

Modification of ATPase Activity in Relation to Azole and Polyenes in *Candida albicans*

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Candida albicans is a widespread opportunistic pathogen in immuno-compromised patients. ATPase activity and protein concentration using spectrophotometric method and antifungal activity by Microtitre / ELISA technique in terms of Minimum Inhibitory Concentration (MIC) was studied to understand the pattern of *Candida albicans*. Minimum Inhibitory Concentration for Clotrimazole and Nystatin showed 6.25µg/ml and 8.0µg/ml "respectively". At optimum pH (7.0) and temperature (28°C), 100% activity of ATPase was recorded. pH showed inverse relationship while temperature showed no remarkable variation of activity. ATPase was found to be 5.0% reduced by Clotrimazole, whereas Nystatin caused 39% inhibition at the optimum pH and temperature.

Key words: *Candida albicans*, Candidiasis, ATPase, Nystatin and Clotrimazole.

Candidiasis is an acute or chronic, superficial or disseminated mycoses caused by the species of *Candida*. *Candida albicans* is commensal of the human oral cavity, the gastrointestinal and vaginal tract. It is most common opportunistic fungal pathogen that causes superficial infections on these sites Cannon *et al.*, (1995). It is a dimorphic fungus that can grow either as yeast or as mycelia. The mycelial form may be required for tissue penetration and therefore may have a role in pathogenesis. Germ tube formation is an early stage of the yeast to mycelia transition and may enhance *Candida* cell adhesion to the host cell surfaces, mediates tissue penetration and help fungus to evade host defenses.

Candida albicans is the most common species of the yeast isolated from patients with oral candidiasis Fidel (2006). *Candida albicans* also causes systemic or life threatening disseminated candidiasis in immuno-compromised host such as patients with cancer or Acquired Immuno Deficiency Syndrome (AIDS) or who have been treated for prolonged period with immunosuppressive agents Bodey 1988, Coleman *et al.*, (1993). Oral candidiasis, the most common opportunistic infection in HIV patients is usually associated with *Candida albicans* Patton *et al.*, (2002) and Mrudula patel *et al.*, (2006) The ability of *Candida albicans* to infect host tissue has variously been attributed to adhesion, dimorphic transition, secretion of hydrolytic enzymes (including proteinases) and influence of ions on the dimorphic transition of *Candida albicans* Frederick *et al.*, (1997) and gradual reduction of host defense mechanism Cannon *et al.*, (1995).

Over the last three decades, there has been an increase in the incidence of candidiasis, which is attributed to the widespread use of antibiotics and immunosuppressive drugs. High doses of antibiotics seem to increase their growth

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and multiplication leading to increased virulence. *Candida* species represent the fourth most common nosocomial bloodstream pathogen during 1993 and accounts for 8% incidences Emori and Gaynes (1993). *Candida* species continue to be "champion" nosocomial pathogen besides other fungal competitors. A surveillance study in the US showed 9% of incidences were of *Candida* species Hilmar *et al.*, (2004). Candidiasis due to *Candida albicans* is AIDS indicative disease Chandler and Selik, (1989). During 1990-91 CDC Atlanta, Georgia reported that AIDS patients suffering from opportunistic candidal infections with high prevalence, and this organism has been recognized as important cause of morbidity and mortality in AIDS patients. During 1985 *Candida albicans* account almost 100% fungal infections in AIDS, Chandler, (1985). *Candida albicans* was the commonest isolate 70% in AIDS with oral candidiasis. Various studies have shown increasing incidences of candidiasis in HIV infected persons in India Ramakrishna *et al.*, (2000), Kaviarasan *et al.*, (2002) and Latif *et al.*, (2004).

In India there is a considerable variation of 9-80% in the incidences reported on the occurrence of *Candida* species in the sputum of patients with pulmonary candidiasis Jain *et al.*, (1982). A study of SSG hospital Vadodara revealed that *Candida* was the commonest isolate (32.6%) followed by *M. tuberculosis* (22.7%) and *Cryptosporidium parvum* (19.8%) Sangeeta *et al.*, (2011).

The availability of antifungal agents, particularly for deep seated *Candida* mycoses are relatively few. Most of potent, currently used antifungals like Amphotericin B, Itraconazole and ketoconazole and fluconazole have some adverse toxic effects.

