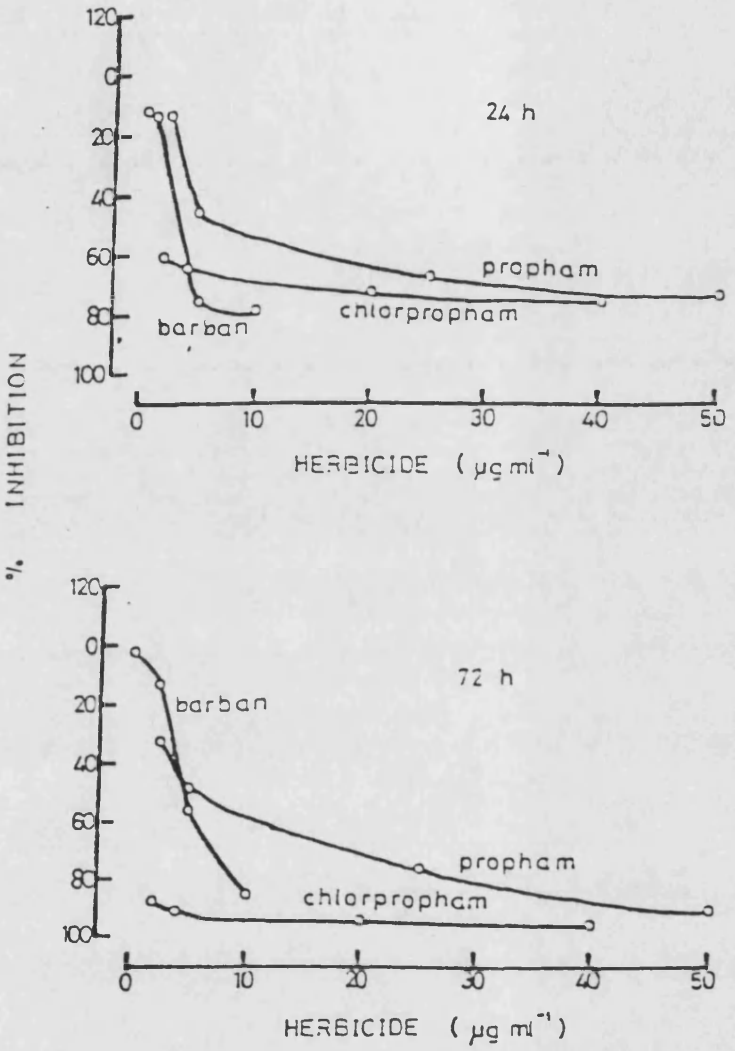


FIG. 82
The dose-response curves for the effect of some phenylcarbamate herbicides on the population growth of Tetrahymena pyriformis (at 24 and 72h) grown in PY medium at 20°C in Erlenmeyer flasks.

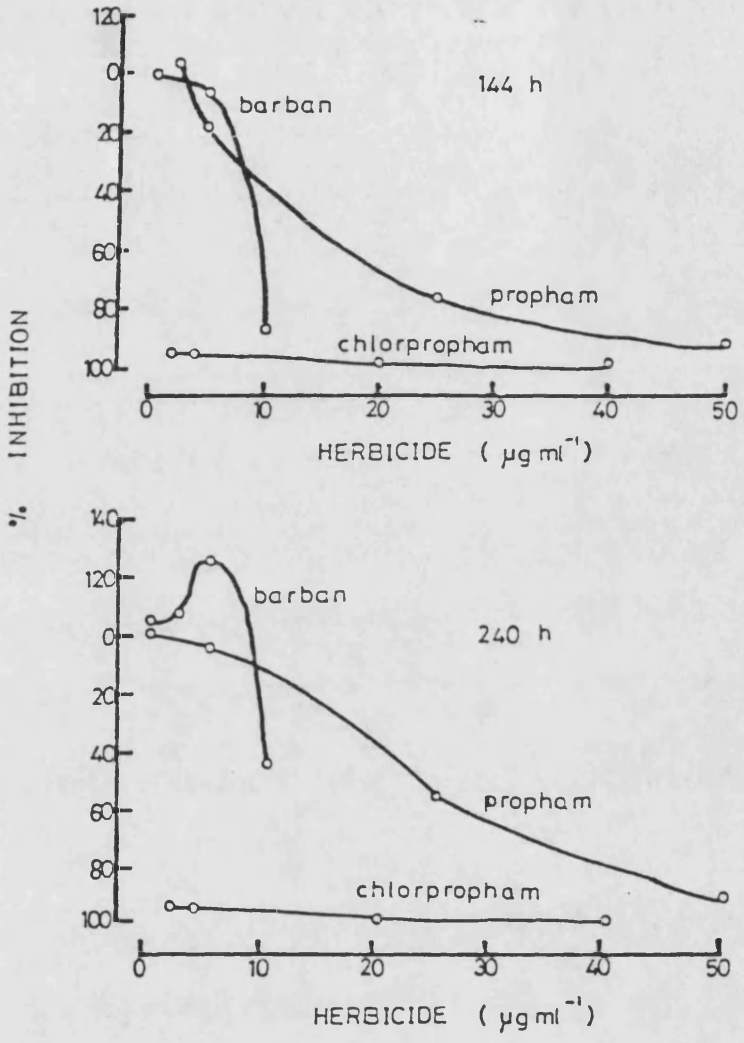


FIG. 83

The dose-response curves for the effect of some phenylcarbamate herbicides on the population growth of Tetrahymena pyriformis (at 144 and 240 h) grown in PY medium at 20°c in Erlenmeyer flasks.

Propham: Inhibition of T. pyriformis by propham was less than that of chlorpropham. Inhibition increased marginally between 24 and 48h (EC_{50} values approx 5 and 6 μgml^{-1} respectively). The EC_{50} value increased linearly after 48h and was approx 13 μgml^{-1} (144h) and 23 μgml^{-1} (240h). The dose-response curve altered from a concave to a convex shape implying recovery of the cells from the inhibitory effects.

Barban: The EC_{50} after 24h for the inhibition of T. pyriformis by barban was approx 3.1 μgml^{-1} and rose to 4.7 μgml^{-1} after 48h. These values rose throughout the experiment (10 μgml^{-1} after 144h and than the compound's solubility after 240h). The shape of the dose-response curve changed from concave (24h) through curvilinear (48h) to convex (144h). Progressive reductions in inhibitory action, at all doses was observed. After 240h significant stimulation of growth occurred. Barban was initially more toxic than chlorpropham or propham but became gradually less inhibitory than either.

44.0 Changes in the response of Tetrahymena pyriformis and Acanthamoeba castellanii to barban

Both organisms 'recovered' from barban treatments (Fig. 84). The EC_{50} values obtained from different culture regimes indicated an increase in the dose of barban required to inhibit 50% of the population of both T. pyriformis and A. castellanii with time.

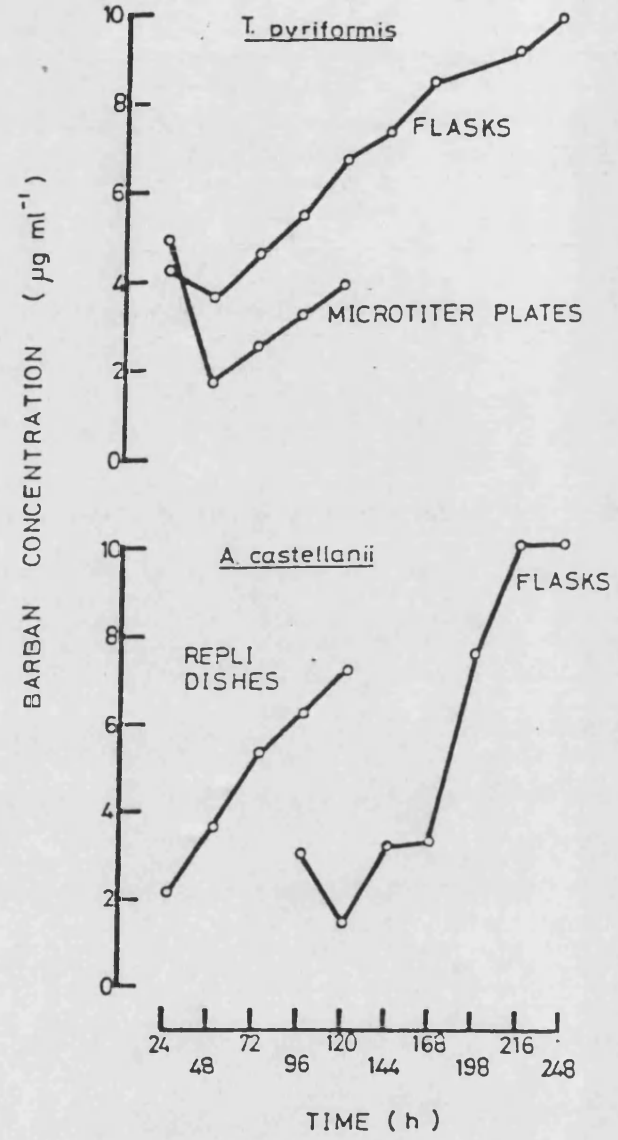


FIG. 84

Changes in the concentration of barban required to inhibit 50% of the population (EC_{50} value) of Tetrahymena pyriformis and Acanthamoeba castellanii in different culture vessels.

Similar curves obtained for chlorpropham and propham gave variable results. Generally A. castellanii 'recovered' slightly from chlorpropham and propham treatments whilst T. pyriformis showed little change with chlorpropham and with propham, an increase in the concentration required to produce the EC_{50}.

45.0 The recovery of Acanthamoeba castellanii population from propham treatment

It was found that A. castellanii populations were able to resume growth after removal from concentrations of propham up to 120 $\mu g ml^{-1}$. However, above 120 $\mu g ml^{-1}$ some decline in cell numbers was seen (Fig. 85). The majority of concentrations above 60 $\mu g ml^{-1}$ induced a delay in the growth of cultures exposed to these levels. The lag-phase increased in response to increased herbicide concentration.

For example, 60 $\mu g ml^{-1}$ delayed substantial recovery by 6h, 70 $\mu g ml^{-1}$ by 12h, 80 $\mu g ml^{-1}$ by 15h and 90 $\mu g ml^{-1}$ propham by 18h. Propham at 50 $\mu g ml^{-1}$ had no effect on the subsequent recovery of growth of A. castellanii. Evidence of induced synchrony of division was found in cultures exposed to 70, 80 and 90 $\mu g ml^{-1}$. With 90 $\mu g ml^{-1}$ this effect was most pronounced with lag-phases between division of 18 and 15h (Fig. 85).

Cells treated with 100, 110 and 120 $\mu g ml^{-1}$ showed poor recovery although with 100 and 110 $\mu g ml^{-1}$ significant

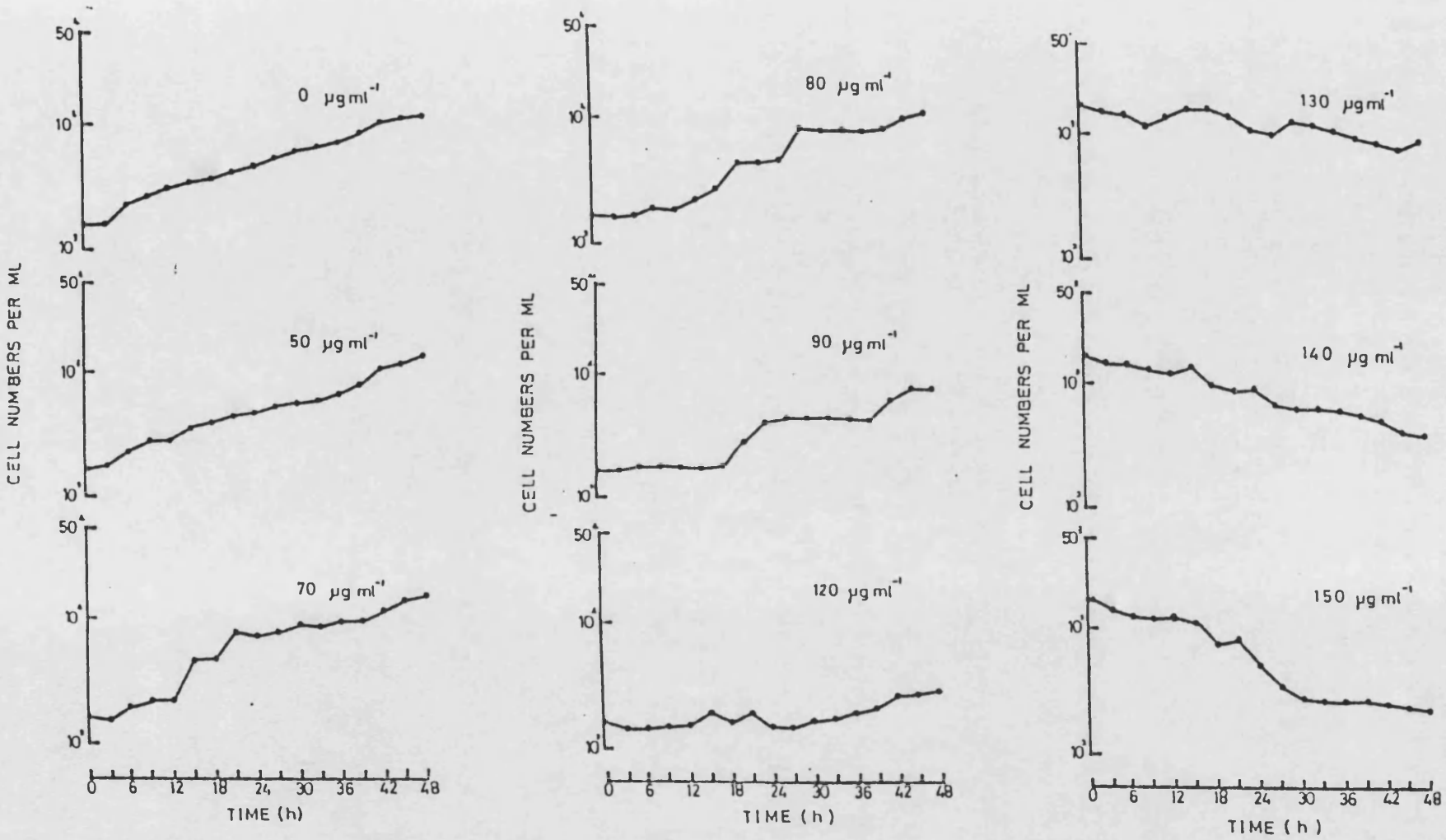


FIG. 85

The growth of Acanthamoeba castellanii after removal from PGY medium containing propham. Cells had been pre-exposed to the stated herbicide concentrations for 48 h.

increases in numbers were seen after 42 and 45h respectively. Cells did not recover from propham treatments above 130 μgml^{-1}. Such treatments caused cell death, numbers decreasing with time.

46.0 The recovery of Tetrahymena pyriformis from some pesticides

Chlorpropham: Cells pre-exposed for 24h to 1,2,4,20 and 40 μgml^{-1} chlorpropham had 'recovery' growth rates which were different from the untreated cells (Fig. 86). Concentrations of 4,20 and 40 μgml^{-1} significantly reduced the recovery growth rates of T. pyriformis (significant at $p=0.01$), whilst cells treated with 2 μgml^{-1} and 1 μgml^{-1} chlorpropham had recovery rates below untreated values which were significant at $p=0.01$ and $p=0.5$ respectively.

