Cell death in the human pathogen *Candida albicans:* effects of garlic (*Allium sativum*), and garlic constituents allyl alcohol and diallyl disulphide.

**Thesis submitted for the degree of
Doctor of Philosophy**

Katey M. Lemar BSc. (Hons)

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Abstract

Garlic extract is very complex, yielding a number of organic sulphur constituents that are thought to be responsible for its anticandidal properties. Many of these are now being investigated in an attempt to determine the mechanisms by which they act.

The effects of fresh and freeze dried extracts of *Allium sativum* (garlic) on the physiology and morphology of *Candida albicans* were compared. Inhibition of growth and loss of structural integrity was observed for both; fresh garlic extract (FGE) has a greater efficacy than garlic powder extract (GPE) as indicated both by its effects on morphology and inhibition of growth.

Gas chromatography-mass spectrometry of extracts was employed to separate and quantify putative inhibitory sulphur-containing components; fresh and freeze-dried extracts yielded the same components but fresh garlic yielded ten times more sulphur constituents.

Cell death mechanisms were investigated by flow cytometry. Low concentrations of allyl alcohol (AA) triggered a necrotic response, whereas an apoptotic type of cell death was observed at higher concentrations ($\geq$6mM). Conversely, low concentrations of diallyl disulphide (DADS) induced apoptosis, whereas higher concentrations ($\geq$6mM) resulted in a necrotic response. Further investigations with using 2-photon microscopy determined that a short 30 min exposure to 0.5mM DADS and then removal, induced 70% cell death (50% necrotic, 20% apoptotic) within 2h; this figure increased to 75% after 4h.

Intracellular levels of reactive oxygen species (ROS), were increased with >10mM menadione, 2mg ml^{-1} GPE, 1mM AA or DADS as measured using dihydrofluorescein and detected by flow cytometry. Two-photon laser scanning microscopy was employed to monitor the intracellular responses of individual *C. albicans* cells after treatment. Changes typical of oxidative stress; NADH oxidation, glutathione depletion and increased reactive oxygen species (ROS), were observed. Additionally, DADS induced a marked enhancement of mitochondrial membrane potential and low respiration rates as could be verified in cell suspensions.

The plasma membrane was monitored by use of the Bis-oxonol dye, DiBaC$_4$(3). Calculation of the electrochemical potential was achieved by application of the Nernst equation. Complete depolarisation was observed with low concentrations of AA, suggesting that for this constituent, the plasma membrane may be a primary target. Effects of garlic extract and diallyl disulphide on plasma membrane were less obvious.

Putative targets for DADS are glutathione-*S*-transferase as determined by *in vitro* kinetics using cell-free extracts; additional targets are likely to be a component prior to Site II in the respiratory electron transport chain as well as ATPsynthase as determined by decreased oxygen consumption and proton production respectively. Known targets for allyl alcohol are alcohol dehydrogenases Adh1 and 2 (in the cytosol) and Adh3 (mitochondrial), although the significant decrease in NAD(P)H after addition of AA is indicative of another mechanism of action.

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Katey M. Lemar, Carsten T. Müller, Sue Plummer, & David Lloyd. (2005). The Plasma Membrane Potential of the Human Opportunistic Pathogen *Candida albicans:* flow cytometric measurement using a bisoxonol probe. Submitted paper.

Abstracts:

Lemar, K. M., Plummer, S., Bahashwan, A., Bowen, I. D., & Lloyd, D. (2002). Programmed Cell Death as a possible mode of action of *Allium sativum* (garlic). Presented at: 1st International Conference on Yeast Apoptosis, University of Minho, Portugal

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Lloyd, D., Salgado, L.E.J., Turner, M.P., **Lemar, K. M.,** Suller, M.T.E., and Murray, D. (2003). An Ultradian clock in continuous cultures of yeast: intracellular timekeeping. Presented at: Yeast, Chandigarh, India.

Lloyd, D., **Lemar, K. M.**, Murray, D., Aon, M.A., Cortassa, S., and O'Rourke, B. (2003). Yeast Respiratory Oscillations in Continuous Cultures and Evaluated by 2-Photon Laser Microscopy. Presented at: VIII congresso de la Sociedad Iberica de Citometria, Madrid, Spain.

Lloyd, D., **Lemar, K. M.,** Murray, D., Aon, M. A., Cortassa, S. C., & O'Rourke, B. (2005). Mitochondrial redox functions in yeast: evaluation by 2-photon laser microscopy. To be presented at: Weber Symposium, Maui, Hawaii.

Abbreviations

AA	Allyl Alcohol
AMD	Allyl Methyl Disulphide
AMS	Allyl Methyl Sulphide
AMT	Allyl Methyl Trisulphide
CMC	Chronic mucocutaneous candidosis
CM-DCF	carboxymethyl-dichlorofluorescein
CM-H$_2$DCFH	5-(-6)-chloromethyl-2',7'-dichlorodihydrofluorescein
CM-H$_2$DCFDA	5-(-6)-chloromethyl-2',7'-dichlorohydrofluorescein diacetate
DADS	Diallyl disulphide
DAS	Diallyl sulphide
DAT	Diallyl trisulphide
DATET	Diallyl tetrasulphide
DMD	Dimethyl disulphide
DMSO	Dimethyl sulphoxide
DMT	Dimethyl trisulphide
FGE	Fresh Garlic Extract
GPE	Garlic Powder Extract
GC-MS	Gas Chromatography-Mass Spectrometry
GSB	Glutathione-bimane fluorescent adduct
GST	Glutathione-S-transferase
HPLC	High Performance Liquid Chromatography
HPTLC	High Performance Thin Layer Chromatography
IC$_{50}$	Inhibitory Concentration for 50% of population
IUD	Intra Uterine Device
LC$_{50}$	Lethal Concentration for 50% of the population
MCB	Monochlorobimane
MeS(O)Me	S-methyl methanthiosulphinate
MIC	Minimum Inhibitory Concentration
MPrD	Methyl Propene disulphide
NCAC	Non-*Candida albicans Candida*
OD	Optical Density
PBS	Phosphate buffered saline
PCD	Programmed cell death
PCR-RFLP	Polymerase Chain Reaction- Restriction Length Polymorphisms
PS	Phosphatidylserine
RAPD-PCR	Random Amplified Polymorphic DNA Polymerase Chain Reaction
ROS	Reactive oxygen species
RT	Retention time
SEM	Scanning Electron Microscopy
TEM	Transmission Electron Microscopy
TLC	Thin Layer Chromatography
TMRE	Tetramethyl rhodamine ethyl ester
TUNEL	Terminal deoxynucleotidyl Transferase Biotin-dUTP Nick End Labelling
2VD	2-Vinyl 1,3-4H-dithiin
3VD	3-Vinyl 1,2-4H-dithiin
YPG	Yeast Peptide Glucose Media

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5.5 Time course of GSB production in *Candida albicans*. Cells harvested and processed as described in M&M were loaded with 40 µM MCB by acute addition of the probe. Cells were imaged at the indicated times after MCB addition in order to follow the kinetics of GSB production (*green* pseudocolor). The progressive vacuolar localization of GSB is quite evident.

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NADH (E) 2mM malate/2mM pyruvate. Successive additions of 0.5mM DADS were added as indicated by black chevrons. Final % inhibition of respiration was as follows: A=0; B=0; C=55; D=25; E=25.

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1. CHAPTER ONE

Introduction

1.1 *Candida albicans*

1.1.1 Background and History

Candida albicans is an opportunistic pathogen causing various forms of candidosis of the skin and particularly, the mucous membranes (thrush), and is quantitatively the most common pathogenic yeast. *C. albicans* has been known since ancient times when Hippocrates (circa 460-377 BC), described two cases of an 'oral condition' in seriously debilitated hosts. However, the yeast responsible for thrush was not discovered until 1839 when Lagenbeck reported it present in the lesions of a patient suffering from typhoid fever, incorrectly relating it to the cause of the disease typhus (Odds, 1979b). The taxonomical position of the fungus was initially within the genus *Monila*; yeasts and moulds responsible for vegetable and fruit decay. Berkhout was responsible for its reclassification in 1923, when yeasts with medical importance were given the generic name *Candida*.

Yeasts of the genus *Candida* are members of the *Cryptococcacae* (Kreger van Rij, 1984). Approximately 200 *Candida* are known (Barnett, 1991), of which around 10% are known to cause infections in humans. *C. albicans* is recognised as being one of the single most common fungal species causing infections, although there are notable exceptions of clinical importance due to infection by non-*Candida albicans Candida* (NCAC) (Fridkin & Jarvis, 1996; Hazen & Brown, 1950; Pfaller, 1996). They do not obviously demonstrate any sexual activity (Kreger van Rij, 1984). DNA fingerprinting techniques (Pujol *et al.*, 1997; Pujol *et al.*, 1993) provide evidence to suggest a clonal mode of asexual reproduction. However, although mating and meiosis have not been observed for *C. albicans*, recent investigations have isolated homologues of genes from *Saccharomyces cerevisiae* that function in both mating (GPA1 and STE20) and meiosis (DMC1), (Hull *et al.*, 2000).

1.1.2 Cytology and Morphology

C. albicans is pleiomorphic and can exist as a budding yeast (blastospore, blastoconidium), a hypha of cylindrical fungal cells known as the mycelial phase, or as pseudohyphae. This developmental heterogeneity is often in response to the environmental conditions, especially temperature and pH (Farah *et al.*, 2000). In the yeast phase, *C. albicans* are 2 to 3μm x 8 to 14μm in size, but hyphae can extend a few hundred micrometres (Saterelli, 1989). Cellular material is deposited close to the apex of the cell surface as the yeast cells bud and continue to enlarge. The bud enlarges and then stops; at this point mitotic nuclear division takes place and a cross-wall is formed before separation to release two individual yeast cells (Fig 1.1). In some cultures, it has been observed that a bud may remain attached to its parent as a pseudo-hypha, resulting in elongated cells. These structures can range from a relatively short length, to extended cells, and can be distinguished from true hyphae by the presence of constrictions present in their septa (Lo *et al.*, 1997).

Different morphologies are used to distinguish *C. albicans* strains; an example of this is demonstrated by the growth of hyphae from yeast cells in a germ tube after incubation in serum at 37°C (Odds, 1979b). True hyphae arise as germ-tubes from yeasts during germination, or as branches from existing hyphae. Mitotic nuclear division occurs with extending hyphae and cross-walls known as septa, are formed at intervals along the hyphal tube. Long branching hyphae can be observed macroscopically, as large white 'clumps' in liquid media. Chlamydospores, yeast-like structures characterised by a thick cell wall, are often seen at the end of hyphae, particularly when the fungus is grown on nutritionally-poor media such as corn-meal agar (Benham, 1931), (Fig 1.2). It has been suggested that these chlamydospores are a

3
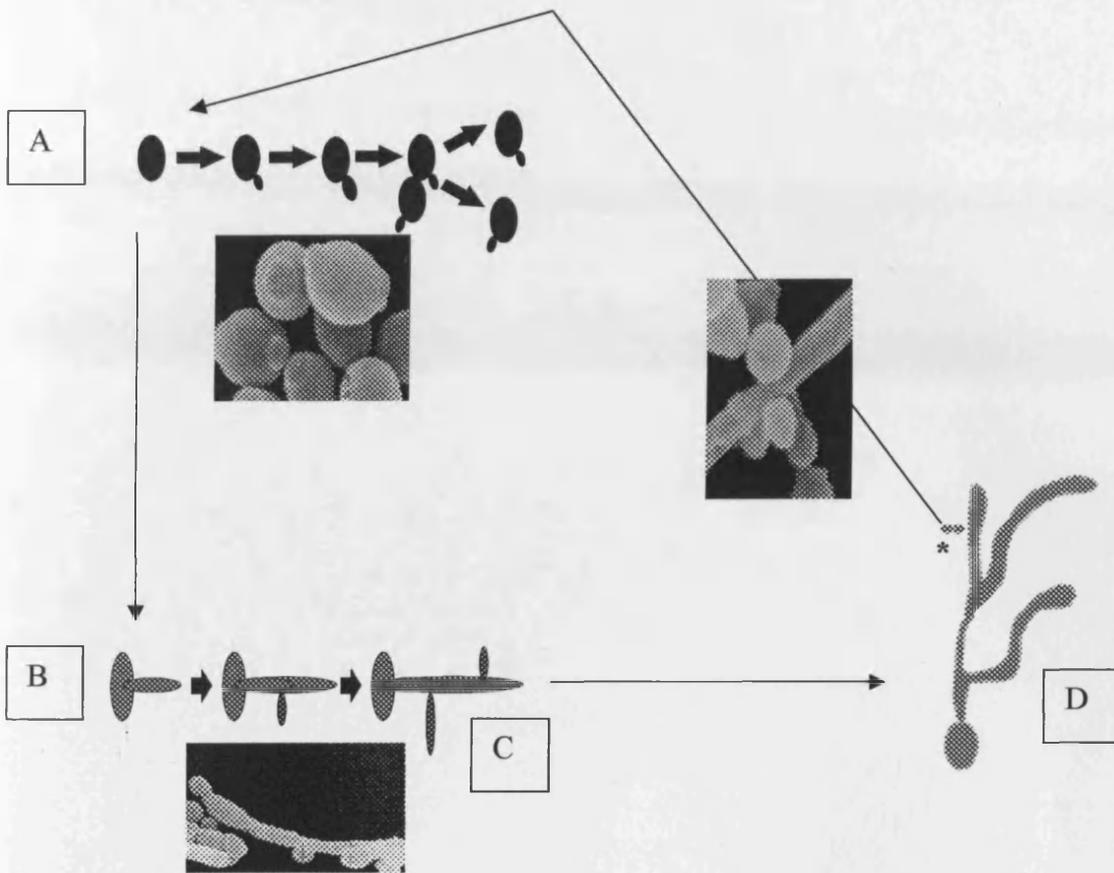
Fig. 1.1.
Life cycle of *Candida albicans* di-morphism (Adapted from Molero *et al.*, 1998). (A) Unicellular blastospores divide by budding. (B) Cylindrical outgrowth form germ tubes after some environmental stimuli (pH/temperature/serum/cell concentration). (C) Germ tubes extend before septa are laid down to form true hyphae. (D) Hyphal branches are produced behind newly formed septa. Secondary blastospores (*) can become separated from the hyphal branch.

Fig. 1.2.
Corn meal agar plate inoculated by the Dalmau technique. Thick-walled chlamydospores are formed. 400X, 25C.
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dormant phase of the fungus (Odds, 1979a). *C. albicans* rapidly undergoes reversible morphogenic transitions between the budding, pseudohyphal and hyphal phases (Brown, 1991). The ability for mycelial transformation from the yeast form is one that is exclusive to *C. albicans* amongst the *Candida* spp. (Calderone & Braun, 1991; Odds, 1988). The process is promoted by a multitude of factors. Morphogenesis is triggered by several signals *in vitro*, many of which probably reflect normal interactions with the host *in vivo*. Recent publications have discussed how the yeast to hyphal transition appears to be caused by one of several virulence attributes that allows *C. albicans* to invade human tissues. It has been suggested that the growth of hyphae is blocked by inactivation of transcription factors in the mitogen-activated protein kinase pathway, but it is unclear as to what other virulence factors may regulate these transcription factors (Brown, 1991).

1.1.3 Biochemistry

The biochemistry of *C. albicans* is typical of that of a lower eukaryote. It possesses both a protein synthetic apparatus (Yamaguchi, 1975), and an oxidative phosphorylation mechanism generally similar to that of *Saccharomyces cerevisiae* (Yamaguchi, 1975).

It is a diploid cell, and its cell wall maintains its shape. The cell wall is a potential target site for antifungal drugs. It is comprised of mainly polysaccharides with small amounts of chitin, protein and lipid (Chattaway *et al.*, 1968). The outermost side of the cell wall appears to house most of the polysaccharide polymers, whereas the remaining components of chitin, protein and lipid are located on the innermost side (Cassone *et al.*, 1979). The outer fibrillar layer contains the antigenic determinants, allowing determination of strains of *C. albicans*; they are also assumed to be important with

regards to the mechanism of adhesion to epithelial cells of the host. There is no

qualitative difference between the cell walls of the morphologic forms of *C. albicans*

(Chattaway *et al.*, 1968). However, investigations that are more recent have

demonstrated varying quantitative differences (Shepherd *et al.*, 1985), particularly in

relation to the amount of chitin present.

1.1.4 Epidemiology

C. albicans can be considered an obligatory commensal of man (van Uden, 1970), and

it is reported that up to 80% of healthy humans harbour *C. albicans* within their oral-

gastrointestinal tracts. Clinical isolates can be obtained from the mouth, vagina, skin

and gut mucosal surfaces of humans, and between 20-64% of this population possess

antibodies to *C. albicans* in their serum (Ryley, 1986). Additionally, *Candida* spp. are

now recognized as causing disease with much greater frequency, and are one of the

most prevalent sources, of nosocomial infections (Verduyn Lunel *et al.*, 1999). The

increasing number of immunocompromised individuals (Sullivan, 1998), may explain

the apparent explosion in fungal diseases, particularly those individuals, both in the

community and hospitals, whose immune systems are impaired as a result of human

immunodeficiency virus (HIV) infection. Conditions such as diabetes, cystic fibrosis

and leukaemia, coupled with the widespread use of steroids and broad spectrum

antibiotics are also likely to be contributing factors (Fridkin & Jarvis, 1996; Pfaller,

1996). Epidemiological techniques such as Random Amplified Polymorphic DNA

Polymerase Chain Reaction (RAPD-PCR) and PCR-Restriction Fragment Length

Polymorphisms (PCR-RFLP) are used to monitor patients held in isolation where

cross-infection has occurred (Fridkin & Jarvis, 1996; Pfaller, 1996). Particularly

prevalent in hospital outbreaks arc exogenously acquired strains, usually originating

from cross-contamination from inanimate objects or the environment (Fridkin & Jarvis, 1996; Pfaller, 1996). Many can be attributed to transfer of isolates from hospital workers to patients (Betrmieux, 1994; Isenberg *et al.*, 1989).

1.1.5 Candidosis

1.1.5.1 Acute pseudomembranous candidosis – oral thrush

Clinical manifestations are usually the appearance of white patches on the buccal mucosa and gum margins (Fig 1.3a). The disease is frequent in debilitated adults. Ill-fitting dentures and poor oral hygiene are large contributing factors, thought primarily to be due to constant irritation and higher levels of saliva, thus allowing easy access and survival of the commensal before eventual colonization and pathogenesis. Symptoms presented are most commonly associated with patients who wear dentures on the upper palate. It may be found in as many as 60% of elderly denture wearers (Odds, 1979b).

1.1.5.2 Acute atrophic candidosis

This condition may follow acute pseudomembranous candidosis and is characterised by erythema of the tongue and gum linings. It is often associated with antibiotic therapy, due to a decrease in normal human commensals of the buccal cavity (Cannon *et al.*, 1995).

1.1.5.3 Vaginal candidosis – vaginal thrush

Characteristic symptoms present white 'curd like' patches on the vulva, vaginal or cervical epithelium. Patients often present evidence of inflammation, acute pruritis and discharge. It is especially common during pregnancy and in women who have reached

Fig. 1.3.

Manifestations of *Candida albicans* a) Oral candidiasis, b) onychomycosis (infection of the nail). Images copyright of Doctorfungus Corporation..

a)

Courtesy of
The Geraldine Kaminski Medical Mycology Library
Produced by: David Ellis and Roland Hermanis
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b)

Image Courtesy of Carlyn Halde
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sexual maturity, particularly those prescribed the oral contraceptive pill and those taking broad range antibiotics (Burns, 1997; Davidson & Mould, 1978; Powell & Drutz, 1983). Vaginitis appears because of an increased number of infecting organisms or an enhanced virulence of candidosis (Hurley, 1975; O'Connor & Sobel, 1986; Sobel, 1985).

1.1.5.3.1 Chronic mucocutaneous candidosis

Chronic mucocutaneous candidosis (CMC) is a rare disease characterized by the presence of *C. albicans* on the skin and nails (onychomycosis) (Fig 1.3b) as well as the mouth (Fig 1.3a) and vaginal mucous membranes (Higgs, 1973). It is most commonly displayed in children under two years of age, is often associated with genetic and endocrinological disorders, and is almost certainly based on defects of cellular immunity (Sutphin, 1943). In serious cases, the skin lesions are widespread and can be disfiguring (Schultz, 1925).

1.1.5.3.2 Systemic candidosis

Systemic candidosis is a very serious condition and is most commonly associated with hospitalized patients who are seriously immunocompromised and debilitated (Epstein & Tuazon, 1981; Henderson, 1986; Paterson, 1985; Spebar & Lindberg, 1979; Warnock, 1982). Infections of the organs may arise from localized primary lesions or from disseminated candidosis where yeasts are spread through the blood-stream infecting several organs. They usually arise endogenously from infections in the gastrointestinal tract, the second most common site for candidal invasion after the oesophagus (Odds, 1988). In these cases there is nearly always a pre-existing gastric lesion which becomes super-invaded by the fungus (Field *et al.*, 1981; Strom *et al.*,

1978). Further colonization of the intestine may cause peritonitis resulting from intestinal perforation (Freund *et al.*, 1979; Radin *et al.*, 1983). *Candida* 'enteritis', *Candida*-related diarrhoea, is also well known, particularly in children (Murphy, 1936), although, as most isolations from stools contain helminthes or protozoa, it is suggested that *Candida* may continue to cause diarrhoea as a secondary pathogen when the primary agent has been eliminated by antibiotics (Alam *et al.*, 1977).

1.1.5.4 Predisposing factors

1.1.5.4.1 Natural factors

Associations between pregnancy and *Candida* are almost exclusively in the form of vaginal candidosis and are known to be more prevalent in pregnant women than non-pregnant women (Odds, 1988). Corticosteroid effects, vaginal pH, and vaginal glycogen levels have all been reported as factors of increasing vulnerability during pregnancy (Sobel, 1985). Neonatal candidosis may be attributed to contraction of infection from the birth canal coupled with an immature immune system, which may promote its virulence. The child's fragile skin, mucosal barriers and underdeveloped bacterial flora all increase susceptibility (Ostrosky-Zeichner, 2000). Old-age is also considered a predisposing factor, but it is likely that cases of candidosis are associated with suppression from other illnesses rather than a direct link to old-age specifically.

1.1.5.4.2 Hormonal factors

Although there is no evidence that hormones promote the growth of *C. albicans* the prevalence of cases linked to use of oral contraceptives has been recognized. It was first reported that oral contraceptives exacerbated vaginal thrush (Bourg, 1964), and this was later confirmed in several publications (Porter & Lyle, 1966; Woenig, 1886;

Yaffee & Grots, 1965). Further investigations have led to the level of oestrogen, and not progesterone, becoming accepted as the predisposing factor (Jackson, 1968). Similarly, the use of the IUD as a contraceptive has been linked to reports of candidosis recurrence and these devices are thought to act as a candidal reservoir (Ferrer, 2000).

1.1.5.4.3 Dietary factors

High intakes of carbohydrate have been linked to oral candidosis (Samaranayake et al., 1989), and subsequent high carbohydrate levels in the gut have been reported to favour the multiplication of yeasts in preference to bacteria (Cormane, 1963). A deficiency in vitamins has also been indicated as a possible predisposing factor in host susceptibility (Samaranayake, 1986). Alcoholism has also been reported as a factor underlying some underlying cases of candidosis (Bardwell et al., 1986).

1.1.5.4.4 Medical factors

Genital Candida infections are reported as a common symptom at the onset of diabetes in men and women due to an increased ability of C. albicans to adhere to diabetic cells (Geerlings & Hoepelman, 1999; Skoglund, 1971). Predisposing endocrine disorders can be linked to chronic mucocutaneous candidosis (Alteras et al., 1969; Giombetti et al., 1971). Increasingly, Candida species are frequently co-pathogens with bacteria in cases of systemic candidosis (as discussed above). Antibiotic treatments depress the endogenous bacterial flora present in a normal healthy host and allow C. albicans to out-compete and overgrow the mucosal surfaces. Importantly, patients treated with antibiotics are already ill and therefore considered 'compromised', increasing the likelihood of colonization and invasive infection (Winner, 1964). Although Winner &

Hurley (1964) have advised caution in accepting the view that extensive use of antibiotics increases the outcome of the yeasts' overgrowth, other reviews report a pre-disposition of deep-seated candidosis from antibiotic therapy (Seelig, 1966a; Seelig, 1966b; Seelig, 1968). Both immunosuppressants and corticosteroids depress inflammatory responses and cell-mediated responses, again encouraging the opportunistic growth of *C. albicans* (Gale, 1982; Schechtman *et al.*, 1986). In many cases, surgery has been reported as being a pre-disposing factor (Bernhardt *et al.*, 1972; Kambayashi *et al.*, 1983) with an increased risk of *Candida* peritonitis after surgery. In-dwelling catheters are also a common port of entry for yeasts to the bloodstream, increasing the chances of systemic candidosis (Williams *et al.*, 1971).

1.1.5.5 Pathogenicity

This organism is considered a highly important and prevalent human pathogen, causing superficial as well potentially life threatening systemic mycoses. Although its invasive mechanisms are still not understood in detail, several factors are believed to be important: protein-protein interactions between *Candida* surface antigens and host cells (Calderone & Braun, 1991; Hostetter, 1994), the morphological form of *C. albicans* (Phan *et al.*, 2000), and the physiological state of the host (Odds, 1988). Infection by this pathogen may rely upon environmental signals within the host niche to regulate the expression of virulence determinants (De Bernardis, 1998).

In tissue samples, the presence of hyphae, pseudohyphae and blastoconidia can be considered pathogenic factors of *C. albicans* (Drago *et al.*, 2000). Other important mechanisms that have been suggested are the production of germination tubes, capsule and cell wall structures, antiphagocytic activity and the production of cytotoxic and histotoxic enzymes (Hube, 2002). The three-stage mechanism of adhesion, blastospore

germination and epithelium invasion are emphasised as a possible cause of recurrence (Drago et al., 2000; Ferrer, 2000). In addition, the ability of C. albicans to translocate through the epithelial mucosa into the lamina-propria and other tissues has been widely described (Alexander et al., 1990; Cole et al., 1988; Deitch et al., 1988). However, this has only recently been reported as an additional pathogenic factor of C. albicans (Drago et al., 2000). Further investigations observed the internalisation of C. albicans in non-typical phagocytic cells, emphasizing that this process may possibly be another factor responsible for recurrent infections (Drago et al., 2000).

The various transitions in morphology of C. albicans have attracted considerable medical interest (Brown, 2002). It has been suggested that the hyphal phenotype is more virulent, due to the misconception that hyphae are more capable of invading host tissue. It has been reported that hyphae may be able to detect and penetrate epithelial breaks or endothelial surfaces (Gale et al., 1996), with other reports isolating monomorphic cultures of yeast cells only from clinical samples (Ernst, 2000). It is now agreed that hyphal cells do in fact promote tissue invasion, but this is certainly not more virulent than yeast cells, which are accepted as promoting dissemination (Ernst, 2000; Feng et al., 1999).

Other virulence factors may be due to an expression of surface antigens that are thought to interfere with the immune response of the host (Cole et al., 1996). This theory led to many studies focussing on the mannoproteins present in the cell wall, which bind human fibrinogen, laminin or fibronectin (Bouali et al., 1986; Bouchara et al., 1988; Klotz & Smith, 1991). The C. albicans gene encoding part of the 58-kDa fibrinogen binding mannoprotein was isolated and its function shown to support the

antigen theory (Lopez-Ribot *et al.*, 1997). Other mannoproteins include the analogues of human β2 integrins, which are able to recognise the complement C3 conversion products iC3b and C3d (Heidenreich & Dierich, 1985; Linehan *et al.*, 1988). Studies on the expression of the iC3b binding protein, in pseudomycelial forms as well as the mycelial and yeast forms, offer a new direction in which to combat the pathogenicity of virulent strains of *C. albicans* (Heidenreich & Dierich, 1985). The complement C3 conversion product, iC3b binding protein, could potentially be targeted with antibodies resulting in reduced adhesion to host mucosal membranes (Lee *et al.*, 1997). Similarly, evidence is emerging that the pathway of disease progression involves a transcriptional programme linking morphogenesis with other virulence traits such as adherence and aspartyl-proteinase production (Brown, 1991).

1.1.5.6 Treatment

Despite its prevalence, candidosis remains difficult to treat. Its wide range of manifestations is partly responsible, with additional problems occurring due to unpleasant side effects and development of resistance.

1.1.5.6.1 Systemic infection treatment

Hazen & Brown (1950) discovered the first antifungal agent, Nystatin powder, a pharmaceutical preparation derived from the soil actinomycete, *Streptomyces noursei* (Hazen & Brown, 1950). This discovery led to the isolation of similar compounds, the polyenes, most notably amphotericin B (Donovick *et al.*, 1955; Stiller *et al.*, 1955). Both preparations are commonly used in medicine today; Nystatin is normally the first treatment given to patients suffering from an intestinal candidosis, whilst amphotericin B remains the 'gold standard' to which other antifungals are compared. However, the

lengthy administration of Nystatin to ensure complete eradication of the candidal infection means this treatment is often unsuitable and impractical. Similarly, the relatively high toxicity and the need for intravenous infusion restricts the use of Amphotericin B. Fever, shaking, hypertension, nausea and vomiting are common unpleasant side-effects which have lead to the need to develop alternative therapy for many invasive mycoses. Due to its ease of administration (orally) and safety, fluconazole is a popular alternative, proving effective against many forms of candidosis (Anaissie, 1991; De Pauw, 1995; Kauffman *et al.*, 1991). However, fluconazole and other current oral administrations have limited efficacy. Fluconazole has a narrow spectrum of activity and itraconazole (in capsule form) has erratic absorption (Ascioglu *et al.*, 2000; De Doncker, 1999). Additionally, the emergence of reduced sensitivity to fluconazole warrants cautious application. Consequently, the incidence of invasive fungal infections has increased and an alternative ketoconazole is administered to patients suffering from recurrent infections; however, its prolonged use can be damaging to the liver.

1.1.5.6.2   Mucosal infection treatment

For external non-invasive mucosal infections, topical treatment with azole creams produces relatively successful results and shows improved efficacy compared to those achieved by orally taken Nystatin. For example, fluconazole is widely available, but is expensive and its over use has seen rapid development of resistance (Cohen, 2002). Similar antifungals, most mild amoebics; clotrimazole, tinidazole and miconazole; display similar effectiveness, are cheaper and importantly show less toxicity towards the liver than ketoconazole, but familiar trends in development of resistance are evident even with these less exploited treatments (Sanglard & Odds, 2002).

Clotrimazole is preferentially and frequently used for the treatment of vaginal candidosis, topically as a cream or systemically in the tablet form. The mechanism of this drug destroys the yeast cell membrane and reduces the adherence to epithelial cells (Bujdakova *et al.*, 1999).

1.1.5.6.3 Development of Resistance

The emergence of resistance to antifungal drugs parallels the prolonged use of azoles in high-risk patients. Most of the clinically-resistant *C. albicans* isolates fail to accumulate fluconazole (Weig *et al.*, 1998), because they do not convert it to its active component, fluorouracil. The newest treatments offered as alternatives to current pharmaceutical preparations, such as lipid and liposomal preparations of amphotericin B, have yet to have the optimal dosage and clinical effects determined. Economic implications are unknown, but their high price hampers their widespread use. New azole and triazole compounds are in development or being clinically evaluated (Kullberg, 1999). Voriconazole is currently the most promising, combining broad antifungal spectrum and a favourable safety profile in both oral and parenteral forms (Uzun *et al.*, 2000). Echinocandin analogues are potentially highly specific and effective against *Candida* (Uzun *et al.*, 2000), particularly those that demonstrate fluconazole resistance (Melkusova *et al.*, 2004).

1.1.5.6.4 Prophylaxis

Due to the increasing evidence of increased resistance to many of the available antifungal treatments (Cohen, 2002), the reduction in the use of broad spectrum drugs is of paramount importance. The use of antifungal agents for the prophylactic treatment is considered highly controversial (Kullberg, 2002), although necessary for

certain groups of patients, particularly those with haematological malignancies (Ascioglu *et al.*, 2000). There has been no definitive study to ascertain the benefits of using topical treatments such as clotrimazole or miconazole (Walsh & Lee, 1993), and in the same study, ketoconazole was observed to reduce the incidence of disseminated candidosis in cancer patients, although it was not proved unequivocally. As discussed earlier, the erratic bioavailabilty of itraconazole capsules limits its use therapeutically and consequently the prophylactic use of fluconazole has become a standard therapy in many transplant centres (Marr *et al.*, 2000). An increasing incidence of fluconazole-resistant isolates has been the unfortunate consequence of this form of treatment, although large studies of randomized trials have not confirmed this association (Goodman, 1992; Menichetti, 1994; Winston, 1993). It is generally accepted that the use of antifungals as prophylactics increases the risk of new strains developing resistance to current treatments and further necessitates the discovery of new remedies to treat *Candida* infections.

1.1.5.6.5 Alternative treatments

Many investigations have turned to natural remedies to aid prophylaxis and also as possible remedies for yeast infections. Various plant metabolites have been purified and adopted widely in pharmaceuticals. Fresh garlic and garlic extract have been shown to kill various yeasts in laboratory sensitivity tests (Ghannoum, 1988; Lemar *et al.*, 2002), with further investigations demonstrating its successful application *in vivo* (Prasad & Sharma, 1980). That there has been no reported developed resistance for garlic in yeasts warrants further investigation into its use as a potential treatment for yeast and other infections

1.2 *Allium sativum* (garlic)

1.2.1.1 Background history

Allium sativum is a member of the Liliaceae family (The Angiosperm Phylogeny Group, 1998), along with onions, chives and shallots and its origin is thought to be in Central Asia. Its common name is derived from the Old English word 'gar' meaning spear like, referring to the shape of the leaves and the word 'lic' being leek, the generic name for *Allium* (Candolle, 1884). The oldest reports to the uses of garlic used as a medicine, date back as early as 2600-2100 BC (Koch, 1996). In these times, garlic was widely recognised as a tonic or remedy for a range of ailments such as skin diseases, rheumatic conditions, haemorrhoids and abdominal diseases (Weiss, 1983.). Weiss also noted that garlic was widely cultivated, suggesting its use prior to these periods.

