ASPECTS OF CELL DEATH AND AUTOLYSIS IN SACCHAROMYCES CEREVISAE

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ABSTRACT

The kinetics of cell death and autolysis of twenty two haploid yeast strains were examined over a period of eight months in wine and synthetic media. Eight distinct patterns of cell death were observed using methylene blue staining and sample plating for viable cells. The rate of death was both yeast strain dependent and influenced by environmental factors such as temperature, nutrient supply and the presence or absence of ethanol.

The activity of extracellular killer yeast toxin concentrated by ultrafiltration was examined under various environmental conditions. Toxin activity was pH and temperature dependent. Concentrations of ethanol greater than 2% completely inhibited killer toxin activity. A difference of 12 hours was detected between a yeast cell becoming incapable of reproduction as the result of killer toxin action and this inability becoming discernible by methylene blue staining. A maximum kill of 97 - 99% was obtained independent of cell or toxin concentration. Toxin induced death was accompanied by the release of arginine and lysine. A bioassay was developed to quantify the amounts of arginine and lysine released.

"I drink it when I'm happy and when I'm sad.
Sometimes I drink it when I'm alone.
When I have company I consider it obligatory.
I trifle with it if I'm not hungry and drink it when I am.
Otherwise I never touch it - unless I'm thirsty."

- Madame Lilly Bollinger

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INTRODUCTION

2.10 AUTOLYSIS

Joslyn (1955) cited four successive stages defined by Lafourcade (1954) of yeast activity during fermentation.

1. Living cells, capable of fermenting sugar and undergoing rapid budding
2. Living yeast cells, in a retarded state, slow reproduction occurring
3. Living cells, incapable of reproducing
4. Dead cells, yielding to autolysis

Autolysis is the process of cellular degradation catalysed by intracellular enzymes after the death of the cell, and is utilized by man to produce the aroma and bouquet that typifies champagne. The key to the champenoise process is the prolonged ageing of bottle fermented champagne in contact with the yeast. Ageing is a slow process traditionally taking four years at low temperatures, during which time autolysis of the yeast cells occurs and cellular products are released into the wine (Margheri, Versini, Serra, Giannotti, Pellegrini and Mattarei, 1984).

During fermentation, amino acids are sequentially removed from the must (Rose and Keenan, 1981). Within the first six days of fermentation there is a drastic reduction in the free amino acid levels with the exception of proline (Suarez, Polo and Llaguno, 1979). The concentration of amino acids increases by 2 to 30% of the initial values in the must by the completion of the fermentation. The increase in free amino acids has been attributed to secretion by yeast and to cellular autolysis (Kluba, Mattick and Hackler, 1978). The most prominent increase is in proline concentration (Ough 1968).

A second explanation is that of passive excretion of amino acids into the wine by living cells (Morfaux and Dupay, 1966).

Yeast autolysis, as determined by a renewed increase of amino acids in the wine, occurs after a latent period of 12 months (Surez et al, 1979). Joslyn (1955) recognised that changes in the flavour and stability of fermenting must can occur even before extensive autolysis of the yeast cells takes place. He found that excretion of amino acids, vitamins and other cellular constituents by viable yeasts in the early stages of autolysis have a noticeable effect on flavour. Substances produced during autolysis have been found to enhance the organoleptic quality of champagne. Changes in the composition of aroma substances was studied using gas chromotography, and the character of sparkling wine was attributed to volatile components of relatively high boiling points (Molnar, Oura and Suomalainen, 1981). It was estimated that the neutral fraction contributed significantly (54.6%) to the total aroma of flavour concentrate isolated from yeast autolysate (Hajslova, Velisek, Davidkk, and Kubelka, 1980).

Volatile compounds are released during the first year of ageing. Upon prolonged ageing the evolution of benzaldehyde is particularly prominent, reaching 4 mg/l after 16 years. The flavour of wine has been shown to be favourably altered by addition of benzaldehyde (Loyaux, Roger and Adda, 1980).

Wine has an elevated pH, and increased concentrations of amino acids, ash phosphates, B vitamins, pantothenic acid, P.D. factor and ribose after nine months ageing on the lees compared to ageing without yeast contact (Margheri, Versini, Della Serra, Giannotti, Pellergrini and Mattarei, 1984). Other substances found to be excreted during autolysis are: peptides, polypeptides, purine and pyrimidene bases, proteases, and other enzymes (Babayan, Latov, Belikov and Kalumyanz, 1984; Joslyn, 1955; Jund, Chevallier and Lacroute, 1977; Molnar, Oura and Suomalainen, 1980). All of the substances released during ageing and the composition of the base wine contribute to the bouquet of the finished champagne.

The release of amino acids into champagne and the changes of composition with time have been most extensively examined. The rate of autolysis and the concentrations of free amino acids in autolysates of different yeast strains is variable despite the similar amino acid compositions of cell protein in different yeast strains (Kluka, 1985; Martini, Martini and Miller, 1979) which explains anomolies between different reports of amino acid concentrations in aged champagne. All reports agree that the overall effect is an increase in total amino acid concentrations in wines aged on the lees. Small increases and small decreases in the amounts of individual amino acids have been observed during ageing (Margheri, Versini and Gianotti, 1984).

Phenolic substances can be formed by means of enzymatic hydroxylation and methylation of aromatic amino acids and by degradation of the resulting products (Hajslova et al, 1980). It has been suggested that the observed increase then decrease of alanine and arginine concentrations between the 12th and 24th months of ageing is due to transformation of amino acids by deamination, with the liberation of ammonical nitrogen; alternatively certain amino acids may act as precursors of aromatic compounds (Feuillat and Charpentier, 1982). Investigation of the influence of the length of contact time showed that the greatest enrichment of amino acids occurs between the 6th and 12th month of ageing. 12% more amino acids were present after six months than in the base wine, and from 12 to 24 months the percentage of free amino acids rose from 24.5% to 25.6%. Major variations occur during the first three months of the secondary fermentation, with amino acid concentrations increasing after 15 months, and surpassing initial levels after 43 months ageing (Suarez et al, 1979).

The distribution of amino acids has been found to be dependent on the actual production method, particularly the extent of yeast contact time. Commercial charmat and champenoise style sparkling wines both have comparable organic acid and alcohol levels, and vary only in total and amino nitrogen composition (Colagrande) and Mazzoleni, 1977). Methionine and tryptophan were found in higher levels in champenoise style wines, while isoleucine, proline and ornithine have higher levels in charmat wines.

A linear relationship exists between the length of storage and the degree of autolysis, with the autolytic rate increasing by 6 to 7 per cent for a 10°C rise in temperature (Molnar et al, 1980). They suggested that elevated temperature may be unfavourable for the organoleptic quality of the wine; as cell components dissolved in the wine are not bound by secondary chemical reactions, the resulting sparkling wine may have a strong yeast flavour. Increasing the temperature caused acceleration of the rate of loss of enzyme activity (Molnar et al, 1980). After 20 days of storage at 10°C a considerable part of the total enzyme activity is lost, and after 80 to 100 days no demonstrable activity was detected that could influence further development of the wine. Protease activity was an exception, in that it increased in activity with storage. Heating of yeast autolysate containing free amino acids and reducing sugar leads to intensive browning (Davidek, Hajslova, Kubelka and Velisek, 1979), increase in alkylpyrazine concentration (a product of browning reactions), and a nutty caramel like character (Hajslova et al, 1980). Organoleptic studies suggested that champagne aged at 10°C had more favourable properties than wine stored at higher temperatures, which was attributed to the presence of volatile substances at the lower temperatures (Molnar et al, 1981).

Reports on the extent of cytolysis associated with autolysis vary. Joslyn (1955) reported loss of protoplasm occurred during autolysis, while the cell wall rigidity was retained. Margheri et al (1984A) reported no hydrolysis of yeast cells during champenoise ageing. But Perez-Leblic, Reves, Martinez and Lahoz (1982) reported autolysis as being the degradation of the cytoplasm and lysis of cell walls. Molnar et al, (1980B) recorded loss of cells as a means of calculating the degree of autolysis.

That autolysis has a genetic basis is evidenced by the mapping of nuclear genes responsible for cell lysis (Stateya and Venkov, 1981). A ten fold difference between the slowest and the most rapidly autolytic strains under the same conditions supports genetic involvement (Kulka, 1953).

This report examines the rate of death of different strains of Saccharomyces cerevisiae. Autolysis is examined using cell death as an indicator of the rate of ageing in wine and synthetic media. No attempt has been made to determine the importance of cellular products to the organoleptic quality of champagne.

2.20 EFFECT OF ETHANOL ON SACCHAROYMCES CEREVISIAE

Saccharomyces cerevisiae strains are widely used in the fermentation of carbohydrates to produce ethanol because of their resistance to ethanol. All strains do not have equal ethanol tolerance, indicating that ethanol tolerance is partly genetic (Gray, 1941). Strains are able to grow in 8% to 12% (v/v) ethanol, survive up to 15% (v/v), and ferment up to 12% (v/v) with some sake strains capable of fermenting 20% (v/v) ethanol (Rose, 1980; Rose, 1983).

Ethanol causes an increase in cytoplasmic membrane fluidity and a decrease in membrane order as the result of a complex combination of physical effects both directly on the membrane and on the membrane environment (Mitchaelis and Mitchaelis, 1982; Janoff and Miller, 1982). Ethanol being a short-chain alcohol is expected to be dominated by its polar function (Ingram and Buttke, 1984), causing an increase in the intracellular pH and a decrease in the strength of hydrophobic interactions disrupting membrane packaging (Rottenberg, Waring and Rubin, 1981). It has been demonstrated that ethanol inhibits sugar uptake in S. cerevisiae by changing the lipid environment of the plasma membrane (Leao and van Uden, 1982). Ethanol increases the membrane fluidity and increases permeability to ions and to small metabolites (Janoff and Miller, 1982). These effects are increased with increasing temperature (Kleinons, Lee, Bord, Haak, and Woods, 1979).

The membrane lipids are also the site of resistance to ethanol. S. cerevisiae strains adapt to ethanol by increasing the membrane mono-saturated fatty acid composition (Bevan, Charpentier, and Rose, 1982). The presence of specific sterols and fatty acid residues enhances resistance to ethanol (Thomas, Hossack and Rose, 1978). The presence of unsaturated fatty acids in media acts as an essential growth factor for brewing yeasts when insufficient oxygen is available for fatty acid biosynthesis (Thompson and Ralph, 1967).

The presence of ethanol may also effect the relationship between the yeast and the physical environment. It has been proposed that the observed decrease of yeast resistance to ethanol as the temperature increases is due to modification of sites located on the cell membrane that determine maximum growth temperature, making the cells more temperature sensitive (von Uden and de Cruz Durote, 1981, cited by Ingram, 1984).

2.30 KILLER YEAST IN SACCHAROMYCES CEREVISIAE

Makower and Bevan (1963) first reported the phenomena of *Saccharomyces cerevisiae* strains which produce an extracellular toxin that is lethal to sensitive cells. The killer character is expressed as one of three distinct phenotypes; killer, neutral or sensitive. Killer cells (K^+,R^+) produce toxin which kills sensitive cells, (K^- R^-). Neutral strains (K^-,R^+) are immune to toxin, and do not produce toxin. To kill, toxins must be structurally distinct from those toxins produced by sensitive strains, since the specific immunity system of the killed strain is active against its own toxin, but not against other toxins (Rodgers and Bevan, 1978). Thirteen classes of killer yeast have been identified using killer and resistant phenotypes (Young and Yagiu, 1978). Three killer groups are found in the genus *Saccharomyces cerevisiae*; K_1, K_2 and K_3. The K_1 toxin kills K_0 sensitive cells and K_2 and K_3 strains (Bevan and Makower, 1963). K_2 strains first discovered in Russian wines are active against K_0 and K_1 strains (Naumov and Naumov, 1973). Toxins of the K_3 group kill K_0, K_1 and K_2 strains (Young and Yagiu, 1978). The different classes of toxins produced can be distinguished on the basis of pH optima for activity, temperature stability, and relative susceptibility to protease action (Young and Yagiu, 1978). Killer mutants have been isolated in which the toxin is defective, and sensitive strains may mutate to resistance (Al - Aidroos and Bussey, cited by Rodgers and Bevan, 1978). Killing activity has also been identified between yeast strains of different genera (Bussey and Skipper, 1975; Bussey and Skipper, 1976; Rodgers and Bevan, 1978; Kandel and Stern, 1979).