Exposure to chlorpropham for 48h increased the t-value for 2,4,20 and 40 μgml^{-1} treatments suggesting greater variance from the untreated but still significant $p=0.01$. A decline in cell numbers was seen with 20 and 40 μgml^{-1} treatments.

The growth rate of cells treated with chlorpropham at 1 μgml^{-1} for 48h was not significantly different from that of the untreated cells, in contrast to the results found with 24h treatments.

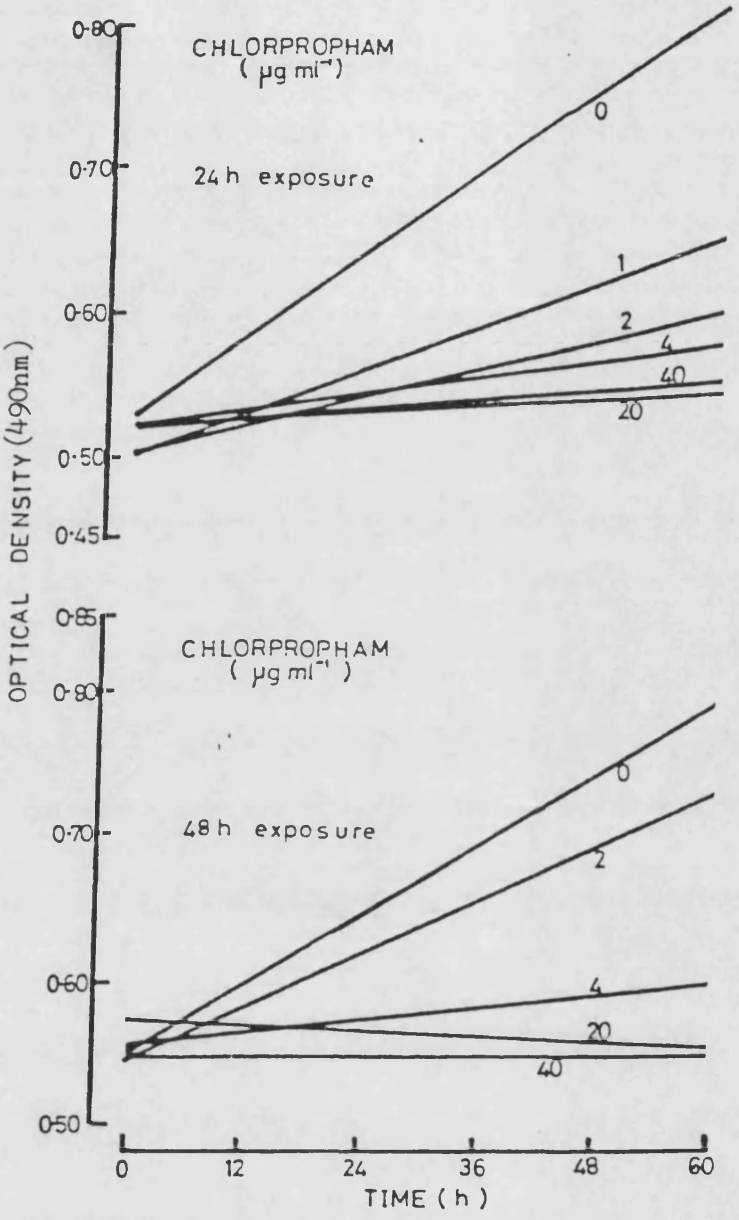


FIG 86

The growth rates of Tetrahymena pyriformis populations after removal from PY media containing chlorpropham. Cells were pre-exposed to the stated concentrations for 24 and 48 h. Only those growth rates which were significantly different from the untreated were plotted.

Observations on cells recovering from chlorpropham treatments showed some of the previously-described morphological effects of chlorpropham. The effects were dose-dependent, increasing in severity with herbicide concentration but decreasing with time.

Not all cells exposed to 40 μgml^{-1} (for 24h or 48h) showed normal morphology. Most possessed no discernable pellicle or cilia and consequently had lysed or were lysing.

The majority of cells pre-exposed to 20 μgml^{-1} chlorpropham were mishapen or possessed 'giant' vacuoles (possibly contractile) after 6h recovery (Plate 19). Some cells were in a state of incomplete division, others were truncated. Most cells, except those dividing, had no cilia. After 12h recovery many rounded cells were observed along with blunt-ended pyriforms, all of these cells had cilia. Some cells developed cytoplasmic extension, which were projected beyond the pellicle but still bounded by a membrane (cytoplasmic 'blebbing'). Some mishapen forms remained in this state after 21h although the largest proportion of cells remained round. Many cells recovered their pyriform shape, although these were smaller than untreated cells of the same age. Cilia were observed on all cells but the occurrence of food vacuoles or any other differentiation in the cytoplasm was often absent. After 63h most cells appeared normal but small, some round cells remained.

PLATE I9.

The appearance of Tetrahymena pyriformis cells after their removal from PY medium containing chlorpropham. Cells were pre-exposed to the stated concentrations for 48 h.

PHOTOGRAPH I.

A cell 6h after removal from PY medium containing 4 μgml^{-I} chlorpropham. The cell is highly vacuolated and mishapen but still retains the cilia.

PHOTOGRAPH 2.

A cell 6h after removal from PY medium containing 4 μgml^{-I} of chlorpropham. Although the cell remains highly vacuolated, normal cell shape has returned.

PHOTOGRAPH 3.

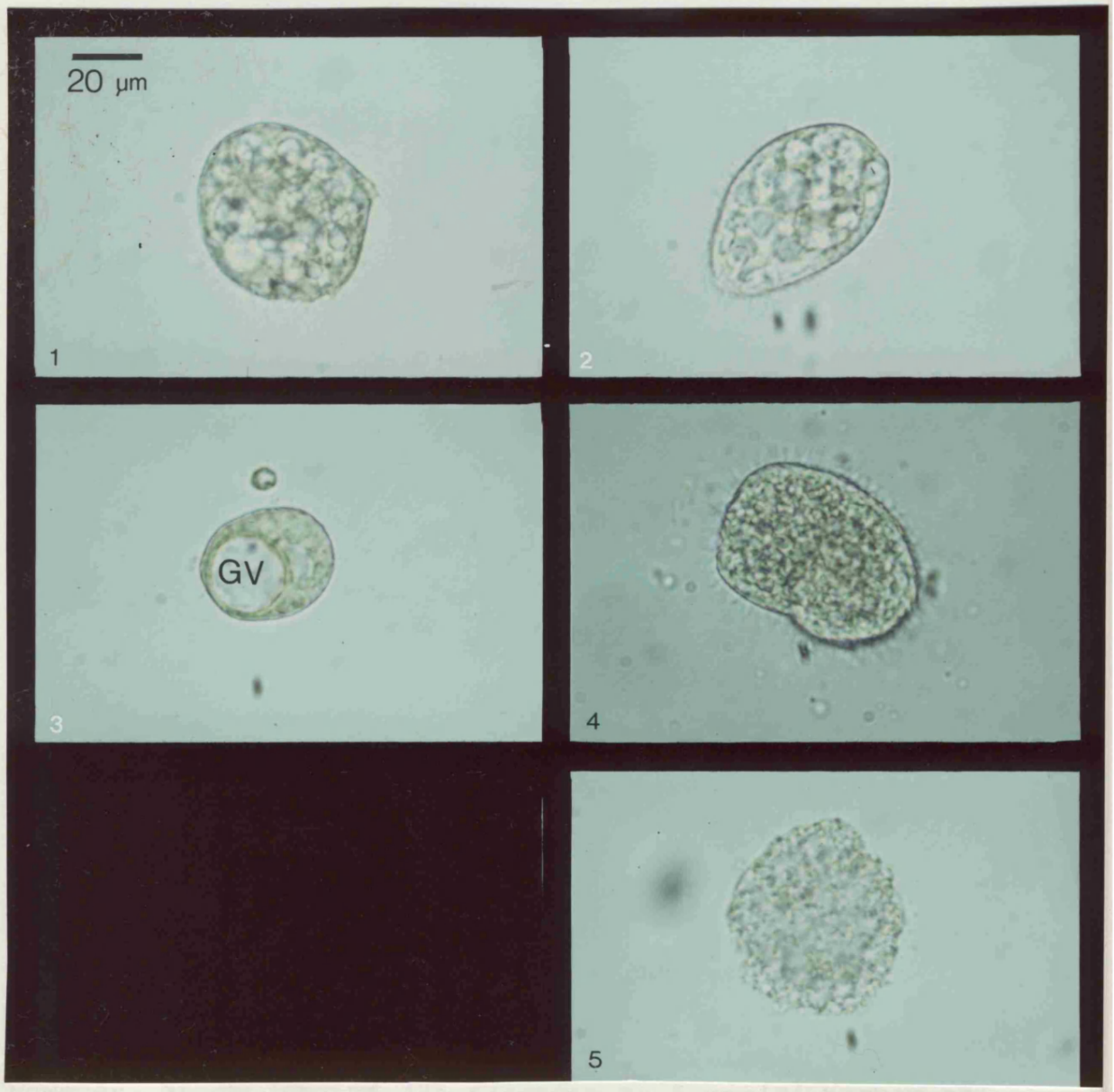
A cell 6h after removal from PY medium containing 20 μgml^{-I} of chlorpropham. The cell remains barrel shaped, small vacuoles are absent and has few cilia. A 'giant' vacuole (GV) at the cell posterior is prominent. This vacuole was thought to be a contractile vacuole.

PHOTOGRAPH 4.

A cell 6h after removal from PY medium containing 20 μgml^{-I} of chlorpropham. The cell has no large vacuole and possesses numerous cilia. They appear to be attempting to divide.

Scale marker in photograph I refers to all the photographs in the plate.

PLATE 19.



PHOTOGRAPH 5.

A cell 6 h after removal from PY medium cotaining 40 $\mu g ml^{-1}$ of chlorpropham. The cell is lysing. Cells were not observed to reccver from such treatments.

Exposure to 4 μgml^{-1} chlorpropham (24h) induced similar morphological effects. Such cells, after 6h, were highly vacuolated, although no 'giant' vacuoles were observed (Plate 19). Some cells were mishapen. Normal division was observed in such cultures and all cells possessed cilia. After 12h an increasing number appeared normal, some were rounded but all had cilia and food vacuoles. All cells were normal after 21h.

After 6h recovery, cells pre-exposed to 1 and 2 μgml^{-1} of chlorpropham showed normal morphology.

Cells exposed to chlorpropham treatments for 48h before removal showed similar morphological effects to 24h treatments except with 20 μgml^{-1} where no recovery of cells occurred.

Barban: Growth rates of cell populations recovering from exposure to barban for 24h at 0.25, 0.5 and 1 μgml^{-1} were not significantly different from the untreated cells. However, after 24h and 48h exposure to 5 μgml^{-1} barban the growth rate of T. pyriformis cells was greatly reduced, significant at $p=0.01$, (Fig. 87). Significant increases in the rate of growth were observed with 0.25 μgml^{-1} ($p=0.01$) and 1 μgml^{-1} ($p=0.05$) after 48h exposure to barban and evidence from comparative analysis (cf. sub-acute toxicity test, Repli-dish) suggested that 0.5 μgml^{-1}-treated cells may have an enhanced growth rate.

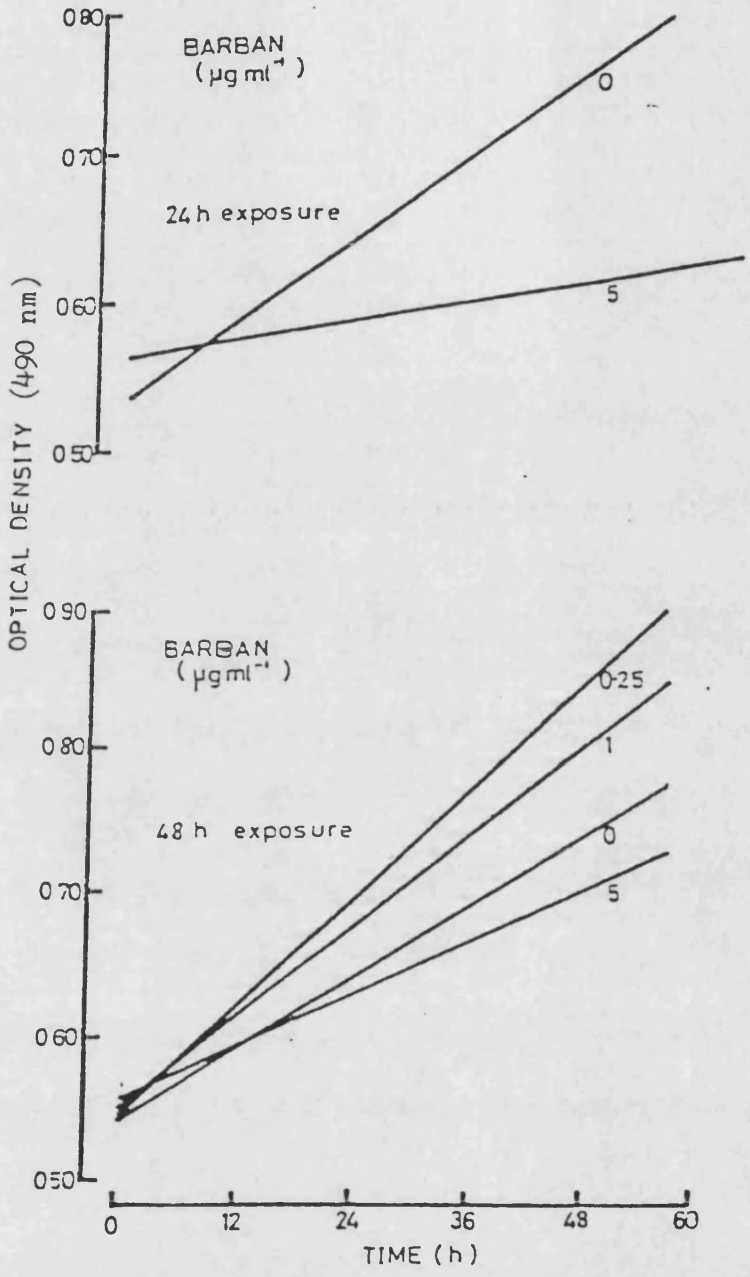


FIG. 87

The growth rates of Tetrahymena pyriformis populations after removal from PY media containing barban. Cells were pre-exposed to the stated concentrations for 24 and 48 h. Only those growth rates which were significantly different from the untreated were plotted.

T. pyriformis cells recovered from all barban treatments without morphological change.

Diuron: The growth rate of cell populations pre-exposed to 15 and 30 $\mu g ml^{-1}$ for 24h were significantly reduced (significant at p=0.01) (Fig. 88). No other treatment affected growth. After 48h exposure stimulation of the growth rate occurred with 3 and 1.5 $\mu g ml^{-1}$. This was significant at p=0.5 and p=0.01 respectively. Again no other treatment affected growth.

Recovery of cells from all diuron treatments occurred within 6h without morphological abnormalities.

Propham: No concentration of propham altered the recovery growth rate of T. pyriformis cells and no morphological defects were observed, except after 6h recovery from 48h exposure to 50 $\mu g ml^{-1}$ when a few rounded cells were observed.

47.0 The transformation of some phenylamide herbicides by Tetrahymena pyriformis and Acanthamoeba castellanii

No detectable amounts of either 3-chloroaniline or aniline were found in the control cultures (T. pyriformis or A. castellanii incubated with no herbicide for 4 and 24h) over the range of cell densities investigated.