The Japanese commonly used garlic as a remedy for colds (Thoms, 1926), and the use of garlic was part of the staple diet of Egyptians (Loret, 1904), later to be adopted by the Israelites (Woenig, 1886). The tendency to use garlic as a treatment for diseases and other common complaints is apparent throughout history across many different continents, its application ranging from intestinal diseases such as the treatment of worms and dysentery by the Egyptians to topical treatment of wounds (Thompson, 1923; Thompson, 1924). Hippocrates prescribed the use of garlic as a diuretic and laxative circa 460-370 BC (Koch, 1996) and in the eleventh century, garlic was specifically mentioned as a treatment for jaundice by Saint Hildegard in her books *Physica* and *Causea et Curae* (Koch, 1996). The transition of its use to Northern Europe was a relatively slow progression; it was not until 1557 that a German physician, Adam Lonitzer, noted that the external application of garlic killed lice and nits, and internally was effective against worms and poisons. The report of four men

who remained free from infection, despite being employed to remove the dead bodies during the 1721 plague in Marseilles, is perhaps the first evidence of the anti-microbial, anti-parasitic and anti-fungal properties of garlic (Hahn, 1996). The tincture of wine and garlic, which became known as '*vinagre des quatre voleurs*', taken by the four men is thought to be the contributing factor behind this phenomenon and further supports the efficacy of garlic during outbreaks of widespread infection (Apitz-Castro, 1987; Block, 1985). More recently, garlic has been used to help combat typhus and dysentery during World War I. Later it was administered prophylactically to treat gangrene and septicaemia during World War II (Spray, 1978).

It has remained prevalent in developing countries as a replacement for, or supplement to, more sophisticated drugs. In Russia, where it is known as "Russian penicillin", it is regularly advised that chewing raw garlic cloves may prevent the common flu, whilst in Mexico it is employed in the treatment of whooping cough (Bolton, 1982). Since the discovery of penicillin by Fleming in 1928 and subsequent development of the drug by Florey, Chain & Heatly (Ligon, 2004) the use of garlic to prevent and treat infection declined, but with resistances to modern antibiotics developing in the organisms they are used to treat, many are turning to natural remedies for the answer. Since this surge of public interest in alternative medicines, scientific investigations have led to a renewed awareness in garlic's medicinal properties. There have been a number of publications commending garlic as a therapeutic tool (see Harris *et al.*, 2002, for review), and many commercial products have been developed to cure a range of ailments and exploit its prophylactic properties.

1.2.1.2 The Chemistry of *Allium*

The pungent aroma characteristic of garlic strongly suggests the presence of sulphur compounds and it was suspected that extraction of these compounds, or their derivatives, would yield the active components. Studies into identifying these components and understanding the action of garlic only began in 1844. The German chemist, Theodor Wertheim subjected whole garlic bulbs to steam distillation, yielding a pungent oil product. The experiment later repeated by Semmler in 1892 yielded a similar product, and the name allyl was proposed for the hydrocarbon compound contained within the oil (Koch, 1996). This terminology is still used today to describe the distinguishing $CH_2=CH-CH_2-$ grouping.

It was not until later that the definitive study on the properties of garlic was performed (Cavallito, 1944). By employing different methods of extraction as outlined by Semmler, they were able to extract and identify compounds diallyl disulphide and diallyl polysulphides. These possessed no apparent anti-bacterial properties, and consequently a less aggressive method of extraction was devised to yield less stable compounds. Through repeated ether extraction at room temperature, an alternative compound, which proved to possess anti-bacterial properties, was eventually produced. This compound was named allicin, and was found to be the oxide of diallyl sulphide.

Allicin is highly unstable (Cavallito, 1944), and is rapidly decomposed when heated. It is relatively stable when refrigerated, but is extremely sensitive to the addition of alkali, losing its anti-bacterial properties. These investigations led to a proposal of a structure for allicin, which is thought to be responsible for the characteristic garlic odour (Fig 1.4).

$$O$$
$$\uparrow$$
$$CH_2=CHCH_2SSCH_2CH=CH$$

$$O$$
$$\|$$
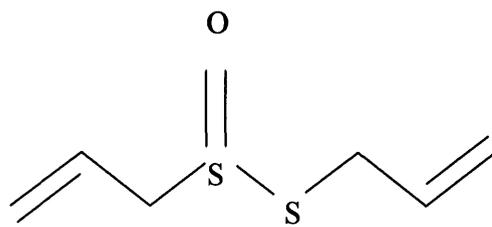
Fig. 1.4. Allicin (2-Propenyl-2-propenethiosulphinate)

Much confusion surrounded allicin due to the fact that its characteristic smell only became evident once the cloves have been crushed or damaged. Investigations performed by Cavalito and co-workers (1944) eventually concluded that allicin must be formed from the breakdown of a parent compound by the action of an enzyme, and that the diallyl sulphides present in the oil extract were formed from allicin. The precursor, alliin (Fig 1.5), and the corresponding alliinase enzyme were isolated by Stoll & Seebeck (Stoll & Seebeck, 1950; Stoll & Seebeck, 1951). Improved extraction methods led to the isolation of several other cysteine sulphoxides and their corresponding thiosulphates (Lawson, 1992).

An even more complex picture emerged after the work of Stoll & Seebeck (1951) reported that alliin had no antibiotic activity unless converted into allicin by alliinase, thus allowing a cascade of events subsequentally yielding the allyl sulphides and cysteine sulphoxides (Stoll & Seebeck, 1951).

The intact cell within garlic cloves contains alliin located in the abundant storage mesophyll cells, whereas, alliinase is located only in the relatively few vascular bundle sheath cells located around the 'veins' (or phloem). Hence, separation of substrate and enzyme involves location in different cell types. On disruption of this arrangement by injury through cutting or crushing, the enzyme and substrate meet and react. Consequently, alliin is decomposed to 2-propenesulphenic acid, which pairs with a second molecule of 2-propenesulphenic to give allicin (Fig 1.6) (Block, 1992). 2-aminoacrylate hydrolyses into its two constituent components: ammonium ions and pyruvate.

$$O$$
$$\text{CH}_2\text{=CHCH}_2\text{SCH}_2\text{CHNH}_2\text{COOH}$$
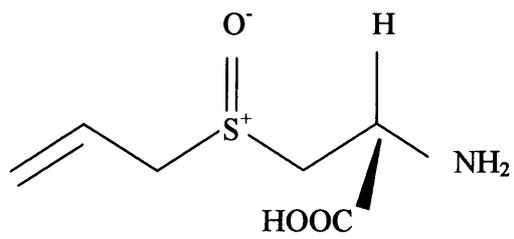
Fig. 1.5. Alliin ((+)-S-allyl-L-cysteine sulphoxide)

$$CH_2=CHCH_2S(=O)CHC(NH_2)COOH \longrightarrow CH_2=CHCH_2SOH + CH_2=C(NH_2)COOH$$

(+)-S-2-Propenyl-L-cysteine
sulphoxide (Alliin)

2-Propenesulphenic
acid

2-Aminoacrylate

$$2 \ [CH_2=CHCHSOH] \xrightarrow{-H_2O} CH_2=CHCH_2S(=O)SCH_2CHCH_2$$

2-Propenesulphenic
acid

Allicin

$$CH_2=CH(NH_2)COOH \longrightarrow NH_4^+ + CH_3COCOOH$$

2-Aminoacrylate

Ammonium
ions

pyruvate

Fig. 1.6.
The investigations of Cavallito, and Stoll & Seebeck (both 1947) displaying the formation of allicin through enzymatic breakdown of the precursor alliin (Adapted from Block, 1992).

Subsequent studies show many garlic components (Cavallito, 1944; Small, 1947). However, arguably the greatest contribution to understanding the chemistry of *Allium* is from the work performed by Eric Block and colleagues. The study, initiated in 1971, explored the properties of allicin and elucidated a detailed scheme of the decomposition of allicin proceeding through a number of different pathways (Fig 1.7). A key step in one pathway resulted in the production of a previously unknown compound, 4,5,9-trithiadodeca-1,6,11-triene-9-oxide. The compound, subsequently named ajoene after the Spanish word for garlic, 'ajo', and has since been shown to be at least as potent as aspirin as an anti-thrombotic agent; it also possesses high antifungal activity (Yoshida *et al.*, 1987).

Later investigations analysed the volatile components from garlic samples cooked by different methods such as frying and baking (Yu, 1993). The dominant compounds observed within this study were the diallyl and trisulphides, although there was no mention of ajoene. However, it was noted that many of thiosulphinates were destroyed or transformed to other compounds via all the cooking processes. It was recognised therefore, that when supplementing a diet with garlic for health reasons, it is important to consider the point at which the garlic is added. Addition of garlic at an early stage in the cooking process results in loss of a greater number of thiosulphinates than if added towards the end of the food preparation

1.2.1.2.1 Anti-microbial activity of garlic

Both extracts of garlic and its many purified components have been demonstrated scientifically to possess a broad spectrum of anti-microbial properties and have recently earned considerable medical recognition. The scope of its activity appears to exceed most of the broad-spectrum antibiotics currently available, as it demonstrates
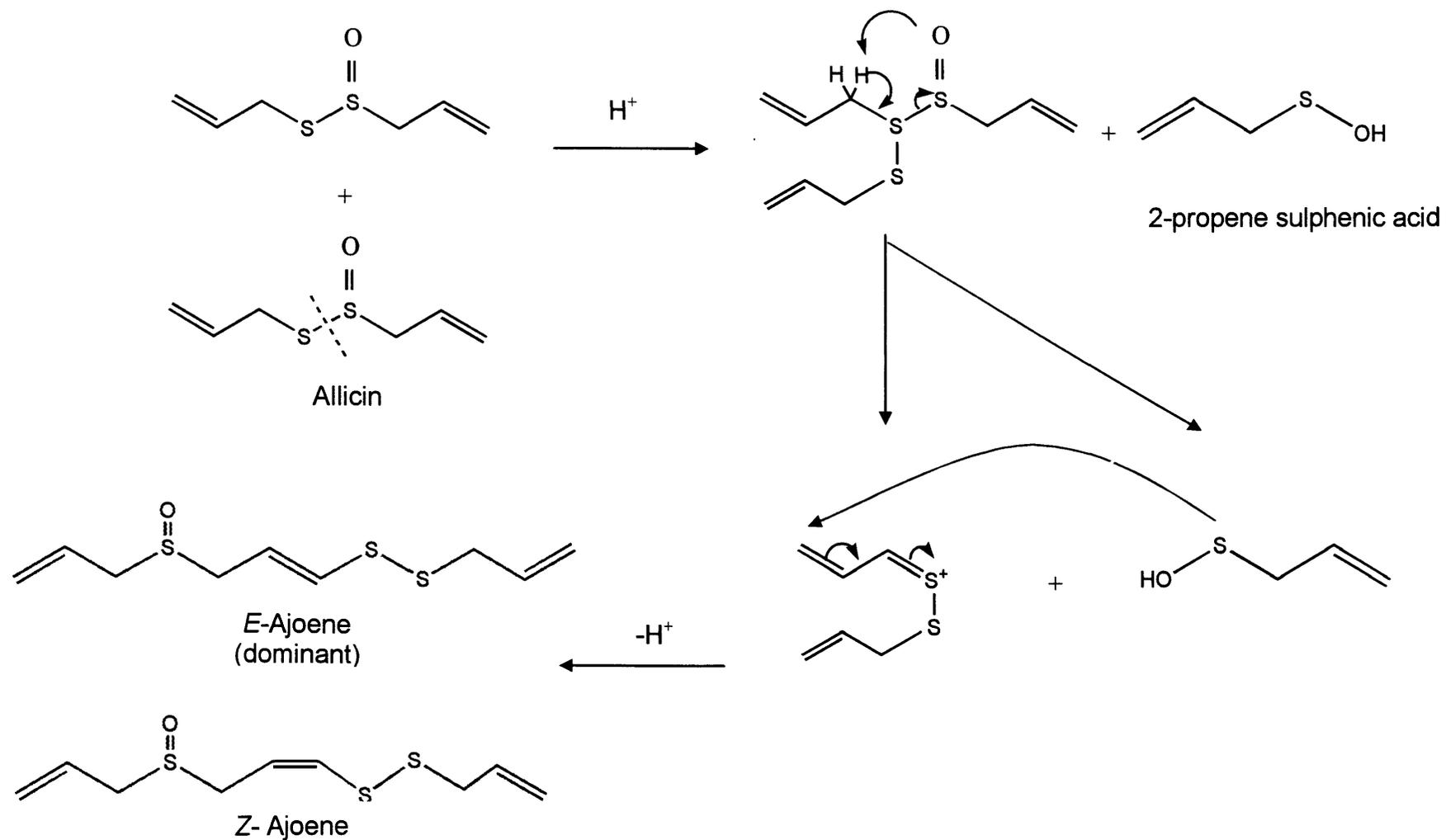
Fig. 1.7. Pathway to show formation of two isomers of Ajoene, *E* and *Z*, the latter possessing a slightly higher potency (Block *et al.*, 1971; 1984; Block 1985). Adapted from Lawson, 1996.

not only anti-bacterial activity, but also proves effective against fungal, parasitic and viral infections (Cavallito, 1944; Jezowa, 1966; Kabelik and Hejtmankova-Uhrova, 1968).

1.2.1.2.1.1 Anti-bacterial activity

Garlic has proven effective against many gram positive, gram negative and acid-fast bacteria. These include *Pseudomonas*, *Streptococcus*, *Proteus*, *Staphylococcus aureus* (Cavallito, 1944), *Escherichia coli*, *Salmonella* (Johnson, 1969), *Klebsiella* (Jezowa, 1966), *Micrococcus*, *Bacillus subtilis* (Sharma *et al.*, 1977), *Clostridium* (DeWit, 1979), *Mycobacterium* (Delaha & Garagusi, 1985) and *Helicobacter* (O'Gara *et al.*, 2000). Differential inhibition occurs due to the composition of bacterial membranes and the permeability of these cells to garlic (Miron *et al.*, 2000). The anti-bacterial efficacy is reduced in samples stored at ambient temperature, but is maintained when stored between 0-4°C indicating thermally instability of the extract (Skyrme, 1997). The mode of action is attributed to the presence of allicin in the extract, which interacts with enzymes, inhibiting lipid, protein and nucleic acid synthesis. Experiments by Rao *et al.*, (1946) proved garlic's efficacy against many strains of *Mycobacteria in vitro*, (Rao, 1946) particularly *M. tuberculosis* (Delaha & Garagusi, 1985). The potency of garlic is similar to pharmaceutical preparations such as streptomycin and isoniazid (Jain, 1994; Jain, 1998). A decrease in number of bacteria in the human mouth flora by up to 99% is reported after gargling with mouthwash containing 10% garlic extract (Elnima *et al.*, 1983). Recent studies using vancomycin resistant bacteria show a large decrease in the MIC of vancomycin in the presence of garlic ranging from 32-256 µg ml^{-1} without garlic and 0.5-1.6 µg ml^{-1} in the presence of garlic (Jonkers *et al.*, 1999). In this case, the mode of action is not entirely known, but has been suggested that

allicin inhibits the enzymes located on transposon Tn*1546*, which codes for vancomycin resistance (Arthur & Courvalin, 1993). In addition, an inhibitory synergism is observed for cysteine after it interacts with the disulphide bonds present in some garlic constituents. Alterations at these sites of vancomycin resistance, caused by the presence of garlic, appear to increase the susceptibility to the antibiotic.

1.2.1.2.1.2 Anti-parasitic activity

Few studies have been performed to establish the mechanisms of action of garlic on protozoa; those reported state effective inhibitory activities against *Opalina sp.*, *Leishmania, Trypanosomes* and *Crithidia* (Reuter, 1994). Many treatments with synthetic compounds or antibiotics are associated with unpleasant side-effects and produce an increasing occurrence of resistance. Consequently, there has been a trend to revert to natural alternatives to reduce ill-effects and adverse reactions. Established knowledge of garlic's anti-parasitic, anti-fungal and anti-bacterial properties, and successful treatment of gastrointestinal diseases with natural remedies (Bolton & Troetal, 1982), led to investigations on garlic's effectiveness against *Entamoeba histolytica, in vitro* (Mirelman *et al.*, 1987; Mirelman *et al.*, 2000). Positive results, with a lethal dose of $50\mu g$ ml^{-1} led to a clinical trial on patients suffering from giardiasis (Soffar & Mokhtar, 1991). Symptoms were eliminated from the patients within 24 hours, and completely removed any indication of *Giardia* from stool samples within 72 hours when administered twice daily at concentration of $1mg$ ml^{-1}. This established garlic as an anti-giardial. Further investigations into the anti-giardial properties of garlic, report an IC_{50} of $14\mu g$ ml^{-1} (Lun *et al.*, 1994), and recent *in vitro* studies support this evidence (Harris *et al.*, 2000; Harris, 2001). Individual components

of garlic extract, allicin, ajoene and various organosulphides were also investigated and found to be effective as anti-protozoals (Lun *et al.*, 1994).

1.2.1.2.1.3 Anti-viral activity

By comparison with other studies associated with the inhibitory effects of garlic, those in relation to viral inhibition are relatively limited. However, there are reports to suggest that garlic is effective *in vitro* against Influenza A and B (Fenwick, 1985; Fenwick & Hanley, 1985) rhinovirus, HIV, herpes simplex 1 (Tsai *et al.*, 1985) and herpes simplex virus 2 (Weber *et al.*, 1992). Benefits against viral pneumonia and rotavirus, where allicin is suggested as the major contributor to the inhibitory effect have been reported (Blake, 1990), but diallyl trisulphide and ajoene have also demonstrated notable activity (Hughes, 1989; Weber *et al.*, 1992). Interestingly, it is the action of ajoene, and not allicin, which is effective against HIV (Tatarintsev *et al.*, 1992). Ajoene acts by inhibiting the integrin-dependent process in Phase I of infection by HIV in the T4 cell cycle. Infection by HIV is inhibited by allyl alcohol and diallyl sulphide (Shoji *et al.*, 1993) and support theories that state only allicin and allicin-derived compounds are active.

1.2.1.2.1.4 Anti-fungal activity

Garlic has been reported as having appreciable activity against both pathogenic fungi and yeast. It can be used as an anti-fungal or anti-mycotic agent and has long been used in folk medicine for the treatment of *Candida* infections, especially those on the skin. Schmidt & Marquardt (1936) first established the fungicidal activity of garlic juice. Almost simultaneously to American authors, Russian authors reported similar findings (Leskinov, 1947). Subsequent studies, mainly with allicin, have reported

successful inhibition of fungal growth (Rao, 1946; Yamada & Azuma, 1977). Kabelik (1970) was among the first to observe the unusually strong effect of garlic against pathogenic yeasts (Kabelik, 1970), however the therapeutic use of garlic is limited to candidosis (Kabelik and Hejtmankova-Uhrova, 1968). The anti-mycotic activity of allicin was found in a study performed by Adetumbi (1985), in which he discovered that garlic reduced the oxygen uptake by the micro-organisms by up to 70% (Adetumbi, 1985). Inhibition of synthesis of nucleic acids, proteins and lipids were observed and was thought to explain the mode of action of garlic (Adetumbi, 1983; 1986). Evidence to support the inhibitory effect of garlic for the treatment of candidosis has been demonstrated in an *in vitro* study where pure allicin was found to be highly active (possessing a minimum inhibitory concentration (MIC) of $7\mu g\ ml^{-1}$, (Hughes, 1991).

The mechanism of action for the inhibition of growth and respiration of *C. albicans*, *Trichophyton cerebriforme* and *Trichophyton granulosum* was assumed to be through inhibition of succinate dehydrogenase (Szymona, 1952). Micro-organisms are much more sensitive to the active components of garlic than higher organisms, as a dilution of garlic juice by 1:1000 inhibited all growth of yeast but had no effect on mammalian tissue cultures (Tynecka, 1974). DiPaolo & Carruthers (1960) performed some model experiments on various strains of yeast and found that enzymically-produced allicin gave total growth inhibition in the original strains; only some mutant strains remained unaffected (Dipaolo & Carruthers, 1960).

Fliermans (1973) reported that the dimorphic soil fungus *Histoplasma capsulatum*, which causes lung disorders in man, was found to be completely destroyed in an

aqueous garlic extract at a dilution as low as 1:400,000, its LD_{50} reported at a 1:127,000 dilution (Fliermans, 1973). These results have been supported by similar publications (Coley-Smith, 1969; Tansey & Appleton, 1975; Timonin, 1950).

1.2.1.2.1.5 Anti-Candidal activity

Specifically relating to *C. albicans*, the inhibition mechanism of growth was investigated by Ghannoum (1988). He found that an aqueous garlic extract caused damage to the outer surface of the cells and several alterations to the lipid content. He concluded that garlic extracts act by oxidising the thiol groups of essential enzymes, an effect attributed to the mode of action of allicin. Further investigation demonstrated a reduced adhesion of *Candida* in the presence of garlic extract (Ghannoum, 1990). The majority of the studies have used the dimorphic yeast pathogen *C. albicans*, as it is commonly related to female genital tract infections (thrush). As the administration of anti-bacterial antibiotics can favour the development of *Candida* infections in buccal, intestinal and vaginal sites, treatment with garlic is a topic of much interest. The treatment of this pathogen can be extremely difficult, as many strains have developed resistance to the few pharmaceutical drugs currently available, such as Nystatin, fluconazole, griseofulvin and amphoterin B (Kabelik, 1970; Tynecka, 1975), often leading to recurrent infection. The toxic properties of these treatments means that simply increasing the dose cannot be used to treat resistant strains, and highlights the need for new, non-toxic anti-candidal therapies. Garlic has been reported to have a greater efficacy than these more common treatments (Tynecka, 1975) and many investigations are now focusing on garlic to treat similar infections.

The potency of aqueous extracts of garlic against *Candida* spp. has been demonstrated *in vitro* in numerous investigations (Barone, 1977; Lau, 1983; Petricic, 1978; Sandhu *et al.*, 1980). Moore & Atkins (1977) looked specifically at twenty-two isolates of *C. albicans* from vaginal infections, and found that all were killed by a 1:128 dilution of crude extract (Moore, 1977). Barone & Tansey (1977) discovered that aside from the lethal properties on cells, lower concentrations of garlic extract also enhanced the conversion from the more virulent yeast to a potentially less pathogenic mycelial phase (Barone, 1977).

1.3 Aims and Objectives

1.3.1 Introduction

Outline the importance of research in the treatment of the opportunistic pathogen *Candida albicans* and introduce *Allium sativum* (garlic) as a potentially effective anti-candidal agent.

1.3.2 Chapter 2

- Investigate the anti-fungal property of garlic against the medically important pathogen *Candida albicans*.

- Determine and compare the efficacy of two different garlic preparations: fresh garlic extract and a commercially available freeze-dried preparation.

- Establish morphological changes after treatment with each preparation.

- Elucidate the individual constituents present in garlic extract, and compare the yields of each constituent from both preparations.

- Select contrasting constituents demonstrating candicidal potential for further investigation.

1.3.3 Chapter 3

- Establish the route by which cell death occurs, necrosis or apoptosis, after treatment with garlic or garlic constituents.

- Determine possible mechanisms of action in *C. albicans*, of targets for garlic extract and selected constituents.

1.3.4 Chapter 4

- Investigate the effects of allyl alcohol and diallyl disulphide on the viability of *C. albicans* cultures.

- Establish morphological changes after exposure to AA and DADS.

- Investigate the effects of allyl alcohol and garlic extract on the functional ultrastructure of *C. albicans*.

- Determine the effects on mitochondrial function: oxygen consumption, mitochondrial membrane potential, and some electron transport chain components.

1.3.5 Chapter 5

- Investigate the effects of diallyl disulphide on the functional ultrastructure of *C. albicans*.

- Determine the effects on mitochondrial function: oxygen consumption, mitochondrial membrane potential, redox potential buffering and some electron transport chain components.

- Establish possible links with increased oxidative stress levels and cell death events.

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2. CHAPTER TWO

Garlic (*Allium sativum*) as an anti-Candida agent: a comparison of the efficacy of fresh garlic and freeze dried extracts.

2.1 Summary

The effects of fresh and freeze dried extracts of *Allium sativum* (garlic) on the physiology and morphology of *Candida albicans* was investigated. Inhibition of growth in glucose-yeast extract-peptone was measured using a multiwell plate reader. Morphological investigations using scanning and transmission electron microscopy indicated loss of structural integrity. Fresh garlic extract has a greater efficacy than garlic powder extract as indicated both by its effects on morphology and inhibition of growth.

Gas chromatography-mass spectrometry of extracts was employed to separate and quantify putative inhibitory sulphur-containing components; fresh and freeze-dried extracts yielded the same sulphur components but the relative yields were greater for FGE than those from GPE.

The ubiquitous opportunistic pathogen, *C. albicans* is sensitive to garlic; resistance to the broad spectrum of active principles present is unlikely so that its anti-candidal effects may provide an important alternative route to chemotherapy.

2.2 Introduction

The opportunistic pathogen *C. albicans* can be found within the oral-gastrointestinal tract in up to 80% of healthy humans. *Candida* species however, are now recognised as causing disease with high frequency and are one of the most prevalent sources of nosocomial infections (Verduyn Lunel *et al.*, 1999). The apparent explosion in fungal infections may be explained by the increasing numbers of immunocompromised patients (Sullivan & Coleman, 1998), e.g., those individuals with impaired immune

systems and those suffering with diabetes, cystic fibrosis and leukaemia. Coupled with widespread use of antibiotics these are all likely to be contributing factors (Pfaller, 1996; Fridkin & Jarvis, 1996).

This human pathogen causes superficial as well as potentially life-threatening mycoses. Although its invasive mechanisms are still not completely understood in detail, several factors are believed to be important: protein-protein interactions between *Candida* cell walls and host mucosal surfaces. The morphological forms of *C. albicans* (Phan *et al.*, 2000) and the physiological state of the host are also considered to be important. In tissue samples, the presence of hyphae, psuedohyphae and blastoconidia can be pathogenic characteristics of *C. albicans* (Drago *et al.*, 2000). Its ability to translocate through the epithelial mucosa (Cole *et al.*, 1988; Alexander 1990) and other tissues has recently been reported as an additional pathogenic factor (Drago *et al.*, 2000).

Watanabe (1966) undertook extensive research into the pharmaceutical value of garlic juice and subsequent reports note garlic as having appreciable activity against pathogenic fungi and yeast. It can be used as an anti-fungal or anti-mycotic agent and has long been used in folk-medicine. Schmidt & Marquardt (1936) first established the efficacy of garlic with epidermorphyte cultures. Later, American and Russian authors reported similar findings (Small *et al.*, 1947; Leskinov, 1947). Allicin, an individual component of garlic, has subsequently been reported as inhibitory against fungal growth. Kabelik was among the first to report the unusually strong effect of garlic against pathogenic yeasts, yet the therapeutic use is limited to candidiasis (Kabelik & Hejtmankova-Uhrova, 1968). The anti-mycotic activity of allicin has been reported to

be due to many different factors including inhibition of succinate dehydrogenase (Syzoma, 1952). This seems likely as oxygen uptake rates in *Mycobacterium tuberculosis* were reduced by 70% in the presence of garlic (Adetumbi, 1985). However, the mechanism of growth of *C. albicans* may be more complex as inhibition of synthesis of nucleic acids, proteins and lipids were also observed (Syzoma, 1952).

2.3 Materials and Methods

2.3.1 Organism and culture

Candida albicans (Berkhout) 3153 was grown in YPG medium (Difco bacto-peptone 2% (w/v), Oxoid yeast extract 1% (w/v) and glucose 2% (w/v)). An early stationary phase culture was used as an inoculum as a 1:100 dilution for 20ml cultures in 100ml conical flasks. Growth was aerobic at 30°C in a rotary shaker (150 rpm). Cell numbers were determined with a Fuschs-Rosenthal haemocytometer slide after dilution in sterile medium. Optical density (A_{450nm}) was measured using a Pye-Unicam SP-1400 spectrophotometer. Stock cultures of *C. albicans* were grown overnight at 30°C and maintained at 4°C on YPG agar, containing 2% (w/v) Oxoid agar.

2.3.2 Garlic Preparations

Garlic powder (GPE) (provided by Cultech Biospeciality Products) and crushed garlic cloves (fresh garlic extract [FGE]) were prepared to the required concentration (w/v) in sterile medium. The wet weight FGE was allowed to stand for 30 min and then both extracts were placed at room temperature for 30 min. Extracts were centrifuged at 3900*g* for 10 min and the supernatant passed through a sterile 0.2µm Millipore filter. Stock solutions of 40mg ml^{-1} were prepared on the same day of testing.

2.3.3 Biocide inhibition

For both types of garlic preparation a concentration range 0-20mg ml^{-1} was tested. 200µl of inoculum was added to 100ml flasks containing 20ml sterile YPG media and placed in a rotary shaker (150rpm) at 30°C. 200µl samples were taken at 90 min intervals, and absorbance (A_{450nm}) measured after appropriate dilution. Measurement was termnated when the control culture showed no further increase in absorption.

2.3.4 Scanning electron microscopy

Cells were fixed in 3% v/v glutaraldehyde in phosphate buffer pH7.4. Dehydration was achieved by 10 min sequential washes of ethanol. A drop was placed onto a round glass coverslip for critical point drying (Balzers CPD 030). The sample was mounted using silver paint (Agar Scientific) onto a 10 x 10mm Jeol type aluminium slab (Agar Scientific). Sputter coating was performed in an Edwards Sputter Coater S150B and image viewed using a Jeol 5200LV scanning electron microscope.

2.3.5 Transmission electron microscopy

Cells were fixed in Cacodylate bufïer pH6.9 containing 1% paraformaldehyde and 2% glutaraldehyde, at 4°C for 1h. Subsequent post-fixing used buffered osmium tetroxide at 4°C for a further 1h. The cells were centrifuged and dehydrated in successive washes of ethanol with a final wash in absolute ethanol for 30 min. The pellet of cells was embedded into Spurr resin and ultrathin sections obtained using a LKB Ultratome III. The sections were mounted onto a 0.5% Pioloform (in chloroform) coated 3.05mm copper grid, stained with 2% aqueous uranyl acetate and 2% lead citrate before imaging on a JEOL 1210 transmission electron microscope.

2.3.6 Gas Chromatography-Mass Spectrometry

1g of ground fresh garlic and garlic powder were prepared separately in 30ml of distilled water. Each suspension was vortexed for 10 min then allowed to stand for 30 min at room temperature before being centrifuged at 3900g for 10 min to give a clear supernatant. The supernatant was then passed through a 0.2μm Millipore filter to remove any particulates and high molecular weight compounds. Samples of 5ml were mixed with 2ml of dichloromethane. The mixture vortexed for 2 min and then placed in a heated water bath (100°C) for 30 min. The mixture was vortexed for a further 1 min before being left to settle at room temperature. The solvent layer was removed carefully and further centrifuged to ensure all water products were removed. A 1ml sample was centrifuged at 9000g for 1 min after which the solvent layer removed using a micro-syringe. All extracts were analysed using a Fisons GC 8000 series with an MD 800 mass spectrometer. Deactivated silica tubing served as a guard column, and a non-polar DB-5ms column selected for maximum separation of compounds. Helium at 40 cm s^{-1} was used as a carrier gas. An on-column splitless injection procedure was used to add 1.2μl, using dichloromethane as a solvent into an injection port at 100°C. The temperature programme, used to separate the components, gave increments of 5°C min^{-1} to 240°C for 3 min. The detector was set at 200°C source temperature and 260°C transfer line with a scan of 35-215 amu in EI+ mode of 70eV. Chromatograms were analysed using Finnigan Masslab version 1.4 with integral NIST and LIBTX libraries. Constituents were either identified by comparison with library analysis data or by mass spectral analysis data obtained for commercial standards.

2.4 Results

2.4.1 Biocide inhibition

Incubation with either Fresh Garlic Extract (FGE) or Garlic Powder Extract (GPE) resulted in a prolongation of lag phase and diminished growth rates during exponential phase of growth of *Candida albicans* (Fig. 2.1 and 2.2). As the concentration of each extract was increased, the growth rates of *C. albicans* decreased. A good correlation was observed for lag and exponential phases performed on different occasions. Highly concentrated extracts demonstrated a greater inhibitory effect in exponential phase than during lag phase. Comparisons between fresh and dried garlic were made and corrected for their different water content. On drying to a constant weight, fresh garlic lost $8 \pm 0.02\%$ mass. IC_{50} values after 10 h exposure for the two extracts were obtained; for FGE this value was 0.29mg ml^{-1} whereas for GPE it was 0.69mg ml^{-1}. FGE therefore exhibited a 42% greater efficacy than GPE.

2.4.2 Fungicidal and fungistatic activity

Fungicidal activity was demonstrated at concentrations greater than 10mg ml^{-1} for FGE (0.32mg ml^{-1}, when corrected for loss of mass on drying). Below this concentration, growth was only inhibited in the presence of FGE (Fig. 2.3). Inoculation into fresh media resulted in normal growth suggesting that concentrations below 10mg ml^{-1} are fungistatic rather than fungicidal. GPE showed similar effects (Fig. 2.4). A higher concentration of 20mg ml^{-1} was required to produce a cidal effect.

2.4.3 Gas Chromatography-Mass Spectrometry

Homogenisation with water followed by controlled heating to 100°C prior to solvent extraction allowed reproducible profiles of constituents to be obtained. Heating the extract at 100°C resulted in decomposition of allicin and thereby allowed the total amount of sulphides present in garlic extract to be quantified.

Figure 2.5 shows the comparison of GC-MS chromatograms showed that the fresh and freeze-dried extracts yielded the same sulphur components but the relative yields were greater for FGE than those from GPE. Comparisons were achieved by calculation of the yield of each component by relative to its most abundant yielded component, 2-vinyl-dithiin.