The antifungals viz. Nystatin and Clotrimazole are still in use for candidal infections. Nystatin is an amphoteric substance derived from *Streptomyces noursei* and contain many double bonds in its chemical structure and hence called a polyene antibiotic. Clotrimazole is a synthetic antimycotic agent that is effective against a variety of human pathogenic fungi. Both the antifungals are less toxic and side effects are very few. Apart from mild gastric intolerance, occasionally nausea, vomiting and diarrhoea may occur after administration. Very little information is available

regarding the mechanism of action of these two antifungals. The drugs combine with the cell membranes and interfere with vital cellular processes like respiration and glucose utilization.

The present work is an approach towards understanding the pathogen and action of these two currently used antifungals. The growth pattern of *Candida albicans* and the effect of Nystatin and Clotrimazole had been studied. The minimum inhibitory concentration of these antifungals had been evaluated to estimate the LD₅₀ concentration by using Microtitre/ELISA technique. The effect of these drugs had also been studied on the functional proteins of *Candida albicans* which is related to the ATPase enzyme activity. The effects of physiological conditions were also studied on the enzymatic profile.

MATERIALS AND METHODS

The isolate was originally cultured from different immuno-compromised patients including tubercular and diabetic patients of Gandhi Medical College, Bhopal.

Candida albicans was isolated from the sputum of a tubercular patient by streak plate method. Many colonies appeared on the plate after 24hrs of incubation at 28°C. On the basis of preliminary and biochemical tests including germ tube, chlamydospore formation test, carbohydrate assimilation and fermentation reaction tests, the organism was characterized as *Candida albicans* by standard method described by G.S. Moore and D.W. Jaciow. The morphology and other characteristics were then compared with the reference strain.

Candida albicans patient isolate designated as G-1 strain was used throughout this study. All the stock cultures were maintained by subculturing on Sabouraud's Dextrose Agar slopes (40% Dextrose, 10% peptone, 20% agar and 0.5% each cycloheximide and chloramphenicol). The incubation temperature used is 28°C and time duration of 24hrs. The culture was maintained freeze dried at 4°C in closed glass vials.

Measurement of growth

The cells of the patient isolate strain G1 were grown in 250 ml Erlenmeyer flasks containing 100ml growth medium inoculated with 1 ml overnight grown inoculum and incubation at 28°C

for 24 hrs aerobically under static conditions.

Growth was recorded in terms of optical density using spectrophotometer at 550nm (Bausch and Lomb).

Antifungal agents and chemicals

The two antifungals were used namely , Nystatin and Clotrimazole. Nystatin was obtained from Sarabhai chemicals and Clotrimazole was obtained from Franco- Indian remedies. Both the antifungals were dissolved in Dimethyl Sulphoxide(DMSO). All chemicals used were of AR grade.

Minimal inhibitory concentration (MIC) determination:

The MIC refers to the minimum quantity of a compound required to inhibit the growth of an organism. Nystatin and Clotrimazole were screened against *Candida albicans* for their inhibitory activity. For MIC determination, the colony Forming Unit (CFU) was determined and inoculum load of 1.0×10^5 cells/ml was used throughout the study. The inhibitory activity was performed by the following method:

Microtitre/Microbroth Dilution Technique/ELISA Technique

Microbroth quantities with drug dilutions were serially transferred with a multichannel appendorf pipette in microbroth plate with 96 (12 x 8) wells. This technique makes the use of measurement of growth by optical density. Observations were made by an automated ELISA reader (Flow labs. Scotland) based on optical density (O.D at 492 nm, matrix 0.2 - 2). Appropriate controls were set accordingly. The MIC was determined in terms of OD value.

Quantitative Evaluation of ATPase Enzyme and Protein:

ATPase enzyme activity was estimated because most of the important effect of the action of antifungals on *Candida* species is directly on the enzyme activity. The enzyme activity was measured in presence of Nystatin and Clotrimazole at their MIC concentration. A comparative analysis of enzyme activity was done in presence of both antifungals separately under different conditions of pH (9.0, 7.0 and 5.0) and temperature (20°C, 28°C and 35°C). pH 7.0 and temperature 28°C was considered as control.