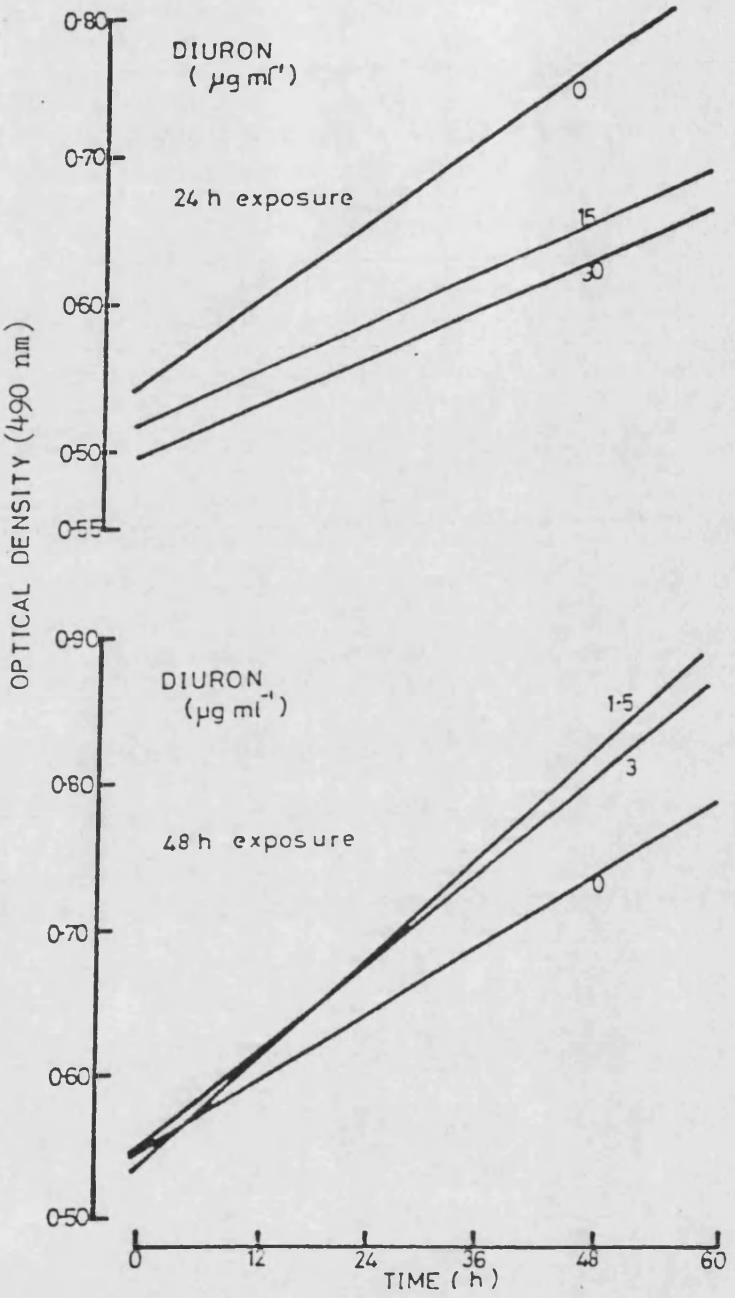


FIG. 88

The growth rates of Tetrahymena pyriformis populations after removal from PY media containing diuron. Cells were pre-exposed to the stated concentrations for 24 and 48 h. Only those growth rates which were significantly different from the untreated were plotted.

Chlorpropham: The formation of 3-chloroaniline from chlorpropham was related to cell number of T. pyriformis (Table 53). The amount of metabolite produced was very low, 0.1% of the herbicide was transformed with 2.2×10^6 cells ml^{-1}, highest cell density tested, after 24h. No activity was seen with cultures incubated for 4h with chlorpropham although a detectable amount of 3-chloroaniline was found with cell fragments incubated over the same period.

Quantities of transformed chemical were again low with A. castellanii (Table 54). Cells grown in successive cultures for 288h in the presence of 10 $\mu g ml^{-1}$ of chlorpropham were not observed to transform greater amounts of chlorpropham than cells cultured on standard medium.

Barban: T. pyriformis-mediated metabolism of barban to 3-chloroaniline was detected (Table 53). Transformational activity was seen in cultures incubated for 4h with barban but the activity in sonicated cells was 2.5x greater over the same period.

The quantity of metabolite produced was lower with A. castellanii (Table 54) than T. pyriformis. Cells grown in successive cultures for 288h in the presence of chlorpropham (10 $\mu g ml^{-1}$), a chlorpropham pre-treatment, had 10x less detectable metabolite than cells grown for the same time on standard PGY.

Propham: The action of T. pyriformis cells on propham was similar to that on chlorpropham, again the quantity transformed was low, 0.10% of propham converted after 24h with 2.2×10^6 cells ml^{-1}.

No activity was seen in 4h-incubated suspensions although aniline was detected in the media after 4h incubation with sonicated T. pyriformis.

The ability of A. castellanii to transform propham was much lower than T. pyriformis (Table 54). Pretreatment over successive cultures with 10 $\mu g ml^{-1}$ of chlorpropham prevented A. castellanii cells from converting propham to aniline.

The transformational ability of T. pyriformis was greater than A. castellanii with all 3 herbicides tested. The ciliate had a greater effect on both the rate and quantity of barban transformed than on either chlorpropham or propham.

Table 53

Formation of aniline compounds from some phenylcarbamate herbicides in Tetrahymena pyriformis suspensions

Cell Number ml^{-1}	Incubation Time (h)	Pesticide ($\mu g ml^{-1}$)	Concentration of metabolite 3 chloroaniline ($\mu g ml^{-1}$)	aniline ($\mu g ml^{-1}$)	Percentage of Parent 3 chloroaniline (%)	Compound aniline (%)
9.2×10^4	24	chlorpropham (40)	0.017	-	0.04	-
	24	barban (10)	0.023	-	0.23	-
	24	propham (50)	-	0.030	-	0.06
	24	control	0	0	-	-
3.1×10^5	24	chlorpropham (40)	0.023	-	0.05	-
	24	barban (10)	0.027	-	0.27	-
	24	propham (50)	-	0.031	-	0.06
	24	control	0	0	-	-
2.2×10^6	24	chlorpropham (40)	0.040	-	0.10	-
	24	propham (50)	-	0.050	-	0.10
7.5×10^5	4	chlorpropham (40)	0	-	0	-
		control	0	0	-	-
	4	barban (10)	0.017	-	0.17	-
	4	propham (50)	-	0	-	0
	4	control	0	0	-	-
7.5×10^5 (sonicated)	4	chlorpropham (40)	0.017	-	0.03	-
	4	barban (10)	0.043	-	0.43	-
	4	propham (50)	-	0.020	-	0.04
	4	control	0	0	-	-

control = Tetrahymena pyriformis cells incubated for 4 or 24h with no herbicide

Table 54

Formation of aniline compounds from some phenylcarbamate herbicides in Acanthamoeba castellanii suspensions

Cell Number ml^{-1}	Incubation Time (h)	Pesticide ($\mu g ml^{-1}$)	Concentration of metabolite 3 chloroaniline ($\mu g ml^{-1}$)	aniline ($\mu g ml^{-1}$)	Percentage of Parent 3 chloroaniline (%)	Compound aniline (%)
9.9×10^6	24	chlorpropham (20)	0.020	-	0.1	-
	24	(40)	0.025	-	0.06	-
		barban				
	24	(5)	0	-	0	-
	24	(10)	0.020	-	0.2	-
		propham				
	24	(25)	-	0	-	0
	24	(50)	-	0.004	-	0.008
	24	control	0	0	0	0
9.9×10^6 pretreated cells*	24	chlorpropham (40)	0.020	-	0.05	-
		propham (50)	-	0	-	0
		barban (10)	0.002	-	0.002	-
	24	control	0	0	0	0

* Cells grown for 288h in PGY + 10 $ugml^{-1}$ chlorpropham

control = Acanthamoeba castellanii cells incubated for 24h with no herbicide

DISCUSSION

48.0 Experimental procedures

The methods used for investigations into the effects of pesticides on protozoa are rarely standardized between different authors. The choice of chemical(s) and organism(s) permits a large number of permutations without replication. In particular, the number of specific chemicals available for experimentation is extremely large and regulated by social, economic and geographical factors.

This inherent problem is further compounded by differences in laboratory culture protocol/procedures and the methods used in determining the interaction of pesticides with protozoa. For example, EC_{50}, LD_{50}, MIC and MAD describe inhibitory effects of a chemical but from very different viewpoints.

Direct comparisons are therefore rarely possible.

49.0 Sub-acute toxicity of pesticides to Acanthamoeba castellanii and Tetrahymena pyriformis with particular reference to experimentation in microtiter plates

Qualitative assessment of the growth of different types of microorganism in microtiter plates have been described

(Darbyshire, 1973; Darbyshire et al., 1974; Cooper et al., 1978; Blaise, Legault & Bermingham, 1982; Lorenz & Krumbein, 1984). Attempts to quantify microbial growth in microtiter plates have been made by direct counting (Weber et al., 1982) ATP bioassay (Blaise et al., 1984) and measurement of optical density (Ekland & Jarmand, 1983; De Girolami et al., 1983; Laughton, 1984). However, no attempt to assess, qualitatively or quantitatively, the effect of toxicants on growth of axenic cultures of protozoa in microtiter plates has been made. It may be noted that Blaise (personal communication) states that Dive suggested the use of microtiter plates for aquatic toxicity tests with Colpidium campylum to him in 1981.

Direct comparisons of the effects of individual pesticides on either T. pyriformis or A. castellanii in microtiter plates with results of other workers using different techniques is therefore impossible. However, comparison of the effects of particular compounds on different organisms may be made on a limited scale from results obtained in the present study.

49.1 Comparison of quantitative and qualitative methods of determining pesticide inhibition of Acanthamoeba castellanii

In the present study there was little correlation between the ranking of toxicity of individual compounds towards

A. castellanii in the two assessment programmes of this investigation (Tables 24 and 46). The EC_{50} and MIC values are not interchangeable and use separate criteria in their determination. Generally the sensitivity of the quantitative method (EC_{10} values) was greater than the qualitative ('band-formation') method in the microtiter plate system. Each lists propham, barban, diuron, chlorpropham and terbutryne as being highly inhibitory to A. castellanii.

Comparison of EC_{50} microtiter plate values with inhibition of 'band-formation' in microtiter plate wells shows the latter's MIC values to detect inhibition of A. castellanii at a lower level.

49.2 Comparison of the effects of pesticides on growth of Acanthamoeba castellanii and Tetrahymena pyriformis in microtiter plate, Repli-dish and flask batch culture systems

T. pyriformis was more susceptible (based on EC_{50} values) to a broader range of chemicals than A. castellanii although the latter was more sensitive at reduced inhibition levels (EC_{10} values). Growth of T. pyriformis was inhibited to a greater degree than A. castellanii by representatives of the phenylcarbamates, Urea and Triazine herbicides, Organophosphorus and Carbamate insecticides and Carbamate fungicides.

Only 3 of 12 compounds inhibited growth of A. castellanii by 50%, suggesting that it would be an unsuitable organism in primary toxicity assessment programmes. However, both chlorpropham and diuron were strongly inhibitory.

The uncharacteristic 'flatness' of the growth curves of A. castellanii in microtiter plates induced by high initial inoculum levels and a slow growth rate are an inherent feature of this system and again mitigate against the use of A. castellanii as a test organism with microtiter plates and the Dynatech MR600 plate reader.

The greater sensitivity of A. castellanii over T. pyriformis to diuron, linuron, propham, terbutryne, malathion, MCPA and pirimicarb (based on EC_{10} values) accounts for the sensitivity of the qualitative method 'band formation' and suggests that this method detects growth inhibition of less than 10%.

As stated previously, the high initial inoculum (3×10^5 cells ml^{-1}) of A. castellanii in microtiter plates produced shallow growth curves, whilst lowering the initial inoculum level to 7×10^4 cells ml^{-1} in qualitative 'band formation' method produced more typical growth curves (Fig. 6). Such shallow growth curves may reflect the physiological state of the cells. Byers (1979) in a review of growth and differentiation in A. castellanii concluded that termination of exponential

growth was probably not due to nutrient deficiency, production of growth limiting factors or accumulation of inhibitors but was more likely to arise from oxygen deficiency. The stationary incubation of large numbers of A. castellanii cells in cylindrical 'U' shaped wells may enhance rapid oxygen depletion and cause a much reduced growth rate and consequent increase in mean generation time.

The apparent lack of sensitivity of A. castellanii to a wide range of pesticides may well be due to the relatively inactive physiological state of cells grown in microtiter plates. A number of authors have observed that action of some pesticides towards protozoa depends on culture age. Changing sensitivity to inhibition by pesticides with increasing culture age was found with A. castellanii (Prescott & Olson, 1972); Euglena gracilis (Poorman, 1973); aquatic protozoan species (Lejazack, 1977), Blepharisma intermedium and Stylonychia notophora (Shivaji et al., 1978a, 1978b) and T. pyriformis (Rup Lal & Saxena, 1979). Marčenko (1980) found that inhibition of cell division in E. gracilis by propham depended on age and nutritional history of the cells in culture.

The assessment of growth according to optical density reading of cell suspensions of A. castellanii at 410nm may further reduce the sensitivity of this toxicity assessment system. Experiments with cell suspension growth in

Repli-dishes showed maximum optical density to occur at 400nm, the Dynatech MR600 has the facility to use this wavelength but this was not available at the time. Such an effect is, however, likely to be slight especially when dealing with such high inoculum levels.

The differences in the response of A. castellanii to inhibition by pesticides in Repli-dishes and microtiter plates probably reflects differences in methodology. In the particular case of the phenylcarbamate herbicides the slower rate of growth of A. castellanii cultured in microtiter plates may also reduce the phenylcarbamates apparent antimitotic effect and contribute to their lack of inhibition in such cultures.

Phenylcarbamate herbicides: Using flask cultures of Colpidium campylum to assess the toxicity of 39 pesticides, Dive et al. (1980) noted that the most toxic chemicals were antimitotic agents or inhibitors of oxidative phosphorylation. The antimitotic mode of action of the phenylcarbamate herbicides has been demonstrated in plants (Ennis, 1948a; Canvin & Friesen, 1959; Mann & Storey, 1966; Helper & Jackson, 1969), the flagellate Ochromonas (Brown & Bouck, 1974), mouse oocytes (Magistrini & Szollosi, 1980), human lymphocytes (Timpson, 1970) and Euglena gracilis (Marčenko, 1980). Lotikar et al. (1968) showed that both chlorpropham and propham uncoupled oxidative phosphorylation in isolated cabbage mitochondria.

In this study chlorpropham was more toxic to both protozoan species than either propham or the metabolites, 3-chloroaniline or aniline. The inhibition of protozoa by these phenylcarbamates and their derivatives parallels their order of toxicity to plants (Clark & Wright, 1970), micro-algae (Wright, 1972, 1975), cyanobacteria and a green alga (Maule & Wright, 1983).

The high degree of toxicity of the phenylcarbamate herbicides to both organisms was confirmed in sub-acute toxicity studies in Repli-dishes. Chlorpropham was the most toxic. The order of inhibition was chlorproham > propham > 3-chloroaniline > aniline > with T. pyriformis and chlorpropham > proham > barban > 3-chloroaniline or aniline with A. castellanii.