2.3.1 Genetics of the Killer System

Two species of cytoplastically inherited dsRNA molecules are seen in killer yeast strains, both of which are necessary for the killer phenotype (Sommers and Bevan, 1969; Berry and Bevan, 1972; Mitchell, Bevan and Herring, 1973). Both dsRNA molecules are separately encapsulated as virus-like particles (Herring and Bevan, 1974; Kane, Pletras and Bruenn, 1979). The larger L-molucule (or P_1) consists of two equal strands of RNA, with a constant molecular weight of 2.5×10^6 to 3.4×10^6, and is found in cells of all killer phenotypes (Wickner, 1980). L dsRNA codes for the major capsid protein, and is present if the m or s plasmids are present (Hopper, Bostian, Rowe and Tipper, 1977). The helper L dsRNS plasmid has no known dependence on other plasmids and confers no known phenotype on the host cell apart from the effect on m dsRNA plasmid maintenance (Tipper and Bostian, 1984). The smaller m plasmid (P_1) codes for the killer toxin polypeptide (Bostian, Hopper, Rogers and Tipper, 1980), and contains the immunity component (Sommers, 1973). The m plasmid is found in all killer toxin producing strains, it has never been found in the absence of the L plasmid. Sensitive strains contain the L plasmid only. K_1, K_2 and K_3 killers carry physically distinct species of mdsRNA with molecular weights of 1.1×10^6 to 1.7×10^6; 1.0×10^6; and 0.87×10^6 respectively (Bevan, Herring and Mitchell, 1973; Vodkin, Katterman and Fink, 1974; Wickner and Leibowitz, 1976; Young and Yagui, 1978). Study of m_1 deletion mutants has shown that $m_1$dsRNA acts as a template coding for a polyadenylate tail needed for protoxin polypeptide translation. Genes have been identified that are responsible for maintenance and replication of the dsRNA sequence (Thiele, Hanning and Leibowitz, 1984). A third type of dsRNA(s) is present in suppressive strains (Vodkin et al, 1974). Killer dsRNA plasmids are maintained at a relatively constant copy number. Transmission occurs by cytoplasmic mixing during budding, mating and other forms of cell fusion. The role of dsRNA in killer systems has been reviewed by Tipper and Bostian (1984).

Chromosomal genes code for proteins involved in the maintenance and regulation of the plasmids, and the expression of the mdsRNA coded proteins (Table 1). The properties of mutations affecting the killer phenotype have been reviewed (Wickner, 1980; Bruenn, 1980). 29 chromosomal genes are involved in the maintenance of the mdsRNA plasmid (mak genes). They are scattered on 15 of the 17 yeast chromosomes. Mutations in any of these mak genes results in loss of killer ability. In each case the mdsRNA is lost, and the L plasmid retained. Each mak gene is thought to have a host specific function independent of the presence or absence of mdsRNA.

Four chromosomal genes (ski 1 to ski 4) regulate killer plasmid replication, a recessive mutation in any one of these genes results

Table 1. Chromosomal Genes Affecting the Killer Character of *Saccharomyces cerevisiae*

I Expression.

kex 1, kex 2; $K_1^- R_1^+$ [KIL-K_1]
kex 2 gene also needed for mating by strains

rex 1 $K_1^- R_1^-$ [KIL-K_1]
regulation of expression

II Killer Plasmid Maintenance

mak 1, mak 3 - mak 28; $K_1^- R_1^-$ [KIL-0]

mak 1 and mak 16 are temperature sensitive for growth

mak 3 and mak 10 mutants lose genetic cytoplasmic determinants [NEX] [HOK] and [EXL]

pet 18 needed for growth and mitochondrial maintenance

spe 2 needed for spermidine and spremine biosynthesis, sporulation and optimum growth

mkt 1 needed for [KIL-K_2] maintenance if [NEX] is present

III Regulation

ski 1 -ski 4; $K_1^{++} R_1^+$ [KIL-K_1]

certain ski mak double mutants are K_1^+ or K_1^{++}; ski mutants can maintain [KIL-sd_1]

KRB 1 $K_1^+ R_1^+$ [KIL-K_1]
dominant: bypasses need for mak 7 or pet 18

Plasmid genes are in brackets in capital letters
Chromosomal genes in small letters.

in an increase in production of killer toxin indirectly by increasing the plasmid copy number, conferring the super-killer phenotype (Toh-e, Guerry and Wickner, 1978; Siddiqui and Bussey, 1981). The ski-5 gene is a recessive mutant that appears to lead to toxin over secretion through a defect in the cell surface (Bussey, Steinmetz and Saville, 1983).

Two chromosomal genes KEX1 and KEX2 in addition to mdsRNA are needed for production and excretion of toxin (Wickner, 1974A). Mutants defective in these genes are K^-R^+ and maintain the m plasmid.

Three chromosomal genes kre1, kre2 and kre3 (killer resistance) are necessary for killing of a sensitive strain (Al-Aidroos and Bussey, 1978). Mutants defective in kre1 or kre2 have decreased binding of the toxin to sensitive cells.

A third class of nuclear mutations affecting the killer character are the rex mutants (regulation of expression). These 'suicide' strains produce toxin but are sensitive to their own toxin. These K^+R^- strains can grow normally above pH 4.8 (Wickner, 1974A).

Mutations of killer plasmid genes may effect plasmid maintenance or production of toxin. The recognised mutant phenotypes are summarised in Table 2 (Wickner, 1981).

2.32 Curing of a Killer Strain

Killer strains may be cured of the ability to produce toxin by growth at 37 to 40°C (Wickner, 1974B), by 5-fluorouracil treatment (Mitchel et al, 1973), or by growth in the presence of 13.3ug per plate of cycloheximide, slightly less than that needed to prevent growth (Fink and Styles, 1972). The resulting clones were mixed or

Table 2. Killer Plasmind Genotypes

[KIL-K_1] and [KIL-K_2]	wild type K_1 and K_2 killer plasmids
[KIL-O]	no killer plasmid
[KIL-n_1]	plasmid conferring resistance to K_1 toxin but not toxin production
[KIL-ts]	Killing is temperature sensitive
[KIL-i]	Confers toxin production but not resistance
[KIL-s]	Defective-interfering plasmid (suppressive) prevents replication of [KIL-K] (Sommers, 1973) (Vodkin et al, 1974)
[KIL-d]	defective maintenance and expression in haploid strains; normal in a/ diploid strains (Wickner, 1976)
[KIL-b]	bypasses need for some mak genes; also confers super-killer phenotype (Toh-e and Wickner, 1980)
[KIL-sk]	confers superkiller phenotype
[KIL-sd]	ski$^-$ dependent plasmid, can be maintained only in ski$^-$ strains (Toh-e and Wickner, 1979)
[KIL-kd]	deletion mutant of a non-essential region of [KIL-k_1]

fully cured, and bred true over repeated trials. Cycloheximide acts by inhibiting protein synthesis on cytoplasmic ribosomes (Hartwell, Hutchinson, Holland and McLaughlin, 1970), which it is assumed blocks plasmid replication while enabling chromosomal and mitochrondrial replication, resulting in the killer plasmid being diluted out over successive generations (Fink and Styles, 1972). All type K_1 and K_3 killers are readily cured by cycloheximide treatment, all type K_2 killers are cured by incubation at elevated temperature. Curing results in the loss of the m plasmid as shown by polyacrylamide gel electrophoresis (Young and Yagia, 1978).

2.33 Purification, Stabilisation and Characterization of Killer Toxin

The extracellular nature of killer toxin was first indicated by the killing of 39% of a sensitive cell culture after a four hour incubation in cell-free filtrates of buffered media in which killer cells had been grown (Bevan and Makower, 1963). A 40% purification of killer toxin from growth media was achieved by fractional precipitation with $(NH_4)_2SO_4$ followed by dialysis, gel filtration and ultrafiltration (Woods and Bevan, 1968). The purified toxin was characterized as an unstable, protease-sensitive macromolecule, stable within the narrow pH range of 4.6 to 4.8. Addition of gelatine slowed down the loss of activity. Addition of 20% glycerol to killer toxin concentrate enables retention of activity indefinitely at 5°C and pH4 to 5 (Ouchi, Kawase, Nakano and Akiyama, 1978).

The possibility that killer toxin may be associated with a yeast virus was eliminated, and the toxin identified as being a protein with a protein to polysaccharide ratio of 3:1 (Bussey, 1972).

Further characterization was achieved by the successful purification of active toxin in high yields by concentration in polyethylene glycol followed by chromatography through glycerol-controlled-pore glass (Palfree and Bussey, 1979). The amino acid composition suggested that the toxin was a monomer with the active unit having a molecular weight of 11470, and activity within the pH range of 4.2 to 4.6. The presence of only two hexose residues per polypeptide supported the theory that the killer toxin was a protein. A more recent purification method avoided the use of harsh treatment and enabled concentration of the toxin from S. cerevisiae strain 28 that was unaltered by the purification procedure (Pfeiffer and Radler, 1982). The killer strain was grown in a modified synthetic-B media at 25°C for three days (Heerde and Radler, 1978). A 2,000 fold concentration was achieved by repeated ultrafiltration, followed by purification by ion-exchange chromatography. The purified toxin contained 111 amino acid residues, comparable to the concentration in the toxin purified by Palfree and Bussey, 1979). The amino acid composition suggested a molecular weight of 14045 for the active unit, and an isoelectric point of 4.5 to 4.8. The total toxin had a molecular weight of 16,000, and a protein to carbohydrate ratio of 9:1, indicating that the toxin is a glycoprotein. They suggested that the use of 4M urea in previous purification methods had caused the removal of the carbohydrate fraction without affecting toxin activity. A considerable fraction of partially purified toxin is inactive, only 10 to 30% is active (Hutchings and Bussey, 1983). 10% of the total extracellular protein is killer toxin (Wickner, 1981).

2.34 Toxin Secretion

The cytoplasm of many toxin producing strains do not appear to contain detectable amounts of toxin, this may be due to extraction methods used. The concentration of intracellular toxin extracted from S. cerevisiae strain 28 is a thousand times more concentrated than extracellular toxin (Pfeiffer and Radler, 1982). Extracellular and intracellular toxins are identical suggesting that killer toxin is produced internally and liberated unmodified into the environment. Toxin is thought to be produced as a protoxin that is processed by proteolytic cleavage (Wickner, 1981). The use of temperature sensitive (sec) mutants has enabled the defining of the secretory pathway of toxin in S. cerevisiae that is similar to secretion pathways found in mammals (Norvick, Ferro, Schekman, 1981). At restrictive temperatures, toxin secretion in sec mutants is blocked, giving evidence that glycosylation, extension of the toxin does occur, prior to the protoxin entering the lumen of the endoplasmic reticulum where cleavage occurs. It has been suggested that glucosylation involves the addition of a non-N-glycosidic-linked polysaccharide that is necessary for efficient toxin secretion (Bussey, Saville, Greene, Tipper and Bostian, 1983).

Two chromosomal genes kex_1 and kex_2 are essential for toxin secretion. Kex mutants retain resistance but the glycosylated protoxin is not properly processed or secreted (Wickner and Leibowitz, 1976; Bussey et al, 1983). Secretion of other proteins with the exception of the α-factor pheromone, are unaffected by kex mutations (Leibowitz and Wickner, 1976).