The ATPase activity was measured by Davies and Brag method (1972) with slight

modification. The cells were harvested in late log phase of growth. Cells were suspended in Tris HCl buffer, and samples were withdrawn at different time intervals i.e. 0, 10, 20, 30, 60 and 90 minutes of incubation. Inorganic phosphate liberated due to ATP hydrolysis catalysed by ATPase activity was estimated spectrophotometrically at 660nm by Bowman and Slayman (1979). Protein was estimated following Lowry's method(1971).

RESULTS AND DISCUSSIONS

Antifungal evaluation by microtitre/elisa technique:

The MIC or LD₅₀ concentration of Nystatin was found to be 8.0µg/ml whereas of Clotrimazole, 6.25 µg/ml concentration. The results show that Clotrimazole was found to more effective against *Candida albicans* as compared to Nystatin as far as MIC is concerned. The present result of MIC using Clotrimazole with *Candida* species support the earlier findings Jacob *et al.*, (1981) in which growth of *Candida* species were inhibited by concentration of 0.78 - 50 µg/ml. The LD₅₀ concentration of Nystatin was found to be similar as reported by Millns and Martin(1996) in the range of 5 - 30 µg/ml. The study of antifungal effects of Nystatin and Clotrimazole on *Candida* species also showed the MIC in the range of 2.2 -18.0 µg/ml Falahati *et al.*, (2005).

Evaluation of atpase activity:

The enzyme system H⁺-ATPase of

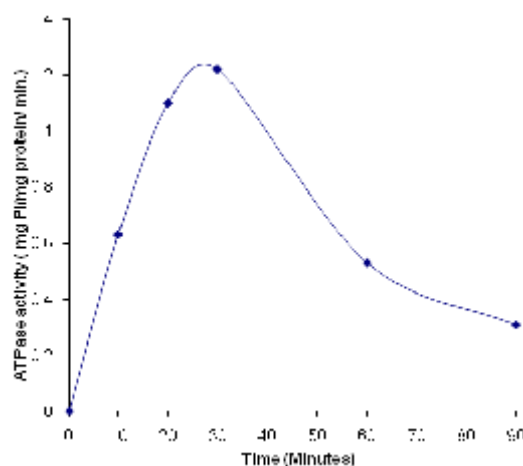


Fig. 1. ATPase activity in *Candida albicans* at pH 7.0 & temperature 28°C in the absence of Nystatin and Clotrimazole

respiratory electron chain was estimated to understand mode of action of antifungals viz. Nystatin and Clotrimazole. The MIC of both antifungals were chosen to measure H^+ -ATPase activity in relation to the time of incubation.

The ATPase specific activity found at pH 7.0 and 28°C (1.22 μ gPi/mg protein/min) was considered as 100% (control). Enzyme activity attained a maximum value after 30 minutes of incubation, thereafter shows a sharp decline (Fig 1). In order to understand the effect of pH, enzyme activity was measured at 9.0, 7.0 and 5.0 pH while effect of temperature was seen at 20, 28 and 35°C.

At optimal conditions, (pH 7.0 and 28°C) ATPase activity was 5% lower in the presence of Clotrimazole whereas Nystatin caused 39% inhibition. Thus, Nystatin is more effective than Clotrimazole in reducing the energy state of fungi. This might be because of the effect that Nystatin is known to become ineffective in aqueous solution Hillas Smith, (1969), therefore less inhibitory effect was shown by Clotrimazole than Nystatin.

At pH 9.0, the ATPase activity was inhibited by 70% in control cells (absence of antifungal agents). The inhibition was potentiated

when cells were treated with Clotrimazole at 6.25 μ g/ml concentration and only 18% ATPase enzyme activity was observed. In contrast, Nystatin (8.0 μ g/ml) practically caused no inhibition and showed similar activity to that without antibiotics.

At pH 5.0, the enzyme activity was reduced to 53% which in the presence of 6.25 μ g/ml Clotrimazole further reduced to 65% while there was not much change in the presence of Nystatin 8.0 μ g/ml.

From the results shown in (Fig 2) it is very clear that antifungal behaved differently under the influence of varying pH i.e. alkaline to acidic.