T. pyriformis was more susceptible to the 'protistatic' and 'proticidal' effects of the phenylcarbamates and had a lower concentration threshold, to inhibition, than A. castellanii. Cell stasis was not unique to T. pyriformis or the phenylcarbamates, for example pirimicarb and propham both had similar effects on A. castellanii.

The comparative EC_{50} values for chlorpropham, propham and barban against A. castellanii in Repli-dishes were 1.4×10^{-4}, 1.6×10^{-4} and 0.23×10^{-4} M respectively indicating that A. castellanii was more sensitive to phenylcarbamate inhibition than the results from microtiter plates suggest.

Prescott & Olson (1972) described the variable effect of propham on A. castellanii, later showing a 66% reduction of growth by 17.9 μgml^{-1} in flask batch culture (Prescott et al., 1977). Marcēnko (1980) noted that 17.9 μgml^{-1} propham also inhibited cell division and growth in Euglena gracilis and at 179 μgml^{-1} was lethal.

In the present study, propham, 5.6 and 7.5 μgml^{-1}, significantly inhibited the growth rates of T. pyriformis and A. castellanii respectively. At 75 μgml^{-1} it induced a lag phase (48h) in A. castellanii cultures and above 113 μgml^{-1} it was lethal. The EC_{50} values were 9 and 30 μgml^{-1} for T. pyriformis and A. castellanii respectively.

Aniline at 250 μgml^{-1} completely inhibited the growth of T. pyriformis (Schultz & Allison, 1979). The EC_{50} value for aniline against T. pyriformis in Repli-dishes in the present study was 154 μgml^{-1}.

Barban, chlorpropham and diuron had an initial toxic action to A. castellanii which preceded a reduced growth rate. A similar action was noted with chlorpropham on T. pyriformis. Other reports on initial toxicity preceding reduced growth in protozoa include amoebicides (Pfaffman & Klein, 1966) malathion (Poorman, 1973) atrazine (Toth & Tomasovicoca, 1973) carbaryl, propoxur and chlorfenvinphos (Lejczak, 1977) and DDT (Rup Lal & Saxena, 1979). The occurrence of such an action with a variety of chemicals

and in a number of species suggest that it is a general response and not specific to the phenylcarbamates.

Insecticides: The mode of action of organophosphorus and carbamate insecticides is through the inhibition of acetylcholinesterase. This enzyme is unknown in T. pyriformis (Hill, 1972; Hutner et al., 1973) which may account for the comparatively low toxicity of malathion, pirimicarb and carbaryl to T. pyriformis. The reason(s) for the enhanced sensitivity of A. castellanii to these compounds, in microtiter plates, or why carbaryl is stimulatory is not known.

Pirimicarb was not very toxic to T. pyriformis in either Repli-dish or microtiter plate batch culture. Nistiar et al. (1981) also noted the low toxicity of another carbamate compound, eserine, to T. pyriformis.

The comparatively low EC_{10} values for malathion and pirimicarb with A. castellanii may be due to the cells, in late exponential growth, having increased enzymic activity (Barnes & Jenson, 1967). This might afford a greater opportunity for the organophosphate and carbamate to interact non-specifically with enzymes other than choline esters. This was a possibility suggested by Dive et al. (1980) for the inhibitory action of organophosphate and carbamate insecticides on Colpidium campylum.

Permethrin, a synthetic pyrethroid insecticide of notably low mammalian toxicity, had no effect on T. pyriformis or A. castellanii.

Miscellaneous pesticides: Differences in the response of different species of protozoa to a single compound have been widely reported (West et al., 1962; Pfaffman & Klein, 1966; Gel'tser, 1968; Gregory et al., 1969; Neméth, 1972; Meredith & Meredith, 1972; Ruthven & Cairns, 1973; Bai & Dilli, 1974; Jeanne-Levain, 1974; Miteva, 1976; Gel'tser & Geptner, 1976; Popovici et al., 1977; Redorko et al., 1977 and Rogerson & Berger, 1981b). Few authors have used identical compounds and even fewer have adopted identical experimental techniques. There are no reports on the use of Repli-dishes and only a few on the use of microtiter plates in studies of the inhibitory activity of pesticides, or any other toxicant, on protozoa. Therefore, the results of this study can only be compared with those of other authors on the broadest of bases.

Carbaryl has variable effects on protozoa. Lejezack (1977) found an LD_{50} value of 10.2 $\mu g ml^{-1}$ (96h) with Paramecium caudatum, growth of A. castellanii was inhibited by 10 $\mu g ml^{-1}$ (Prescott & Olson, 1972), the minimum active dose for Colpidium campylum was 10 $\mu g ml^{-1}$ (Dive et al., 1980) and 1 $\mu g ml^{-1}$ inhibited growth of

A. castellanii was inhibited by 10 μgml^{-1} (Prescott & Olson, 1972), the minimum active dose for Colpidium campylum was 10 μgml^{-1} (Dive et al., 1980) and 1 μgml^{-1} inhibited growth of Euplotes spp. by 50% (Weber et al., 1980). In the present study carbaryl at 11.4 and 38 μgml^{-1} inhibited population growth (96h) of T. pyriformis by 10 and 50% respectively and stimulated the growth of A. castellanii.

The minimum active doses of MCPA, fenuron and malathion were greater than 10 μgml^{-1} with C. campylum (Dive et al., 1980), whilst in this study EC_{50} values for T. pyriformis were 100, 700 and 110 μgml^{-1} respectively.

Poorman (1973) noted that concentrations of malathion between 1 and 100 μgml^{-1} were toxic to Euglena gracilis over 24h while Weber et al. (1982) reported an LD_{50} of 1 μgml^{-1} with Euplotes spp. The malathion EC_{50} value with T. pyriformis in the present study was 110 μgml^{-1}.

The above results suggest that T. pyriformis is not as sensitive to the inhibitory action of pesticides as other ciliated protozoa. However, a number of reports, based on direct comparisons, show T. pyriformis to exhibit a greater sensitivity than other protozoa. Cooley & Keltner (1970) found T. pyriformis to be more sensitive to DDT than either Paramecium multinucleatum, P. bursaria or Euglena gracilis. Nemèth (1972) described more 'distinct

inhibition' of growth with blood sera to T. pyriformis than P. caudatum and Jeanne-Levain (1974) found T. pyriformis to be inhibited to a greater extent by the insecticide Lindane than were either E. gracilis or a dinoflagellate. Colpidium campylum and T. pyriformis were both more susceptible to PCB's than E. gracilis (Dive et al., 1976).

Dose-response curves: Few authors have presented dose-response curves from their data for the action of toxicants on protozoa. Weber et al. (1980) described mortality curves, (technically, % inhibition) for Euplotes spp. treated with four pesticides. The general shapes of these curves reflected those obtained with T. pyriformis in both Repli-dish and microtiter plates in the present study, although Euplotes spp. appear more sensitive to malathion and carbaryl than did T. pyriformis.

In this study dose-response curves were negatively sigmoidal with the ciliate and curvilinear for A. castellanii in experiments in both microtiter plates and Repli-dishes. Such curves are similar to those proposed by Ord (1979) to theoretically represent whole cell mortality due to combined nuclear and cytoplasmic damage in Amoeba proteus.

The dose-dependent action of pesticides against both protozoa are typical of such chemicals and have been

demonstrated to occur with T. pyriformis (Cooley & Keltner, 1970; Thrasher & Adams, 1972; Rankin et al., 1977; Rup Lal & Saxena, 1979; Schultz & Allison, 1979; Nistar et al., 1981); A. castellanii (Prescott & Olson, 1972; Prescott et al., 1977); P. caudatum (Lejazak, 1977); E. gracilis (Poorman, 1973; Marcenko, 1980) and Blepharisma intermedium (Shivaji et al., 1979a, 1979b, 1979d).

The validity of comparing protozoan sensitivities to toxic chemicals is questioned when different experimental procedures are used. Dive et al., (1980) and Weber et al., (1982) used monaxenic cultures, not axenic as in the present study. Prescott & Olson (1972) and Poorman (1973) used Erlenmeyer flasks, not microtiter plates and none of these authors employed optical density to assess growth.

49.3 Growth stimulatory effects of pesticides.

Stimulatory effects of pesticides on protozoa have also been observed previously. The herbicide 2,4-D was initially inhibitory to the growth of A. castellanii at 100 $ugml^{-1}$ but after 96h increased growth rates were observed (Prescott & Olson, 1972). Lower concentrations also stimulated growth but without the preceding inhibitory action. Results from the present study with both organisms, suggest that generally those compounds which are inhibitory at high concentrations stimulate

growth at low concentrations. Examples of this phenomenon are the action of chlorpropham, propham, barban, aniline, 3-chloroaniline, diuron, linuron, fenuron, isoproturon, cyanazine, terbutryne, pirimicarb, carbaryl, MCPA, glyphosate and ethirimol on T. pyriformis in microtiter batch culture. Generally this action is reduced or absent with A. castellanii.

49.4 The use of optical density to assess the growth of Tetrahymena pyriformis

The use of optical density to determine population growth of Tetraphymena spp.is not generally favoured. Slater & Elliot (1951) questioned its use in determining total cell number with Tetrahymena gelei. Their results indicated that optical density reflected neither the number nor volume of individual cells in a directly proportional manner. Changes in cell size were also observed with different culture age. Evidence from studies with other pesticides; malathion (Duff & Hill, 1970) lindane (Jeanne-Levain, 1974) mercuric chloride (Tingle et al., 1973) benomly (Rankin et al., 1977) metepa (Shivaji et al., 1979) and DDT (Rup Lal & Saxena, 1979) suggest that a variety of pesticides can induce size and volume changes in T. pyriformis. Alterations in the dimensions of T. pyriformis exposed to adrenergic blocking agents (Iwata et al., 1975) cigarette smoke (Gray & Kennedy, 1974) fungal toxins (Hayes et al., 1976) antioxidants (Surak et al., 1976) phenol (Schultz & Dumont, 1977) Dimethyl sulfoxide

(DMSO, Nilsson, 1977) antibiotics (Tanida et al., 1980) and acridine (Schultz et al., 1981) argue that cell size and volume changes may be a generalised non-specific response to stress in this organism (Schultz & Dumont, 1977) or be the result of a number of complex triggers which all affect cell division and indirectly induce morphological changes (cf morphology discussion). Irrespective of the cause such effects render the conversion of optical density values to cell numbers unreliable.

Partial disruption by sonication of cells may validate results but it is impracticable in microtiter plates. In this study optical density was employed to measure growth by establishing differences in population optical densities between untreated (control) and treated cultures. This has been used by Iwata et al. (1967) Cooley et al. (1972, 1973) Schultz & Allison (1979) and Wallace & Holmund (1980). Comparisons of growth curves obtained in the present study for both optical density and haemocytometer counts for both T. pyriformis and A. castellanii in microtiter wells has revealed a good correlation (significant at p=0.01).

With T. pyriformis the relatively high initial inoculum, plus the sophisticated detection system of the Dynatech MR600 may reduce the non linearality introduced by cell size and volume changes in other systems. Each well is

'read' 32x by this instrument before the mean optical density value is displayed. With 'dense' cultures the sheer number of cells combined with the extreme sensitivity of the machine may minimise the distortion introduced by individual cell volume changes.

49.5 Microtiter plate readers

The use of microtiter plates in conjunction with plate readers offers a number of advantages: it is economic, saving on time, labour and materials; it is reliable, giving quantifiable, highly replicable and reproducible results, and is sensitive, detecting growth stimulation, inhibition or recovery. There is also the potential for semi-automation by the use of micro-computers to record, store and analyse data while still retaining the option for direct microscopic observation on samples taken from wells.

The flexibility of the experimental procedure, (Section 14.0), lends itself to the investigation of growth-affecting compounds in general, whether against protozoa or other microorganisms or cell cultures. The use of such a system is not simply restricted to growth assessment. Indeed, any process which can be detected by colourmetric or turbidimetric change can be monitored.

When used with T. pyriformis the technique offers a simple standardised method to assess the inhibitory activity of

pesticides on protozoa which, through computer disk storage, facilitates cataloging and direct comparison of data within and between experiments.

50.0 Chronic effects of some phenylcarbamate herbicides on Tetrahymena pyriformis and Acanthamoeba castellanii

On the evidence from sub-acute toxicity tests in Repli-dishes and microtiter plates, the phenylcarbamate herbicides were the most toxic pesticide to both species. However, in such experiments the herbicide concentrations employed did not represent probable environmental concentrations, but were designed to elucidate both the stimulatory and inhibitory effects of the compound (its 'activity profile'). To provide evidence of the chronic effects of phenylcarbamates, both species were batch-cultured in flasks for 10 to 14d in media containing herbicides at concentrations approximating (in $\mu g ml^{-1}$) to field application rates. These estimated field concentrations (EFC's) are idealistic and care must be exercised in assessing the importance of their action.

Such experiments assist in defining a pesticide's effect in terms of concentration and exposure.

Chlorpropham: The lethal action of chlorpropham with T. pyriformis was evident at concentrations above 2 $\mu g ml^{-1}$.

In sub-acute toxicity tests the 'activity profile' of chlorpropham with this organism in this study was shown to consist of stimulation (0.001 - 0.01 μgml^{-1}), dose-dependent inhibition (0.1 - 1.0 μgml^{-1}), inhibition of cell division (3.2 - 5.0 μgml^{-1}) and a lethal action at 15.9 μgml^{-1}. In chronic exposure tests only a lethal effect was observed at a concentration 'equivalent' to half the 'estimated field concentration', 2 μgml^{-1}.

Prolonged exposure of T. pyriformis to low concentrations of chlorpropham resulted in the ciliate responding in a way which mimicked the acute lethal effect of high concentration of the chemical in sub-acute toxicity tests.

Even using conservative estimates of the amount of pesticidal chemical biologically available to interact with soil protozoa, the result of this study indicates a probable inhibitory action of chlorpropham to ciliates in situ. Similarly, the results indicate possible, though less likely, inhibitory effects on soil amoebae. Again, prolonged exposure of A. castellanii to low concentrations of chlorpropham mimicked the acute effect of higher concentrations in short-term tests.

Propham: Chronic toxicity tests indicated dose-dependent inhibitory effects of propham on T. pyriformis.

In sub-acute toxicity tests, dose-dependent inhibition of

the growth rate occurred with concentrations of 5.8 - 22.4 μgml^{-1}, with chronic exposure, 25 and 50 μgml^{-1} induced inhibition of cell division but 2.5 and 5.0 μgml^{-1} did not affect growth.

Although propham depressed cell numbers at 0.5x and normal EFC's over 6d significant differences were not detected beyond this point, suggesting recovery. Indications of recovery were also observed in sub-acute studies.

Propham caused a decrease in number of A. castellanii over 24h (25 and 50 μgml^{-1}) but permitted reduced growth over 13d. At 2.5 and 5.0 μgml^{-1} the initial lethal action was observed but there was no reduction in subsequent growth rate.

The action of propham at and below EFC's suggest the possibility that this herbicide might have inhibitory effects on amoebae in soil, but these would be less severe than those caused by chlorpropham.