2.35 Mechanism of Toxin Action and Effect on Sensitive Cells

Three successive stages are recognised for killer toxin action on sensitive cells leading to cell death. The process takes 3 to 4 hours (Middlebeek, van de Laar, Hermans, Stumm and Vogels, 1980).

I Binding of the toxin to primary cell wall binding sites, requires kre 1 and kre 2 gene products

II Transmission of the toxin to reactive sites in the plasma membrane

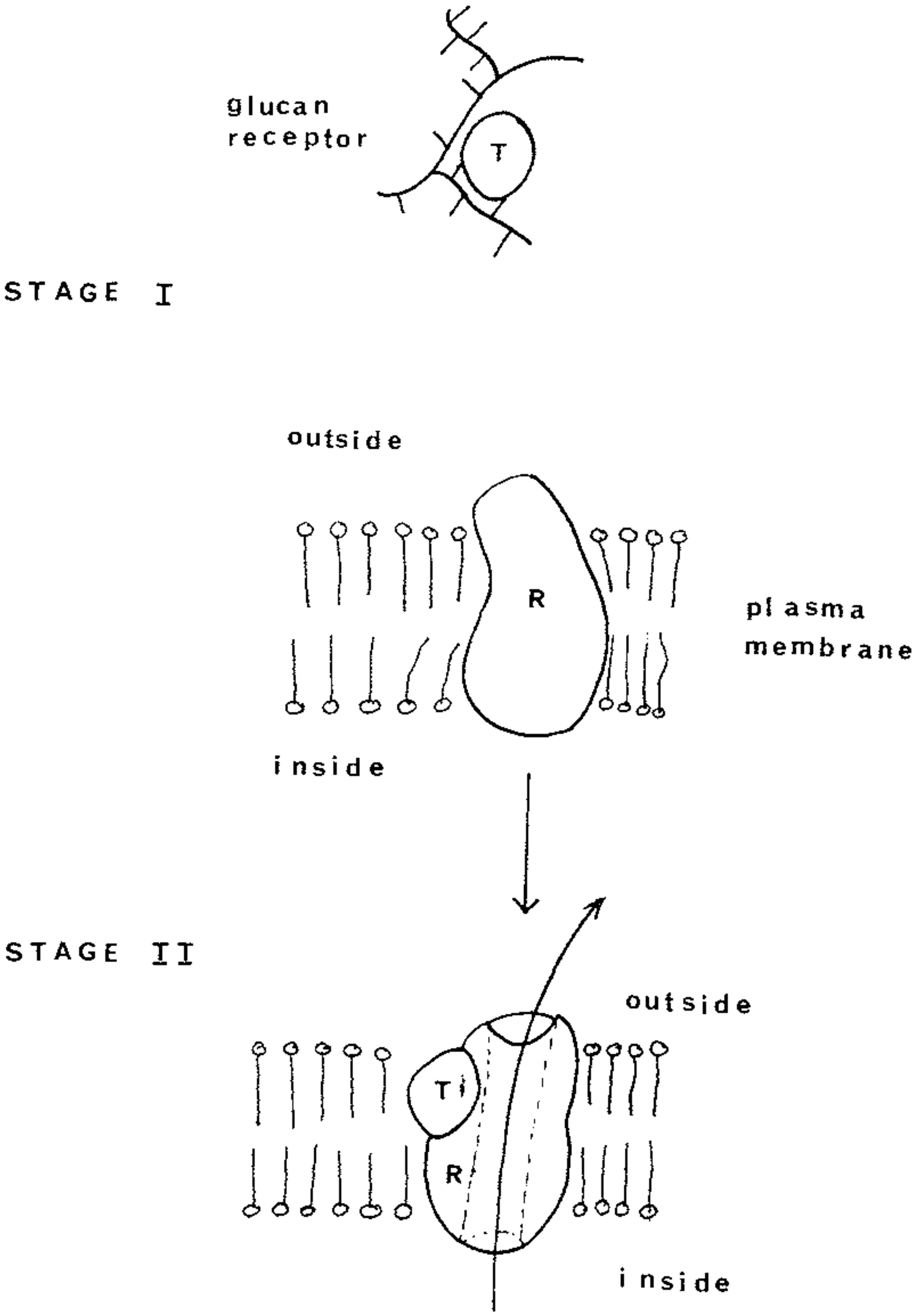
III Functional change

Figure 1 shows a two stage model of toxin action (Bussey, 1981).

Toxin binds to β-(1-6) D glucan receptor sites on the cell wall by an energy dependent reaction (Hutching and Bussey, 1983). Binding is inhibited in the absence of metabolic energy and at low temperatures (Middlebeek, Laar et al, 1980). Examination of toxin binding to S. cerevisiae strain S14A showed that each cell has 1.1×10^7 cell wall receptor sites, but binding of only 2.8×10^4 toxin molecules is needed to kill a cell (Bussey, Saville, Hutchings and Palfree, 1979). Toxin binds to sensitive and resistant cells. Sensitive mutants lacking cell wall receptor sites cannot bind toxin, so are resistant to killing. When spheroplast cultures of these mutants are treated with toxin, killing occurs, suggesting that binding of the toxin to the cell wall is necessary to allow killer passage through the cell wall to active sites on the cell membrane. Only 1% of the toxin bound to the cell wall is transferred to the membrane and is active in the killing (Bussey, Sherman and Sommers, 1973). Binding of toxin to the plasma-membrane results in changes of membrane permeability to small molecules allowing release of potassium ions, ATP and other

Figure 1. Two Stage Model of Toxin Action

T indicates toxin molecule; R, the glucan membrane receptor.


glucan receptor
T
STAGE I
outside
R
plasma membrane
inside
STAGE II
outside
Ti
R
inside

small molecules allowing release of potassium ions, ATP and other small molecules. Coinciding with leakage is acidification of the cell interior and inhibition of active transport of amino acids (Middlebeek, Laar et al, 1980; Bussey and Skipper, 1975; Bussey and Sherman, 1973). Intracellular changes observed are the co-ordinate inhibition of protein and nucleic acid synthesis and inhibition of D-glucose incorporation into macromolecules (Bussey and Sherman, 1973). Increased turbidity of whole and spheroplast cultures is observed due to a reduction of cell volume coinciding with loss of intracellular ATP (Bussey, 1974). No cell lysis is observed even after several days (Bussey, 1972).

Toxin binds immediately to the cell wall with a lag period of 50 to 90 minutes before physiological changes occur (Skipper and Bussey, 1977; Middlebeek, Stumm and Vogels, 1980). Further evidence suggests that binding of toxin immediately disrupts the electrochemical proton gradient across the plasma membrane causing inhibition of leucine transport and blocking proton excretion (de la Peña, Barros, Gascón and Ramos, 1980). It is proposed that the observed effects are due to the irreversible formation of ion-permeable channels in the phospholipid bilayer membrane, similar to the mode of action of bacterial colicins of the E1 functional class. A single toxin molecule may be responsible for channel formation. The formation of a channel changes the existing membrane potential, disrupting the internal negative potential required for amino acid transport (Kagen, 1983).

Sensitive cells enter a transient state after toxin binding, at this stage killing may be enhanced by the addition of ADP (Kotani, Shinmyo and Enatsu, 1977). Alternatively cells may be rescued by appropriate treatments such as incubation in yeast extract-peptone media supplemented with Ca^{2+} (Kotani et al, 1977); by removal or inactivation of the toxin at elevated pH (Bussey, 1972); or by adjustment of the media to physiological conditions (pH 6.5, 50mM KCl) (Middlebeek, Crutzen and Vogels, 1980). Rescue of cells by physiological conditions implies that the loss of K^+ and H^+ and subsequent decrease of the internal pH, is responsible for the lethal effect of killer toxin (Kagen, 1983). Rescue of intoxicated cells may be accomplished only before functional damage has occurred (Middlebeek, Laar, 1980).

2.36 Toxin Immunity

The immunity component is presumably a protein coded for by the m plasmid, but is distinguished as a separate activity from toxin production. The rex_1 gene is also implicated in immunity, but its role is not understood. The mechanism of immunity is unknown. It is thought that immunity probably occurs at stage II of the killing process, and that the immunity protein may prevent membrane damage by inhibiting toxin recognition of the membrane receptor (Bussey, 1980).

2.37 Commerical Application of Killer Wine Yeasts

In the fermentation of wine a specific strain is added to the must to assure the desired quality of the product. Contamination by wild yeast in wine fermentation can be a serious problem resulting in off flavours and even killing of sensitive wine yeasts by wild killer strains. Although many commercially available wine yeasts are killers, little attempt has been made to seek practical wine applications for killers, presumably due to the low pH of grape juice at which the killer effect is depressed. Inhibition of growth of contaminant killer sensitive cells in grape juice has been successfully achieved by cytoduction of super-killer RNA plasmids into a wine yeast (Seki, Choi and Ryu, 1985). Crossing of killer and wine strains has enabled the production of killer hybrids for use as starter cultures. Using such hybrids, sensitive S. cerevisiae strains can be killed during fermentation (Shodo, 1984).

This study examines the effect of temperature, pH and ethanol on the killing of K_0 cells by concentrated K_1 toxin to examine the potential application of killer toxin to induce natural ageing of charmat style champagnes.

MATERIALS AND METHODS

3.1 Identification and Origin of Yeast Strains Used

GW8021	Champagne yeast, Yalumba wines, Australia
GS strains 1 to 22	Haploid yeast, f_1 generation of GW8021
[1]K_0	5X47[KIL-K_0] a/ his1/+ trp1/+ ura3/+ diploid
[1]K_1	A364[KIL-K_1] a ade1, ade2, lys2, tryl, his7, ura1, mk+1 haploid
[1]K_2	1384[KIL-K_2] his4 mk+$^+$ [NEX-0] haploid
[2]K_3	K3GRS [KIL-K_3] a arg$^-$ haploid
[3]a	a mating type tester
[3]	α mating type tester
[3]ARG	arginine auxotroph
[3]LYS	lysine auxotroph

[1] provided by R.B. Wickner
Laboratory of Biochemical Pharmacology
National Institute of Arthritis, Metabolism and Digestive Diseases
Bethesda
Maryland

[2] provided by A L Extramera

[3] provided by R J Thornton
Massey University
Palmerston North

3.2 Standard Media and Reagents

Amino Acid Stock Solutions

Adenine	20 mg	F
Arginine	20 mg	A
Histidine	10 mg	A
Leucine	30 mg	A
Lysine	80 mg	A
Methonine	20 mg	A
Serine	375 mg	A
Threonine	350 mg	A
[2]Tryptophan	20 mg	F
[1,2]Tyrosine	20 mg	F
Phenylalanine	20 mg	A
Uracil	20 mg	A
Homoserine	75 mg	F
Aspartic acid	100 mg	F
Valine	60 mg	A

Make up to 1 litre using distilled water.

F filter sterilize

A autoclave 10 p.s.i. pressure for 15 minutes

1. Keep at room temperature.
2. Wrap in foil, pH using 2cm^3 of 0.1M HCl for every 200 cm^3 of amino acid solution.

Stock solutions sterilized and kept at 5°C or room temperature. 2 cm^3 of 1M NaOH added for every 200 cm^3 of amino acid solution.

To prepare a total amino acid solution, add 5 cm^3 of each solution into a sterile bottle. Use 5 cm^3 of total amino acid solution for every 100 cm^3 media.

0.1M Citrate-Phosphate Buffer pH 4.8

Citric acid	6.3045 g
Sodium phosphate anhydrous	8.517 g

Dissolve each in 300 cm^3 of deionized water. Take approximately 252 cm^3 of citric acid solution, and adjust to pH 4.8 with sodium phosphate solution. Make up to a total of 1 litre.