2.4.4 Scanning electron microscopy

Growth in YPD medium resulted in growth of globular or short ovoid cells that displayed characteristic bud scars (Fig 2.6a). Blebbing in cells was evident at low garlic extract concentrations (Fig. 2.6b), whereas growth with more concentrated solutions resulted in pit formation suggesting localised collapse of the cell wall (Fig. 2.7). Cells incubated in the presence of garlic extract demonstrated a greater tendency to clump than observed in control cultures.
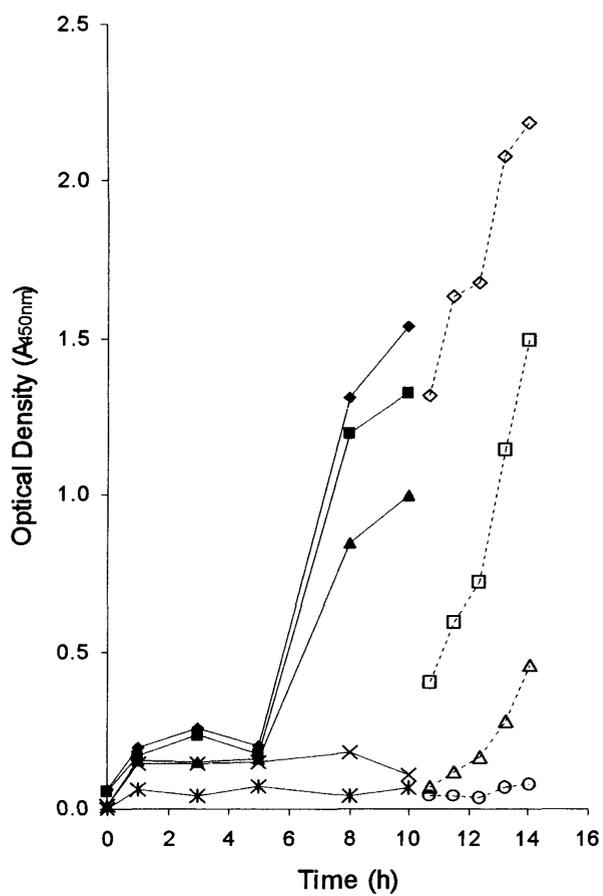
Fig. 2.1.
Inhibitory effects of Fresh Garlic Extract on the growth of *Candida albicans*. Concentrations: (♦) 0mg ml^{-1}, (■) 0.5mg ml^{-1}, (▲) 1mg mg^{-1}, (✕) 1.5mg ml^{-1}, (✱) 2mg ml^{-1}, (◇) 0mg ml^{-1}, (□) 1 mg ml^{-1}, (△) 1.5mg ml^{-1}, (O) 1.75mg ml^{-1}.
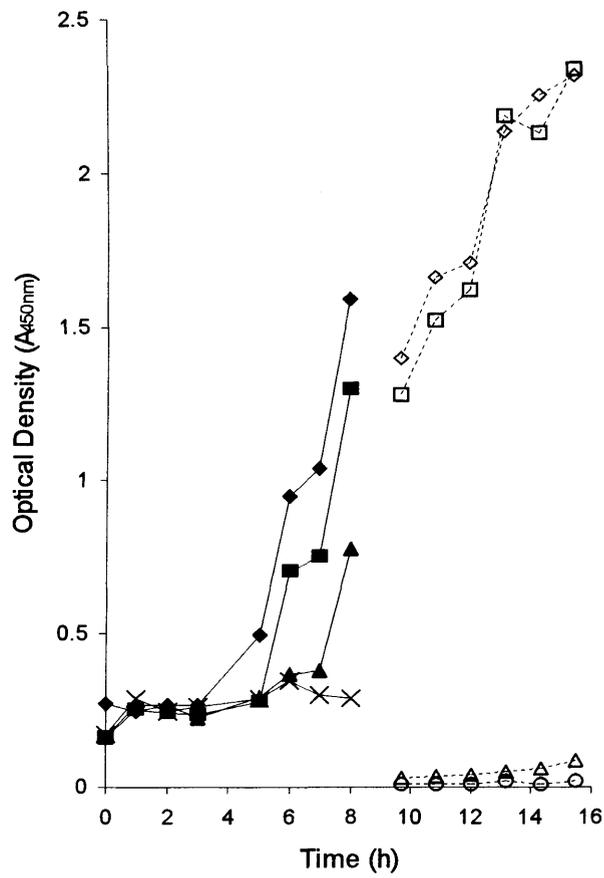
Fig. 2.2.

Inhibitory Effects of Garlic Powder Extract on the growth of *Candida albicans*. Concentrations: (◆)
0mg ml^{-1}, (■) 1mg ml^{-1}, (▲) 2mg ml^{-1}, (×) 10mg ml^{-1}, (◇) 0mg ml^{-1}, (□) 0.5mg ml^{-1}, (△) 1.0mg ml^{-1},
(○) 1.5mg ml^{-1}.
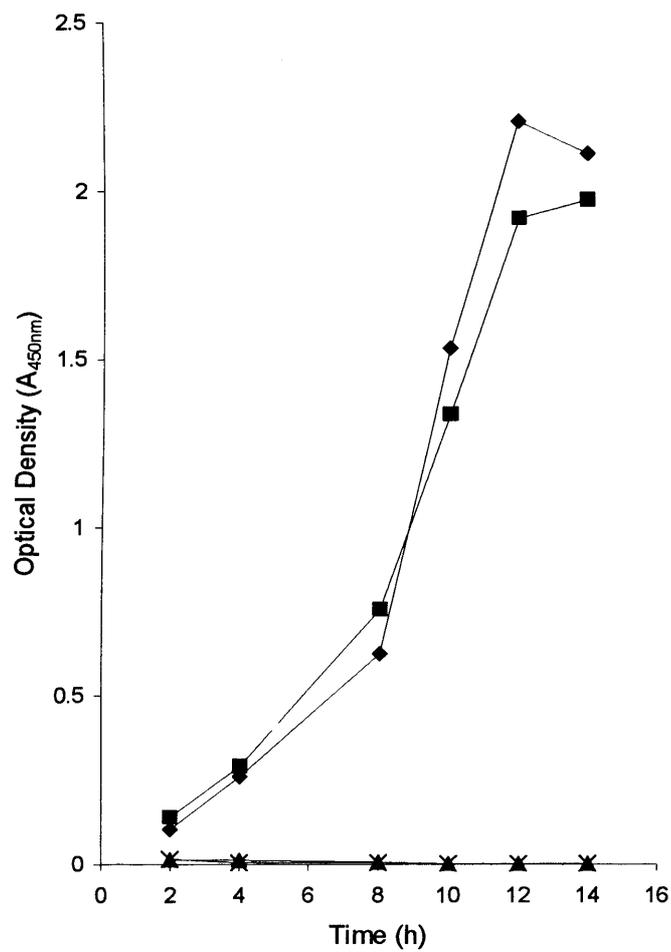
Fig. 2.3.
Growth of *Candida albicans* in fresh media after incubation for 24h in Fresh Garlic Extract.
Establishment of fungistatic and fungicidal properties. Concentrations: (◆) 0mg ml⁻¹, (■) 2mg ml⁻¹, (▲) 10mg ml⁻¹, (✕) 20mg ml⁻¹.
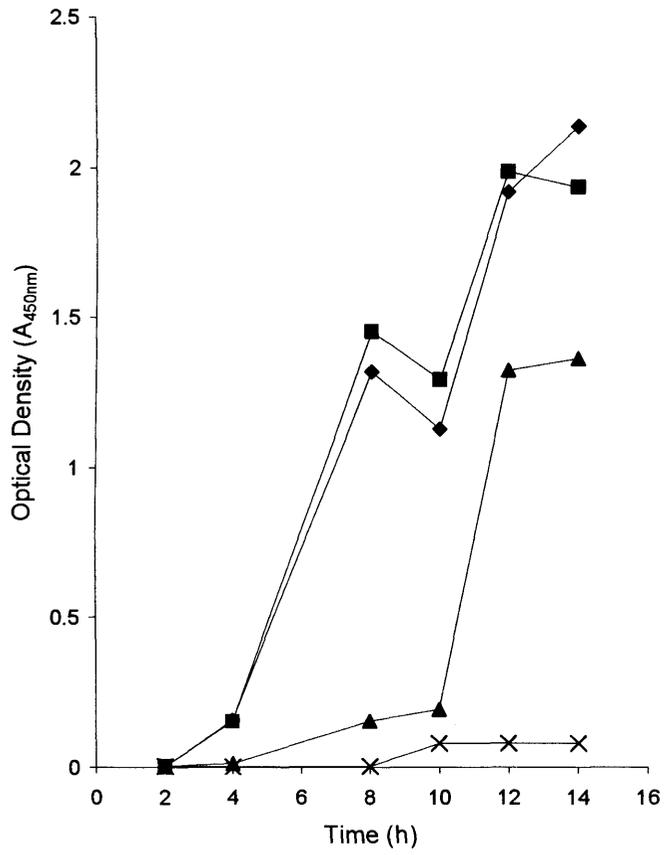
Fig. 2.4.

Growth of *Candida albicans* in fresh media after incubation for 24h in Garlic Powder Extract. Establishment of fungistatic and fungicidal properties. Concentrations: (♦) 0mg ml^{-1}, (■) 2mg ml^{-1}, (▲) 10mg ml^{-1}, (✕) 20mg ml^{-1}.
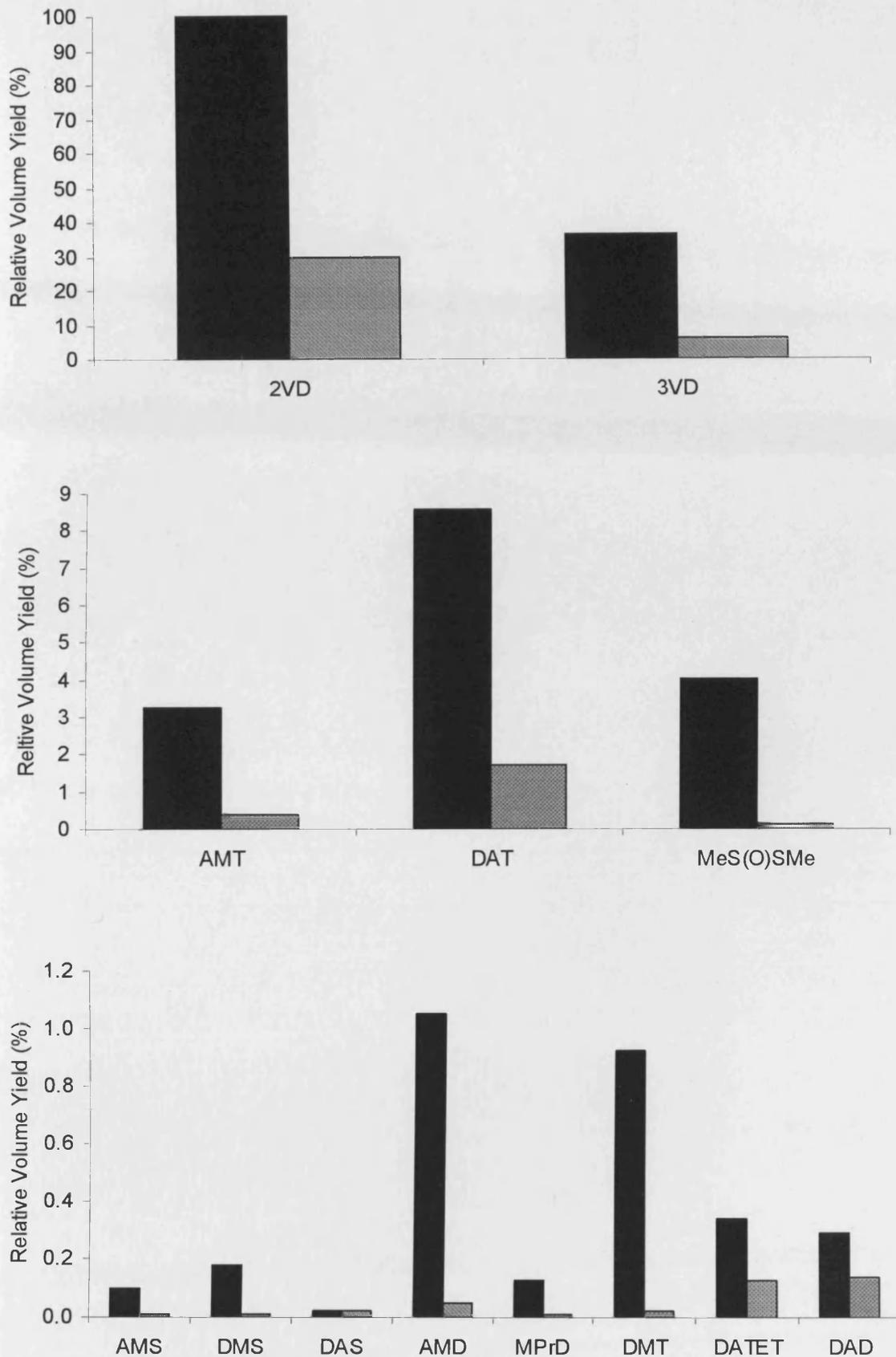
Fig. 2.5.
Comparison of yields of sulphur compounds from GC-MS analysis of Fresh Garlic Extract (black) and Garlic Powder Extract (grey) with respect to production of 2-vinyldithiin from FGE taken as 100% yield. Graphs separated due to large variation in scale.

Fig. 2.6.
Scanning electron micrograph of *Candida albicans* cultured in (a) fresh YPG media, and (b) Fresh Garlic Extract (1mg ml^{-1}). Bar length 5μm x5,000.
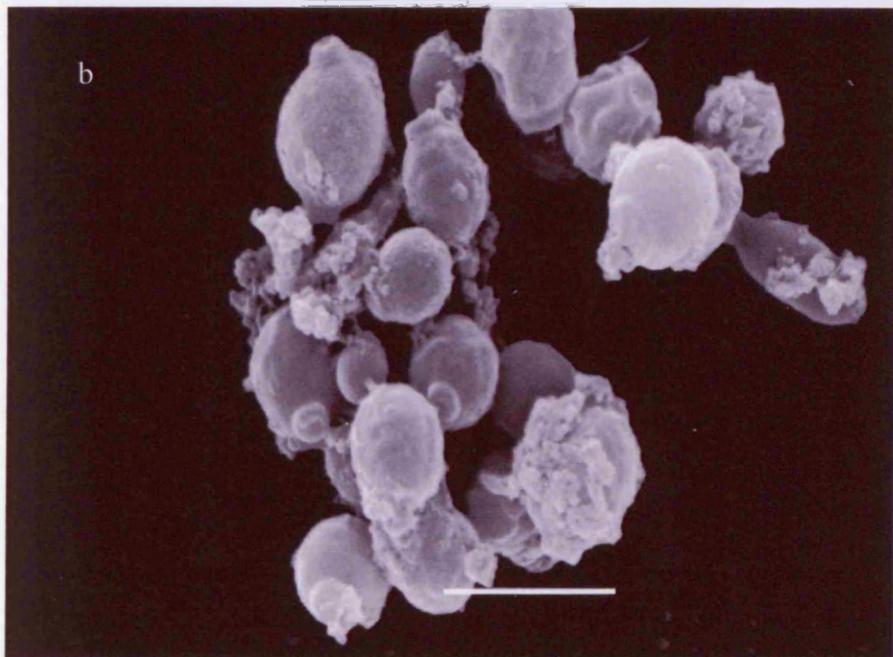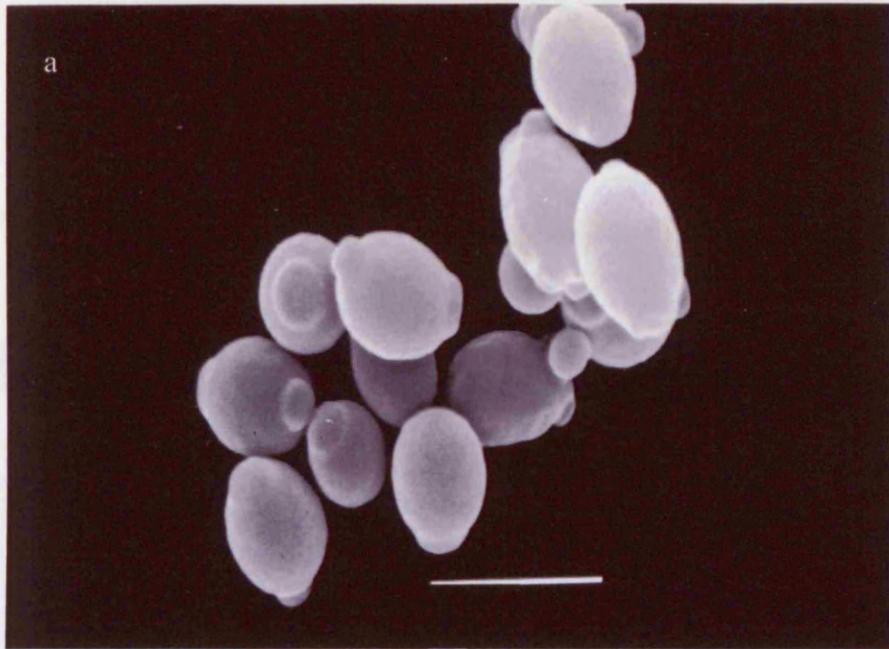
Fig. 2.7.
Scanning electron micrograph of formation of pits on the surface of *Candida albicans* cells. Bar length 1μm, magnification x10,000.

2.4.5 Transmission electron microscopy

Incubation of cells with fresh garlic affected the internal organisation of *C. albicans* (Fig. 2.8). The most noticeable change, the formation of vacuoles in the cytoplasm is observed even at low concentrations (1mg ml^{-1}) (Fig 2.8 b, but more evidently at higher concentrations (20mg ml^{-1}) (Fig 2.8c). The cytoplasm became more granular in appearance. Some membrane damage was evident after growth in the presence of the biocide as crisp cell membrane definition was diminished (Fig. 2.9). They also appeared to be less dense suggesting inhibition in cell membrane electron density.

2.4.6 Discussion

The anti-microbial properties of garlic can be exploited as an effective alternative to those of more common pharmaceutical preparations. A broad range of effects, in particular the observed antifungal properties, are now being investigated as well as investigations into the breakdown products of organosulphur compounds for their specific inhibitory effects. The mechanisms of action of garlic constituents *in vivo* still require much research.

The assumption that all of the antimicrobial properties were produced by allicin has been supported by many *in vitro* studies (Di Paolo & Carruthers, 1960; Bouchara *et al.*, 1990; De Pauw *et al.*, 1995). However, the effects observed *in vivo* are more likely to arise from the metabolic products of degradation. Although allicin itself has shown first-pass clearance in the liver, thereby maintaining concentrations high enough to cause considerable pathogen cell damage, it fails to reach systemic levels as high as those demonstrated by its degradative metabolic components, primarily due to its short half-life in biological tissue. However, there is no doubt that allicin plays a major role in the activity of garlic and its inhibitory mechanisms have been proposed.

Fig. 2.8.
Transmission electron micrograph of *Candida albicans* incubated for 24 hours at 30°C in (a) fresh YPG Broth (b) 1mg ml^{-1} Fresh Garlic Extract (c) and 30mg ml^{-1} Fresh Garlic Extract. Magnification x6250.
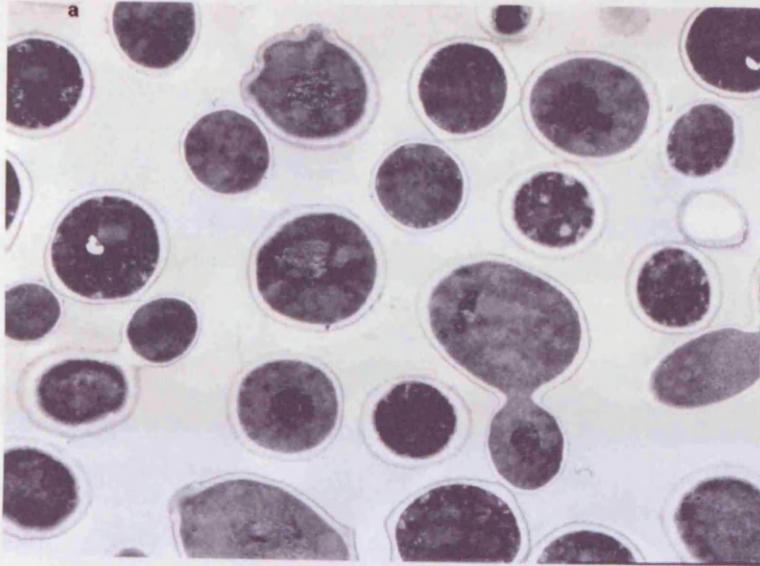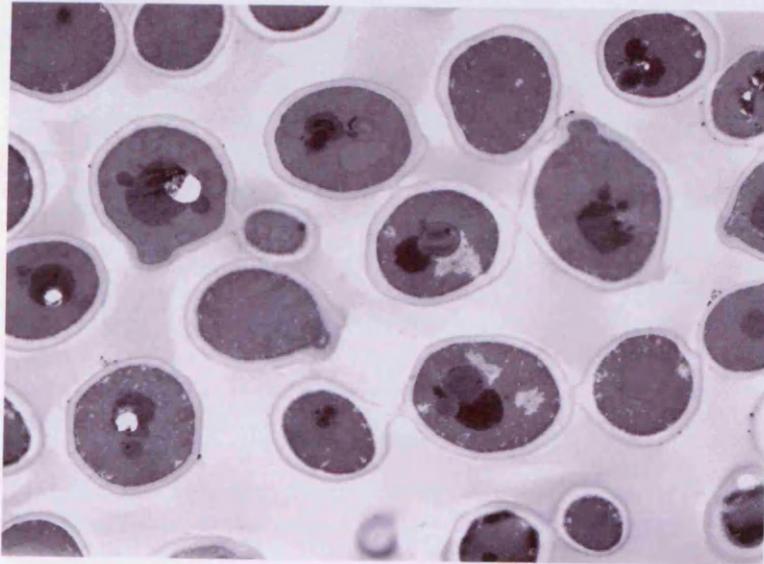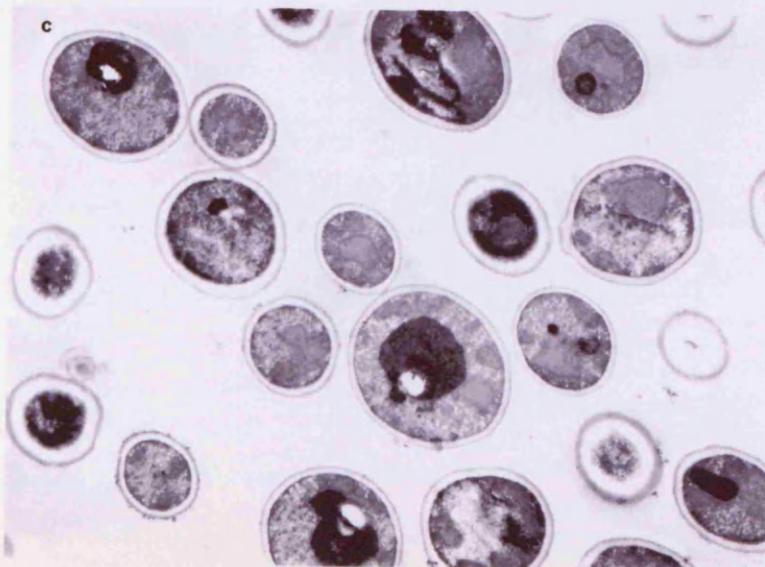
Uptake mechanisms have seen little investigation, but research using various bacterial species demonstrates that lipid differences between species may alter the permeability to allicin and garlic constituents (Salton, 1964). This may explain the increased levels of garlic extract required to produce inhibition of growth in *C. albicans*. IC_{50} values for freeze dried extract on *Escherichia coli* at 10 h has been demonstrated to be as high as $1.2mgml^{-1}$ (S. Cottrell, pers. comm.). The IC_{50} value at 10h for *C. albicans* was demonstrated to be only $0.7mg\ ml^{-1}$. This may be due to the bacterial peptidoglycan wall being less permeable than the yeast cell wall. Results for *Giardia intestinalis* (Harris *et al.*, 2000), IC_{50} 24 h, $0.29mg\ ml^{-1}$, suggest that the IC_{50} at 10 h would be much lower than that observed for *C. albicans* and is most likely explained due to the absence of a cell wall. Garlic has been reported to affect the lipid constituents of the outer surface of *C. albicans* (Ghannoum, 1988) and the fungicidal membrane of *Paracoccidiodes* spp. (San Blas *et al.*, 1993) and correlates to the observations in this investigation. In addition to the probable difference in lipid content in the yeast cell, the presence of the rigid cell wall also provides an additional barrier. The cell wall also possesses a high proportion of carbohydrate that may lack the thiol groups shown to be targets for allicin. Consequently, a higher concentration of garlic may be required before the cell itself accommodates an inhibitory concentration of garlic. The loss of cytosolic components, as demonstrated by electron microscopy, may be explained by the attack of the garlic components on the cell membrane targets. It is evident that much of the cell membrane integrity is damaged; this results in escape of much of the cytoplasm, formation of pits and eventual cellular collapse. Oxygen consumption of the organism was measured using a polarographic (Clark) oxygen electrode. Addition of 100-fold increased concentration of either extract produced no detectable affect on respiration. It may be concluded therefore, that the primary target of anti-candidal

activity in *C. albicans* does not appear to be on the mitochondrial electron transport chain.

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3. CHAPTER THREE

The effects of *Allium sativum* (garlic), and garlic constituents allyl alcohol and diallyl disulphide on *Candida albicans*, as assessed by flow cytometry.

3.1 Summary

Flow cytometry was utilised to investigate the effect of garlic extract on populations of the medically important pathogen *Candida albicans*. Mechanisms of cell death were investigated by use of the apoptotic probe, fluorescein labelled-annexin-V and propidium iodide, which live cells are impermeant to. Evidence for both apoptotic and necrotic populations was obtained. Low concentrations of allyl alcohol (AA) triggered a necrotic response, whereas an apoptotic type of cell death was observed at higher concentrations ($\geq$6mM). Conversely, low concentrations of diallyl disulphide (DADS) induced apoptosis, whereas higher concentrations ($\geq$6mM) resulted in a necrotic response. Intracellular levels of reactive oxygen species (ROS) after treatment with ROS generating compounds (e.g.: menadione or hydrogen peroxide), or garlic were measured by use of the fluorescent probe dichlorodihydrofluorescein. Cellular levels were increased with 10mM menadione and garlic powder extract, AA and DADS. Finally, plasma membrane integrity was monitored by use of the fluorimetric lipophilic "slow" voltage-sensitive Bis-oxonol dye, DiBaC$_4$(3). Calculation of the plasma membrane electrochemical potential was achieved by application of the Nernst equation to the distribution of this fluorophore between the outside and the inside of the organism. Complete depolarisation was observed with low concentrations of AA, suggesting that for this constituent, the plasma membrane may be a primary target. Effects of garlic extract and diallyl disulphide on plasma membrane were less obvious.

3.2 Introduction

The rising incidence of *C. albicans* infections is most likely linked to the increasing occurrence of immuno-debilitating conditions such as cancer, AIDS, transplantations, and diabetes. Linked to this is the over-prescription of the current limited available

drugs (Sanglard & Bille, 2002). This situation has triggered the search for new antifungal agents with novel modes of action, many of which target different cellular processes involved in the biosynthesis of components required for growth (Sanglard & Bille, 2002). However, these modern solutions often only target one single biosynthetic site, e.g.: 5-fluorocytosine (5-FC), which inhibits DNA synthesis and amphotericin B, which binds to ergosterol. Reports of some emerging resistances are outlined in Table 1.

An area of current interest is the resurgence of traditional plant preparations to help solve developing resistance problems. That there is no reported resistance to the activity of *Allium sativum* (garlic) in bacterial species (Skyrme, 1996) makes garlic extract an attractive therapeutic tool in the treatment of *C. albicans* infections. Garlic extract has proved to be fungicidal to *C. albicans* cultures (Ghannoum, 1990; Lemar *et al.*, 2002); *in vivo* experiments remain to be performed. The garlic constituents allyl alcohol and diallyl disulphide, have also been reported as being antifungal (Avato, 2000), but no study with *C. albicans* so far has investigated the potential targets of garlic, or determined their modes of action.

Flow cytometry was described as a technique awaiting discovery by microbiologists (Lloyd, 1993), but since then this technique has been widely applied to a variety of different aspects of microbiology (Shapiro, 2003). The technique is ideally suited for the rapid and accurate analysis of entire populations of organisms and has the potential to reveal immediate or short term effects on specific cellular processes as well as longer term consequences such as loss of cell viability and death (Carter *et al.*, 1993). Previous investigations have successfully used flow cytometry by measuring side

Table 3.1

Overview of some antifungal agents (traditional and new), mode of action and emerging resistance. Table adapted from (Sanglard & Bille, 2002).

Antifungal agent(s)	Mode of Action	Mechanism of resistance
Polyenes (amphotericin B, nystatin)	Binding to ergosterol	-Absence of ergosterol required for membrane integrity -Increased catalase activity
5-fluorocytosine (5-FC)	Inhibition of DNA synthesis	-Defect in cytosine permease -Deficiency in enzymes required for 5-FC metabolism -Deregulation of pyrimidine pathway
Azoles (ketoconazole, itraconazole, fluconazole, voriconazole etc)	Inhibtion of *ERG11*, lanosterol formation leading to depleted membrane integrity	-Alterations in ergosterol biosynthetic pathway -Enhanced efflux mediated by multidrug transporters
Cationic antimicrobial peptides	Pore formation in cellular membranes	Loss of mitochondrial function

scatter and permeability to propidium iodide (PI), to assess the viability of *C. albicans* in the presence of a variety of antifungal preparations (Green *et al.*, 1994). Subsequent work has relied primarily on dye exclusion determinations, such as those with PI (Ramani *et al.*, 1997; Wenisch *et al.*, 1997; Green., 1999) and FUN-1 (available from Molecular Probes, Haugland, 2002). Variations of these experiments utilise membrane potential dyes as viability indicators (Ordonez *et al.*, 1995; Peyron *et al.*, 1997, 2000), and correlate well with standard methods of viability determination (Wenisch *et al.*, 2001; Ordonez *et al.*, 1995).

In this study, we exploit the rapid and precise data collection as allowed by modern flow cytometers to observe the effects of garlic on *C. albicans*. Specific dyes were utilised to monitor cellular processes. Apoptotic and necrotic states of cell death were investigated using fluorescein labelled annexin-V and propidium iodide respectively, as detailed in previous investigations (Madeo *et al.*, 1997; Ludovico *et al.*, 2001). Dichlorodihydrofluorescein (DCF) was used to investigate the production of reactive oxygen species (ROS). Plasma membrane integrity (Ψ_{pm}) of the organism was investigated by use of the fluorimetric lipophilic Bis-oxonol dye, $DiBaC_4(3)$ (Krasnai *et al.*, 1995; Lemar *et al.*, 2005a).

3.3 Methods and Materials

3.3.1 Organism and Culture

Candida albicans (Berkhout) 5134 was grown in YPG medium (Difco bacto-peptone 2% (w/v), Oxoid yeast extract 1% (w/v) and glucose 2% (w/v)). An early stationary phase culture was used as an inoculum and a 1:100 dilution for 20ml cultures in 100ml conical flasks. Growth was aerobic at 30°C in a rotary shaker (150rpm). Stock cultures

of *C. albicans* were grown overnight at 30°C and maintained at 4°C on YPG agar, containing 2% (w/v) Oxoid agar. For each experiment cell suspensions were cultured to exponential phase before addition of challenge agent. Cells were harvested at 4000g for 5 min ar·d washed twice in distilled water. After the second wash, organisms were resuspended in phosphate buffered saline (PBS, pH5.6)

3.3.2 Garlic preparations.

Garlic powder (GPE; provided by Cultech Biospeciality Products, Swansea, Wales, UK) was prepared to the required concentration (w/v) in sterile growth medium. After 30 min the extract was centrifuged at 3900g for 10 min and the supernatant passed through a sterile 0.2-μm filter (Millipore, Watford, UK). Stock suspensions were prepared on the same day of testing in PBS (pH5.6). Concentration ranges were prepared for DADS in the solvent DMSO (dimethyl sulphoxide), and AA in PBS (phosphate buffered saline, pH5.6).

3.3.3 Fluorescence Measurements:

3.3.3.1 Analysis of apoptotic and necrotic cell:

Cell death was analysed using Alexa Fluor 488 annexin V/propidium iodide (Vybrant Apoptosis Assay Kit #2, Molecular Probes, Inc. OR. USA). The Alexa Fluor 488 conjugated to annexin-V interacts with externalised phosphatidyl-serine in apoptotic cells, producing a bright green fluorescence whereas necrotic ones are stained red by propidium iodide which live cells are impermeant to.

Digestion of the cell walls to form spheroplasts was achieved by incubating the cells in a mixture of enzymes modified from Phillips *et al.*, (2003). Cultured cells were harvested by centrifugation (2,000g) and washed twice in PBS (pH5.6) before re-

suspension in 0.5 ml pre-digestion buffer (50mM K_2HPO_4/5mM EDTA/50mM DTT:

pH 7.2) for 30 min a 30°C. Digestion of the cell wall was achieved at 30°C after re-

suspending the cells in 0.5ml of digestion solution (50mM KH_2PO_4/40mM 2-

mercaptoethanol/3μg ml^{-1} chitinase/1.8μg ml^{-1} lyticase (stock 200-1,000 units.g^{-1})/12μl

β-glucuronidase/20μl glusulase (Perkin Elmer, Bucks, U.K) in 2.4M sorbitol, pH7.2.

Control cells were incubated for 30 min, whereas those exposed with either AA or

DADS, were only digested for 20 min. Protoplasts were washed in modified annexin

binding buffer (MABB) (1.2M sorbitol/10mM Hepes/NaOH/40mM NaCl/50mM

$CaCl_2$, pH7.4). Annexin-V binding assays were performed according to the protocol of

Madeo et al. (1997), but suspended in MABB and 20 μl ml^{-1} annexin reagent

preloaded and incubated at room temperature in the absence of light for 15 min.

Spheroplasts were centrifuged at 1700g and washed in MABB before addition of 20μg

ml^{-1} PI. Fluorescence distributions were obtained from 100,000 events per cell sample

through the FITC filter, (518nm), for measurement of annexinV-FITC fluorescence

and rhodamine filter, (620nm), for measurement of PI, using a FACScalibar flow

cytometer (BD Biosciences, Oxford, UK). The fluorescence of gated cell populations

was analyzed using validated analysis software, WinMDI 2.8. Fluorescence intensity

of each dye was plotted against each other to distinguish between apoptotic and

necrotic populations of cells (Fig 3.1).