Marcenko (1980) found that propham at 17.9 μgml^{-1} halted cell division in Euglena gracilis cultures while Prescott et al. (1977) also reported 17.9 μgml^{-1} to reduce A. castellanii populations by 66% after 6d. Given the suspected greater sensitivity of T. pyriformis over E. gracilis to toxic chemicals (Keltner & Cooley, 1970; Jeanne-Levain, 1977) and that inhibition of population

growth by 50% was observed after 4d with 11 μgml^{-1} propham in this study the results appear consistent with those of other authors.

Barban: Except at 10 μgml^{-1}, barban had no inhibitory effect on T. pyriformis over 6d. It therefore seems that it is unlikely to affect ciliate numbers in soil. Evidence of the recovery of A. castellanii population from barban's initial inhibitory action at EFC's in this study also suggests that prolonged depression of soil protozoan numbers would not be expected.

Both organisms showed evidence of recovery from barban's initial inhibitory effect. The herbicide 2,4-D stimulated growth of A. castellanii at 0.1 - 1.0 μgml^{-1} (Prescott & Olson, 1972). They concluded that the amoeba was degrading 2,4-D and using it as a carbon and/or energy source.

Many microorganisms have the ability to hydrolyse phenylcarbamate (Cripps & Roberts, 1978). Kaufman (1967) isolated Pseudomonas, Agrobacterium, Flavobacterium and Achromobacter spp. which could degrade chlorpropham. Clark & Wright (1970a, 1970b) isolated chlorpropham and propham-degrading species of Arthrobacter and Achromobacter while Wright & Forey (1972) demonstrated the conversion of barban to 3-chloroaniline by a soil Penicillium sp.

Notably less toxic than either chlorpropham or barban, their metabolite 3-chloroaniline significantly stimulated the growth rate of T. pyriformis at concentrations below 3.2 $\mu g ml^{-1}$ in the present study.

51.0 The mode of action N-phenylcarbamage herbicides

Having established the inhibitory influence of some phenylcarbamates on the representative protozoa, further investigations concentrated on the possible sub-lethal effects of these chemicals on certain physiological processes. Of particular interest was the action of chlorpropham and propham on T. pyriformis. Their inducement of morphological abnormalities and inhibition of cell division reflected the reported mode of action of phenylcarbamates in a number of other cell systems.

51.1 Mode of action of the N-phenylcarbamages in other cell systems

The N-phenylcarbamage herbicides affect growth and physiological events in a number of eukaryotic organisms (cf Introduction). In general cell division appears to be most severely affected.

The earliest report on propham-induced failure of cytokinesis in plants, Ennis (1948a), noted multinucleated cells with greatly increased chromosome numbers and it was

concluded that this form of endopolyploidy could only result from repeated nuclear divisions without separation. The presence of tripolar and polypolar anaphase cells also suggested that the spindle mechanism was affected. Morphologically the cells were large, swollen, highly vacuolated and often mishapen.

Such mitotic abnormalities in chlorpropham, propham and barban-treated barley coleoptiles were thought to arise from inhibition of protein synthesis (Mann, Jordan & Day, 1965). They considered that such N-phenylcarbamate-treated cells could not synthesize spindle protein and therefore failed to divide. Newly formed cells, unable to synthesize a complete enzymatic apparatus, would rapidly die while mature cells unable to replace essential enzymes once they were depleted would also die. However, using cinemicrographic recordings of the action of propham on Haemanthus katherinae, Helper & Jackson (1969) showed the failure of chromosomes to align at metaphase. The chromosomes aggregated into several interconnected micronuclei and coalesced into a single polyploid. The mitotic spindle apparatus was clearly implicated as the target of propham action. Helper & Jackson (1969) found that in contrast to colchicine, propham did not destroy the spindle microtubules but led to their re-orientation. Through disruption of the orientation of spindle microtubules propham would inhibit growth and enlargement in any system which requires cell division.

Such a theory does not explain the inhibition of photolysis in isolated cabbage mitochondria by chlorpropham (Moreland & Hill, 1959) or the inhibition of gibberellin-induced synthesis of α amylase in barley seedlings by barban (Mann et al., 1967). Moreland & Hill (1959) suggested that chlorpropham may hydrogen bond to peptide linkages and thus affect the secondary or tertiary structure of proteins leading to alteration of their conformation-dependent activity. It is probable that the total effect of phenylcarbamate herbicides involves more than one site of action (Ashton et al., 1977).

The anti-mitotic action of the N-phenylcarbamates in plants led Helper & Jackson (1969) to postulate that mitosis in other systems may also be inhibited. Subsequent investigations have shown that the N-phenylcarbamages inhibit mitosis in human lymphocytes (Timpson, 1970), the algae Oedogonium cardiacum (Coss & Pickett-Heaps, 1974), Ochromonas (Brown & Bouck, 1974) and Euglena gracilis (Marcenko, 1980) and the yeast Schizosaccharomyces pombe (Walker, 1982). In the present study the halting of cell division in T. pyriformis and the appearance of an extended lag-phase in A. castellanii populations imply chlorpropham also inhibits cell division in these organisms.

Propham prevented the assembly of microtubulins and/or the depolymerisation of existing microtubulins and to increase the number of microtubular organising centres (MTOC's) in the nucleus of Oedogonium cardium (Coss & Pickett-Heaps,

1974). They considered that the herbicide acted on the MTOC's themselves rather than the microtubulin sub-units. Later Coss et al. (1975) found that propham did not bind to chick brain tubulin in vitro or to affect the re-assembly of microtubules unlike colchicine, colcemid (a commercial colchicine preparation) and vinblastine sulfate. Magistrini & Szollosi (1980) reported that propham had no effect on the polymerization of microtubules in mouse oocytes in contrast to colchicine and vinblastine, although all three blocked cells at metaphase. Propham was thought to block microtubule assembly or to disturb microtubule orientation thus provoking the formation of multipolar spindles, a view also held by Coss et al., (1975). In contrast, Brown & Bouck (1974) concluded that propham bound microtubule sub-units causing conformational changes which permitted re-assembly into macrotubules only.

Propham inhibited both protein and RNA synthesis in HeLa cells (Myhr, 1973), although chlorpropham selectively inhibited RNA synthesis only. However, the results of Brown & Bouck (1974) showed that propham rapidly and reversibly inhibit microtubule assembly without affecting total protein synthesis. From the foregoing background of information concerning the physiolical effects of phenylcarbamate herbicides it might be expected that in protozoa a number of 'targets' would be affected.

Time did not permit investigation of these during the present study, but there are clear pointers from the literature and findings in the present work as to directions which might now be followed. Specifically I would propose that investigation be made of the effects of the phenylcarbamates on mitosis, microtubule assembly and protein synthesis in protozoa particularly obtaining mitotic indexes and information on chromosome movement. High voltage electron microscopy may assist in determining the site of action of these herbicides by revealing MTOC's in the protozoa and in addition work on invitro and invivo binding of microtubules and inhibition of oral band regeneration may help develop an overall view of the mode of action of phenylcarbamates in these organisms.

52.0 Some other (physiological) effects of phenylcarbamates on protozoa

52.1 Respiration

It was found in the present study that chlorpropham (and diuron, a phenylurea) had no apparent affect on respiration in T. pyriformis. However, inhibition of oxygen uptake by phenylcarbamates has been observed in isolated plant mitochondria (Lotlikar, Remment & Freed, 1968; Macherval, Ravanel & Tissat, 1982) and similarities between the response of plant and T. pyriformis mitochondria to another herbicide (2,4,5-T) have been made (Silberstein & Hooper, 1975).

Other pesticides have been shown to inhibit respiration in T. pyriformis. These include malathion (Duff & Hill, 1970), parathion and pentachlorophenol, an uncoupler of oxidative phosphorylation (Slabbert & Morgan, 1982).

52.2 Encystment

Chlorpropham had no effect on encystment in A. castellanii. Encystment is a period of visible nuclear and nucleolar activity (Griffiths, 1970) and inhibitors of protein synthesis, eg chloramphenicol, tetracycline and other metabolic inhibitors (2,4-dinitrophenol and malonate) have been shown to prevent encystment (Band, 1963; Griffiths & Hughes, 1969). However, some inhibitors of DNA or protein synthesis induce encystment in Acanthamoeba (Griffiths, 1970). Earlier reports from this laboratory (Bradley, 1979; Irwin, 1980) found encystment in A. castellanii treated with chlorpropham to be greater than in control cultures, suggesting that chlorpropham may induce encystments albeit at a low level.

Altered cell permeability during encystment (Band, 1963; Griffiths & Hughes, 1969) and a decrease in phagocytotic activity prior to exocyst appearance (Griffiths, 1970) may restrict entry of chlorpropham into the cell and subsequently restrict the herbicide's inhibitory effects. This may be the reason for the lack of effect of chlorpropham on A. castellanii with regard to encystment in this present study. The cessation of cell division during encystment may effectively remove the prime site of

action of phenylcarbamates.

52.3 Excystment

Excystment of A. castellanii was delayed by barban but chlorpropham and propham had no effect. The process of excystment has not been as extensively investigated as encystment, but the activities of a number of physiological processes have been shown to increase in excysting cells. For example, oxygen consumption (Chamber & Thompson, 1973) and acid phosphatase activity (Stratford & Griffiths, 1971). The nature of the inhibition due to barban has not been determined. There are no reports on the effects of pesticides on excystment (or encystment) although Gel'tser (1967) found toxins from Penicillium spp. to totally suppress excystment in A. castellanii.

52.4 Motility

At five times the estimated field concentration, chlorpropham, propham and barban (and diuron) decreased motility of T. pyriformis.

Decreased motility in ciliates has been observed in the presence of a number of different agents, including high hydrogen ion concentrations (Mills, 1931), chlorpromazine (Dryl & Masnyk, 1971), detergents (Bujwid-Cwik & Dryl,

1971; Brutkowska & Raukaba, 1974), cadmium (Berquist, 1974), methyprylin, a sedative (Durojaiye, 1979) and antibiotics (Tanida et al., 1980).

In this study diuron (a phenylurea) at 15 μgml^{-1} alone stimulated motility in T. pyriformis before inhibiting it whilst chlorpropham (20 μgml^{-1}) induced periodic ciliary reversals (PCR), pivoting and characteristic 'avoidance behaviour' before inhibiting motility. Similar but less severe effects were observed with propham and barban.

Detergents initially increased motility and induced PCR's and avoidance reactions in Paramecium caudatum, P. aurelia and Stentor coeruleus (Bujiwid-Cwik & Dryl, 1971; Brutkowska et al., 1974). 'Rotary movement' (pivoting?) in T. pyriformis cells treated with adrenergic blocking agents has also been reported (Iwata et al., 1967).

The author is not aware of other reports on inhibitory and stimulatory effects of pesticides on motility in protozoa.

Anasamitocins, which have a specific action on microtubular systems, have been shown to interact with ciliary microtubules reducing motility and preventing cilia regeneration in T. pyriformis (Tanida et al., 1980). Other compounds which prevent polymerization of

microtubule proteins into tubules (antimitotics) have also been shown to interface with regeneration of oral ciliature in Stentor coeruleus. These include the fungicide griseofulvin (Margulis, Neviackas & Banerjee, 1969), colchicine (Neviackas & Margulis, 1969), the herbicides trifluralin (Banerjee et al., 1975) and propham (Margulis & Banerjee, 1969; Sarras & Burchill, 1975), melatonin (Banerjee et al., 1972) and colcemid, vinblastine sulfate and xylocaine (Sarras & Burchill, 1975). Inhibition of protein synthesis during G2 growth phase prevents mitosis and also prevents the development of oral primordia in T. pyriformis (Frankel, 1967).

The effect of phenylcarbamate herbicides (or other pesticides) on protein synthesis in T. pyriformis and A. castellanii was not investigated in this study.

The mechanism of band formation and oral regeneration in Stentor is believed to be the same as that involved in mitotic chromosome movement (Banerjee et al., 1972). Microtubules, involved in cilia and flagellar movement, are also affected by antimitotic agents, though they appear less sensitive to the disorganisational effects than oral ciliature (Tanida et al., 1980). A link between inhibition of cilia regeneration, inhibition of cell division, decreased cell motility and the mode of action of antimitotic compounds (including phenylcarbamate herbicides) appears probable.

The use of pattern formation in Petri-dish culture to detect gross inhibition of population movement provides a rapid, simplistic (if amusing) observational technique, the usefulness of which may be limited by its simplicity. The use of counting chambers to quantify motility in ciliates offers a simple, though laborious, method to monitor the effects of toxicants on protozoan motility.

52.5 Food vacuole formation

T. pyriformis forms food vacuoles continually except during cell division when it was observed to cease for at least 40 min (Chapman-Anderson & Nilsson, 1969). During this time partial resorption of the parental oral apparatus occurred, marking a cessation in carmine particle uptake. The authors concluded that there exists a strong correlation between cell division and lack of food vacuole formation in T. pyriformis.

The cessation of cell division in T. pyriformis populations treated with 3.2 $\mu g ml^{-1}$ chlorpropham, in sub-acute toxicity tests, is linked with the cessation of food vacuole formation (24h) with 4 $\mu g ml^{-1}$. Concentrations of the herbicide below 3.2 $\mu g ml^{-1}$ showed a dose-dependent inhibitory effect on both the growth rate and food vacuole formation. Increasing concentrations progressively inhibited both activities. Concentrations above 15.9 $\mu g ml^{-1}$ were lethal to the ciliate after 24h

whilst complete inhibition of food vacuole formation occurred after 6h with 20 μgml^{-1} chlorpropham.

Ricketts & Rappitt (1975) also reported dose-dependent inhibition of food vacuole formation and cell division in T. pyriformis treated with cycloheximide and puromycin. They considered that food vacuole formation depended on a continuous supply of proteinaceous material, of which there is only a small cellular store. The observed delay between inhibition of protein synthesis and cessation of food vacuole formation was explained by the time taken for this reservoir to be depleted before complete blocking of food vacuole formation occurred. In the present study such a delay appeared between the start of cell division inhibition and the onset of the prevention of food vacuole formation in chlorpropham-treated cells.

The dose-dependent order of inhibition of food vacuole formation by chlorpropham, propham and barban corresponds to their respective inhibition of growth in T. pyriformis. The asynchrony of division within such populations could account for the gradual inhibition of growth with each concentration.

Chronic exposure of T. pyriformis to low concentrations of chemical inhibited food vacuole formation in such a way as to mimic the acute inhibition of food vacuole formation observed with higher concentrations. The stimulatory

effect of low doses of propham and barban on food vacuole formation coincides with their stimulatory effects on growth.

The appearance of increased generalised vacuolation in the cytoplasm of ciliates treated with antimitotics and cell division inhibitors has been reported with Colchicine (Neviackas & Margulis, 1969), benomyl (Rankin et al., 1972), elatonin (Banerjee et al., 1972), colcemid, vinblastine sulfate, propham and xylocaine (Sarras & Burchill, 1975). Increased vacuolation also occurred with malathion (Duff & Hall, 1970), cadmium (Berquist, 1974), metepa (Shivaji et al., 1975, 1978a, 1979) and fungal toxins (Gel'tser, 1967). Similar observations were noted with T. pyriformis at concentrations of chlorpropham that inhibited food vacuole formation. The nature of such vacuoles is unclear. They may arise from impairment of the cell's water expulsion system (Sarras & Burchill, 1975) or signify development of autophagic vacuoles or be the result of lysosomal action on pre-formed food vacuoles.