Complete Mineral Media

Glucose	100 g
NH_4Cl	1 g
$CaCl_2.2H_2O$	0.7 g
KCl	0.6 g
Na_2SO_4	0.3 g
$MgCl_2.6H_2O$	0.4 g
$Na_2HPO_4.7H_2O$	1.8 g
Citric acid	1.8 g

Make up to 1 litre using distilled water. Sterilize for 15 minutes 70kPa (10 p.s.i.)

i Mineral Stock Solution

H_3BO_3	50 mg
$MnCl_2.4H_2O$	46.8 mg
$FeCl_3.6H_2O$	20 mg
$Na_2MoO_4.2H_2O$	20 mg
ZnCl	19 mg
KI	10 mg
$CuCl_2.2H_2O$	2.7 mg

Make up to 100 cm^3 using distilled water. Add 1 cm^3 to each litre of complete mineral media before sterilization.

ii Vitamin Stock Solution

Inositol	200 mg
Thiamine HCl	40 mg
Pyridoxine HCl	40 mg
Biotin	0.2 mg
Calcium pantothenate	20 mg
Folic acid	4 mg
Vitamin B_{12}	10 mg

Make up to 100 cm^3 using distilled water, and filter sterilize. Add 1 cm^3 to each litre of sterile complete mineral media.

Fermentation Syrup

Glucose	500 g
Yeast nitrogen base without amino acids (Difco Labs)	20 g

Make up to 1 litre and sterilize for 15 minutes at 70kPa (10 p.s.i.)

Magdala Red Agar

Add 10 p.p.m. phloxin type magdala red to M.Y.G.P. and sterilize for 15 minutes at 70 kPa (10 p.s.i.) (Cutz, Heslot, Leupold and Laprieno, 1974).

Minimal Media

Yeast nitrogen base without amino acids	7 g
Glucose	20 g

Make up to 1 litre using distilled water. Sterilize for 15 minutes at 70kPa (10 p.s.i.).

Yeast Morphology Agar (MYGP)

Malt extract (Difco Labs)	3 g
Yeast extract (Difco Labs)	3 g
Bacto peptone (Difco Labs)	5 g
Glucose (Baker Chemical Co.)	10 g
Agar	30 g

Make up to 1 litre using distilled water. Sterilize at 70kPa (10 p.s.i.) for 15 minutes. For M.Y.G.P. broth, omit agar.

For M.Y.G.P. buffered to pH 4.8, dissolve the ingredients in 300 cm^3 of water, and buffered to pH 4.8 using 0.1M citrate and 0.1M sodium phosphate, then the volume adjusted to 1 litre.

Pre-Sporulation Media - (Glucose Nutrient Agar)

Glucose	50 g
Nutrient agar	23 g
Yeast extract	10 g
Agar	5 g

Make up to 1 litre using distilled water, then dispense into clean bijou bottles, sterilized at 70kPa (10 p.s.i.) for 10 minutes and slope to set.

Sporulation Media - (Potassium Acetate Agar)

Potassium acetate	10 g
Yeast extract	30 g
Agar	5 g

Make up to 1 litre using distilled water, boil to dissolve the agar, then dispense into clean bijou bottles and autoclave at 70kPa (10 p.s.i.) for 10 minutes and slope to cool.

Synthetic Wine - 10% Ethanol

Absolute Ethanol	100 g
Tartaric acid	7 g

Dissolve tartaric acid in 500 cm^3 of distilled water, and sterilize at 70kPa (10 p.s.i.) for 15 minutes. Let cool, then add ethanol and make up to 1 litre using sterile distilled water.

Synthetic - B Media

Glucose	50 g
DL-malate	20 g
Sodium citrate dihydrate	0.5 g
Inositol	0.04 g
Yeast extract	3 g
Peptone	5 g

Adjust to pH 4.8 using 0.1M KOH. Make up to 1 litre, and sterilize at 70kPa (10 p.s.i.) for 15 minutes.

Methylene Blue 0.02%

methylene blue	0.02 g
sodium citrate dihydrate	2.0 g

Dissolve in 10 cm^3 of distilled water. Filter and dilute the filtrate to a total of 100 cm^3 with distilled water.

Saline

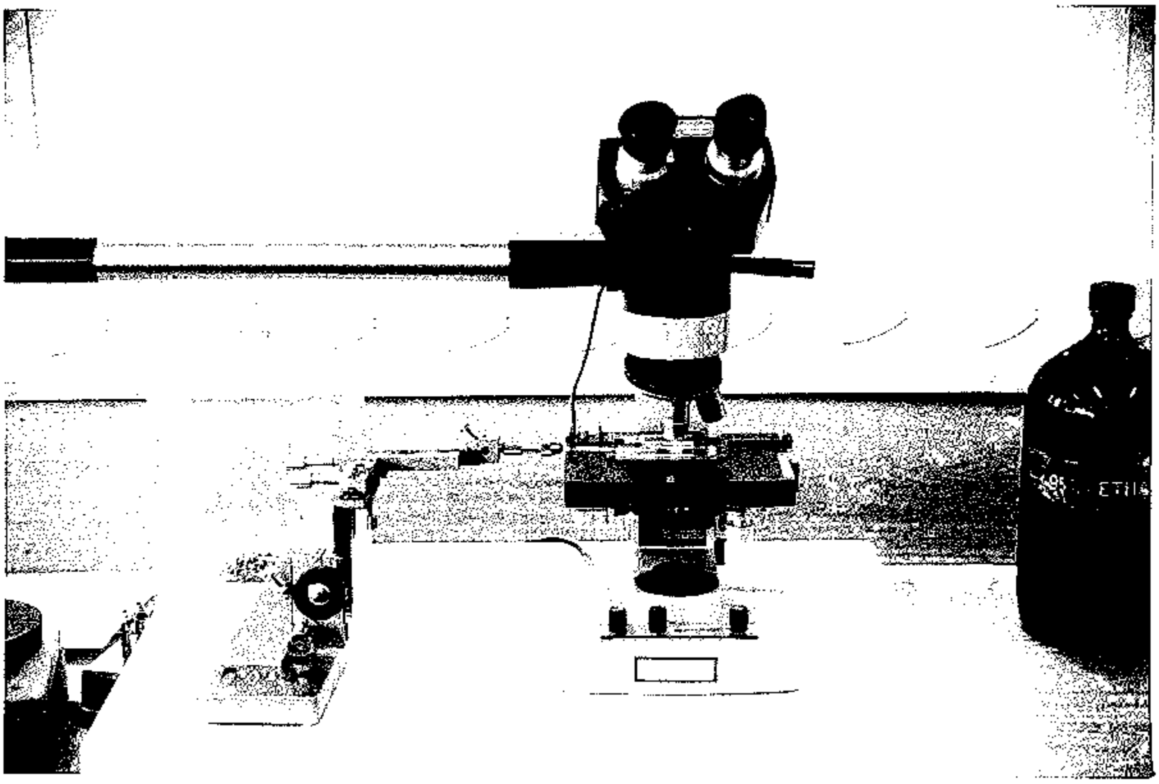
NaCl	8.5 g dissolved in 1 litre of water

3.3 Yeast Hybridisation Procedure

Conventional techniques for yeast hybridisation were used to obtain haploid strains from the champagne strain GW8021 (Mortimer and Hawthorne, 1969; Thornton and Eschenbruch, 1976).

For micromanipulation a Proir manual yeast micromanipulator was used, and a Reichert-Jung micro star 110 fixed stage microscope. Both were placed on an antivibration table. The micromanipulator fitted with a blunt ended glass needle was controlled by the left hand, the right hand was used to control the microscope (Plate 1). The spore suspension to be dissected was spread on an agar slab, placed inverted on a perspex dissection chamber (Plate 2).

Plate 1. Micromanipulator



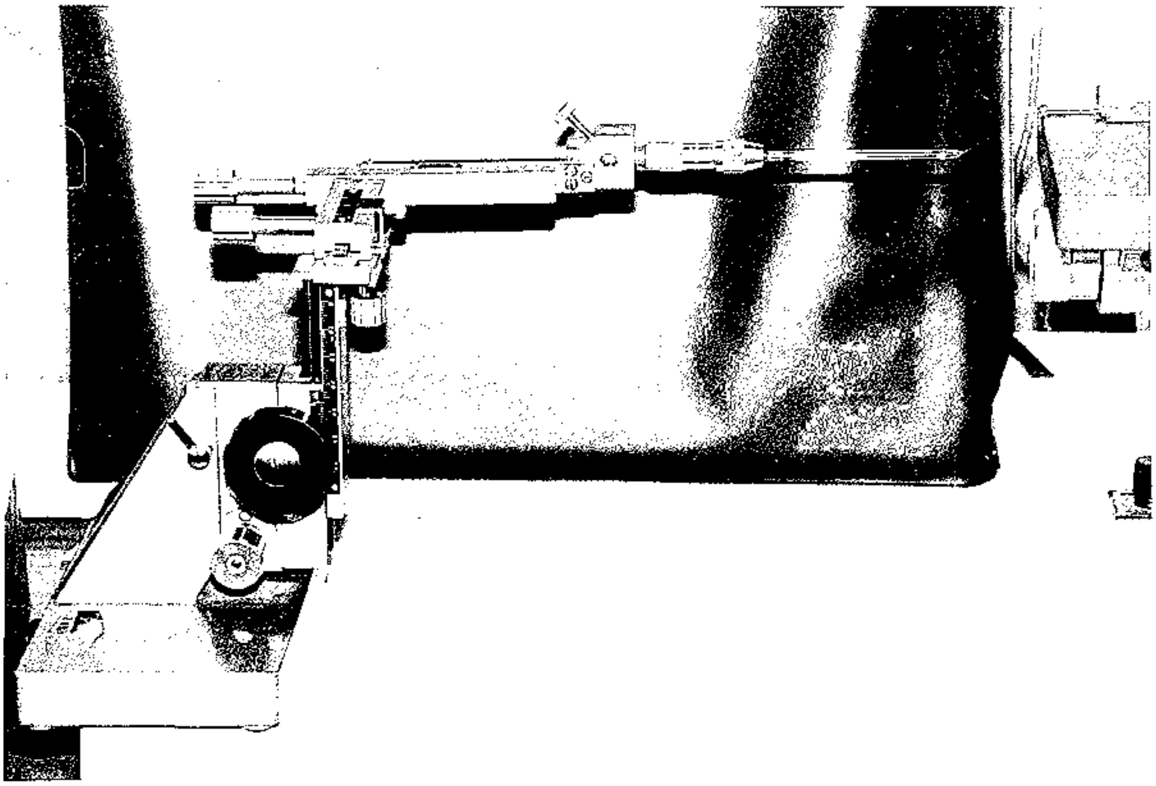
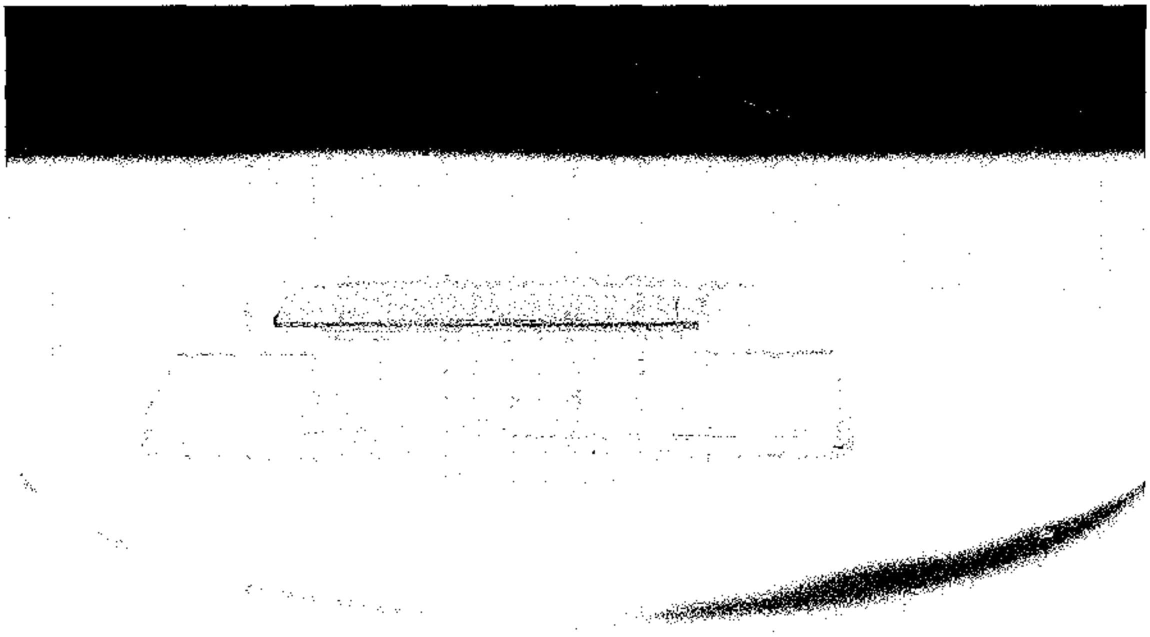
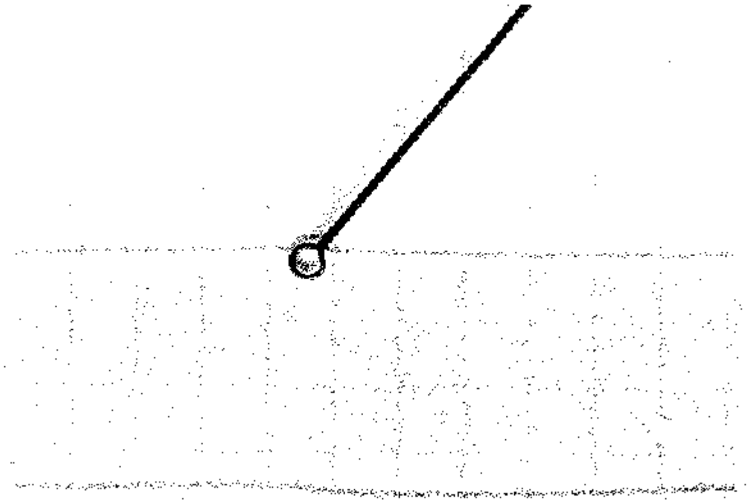