3.3.3.2 Analysis of intracellular Reactive Oxygen Species.

Intracellular events in a population of C. albicans were analysed using DCF as a

marker for ROS. Known ROS generators (menadione, tetra butyl-hydrogen peroxide

(tb-H_2O_2), hydrogen peroxide (H_2O_2)), or GPE, AA or DADS were added to
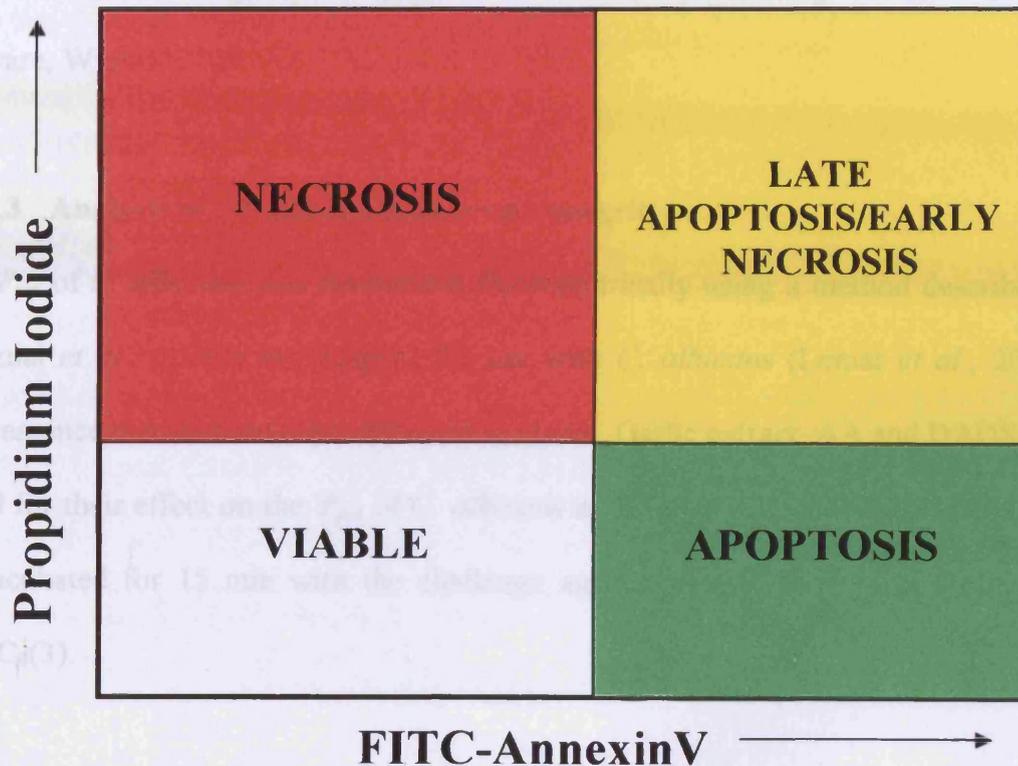
Fig. 3.1.
Diagrammatic representation of flow cytometric distributions for apoptotic/necrotic cell death investigations.

exponential phase cultures and incubated at 30°C for 15min. Cells were harvested by centrifugation (5min; 2000g), and resuspended in PBS (pH5.6). The fluorescence of gated cell populations was analyzed using validated analysis software, WinMDI 2.8. The fluorescence of gated cell populations was analyzed using validated analysis software, WinMDI 2.8.

3.3.3.3 Analysis of cell plasma membrane integrity.

The Ψ_{pm} of *C. albicans* was determined fluorometrically using a method described by Krasznai *et al.*, (1995) and adapted for use with *C. albicans* (Lemar *et al.*, 2005a). Fluorescence distributions were obtained as above. Garlic extract, AA and DADS were tested for their effect on the Ψ_{pm} of *C. albicans* at different concentrations. Cells were pre-incubated for 15 min with the challenge agents prior to the addition of 1.0μM DiBaC$_4$(3).

Estimation of Ψ_{pm}: As a lipophilic anion the dye distributes across the plasma membrane in accordance with the Nernst equation, $\Psi_{pm}=(RT/F).\ln(Din/Dout)$, where R represents the universal gas constant (J K^{-1} mol^{-1}), T, the absolute temperature (K), F, the Faraday constant (C mol^{-1}), and D$_{in}$ and D$_{out}$, the intra- and extracellular dye concentrations, respectively. D$_{in}$ was estimated by flow cytometry. A calibration curve relating cell fluorescence intensity to D$_{in}$ was obtained using cells that had been heat-fixed at 60°C for 15 min in order to eliminate the Ψ_{pm} (heat fixation did not significantly affect the auto-fluorescence of the cells). Under these conditions, $\Psi_{pm}=0$ when D$_{in}$=D$_{out}$. The cells were suspended in media having a range of bis-oxonol concentrations (0.1 − 3.2μM) and their fluorescence measured by flow cytometry. Cell fluorescence was plotted as a function of D$_{out}$ (=D$_{in}$) and this curve was then used for

the estimation of Din (and hence Ψ_{pm}, via the Nernst equation) from the measured fluorescence of live cells suspended under different ionic conditions and in the presence and absence of inhibitors. The larger the value for D_{in} the more positive the value of Ψ_{pm}.

3.4 Results

3.4.1 Analysis of apoptotic and necrotic cells:

Cells from control cultures of *C. albicans* (Fig 3.2ai) were observed to have the largest population of cells with fluorescence in the 'viable' quadrant of the histogram (refer to Fig. 3.1). A small number of cells had bound annexin-V, suggesting apoptotic cell death, as indicated by fluorescence detection in the green quadrant. Similarly, a small number of necrotic organisms were evident as indicated by the red fluorescence of PI. Both apoptotic and necrotic cell death routes are observed in *C. albicans* after treatment with the garlic constituents AA and DADS (Fig. 3.2). Increasing concentrations of each antifungal agent increases the proportions of cells undergoing each type of cell death. Low concentrations of AA (up to 1mM), cause cells to undergo necrosis (Fig 3.2aii), whereas, higher doses (>6mM), generate an apoptotic cell death response as indicated by the population of cells fluorescing green (Fig 3.2aiii). Conversely, low concentrations of DADS (up to 1mM) induce an apoptotic cell death (Fig 3.2bii), and increased concentrations induce cells into necrosis (Fig. 3.2bii and iii).

3.4.2 Analysis of intracellular Reactive Oxygen Species

An increase in DCF fluorescence (left to right on histogram), is indicative of increased intracellular ROS. This was observed after addition of 10mM menadione (Fig 3.3ai).

By comparison there was no apparent increase in fluorescence after addition of 100mM concentrations of either H_2O_2 or tb-H_2O_2, (Fig 3.3aii and iii). Various garlic treatments, GPE, DADS or AA added to separate cultures resulted in increased ROS levels in all cases (Fig 3.3bi-iii); there was no apparent dose-dependence.

3.4.3 Analysis of plasma membrane potential.

Increasing the extracellular DiBaC$_4$(3) concentration resulted in an increase in cell fluorescence for both live and heat-treated cells. These increases were monitored accurately by flow cytometry (Fig. 3.4a). The relation of cell fluorescence and intracellular dye concentration for heat fixed cells suspended in NMG-Cl solution is shown in the calibration curve (Fig. 3.4b). Shifts in fluorescence, after addition of GPE, AA and DADS indicate changes in Ψ_{pm} (Fig. 3.5); these could be quantitated with the aid of the calibration curve (Fig 3.6). Even at low doses of AA the plasma membrane was completely depolarised, suggesting that the plasma membrane is a primary target for AA. A dose-dependent response was observed for both DADS and GPE; fluorescence intensity increased with increased concentration. Addition of 6.25 mg ml^{-1} GPE (slightly above the MIC value of 5mg ml^{-1}), was required before complete depolarisation was observed. Similarly, the MIC for DADS (0.5mM) was exceeded fifty-fold, before complete depolarisation of the plasma membrane was observed. The production of sub-populations of cells, which are also not dose-dependent, were evident after treatment with the lowest doses of each agent (Fig. 3.5).
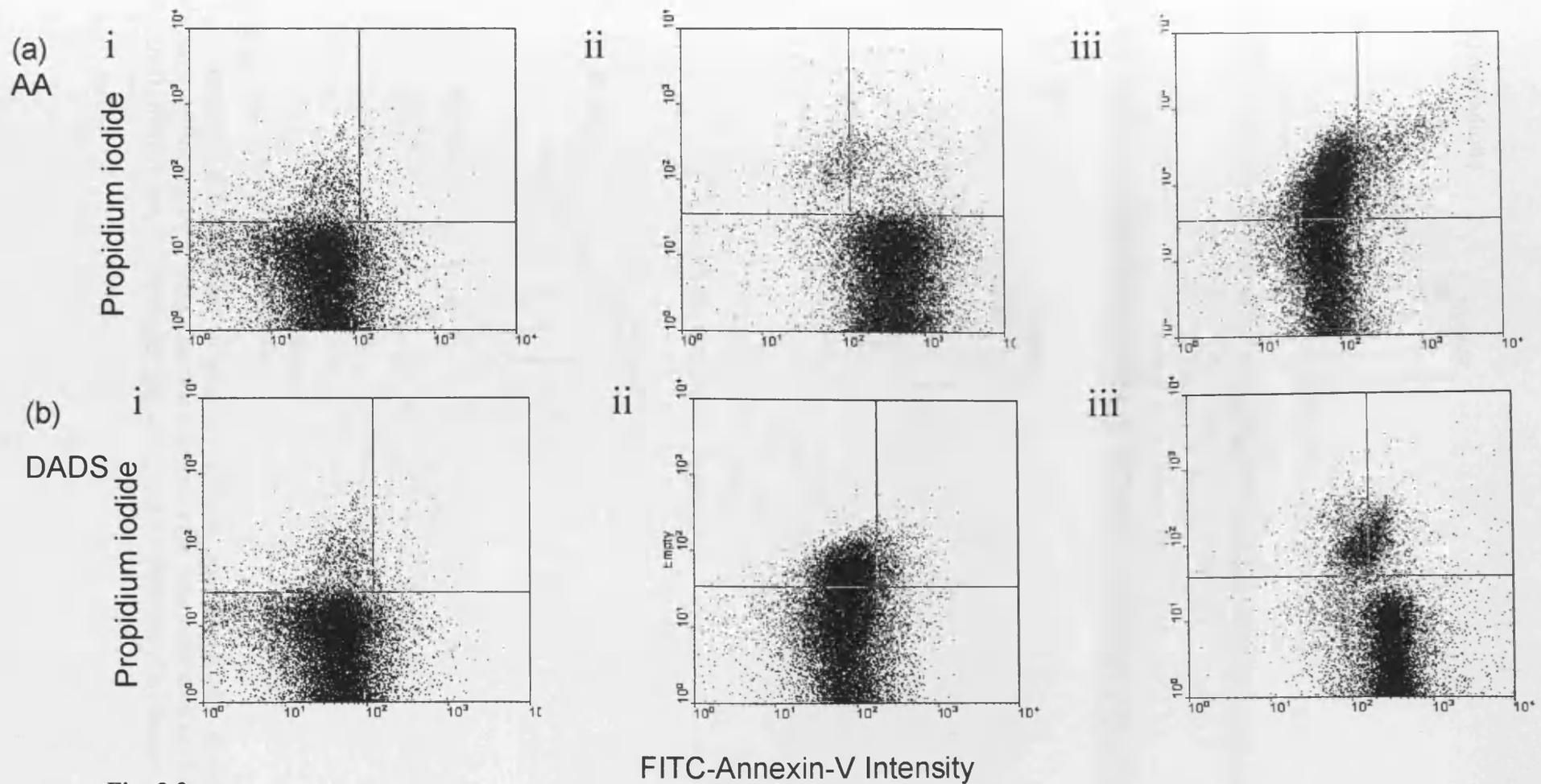
Fig. 3.2

Cell death investigation. Flow cytometric distribution dot plots for *Candida albicans* after treatment with (a) allyl alcohol: i) 0mM (control); ii) 1mM; iii) 6mM. (b) diallyl disulphide: i) 0mM (control); ii) 1mM; iii) 6mM.

(i) menadione

10.0mM
5.0mM
2.5mM
1.25mM
0.6mM
0mM

Cell Count

Fluorescence Intensity

(ii) H_2O_2

100mM
50mM
25mM
12mM
6mM
0mM

Cell Count

Fluorescence Intensity

(iii) tb H_2O_2

100mM
50mM
25mM
12mM
6mM
0mM

Cell Count

Fluorescence Intensity
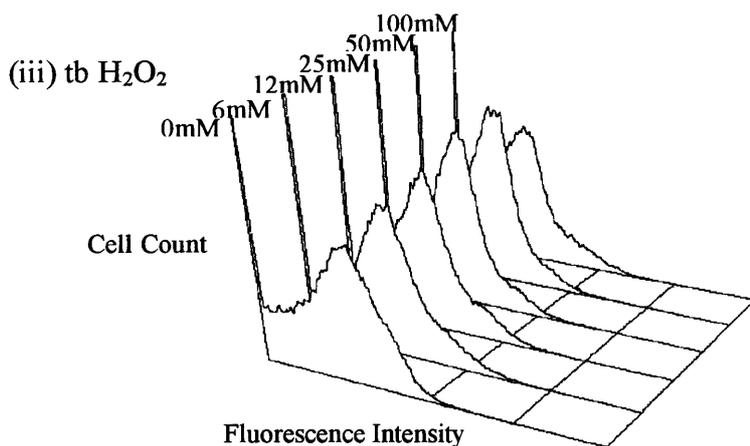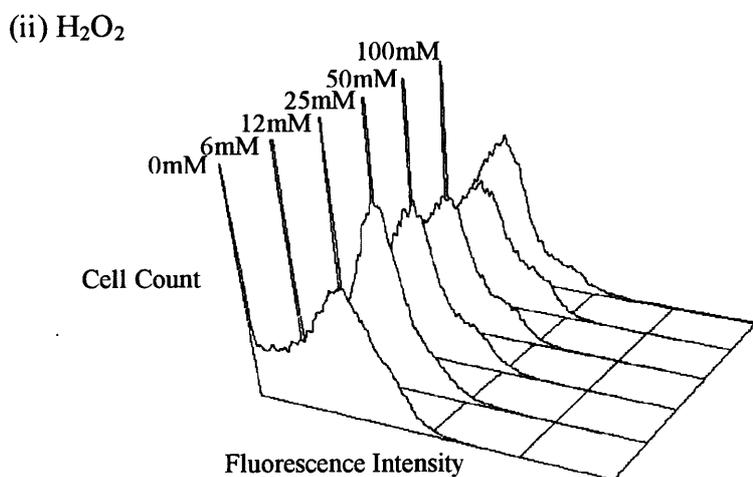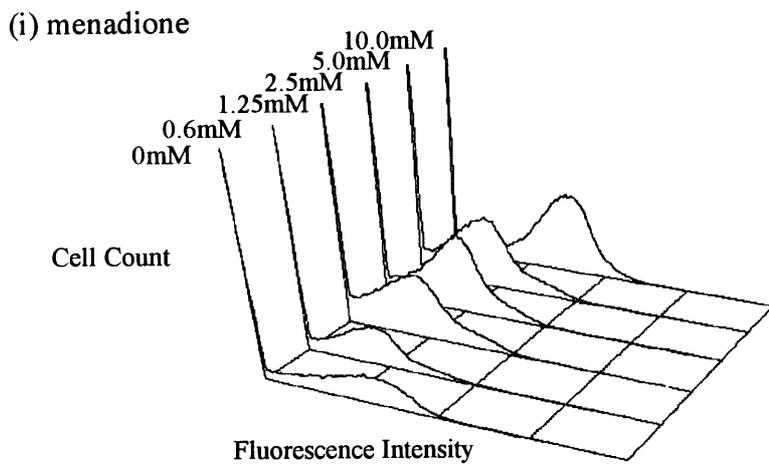
Fig. 3.3a

Flow cytometric determination of reactive oxygen species (ROS). Representative
histograms of fluorescence from *Candida albicans* after treatment with (a) ROS
inducers (i) menadione, (ii) hydrogen peroxide, and (iii) tetrabutyl-hydrogen peroxide.
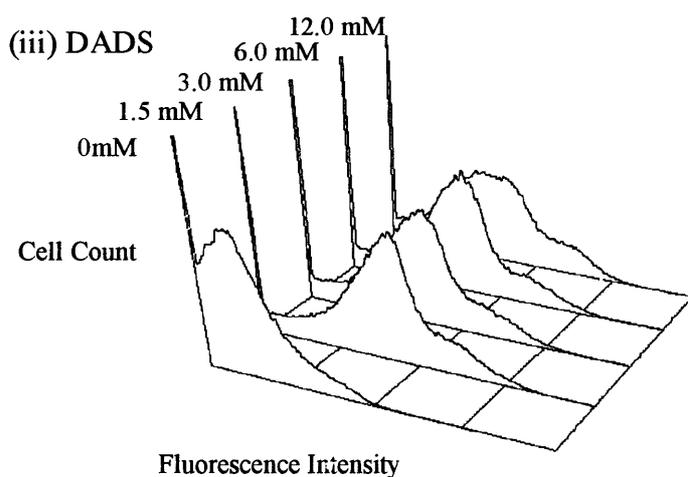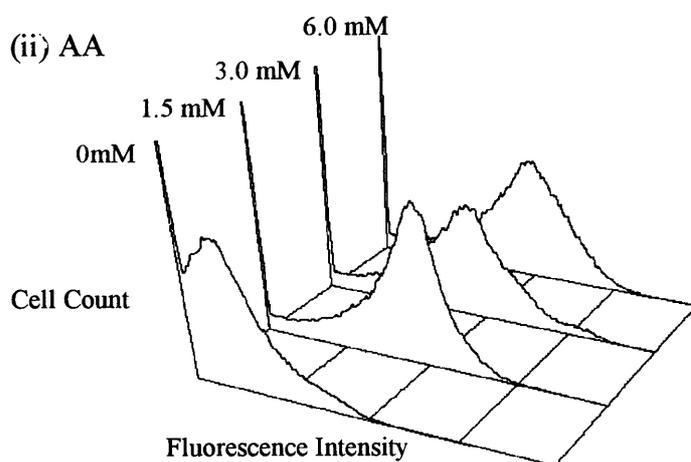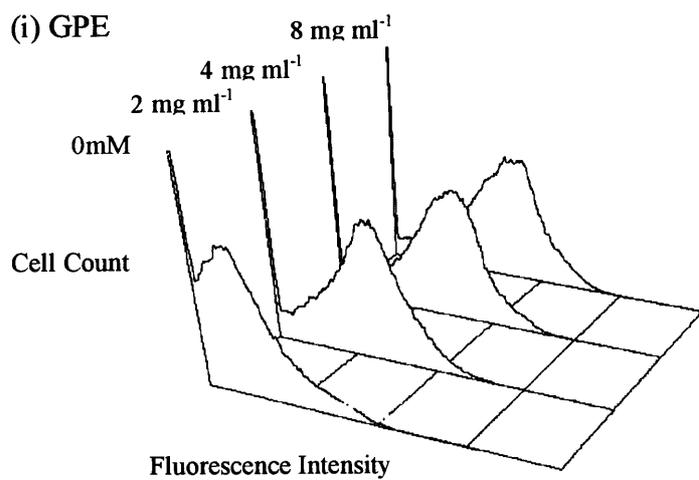
Fig. 3.3b
Flow cytometric determination of reactive oxygen species (ROS). Representative histograms of fluorescence from *Candida albicans* after treatment with (b) Garlic constituents (i) garlic powder extract, (ii) allyl alcohol, and (iii) diallyl disulphide.

a)

b)
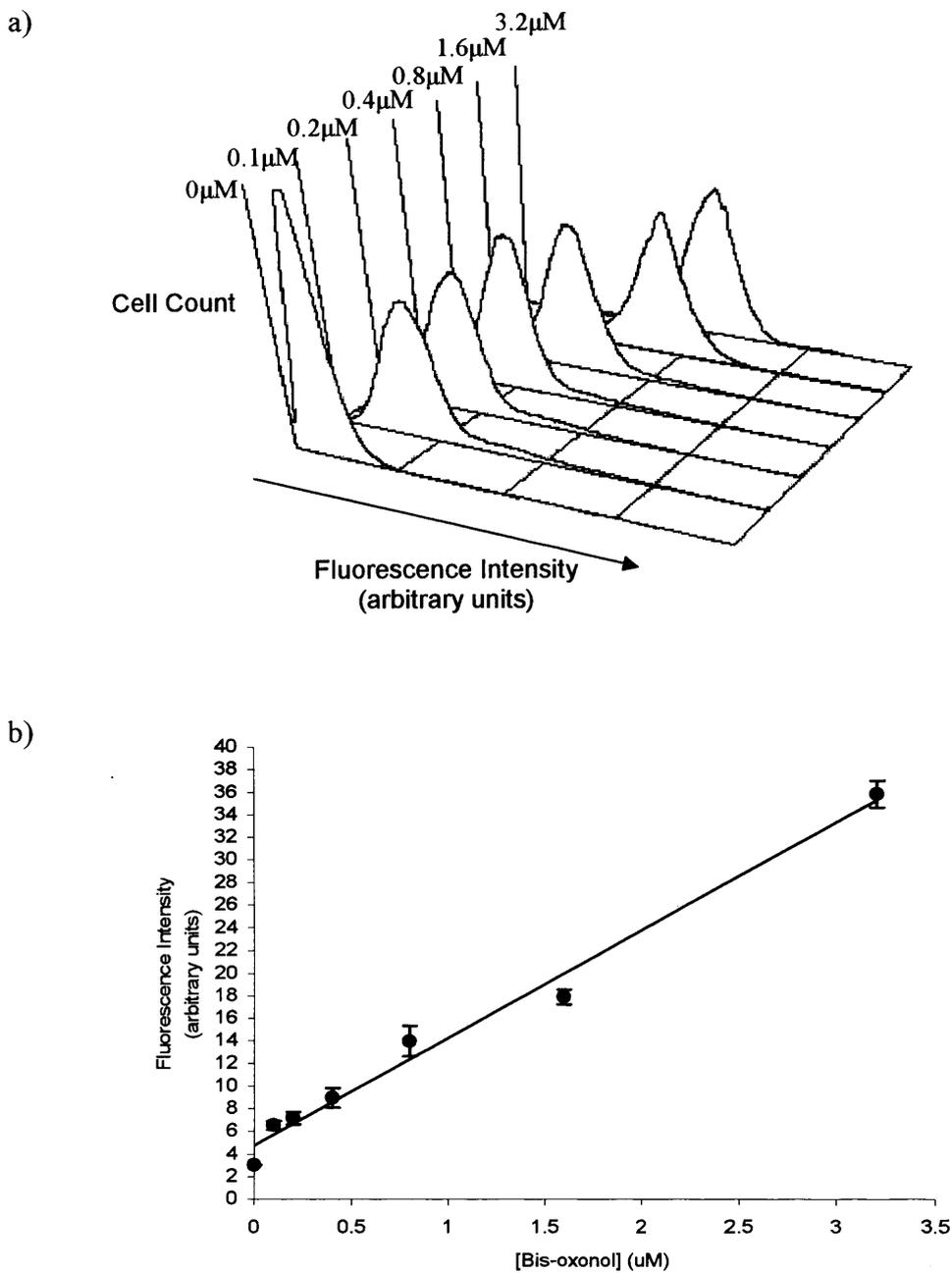
Fig. 3.4.

Flow cytometric determination of Ψ_{pm}. (a) Flow cytometric histogram of heat killed *C. albicans* suspended in NMG Cl solution and stained with increasing concentrations of bis-oxonol. (b) Calibration curve showing intracellular fluorescence intensity of heat killed cells as a function of extracellular dye concentration. The data are averaged from three separate experiments and are shown ±S.E.M. A shift to the right indicates an increase in fluorescence, which translates to a less negative membrane potential.

(i) GPE

25 mg ml⁻¹
12.5 mg ml⁻¹
6.25 mg ml⁻¹
3.0 mg ml⁻¹
1.5 mg ml⁻¹
0mg ml⁻¹

Cell Count

Fluorescence Intensity

(ii) DADS

12.5mM
6.25mM
3.125mM
0mM

Cell Count

Fluorescence Intensity

(iii) AA

25mM
12.5mM
6.25mM
3.125mM
0mM

Cell Count

Fluorescence Intensity
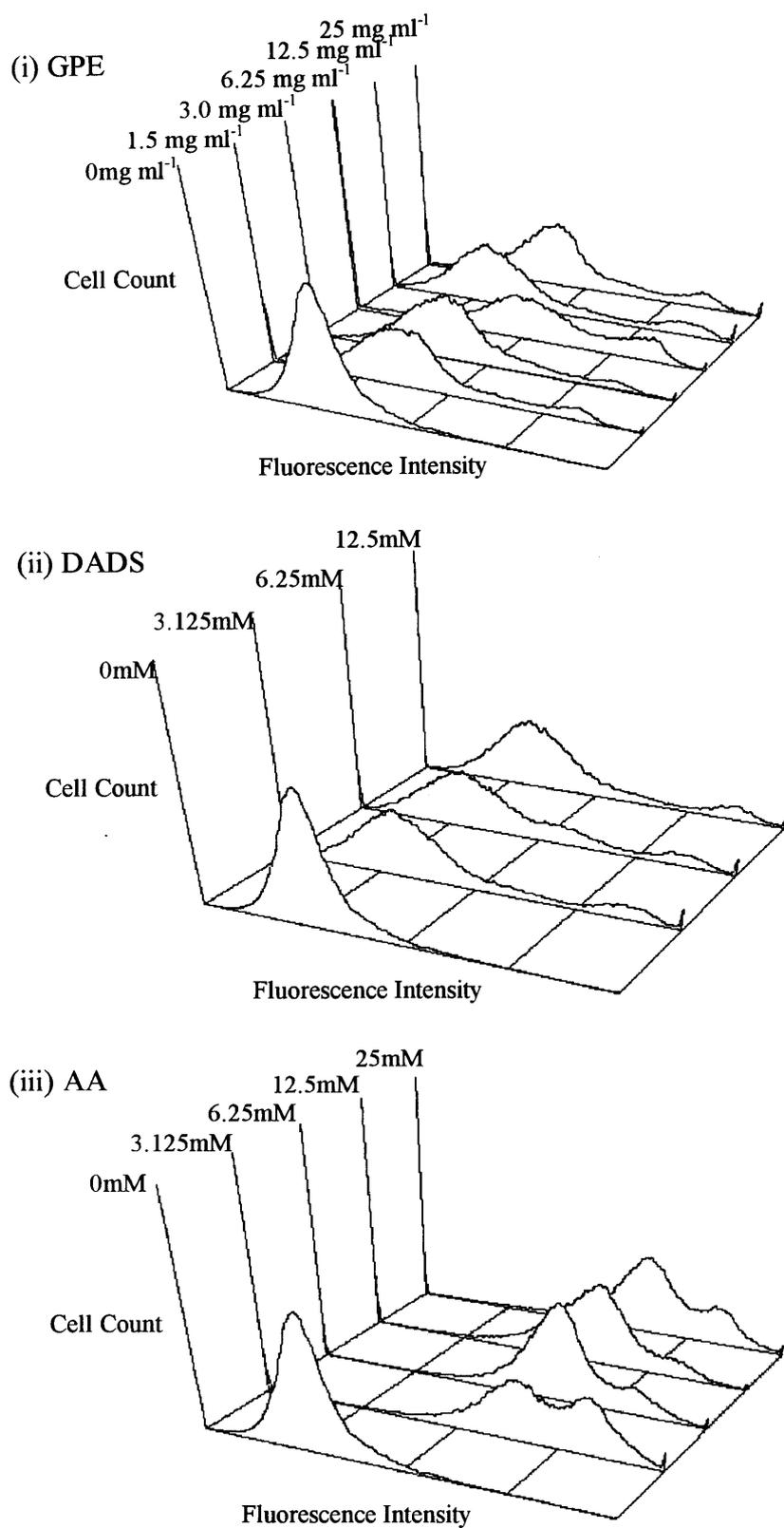
Fig. 3.5.
Fluorescence imaging of DiBaC₄(3) to measure the effect on the plasma membrane potential of *Candida albicans* cells treated with (i) garlic powder extract (ii) diallyl disulphide (iii) allyl alcohol.

The scope of physiological investigations using flow cytometric analysis in mixed cell systems are tangible (Davey, 1993, Shapiro, 2003). Combining flow cytometry and the development of new fluorescent probes means that investigations have the ability to evaluate cell viability, with respect to parameters of subcellular function such as function of the membrane.
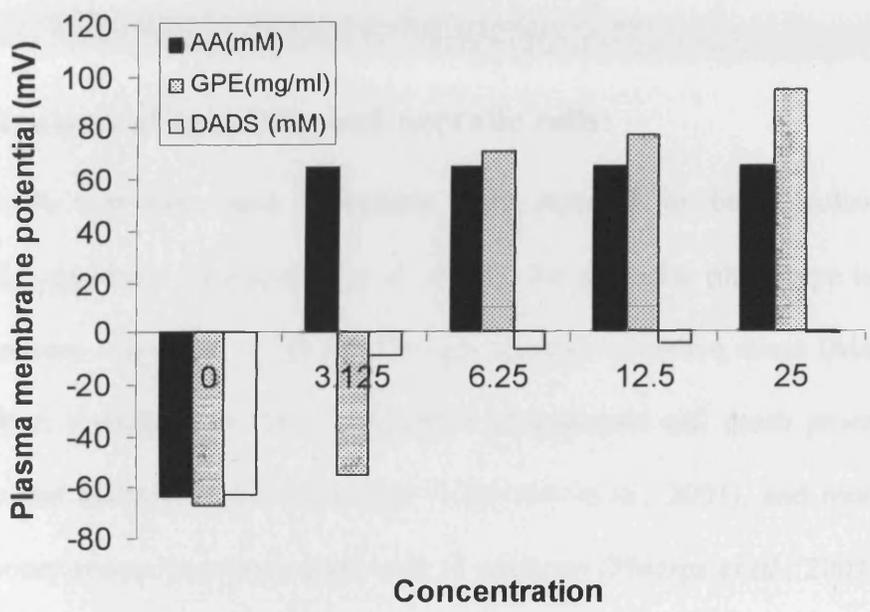
Fig. 3.6.
Effect of garlic constituents on plasma membrane potential of *Candida albicans.*
Concentration units as indicated in figure caption.

3.5 Discussion

The scope of microbiological investigations using flow cytometry continues to expand and provide new insights (Lloyd, 1993, Shapiro, 2003). Combining flow cytometry and the development of new fluorescent probes means that investigations into the details of population heterogeneity with respect to processes of sub-cellular organisation and function can be undertaken.

3.5.1 Analysis of apoptotic and necrotic cells:

Cell suicide responses have previously been reported in both prokaryotic and unicellular organisms (Matsuyama *et al.*, 1999). An apoptotic phenotype in the yeast *Saccharomyces cerevisiae* is induced through elevated oxidative stress (Madeo *et al.*, 1997). Other investigations have identified a programmed cell death process closely linked to this apoptotic yeast phenotype (Ludovico *et al.*, 2001), and more recently similar observations have been made with *C. albicans* (Phillips *et al.*, 2003; Lemar *et al.*, 2003; 2005b,c). It is not unlikely therefore, that the garlic constituents studied induce the apoptotic route by causing elevation of oxidative stress. However, that both necrotic and apoptotic responses after treatment with allyl alcohol and diallyl disulphide were evident is perhaps indicative of the complex range of processes involved in their induction of cell death in *C. albicans*. In this investigation the different death responses are shown to be dependent on the concentration and the length of exposure to DADS or AA. There is some indication that a further complex mechanism is invoked by AA, whereby initial apoptotic control is lost and a secondary necrotic state prevails (Fig 3.2a ii-iii). Of interest therapeutically, is the relatively low doses required to trigger the cells into a death response, be it apoptotic or necrotic. The use of higher concentrations to elicit a candicidal response may be unnecessary, and

would thereby reduce the possible damage to mammalian tissue that these constituents might cause (Nalgelkerke, 1991; Tygstrup, 1997; Koch, 1996). Further investigations would be necessary in order to fully understand these mechanisms. DADS and AA are only two of the many constituents present in garlic extract. Others also have biocidal properties attributed to them. Ajoene, allicin and sulphide constituents such as diallyl sulphide, triallyl sulphide and allyl sulphide all have reported effective antimicrobial activity (Lun *et al.*, 1994; Naganawa *et al.*, 1996; Chung *et al.*, 1998; Ankri, 1999; Avato, 2000; Tsao & Yin, 2001); ajoene and allicin in particular, exhibit anticandidal activity (Yoshida *et al.*, 1987; Ledezma *et al.*, 1996; Yamada & Azuma, 1977). The 'cocktail' of these constituents found naturally in garlic extract, and the concentrations that they represent, may also contribute to the eventual decline of cell viability and ultimate route of cell death. Further investigation into each individual constituent, the concentration present in garlic extract and the resulting effects that each causes is necessary.

3.5.2 Analysis of intracellular Reactive Oxygen Species.

Investigation by Fröhlich & Madeo (2000) proposed that apoptosis originated in unicellular organisms as an altruistic response to severe oxidative damage. Oxidative stress is tolerated by the cell by elevation of levels of reduced glutathione (GSH) and also through protective enzyme reactions involving, for example, catalase and superoxide dismutase. Perturbations caused by the presence of garlic, on these or similar processes, may further 'stress' the cell and invoke a cell death response. The extent to which the cell is compromised may decide the ultimate route by which cell death occurs. Neither ≤100mM H_2O_2, nor tb-H_2O_2 resulted in symptoms of elevated oxidative stress, and this suggests that the cells were able to successfully withstand the

increased oxidative burden placed upon it. It is unusual, however, that such high levels

of peroxide are not toxic; reports for *S. cerevisiae* give tolerances <10mM for H_2O_2. It

is possible that the plasma membrane is not permeant to H_2O_2, however use of organic

H_2O_2 (tb-H_2O_2), would normally overcome this problem. That stationary phase

organisms are more able to withstand high levels of oxidative stress than those in

exponential growth phase may provide a possible explanation (Jamieson *et al.*, 1994).

After exposure to menadione however, elevated ROS levels were observed (Fig. 3.3

ai). Menadione induces oxidative stress by redox cycling in the presence of O_2,

resulting in the generation of superoxide radical anions (Chaput *et al.*, 1983; Chaput &

Sels, 1987; Halliwell & Gutteridge, 1984). It is possible therefore that above 10mM,

superoxide dismutase becomes saturated and therefore unable to disproportionate the

superoxide ion (O_2^-) to water and the less damaging peroxide.

Different responses to superoxide and peroxide anions in *S. cerevisiae* are recognised

(Flattery-O'Brien *et al.*, 1993), and are therefore, unexceptional observations for the

different radical generators in *C. albicans* (Fig. 3.3). Research has concluded that

adaptive responses to oxidative stress after pre-treatment with oxidants are evident in

both *S. cerevisiae* and *C. albicans* (Jamieson, 1992; Jamieson *et al.*, 1996). *C. albicans*

is observed to be more tolerant than *S. cerevisiae*, withstanding concentrations of

100mM H_2O_2 (Jamieson *et al.*, 1996). Although, there was no pre-treatment in the

experiments performed in this instance, it may be that the duration of exposure

necessary for the effects of peroxide to increase oxidative stress levels was not

reached.