The pH profile plays an important role on the ATPase activity. It has been established that at acidic pH in *E. coli* Δ pH, a component of electrochemical gradient plays a major role, whereas at alkaline pH(9.0) Δ ψ is a major part Padan *et al.*, (1976) and Ramos *et al.*, (1976). It is also known that at acidic pH, component of proton electrochemical gradient is abolished i.e. at μH^+ by exchange of OH^- for Cl^- , which may be responsible for an observed hypersensitivity of ATPase activity to pH variation Padan *et al.*, (1976). The inhibition of ATPase activity by antifungals in

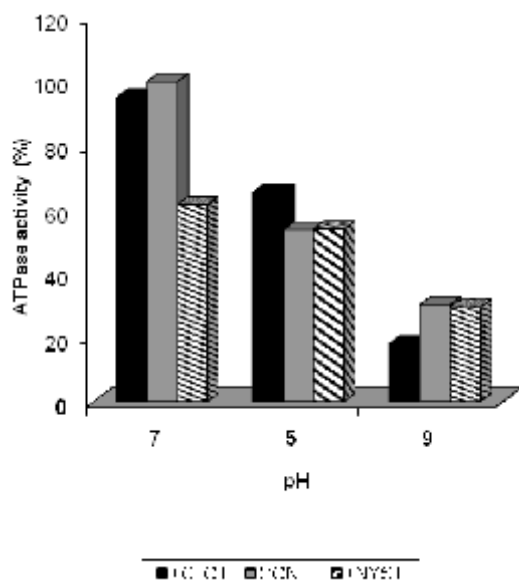


Fig. 2. Effect of pH on percent ATPase activity in the absence of antifungal (control) and in the presence of Clotrimazole and Nystatin. (100% ATPase activity = 1.22mgPi/mg protein/min.) at pH 7.0 & temperature 28°C

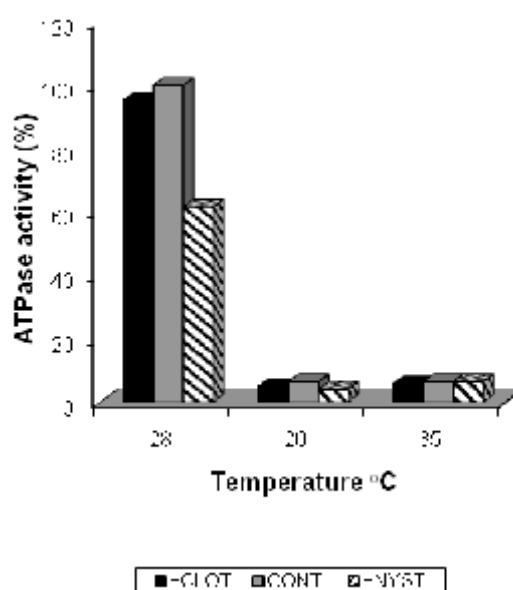


Fig. 3. Effect of temperature on percent ATPase activity in the absence of antifungal (control) and in the presence of Clotrimazole and Nystatin. (100% ATPase activity=1.22mgPi/mg protein/min.) at pH 7.0 & temperature 28°C.

alkaline medium than in acidic medium could be attributed to the fact that Na⁺ - H⁺ antiporter in *Candida* membranes tends to stop at alkaline pH but not at acidic pH resulting in dissipation of proton motive force (pmf) involved in various physiological processes.

The ATPase activity was 94% reduced in the absence of antifungal agents at 20°C. However, both the antifungals showed no remarkable difference in ATPase activity. Similarly at 35°C temperature, ATPase activity was observed to be 7.0% in the presence as well as absence of antifungal agents. From the present observation it can be concluded that the reduction in ATPase activity as well as inactivation of antifungals might have served in remarkable reduction of ATPase activity (Fig.3). Low temperature generally slows down cellular metabolism while, higher temperature increases the rate of cell activities. The effects of extremes of temperature on microbial growth may be generally explained on the basis of inactivation of enzymes or other vital cell structures Bloom *et al.*, (1986) and Hurst *et al.*, (1975).

The inhibitory effects of heat stress on ATPase activity are thought to result from the damage of proteins and permeabilization of membranes Magger *et al.*, (1993), Watson (1987) and Benschoter and Ingram (1986), particularly the plasma membrane leading to dissipation of transmembrane H⁺ gradient and to a decrease in intracellular pH Weitzel *et al.*, (1987).