Plate 2. Dissection Chamber for Micromanipulation





3.3.1 Preparation of Yeast Strains for Micromanipulation

Pure cultures of GW8021 were streaked onto glucose nutrient agar slopes and incubated at 30°C for 40 hours, then transferred to potassium acetate slopes and incubated at 20°C for four to five days until sporulation was seen by the presence of asci in a wet mount by light microscopy (Plate 3). If sporulation failed to occur, cells from P.A. slopes were streaked on G.N.A. and the cycle repeated. The transfer from a nutritionally rich media (G.N.A.) to starvation media (P.A.) triggers sporulation.

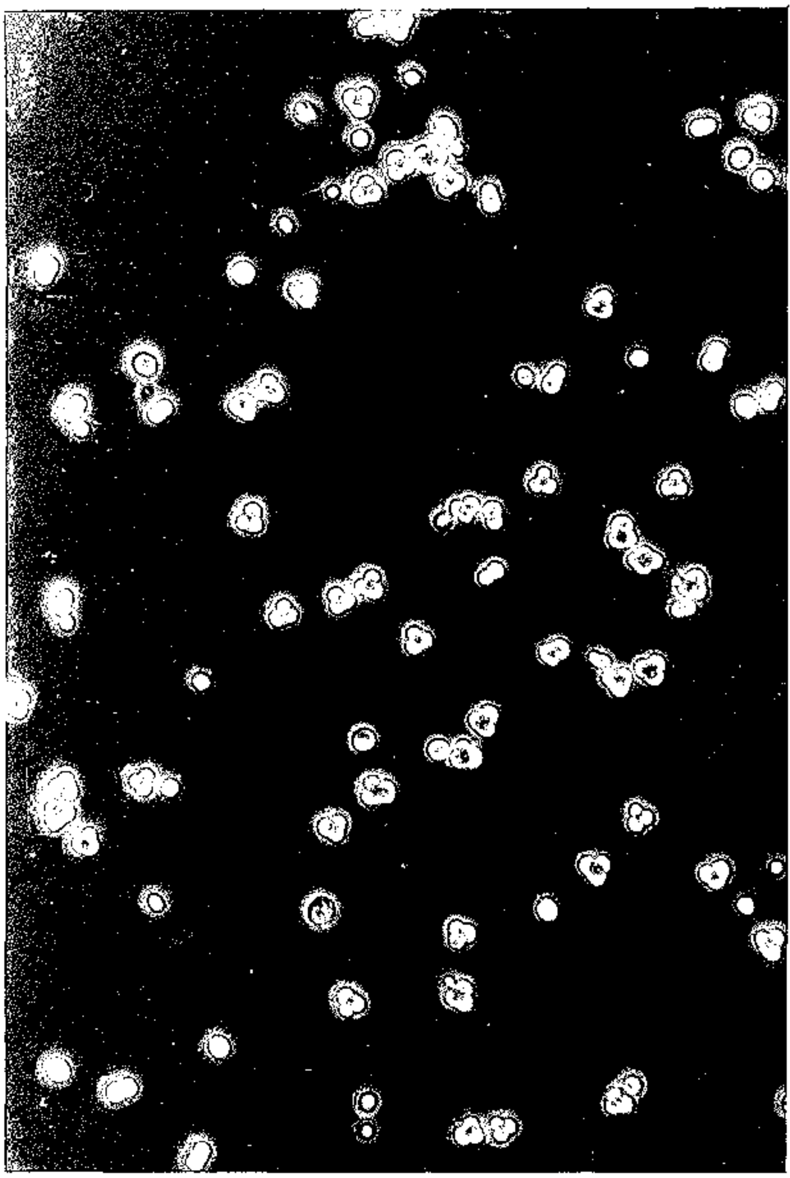
A toothpick of the sporulating culture was placed in 0.2 cm^3 of snail juice enzyme (glucuronidase, Sigma Chemicals, diluted 1/5 in sterile distilled water) and incubated for 20 - 25 minutes at 30°C. This treatment allowed partial digestion of the asci wall and enables dissection of tetrads (Johnston and Mortimer, 1959). After enzyme treatment, the spore suspension may be kept at 5°C for later use.

3.3.2 Micromanipulation Procedure

In preparation for micromanipulation, universal bottles containing 10 cm^3 of sterile 3% M.Y.G.P. agar were placed in beakers of boiling water over a bunsen to redissolve the agar. 22 x 50 mm glass cover-slips were sterilized in ethanol, then flamed, and placed on an ethanol sterilized bench top, covered by a sterile glass petri dish. 0.7 to 0.9 cm^3 aliquots of molten agar were aseptically pipetted onto the coverslips. When the agar had set, 3 to 5 mm of agar was trimmed from the edge of each slab to enable positioning of the slabs on the dissection chamber. Dissection slabs were used within two hours of

Plate 3. Spore Suspension

The arrow indicates a 4 spore asci.



preparation before dehydration occurred.

The enzyme treated sporulating culture was streaked along one edge of the length of the agar slab.

Needles for dissection were prepared from 3 mm soda glass rods, two of which were heated over a bunsen flame, melted together, then quickly pulled apart at a slight angle. The dissection needle was centred in the microscope field prior to micromanipulation. The agar slab was then placed inverted on the dissection chamber. Micromanipulation was carried out at 300 magnification. The needle was controlled by the micromanipulator, in the vertical plane only, and movement of the agar slab controlled by the microscope stage controls.

After micromanipulation, the agar slab was lifted off the coverslip using a sterile spatula, and the agar placed on a M.Y.G.P. agar plate, culture side upward. The plates were incubated at 30°C.

3.3.3 Micromanipulation of Yeast Spores

An ascus was isolated from the streak and moved to a noted co-ordinate on the agar slab by bringing the needle and ascus into contact and either picking up the ascus or dragging it across the agar. By gently tapping the needle, the ascus wall was broken, and the four spores separated in a line 2 mm apart. Separate asci were dissected in this way, and placed 3 mm apart along the agar slab.

3.3.4 Micromanipulation of Zygoes

Zygotes were isolated from a streak in the same way and separated in the same way.

3.3.5 Selection of Haploids

Due to meiotic recombination, and unequal separation of chromosomes, spore clones resulting from the triploid GW8021 may be haploid, diploid or aneuploid. To select haploid clones, the spore clones were tested with mating tester strains. Those clones which mated with only one tester strain as seen by the presence of zygotes in wet microscope mounts, were considered to be haploids, and were selected for autolysis trials (Plate 4).

3.4 Determination of Cell Concentration

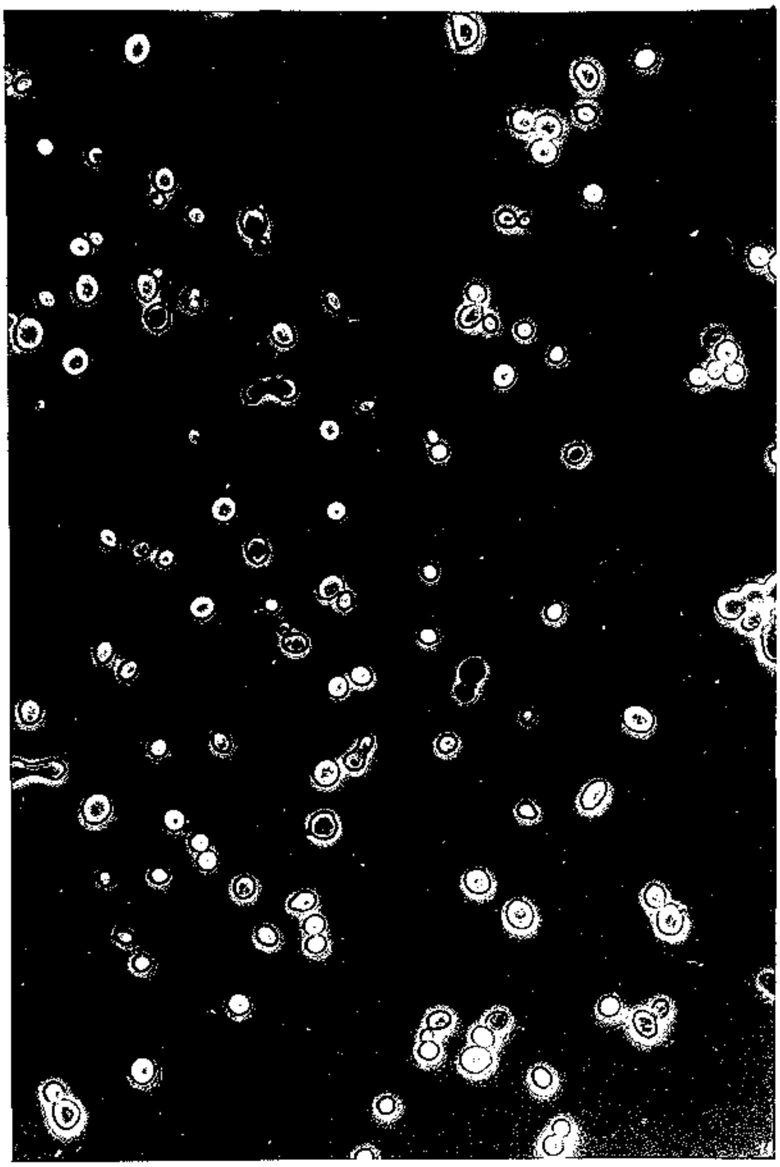
Cell numbers were estimated using a hemacytometer. Total and viable cell numbers were determined by diluting an aliquot of cells in methylene blue; dead cells stained blue, viable cells appeared unstained.