Increase in the level of free radicals generated by normal respiratory functions may

increase the burden of oxidative stress above the threshold that the organism can

tolerate. The increase in oxidative stress after addition of GPE, AA or DADS, may possibly be used to indicate the early onset of cell death; ROS levels are increased at levels known to induce cell death in *C. albicans* (Figs. 3.5i, ii, iii and Fig 3.2a, b). Further investigation would be required to ascertain whether the addition of garlic, or either of its constituents, cause perturbations in ROS levels directly or, instead, target the anti-oxidant defence system. Depletion of components, such as glutathione, that would otherwise eliminate the toxic intermediates and prevent ROS accumulation, may be an alternative target and produce similar effects.

3.5.3 Plasma membrane investigation.

Membrane potential across the membrane of all cells is essential for uptake of nutrients and intracellular pH regulation. As an indispensable energizing process for essential biological functions of cells, Ψ_{pm} is an attractive target for drug therapy. Previous investigations have observed a highly significant depolarisation caused by DCCD (Lemar *et al.*, 2005), and suggested that maintenance of the Ψ_{pm} involves a H+ pump. Although, a complete depolarisation of Ψ_{pm} was not observed in this previous investigation (i.e.: from a resting potential of -60mV to 0mV), that DCCD caused depolarisation to 3mV, suggests that an antibiotic or synthetic compound targeting the yeast Ψ_{pm}-generating plasma membrane components could potentially be a useful candicidal agent.

It is likely that the plasma membrane is a primary target for AA, as indicated by complete depolarisation after addition of low doses of AA. The MIC of AA for *C. albicans* is reported as 1mM (personal communication, O. Passa, 2002), although not tested here, the immediate depolarisation after addition of only 3 fold higher than the

MIC, suggests that *C. albicans* is not able to tolerate such concentrations. As such low doses of AA are required to elicit complete depolarisation of the plasma membrane potential it is feasible that the effect of GPE on the plasma membrane may well be attributed to the presence of AA in the extract. On the other hand, relatively high concentrations of DADS (>25mM) were required before depolarisation was observed. These observations suggest that the candicidal properties of this constituent are due to other factors unrelated to targeting of the plasma membrane. It is possible that DADS may cause depolarisation only in those cells already highly compromised; this is perhaps suggested by the existence of a subpopulation of cells present even with low doses of DADS well below that of the MIC values. These sub-populations are evident after treatment with AA and GPE. It is possible that these populations represent organisms that are in a hyperpolarised state. However, it is more likely that these are cell ghosts; acute damage to the plasma membrane of cells allows the dye to bind tightly to the intracellular organelle membranes and results in an intense fluorescence. This phenomenon has been observed previously in plasma membrane investigations with yeast (Lloyd *et al.*, 2002) and *Giardia intestinalis* (Lloyd *et al.*, 2000).

Complex controls, although undetermined here, are involved in apoptotic and necrotic responses triggered in *C. albicans* after exposure to garlic and certain constituents. An early sign of cell death induction may potentially be indicated by the elevation of intracellular ROS. Further investigation using more specific fluorogenic probes, such as dihydroethidium to monitor levels of superoxide anion, should be performed (Carter *et al.*, 1994). A target for AA is at the plasma membrane. The target of DADS is not precisely determined in this study, but it is likely to be linked to elevation of oxidative stress through generation of intracellular ROS, potentially associated with impaired

mitochondrial function. Investigations into the effects of GPE, AA or DADS on the protective mechanisms (*e.g.:* catalase and superoxide dismutase activity) and redox buffers (GSH-GSSG cycling) should also be undertaken.

3.6 References

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4. CHAPTER FOUR

Garlic (*Allium sativum*) and allyl alcohol produce oxidative stress in *Candida albicans*.

4.1 Summary

Both the growth and respiration of *Candida albicans* are sensitive to extracts of *Allium sativum* and investigations into the anti-candidal activities are now focussing on the purified constituents for the determination of the targets of inhibition. Of particular interest is allyl alcohol (AA), a metabolic product accumulating after trituration of garlic cloves. Putative targets for allyl alcohol were investigated by monitoring changes in intracellular responses after exposure of *C. albicans* cells to AA or a commercially available garlic extract. We used two-photon laser scanning microscopy and other techniques. Changes typical of oxidative stress; NADH oxidation and glutathione depletion and increased reactive oxygen species (ROS), were observed microscopically and by flow cytometry. Known targets for allyl alcohol are alcohol dehydrogenases Adh1 and 2 (in the cytosol) and Adh3 (mitochondrial), although the significant decrease in NAD(P)H after addition of AA is indicative of another mechanism of action.

4.2 Introduction

Garlic (*Allium sativum*) has been used medicinally since before the time of the Sumerian civilization (2600-2100 BC), by when it was already widely cultivated in India and China (Harris, 2001). Traditionally used as an antimicrobial agent, garlic has been reported also to modulate cardiovascular and immune functions as well as having antioxidant and anticancer properties. Garlic constituents were first investigated by Wertheim (1844); more definitive studies, showed that whereas steam distillation gave diallyl disulphide, more gentle extraction using ethanol at 25°C or at sub-zero temperature released allicin and alliin respectively (Cavallito & Bailey, 1944). That the latter were examples of a number of cysteine sulphoxides and corresponding

thiosulphinates was more recently demonstrated (Lawson, 1996; Stoll & Seebeck, 1950; 1951).

Garlic as an antibacterial (Sharma *et al.*, 1977; Skyrme, 1997) has been the focus of detailed investigations recently on *Helicobacter pylori* (O'Gara *et al.*, 2000; Ross *et al.*, 2001). Differential inhibitory effects against *Escherichia coli* and *Lactobacillus casei*, whereby a 10-fold greater sensitivity was evident in the former (Rees, 1993) have been studied in more detail with respect to the different membrane structures of Gram positive and negative bacteria (Cottrell, 2003). Antiprotozoal studies include those on *Entamoeba histolytica* (Mirelman *et al.*, 1987; Reuter, 1994), and *Giardia intestinalis* (Harris *et al.*, 2000); the anticandidal effects (Lemar *et al.*, 2002; 2003) of garlic similarly include a wide range of ultrastructural lesions by affecting cytoplasmic membranes, organelles and cytoskeletal organization. The widespread efficacy of this plant extract as an antimicrobial may well depend upon the multiplicity of intracellular targets that its constituent active components inactivate; that no example of acquired microbial resistance to garlic has been reported may also stem from its diverse modes of action. Work with *G. intestinalis* (Harris *et al.*, 2000) and *C. albicans* (Lemar *et al.*, 2002) suggest that two of the simplest garlic constituents, diallyl disulphide and allyl alcohol (AA), are amongst the most potent. The latter can be isolated by a process of steam distillation (Wertheim, 1844). A well-researched mechanism of AA toxicity is by its inhibition of alcohol dehydrogenase after its conversion to acrolein (Rando, 1974). Acrolein (crotonaldehyde) and AA were the most toxic of twenty low boiling point compounds tested on the yeast *Candida utilis* (Sestakova *et al.*, 1976). More recent investigations have indicated that AA itself, is not toxic to cells of the methylotrophic yeast *Pichia pastoris*, but it is toxic when oxidised via an alcohol

oxidase pathway to acrolein (Johnson *et al.*, 1999). In rats, *in vivo*, AA is metabolised by liver alcohol dehydrogenase to the toxic aldehyde, acroleine (Mapoles *et al.*, 1994). Severe damage to the microtubules of rat hepatocyte mitochondria after exposure to AA (Vengerovskii *et al.*, 1989) is observed concomitantly with the depletion of glutathione (Nagelkerke *et al.*, 1991). However, recent studies reveal that 100µl AA per kg body weight in rats fed by gastric tube is not toxic to hepatocytes as determined by the RNA extraction (Tygstrup *et al.*, 1997). Resultant effects observed are disruption to the cellular kinetics, redox balance and electrophoretic mobility (Wills & Phelps, 1978). Acrolein is also known to deplete intracellular stores of glutathione and cause peroxidation of cellular lipids. This affects the permeability of the membrane and consequently determines the viability of the cell (Glascott *et al.*, 1996).

In this paper we investigate the biocidal mechanisms of AA on *C. albicans* by assessing its effects on cell physiology and also on morphology using optical and electron imaging.

4.3 Methods and Materials

4.3.1 Organism and culture

Candida albicans (Berkhout) 5134 was grown in YPG medium (bacto-peptone 2% (w/v), Difco, Michigan, USA; yeast extract 1% (w/v), Oxoid, Hampshire, U.K, and glucose 2% (w/v). An early stationary phase culture was used as an inoculum (1:100 dilution in 50 ml) in 100 ml conical flasks. Growth was aerobic at 30°C in a rotary shaker (100g). Cell numbers were determined with a haemocytometer slide (Fuschs-Rosenthal, London, UK) after dilution in sterile medium. Optical density ($A_{450\ nm}$) was measured using a spectrophotometer (SP-1400; Pye-Unicam, Cambridge, UK). Stock

cultures of *C. albicans* were grown overnight at 30°C and maintained at 4°C on YPG agar containing 2% (w/v) agar (Oxoid).

4.3.2 Garlic Preparations

Garlic powder (GPE; provided by Cultech Biospeciality Products, Swansea, Wales, UK) was prepared to the required concentration (w/v) in sterile growth medium. After 30 min the extract was centrifuged at 3900*g* for 10 min and the supernatant passed through a sterile 0.2-μm filter (Millipore, Watford, UK). Stock suspensions were prepared on the same day of testing in PBS (pH5.6) or appropriate media as stated. Concentration ranges for biocide assays for AA (0-10mM) were prepared in sterile growth medium.

4.3.3 Scanning electron microscopy

Cells were fixed in 3% (v/v) glutaraldehyde in PBS (pH7.4). Dehydration was by 10-min sequential washes of 10% incremental rises of % vol. ethanol. A drop was placed onto a round glass coverslip for critical point drying (CPD 030; Balzers, Lerchtenstein) The sample was mounted onto an aluminium slab (10 x 10mm Jeol type; Agar Scientific) using silver paint (Agar Scientific, Essex, UK). Sputter coating was performed in a sputter coater (S150B; Edwards, West Sussex, UK) and the image viewed using a scanning electron microscope (5200LV; Jeol, Herts, UK).

4.3.4 Transmission electron microscopy

Cells were fixed in cacodylate buffer, pH6.9, containing 1% (v/v) paraformaldehyde and 2% (v/v) glutaraldehyde, at 4°C for 1 h. The cells were centrifuged and dehydrated in successive washes of ethanol for 30 min. The pellet of cells was embedded in Spurr

resin and ultrathin sections obtained (Ultratome III; LKB, Stockholm, Sweden). The sections were mounted onto a 0.5% Pioloform (in chloroform)-coated 3.05mm copper grid then stained with 2% aqueous uranyl acetate and 2% lead citrate before imaging on a transmission electron microscope (1210; Jeol).

4.3.5 Whole cell respiration

Organisms in growth medium were harvested in exponential phase. Cells were washed and re-suspended in 500µL PBS, pH6.4. Measurement of glucose supported O_2 consumption was performed at 30°C in a thermostatically controlled closed electrode system (Rank, Bottisham, Cambridge, UK) using a Teflon membrane inert electrode. Cell suspensions (40µl) were injected into 2ml PBS (pH5.6) and continually stirred at 150rpm.

4.3.6 Redox Investigation

4.3.6.1 Preincubation and conditions for microscopic examination.

Early stationary phase cells were harvested and resuspended in PBS (pH5.6). Samples (0.5ml) were preloaded with 200nM tetramethyl rhodamine ethyl ester (TMRE) at 37°C for 30 min before sample removal to the observation chamber and supplemented with 10mM glucose. The dish containing the yeasts was equilibrated with unrestricted access to atmospheric oxygen on the stage of a Nikon E600FN upright microscope which was maintained at 30°C.

Fluorescent probes for two-photon laser scanning microscopy. The cationic potentiometric fluorescent dye, TMRE, was used to monitor changes in mitochondrial membrane potential, $\Delta \Psi_m$. The large negative potential gradient across the inner mitochondrial membrane results in the accumulation of TMRE within the matrix

compartment according to its Nernst potential (Loew *et al.*, 1993). Reactive oxygen species (ROS) production were monitored with the ROS-sensitive fluorescent probe 5-(-6)-chloromethyl-2',7'-dichlorodihydrofluorescein diacetate (CM-H_2DCFDA, 10μM). The acetate group of CM-H_2DCFDA is hydrolyzed by esterases when it enters the cell and is trapped inside as the non-fluorescent 5-(-6)-chloromethyl-2',7'-dichlorodihydrofluorescein (CM-H_2DCFH). CM-H_2DCFH was chosen because, unlike underivatized dichlorodihydrofluorescein, it is well retained in cells (Xie *et al.*, 1999) and, in this case, in the mitochonarial matrix (Aon *et al.*, 2003). Oxidation of CM-H_2DCFH by ROS, particularly by hydrogen peroxide (H_2O_2) and hydroxyl radical (Vanden Hoek *et al.*, 1997a; 1997b), yields the fluorescent product carboxymethyl-dichlorofluorescein (CM-DCF), and in an indirect manner, measures mitochondrially-produced O_2^- that has dismutated to H_2O_2 through the action of superoxide dismutase (Chance *et al.*, 1979; Turrens *et al.*, 1985).

Glutathione was monitored intracellularly by production of the fluorescent adduct, glutathione-bimane (GSB) (Kosower & Kosower, 1987) as a result of the reaction of the cell permeant monochlorobimane (MCB, 50 μM) with reduced glutathione (GSH) catalysed by glutathione-*S*-transferase (GST) (Cortassa *et al.*, 2004). Reduced nicotinamide nucleotides were monitored by their autofluorescence under the imaging conditions optimised previously (Aon *et al.*, 2003).

4.3.6.2 Image acquisition and analysis.

Images were recorded using a two-photon laser scanning microscope (Bio-Rad MRC-1024MP) with excitation at 740nm (Tsunami Ti:Sapphire laser, Spectra-Physics) as described before (Aon *et al.*, 2003). Briefly, owing to the overlap in the cross sections for two photon excitation of the three fluorophores of interest (Xu *et al.*, 1996)

(TMRE, CM-DCF, and NADH or GSB) this wavelength permitted recording of $\Delta\Psi_m$, ROS production, and NAD(P)H or GSH simultaneously. The red emission of TMRE was collected at 605±25nm; the green emission of CM-DCF was recorded at 525±25nm; and the blue emission of GSB detected at 480±20nm. NADH emission was collected as the total fluorescence <490nm. At 3.5s or 30 sec intervals as indicated, 512 x 512 pixel 8-bit grayscale images of the three emission channels were collected simultaneously and stored.

While performing kinetics of GSB production in cells, about 10 images were recorded to get the cellular background of NADH before acute addition of 50 µM MCB. This was necessary due to the fact that the NAD(P)H and GSB emissions were collected at similar wavelengths, i.e. 480-490nm (Aon *et al.*, 2004; Cortassa *et al.*, 2004). At steady state, the NAD(P)H fluorescence levels (n=14) represented 31±1.2% of the maximal GSB fluorescence levels attained (n=14) indicating an increase of ~3-fold respect to the background. Images were analyzed offline using ImageJ software (Wayne Rasband, National Institutes of Health, http://rsb.info.nih.gov/ij/).

4.3.7 Fluorometric kinetic studies.

The effect of AA and GPE on purified glutathione-*S*-transferase (GST) as a function of GSH concentration was further investigated (Sigma, 6 U/ml final concentration). Increasing concentrations of GSH (in phosphate buffer saline [PBS] pH5.6) in the absence and presence of AA (0.5mM/1.0mM) and GPE (5 mg ml^{-1}/10 mg ml^{-1}) was added to 3ml PBS (pH5.6); a subsequent addition of 50µl GST was used to commence the reaction. Monochlorobimane (MCB, 2µM final concentration; λmaxexcitation 391 nm; λmax emission 491 nm) was used as previously described (Aon *et al.*, 2003), and
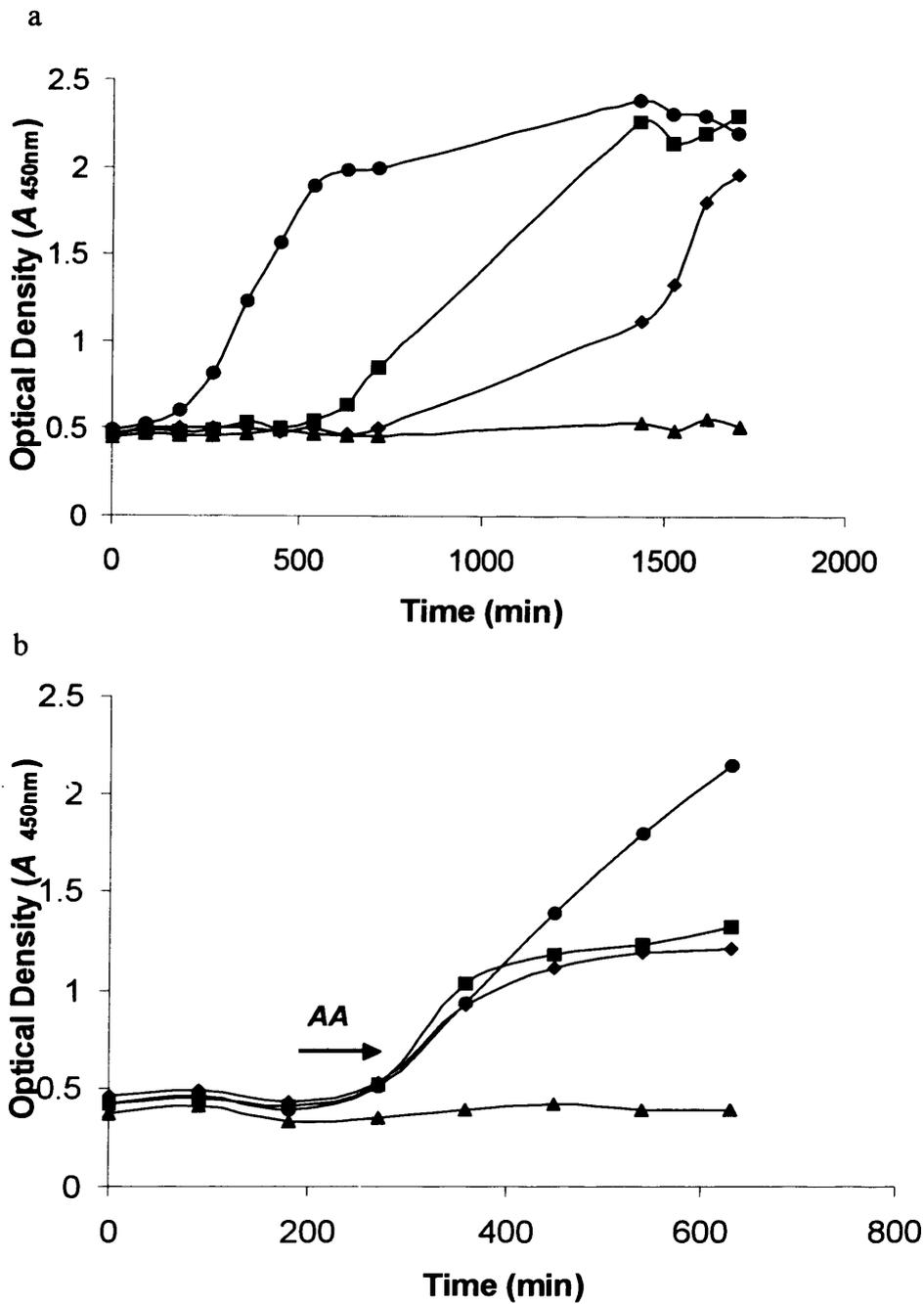
Fig. 4.1.
Effect of allyl alcohol on growth of *Candida albicans* after addition at (a) early stationary phase. Concentrations (mM): ● 0; ■ 0.25; ◆ 0.5; ▲ uninoculated control. (b) mid-exponential phase of growth (as indicated by arrow), concentrations (mM): ● 0; ■ 1.0; ◆ 1.5; ▲ uninoculated control.

However, these AA-inhibited cultures eventually achieved the same maximum cell numbers as the controls (Fig. 4.1a). Doses of AA greater than 0.5mM resulted in complete inhibition (data not shown). Established cultures of $C.$ $albicans$ were able to tolerate higher concentrations of AA; when added in mid-exponential phase, inhibition of growth was observed only at concentrations $\geq$ 1mM AA (Fig 4.1b). The IC_{50} for AA is 1mM at 10 h, and the MIC value was 0.1mM. Colony growth on YPD agar indicated that AA is fungistatic at concentrations above 1mM but fungicidal at concentrations above 2mM.

4.4.2 Scanning Electron Microscopy.

Aerobic growth of $C.$ $albicans$ cells in YPD media at 30°C gave typical yeast cells displaying the characteristic bud scars (Fig. 4.2a). Development of pseudohyphae (chains of elongated un-separated blastospores) was only observed in cultures containing low concentrations of AA (Fig. 4.2b). The external morphology of the cells after addition of 0.1 mM AA (MIC) did not appear as smooth when compared to untreated cells; this indicates disruption of cell wall composition, a possibly with loss of cytosolic components and (Fig. 4.2a and b). Increasing the AA concentration further distorted the cell wall surface (Fig. 4.2c and d). Pseudohyphae were not observed at higher concentrations of AA. Neither untreated nor treated cultures produced true hyphae (presence of septa between elongated cells).

Fig. 4.2.
Scanning electron micrographs of *Candida albicans* cultures (a) untreated culture, treated with allyl alcohol (b) 0.1mM, (c) 1.0mM, (d) 10mM.

(a)

Acc.V Spot Magn Det WD Exp   5 µm
25.0 kV 3.0 2004x SE 12.3 18   C. albicans/control#24h

(b)

Acc.V Spot Magn Det WD Exp   2 µm
25.0 kV 3.0 6530x SE 12.9 14   C. albicans#10mM#24h

(c)

Acc.V Spot Magn Det WD Exp   5 µm
15.0 kV 4.0 2008x SE 12.8 28   Candida albicans#0.1#24h

(d)

Acc.V Spot Magn Det WD Exp   5 µm
25.0 kV 3.0 3130x SE 12.8 22   C. albicans#10mM#ex
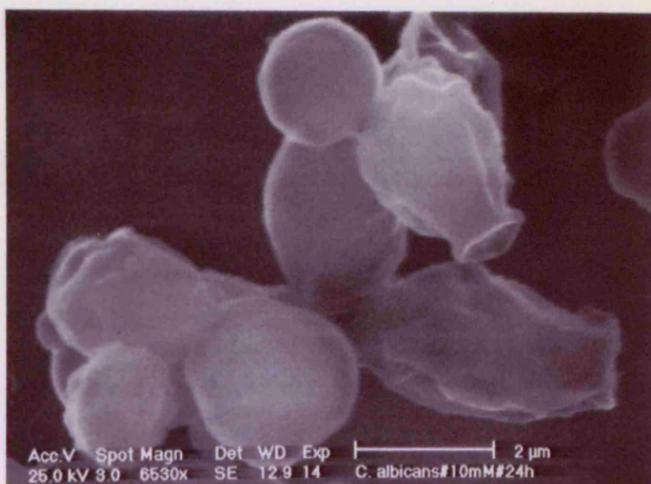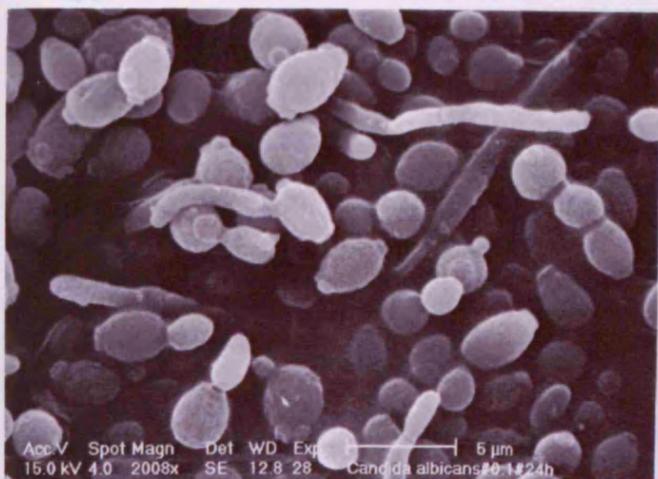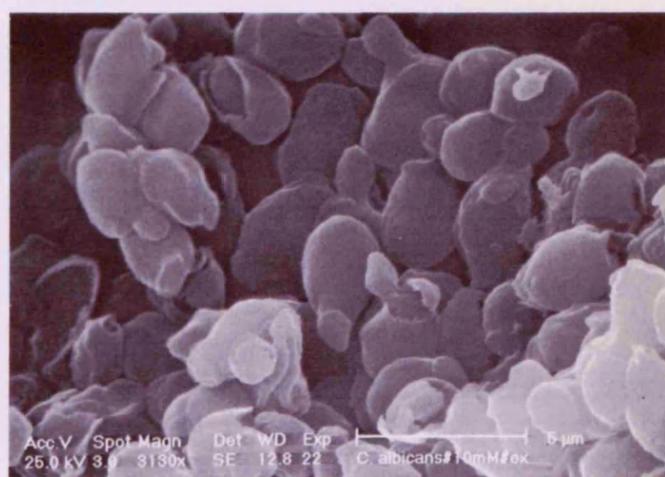
4.4.3 Transmission Electron Microscopy.

Incubation of *C. albicans* in the presence of AA caused notable alterations in the internal morphology (Fig. 4.3). At high concentrations of AA (10mM) (Fig. 4.3b), peripheral vacuoles were evident that were not visible in organisms from the control cultures (Fig 4.3a). A more granular appearance in the cytoplasm and some loss in definition of the cell wall were observed at higher concentrations of AA. Unusually shaped mitochondria were also visible at high doses of AA (Fig. 4.3a).

4.4.4 Cellular respiration.

Oxygen consumption rates increase as oxygen concentration decreases, down to about 50% of air saturation value (Fig. 4.4).

Successive additions of 5mM AA to that of glucose supported respiration in exponential phase cells did not alter the rate of oxygen consumption. The final concentration was 20mM AA, 200 times more concentrated than the MIC for AA. Successive additions of 1.2mg ml^{-1} GPE (exponential phase cells, glucose supported respiration) had no effect up to a concentration of 2.4mg ml^{-1}. Inhibition in respiration was observed at concentrations $\geq$ 3.6mg ml^{-1}. A final concentration of 4.8 mg ml^{-1} GPE caused 18% reduction of the rate of oxygen consumption.

4.4.5 Confocal Imaging of Redox mechanisms.

We monitored key intracellular variables such as mitochondrial membrane potential, $\Delta\Psi$m, ROS, and reduced glutathione (GSH), with fluorescent probes (TMRE, CM-DCF, and MCB), whereas the NAD(P)H redox status was imaged using autofluorescence (Fig. 4.5). A significant increase in $\Delta\Psi$m was evident after treatment

Fig. 4.3.
Transmission electron micrograph of *Candida albicans* cultures (a) untreated, or (b) treated with10mM allyl alcohol; increased granulation of cytosol and appearance of peripheral vacuoles (white arrows). Abnormally shaped mitochondria indicated by grey arrows.

a
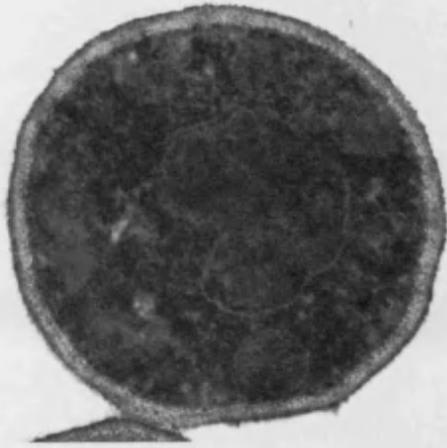
b
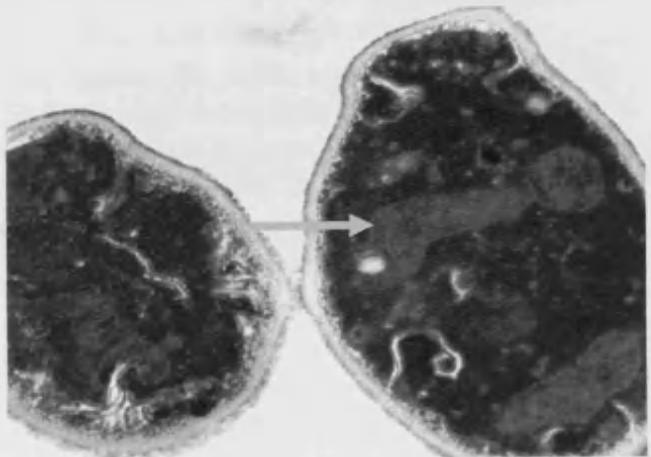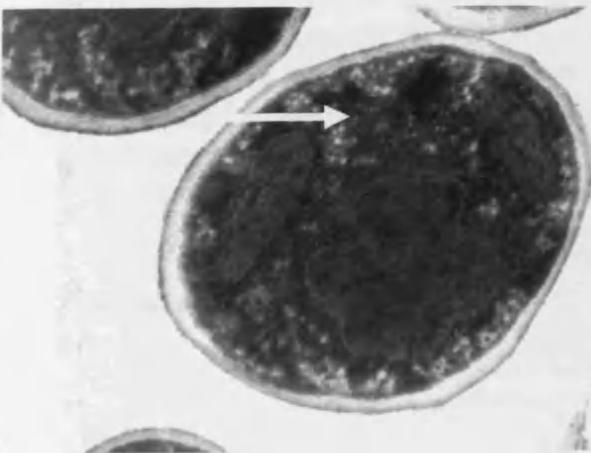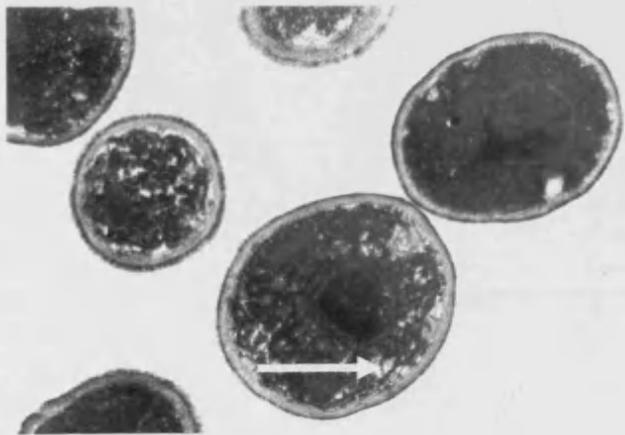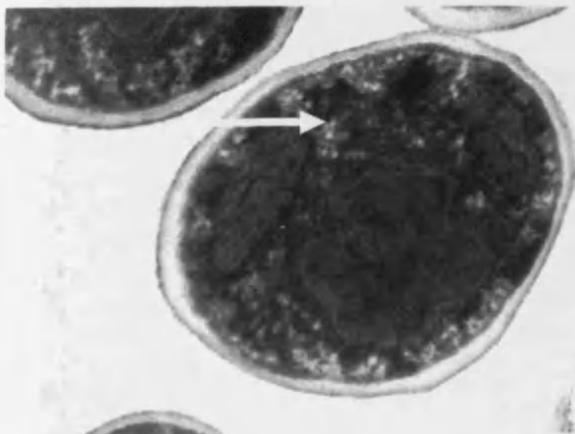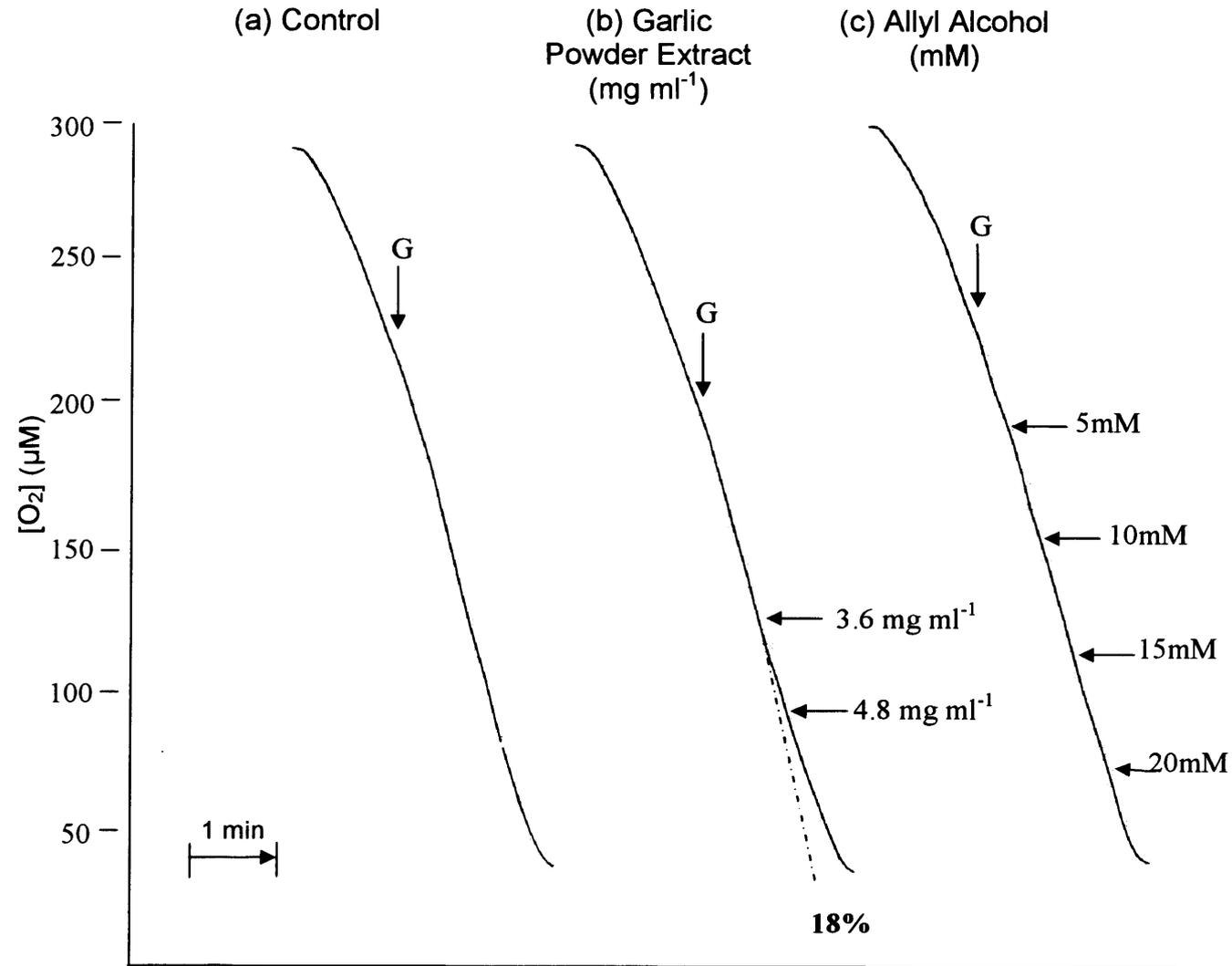
Fig. 4.4.
Oxygen consumption of organisms harvested from an exponentially growing culture of *Candida albicans*. Endogenous and exogenous rates (addition of 100mM glucose indicated 'G' vertical arrow) are shown for (a) control (b) garlic powder extract and (c) allyl alcohol. Horizontal arrow indicates additions of garlic extract; adjacent numbers indicate concentration. Bold number underneath the trace indicates the % inhibition at a final concentration of 4.6 mg ml^{-1} GPE. Cell density was 8 x 10^6 cell ml^{-1}. Additions of allyl alcohol are not shown as no inhibition is evident. Traces shown are typical of results with three batches of cells. Dotted line indicates rate of oxygen consumption if no inhibition had occurred.