A link between the inhibition of food vacuole formation and slowing of ciliary movement in Colpidium campylum treated with various hydrogen ion concentrations has been established (Mills, 1931). Durojaije (1979) concluded that any agent which interferes with ciliary beating can indirectly cause starvation, the cilia are prevented from

forming the currents which carry the food particles to the oral cavity. However, inhibition of food vacuole formation would result from incomplete cell division, as food vacuoles are not formed during division. Nilsson (1974) found neither cell motility nor cell division to be affected by DMSO, a solvent that completely inhibited food vacuole formation. DMSO was thought to interfere with cellular energy levels or with the proper functioning of the cell membrane (Nilsson, 1977, 1980). Malathion, reported to interfere with lipid synthesis in T. pyriformis (Duff & Hall, 1970), had no affect on food vacuole formation in the present study.

Inhibition of food vacuole formation may be linked to the probable antimitotic mode of action of the phenylcarbamates. By preventing cell division the herbicides would also inhibit food vacuole formation. The action of the phenylcarbamates in reducing motility in the ciliate may be consistent with their proposed site of action, the MTOC, in that ciliary movement and cell division are both dependent on the correct functioning of MTOC. However, the possibility that the phenylcarbamate herbicides prevent food vacuole formation by limiting the movement of oral ciliature and thus the movement of food particles into the oral cavity cannot be dismissed.

52.6 Contractile vacuole formation

Many agents adversely affect the activity of contractile

vacuole complexes (Patterson, 1980). Compounds inhibiting the correct functioning of contractile vacuole in T. pyriformis include: mercuric chloride (Tingle et al., 1973), adrenergic blocking agents (Schorr & Baggs, 1973), phenol and phenolic antioxidants (Surak et al., 1976; Shultz & Dumont, 1977), DMSO (Nilsson, 1974, 1977) and acridine (Schultz et al., 1981). Each compound was also reported to inhibit cell division, cell motility and induced rounding up in T. pyriformis cells.

In this study chlorpropham inhibited contractile vacuolar output, induced cell rounding, reduced cell motility and inhibited cell division. Observations on the contractile vacuoles of chlorpropham-treated cells, over 9h, showed that vacuolar output decreased with time. The effect on contractile vacuole formation was believed to arise from the action of chlorpropham on microtubules preventing their assembly or orientation. However, colchicine, another antimitotic, has no effect on contractile vacuole function (Patterson, 1980). This may be a reflection on the proposed different sites of action of the two compounds, chlorpropham acting directly on the MTOC involved in vacuolar contractions.

Observations on Tetrahymena cells treated with 20 $\mu g ml^{-1}$ over 72h revealed 1-4 giant vacuoles. The majority of cells had only 1 giant vacuole which occupied up to 90% of the cell volume. Although such vacuoles were not seen to

contract observations on similarly treated cells over 9h showed the development of giant contractile vacuoles of a similar size and location. Such vacuoles did contract but at a rate which slowed with increased exposure to the herbicides. It was believed that eventually such vacuoles lost the ability to contract, the transition occurring after between 9 and 24h exposure. The appearance of 3-4 such giant vacuoles in one cell is puzzling and may possibly signify extensive cytological damage inducing the formation of autophagic vacuoles leading to increased water diffusion across the membrane and/or increased cell permeability. Such cells did not recover from this condition. Similar effects were observed in a Paramecium sp. treated with fungal toxins (Gel'tser, 1967).

53.0 The effects of some phenylcarbamates on protozoan morphology and cytology

53.1 Morphological changes

53.1.1 Light microscopy

Light microscopy observations showed spherical cells to occur in populations of T. pyriformis treated with EFC's of chlorpropham and propham but not barban. The rounding up in treated cells was dose-dependent and consistent with the chronic inhibitory action of these chemicals on growth.

A transient decrease in cell size correlated with the initial toxic action of barban (24h) on T. pyriformis. Rounding up was believed to result from the antimitotic action of phenylcarbamates, the cells rounding as a substitute for division. However, an attractive site of action could have been the cortical microtubules, incorrect alignment of which could lead to pellicle instability and collapse to form a 'minimum surface' (a sphere). Microtubules act as cytoskeletons in the formation and maintenance of cell shape in many cell systems (Marchant, 1979; Marchant & Hines, 1979). By acting on these cortical microtubules chlorpropham and propham may cause rounding. Propham caused partial shape loss in Ochromonas (Brown & Bouck, 1974) and Schizosaccharomyces pombe (Walker, 1982) at 42 and 50 μgml_{-1} respectively. Chlorpropham at 5 μgml^{-1} altered morphology in Chlamydomonas reinhardii causing loss of flagella, cell rounding and the development of a multilayered envelope (Maule & Wright, 1983). The authors concluded that if chlorpropham affects microtubule assembly then both cytokinesis and karyokinesis would be highly disorganised. In the present study propham (25 μgml^{-1}) and chlorpropham (2 μgml^{-1}) induced rounding in T. pyriformis. Ansamitocin, another antimitotic, also caused loss of cell shape in T. pyriformis at 5 μgml^{-1} (Tanida et al., 1980). It is possible that previous reports on cell rounding in T. pyriformis may be attributable to a specific action on cell division.

The rounding up of T. pyriformis cells may be the result of incomplete cell division (Wantabe, 1971). Cell rounding, in T. pyriformis, has been induced by a number of chemicals that also inhibit cell division (Iwata et al., 1967; Banerjee et al., 1972; Surak et al., 1976; Schultz & Dumont, 1977; Tanida et al., 1980; Schultz et al., 1981). Other authors believed cell rounding in this organism to be a behavioural response induced by a number of chemicals which have a non-specific mode of action (Surak et al., 1976; Rup Lal & Saxena, 1979; Shivaji et al., 1979).

Cell rounding has also been observed in T. pyriformis treated with a number of pesticides including lindane (Jeanne-Levain, 1974) DDT (Rup-Lal & Saxena, 1979) metepa (Shivaji et al., 1979) and with other compounds such as cigarette smoke (Gray & Kennedy, 1974) and fungal toxins (Hayes et al., 1976). Observations during the present study showed that rounding up in T. pyriformis was not induced by storage of cells for 96h in ¼ strength Ringers solution. As such ageing and semi-starved cells did not round up it suggests that spherical cells are formed in response to a chemical (toxic) effect.

It is possible that previous reports of cell rounding in T. pyriformis may be attributable to a specific chemical action on cell division. In T. pyriformis cells exposed to DDT the lipophilic nature of the chemical may cause membrane alterations which could lead to amino acid

starvation, ultimately preventing protein synthesis, inhibiting cell division and induce rounding up (Rup Lal & Saxena, 1979). Most heavy metals are capable of forming ligands; mercury, for example, has a known high affinity for sulfydryl groups (Thrasher & Adams, 1972). Conformation changes due to the binding of microtubule proteins with heavy metals may lead to inhibition of cell division and formation of spherical cells (Bowles & Wolfson, 1976). This could also account for the inhibitory action of mercuric chloride on motility (Tingle et al., 1983) and cilia regeneration (Thrasher & Adams, 1972) in T. pyriformis.

Conformational changes in proteins due to the introduction of metepa into a suitable nucleophilic receptor (such as the carboxyl, sulfhydryl or terminal amino group of proteins) may have been the cause of rounding up of T. pyriformis cells treated with this pesticide (Shivaji et al., 1975; 1978a; 1978b; 1979). Similarly with DMSO, effects on ATP turnover in the cell or conformational changes in proteins could lead to starvation, inhibition of cell division and induce rounding (Nilsson, 1977).

The chemical induction of cell rounding in T. pyriformis may therefore be indicative of direct or indirect action on cell division.

In the present study A. castellanii cells in the presence of 40-60 $\mu g ml^{-1}$ chlorpropham developed enlarged cell membranes. Maule & Wright (1983) observed chlorpropham

(5 μgml^{-1}) to induce a multilayered envelope which surrounded Chlamydomonas reinhardii cells. They believed this effect to be due to the chemical's action on somatic microtubules. The general effects of chlorpropham on algae and amoebae appear similar but the evidence is not strong enough to implicate microtubules as the sole site of action in both organisms. For example, Gel'tser (1967) found a similar 'dissolution' of the plasmalemma in A. castellanii to arise after treatment with fungal toxins. The possibility exists that such an effect is the result of chemical action on sites other than microtubules.

There is no evidence to suggest that the cellular 'envelope' observed in this study in chlorpropham-treated A. castellanii cells is associated with abortive encystment.

53.1.2 Electron microscopy

Scanning electron micrographs confirmed light microscope observations on T. pyriformis cells affected by herbicides. The appearance of large surface depressions observed in both thin section micrographs and with SEM were thought to represent collapsed enlarged contractile vacuoles, an artefact of preparation.

Loss of cilia has been reported in T. pyriformis treated with phenolic antioxidants (Surak et al., 1976) and

acridine (Schultz et al., 1981). Alteration to the pellicle surface by the partitioning of the hydrophilic acridine into the highly lipid membrane has been found in T. pyriformis exposed to acridine (Schultz et al., 1981) and the formation of 'club' like cilia was observed in T. pyriformis treated with methyprylon (Durojaiye, 1979). Cilia abnormalities have been reported in other species treated with xenobiotics, for example, Stentor coeruleus treated with griseofulvin (Margulis et al., 1969) and aminoglycoside antibiotics (Altman et al., 1974) and Paramecium sp. exposed to simazine (Gel'tser, 1967).

In the present study structural changes did not physically block the contractile vacuole pores in T. pyriformis treated with chlorpropham although DMSO was thought to influence contractile vacuole function at the level of the pore mechanism (Nilsson, 1974).

The increased generalised vacuolation in T. pyriformis cells treated with chlorpropham found in this present study may arise from physical and chemical alterations in the pellicle and lipid membrane and subsequent increase in diffusion into the cell. Physical alteration to the pellicle were observed in this study with 20 $\mu g ml^{-1}$ chlorpropham.

Reductions in the oral ciliature of chlorpropham-treated cells may reflect the inhibitory action of

phenylcarbamates on oral regeneration processes and be linked to its antimitotic action through its inhibition of MTOC. A correlation between inhibition of oral regeneration and an antimitotic action has been reported in a number of ciliate protozoa treated with antimitotic compounds (Margulis et al., 1969; Margulis & Banerjee, 1969; Banerjee et al., 1975; Sarras & Burchill, 1975). As chlorpropham at 4 $\mu g ml^{-1}$ inhibited cell division, loss of, or a reduction in, oral ciliature may reflect the partial resorption of parental oral apparatus that occurs during division (Chapman-Anderson & Nilsson, 1969). However, the stage at which chlorpropham arrests division is not known.

53.2 Cytological changes

53.2.1 Electron microscopy

Transmission electron micrographs showed that T. pyriformis cells treated with chlorpropham contained damaged mitochondria. The mitochondria were round and had migrated to the cell centre. With higher concentrations of chlorpropham (20 $\mu g ml^{-1}$) the mitochondria appeared swollen with little internal organization. Some had lost their integrity. Similar effects in T. pyriformis have been reported with cadmium (Berquist, 1974), mercury (Tingle et al., 1973), acridine (Schultz et al., 1981) and cigarette smoke (Gray & Kennedy, 1974) and in Stentor coeruleus with aminoglycoside antibiotics (Altman et al., 1974).

In chlorpropham-treated cells the damage to mitochondria was found to be dose-dependent, increasing concentrations of the herbicide affecting density of the mitochondrial ground matrix, organization of cristae and mitochondrial shape. Such extensive damage could affect a number of energy dependent cellular functions eg food vacuole formation, motility, contractile vacuole function and cell division. Tingle et al. (1973) proposed that in damaging mitochondria, mercury may cause decreased production of ATP and thus reduce osmoregulation. Overlarge contractile vacuoles were not, however, reported with such treatments.

The detrimental affects of chlorpropham on mitochondria are inconsistent with its lack of action on respiration, although the time course of the onset of mitochondrial damage is not known, it may lie outside the first 3h of contact with the herbicide.

Chlorpropham at 4 $\mu g ml^{-1}$ (EFC) caused macro-nuclear abnormalities in T. pyriformis. The ground matrix of the nucleoplasm was less dense, chromatin bodies condensed and the nucleus became spherical. At 2 $\mu g ml^{-1}$, nuclear inclusions appeared to be concentrated to one side of the nucleus. Such abnormalities have been noted in T. pyriformis treated with mercuric chloride (Tingle et al., 1973), the fungicide benomyl (Rankin et al., 1977), DMSO (Nilsson, 1977) and phenolic antioxidants (Surak et al., 1976), while elongation and breakage of the cylindrical

macronucleus occurred in Frontonia leucas treated with the insecticide parathion (Bai & Dilli, 1974). In each case such effects correlate with inhibition or abnormal cell division. In particular, displacement of chromatin bodies, not believed to result from centrifugation, may suggest a suppressive effect on nucleic acid function and synthesis (Tingle et al ., 1973).

Trifluralin, an antimitotic, also caused macronuclear aberrations in Stentor coeruleus (Banerjee et al., 1975). These authors report that Paulin (1975) had discovered that microtubules are involved in macronuclear morphogenetic processes. The role of chlorpropham as an antimitotic is not inconsistent with the above findings and may explain nuclear aberrations seen in treated T. pyriformis cells.

54.0 Recovery from the growth-inhibiting and morphological effects of phenylcarbamates

The ability of T. pyriformis cells to recover on removal from chlorpropham solutions was related to concentration and length of exposure. Generally cells treated with low doses for 24h assumed normal morphology earlier than cells exposed to higher concentrations for the same duration or similar low doses for longer periods. However, permanent halting of cell division was induced with concentrations greater than 4 $\mu g ml^{-1}$ of chlorpropham for 48h whilst recovery was observed in all other herbicide treatments.

Inhibition of growth and oral regeneration has also been shown to be reversible in a number of ciliates with a number of chemicals, for example propham (Margulis & Banerjee, 1969), colchicine (Neviackes & Margulis, 1969), griseofulvin (Margulis et al., 1969), vinblastine sulfate (Stone, 1968) and Taxol, a microtubule stabilizing agent (Baum et al., 1981).

Transferring A. castellanii cells from propham solutions to fresh PGY medium induced a tendancy to synchronous division. The effect was dose-dependent, maximum effect 70-90 $\mu g ml^{-1}$. Above this concentration cell division was arrested and lethal effects were noted. Below 70 $\mu g ml^{-1}$ propham, division was asynchronous.

Synchronously dividing populations have been obtained by removal of Schizosaccharomyces pombe from chlorpropham solutions, 50 $\mu g ml^{-1}$ (Walker, 1982) and T. pyriformis from ansamitocin solutions (Tanida et al., 1980). Synchronous division in T. pyriformis has also been induced by heat, cold, centrifugation, colchicine, hypoxia, starvation and vinblastine (Everhart, 1972). The results appear to be consistent with inhibition of cell division by chlorpropham and propham.

55.0 Transformation of phenylcarbamate herbicides

The evidence that T. pyriformis and A. castellanii transformed chlorpropham, propham and barban to their

respective aniline metabolites is slight. Chlorpropham and propham are transformed to 3-chloroaniline and aniline, respectively, by a number of soil microorganisms, includeing bacteria (Clark & Wright, 1970a, 1970b) and micro-algae (Wright & Maule, 1982).