3.4.1 Hemacytometer Counting Procedure

A drop of the stain mixture of yeast cells was allowed to flow under a heavy glass coverslip of the hemacytometer and completely fill the space over the ruled area. Excess suspension was removed with filter paper, and a minute was allowed to let the yeast settle before counting. Four large squares of the improved neubauer counting chamber were counted, each square had an area of 1 mm^2, consisting of sixteen small squares and was 0.100 mm deep. All cells lying on the top and right boundary lines were counted but not cells touching the bottom and left boundary lines; this convention avoided counting the same cell

Plate 4. Zygote Suspension

The arrow indicates a zygote.



twice. Budding yeast cells were counted as one cell. The yeast culture was diluted to give a count of no greater than 300 to 400 cells in the total area counted.

0.02% methylene blue was used in a one to one ratio with yeast suspension to determine the number of dead cells in a culture. After being allowed to stand for five minutes the suspension was counted using the above procedure. Blue stained cells were scored as dead, budding cells were counted as viable if one cell was unstained as, if using a plate counting method, these cells would give rise to a colony and be counted as a viable cell.

3.4.2 Calculation of Cell Concentration

The total cell number was calculated using Formula 1.

Formula 1

$$\frac{\text{Total number of yeast cells} \times \text{dilution factor} \times 10{,}000}{\text{Number of 1 mm}^2\text{ areas counted}}$$

The percentage of dead cells were calculated using Formula 2.

Formula 2

$$\%\text{ dead cells} = \frac{\text{number of stained cells} \times 100}{\text{Total number of cells}}$$

The survival ratio (So) was calculated using Formula 3.

Formula 3

$$So = \frac{\text{Total number of cells - number of dead cells}}{\text{Total number of cells}}$$

3.5 Autolysis Experiment (I)

The initial autolysis was designed to examine the time taken by 5 haploid yeast strains to die and autolyse in nutrient deficient media.

3.5.1 Media Preparation

Three types of nutrient deficient media were selected, sterile distilled water, spent synthetic media, and base wine. Complete mineral media and grape juice were inoculated with yeast, and incubated at 30°C to ensure that all the nutrients were used. The cells were removed by centrifugation. All media were filter sterilized, and dispensed in 100 cm^3 volumes into 10 sterile flasks.

3.5.2 Inoculation and Sampling Procedure

Yeast strains were incubated in complete mineral media at 30°C for 48 hours. Total cell number was determined by hemacytometer. 1×10^7 cells/cm^3 were inoculated into each of the three media in duplicate giving final cell concentrations of 1×10^3 cells/cm^3.

Sampling was initially carried out every five days. Hemacytometer counts were used to determine total cell numbers, and viable cell numbers determined by plate counts.

3.6 Autolysis Experiment (II)

The second autolysis experiment was designed to examine 22 GS haploid strains in M.Y.G.P. to select those strains which died or autolysed rapidly.

3.6.1 Strain Preparation and Inoculation

22 haploid GS strains were inoculated into M.Y.G.P. broth and incubated for 48 hours at 30°C. Methylene blue staining was used to count viable and dead cells. Each strain was inoculated in duplicate into M.Y.G.P. to give a viable cell count of 10^3 cells per cm^3.

3.6.2 Sampling and Analysis of Autolysis

The yeast populations were counted monthly; viable and dead cell numbers were determined using the methylene blue staining method or by plate counts.

3.7 Determination of Growth Curves

GS18 and GS20 were inoculated into 10 cm^3 of M.Y.G.P. and incubated at 30°C for 48 hours. Viable and dead cell counts were recorded, and each strain inoculated in duplicate into 100 cm^3 of M.Y.G.P. to give viable counts of 10^5 cells per cm^3. The flasks were incubated at 30°C.

0.1 cm^3 samples were taken every four hours and total, viable and dead cell counts recorded. Samples were taken until stationary phase had been maintained for 12 hours.

Cell concentrations were plotted on semi-log paper.

3.8 Examination of Amino Acid Leakage During Autolysis

Amino acid leakage associated with autolysis was studied at 15°C and 30°C in minimal media.

Haploid yeast strains GS18 and GS20 were grown in 10 cm^3 of M.M. at 30°C, viable and dead cell concentrations recorded, and inoculated into 8 1 litre flasks of M.M. to give viable cell counts of 10^5 cells per cm^3. Duplicate flasks of each strain were incubated statically at 15°C and 30°C.

Initial sampling was carried out daily. 3 cm^3 of shaken culture were removed, filter sterilized into sterile vials, and held at 5°C before being analysed for amino acid concentration by H.P.L.C. Further 0.1 cm^3 samples were removed, diluted in methylene blue, and viable and dead cell counts recorded.

3.9 Examination of Autolysis in Wine

To investigate the changes of viable and dead cell numbers in wine, yeast cultures were inoculated into 50 cm^3 of wine containing 5 cm^3 fermentation syrup to give viable counts of 10^5 cells/cm^3. The wine was tested for ethanol concentration by enzymatic assay and the SO_2 level checked. The flasks were incubated statically at 15°C with fermentation traps.

3.9.1 Acclimitization Procedure

GS strains were adapted to growth in 12% (v/v) ethanol. A five day fermentation of 10% glucose at 25°C produces 4% ethanol. The fermentation was repeated four times transferring cells from an ethanol free media to media containing 4%, 8%, 10%, then 12% (v/v) ethanol.

3.9.2 Determination of Sulphur Dioxide Levels

Determination of free and bound SO_2 levels in wine were carried out using the technique of Rankine and Pococh (1970). Total SO_2 may be determined by one analysis, or by addition of bound and free SO_2 concentrations.

i Reagents

Hydrogen peroxide:

10 cm^3 of 3% H_2O_2 was added to 80 cm^3 of deionized distilled water and 0.5 cm^3 of 0.1% methyl red indicator. 0.01M sodium hydroxide was added until the solution just turned yellow, then the total volume made up to 100 cm^3 in a volumetric flask using distilled water. This solution was made up fresh on the day of each determination.

Phosphoric acid:

280 cm^3 of concentrated (90%) phosphoric acid was added to 720 cm^3 of distilled water and mixed well.

ii Estimation of Free SO_2

10 cm^3 of the yellow 0.3% H_2O_2 solution was pipetted into a 50 cm^3 round bottomed flask. The flask was then connected to the bubbling tube, ensuring that the clamp was on, and that the vacuum lead was connected. 20 cm^3 of wine was pipetted into a second round bottomed flask and, while on a tilt, 10 cm^3 of 25% phosphoric acid solution was added. This flask was connected to the blower tube. Water was then run through the condenser at a fast rate, and the water jet pump turned on to allow air to be drawn through the system at a rate of 1 litre/minute, as checked with a gas rotometer. Aspiration in this way was continued for 10 minutes. When free SO_2 is present, the H_2O_2 indicator will turn red (Plate 5).

After aspiration was complete, the flask containing the indicator was lowered, and the bubbler tube rinsed with a little distilled water into the flask. The contents of the flask were titrated with 0.01M H_2O_2. End point was reached when the indicator turned the initial yellow colour.

iii Estimation of Bound SO_2

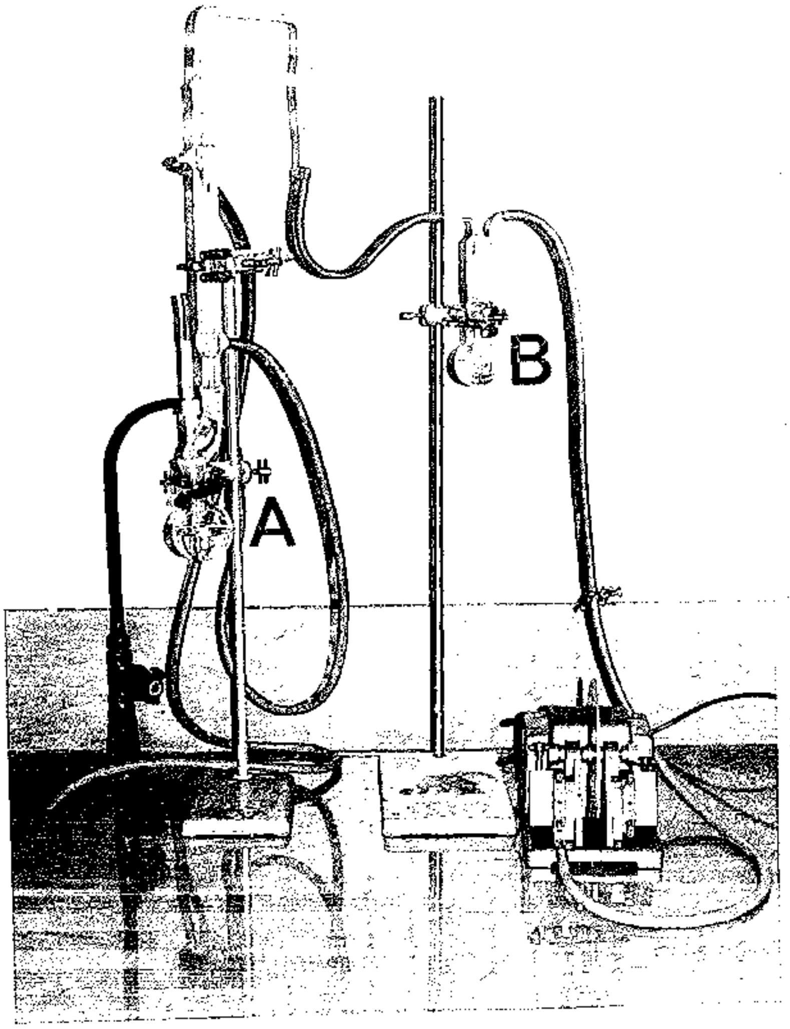
The bound SO_2 concentration can be determined on the same sample by using fresh indicator solution and aspirating for 10 minutes while the sample is kept boiling. Timing of aspiration begins when the sample boils.

iv Calculation of SO_2 Concentration

1 cm^3 of 0.01M NaOH is used in the titration for every 0.32 mg of

Plate 5. Appartus for SO_2 Determination

The wine sample is added to flask A; Flask B contains H_2O_2 indicator solution


A
B

SO_2 present. For a 20 cm^3 sample, the titration figure in cm^3 multiplied by 16 gives mg/l (p.p.m.) SO_2.

3.9.3 Enzymatic Ethanol Assay

i Reagents

Triz/lyzine buffer

Trizma base	72.68 g
Lysine monohydrochloride	73.08 g

Adjusted to pH 9.7 with 4M NaOH, then made up to a total volume of 1 litre. Stored in a fridge.

Cocktail - for 30 assays

NAD^+ grade III	45 g
Deionized water	42 g
Buffer	52 g

The cocktails were made up fresh on the day of the assay and stored on ice until used.

Alcohol dehydrogenase

15 mg dissolved in 1 cm^3 of water and stored on ice until used.

4 mM ethanol standard

Wine samples diluted 1/500

ii Assay Procedure

For the assay 3 cm^3 of cocktail were used per cuvett, 50uL of sample added, mixed, and absorbance at 340nm against a water blank recorded. A reference blank was used, containing no sample, to adjust for cocktail absorbance change.

20uL of alcohol dehydrogenase was added to each cuvett, mixed, and allowed to stand for 2 minutes, then the absorbance at 340nm read. The following calculation was used to determine the amount of ethanol in the samples in mM.

$$\frac{\text{Final absorbance} - (\text{Initial absorbance} + \text{A Abs of Blank})}{6.22} \times \frac{\text{Total volume}}{\text{Sample volume}}$$

$$= \text{Abs} - \text{Abs (blank} \times 9.8714)$$

% ethanol = mM ethanol x 2.88

3.9.4 Sampling

0.1 cm^3 samples were taken every four weeks, diluted in methylene blue and total, viable and dead cell numbers determined by microscope counts.