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Fig. 4.5.

Fluorescent monitoring of early stationary phase *Candida albicans* (a) control and treated cells (b) 5mg ml^{-1} GPE and (c) 1mM allyl alcohol.

Images show:

      (i)   mitochondrial membrane potential,
      (ii)   ROS,
      (iii)   NAD(P)H auto fluorescence, and
      (iv)   reduced glutathione with two-photon scanning laser microscopy.

Imaging conditions are as described in M&M. Laser conditions and fluorescent probes:

      For (i) tetramethyl rhodamine ethyl ester (TMRE); (a) untreated control cells: 5x accumulated image, 80% laser intensity, (b) 5 mg ml^{-1}GPE: 3x accumulated image, 40% laser intensity, and (c) 1mM AA: no accumulation 40 % laser intensity.

      For (ii) CM-DCF (iii) NADH auto-fluorescence, and (iv) MCB, qall conditions were the same; (a) untreated control cells, (b) 5 mg ml^{-1} GPE, and (c) 1mM AA were all 3x accumulated image, 40% laser intensity.

	(a) Control	(b) GPE	(c) AA
(i) TMRE			
(ii) CM-DCF			
(iii) NADH			
(iv) GSB			
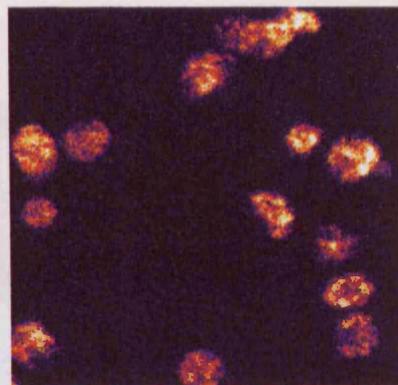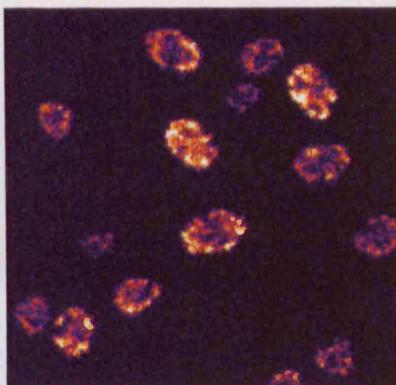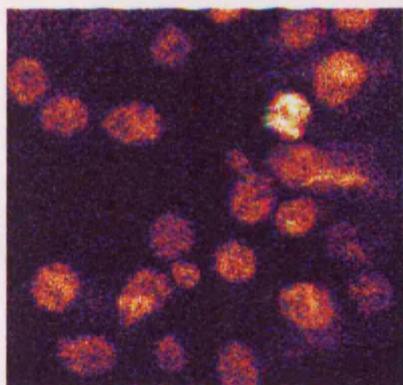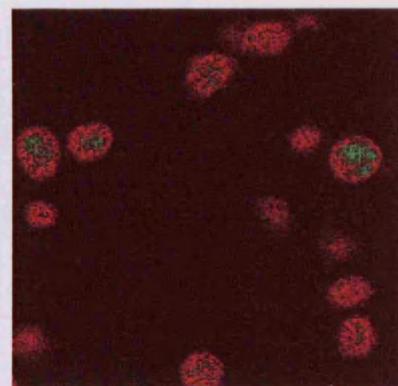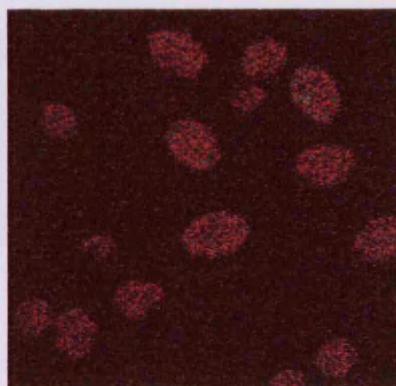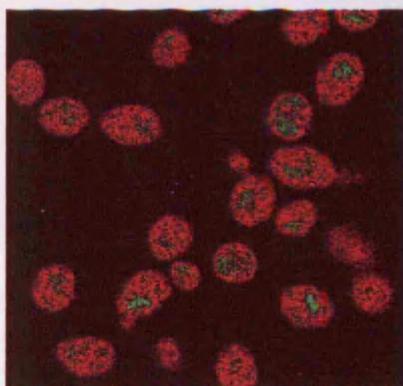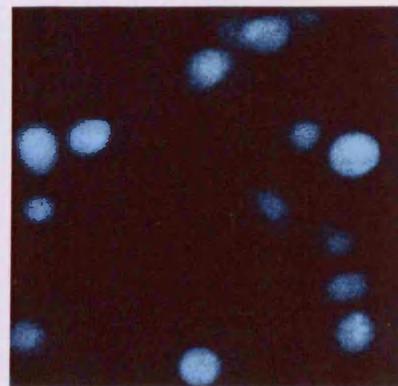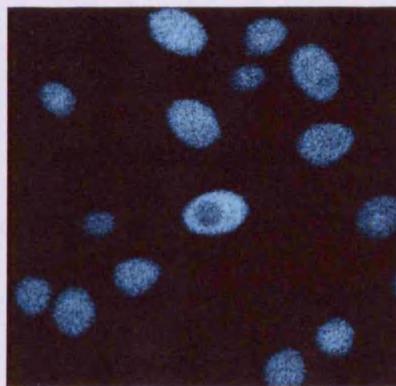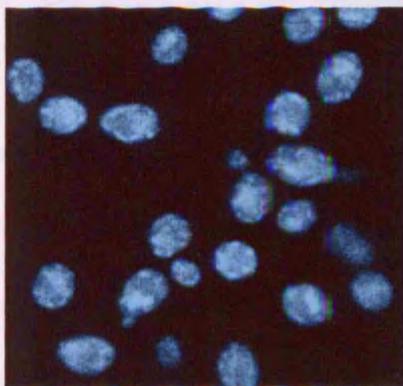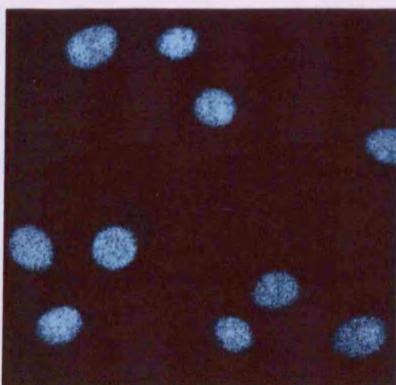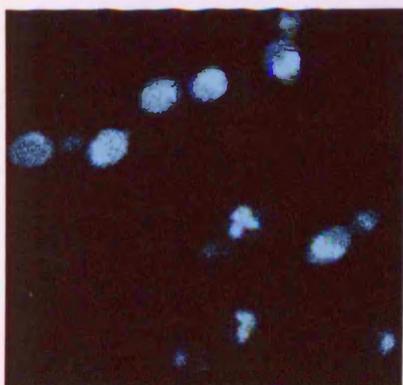
with 1mM AA as compared to controls or exposure or 5 mg ml^{-1} GPE (Fig. 4.5i). A pattern of effects characteristic of oxidative stress was induced, (i.e. high ROS, and a low level of GSH) was induced by addition of 1mM AA (the IC$_{50}$ value for this compound) to early stationary phase cells (Fig. 4.5). Addition of 5mg ml^{-1} GPE caused a similar increase in ROS and depletion of GSH, but also a decrease in NADH autofluorescence was observed. A similar response was exhibited by cells harvested from exponential phase of growth (not shown).

Low levels of ROS were localised in the mitochondria in control cells (Fig. 4.5aii); increased CM-DCF fluorescence was observed in both mitochondrial and cytoplasmic compartments after treatment with either GPE or AA (Fig. 4.5bii and 4.5cii). Autofluorescence of mitochondrial NAD(P)H was intense in control samples, but this signal was attenuated by oxidation after addition of GPE and AA (Fig. 4.5biii and 4.5ciii). Quantification of auto-fluorescence, as mean values of the pixel intensities, from mitochondria as compared to cytosolic NADH after AA addition is illustrated in Fig. 4.6. A very significant decrease of NAD(P)H fluorescence was registered in both cytosolic and mitochondrial compartments as a result of the cells treatment with AA. Formation of glutathione-bimane product (GSB) used to monitor glutathione levels before and after addition of challenge agents indicated a general decrease in cell fluorescence (Fig. 4.5iv); AA gave the most significant decrease in GSB fluorescence (21±0.7 a.u., n=10), to below the levels of NAD(P)H autofluorescence control (55±0.9 a.u., n=14). The effect of GPE was less significant (Fig. 4.7).
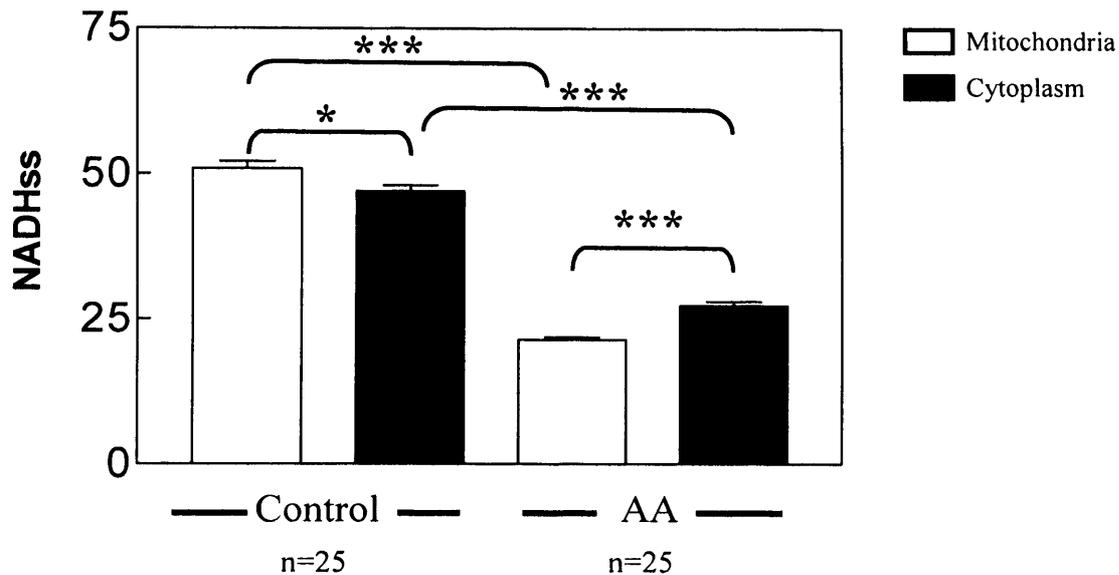
Fig. 4.6

Effect of allyl alchol(AA) on the cellular redox environment. The intensities of NAD(P)H autoflourescence in the mitochondrial and cytoplasmic compartments were determined in *Candida albicans* cultures and harvested in early stationary phase as described under M&M. Quantification was performed in cells in the absence (control) or presence of 1mM allyl alcohol. Cells loaded with TMRE allowed determination of the regions of interest corresponding to mitochondria or cytoplasm in order to quantify autofluorescence in both compartments. The brackets above the bars indicate the statistical significance corresponding to different compartments and/or the absence or presence of AA, ***, $p < 0.001$; *, $p < 0.05$.
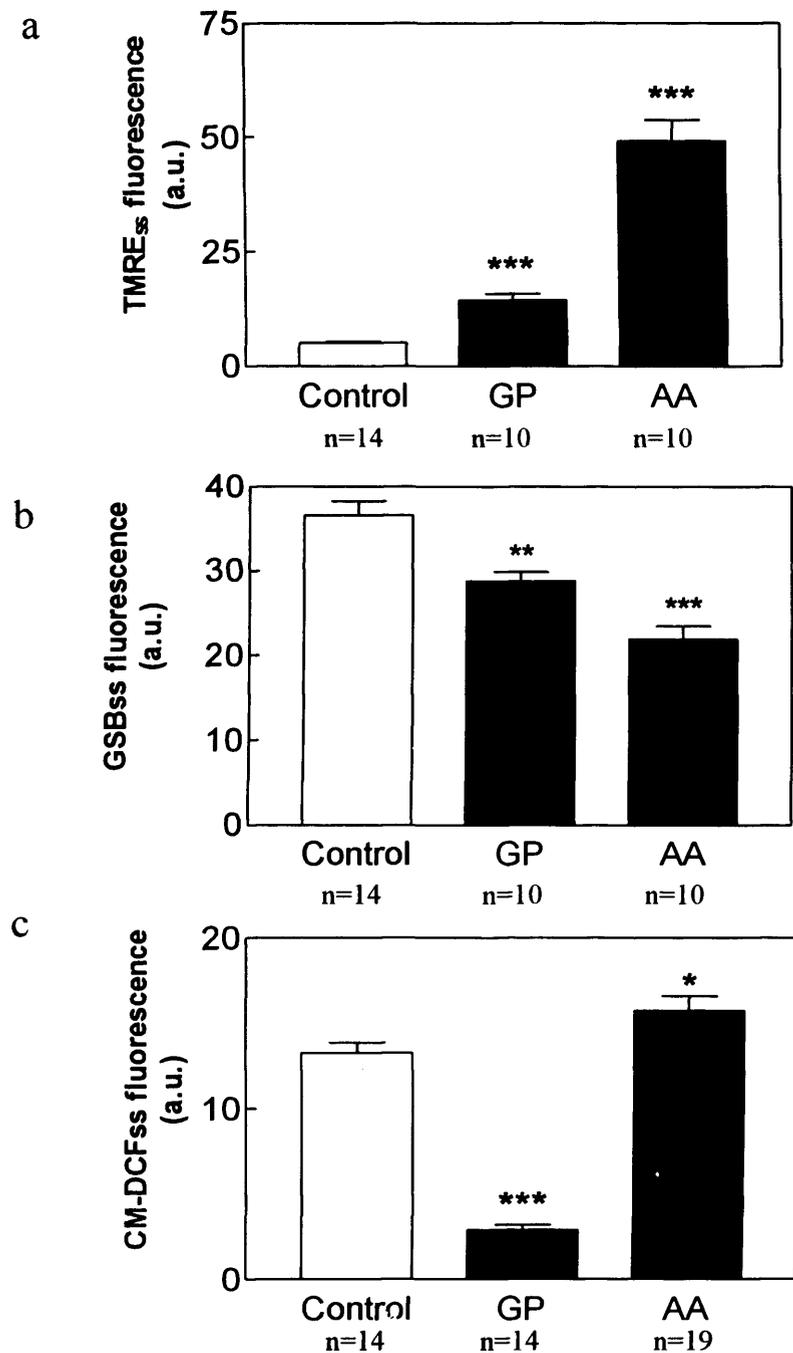
Fig. 4.7.
Effects of garlic components on fluorescence signals from two photon microscopy images. *Candida albicans* cells were harvested in early stationary phase as described under M&M and exposed to different garlic components at room temperature. The steady state levels of fluorescence (as the mean pixel intensity of a normal distribution) from TMRE, GSB and CM-DCF probes after 30 min treatment with 1mM AA, or 5 mg ml^{-1} GPE were determined ***, $p<0.001$; **, $p<0.01$; *, $p<0.05$ versus control.

4.4.6 Glutathione-*S*-transferase activity.

That GST was not a target for AA or GPE was confirmed by fluorimetric analysis of the reaction of the purified enzyme. Inhibition of the enzyme GST was not evident on increasing concentrations of either AA or GPE (results not shown). This suggests that GST is not one of the intracellular targets of AA or GPE.

4.4.7 Flow Cytometric analysis.

The fluorogenic compound CM-H₂DCFH is oxidised by ROS to give a fluorescent product as described in the methods section. Control suspensions of *C. albicans* loaded with this fluorogen showed only autofluorescence at 530nm, whereas cells treated with AA and GPE fluoresced much more brightly. The fluorescence intensities were observed to increase as the concentrations of either challenge agent was increased (Fig. 4.8).

4.5 Discussion.

These results further confirm that some garlic components possess significant anticandidal properties (Lemar *et al.*, 2002) and that AA, a metabolic breakdown product of allicin plays a major role in the inhibition of growth of *Candida albicans*.

Allicin is considered one of the principal anti-microbial constituents of triturated garlic cloves (Cavallito, 1944), and although it has microbiocidal *in vitro* activity (Ankri & Mirelman, 1999), its half life *in vivo* in tissues is too short to ensure considerable pathogen damage (Koch, 1996). Systemic levels of its degraded metabolic components are reported to be relatively high (Egen-Schwind *et al.*, 1992), and researchers have
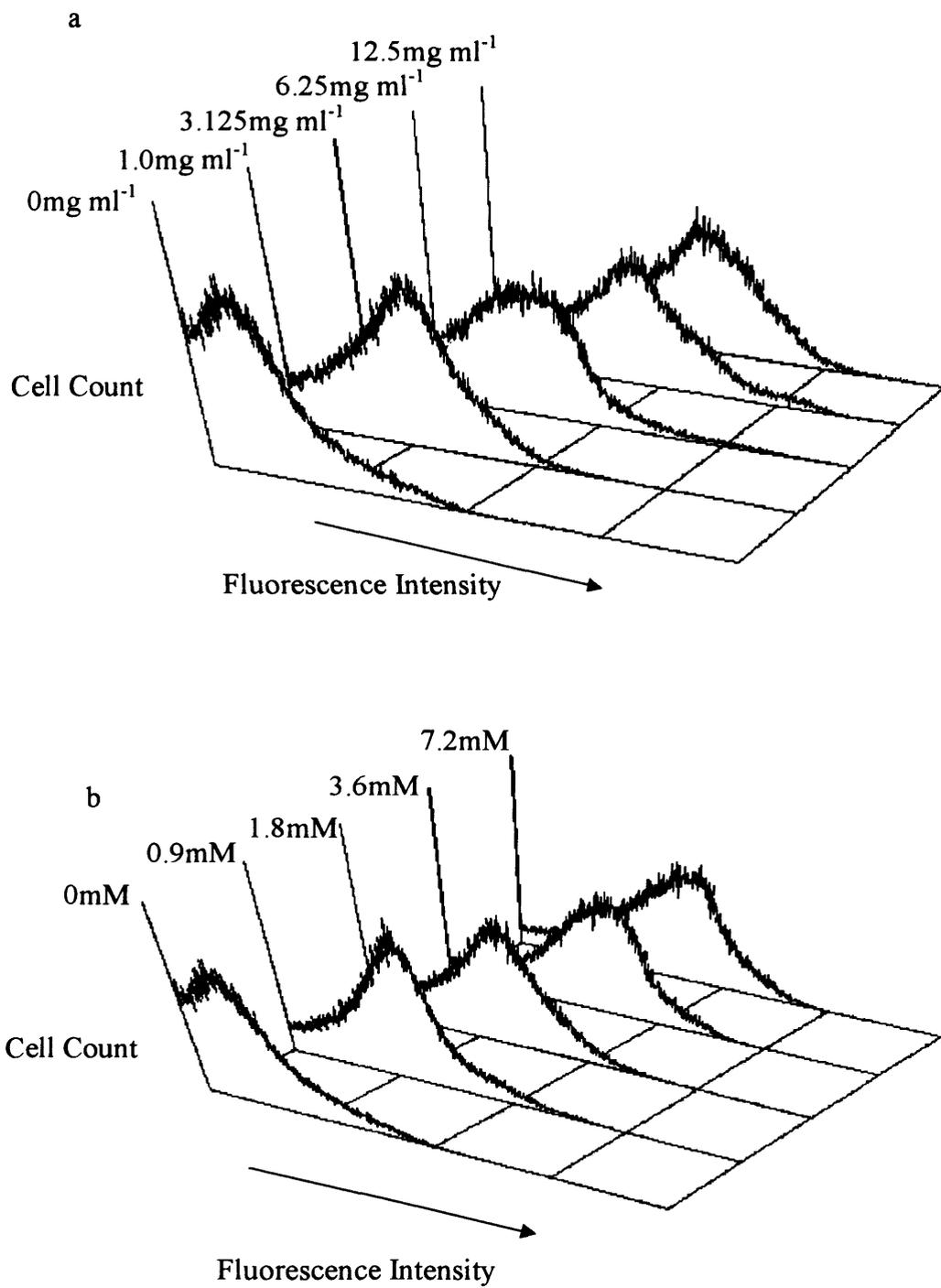
Fig. 4.8.
Flow cytometric determination of reactive oxygen species (ROS). Representative histograms of fluorescence from *Candida albicans* after treatment with (a) garlic powder extract, and (b) allyl alcohol.

now turned their interest to these constituents as possessing considerable anti-microbial properties. AA is found in exhaled air after ingestion of freeze-dried garlic tablets (Laasko, 1989). A known hepato-toxicant, its use in pure form would have to be administered carefully; dosage up to 100µl AA per kg body weight in rats after administration by gastric tube presents no toxicity to hepatocytes (Tygstrup *et al.*, 1997). Conversion of AA to acrolein can lead to subsequent inhibition of alcohol dehydrogenases (Rando, 1974). *Saccharomyces cerevisiae* has 20 alcohol dehydrogenases (Kruckerberg, 2004), but the best understood and possibly the more important enzyme targets involved in AA toxicity are the cytosolically located Adh 1 and 2, involved in ethanol formation and growth on ethanol respectively, and Adh 3, which is mitochondrial. The functions of Adh 3 are not entirely understood, but recent investigations postulate that it is involved in a redox shuttle, transferring mitochondrial NADH to the cytosol (Bakker *et al.*, 2000). Improvements in methods for measuring mitochondrial redox state (see (Chance, 2004) for a review) have allowed us to detect a decrease in both cytosolic and mitochondrial NADH. Results suggest that although it is possible that all three of these enzymes are targeted by AA, another site of action is involved to produce this effect (Fig. 4.5ciii). Depletion of NAD(P)H, the co-substrate for glutathione reductase would diminish the recovery of the GSH pools in both compartments. As AA depletes glutathione (Glascott *et al.*, 1996), one would predict an increase in ROS. Confocal imaging and quantitative analysis does indeed indicate the depletion of glutathione and increase in ROS when cells are treated with AA (Figs. 4.5, 4.7, 4.8). Morphological evidence obtained using electron microscopy of cells treated with AA, also indicates that mitochondria may be a target for AA (Fig. 4.3). However, decreased respiration, as observed with garlic extract and other garlic constituents (unpublished data), was not observed for AA. This may be explained in

glucose grown organisms, where alcohol dehydrogenase function is not implicated in glucose respiration. Furthermore, when organisms are grown in the presence of excess glucose alcohol dehydrogenase is inactivated (Gonzalez *et al.*, 2000). The conversion of AA to the toxic aldehyde, acrolein would therefore not occur, and may indicate that exposure to AA in this study impair *C. albicans* viability through mechanisms not directly associated with mitochondrial function.

The enzyme glutathione-*S*-transferase, associated with maintaining redox balance, is a target of a different garlic constituent, diallyl disulphide (DADS) (unpublished data). Experiments here demonstrate that it is not a target for AA (results not shown). This is perhaps unsurprising, as the two compounds are of quite different structures. However, this observation assists in our understanding of why GPE has such a broad spectrum of activity. The production of multiple metabolic constituents by the plant (many of which remain to be explored), which have many different targets that all act in concert to inhibit the diverse processes essential for an organism's viability. Its extensive repertoire of inhibitory constituents makes garlic an interesting alternative to single-site specific antibiotics or synthetic organic compounds for combating *Candida* infections.

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5. CHAPTER FIVE

Diallyl disulphide depletes glutathione in *Candida albicans*: oxidative stress mediated cell death studied by two-photon microscopy.

5.1 Summary

Using 2-photon scanning laser microscopy we investigated the effect of an *Allium sativum* (garlic) constituent diallyl disulphide (DADS), on the functional ultrastructure of the opportunistic pathogen, *Candida albicans*. A short 30 min exposure to 0.5 mM DADS and then removal, induced 70% cell death (50% necrotic, 20% apoptotic) within 2h; this figure increased to 75% after 4h. We attempted to dissect the early intracellular events associated with cell death that were triggered by DADS. Simultaneous monitoring using 2 photon confocal microscopy, of mitochondrial membrane potential ($\Delta\Psi_m$), reactive oxygen species (ROS), and NADH or reduced glutathione (GSH) pools, was performed aerobically after the addition of 0.5mM DADS. Decreased intracellular GSH and concomitantly elevated ROS levels were observed. Additionally, DADS induced a marked enhancement of $\Delta\Psi_m$ and low respiration rates as could be verified in cell suspensions. *In vitro* kinetic experiments in cell-free extracts suggest that glutathione-*S*-transferase (GST) is one of the intracellular targets of DADS, with additional targets likely to be a component prior to Site II in the respiratory electron transport chain as well as ATP synthase as determined by decreased oxygen consumption and proton production respectively. The results obtained indicate that DADS is an effective antifungal agent able to trigger cell death in *Candida* by eliciting oxidative stress as a consequence of thiol depletion and that its efficacy as an antifungal agent depends on this primary mechanism.

5.2 Introduction

The opportunistic human pathogen *Candida albicans* is associated with a range of clinical conditions. The organism most commonly disseminates from vaginal and oral mucosal infections to a more complex and life-threatening systemic condition

particularly manifest in immuno-compromised patients such as those suffering from AIDS or undergoing chemotherapy (Cutler, 1991; Odds, 1988). A limited range of anti-fungal treatments are available, but these are often associated with unpleasant side effects. The increasing resistance to favoured treatments using fluconazole or amphotericin ensures that new investigations into the treatment of fungal infections are essential.

We believe that decisive breakthroughs in the search of novel antifungal agents will become associated with the identification of the most sensitive cellular variables; impairment of these may lead to cell death. Oxidative stress is among one of the most sensitive predictors of cell death in many organisms and cellular systems. Reactive oxygen species (ROS) are frequently associated with cytotoxicity, often involving the passive attack on cellular components in conditions where oxidative stress is the initiating stimulus for apoptosis. Cell death is assumed to be triggered as the eventual result of cumulative oxidative damage (Poljak, 2003). High concentrations of ROS trigger biochemical mechanisms (Halliwell, 1999), which in turn promote the morphological changes, nuclear fragmentation, chromatin condensation, cellular swelling (blebbing), and biochemical effects (phosphatidyl-serine externalisation) commonly associated with the apoptotic route of death (Bowen, 1993; Schwartzman & Cidlowski, 1993; Wyllie, 1980). These characteristic morphological markers and biochemical changes have been observed in the brewing yeast *Saccharomyces cerevisiae* although the mechanisms and their genetic control is less understood (Madeo *et al.*, 1997).

C. albicans death may be caused by lethal external forces leading to apoptosis or necrosis, whose triggering is recognised as a potentially therapeutic tool. This response is commonly elicited after exposure to environmental and physiological stresses, (Del Carratore *et al.*, 2002; Frohlich, 2000; Huh *et al.*, 2002; Ludovico, 2001; Madeo *et al.*, 2002a; Madeo *et al.*, 2002b; Madeo, 1999) and in cases such as aged or mutated organisms (Laun *et al.*, 2001; Severin & Hyman, 2002). A demonstration of apoptosis after oxidative stress following exposure to acetic acid and hydrogen peroxide was recently reported for *C. albicans* (Phillips *et al.*, 2003).

Pharmacological properties of plant extracts are well documented and their underlying mechanisms are gradually being understood. Their widespread efficacies as antimicrobials may well depend upon the multiplicity of intracellular targets that become inactivated. For *Allium sativum* (garlic), no example of acquired microbial resistance has been reported (Harris *et al.*, 2000; Lawson, 1996; Reuter, 1996); this may also stem from its diverse modes of action. Work with *Giardia intestinalis* (Harris *et al.*, 2000) and *C. albicans* (Lemar *et al.*, 2002) suggests that two of the simplest garlic constituents, allyl alcohol (AA) and DADS, are amongst the most potent. Within mammalian systems the application of DADS has been successful at inhibiting cell proliferation in human colon, skin and liver tumours (Sundaram & Milner, 1996). More specifically, apoptotic induction by DADS was reported in leukaemia cells via activation of the caspase-3 pathway (Park *et al.*, 2002). Elevated levels of ROS following DADS exposure is highlighted as the primary cause for apoptotic cell death in neuroblastomas (Filomeni *et al.*, 2003).

In this paper, we investigate the effects of DADS on cell death in *C. albicans*. Some of the intracellular targets and mechanisms involved are analysed. A main finding is that transient and short exposure to DADS elicits up to 75% cell death within 4 h by either necrosis or apoptosis. The deadly effects of DADS seem to be mediated by oxidative stress, suggesting its potential usefulness in the therapeutic induction of cell death in *C. albicans*.

5.3 Methods and Materials

5.3.1 Organism and culture.

Candida albicans (Berkhout) 5134 was grown in YPG medium (bacto-peptone 2% (w/v), Difco, Michigan, USA; yeast extract 1% (w/v), Oxoid, Hampshire, U.K, and glucose 2% (w/v). An early stationary phase culture was used as an inoculum (1:100 dilution in 50 ml) in 100 ml conical flasks. Growth was aerobic at 30°C in a rotary shaker (150rpm). Cell numbers were determined with a haemocytometer slide (Fuschs-Rosenthal, London, UK) after dilution in sterile medium. Optical density ($A_{450\,nm}$) was measured using a spectrophotometer (SP-1400; Pye-Unicam, Cambridge, UK). Stock cultures of *C. albicans* were grown overnight at 30°C and maintained at 4°C on YPG agar containing 2% (w/v) agar (Oxoid).

5.3.2 Garlic preparations.

Garlic powder (GPE; provided by Cultech Biospeciality Products, Swansea, Wales, UK) was prepared to the required concentration (w/v) in sterile medium and placed at room temperature for 30 min. The extract was centrifuged at 3900*g* for 10 min and the supernatant fluid passed through a sterile 0.2-μm filter (Millipore, Watford, UK).

Stock solutions of 40mg ml^{-1} were prepared on the same day of testing. DADS was prepared in DMSO and AA in PBS (pH 5.6).

5.3.3 Analysis of apoptotic and necrotic cells.

Cell death was analysed using Alexa Fluor 488 annexin V/propidium iodide (Vybrant Apoptosis Assay Kit #2, Molecular Probes, Inc. Or. USA). The Alexa Fluor 488 conjugated to annexin-V interacts with externalised phosphatidyl-serine in apoptotic cells, producing a bright green fluorescence whereas necrotic ones are stained red by propidium iodide which live cells are impermeant to.

Digestion of the cell walls to form spheroplasts was achieved by incubating the cells in an enzyme cocktail modified from Phillips *et al.*, (2003). Cultured cells were harvested by centrifugation (2,000g) and washed twice in PBS (pH 5.6) before re-suspension in 0.5 ml pre-digestion buffer (50mM K$_2$HPO$_4$/5mM EDTA/50mM DTT: pH 7.2) for 30 min at 30°C. Digestion of the cell wall was achieved at 30°C after re-suspending the cells in 0.5ml of digestion solution (50mM KH$_2$PO$_4$/40mM 2-mercaptoethanol/3μgml^{-1} chitinase/1.8μg.ml^{-1} lyticase (stock 200-1,000 units.g^{-1})/12μl β-glucoronidase/20μl glusulase (Perkin Elmer, Bucks, U.K) in 2.4M sorbitol, pH7.2. Control cells were incubated for 30 min, whereas those exposed with any challenge agent were only digested for 20 min. Protoplasts were washed in modified annexin binding buffer (MABB) (1.2M sorbitol/10mM Hepes/NaOH/40mM NaCl/50mM CaCl$_2$, pH 7.4). Annexin-V binding assays were performed according to the protocol of Madeo *et al.* (1997) in MABB and 20 μlml^{-1} annexin reagent preloaded and incubated at room temperature in the absence of light for 15 min. Spheroplasts were centrifuged at 1700g and washed in MABB before addition of 20 μgml^{-1} PI.

5.3.4 Redox Investigation: intracellular events.

5.3.4.1 Preincubation and conditions for microscopic examination.

Early stationary phase cells were harvested and resuspended in PBS (pH 5.6). Samples (0.5ml) were preloaded with 200nM TMRE at 37°C for 30 min before sample removal to the observation chamber and supplemented with 10mM glucose. The dish containing the yeasts was equilibrated with unrestricted access to atmospheric oxygen on the stage of a Nikon E600FN upright microscope which was maintained at 30°C.