56.0 An overall view of the mode of action of phenylcarbamates in protozoa and further general comments

The mode of action of chlorpropham, propham and barban in A. castellanii and T. pyriformis seems to be consistent with their reported role as antimitotics in other cell systems. They inhibit population growth, cell division, food vacuole formation and motility in the protozoa and cause morphological and cytological changes consistent with previous reports on the effects of phenylcarbamates on Chlamydomonas reinhardii (Maule & Wright, 1983), Euglena gracilis (Marcenko, 1980) and Schizosaccharomyces pombe (Walker, 1982) and antimitotics in general. Experiments to determine their effect on mitotic index, inhibition of oral regeneration and the competitive use of other microtubule inhibitors to detect site of action would assist further elucidation of the effects of phenylcarbamates on protozoa.

The assessment of the potential hazard of a chemical is based upon the knowledge of both its inherent toxicological properties and its environmental fate (Cairns, 1980). However, environmental toxicity or hazard

is not implicit in the demonstration of toxicity in the laboratory, while even direct observations on the environmental impact of a chemical in itself does not assist in predicting future hazard. In determining the consequences to protozoa of the application of a pesticide to the environment there is a role for both single species studies in the laboratory and the more ecologically realistic investigations on mixed protozoan populations *in situ*.

A systematic approach to the effect of pesticides on protozoa in culture has been devised but in failing to extend this investigation beyond the laboratory this study presents only one side of the complex issue of the interactions of pesticides with free-living protozoa. Confirmation of these results should now be obtained through model ecosystem and field studies.

The suitability of *T. pyriformis* for use as a 'model' for toxicant studies has been cited by other authors, notably Hutner (1963), Hutner *et al*. (1973) and Corliss (1976). Its use as a 'model' for pesticide studies is further endorsed by this present study, particularly its use in conjunction with Repli-dishes and microtiter plates in initial sub-acute toxicity studies.

In conclusion, the evidence presented suggests that some pesticides, notably the phenylcarbamate herbicides have

detrimental effects upon growth and physiological processes within certain species or classes of free-living protozoa.

Given that this might occur *in situ* and accepting the suggested role of protozoa in the soil (Clarholm, 1981; 1984) the repercussions of inhibitory effects by pesticides against protozoa could be felt at many different trophic levels.

APPENDIX

Appendix 1

Definitions for use in toxicity testing

The definitions given in section 12.0 are generally applied to the evaluation of pesticides against rodents and mammals in accordance with governmental legislation. In applying such definitions to the effect of toxicants on cultures of micro-organisms it is necessary to place these terms in context and to remember that the index of toxicity is not primarily mortality (though this is often the case) but inhibition of growth.

The 'development phase' and 'life span' in rodents refers to different periods of time within individuals from a sample population. With axenic cultures of protozoa such terms refer to different periods of a population culture, determined by samples. The substitution of cell cycle events for 'life span' studies would be possible, especially if synchronous cultures were used. Out of necessity such experiments would be short in duration and simply not applicable to sub-acute and chronic toxicity testing. Synchronous cultures in themselves are of benefit when determining the mode of action of a chemical but the stress created in inducing synchrony and the rapid deterioration to asynchrony are undesirable when assessing the effects of an unknown toxicant.

In acute toxicity testing in general, the duration of the experiment is defined in relation to the length of the sub-acute and chronic tests. With micro-organisms, in order to register any inhibition of growth or mortality a prime consideration is the mean generation time, the duration of which is a function of the culture conditions employed. However, this pre-supposes that toxicity/lethality are being determined according to the ability/inability of the micro-organism to multiply. It would be possible to use cytochemical or viable stain procedures but their use is limited to the establishment of mortality only. Thus of necessity the length of acute toxicity tests with protozoan cultures may be proportionally longer than is normal with larger organisms.

McEwan & Stephensen (1979) defined the development phase, in rodents, as being that period of the life span before sexual maturity is reached. In protozoan cultures the development of the population is restricted to the exponential phase (the lag-phase may be absent if the inoculum is taken from an actively growing culture, as is the case with Tetrahymena (Sleigh, 1979). Thus the duration of the sub-acute toxicity tests are determined by the length of the exponential phase of the culture and the chronic toxicity tests last through all the phases of a culture.

In sub-acute tests an 'effective concentration' of a pesticide is that level required to produce the response under investigation (growth inhibition, mortality, etc). In chronic toxicity testing the reasons for considering a concentration of a chemical to be 'relevant' are varied again depending on the objectives of the test and the methods employed to examine them.

Appendix 2

Defining relationships between chemical concentration and growth rates

The differences between the growth rate of populations of cells treated with pesticides and those of untreated controls are shown by plotting the appropriate regression lines. The significance of these differences can be determined by performing student t-tests on the slopes of the regression lines. By comparing the slopes of each treatment one with another, a matrix can be constructed which readily detects trends not seen in simple line plots.

The following are illustrative examples which describe some of the patterns seen.

Key

-	not significantly different
+	significantly different
C	control untreated populations of cells
t 1-5	hypothetical increasing concentrations of pesticide treated cell popluations

Example 1

No treatment growth rate differs significantly from the control
No treatment growth rate differs significantly from another
No significant values, no differences in growth rates

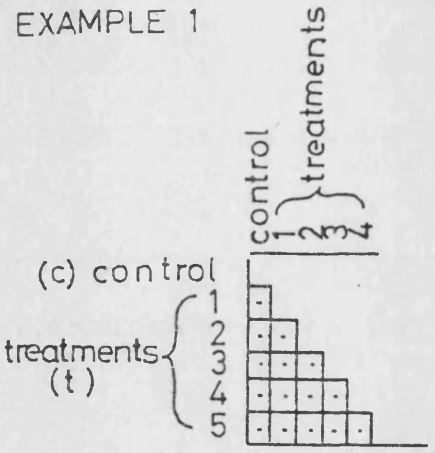
Example 2

All treatment growth rates differ significantly from the control
No treatment growth rate differs significantly from another
Suggestion that treatment 1 is the start (threshold) of inhibitory action

Example 3

Only treatment 5 is significantly different from the control
Treatment 5 is significantly different from all other treatments, which do not differ from each other
Suggestion that treatment 5 is the start (threshold) of inhibitory action

EXAMPLE 1



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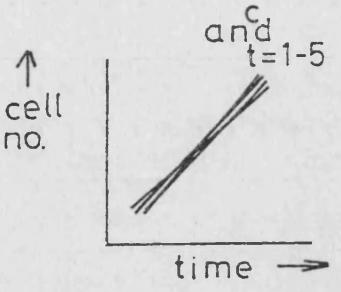
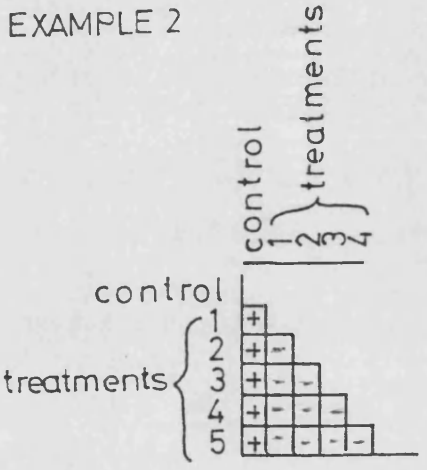


GROWTH RATES

EXAMPLE 2



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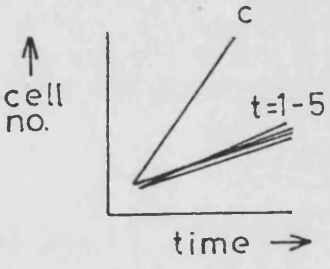
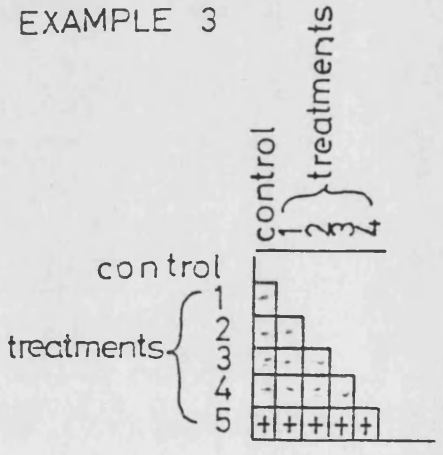


GROWTH RATES

EXAMPLE 3



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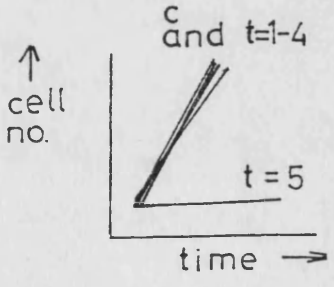


GROWTH RATES

Example 4

Treatments 3-5 are significantly different from the control
Treatments 1 and 2 are significantly different from treatments 3-5 but are not significantly different from the control
Treatments 3-5 are not significantly different from each other
Suggestion that treatment 3 is the threshold of inhibitory action

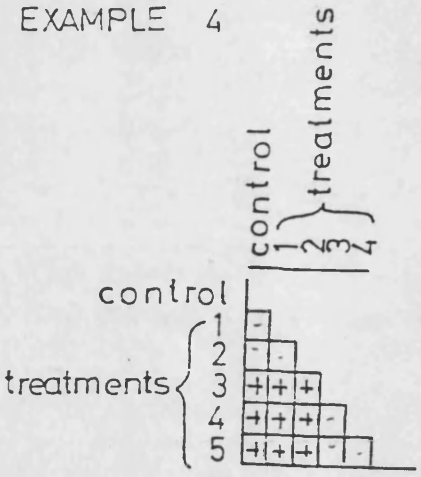
Example 5

No treatment differs significantly from the control (despite the appearance of the growth rates)
Treatment 2 however is significantly different from treatments 3 and 5
Although no stimulatory action is detected against the control the evidence points to a stimulation of growth by treatment 2

Example 6

No treatment differs from the control
Treatments 2 and 3, however, differ significantly from treatments 4 and 5
Treatment 2 is also significantly different from treatment 3

EXAMPLE 4



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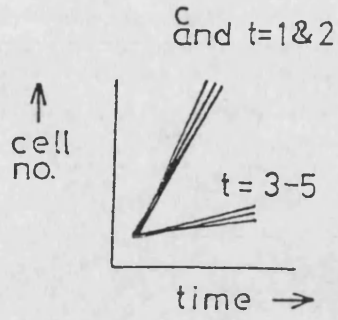
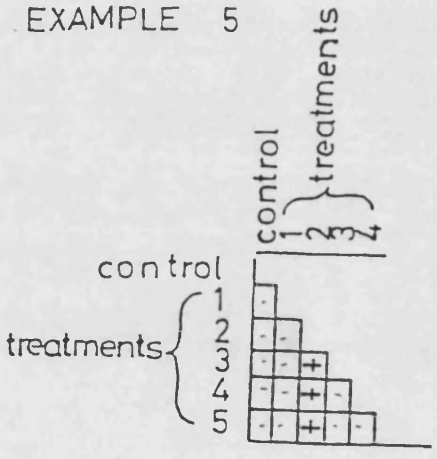


GROWTH RATES

EXAMPLE 5



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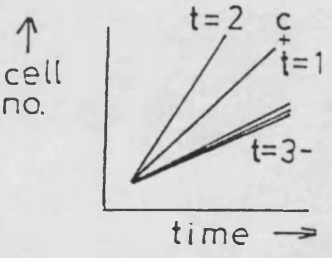
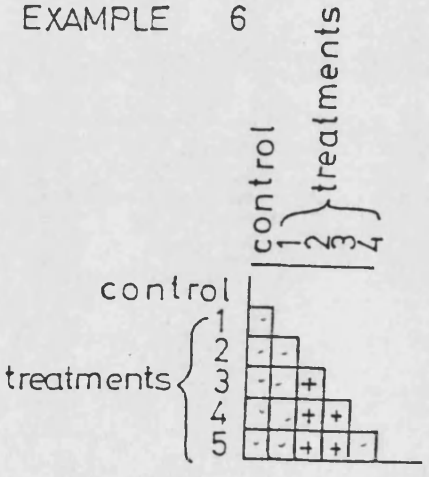


GROWTH RATES

EXAMPLE 6



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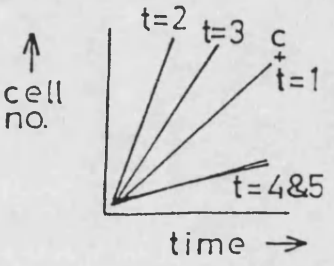

GROWTH RATES

Although no stimulatory action is detected against the control the results suggest some stimulatory action which starts with treatment 2

The majority of relationships are combinations of the above. The significance of the differences are therefore graded: * = significant, ** = very significant, *** = highly significant

Appendix 3

Evaluation of the sub-acute toxicity of some pesticides and metabolites to Tetrahymena pyriformis and Acanthamoeba castellanii using the Repli-dish culture techniques : Analyses of variance for each sampling point

3.1

The analysis of variances, over time, for Tetrahymena pyriformis in Repli-dishes treated with chlorpropham (concentration range 0 to 5.0 $\mu g ml^{-1}$)

Time (h)	Source	ss	df	mss	F value
24	treatment	6013.60	6	1002.27	21.08
	error	2995.60	63	47.55	
	total	9009.20	69		
48	treatment	73293.94	6	12215.66	116.64
	error	6598.00	63	104.73	
	total	79891.94	69		
72	treatment	293342.74	6	48890.46	168.01
	error	18333.20	63	291.00	
	total	311675.94	69		
96	treatment	602876.37	6	100479.40	248.14
	error	25510.90	63	404.93	
	total	628387.27	69		

key

ss = sum of squares
df = degrees of freedom
mss = mean sum of squares

3.2

The analysis of variances, over time, for Tetrahymena pyriformis in Repli-dishes treated with chlorpropham (concentration range 0 to 60 μgml^{-1})

Time (h)	Source	ss	df	mss	F value
24	treatment	137055.06	6	22842.51	81.30
	error	25567.64	91	280.96	
	total	162622.70	97		
48	treatment	264736.86	6	44122.81	158.06
	error	25408.14	91	479.16	
	total	290140.00	97		
72	treatment	413886.91	6	68981.15	59.08
	error	101575.94	87	1167.54	
	total	515462.85	93		
96	treatment	377585.26	6	62930.88	81.73
	error	64679.70	84	770.00	
	total	442264.96	90		

3.3

The analysis of variances, over time, for Tetrahymena pyriformis in Repli-dishes treated with 3-chloroaniline

Time (h)	Source	ss	df	mss	F value
24	treatment	1078.30	7	154.00	7.47
	error	659.10	32	20.59	
	total	1737.40	39		
48	treatment	15908.90	7	2272.70	16.45
	error	4419.90	32	138.12	
	total	20328.80	39		
72	treatment	122420.80	7	17488.60	59.44
	error	9419.79	32	9414.79	
	total	131835.59	39		
96	treatment	124161.10	7	17737.30	24.70
	error	22967.50	32	717.70	
	total	147128.60	39		

3.4

The analysis of variances, over time, for Tetrahymena pyriformis in Repli-dishes treated with propham (concentration range 0 to 5.0 μgml^{-1})

Time (h)	Source	ss	df	mss	F value
24	treatment	3580.40	6	199.85	3.52
	error	1199.09	63	56.83	
	total	4779.49	69		
48	treatment	25242.29	6	4207.05	9.14
	error	28994.80	63	460.23	
	total	54237.09	69		
72	treatment	95656.09	6	15942.68	24.04
	error	41776.90	63	663.13	
	total	137432.99	69		
96	treatment	99711.54	6	16618.59	23.65
	error	44196.40	63	701.53	
	total	143907.94	69		