3.10 Killer Cross Reactions

All of the killer strains used were tested for killer phenotype using a plate assay. Reactions with standard killer strains were used to determine the killer phenotype of other yeast strains.

Every strain to be tested was spotted onto a master plate and incubated at 30°C for 48 hours. Each master plate contained a spot culture of each strain to be tested and replicas made, one copy of the master plate for each strain.

Each strain was inoculated into M.Y.G.P. pH 4.8 broth and incubated at 30°C for 24 hours. 0.1 cm^3 of each 24 hour broth culture was spread onto M.Y.G.P. (pH 4.8) agar plates, so that each plate contained a lawn of approximately 10^6 cells. These plates were incubated at 30°C for three hours to enable the cells to enter exponential phase. After incubation, a master plate was replica plated on to each tester strain lawn, using a velvet block. The plates were incubated at 25°C for 48 hours, then examined for killing reaction. Positive killing reactions were seen as a zone of inhibition around the spot culture.

3.11 Production of K_1 Toxin and Concentration

S. cerevisiae K_1 strain was grown statically at 20°C in 2 litres of synthetic B-media, pH 4.8 adjusted with 0.1M KOH, as modified by Pfeiffer and Radler (1982) from the media of Heerde and Radler (1978). After three days incubation, the yeast cells were removed by centrifugation at 12,000 r.p.m. in a Sorvall RC-5B refrigerated superspeed centrifuge using a G.S.A. rotor. The supernatant was passed through a 0.45u filter to remove excess cells, and concentrated through a Diaflo Amicon ultrafiltration membrane with an exclusion limit 10,000 M.W. The total volume was concentrated to a final volume of approximately 55 cm^3 which corresponded to a 36 fold concentration. 20% glycerol was added to the killer concentrate to stabilise the killer protein (Ouchi et al, 1978; and Bussey 1982). The concentrate was stored at 5°C.

3.11.1 Curing of a K_1 Killer

1.3 mg of cyclohexamide were dissolved in 100 cm^3 of distilled water and sterilized at 70kPa (15 p.s.i.) for 15 minutes. 1 cm^3 aliquots of this stock solution were added to sterile petri dishes and mixed with 20 cm^3 of sterile molten M.Y.G.P. When cool, 0.1 cm^3 of of a K_1 suspension were spread in duplicate on to the cyclohexamide plates, and on to control M.Y.G.P. plates lacking cyclohexamide. The plates were incubated at 30°C. After a 70 hour incubation, 25 colonies from the cyclohexamide plates and 13 control colonies were selected and spotted on to duplicate M.Y.G.P. plates and incubated at 30°C for 48 hours.

The selected colonies were tested for killing ability by the killer reaction plate assay. Strains which showed no zones of inhibition, indicating loss of killer activity, were streaked; single colonies selected and retested. The procedure was repeated three times interspersed at three day intervals, at 30°C, before strains were accepted as having been cured.

3.11.2 Determination of Protein in Killer Toxin concentrate

Protein concentration was determined using the method of Ehresmann, Imbault and Well (1973). Protein standards were made by diluting Bovine Serum Albumin to concentrations of 250ug, 100ug, 50ug and 25ug. The sample was diluted to give an absorbance reading corresponding to less than the 100ug standard. U.V. absorbance was read at 228.5 nm and 234.5 nm using a Cecil 272 Ultraviolet Spectrophotometer and a tungstan lamp. Equation 12.1 was used to determine the detected concentration of protein.

Equation 12.1 $\text{abs} \times 10^3 \times 0.318 = \text{ug protein/cm}^3$

A standard curve was drawn and used to determine the actual protein concentration in the killer concentrate. The protein concentration in the supernatant of a parallel cured strain was used to distinguish toxin protein from other extracellular protein.

3.11.3 Determination of Killing Activity of Toxin

Each batch of killer toxin concentrate was tested for killing activity in both liquid and solid media.

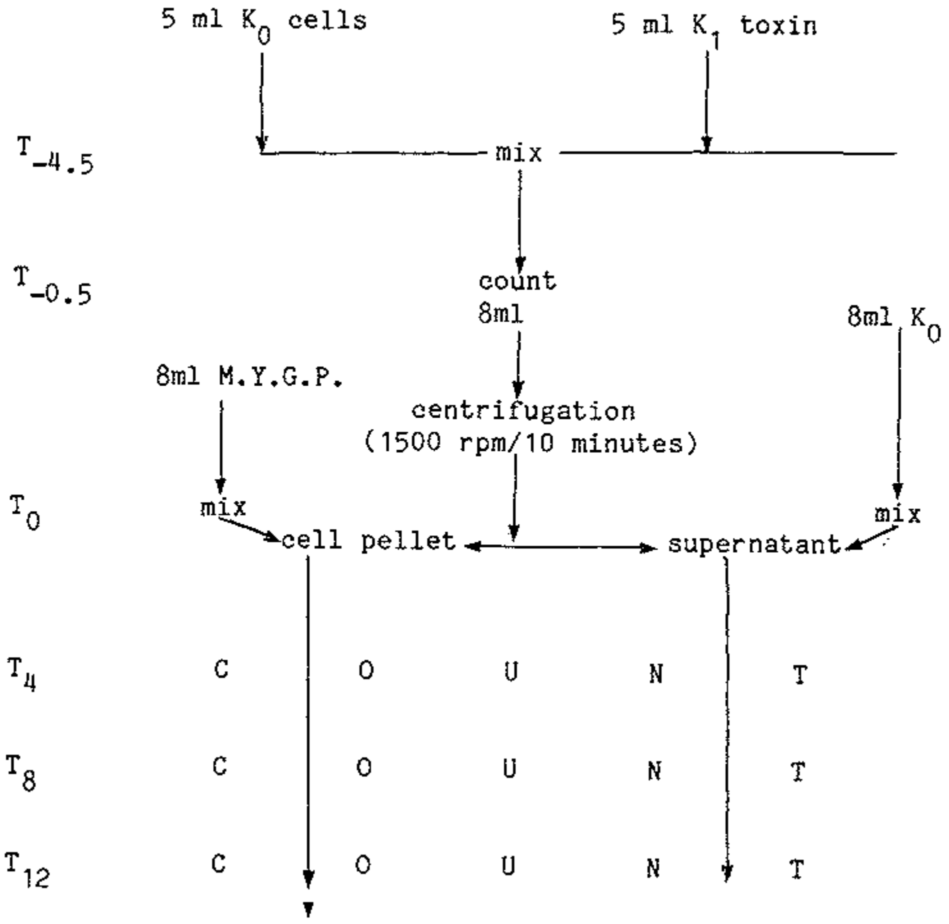
For killing in liquid media, 0.1 cm^3 of the toxin was incubated with 0.1cm^3 of exponential K_0 cells in duplicate at 30°C and the killing activity scored after a four hour incubation.

Assay of killing activity in solid meda was carried out using a modified version of the killer reaction test. 0.2 cm^3 of the toxin were aseptically pipetted into 10 mm wells in M.Y.G.P. plates seeded with K_0 cells; the zones of inhibition after two days incubation at 30°C were measured and recorded.

The effect of addition to P.M.S.F. to give a concentration of 0.2 nM was examined to study increase stability of the toxin.

The role of active toxin in the supernatant after a four hour incubation with K_0, as opposed to the time taken for a cell to die once it is infected with toxin, was examined using the procedure shown in Figure 2.

Figure 2. Procedure to Examine Toxin Activity of Supernatant


5 ml K0 cells
5 ml K1 toxin
T-4.5
mix
T-0.5
count
8ml
8ml K0
8ml M.Y.G.P.
centrifugation
(1500 rpm/10 minutes)
T0
mix
cell pellet
supernatant
mix
T4
C O U N T
T8
C O U N T
T12
C O U N T

3.11.4 Calculation of Killer Activity

Estimation of multiplicity and active killer units of killer toxin were obtained using poisson distribution, a standard formula used for estimation of viral infection of cells (Davis, Dalbeco, Eisen and Ginsberg, 1980).

The multiplicity of infection (m) is taken as the average number of toxin molecules infecting each cell. Only active molecules are considered; inactive molecules or molecules which do not enter a cell are neglected. From studies of killing kinetics Bussey (1972) estimated that one lethal unit of killer toxin (the amount needed to kill one cell) contains approximately 10,000 molecules. Arbitrarily, one killer unit kills one cell.

m is related to the total number of killer molecules (N) and of sensitive yeast cells (C), by the Formula 4.

Formula 4

$m = aN/C$

where a is the proportion of killer molecules that initiates infection

The proportion (K) of cells infected by K killer molecules is given by the poisson distribution, assuming that the cells are all identical in the ability to be infected. According to the poisson distribution -

Formula 5

$$S/S(K) = \frac{e^{-m}m^{k}}{K}$$

The value of m can be determined experimentally when the total number of cells and the number of infected cells are known. When K = 0, Formula 6 is used.

Formula 6, Bussey and Sherman (1973)

$S/S_0 = e^{-m}$

therefore $m = -\ln S_0$

where ln stands for the natural logarithm and the survival ratio (S/S_0) is determined by Formula 7.

Formula 7

$$S/S_0 = \frac{\text{total number of cells - number of infected cells}}{\text{total number of cells}}$$

To measure the concentration of active killer units in a sample, Formula 8 is used.

Formula 8

$aKu = -C \ln S/S_0$

where C is the total number of cells and aKu is the number of active killer units

In killing experiments, killer killed cells are scored as those cells dead after four hours incubation with killer toxin. A proportion of the killer treated cells are dead at the start of the experiment, or die during the experiment without being killed by toxin. To correct for these cells, control suspensions of sensitive cells were held under experimental conditions without toxin. The concentrations of false a.k.u. in the controls were calculated and subtracted from the a.k.u. obtained for killer treated cells to give a corrected active killer unit concentration (c.a.k.u.).

3.12 Efficiency of Killing at Different pH's

Killing activities of concentrated K_1 toxin were tested at different pH levels and at different temperatures.

M.Y.G.P. tubes were prepared, the media buffered using sodium phosphate and citric acid to pH 2, 3, 4, 4.8 and 5. The day before each assay, one tube of each pH was inoculated with 0.1 cm^3 of K_0 culture, and incubated at 30°C for 16 to 18 hours to let the cells reach exponential phase.

For each assay, 0.1 cm^3 of K_0 culture of each pH was mixed in duplicate with 0.1 cm^3 of K_1 toxin concentrate and incubated at the designated temperature. Control tubes were included for each pH containing 0.2 cm^3 of undiluted K_0 cells. The assay was repeated five times, with tubes being incubated for 4 hours at 5, 15, 20, 25 or 30°C. After incubation, the concentrations of dead and viable cells were determined using methylene blue counting procedure. Multiplicity and corrected active killing units were calculated for each experimental tube.

3.13 Effect of Ethanol on Killing Activity of K_1 Toxin

The effect of different concentrations of ethanol on killing at different temperatures in M.Y.G.P. was studied to establish the maximum ethanol at which killing would occur.

3.13.1 Media Preparation

Double strength M.Y.G.P. was used to prepare 10 cm^3 tubes containing various amounts of ethanol (v/v) as shown in Table 3. Filter sterilized ethanol was added to the sterile M.Y.G.P. and phosphate-citrate buffer mixture at the time of inoculation, and the tubes sealed with parofilm paper.

3.13.2 K_0 Strain Preparation

For the ethanol assays a K_0 strain was used that had been adapted to growth in 12% (v/v) ethanol using acclimitization procedure in section 3.91.