5.3.4.2 Fluorescent probes for two-photon laser scanning microscopy.

The cationic potentiometric fluorescent dye, TMRE, was used to monitor changes in $\Delta\Psi_m$. The large potential gradient across the inner mitochondrial membrane results in the accumulation of TMRE within the matrix compartment according to its Nernst potential (Loew et al., 1993). ROS production was monitored with the ROS-sensitive fluorescent probe 5-(-6)-chloromethyl-2',7'-dichlorohydrofluorescein diacetate (CM-H_2DCFDA 10µM). The acetate group of CM-H_2DCFDA is hydrolyzed by esterases when it enters the cell and is trapped inside as the non-fluorescent 5-(-6)-chloromethyl-2',7'-dichlorodihydrofluorescein (CM-H_2DCFH). CM-H_2DCFH was chosen because, unlike underivatized dichlorohydrofluorescein, it is well retained in cells (Xie et al., 1999) and, in our case, in the mitochondrial matrix (Aon et al., 2003). Oxidation of CM-H_2DCFH by ROS, particularly by hydrogen peroxide (H_2O_2) and hydroxyl radical (Vanden Hoek et al., 1997), yields the fluorescent product CM-DCF, and in an indirect manner, measures mitochondrially-produced O_2^- that has dismutated to H_2O_2 through the action of superoxide dismutase (Chance et al., 1979; Turrens et al., 1985).

Glutathione was monitored intracellularly by production of the fluorescent adduct, glutathione-bimane (GSB) (Kosower & Kosower, 1987) as a result of the reaction of

the cell permeant monochlorobimane (MCB, 50 μM) with reduced glutathione (GSH)
catalysed by glutathione-S-transferase. Reduced nicotinamide nucleotides were
monitored by their autofluorescence under the imaging conditions optimised
previously (Aon *et al.*, 2003).

5.3.4.3 Image acquisition and analysis.

Images were recorded using a two photon laser scanning microscope (Bio-Rad MRC-
1024MP) with excitation at 740nm (Tsunami Ti:Sapphire laser, Spectra-Physics) as
described before (Aon *et al.*, 2003). Briefly, owing to the overlap in the cross sections
for two photon excitation of the three fluorophores of interest (Xu *et al.*, 1996)
(TMRE, CM-DCF, and NADH or GSB) this wavelength permitted recording of $\Delta\Psi$m,
ROS production and NAD(P)H or GSH simultaneously. The red emission of TMRE
was collected at 605±25nm; the green emission of CM-DCF was recorded at
525±25nm; and the blue emission of GSB detected at 480±20nm. NADH emission
was collected as the total fluorescence <490nm. At 3.5s or 30 sec intervals as
indicated, 512 x 512 pixel 8-bit grayscale images of the three emission channels were
collected simultaneously and stored.

While performing kinetics of GSB production in cells, about 10 images were recorded
to get the cellular background of NADH before addition of 50 μM MCB. This is
essentially due to the fact that the NAD(P)H and GSB emissions were collected at
similar wavelengths, i.e. 480-490nm (Aon *et al.*, 2004; Cortassa *et al.*, 2003). At
steady state the NAD(P)H fluorescence levels (n=14) represented 31%±1.2% of the
maximal GSB fluorescence levels attained (n=14) indicating an increase of ~3-fold
respect to the background. Images were analyzed offline using ImageJ software
(Wayne Rasband, National Institutes of Health, http://rsb.info.nih.gov/ij/).

5.3.4.4 Statistical analysis

Data were analyzed with the software GraphPad Prism (Ver. 2; San Diego, Ca.). The statistical significance of the differences between treatments presented as mean ± SEM, (95% confidence interval) was evaluated with a t-test (small samples, unpaired t-test with two tail P-values). The nonlinear regression analysis of the GST kinetic data was performed with a Levenberg-Marquardt algorithm (Microcal(TM) Origin, Northampton, MA). The normality of the data was tested with a Kolmogorov-Smirnov test (GraphPad Prism).

5.3.5 Fluorometric kinetic studies.

The effect of DADS as a function of GSH concentration was further investigated using purified glutathione-S-transferase (GST) and GSH. Increasing concentrations of GSH (in phosphate buffer saline [PBS] pH5.6) in the absence and presence of DADS (0.5mM/1.0mM) was added to 3ml PBS (pH5.6); a subsequent addition of 50µl GST (Sigma, 82 units/mg protein) was used to initiate the reaction. Monochlorobimane (MCB, 2µM final concentration) (Em. 391 nm: Ex. 491 nm) was used as previously described (Aon *et al.*, 2003), and fluorescence measured using a Cary Eclipse Fluorescence Spectrophotometer (Varian, Victoria, AU.).

5.3.6 Cellular respiration.

5.3.6.1 Isolated Mitochondria:

C. albicans was grown in Yeast extract medium containing 2% glycerol. Mid-exponential phase and stationary phase (after 24 h growth). Cell pellets were washed twice in PBS (pH6.4) and suspended in an ice-cold isolation buffer (1.4M sorbitol/50mM K_2HPO_4/10mM $MgCl_2$/1mM EGTA). The cell suspension was

disrupted (Braun homogeniser) using 0.4mm diameter acid washed glass beads for 2 x

1min intervals interspersed by cooling on ice (1:1:1 beads:cell suspension:free-space).

Homogenate was centrifuged 3900g for 5 min at 4°C to remove whole cells and

remaining glass beads. The supernatant was further centrifuged at 9000g at 4°C for 20

min to harvest mitochondria. The pellet (mitochondria) was gently resuspended and

washed in ice-cold isolation buffer, prior to final harvesting (800g/4°C/20min) and

resuspension in ice-cold isolation buffer. Isolated mitochondrial suspensions (50μl)

were incubated in a closed electrode system (Rank, Bottisham, Cambridge, UK)

containing PBS (4ml) at 30°C for measurements of respiration. Oxygen consumption

was measured using 1mM NADH, 10mM potassium succinate, 10mM 2-oxo-glutarate,

2mM pyruvate, 2mM malate and a mixture of 2mM malate/2mM pyruvate. ADP was

used at a final concentration of 10 mM to ensure state 3 was maintained throughout the

entire duration of the experiment.

5.3.6.2 ATPase activity.

ATPase was assayed by proton release measured on adding cell free extract to a stirred

buffer (10mM Tris/H_2SO_4/4mM ATP/6mM $MgSO_4$) pH6.9 at 25°C. A trace of

carbonic anhydrase prevented drift of pH caused by absorption of atmospheric CO_2.

An EIL combination pH-electrode connected to a Johnson Foundation pH-meter

monitored the decreasing pH in the reaction mixture (total volume 2ml). The output of

the meter was connected via an amplifier to a 50mV potentiometric recorder.

Calibration was by adding known volumes of 100mM HCl (Lloyd & Edwards, 1976).

fluorescence measured using a Cary Eclipse Fluorescence Spectrophotometer (Varian, Victoria, AU.).

4.3.8 Analysis of Intracellular ROS using Flow Cytometry.

Fluorescence distributions were obtained from 100,000 events per cell sample through a bandpass filter FL1 using a FACScalibar flow cytometer (BD Biosciences, Oxford, UK). Forward light scatter and side scatter were measured and used for gating the data collection. The fluorescence of gated cell populations was analyzed using validated analysis software, WinMDI 2.8. Intracellular events in a population of *C. albicans* were analysed using CM-H$_2$DCFH as a marker for ROS as described previously (Aon *et al.*, 2003).

4.3.9 Materials.

TMRE, CM-H$_2$DCFDA and MCB were purchased from Molecular Probes, Inc. Or. USA. All other reagents, including enzymes, were from Sigma-Aldrich unless specified. Garlic Powder was from Cultech Biospeciality Products, Swansea, UK.

4.4 Results

4.4.1 Growth inhibition.

Incubation with AA resulted in an extended lag phase and inhibition of growth during exponential phase (Fig. 4.1). Low concentrations of AA present throughout the experiment extended the time which the culture takes to establish; increases in AA concentrations resulted in decreases in growth rate doubling time in control cultures, (0mM AA: 4h; at 0.25mM AA: 8.3h; and at 0.5mM AA: 10.5h).

5.3.7 Materials.

TMRE, CM-H₂DCFDA and monochlorobimane were purchased from Molecular Probes, Inc. Or. USA. All other reagents, including enzymes and DADS, were from Sigma-Aldrich unless specified. Garlic Powder was from Cultech Biospeciality Products, Swansea, UK.

5.4 Results.

Compounds that affect the intracellular thiol redox balance are known to trigger oxidative stress. DADS, present in the garlic extract, is a potential thiol oxidant able to influence the thiol:disulfide redox potential and through this the activity of numerous proteins in the cell. Preliminary experiments showed that DADS triggered oxidative stress in either stationary or exponential phase cultures of *C. albicans*. Thus we decided to investigate its potential deadly effects on this pathogen.

5.4.1 Cell death triggered by DADS.

We exposed cells, harvested from early stationary phase of *C. albicans* cultures, transiently (30 min) to 0.5 mM DADS and investigated its effect on cell death. The kinetics of appearance of apoptotic and necrotic cells was followed for up to six hours after the short exposure to DADS (Fig. 5.1a). These studies were performed by two-photon scanning laser microscopy using exposure of phosphatidylserine (PS) as an early apoptotic marker and propidium iodide (PI) as a marker of non-viable (necrotic) cells (Fig. 5.1b). Cell viability was also assessed by endogenous NAD(P)H fluorescence (Fig. 5.1b).

An immediate increase to 15% in necrotic cells, but not apoptosis, was observed during the transient 30 min exposure to 0.5mM DADS (Table 1). The percentage of

Fig. 5.1
Kinetics of cell death induction in *Candida albicans* after a transient treatment with 0.5 mM DADS. a) Culture grown *C. albicans* cells were harvested in early stationary phase as described under M&M and exposed to 0.5 mM DADS for 30 min at room temperature while untreated cells remained as control. At the times specified after treatment, the cells were washed and subjected to protoplasting as described under M&M. Once protoplasted, the cells were resuspended in Annexin-binding buffer and incubated for at least 30 min with 10 μl of Alexa Fluor 488 annexin V (component A) and 5 μl of propidium iodide (component B) from the Vybrant Apoptosis Assay kit #2 (see M&M) on coverslips coated with polylysine in a thermostated chamber at 30°C with unrestricted access to atmospheric oxygen on the stage of a Nikon E600FN upright microscope.

b) Images of green and red fluorescence, and NAD(P)H autofluorescence were recorded as described under M&M. Representative snapshots of necrotic (red), apoptotic (green), or viable (blue) cells are shown after 4h (top) or 6h (bottom). In all cases, cells viability and integrity was further tested by NAD(P)H autofluorescence and transmitted light images from the same fields. Key to abbreviations: A, apoptotic; N, necrotic; V, viable; DADS, diallyl disulphide.
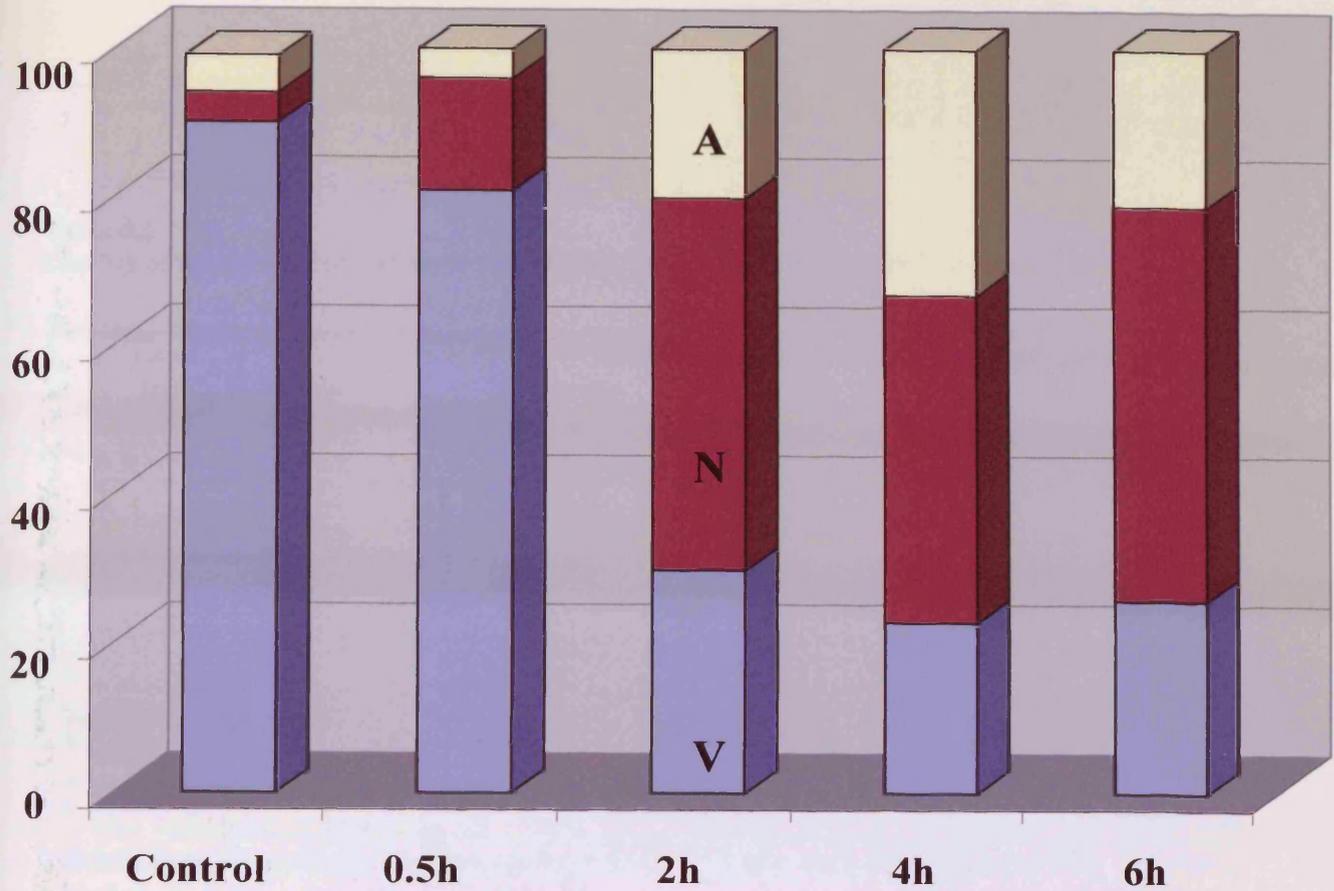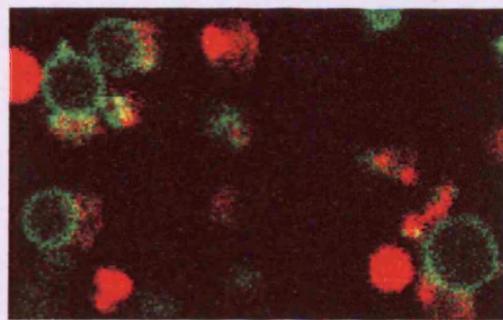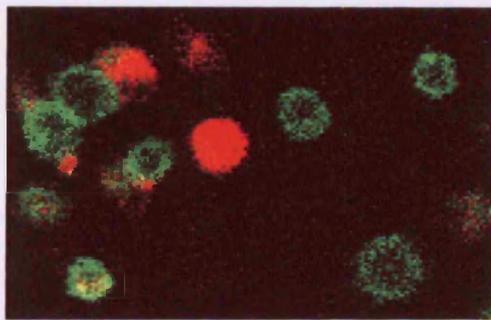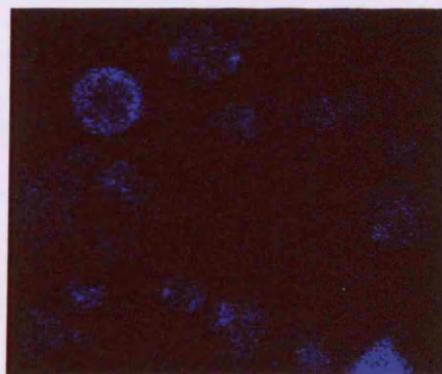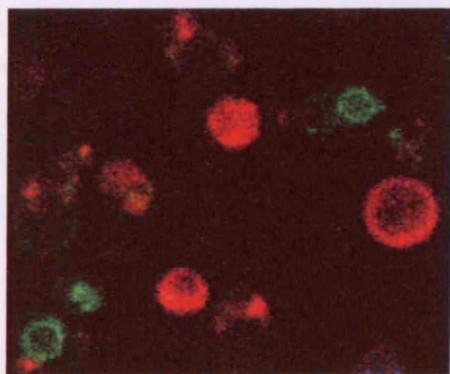
4h after DADS

6h after DADS

Table 5.1.
Kinetics of appearance of apoptotic and necrotic yeast cells after 30 min DADS treatment.

Time (h)	Viable	Cells (%) Necrotic	Apoptotic	Total number of cells
Control[#]	91±2.5[§]	4±1.5	5±0.9	219 (n=8)*
0.5	81±0.7	15±1.4	4±1.4	272 (n=8)
2	30±8.6	50±4.5	20±4.5	674 (n=9)
4	23±5.3	44±7.6	33±5.9	504 (n=9)
6	26±3.6	53±3.6	21±2.6	771 (n=15)

Footnotes to the table:
[§] ±SEM.
* n, is the number of microscopic fields observed.
[#] The following percentage of viable (V), necrotic (N), or apoptotic (A) cells was determined in a parallel culture *without* DADS: Time zero [308 cells; n=10]: V= 90±2; N=5±1.3; A=5±1.5; Time 2h [334 cells; n=7]: V=96±0.6; N=2±0.5; A=2 ±0.5; Time 4h [215 cells; n=7]: V=95±1.7; N=1±0.4; A=4±1.

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necrotic cells increased up to 50% within the next 2 h in the absence of DADS and was paralleled by a significant cell engagement in apoptosis at 2 h (20%) and even further at 4h (33%) (Fig. 5.1a, Table 5.1) as could be judged by the bright green peripheral fluorescence of PS exhibited by yeast (Fig. 5.1b).

5.4.2 Redox investigation:

5.4.2.1 Early intracellular events triggered by garlic components.

Exposure of *C. albicans* to 0.5mM DADS triggered rapid intracellular processes relative to those observed in untreated cells. We monitored key intracellular variables such as mitochondrial membrane potential, $\Delta\Psi_m$, ROS, and reduced glutathione (GSH), with TMRE, CM-DCF, and MCB fluorescent probes, respectively, whereas the NAD(P)H redox status was imaged through autofluorescence (Fig. 5.2). Quantification of signals from each fluorescent probe, as mean values of a normal distribution of the pixel intensities, is illustrated in Fig. 5.3. When *C. albicans* cultures in early stationary phase were treated with 0.5mM DADS a pattern of effects characteristic of oxidative stress was induced, i.e. high ROS, low GSH, and a decrease in NAD(P)H, (Fig. 5.2 and Fig. 5.3b, c); a similar response was observed in exponential phase cells (not shown). Low levels of ROS were localised in the mitochondria in control cells (Fig. 5.2b); increased CM-DCF fluorescence was observed in both mitochondrial and cytoplasmic compartments after treatment with DADS (Fig. 5.2b) and to a lesser extent AA and GPE (not shown). Autofluorescence of mitochondrial NAD(P)H was intense in control samples, but this signal was attenuated by oxidation after addition of garlic constituents (Fig 2c). Observation of the formation of glutathione-bimane product (GSB) used to monitor glutathione levels before and after addition of challenge agents indicated a general decrease in cell

Fig. 5.2.
Fluorescent monitoring of $\Delta \Psi_m$, ROS, NAD(P)H, and GSH from *Candida albicans* with two-photon microscopy. Images of *C. albicans* early stationary phase cells were obtained as described in M&M. Representative images of control and cells treated with 0.5mM DADS after 30 min are shown.
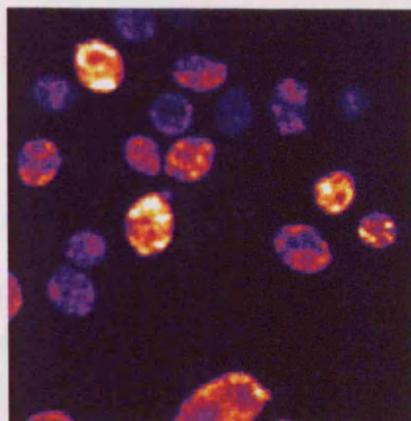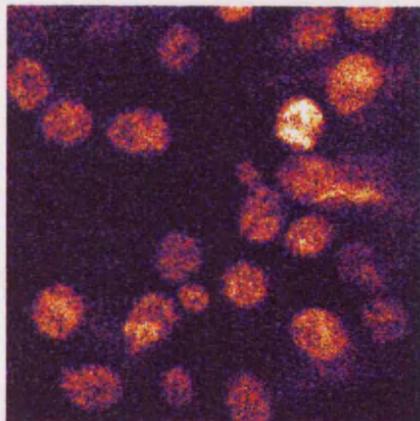
	Control	DADS	
a			$\Delta\Psi_m$ (TMRE)
b			ROS (CM-DCF)
c			NAD(P)H
d			GSB (MCB)
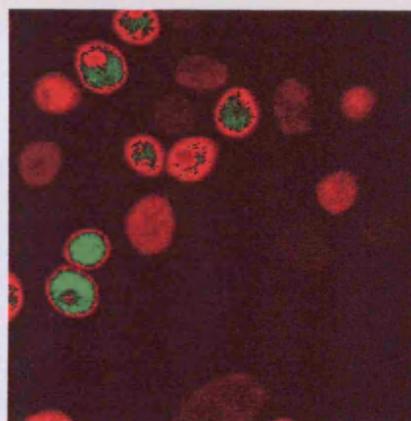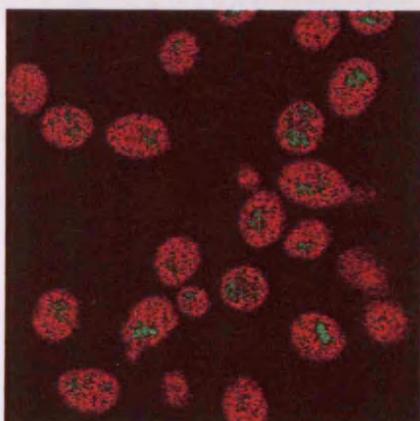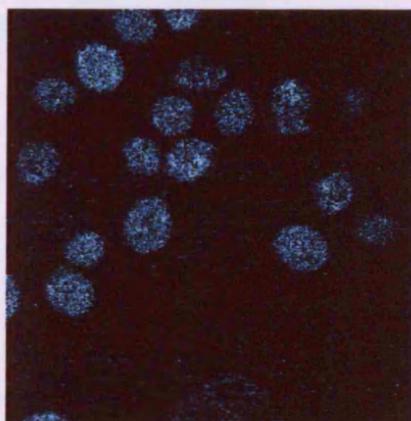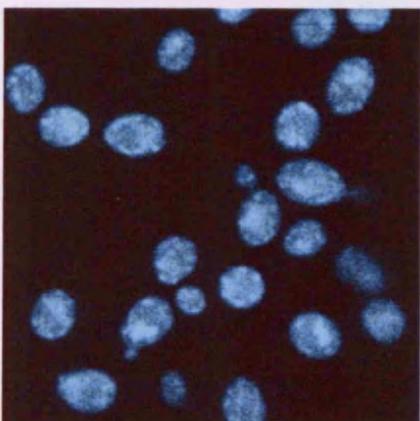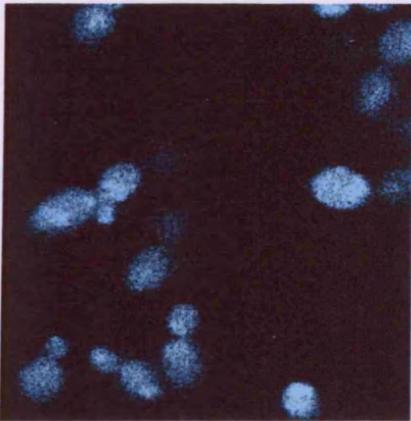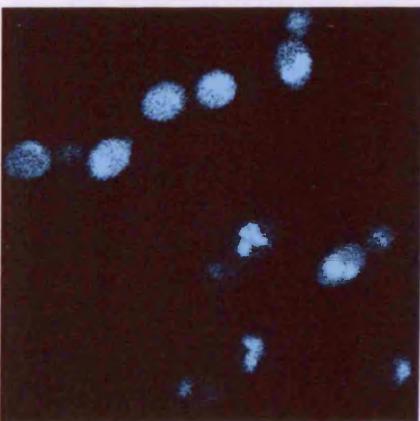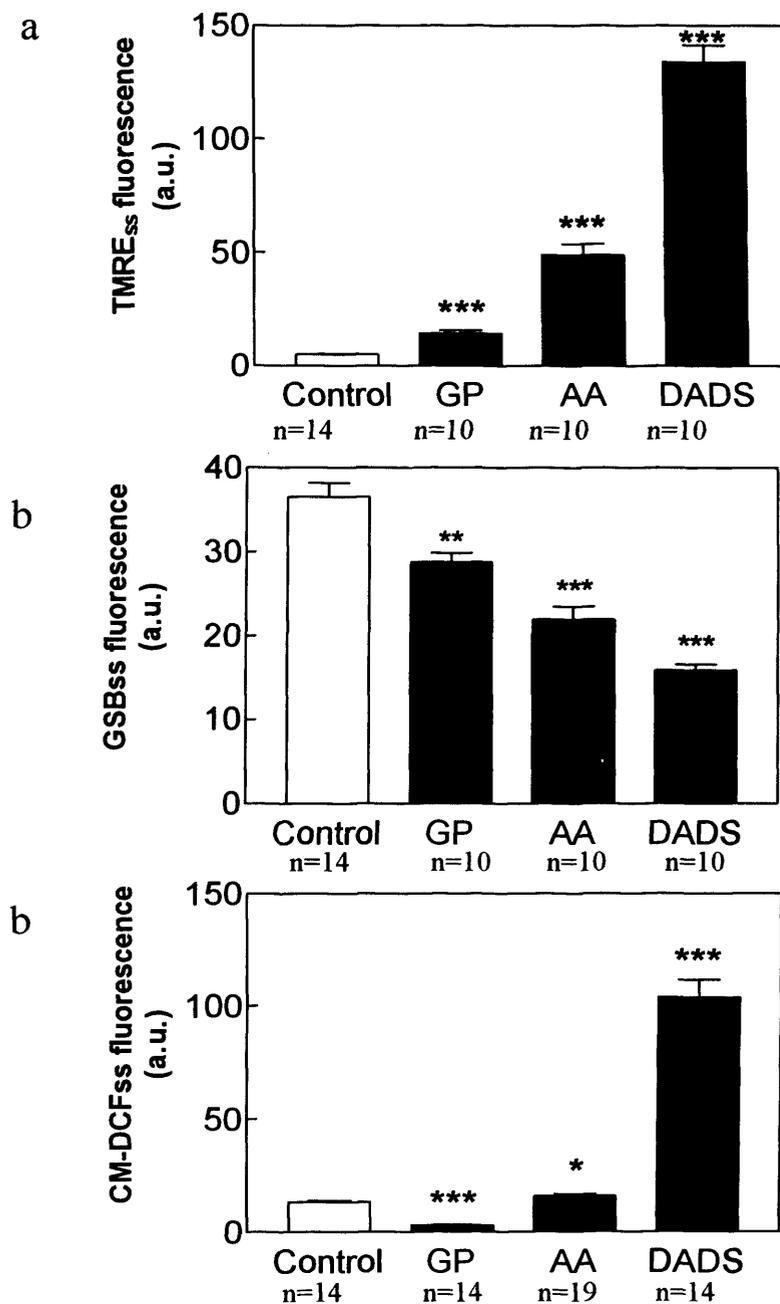
Fig. 5.3.

Quantifying the effects of garlic components on different cellular variables from two photon microscopy images. Culture grown *Candida albicans* cells were harvested in early stationary phase as described under M&M and exposed to different garlic components at room temperature. The steady state level of fluorescence (mean of maximal fluorescence intensity) from TMRE, GSB and CM-DCF probes after 30 min treatment with 0.5mM DADS, or 1.0mM AA, or 5 mg ml^{-1} GP were determined. ***, p<0.001; **, p<0.01; *, p<0.05 *versus* control. Key to abbreviations: AA, allyl alcohol; GP, garlic powder.

fluorescence (Fig. 5.2d). Most significantly, DADS decreased the GSB fluorescence (16 ± 0.7, n=10) below the levels of NAD(P)H autofluorescence control (55 ± 0.9, n=14). Effects of AA and GPE were less significant.

A dramatic and significant increase in $\Delta\Psi_m$ was evident after treatment with DADS as compared to controls or exposure to 0.5mM AA or 5 mg.ml^{-1} GPE (Fig. 5.3a).

5.4.2.2 Mechanistic studies of the effect of DADS.

The intracellular targets of DADS were more thoroughly investigated. GST catalyzes the conversion of MCB and GSH to the fluorescent adduct GSB. In cells, this conversion takes place within minutes, and to a great extent the fluorescent product GSB appeared to be rapidly translocated to the vacuolar compartment where it accumulates (Fig. 5.4). The MCB loading by yeast was not influenced by 30 min preincubation with DADS (n=20; control cells without DADS preincubation=42.4 ± 1.4 versus cells preincubated 30 min with 0.5 mM DADS=44.0 ± 1.8). By the time MCB uptake reaches plateau (15 min loading with 50μM MCB; see Fig. 5.4) no significant differences could be detected between both groups of cells. However, when 0.5mM DADS was added to MCB-loaded yeast, the whole cell fluorescence (mitochondrial and cytosolic combined) was significantly reduced by 27% within 5 min (n=20; baseline=37.5 ± 1.3 versus DADS after 5 min=27.4 ± 1.0) (Fig. 5.5).

Fluorometric analysis of kinetics measured with the purified enzyme was used to establish whether GST could be a target of DADS. Inhibition of GST is evident on increasing concentrations of DADS (Fig. 5.6). The maximal rate of GSB production decreased by 40% on addition of 0.5mM DADS. Similar experiments demonstrated that DADS does not oxidize GSH itself directly (results not shown). Taken together these results suggest that GST is at least one of the intracellular targets of DADS.
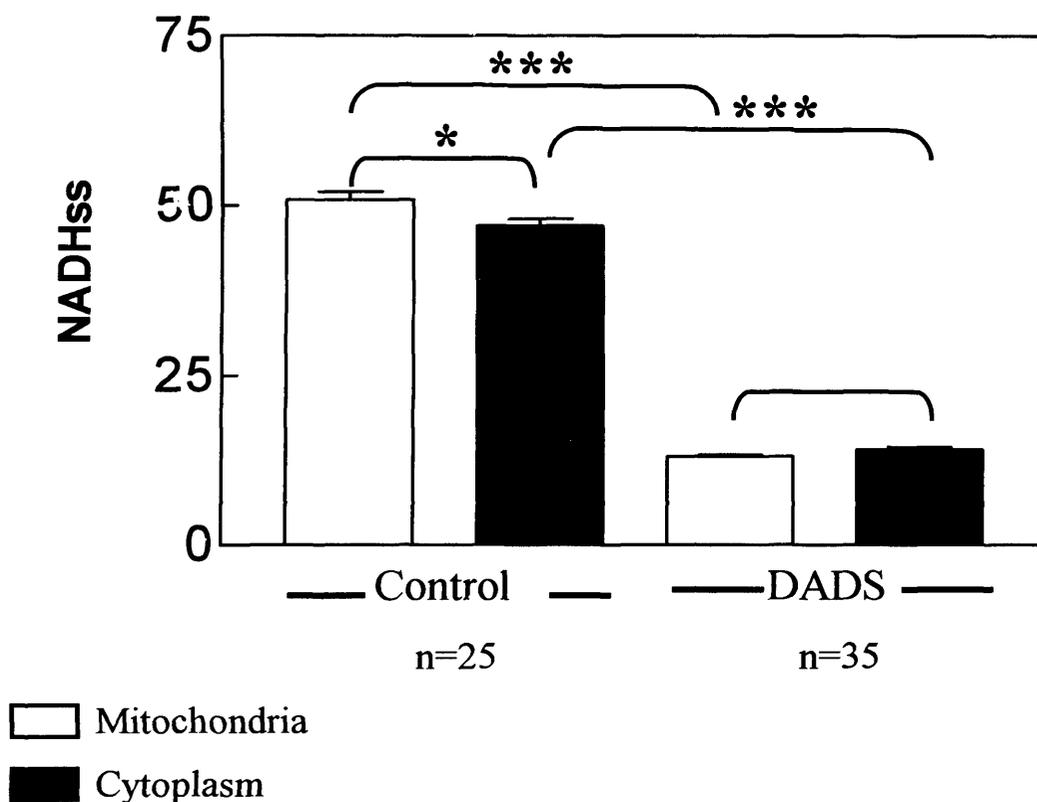
Fig. 5.4.
Effect of diallyl disulphide (DADS) on the cellular redox environment. The intensities of NAD(P)H autofluorescence in mitochondrial and cytoplasmic compartments were determined in culture grown *C. albicans* cells and harvested in early stationary phase as described under M&M. Quantification was performed in cells in the absence (control) or the presence of 0.5nM DADS. Cells loaded with TMRE allowed determination of the regions of interest corresponding to mitochondria or cytoplasm in order to quantify autofluorescence in both compartments. The brackets above the bars indicate the statistical significance corresponding to different compartments and/or the absence or presence of DADS. ***, p<0.001; *, p<0.05; no star, p>0.05.