3.5

The analysis of variances, over time, for Tetrahymena pyriformis in Repli-dishes treated with propham (concentration range 0 to 22.4 μgml^{-1})

Time (h)	Source	ss	df	mss	F value
24	treatment	232313.74	6	38718.96	85.83
	error	28419.70	63	451.11	
	total	260733.44	69		
48	treatment	1206487.04	6	201081.17	210.63
	error	58235.02	61	954.67	
	total	1264722.06	67		
72	treatment	2104807.60	6	350801.27	216.35
	error	98906.68	61	1621.42	
	total	2203714.28	67		
96	treatment	3047807.77	6	507967.96	118.67
	error	26961.60	63	4280.66	
	total	3317489.37	69		

3.6

The analysis of variances, over time, for Tetrahymena pyriformis in Repli-dishes treated with aniline

Time (h)	Source	ss	df	mss	F value
24	treatment	930.8	7	132.9	3.68
	error	1155.2	32	36.1	
	total	2086.0	39		
48	treatment	18897.30	7	2699.6	24.10
	error	3576.30	32	111.7	
	total	22473.60	39		
72	treatment	84350.30	7	12050.0	28.61
	error	13474.70	32	421.1	
	total	97825.00	39		
96	treatment	46963.50	7	6709.0	6.8
	error	31370.30	32	980.3	
	total	78333.80	39		

3.7

The analysis of variances, over time, for Tetrahymena pyriformis in Repli-dishes treated with pirimicarb

Time (h)	Source	ss	df	mss	F value
24	treatment	82499.07	11	7499.92	70.77
	error	11444.80	108	105.97	
	total	93943.87	119		
48	treatment	180419.57	11	16401.78	51.86
	error	34154.80	108	316.25	
	total	214574.37	119		
72	treatment	385379.60	11	35034.51	124.12
	error	30483.20	108	282.00	
	total	415862.80	119		
96	treatment	564415.87	11	45688.13	79.78
	error	61846.40	108	572.65	
	total	564415.87	119		

3.8

The analysis of variances, over time, for *Acanthamoeba castellanii*, in Repli-dishes treated with chlorpropham

Time (h)	Source	ss	df	mss	F value
24	treatment	22989.98	7	3284.20	28.35
	error	3706.40	32	115.83	
	total	26695.38	39		
48	treatment	51797.60	7	7399.66	47.06
	error	5032.00	32	157.25	
	total	56829.60	39		
72	treatment	82317.60	7	11759.66	88.03
	error	4274.80	32	133.59	
	total	86592.40	39		
96	treatment	99776.00	7	14253.71	34.78
	error	13113.60	32	409.80	
	total	112889.60	39		
120	treatment	43307.38	7	6186.77	17.98
	error	11010.00	32	344.06	
	total	54317.38	39		
144	treatment	250038.00	7	35719.71	71.41
	error	16005.60	32	500.18	
	total	266043.60	39		
168	treatment	503462.58	7	71923.23	69.19
	error	33264.80	32	1039.53	
	total	536727.38	39		

3.9

The analysis of variances, over time, for Acanthamoeba castellanii, in Repli-dishes treated with 3-chloroaniline

Time (h)	Source	ss	df	mss	F value
24	treatment	29945.10	7	4277.9	19.83
	error	6902.70	32	215.7	
	total	36847.80	39		
48	treatment	49939.90	7	7134.27	18.53
	error	12315.10	32	384.80	
	total	62255	39		
72	treatment	7437.9	7	1062.57	1.88
	error	18016.3	32	652.60	
	total	254542	39		
120	treatment	28993.5	7	4141.9	7.44
	error	17805.5	32	556.4	
	total	467990	39		

3.10

The analysis of variances, over time, for Acanthamoeba castellanii, in Repli-dishes treated with propham

Time (h)	Source	ss	df	mss	F value
24	treatment	68815.38	7	9830.77	23.91
	error	13158.40	32	411.20	
	source	81973.78	39		
48	treatment	150229.50	7	21461.36	55.79
	error	12309.60	32	384.68	
	source	162539.10	39		
72	treatment	234253.38	7	33464.77	41.14
	error	26027.60	32	813.36	
	source	260280.98	39		
96	treatment	803296.98	7	114756.71	39.62
	error	92684.00	32	2896.38	
	source	895989.98	39		
120	treatment	1823151.38	7	260450.20	91.88
	error	90709.00	32	2834.63	
	source	1913859.38	39		
144	treatment	1638228.80	7	234032.69	115.41
	error	64891.60	32	2027.86	
	source	1703120.40	39		

3.11

The analysis of variances, over time, for Acanthamoeba castellanii, in Repli-dishes treated with aniline

Time (h)	Source	ss	df	mss	F value
24	treatment	8098.90	7	1156.90	7.54
	error	4908.70	32	153.30	
	total	13007.60	39		
48	treatment	11335.10	7	1619.30	5.22
	error	9928.30	32	310.26	
	total	21263.40	39		
72	treatment	7494.80	7	1070.0	1.37
	error	24908.70	32	778.3	
	total	32403.50	39		
120	treatment	38634.20	7	5519.10	5.62
	error	31402.70	32	981.30	
	total	70037.90	39		

3.12

The analysis of variances, over time, for Acanthamoeba castellanii, in Repli-dishes treated with barban

Time (h)	Source	ss	df	mss	F value
24	treatment	25537.42	7	3648.20	25.44
	error	4445.55	31	143.40	
	source	29982.97	38		
48	treatment	44014.04	8	5501.76	14.21
	error	13939,60	36	387.21	
	source	57953.64	44		
72	treatment	48881.38	7	6983.05	20.50
	error	10899.60	32	340.61	
	source	59780.98	39		
96	treatment	98212.98	7	14030.43	28.46
	error	15766.40	32	492.70	
	source	113979.38	39		
120	treatment	100243.20	7	14320.46	7.42
	error	61780.40	32	1930.64	
	source	162023.60	39		

3.13

The analysis of variances, over time, for Acanthamoeba castellanii, in Repli-dishes treated with diuron

Time (h)	Source	ss	df	mss	F value
24	treatment	8587.20	8	1073.40	9.27
	error	4166.80	36	115.74	
	total	12754.00	44		
48	treatment	17100.30	7	2442.90	8.13
	error	9613.20	32	300.41	
	total	26713.50	39		
72	treatment	17420.80	7	2488.69	6.84
	error	11646.80	32	363.96	
	total	29067.60	39		
96	treatment	28787.38	7	4112.48	9.08
	error	14491.60	32	452.86	
	total	43278.98	39		
120	treatment	8423.69	7	1203.38	1.01
	error	36960.00	31	1192.26	
	total	45383.69	38		

3.14

The analysis of variances, over time, for Acanthamoeba castellanii, in Repli-dishes treated with permethrin

Time (h)	Source	ss	df	mss	F value
24	treatment	12787.50	7	1826.79	16.78
	error	3484.40	32	108.89	
	total	16271.90	39		
48	treatment	10146.00	7	1449.43	4.62
	error	10031.60	32	313.49	
	total	20177.60	39		
72	treatment	9509.78	7	1358.54	4.40
	error	9879.20	32	308.73	
	total	19388.98	39		
96	treatment	9269.18	7	1324.17	1.64
	error	25915.60	32	809.86	
	total	35184.78	39		
120	treatment	8177.98	7	1168.28	1.02
	error	36630.80	32	1144.71	
	total	44808.78	38		

3.15

The analysis of variances, over time, for *Acanthamoeba castellanii*, in Repli-dishes treated with asulam

Time (h)	Source	ss	df	mss	F value
24	treatment	39601.78	7	5657.40	6.10
	error	29678.00	32	927.44	
	total	69279.78	39		
48	treatment	6052.00	7	864.57	1.80
	error	15355.60	32	479.86	
	total	21407.60	39		
72	treatment	5019.50	7	717.07	0.66
	error	34562.40	32	1080.08	
	total	39581.90	39		
96	treatment	3706.98	7	529.57	0.53
	error	32224.00	32	1007.00	
	total	35930.98	39		
120	treatment	39989.18	7	5712.74	4.01
	error	45635.60	32	1426.11	
	total	85624.78	39		

Appendix 4

Evaluation of the sub-acute toxicity of some pesticides and metabolites to Tetrahymena pyriformis and Acanthamoeba castellanii using the Repli-dish culture technique : Equations of linear regressions for the exponential phase of growth

4.1

The equations of the linear regression for Tetrahymena pyriformis in Repli-dishes treated with chlorpropham

Herbicide Concentrations (µg/ml)	Linear Regressions	Correlation Coefficients
0	y = 990.7 (x) + (-2111.2)	0.99
0.001	y = 1503.2 (x) + (-8933.4)	0.99
0.01	y = 1444.9 (x) + (-8133.4)	0.99
0.1	y = 695.3 (x) + (-1755.6)	0.99
1.0	y = 113.4 (x) + 2944.5	0.96
5.0	y = 13.4 (x) + 3244.4	0.86
0	y = 2020.1 (x) + 38682.4	0.97
3.2	y = -45.1 (x) + 27935.4	0.74
15.9	y = -174.9 (x) + 21835.5	0.59
31.8	y = -304.8 (x) + 21890.1	0.28
47.7	y = -176.4 (x) + 13345.8	0.06
63.7		

4.2

The equations of the linear regression for Tetrahymena pyriformis in Repli-dishes treated with 3-chloroaniline

Concentration (µg/ml)	Linear Regressions	Correlation Coefficients
0	y = 2085.6(x) + (-2278.4)	0.98
0.06	y = 2245.4(x) + (-4834.3)	0.98
0.6	y = 2481.9(x) + (-9211.7)	0.97
3.2	y = 2780.1x) + (-8500.7)	0.98
15.9	y = 1257(x) + 10743.6	0.99
31.8	y = 1006.9(x) + 8166.1	0.99
47.7	y = 661.5(x) + 12543.6	0.98
63.7	y = 452.3(x) + 10966.1	0.97

4.3

The equations of the linear regression for Tetrahymena pyriformis in Repli-dishes treated with propham

Concentration (µg/ml)	Linear Regressions	Correlation Coefficients
0	y = 3914.4 (x) + 13388.5	0.99
1.1	y = 3494.5 (x) + 11866.3	0.99
5.6	y = 1912 (x) + 14733	0.99
11.2	y = 1499.5 (x) + 12044	0.99
16.8	y = 880.5 (x) + 13021.9	0.98
22.4	y = 602.7 (x) + 10605.3	0.98
0	y = 1229.1 (x) + (-2889)	0.99
0.001	y = 1424.8 (x) + (-4944.5)	0.99
0.01	y = 1443.0 (x) + (-3644.3)	0.99
0.1	y = 1558.8 (x) + (-8588.9)	0.98
1.0	y = 1295.6 (x) + (-4568.5)	0.99
5.0	y = 922.7 (x) + (-4466.6)	0.99

4.4

The equations of the linear regression for Tetrahymena pyriformis in Repli-dishes treated with aniline

Concentration (µg/ml)	Linear Regressions	Correlation Coefficients
0	y = 2085.6(x) + (-2278.4)	0.98
0.15	y = 1942.1(x) + (-500.8)	0.98
1.5	y = 2023.6(x) + 121.7	0.99
7.5	y = 1942.1(x) + 1943.8	0.99
37.5	y = 3294.9(x) + (-11367.5)	0.98
75	y = 2393.1(x) + (-5234.4)	0.99
113	y = 2403.3(x) + (-6212)	0.98
150	y = 622.7(x) + 9610.5	0.97

4.5

The equations of the linear regression for Tetrahymena pyriformis in Repli-dishes treated with pirimicarb

Pirimicarb (µg/ml)	Linear Regressions	Correlation Coefficients
0	y = 1042.3 (x) + 15258.3	0.99
0.1	y = 1159.9 (x) + 13969.5	0.99
1.0	y = 1324.2 (x) + 14247	0.99
5.0	y = 1062.7 (x) + 15413.8	0.99
10	y = 1066.4 (x) + 13058.4	0.99
50	y = 988.6 (x) + 10969.3	0.99
250	y = 571.0 (x) + 10225	0.99
500	y = 318.2 (x) + 10436	0.96
750	y = 141.4 (x) + 10247.1	0.92
1000	y = 27.5 (x) + 10458.2	0 83

4.6

The equations of linear regression for Acanthamoeba castellanii in Repli-dishes treated with chlorpropham

Concentration (µg/ml)	Linear Regression	Correlation Coefficient
0	y = 3133.4(x) + 66212.1	0.98
0.06	y = 2321.1(x) + 80934.5	0.96
0.6	y = 2380(x) + 73004.9	0.97
3	y = 1966.5(x) + 65479.5	0.96
15	y = 1323.6(x) + 65959.6	0.94
30	y = 1197.4(x) + 63181.5	0.94
45	y = 857.5(x) + 66893.9	0.92
60	y = 723.9(x) + 53616.9	0.98

4.7

The equation of the linear regression for Acanthamoeba castellanii in Repli-dishes treated with 3-chloroaniline

Concentration (µg/ml)	Linear Regression	Correlation Coefficients
0	y = 1742.4 (x) + 94213.7	0.93
0.06	y = 1255.3 (x) + 77471	0.92
0.6	y = 1913.9 (x) + 70930.9	0.95
3.2	y = 1180.6 (x) + 60400.4	0.91
15.9	y = 2235.9 (x) + (-2023.3)	0.98
31.8	y = 2388.4 (x) + (-3791)	0.99
47.7	y = 1888.7 (x) + 25080.3	0.99
63.7	y = 1546.7 (x) + 33497.7	0.99

4.8

The equations of linear regression for Acanthamoeba castellanii in Repli-dishes treated with propham

Concentration ($ugml^{-1}$)	Linear Regression	Correlation Coefficient
0	y = 1940.7(x) + 52311.2	0.98
0.15	y = 1784.7(x) + 23799.8	0.98
1.5	y = 1726.4(x) + 31511	0.98
7.5	y = 1669.4(x) + 14466.8	0.99
37.5	y = 973.6(x) + 24933.4	0.97
75	y = 379.1(x) + 40888	0.98
113	y = -63.5(x) + 54555	0.79
150	y = -207.8(x) + 52107.3	0.67

4.9

The equations of linear regression for Acanthamoeba castellanii in Repli-dishes treated with barban

Concentration (µg/ml)	Linear Regression	Correlation Coefficient
0	y = 1996 (x) + 81717	0.95
0.008	y = 1331 (x) + 73080.6	0.93
0.08	y = 1220.5 (x) + 73080.5	0.93
0.4	y = 3786.8 (x) + (-69848.5)	0.98
1.9	y = 2403.2 (x) + (-6212)	0.99
3.9	y = 1890.7 (x) + (-909.5)	0.99
5.8	y = 1537.2 (x) + 2879	0.99
7.7	y = 1782.4 (x) + (-23257.5)	0.98

4.10

The equations of linear regression for Acanthamoeba castellanii in Repli-dishes treated with diuron

Concentration (µg/ml)	Linear Regression	Correlation Coefficients
0	y = 2103.3(x) + 47903.8	0.98
0.03	y = 2360.1(x) + 3637	0.99
0.3	y = 2103.3(x) + 47903.8	0.99
1.5	y = 2766.8(x) + (-23703)	0.99
7.4	y = 1969.7(x) + 3509.5	0.99
14.7	y = 2314.8(x) + (-37021)	0.99
22.1	y = 2027.6(x) + (-22248)	0.99
29.4	y = 1875.0(x) + (-12273.5)	0.99

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