The enhancement of temperature from 20 to 35°C, ATPase activity was found to be ceased because of denaturation of the functional proteins in the influence of high temperature. The influence of temperature may also result in the dimorphic transition of *Candida albicans* Frederick *et al.*, (1997). ATPase activity was observed at 35°C in the absence of Clotrimazole and Nystatin at lower levels. However, ATPase was found to be more sensitive to Nystatin than Clotrimazole even at different observed pH levels.

From the present observation, it can be concluded that the target site of the host cell appears to be energy generation pathway in case of Nystatin. In contrast, Clotrimazole was not found to be directly involved in modulating ATPase activity. In the present experimentation, the MIC indicates that Clotrimazole was effective as compared to Nystatin, but it may have different

target site, apart from ATPase. Clotrimazole and Nystatin has therapeutic importance in pharmaceutical applications for the recovery of candidiasis, still recovery of disease in tropical countries appears to be challenging. The fluctuating temperature conditions in tropical countries make the host susceptible to *Candida* species infections.

In the present investigation it can be concluded that the mode of action for Nystatin differs extensively than Clotrimazole. Further more, the effectiveness of the drug proved by the present observation can also be confirmed by growth inhibition studies because at 8.0 µg/ml Nystatin shows 50% inhibition of pathogen whereas the same inhibition was observed only at 6.25 µg/ml concentration of Clotrimazole in laboratory conditions.

Therefore, it can be concluded that both antibiotics applied for candidiasis works biologically by two different processes.

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REFERENCES

1. Cannon R.D, Holmes A.R., Mason A.B., Monk B.C. Oral candida: clearance, colonization or candidiasis. *J. Dent. Res.* 1995; **74** :1152.
2. Fidel, P.L., jr. Candida-host interaction in HIV disease: relationship in oropharyngeal candidiasis. *Adv.Dent.Res.* 2006; **19** :80-84.
3. Bodey G.P. Fungal infections in cancer patients. *Ann. N.Y. Acad. Sci.*, 1988; **544** :431.
4. Coleman, D. C., Bennett, D.E., Sullivan, D.J., Gallagher, P.J., Henman, M. C., Shanley, D.B., Russell, R.J. Oral candida in HIV infections and AIDS : New perspective /new approaches. *Crit.Rev.Microbiol.* 1993; **19** :61.
5. Patton, L. L., Phelan, J. A., Ramos-Gomez, F. J., Nittayananta, W., Shiboski, C. H. & Mbuguye, T. L. Prevalance & classification of HIV associated oral lesions. *Oral Dis.* 2002; **8** (suppl.2), 98-109.
6. Mrudula Patel, Joanne, T., Shackleton and Maeve, M.Coogan. Effect of antifungal treatment on the prevalence of yeast in HIV