Fig. 5.5.
Time course of glutathione-bimane (GSB) production in *Candida albicans*. Cells harvested and processed as described in M&M were loaded with 40μM MCB by acute addition of the probe. Cells were imaged at the indicated times after MCB addition in order to follow the kinetics of GSB production (*green* pseudocolor). The progressive vacuolar localization of GSB is quite evident.
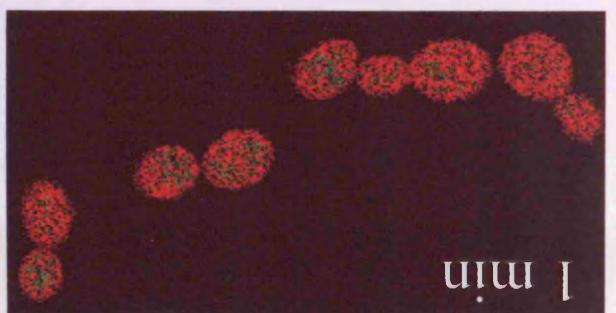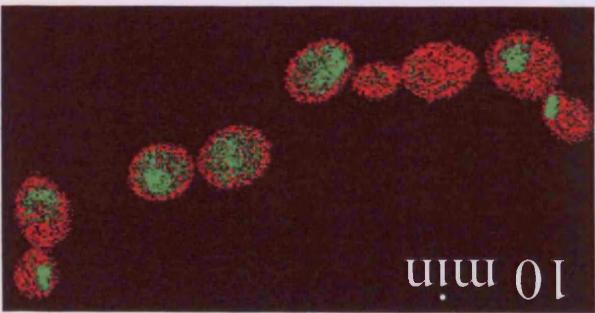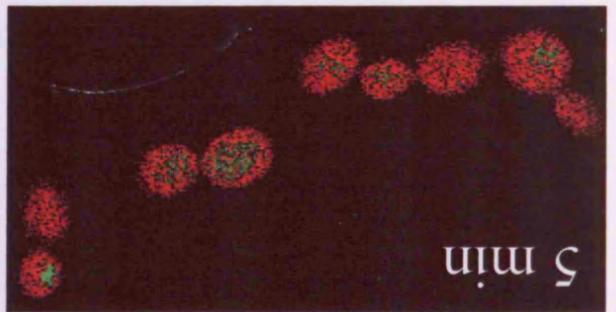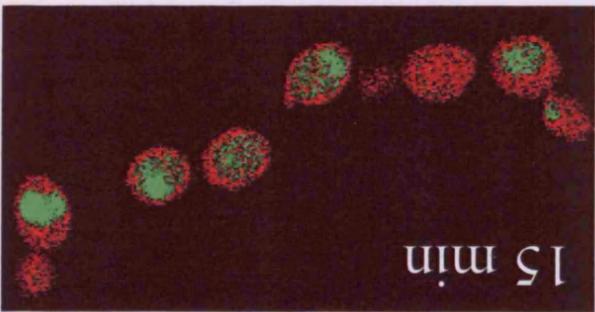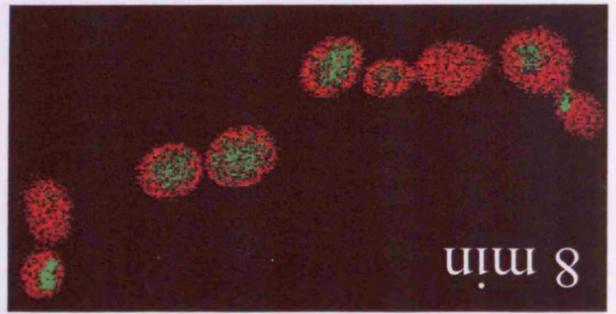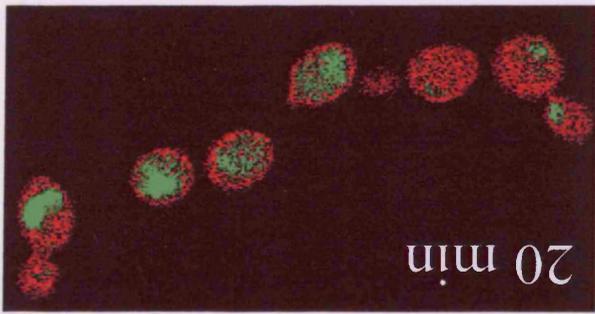
The rapid and significant increase in $\Delta\Psi_m$ effected by DADS was remarkable. We hypothesized that this high $\Delta\Psi_m$ could be related with the induction of state 4 in mitochondria (high $\Delta\Psi_m$, low respiration). In whole organisms, oxygen consumption was decreased by 50% after addition of 1mM DADS, and complete inhibition of respiration was observed at 2mM DADS (data not shown). In suspensions of uncoupled mitochondria, the rate of O_2 uptake was progressively reduced on successive additions of 0.5mM DADS, in the presence of 2-oxo-glutarate, malate and glutamate or NADH, but not with succinate driven respiration (Fig. 5.7b). When using coupled mitochondria and succinate as a respiratory substrate, inhibition of respiration after addition of DADS is observed. Oxygen consumption is reduced by 10% after addition of 0.5mM, and further inhibition observed after successive additions of DADS (up to 1.5mM) (Fig. 5.7a). Inhibition caused by 5µM oligomycin (15% decrease in oxygen consumption), was reversed by addition of 0.5µM CCCP, however, there was no release from DADS inhibition after similar additions of CCCP (Fig. 5.7a). According to our hypothesis, DADS inhibits mitochondrial respiration at two different sites. Previous investigations using *C. albicans* have demonstrated that the matrix-facing NADH-Q oxidoreductase posesses rotenone sensitivity and our results suggest that DADS may behave in a similar manner to this site I inhibitor (Helmerhorst, 2002). A second putative target for DADS is ATP synthase, as its inhibition induces state 4 in mitochondria. DADS inhibited ATP synthase with an IC_{50} of 5.3 nmoles/mg protein. Comparatively, oligomycin (a well known inhibitor of ATP synthase) was less effective than DADS (IC_{50}=16.5 nmoles/mg protein). The inability of CCCP to uncouple mitochondria in the presence of DADS suggests that this thiol reagent could have targeted other inner mitochondrial membrane proteins, most likely by irreversibly changing the oxidation status of critical cysteine residues; previous

Fig. 5.7.

Effect of DADS on mitochondrial respiration. a) Mitochondrial respiration driven by succinate (black vertical arrow). 50µl mitochondria added at light grey arrow. 10mM ADP added at black horizontal arrow. Inhibitors were added as indicated: 5µM oligomycin (grey chevron) and 0.5mM DADS (black chevron) and the uncoupler 0.5µM CCCP (open chevron). Percentage inhibition after each addition is indicated by figures in bold typescript underneath. b) Uncoupled mitochondrial respiration driven by different substrates. 50µl mitochondria added at light grey arrow. Additions of respective respiratory substrate added at black vertical arrow. 10mM ADP added at black horizontal arrow (A) 1mM Succinate control (B) 1mM Succinate test (DADS added) (C) 10mM 2-oxo-glutarate (D) 10mM NADH (E) 2mM malate/2mM pyruvate. Successive additions of 0.5mM DADS were added as indicated by black chevrons. Final % inhibition of respiration was as follows: A=0; B=0; C=55; D=25; E=25.

studies have shown that an alteration in oxidation decreases the ability of the protein to rearrange and stabilise, severely retarding disulfide formation (Gilbert, 1997; Schafer & Buettner, 2001).

5.5 Discussion.

One of the garlic components studied here, DADS, produced cell death in a high percentage of the population. More importantly, the highly effective action of DADS on *C. albicans* was manifested as a long-term effect (2-4 h) after a relatively short (30 min) time exposure (Fig. 5.1).

5.5.1 Glutathione depletion and increased oxidative stress in *C. albicans* treated with DADS.

Early on, during the 30 min treatment with DADS, several intracellular processes related to oxidative stress were activated. Concomitant to a generalized and significant increase in ROS production at the cellular level, was the evident depletion of glutathione and a general oxidation of the NAD(P)H pool after treatment with DADS (Figs. 5.2 – 4). It appears that at least part of the decrease in the fluorescent adduct (GSB) associated with the GSH probe, could be due to inhibition of glutathione-*S*-transferase by DADS. Depletion of the NAD(P)H, the substrate for glutathione reductase diminishes the recovery of the GSH pools in both compartments.

Garlic powder possibly has an attenuating effect on the individual garlic constituents present in its extract, as intracellular events appear to be less damaging (Fig. 5.3).

The antioxidant defences of *C. albicans* are considered paramount for this organism to exhibit full virulence and to resist the host immune response. Thus, GSH is thought to

play a role in the ability of this pathogen to defend itself against ROS and grow in the host successfully. Strong support for this idea originated from research performed using auxotrophic mutants of *C. albicans* for GSH (Baek *et al.*, 2004). These authors showed that mutants of *C. albicans* for glutamylcysteine synthetase (GCS1), an essential enzyme in GSH synthesis, when depleted of GSH undergo apoptosis. Our results are in agreement with these results and further suggest that a transient depletion of the GSH pool was devastating for *C. albicans*.

5.5.2 Mechanism of inhibition of mitochondrial respiration by DADS.

Mitochondrial damage is likely to play a pivotal role in the cell death decision. ROS generation by mitochondria (the major site of free radical generation) together with localised enrichment of anti-oxidants e.g.: GSH, SOD, result in mitochondria mediating a wide variety of controlled biological functions including apoptosis. Altered redox state as a result of mitochondrial respiratory chain inhibition, and loss of $\Delta\Psi_m$ are likely outcomes of treatment with various garlic constituents. Ajoene (20μM), another chemical that can be isolated from garlic is known to dissipate the mitochondrial membrane potential and activate caspases involved in programmed cell death in mammalian cells (Dirsch *et al.*, 2002).

The oxidative stress observed in *C. albicans* after DADS treatment was paralleled by a marked increase in mitochondrial membrane potential (Figs. 5.2 and 5.3) not a decrease as previously reported (Dirsch *et al.*, 2002). However, the characteristics of high membrane potential and low respiration correspond to mitochondria in state 4 and suggest that DADS may target a site on the respiratory electron transport chain. Indeed, DADS was able to inhibit respiration in isolated mitochondria (Fig. 5.7) and in

whole cells (data not shown). ATP synthase inhibition in isolated mitochondria is observed in a manner similar to oligomycin (refer to IC_{50} values as above). The latter further supports our interpretation that DADS induces state 4 in *Candida* mitochondria by its inhibitory effects on ATP synthase. As there is no release from the DADS inhibition on addition of the uncoupler CCCP in whole cells, another target of DADS that lies on the respiratory chain prior to the ubiquinone pool is indicated. This is shown by the lack of sensitivity of succinate-driven respiration after acute additions of DADS in isolated mitochondria (Fig. 5.7). Our results obtained with isolated mitochondria suggest that DADS may inhibit mitochondrial respiration at two different sites: ATP synthase and upstream of complex II. The former site of DADS action is suggested by its drastic inhibition of ATP synthase as compared with oligomycin (i.e. three-fold lower IC_{50}'s were observed in the presence of DADS). The latter is in agreement with our interpretation that DADS indues state 4 in *Candida* mitochondria by its inhibitory effects on ATP synthase. However, that there was no release from DADS inhibition on addition of the uncoupler CCCP, suggests that another target of DADS lies on the respiratory transport chain prior to the ubiquinone pool (Fig. 5.8). This is shown by the lack of sensitivity of succinate-driven respiration in uncoupled mitochondria after acute additions of DADS (Fig. 5.7b, trace b). Previous investigations using *C. albicans* have been demonstrated that the matrix-facing NADH-Q oxidoreductase possesses rotenone sensitivity and our results suggest that DADS may have behaved in a similar way (Helmerhorst *et al.*, 2002). These early effects of DADS on increasing *Candida* mitochondrial membrane potential along with oxidative stress are partially different to observations performed in other systems in which a decrease of the mitochondrial membrane potential is an early event initiated by oxidative stress (Richter, 1998). It is likely that even small perturbations may alter
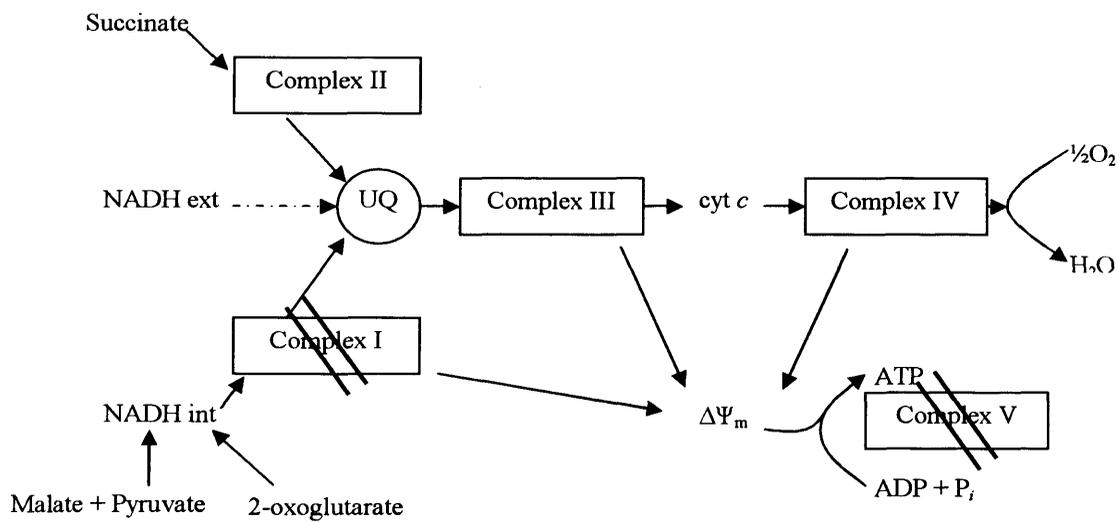
Fig. 5.8.
Schematic diagram indicating potential sites of inhibition by diallyl disulphide
(DADS), (double cross hatch lines).

the cascade of events resulting in apoptotic cell death or at least, a decrease in functional capability.

5.5.3 Cell death in *C. albicans* by necrosis and apoptosis following treatment with DADS.

The immediate necrotic response in a cell sub-population, followed by a chronic apoptotic death of a large percentage of cell population is potentially an extremely important phenomenon, particularly as exposure to a low concentration DADS (0.5mM) was transient, only 30min and being removed thereafter. Although the effect of DADS on the GSH pool seems to be at the cellular level (Fig. 5.5), the efficacy of DADS beneficial capacity as a therapeutic agent is likely to be due to its specific targeting of mitochondrial GSH pool; the consequences of this attack are predictors of the onset of cell death in *C. albicans*. Taken together, these results point out that the drastic increase in mitochondrial membrane potential observed in intact cells by two photon microscopy (Figs. 5.2 and 5.3) was mediated by effects of DADS on mitochondrial respiration. Moreover, the higher ROS levels in the presence of DADS are likely to be due to a decrease in the antioxidant defences, i.e. GSH (Figs 5.3 and 5.5).

The depletion of GSH is perhaps the more relevant and controlling factor, as the ability to minimise oxidative stress is thereby diminished. Although the effect of DADS beneficial capacity as a therapeutic agent is likely to be due to its specific targeting of the mitochondrial NAD(P)H and GSH pools, the consequences of this attack are predictors of the onset of cell death in *C. albicans*. The inability of mitochondria to remove these damaging free radicals results in their accumulation to above a threshold where continuing cell viability is impossible. Furthermore, as DADS induces a drastic

oxidation of the mitochondrial NAD(P)H pool (Fig. 5.2 and 5.4) and further reduction of GSSG by glutathione reductase is prevented; this exacerbates the detrimental effect on the cell. In this scenario, the eventual route to death appears to be dependent on the concentration of the initial exposure to the agent.

5.5.4 Concluding remarks.

Taken together, the results obtained indicate that increased oxidative stress through decreased GSH levels mediate the drastic death response induced by DADS in *C. albicans*. Impairment of mitochondrial respiration, elicited by the action of DADS on ATP synthase and a site close to the rotenone-sensitive site within complex-I, also appear to be involved in the death response. In summary, we have described a promising therapeutic agent for *C. albicans*.

5.6 References

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6. CHAPTER SIX

6.1 Introduction

This thesis has investigated several areas:

- Anti-fungal properties of garlic and identification of constituents, present in garlic extract that are potentially responsible for its efficacy. Selection of two chemically contrasting constituents allyl alcohol and diallyl disulphide for further investigation (Chapters 2-4).

- Cell death routes/mechanisms; apoptosis and necrosis induction (Chapters 3-5).

- Potential targets in *C. albicans* of garlic extract, allyl alcohol and diallyl disulphide. (Chapters 3-5).

Main over-riding topics will be discussed here, as detailed discussions are covered in the individual chapters.

6.1.1 Anti-fungal properties of Garlic

Candidal infections are becoming intrinsically more and more difficult to treat due to inadequacies of the commonly-used anti-fungal drugs as well as limited clinical experience available for many of the newer drugs. Amphotericin B remains the treatment of choice for invasive fungal infections, but alternative treatments are sought because of the rising incidence of pathogen resistance and the serious side effects of the drug, particularly its nephrotoxicity (Bell *et al.*, 1962; Churchill & Seely, 1977). Relatively 'safe' antifungals such as fluconazole are not broad spectrum enough to be of use to a significant percentage of patients who would be deemed as requiring antifungal prophylactic treatment. Early intervention against fungal disease is one of the best predictors of treatment success.

It is for these reasons that an ideal antifungal agent would be broad spectrum, inexpensive, free from negative side effects, and synergistic with existing treatments. It would also be a treatment unlikely to induce resistance in the infective organism, and it would be able to stimulate a cellular immune response. Such an ideal agent would also make an ideal prophylactic agent, giving protection to at-risk clinical groups (Davis, 2005).

The antifungal, antibacterial and anticancer properties of garlic and its derivatives are well known (Ghannoum, 1988; Lemar *et al.*, 2002, Sundaram & Milner, 1996), but only now are investigations being undertaken to understand the mechanisms of action of garlic extract and individual constituents (Shen *et al.*, 1996; Davis *et al.*, 1990).

Garlic and its antifungal derivatives appear to satisfy many of the criteria for useful antifungal agents. Garlic and constituents are cheap, readily available, and appear to be effective. *In vitro* studies have demonstrated broad spectrum antifungal activity of various consituents; allicin has an MICs of 2–16 $\mu g\,ml^{-1}$ against 31 clinical isolates of *Aspergillus fumigatus* (Pai & Platt, 1990; Shadkchan *et al.*, 2004), MICs for 'allitridium' (a synthetic garlic compound) in the range of 4-16 $\mu g\,ml^{-1}$ for activity against *Scedosporium prolificans* (Davis & Perrie, 2003a) and of less than 0.25 $\mu g\,ml^{-1}$ for activity against *Cryptococcus neoformans* (Davis & Perrie, 2003b). Furthermore, according to reports, allitridium is non-toxic to humans; it has been successfully used intravenously on a daily basis for over a month (Davis, 2005), and is synergistic with amphotericin B *in vitro* (Shen *et al.*, 1996). In addition garlic appears to be immunomodulatory, enhancing cellular immunity within the host (Davis, 2005).

Aqueous garlic extract was found to inhibit the growth of *C. albicans* (Ghannoum, 1988). Further investigations established that lipid synthesis of the yeast was blocked, which in turn, inhibited both protein and nucleic acid synthesis (Adetumbi *et al.*, 1986) Interestingly, a later study on the inhibition of human cholesterol synthesis by various allicin breakdown products, including diallyl trisulfide and *S*-allyl cysteine (a sulphur-containing, biologically-active water soluble allicin breakdown product), implicates them as inhibitors of squalene monooxygenase, an essential enzyme for the biosynthesis of cholesterol (Gupta & Porter, 2001). Squalene monooxygenase is also important in the formation of the fungal plasma membrane. The allylamines (a class of antifungal compounds containing a allyl group naturally found in garlic), cause squalene to accumulate, preventing the formation of ergosterol, essential for the plasma membrane synthesis (Balkis *et al.*, 2002). Cell 'blebbing' and other morphological abnormalities evident in our experiments, may well be attributed to a similar inhibitory mechanism.

6.1.1.1 Selection of allyl alcohol and diallyl disulphide.

The choice of allyl alcohol and diallyl disulphide for our investigations was made in order to compare two chemically different constituents that were both reportedly inhibitory to microbial species. Diallyl disulphide is also the predominant organic sulphur compound in garlic extract (Milner, 2001; Bose *et al.*, 2002), and the general reaction between sulphide-containing molecules and sulphydryl (SH) groups in amino acids and cellular proteins within these organisms has been linked to its antimicrobial activity (Naganawa *et al.*, 1996; Ross *et al.*, 2001). Allyl alcohol does not contain a sulphur atom, but has been shown to be an effective antigiardial (Harris *et al.*, 2000). It is known inhibitor of alcohol dehydrogenase, and much of its activity in *C. albicans* is likely to be attributed to this mechanism.

Morphological abnormalities effected by DADS and AA in comparison to those produced by garlic extract, can be attributed to the relative concentrations of each constituent present in the extract. Definitive studies must be performed to determine the concentrations of active components produced after trituration for each occasion when an extract (fresh or freeze-dried) is prepared.

6.1.2 Cell death Mechanisms.

Necrosis is a passive and degenerative process by which cells and their organelles swell and break open, releasing their contents, which in turn, can potentially damage neighbouring cells. This response is triggered when the viability of the cell is completely compromised after exposure to a gross injury, and is often associated with exposure of organisms to high doses of cytotoxic agents (Darzynkiewicz *et al.*, 1995).

Apoptosis is a programmed mode of cell death, and depends on the subsequent activation of specific cellular processes (*i.e.* at the plasma membrane, or within mitochondria), gene transcription, and protein synthesis. This sequence of events ultimately leads to the activation of caspases, which, in turn, partially hydrolyse specific cellular proteins. The possibility that these pathways of control could be exploited for therapeutic treatment has been a focus of much research in recent years.

Garlic-derived sulphur constituents, including diallyl sulphide (DAS), diallyl disulfide (DADS) and diallyl trisulphide (DATS), have been shown to offer significant protection against highly proliferative tumour cells in animal models (Wargovich *et al.*, 1992; Wattenberg *et al.*, 1989; Sumiyoshi and Wargovich, 1990; Schaffer *et al.*, 1996). Recent investigations suggest that diallyl trisulphide possesses a greater activity than diallyl

disulfide, (and DAS thereafter) (Tsao & Yin, 2001; Xiao *et al* 2004); potentially due simply to an additional sulphur atom available for interaction with the targeted organism or yeast cell. Further investigation would be required to determine whether this observation can be extrapolated to the anticandidal efficacy.

More specifically, sulphur constituents cause inhibition of proliferation of cultured cancer cells by causing apoptosis and/or cell cycle arrest (Sundaram & Milner, 1996; Knowles & Milner, 1998, 2001; Nakagawa *et al.*, 2001; Filomeni *et al.*, 2003; Xiao *et al.*, 2003a). The first report of apoptosis induction by DADS was observed in human colon cancer cells (Sundaram & Milner, 1996). Even though these studies have provided convincing evidence to implicate apoptosis induction by DADS and DATS the sequence of events leading to proapoptosis effects of sulphides is poorly defined, and scientists are utilising more simple models, involving yeasts, as an aid to studying apoptosis. It is still unclear however, why a unicellular organism like yeast possesses a suicide program.

Until the mid-90's, all apoptosis research had been conducted with animal cells. With the discovery that *S. cerevisiae* provides an extremely convenient 'model' for investigation of the genetics and biochemistry of the apoptotic mechanism, an explosion of interest in cell death mechanisms in lower eukaryotes has occurred (Moreira *et al.*, 1996; Matsuyama *et al.*, 1997; Lewis, 2000; Tan *et al.*, 2001).

In recent years, it has been convincingly shown that a number of other unicellular organisms also possess the ability to undergo programmed death (Lam *et al.*, 1999; Raff, 2000; Skulachev, 2001, 2002; Madeo *et al.*, 2002). In particular, the yeast *Saccharomyces cerevisiae* dies in an apoptosis-like way in response to various harsh

treatments (Madeo *et al.*, 1999; Ligr *et al.*, 2001; Ludovico *et al.*, 2001), and similar observations are reported for *C. albicans* (Phillips *et al.*, 2003). It has been argued that a physiological role of suicide in a unicellular organism is to increase the fitness and dynamicity of the whole cell community (see Skulechev, 2001, for review). In other words, altruistic yeast cell death could possibly be physiologically relevant only when yeast cells are part of a community (Knorre *et al.*, 2005).

Intercellular communication via sexual pheromones causes *S. cerevisiae* to die in a way resembling apoptotic death in higher cells (Severin & Hyman, 2002). This phenomenon has been linked to programmed cell death (PCD) in yeast populations, whereby when lots of mating partners are available, the elimination of a cell unable to mate could be beneficial for the population (Skulachev, 2002; Severin & Hyman, 2002). Although there is some evidence of a mating cycle in *C. albicans*, (Hull *et al.*, 2000) it is generally accepted as having 'asexual' reproduction and therefore an altruistic response is less easy to explain.

Furthermore, exposure of yeast cells to certain oxidants results in rapid accumulation of reactive oxygen species (ROS), a common trait in organisms that undergo apoptosis (see Skulachev, 1999, for review). Depletion in glutathione and other pools of redox components is intrinsically linked to elevation in ROS, and often it is difficult to determine which is the cause or consequence of the other. Recent investigations however, have observed that *S. cerevisiae* grown in glycerol-based media accumulate ROS, while those grown in raffinose-based media (therefore possessing poorly-energized mitochondria) are resistant to acetate-induced ROS formation (Knorre *et al.*, 2005). A

specific mitochondria-dependent cell death cascade is assumed to be initiated as cell death was observed soon after (Knorre *et al.*, 2005).

Specifically, it is likely that the apoptotic responses observed in *C. albicans* after treatment with garlic and its constituents can be explained by similar hypotheses. Exposure to DADS increased ROS levels in *C. albicans*, and these observations are consistent with previous results obtained with DADS, which suggest that it may represent an oxidizing agent able to induce oxidative stress-mediated cell death (Filomeni *et al.*, 2003). Interestingly, studies in another pathogenic yeast, *Cryptococcus neoformans*, have shown that DADS treatment does not alter glutathione redox state or related enzymes activity, (Shen *et al.*, 1996), suggesting that it is through the direct generation of ROS, rather than alteration of antioxidant defence system, that may be responsible for DADS-induced oxidative stress (Xiao *et al.*, 2004).

S. cerevisiae lack the main regulators of apoptosis (caspases, Bcl-2 family members) found in mammals (Fleury *et al.*, 2002), and until recently, apoptotic-like cell death observed in yeast was disputed. Some mammalian pro- or anti-apoptotic proteins induce or prevent cell death in *S. cedrevisiae*, as well as certain mutations and after addition of various toxicants (Madeo *et al.*, 1999, Ludovico *et al.*, 2001). A caspase, which is activated by H_2O_2 or aging, is required for the protein-synthesis-dependent death of *S. cerevisiae* and thus, a specific apoptosis-mediating protein was identified for the first time in a yeast (Madeo *et al.*, 2002). Several labs have now observed that yeast exhibit other characteristics similar to those of apoptotic mammalian cells, including externalized phosphatidylserine (PS) on the outer leaflet of the plasma membrane (detected by Annexin V binding), DNA fragmentation (detected by TUNEL (Terminal

deoxynucleotidyl Transferase Biotin-dUTP Nick End Labeling) assays), and chromatin condensation. Use of these criteria to support the existence of "apoptosis" in yeast is still debated, partly because it is not known if the events responsible for the "apoptotic" morphology contribute in any way to yeast cell death. Exposure of PS on the surface of a dying *S. cerevisiae* or *C. albicans* cells (Madeo *et al.*, 2002; Phillips *et al.*, 2003), may not be a signal for engulfment by neighboring cells, as suggested for animal tissues, and it has been suggested that a deteriorating yeast cell simply fails to continually flip PS back into the plasma membrane (Harwick & Cheng, 2004).

6.1.3 Identification of targets of garlic extract, DADS and AA.

At least three general mechanisms of mitochondrial involvement in apoptotic induction are known in higher eukaryotes (Green & Reed, 1998) and their effects may be interrelated; (i) disruption of electron transport, oxidative phosphorylation, and adenosine triphosphate (ATP) production; (ii) release of proteins that trigger activation of caspase family proteases as discussed earlier; and (iii) alteration of cellular reduction-oxidation (redox) potential as discussed previously.

During the course of normal aerobic conditions, and after exposure to radical-generating compounds, cells are exposed to a range of reactive oxygen species. Such ROS can damage a wide range of macromolecules in the cell including nucleic acids, proteins, lipids, eventually leading to cell death. Cells contain various antioxidant defence mechanisms to protect against ROS, including enzymes such as catalase, glutathione peroxidase and superoxide dismutase as well as compounds which can bind and inactivate ROS directly such as glutathione and uric acid (Grant & Dawes, 1996; Stephen & Jamieson, 1996). Oxidative stress occurs when a proportion of ROS evade or overcome

this defence system, resulting in oxidative damage and in some cases cell death. Yeast

cells, in response to oxidative challenges are no different and increase the synthesis of a

number of enzymes involved in detoxification of ROS including glutathione reductase

and thioredoxin (Grant *et al.*, 1997). Garlic, AA and DADS have been shown in these

investigations to deplete these defensive pools that would normally serve to remove the

harmful ROS; this results in a fatal accumulation of ROS and ultimately leads to cell

death.

The targetting of mitochondria by garlic and the individual constituents was investigated.

The effects on oxidative phosphorylation were not completely elucidated, but only DADS

decreases the rate of oxygen consumption in whole cells. Perturbation of the Ψ_m, was most

evident after addition of DADS, as indicated by the hyperpolarised state (Fig 5.2).

Attempts to elucidate specific sites that DADS is targetting have led us to believe there

are at least two points of inhibition, one at a site upstream of Complex I and the other at

ATPsynthase; investigations to determine which subunit is inhibited remain to be

performed. These results however, further substantiate reports that apoptosis does indeed

occur in unicellular organisms via mechanisms similar to those in higher eukaryotes.

It has been reported that *C. albicans* contains a variety of respiratory systems whose

expression depends on growth conditions (Helmerhorst *et al.* 2002); these are not present

in other yeasts such as *S. cerevisiae* or *Schizosaccharomyces pombe* (Schatz & Racker,

1966; Balcavage & Mattoon 1967). The alternative oxidoreductase pathway (AOX) is

terminated by an alternative terminal oxidase that reduces oxygen to water, but is

distinguished by its insensitivity to cyanide and antimycin A and its sensitivity to

hydroxamic acids such as salicyl hydroxamic acid (SHAM). It is induced by respiratory

chain inhibitors that act downstream from coenzyme Q, or by oxidative stress, suggesting a role in the protection against oxidative damage (Minagawa *et al.*, 1992; Popov *et al.*, 1997; Maxwell *et al.*, 1999). *C. albicans* has the ability to regulate the expression of individual complexes by the aportionment of electrons between these two respiratory chains (classical and alternative oxidase pathways) and Helmerhorst *et al.*, (2002) further suggested that this may be the reason why *C. albcians* cells can survive under conditions of oxidative stress; recent investigations with *C. albicans* and *C. parapsilosis* further support this theory (Cavalheiro *et al.*, 2004). It is possible that elevated levels of ROS after exposure to one or more of the garlic constituents may induce the AOX pathway, and further investigations would be necessary to investigate this possibility.

Of additional interest is the discoveiy of a rotenone sensitive and proton-pumping NADH-Q oxidoreductase similar to mammalian Complex I in *C. albicans* (Helmerhorst *et al.*, 2002). This research demonstrates that genes for Complex I are expressed and functional in *C. albicans*. This is another potential therapeutic target against this opportunistic pathogen, and that targeting of Complex I by DADS is feasible. Further support that Complex I may be the site of acion of DADS comes from research investigating the effects of perfumes as mitochondriotoxins (Griffiths, D.E., personal communication to Lloyd, D). Many perfumes are able to cross membranes by virute of their lipophilic nature, and in turn may react with organelles (Sikkema *et al.*, 1995), and studies with β-pinene (Uribe *et al.*, 1984), and cinnamaldehyde (Usta *et al.*, 2002) report a reduction in $\Delta\Psi_m$. With many perfumes being derived from plant materials it is therefore not unlikely that similar inhibitory properties may be expected with garlic-derived constituents. DADS, however, appears to hyperpolarise $\Delta\Psi_m$, therefore further investigation would be required to explain this apparent contradiciton. Reports of chemical fragrants interacting

at other mitochondrial sites (Griffiths, DE, personal communication to Lloyd, D), including $F_1F_0ATPase$ (ATPsynthase), may also support our results that garlic constituents inhibit ATPsynthase.

A mechanism for AA inhibition most likely involves alcohol dehydrogenase; where conversion to acrolein, which reacts with glutathione, is followed by peroxidation of cellular lipids. Ultimately the viability of an organism is affected, as the permeability of the plasma membrane is detrimentally impaired. This is confirmed by our investigations that the Ψ_{pm} is immediately depolarised after addition of low concentrations of AA. Again the extent to which garlic acts will be dependent on the level of AA present in the extract.

6.2 In conclusion

The efficacy of garlic is likely to depend on a complex combination of the many different constituents present in the extract, each acting on different targets. That the extract undergoes a continual chain of degradative events, ever yielding a succession of potentially active constituents, may contribute to the fact that, so far no developing resistance has been observed. These complex mechanisms of action and variety of targets, makes garlic an attractive tool for use in combating Candida infections. Garlic derivatives could make safe and effective antifungal prophylactics and treatments. The recent dramatic increase in the studies of the anticancer properties of garlic will further increase its popularity. Once the relative safety of the constituents and derivatives has been established, it will be only a small step to seriously look at the possibility of their use as antifungals.

6.3 Further Work

- Concentrations of allyl alcohol and diallyl disulphide present in garlic extract should be determined at the point of experimentation. This can be achieved through accompanying GC-MS analysis and use of an internal standard (*e.g.:* methyl propyl sulphide) for quantification (Harris, 2001; Cottrell, 2003).

- Use of the fluorimetric probe, diethidium can be used to monitor superoxide levels directly and determine levels attributable to the increasing ROS levels that lead to cell death.

- Induction of a hyperpolarised mitochondrial potential after DADS addition, contradicts the effects of DADS reported in some literature. Further investigation is necessary to explain this observation. The dye TMRE is electrophoretically accumulated within the inner membrane of the mitochondria due to its positive charge. Its redistribution to the cytosol is observed on depolarisation of the mitochondrial membrane. However, fluorescent probes undergo a phenomenon called 'dequenching', which produces a paradoxical surge of fluorescence on exudation of the dye from its membrane bound location where its high local concentration has led to quenching by dimerisation (Duchen *et al.*, 2003). Polarization-depolarization of mitochondrial membranes should be further investigated using this method.

- Inhibition of ATPase can occur by targetting either of one of the two components F_1 or F_0. Distinguishing at which sub-unit DADS acts is necessary. Use of the purified F_1 'head-piece' and inhibitors such as oligomycin (F_1), as a control should be performed.

- In this study focus was primarily on the major non-enzymic defence mechanisms against ROS (glutathione). Further work should determine the effects on the

proctective enzymes whose function would normally serve to mobilse or remove the ROS, *e.g.*, superoxide dismutase and catalase etc.

- Apoptotic studies in yeasts are the focus of much attention, and improved experimental prcoedures allow more conclusive evidence that programmed cell death does indeed occur in these unicellular organisms. Standard criteria could be checked (*e.g.*: TUNEL assays, assessment of Ca^{2+} loss and DNA laddering) could be performed to further confirm results described in this investigation. More recent work has monitored changes in mitochondrial cardiolipin distribution as an indicator of apoptosis and could also be exploited (Garcia Fernandez *et al.*, 2002; Lloyd *et al.*, 2005).

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