- infected subjects. *Journal of medical microbiology*, 2006; **55**, (9): 1279-1284.
7. Frederick, D. Northrop., Srdjan, Kjubojevic and Julia, M. Davies. Influence of Na⁺ and anions on the dimorphic transition of *Candida albicans*. *Microbiol*, 1997; **143**: 3757-3765.
 8. Emori, T.G and Gaynes, R.P. An overview of nosocomial infections including the role of microbiology laboratory. *Clinical Microbiology Reviews*, 1993; **6**: 428.
 9. Hilmar Wisplinghoff., Tammy Bischoff., Sandra, M. Talent., Herald Seifart., Richard ,P. Wenzel and Michael B. Edwards. Nosocomial bloodstream infection in US hospitals : Analysis of 24,179 cases from a prospective nation wide surveillance study. *Clin.Infect.Dis*. 2004; **39**(3): 309-317.
 10. Chandler, F.W and Selik R.M. Epidemiology of AIDS and its opportunistic infections. In 3rd Symposium. *Topics in Med. Myc. Mycoses in AIDS patients* (1989), p-29-32(abstract).
 11. Chandler, F. W . Pathology of mycoses in patients with AIDS. In *current Topics in Med. Myc (eds.) MR Mc Ginnis*. vol 1 Springer Verlag, N.Y Berlin (1985) pp 123.
 12. Ramakrishna, B., Tendolkar, U. M., Varaiya, A., Dhurat, R., Geogate, A. Fluconazole isolates of *Candida* species for HIV positive patients. *Ind.J.Med.Microbiol*. 2000; **18** : 30-32.
 13. Kaviarasan, P.K., Tappa, D. M., Jaishankar, T. J., Sujatha. S. Candidiasis in HIV infected patients, clinical and microbiological study. *Natl.Med.J.India*. 2002; **15** : 51-52.
 14. Lattif, A. A., Banerjee, U., Prasad, R., Biswas, A., Wig, N & Shama, N. Susceptibility pattern and molecular type of oropharyngeal lesions of Immuno deficient virus patients. *J.Clin.Microbiol*. 2004; **42**: 1260-1262.
 15. Jain, S. K., Agrawal, R. L., Sharma, D. A., Agrawal, M. M. *Candida* in pulmonary tuberculosis. *Journal of post graduate medicine*, 1982; **28**(1) : 24-29.
 16. Sangeeta D Patel., Dipa M Kinariwal & Tanuja B Javedekar. Clinico-microbiological study of opportunistic infections in HIV seropositive patients. *Indian. J. Sex Trans.Dis*. 2011; **32** (2) : 90-93.
 17. G.S. Moore and D.W. Jaciow. Mycology for the clinical laboratory, yeast identification. (1977). Reston Publishing Comp. Inc. Prentice Hall Comp. Reston. Virginia, 169.
 18. Davies, P.L., and Brag, P. D. Properties of a soluble (Na⁺ and Mg⁺² activated ATPase released from *E.coli* membrane. *Biochem.Biophys.Acta*. 1972; **266**: 273.
 19. Bowman, B. J & Slayman, C.W. The effects of vandate on the plasma membrane ATPase of *Neurospora crassa*. *J.Biol.Chem*. 1979; **254**: 2928-2934.
 20. Lowry, O. H., Carter. J. Wood, J. B., and Glasser, L. The effect of carbon and nitrogen sources on the level of metabolic intermediates of *E.coli*. *J.Biol.Chem*. 1971; **193**: 265.
 21. Jacob, Z., Seema Wahab, M. Ghosh and O.P. Srivastava. Superficial mycoses and in vitro sensitivity of dermatophytes and *Candida* species. *Ind.J.Med.Res*, 1981; **74**: 365.
 22. Millns, B., Martin, M.V. Nystatin pastilles and suspension in the treatment of oral candidosis. *British- Dental journal*, 1996; **181**: 6 : 209.
 23. Falahati, M., M. Mahmoodian., M. Mir., Mohammed, A. Roodaki., M. J. Shariat. Study of antifungal effects of Nystatin, Clotrimazole and Fluconazole. *Razi.Jour.Med.Sci*. 2005; **12**(4): 99-106.
 24. Hillas Smith. Antibiotics in clinical practice. *Pitman Press Bath Publication*, 1969; pg-146.
 25. Padan, E., Zilberstein, D and Rottenberg, H. The proton electrochemical gradient in *E.coli* cells. *Eur. J.Biochem*. 1976; **63**: 533.
 26. Ramos, S., Schuldiner, S and Kaback, H. R. The electrochemical gradient of protons and its relationship to active transport in membrane vesicles. *Proc.Natio.Acad.Sci.USA*. 1976; **73**: 1892.
 27. Bloom M.S.S., Vanboeglan .R., Neidhardt, F., Brot, N and Weissbach, H. In vitro effect of the *E.coli* heat shock regulatory protein on the expression of heat shock genes. *J.Bacteriol*. 1986; **166** : 380.
 28. Hurst, A., Hughes, A., Duckworth, M and Baddiley, J. Loss of D-alanine during sublethal heating of *Staphylococcus aureus* and magnesium binding during repair. *J.Gen.Microbiol*. 1975; **89**: 277.
 29. Magger, W. H and Ferreiria, M. P. Stress response of yeast. *Biochem.J*. 1993; **290**: 1.
 30. Watson, K., Rose, A.H and Harrison, J. S(ed). *The yeasts* vol.2. Academic Press, London. 1987; p 41.
 31. Benschoter, A. S & Ingram, L. O. Thermal tolerance of *Zymomonas mobilis* : temperature induced changes in membrane composition. *Appl. Enviro. Microbio*. 1986; **51**: 1278.
 32. Weitzel, G., Pilatus, V and Rensing, L. The cytoplasmic pH, ATP content and protein synthesis during heat shock protein induced treatment in yeast. *Exp.Cell.Res*. 1987; **170**: 649.