3.13.3 Assay Procedure

0.1 cm^3 of acclimitized K_0 cell suspension was inoculated into media containing 0 to 12% ethanol (v/v) and incubated at 30°C for 15 to 18 hours. 0.1 cm^3 samples of each ethanol concentration were mixed in duplicate with 0.1 cm^3 of K_1 toxin concentrate. 0.2 cm^3 of K_0 cell suspensions at each ethanol concentration were used as controls. After four hours incubation at 5, 15, 20, 25 or 30°C, the samples were counted using the methylene blue counting procedure, and the multiplicity and corrective active killer units calculated.

3.13.4 The Direct Effect of Ethanol on Toxin

To examine the direct effect of ethanol on toxin, 2 cm^3 aliquots

Table 3. Assay Tubes for Effect of Ethanol on the Toxin Reaction

Alcohol concentration %	0	2	4	6	8	10	12
Double Strength M.Y.G.P. pH4.8 cm^3	5	5	5	5	5	5	5
Phosphate-citrate buffer pH4.8 cm^3	4.9	4.7	4.5	4.3	4.1	3.9	3.7
Absolute ethanol cm^3	0	0.2	0.4	0.6	0.8	1.0	1.2

of toxin were diluted in 10 cm^3 of water containing various amounts of ethanol (0, 2, 4, 6, 8, 10% v/v) and incubated at 30°C for 30 minutes. To remove the ethanol, each sample was reduced to 2 cm^3 by ultra-filtration at 5°C, washed in 8 cm^3 of buffer, then reduced to less than 2 cm^3. The volume of each sample was adjusted to 2 cm^3 by weight (2 cm^3 of toxin weighed 2.30 g).

The remaining activity of the toxin was determined by mixing 1:1 with a 16 hour K_0 cell culture and incubated at 30°C. After a four hour incubation the number of cells killed was scored by hemocytometer counting and methylene blue staining.

3.14 Amino Acid Leakage from Killed Cells

Amino acid auxotrophs were tested for sensitivity to K_1 toxin using the standard killer assay procedure. Arginine and lysine requiring mutants were found to have K_0 phenotype.

The minimum concentration of amino acids required for growth was examined by following growth by optical density readings at 650 nm for a three day period.

3.14.1 Examination of Killing and Regeneration

Both auxotrophs were inoculated into minimal media that lacked amino acids. The suspensions were diluted 1:1 with K_1 toxin, incubated statically at 30°C and counted every four hours. The killing and regeneration pattern was compared with control cultures lacking toxin, and with patterns obtained when the experiment was repeated using a standard K_0 strain.

3.14.2 Amino Acid Leakage

The amount of arginine needed to support the regeneration seen was determined in minimal media supplemented with various amounts of arginine. An inoculum of 1×10^6 arg^-cells (the number of viable cells present after killing was complete) was used, then the growth and final cell yield calculated by hemocytometer counts. For lys^- estimates, an inoculum of 9×10^6 cells per cm^3 was used, and growth followed in the same way.

RESULTS

4.1 Autolysis

The mating types of 35 spore clones, isolated by the micromanipulation of the asci of a triploid champagne yeast GW8021, were determined. Those clones which mated with only a or α mating type tester strains were given a GS strain number as shown in Table 4. These GS strains are presumptive haploids or, at worst, aneuploids. Each GS strain was tested for killer phenotype by the plate assay system. The GS strains showed the neutral phenotype of the parent strain GW8021, in that toxin was not produced by any strain, and all strains were resistant to the three types of killer toxin.

Five GS strains were used for initial comparison of death and autolysis in nutrient deficient media. The fate of these strains in synthetic media, wine and distilled water was examined by hemacytometer and plate counts (Tables 5, 6 and 7). A slight increase in total cell number was seen before a decline in viable cell number. After a period of 122 days, disintegration of the cells made accurate cell counting impossible. By nine months, the cells had crystallised into hard black crystals as shown in Plate 6.

To investigate strain comparisons more accurately, all 22 strains were inoculated into M.Y.G.P. broth. The growth and death of these strains at 15°C was observed for a period of eight months. Cell numbers were originally estimated using methylene blue staining and hemacytometer counts, but after 12 weeks disintegrating cells no longer stained blue, so plate counts were used to determine viability. The strains studied have been grouped into eight classes, each

Table 4. Mating Types of GS Strains

A positive mating reaction (+) with a single mating type tester strain indicatess the opposite spore clone mating type.

Spore Clone	Mating Tester		Mating Type	Strain
Number	a	α		
1	-	+	a	GS1
2	-	+	a	GS2
3	+	-	α	GS3
4	+	-	α	GS4
5	+	-	α	GS5
6	-	-	-	
7	-	+	a	GS6
8	-	-	-	
9	-	-	-	
10	-	-	-	
11	-	-	-	
12	-	-	-	
13	-	-	-	
14	-	+	a	GS7
15	-	+	a	GS8
16	-	-	-	
17	-	+	a	GS9
18	+	-	α	GS10
19	-	+	a	GS11
20	-	-	-	
21	-	+	a	GS12
22	-	-	-	
23	-	+	a	GS13
24	+	-	α	GS14
25	+	-	α	GS15
26	-	+	a	GS16
27	-	-	-	
28	-	+	a	GS17
29	+	-	α	GS18
30	-	-	-	
31	-	+	a	GS19
32	-	+	a	GS20
33	+	-	α	GS21
34	-	+	a	GS22
35	-	-	-	

Table 5. Growth in Nutrient Deficient Synthetic Media

Viable and total cell concentrations per cm^3 of synthetic media.

Day	GS1		GS2		GS3	
	viable	total	viable	total	viable	total
0	3.7×10^{5}	9.7×10^{6}	2.0×10^{5}	6.08×10^{5}	3.0×10^{5}	9.7×10^{5}
5	4.1 10^{5}	9.7×10^{5}	2.0×10^{6}	9.1×10^{6}	4.9×10^{6}	9.3×10^{6}
10	1.8×10^{6}	2.3×10^{6}	1.0×10^{4}	5.6×10^{4}	3.0×10^{4}	5.3×10^{6}
15		8.0×10^{6}	1.1×10^{4}		1.8×10^{5}	
20	2.5×10^{7}	1.8×10^{7}	1.0×10^{3}	2.0×10^{6}	5.7×10^{5}	1.7×10^{7}
25	2.9×10^{7}	2.14×10^{7}	3.5×10^{1}	2.5×10^{6}	3.2×10^{4}	1.5×10^{7}
53			1.0×10^{2}	4.5×10^{6}	1.0×10^{4}	2.0×10^{7}
81	7.0×10^{6}		5.0×10^{6}		2.4×10^{6}	2.3×10^{7}
112	3.8 x107		3.5×10^{7}		3.8×10^{7}	

Day	GS5		GS8	
	viable	total	viable	total
0	8.9×10^{4}	2.5×10^{5}	2.2×10^{5}	1.0×10^{6}
5	7.9×10^{5}	1.9×10^{6}	3.0×10^{6}	4.9×10^{6}
10	1.0×10^{5}	4.3×10^{6}	1.6×10^{5}	8.3×10^{6}
15	3.7×10^{6}		3.7×10^{5}	
20	1.8×10^{6}	6.1×10^{6}	2.0×10^{5}	8.9×10^{6}
25	4.3×10^{5}	4.6×10^{6}	2.5×10^{5}	9.3×10^{6}
53	0	8.5×10^{6}	0	9.7×10^{6}
81	0	6.2×10^{6}	0	1.2×10^{7}
112	0	9.0×10^{6}	0	7.1×10^{6}

Table 6. Growth in Base Wine

Viable and total cell concentration per cm^3 of base wine

Day	GS1		GS2		GS3	
	viable	total	viable	total	viable	total
0	1.3×10^{5}	6.8×10^{5}	1.4×10^{5}	5.3×10^{5}	4.6×10^{5}	1.0×10^{6}
5	2.0×10^{4}	7.0×10^{5}	8.5×10^{4}	5.0×10^{5}	1.0×10^{6}	1.5×10^{6}
10	2.0×10^{4}	5.3×10^{5}	8.0×10^{4}	2.5×10^{6}	6.3×10^{5}	3.3×10^{6}
15	1.8×10^{6}	3.6×10^{5}	5.8×10^{5}	5.0×10^{6}	2.4×10^{6}	3.3×10^{6}
20	4.5×10^{6}	8.0×10^{6}	1.0×10^{6}	5.6×10^{6}	7.0×10^{5}	5.3×10^{6}
25	4.7×10^{6}	7.0×10^{6}	1.9×10^{4}	7.0×10^{6}	1.0×10^{2}	5.8×10^{6}
53	0	9.5×10^{6}	0	6.6×10^{6}	0	5.0×10^{6}
79	0	1.2×10^{7}	0		0	
112	0	1.6×10^{7}	0	9.5×10^{6}	0	8.77×10^{6}

Day	GS5		GS6	
	viable	total	viable	total
0	3.1×10^{5}	4.1×10^{5}	3.1×10^{5}	5.7×10^{5}
5	1.8×10^{5}	4.9×10^{5}	1.1×10^{5}	6.5×10^{5}
10	1.0×10^{4}	4.0×10^{5}	2.0×10^{5}	6.0×10^{5}
15	9.5×10^{3}	6.3×10^{5}	6.8×10^{6}	6.0×10^{6}
20	1.1×10^{4}	5.7×10^{5}	1.0×10^{5}	7.2×10^{6}
25	5.0×10^{3}	1.1×10^{6}	3.3×10^{5}	7.0×10^{6}
53	0	7.8×10^{6}	0	8.9×10^{6}
79	0		0	
112	0	1.0×10^{7}	0	1.2×10^{7}

Table 7. Growth in Distilled Water

Viable and total cell concentrations per cm^3 of distilled water.

	GS1		GS2		GS3	
Day	Viable	Total	Viable	Total	Viable	Total
0	$4x10^4$	$5.6x10^5$	$1.1x10^5$	$4.4x10^5$	$5.9x10^5$	$1x10^6$
5	$2x10^4$	$9.8x10^5$	$2.1x10^6$	$1.7x10^6$	$3.2x10^5$	$1.1x10^6$
10	$1.9x10^4$	$7.9x10^5$	$1.2x10^5$	$1.6x10^6$	$4.4x10^4$	$8.0x10^5$
15	$5.0x10^5$	$1.5x10^6$	$8.5x10^2$	$6.2x10^6$	$7.5x10^5$	$5.0x10^5$
20	$1.1x10^6$	$1.5x10^6$	$1.2x10^3$	$6.2x10^6$	$4.4x10^5$	$8.1x10^5$
25	$1.0x10^6$	$4.1x10^6$	$1.1x10^4$	$1.6x10^6$	$2.4x10^5$	$1.1x10^6$

GS5		GS6	
Viable	Total	Viable	Total
$3.3x10^5$	$5.2x10^5$	$1.3x10^5$	$9.4x10^5$
$6.0x10^5$	$9.5x10^5$	$4.1x10^5$	$7.7x10^5$
$3.6x10^5$	$1.2x10^6$	$1.9x10^5$	$3.2x10^6$
$6.2x10^5$	$1.7x10^6$	$8.4x10^5$	$6.9x10^5$
$2.6x10^5$	$1.6x10^6$	$3.2x10^5$	$8.3x10^5$
$1.2x10^4$	$1.7x10^6$	$1.5x10^5$	$8.5x10^5$

Plate 6. Crystallized Yeast Cells from Base Wine

Actual size of crystallized yeast.

representing an observed pattern of death. These classes are shown in Figure 3, while the actual values for each strain are in Appendix I. The disappearance and subsequent reappearance of dead cells of group G is shown by a break in the dead cell graph line. Plate 7 illustrates the appearance of selected strains after eight months ageing.

Figure 3. Growth and Death of GS Strains for 8 Months in M.Y.G.P.

Each graph represents the pattern of death seen for the named strains.

A: G.S. 1, 6, 15

B: G.S. 3, 7, 9, 12, 13, 16

C: G.S. 18, 20, 22

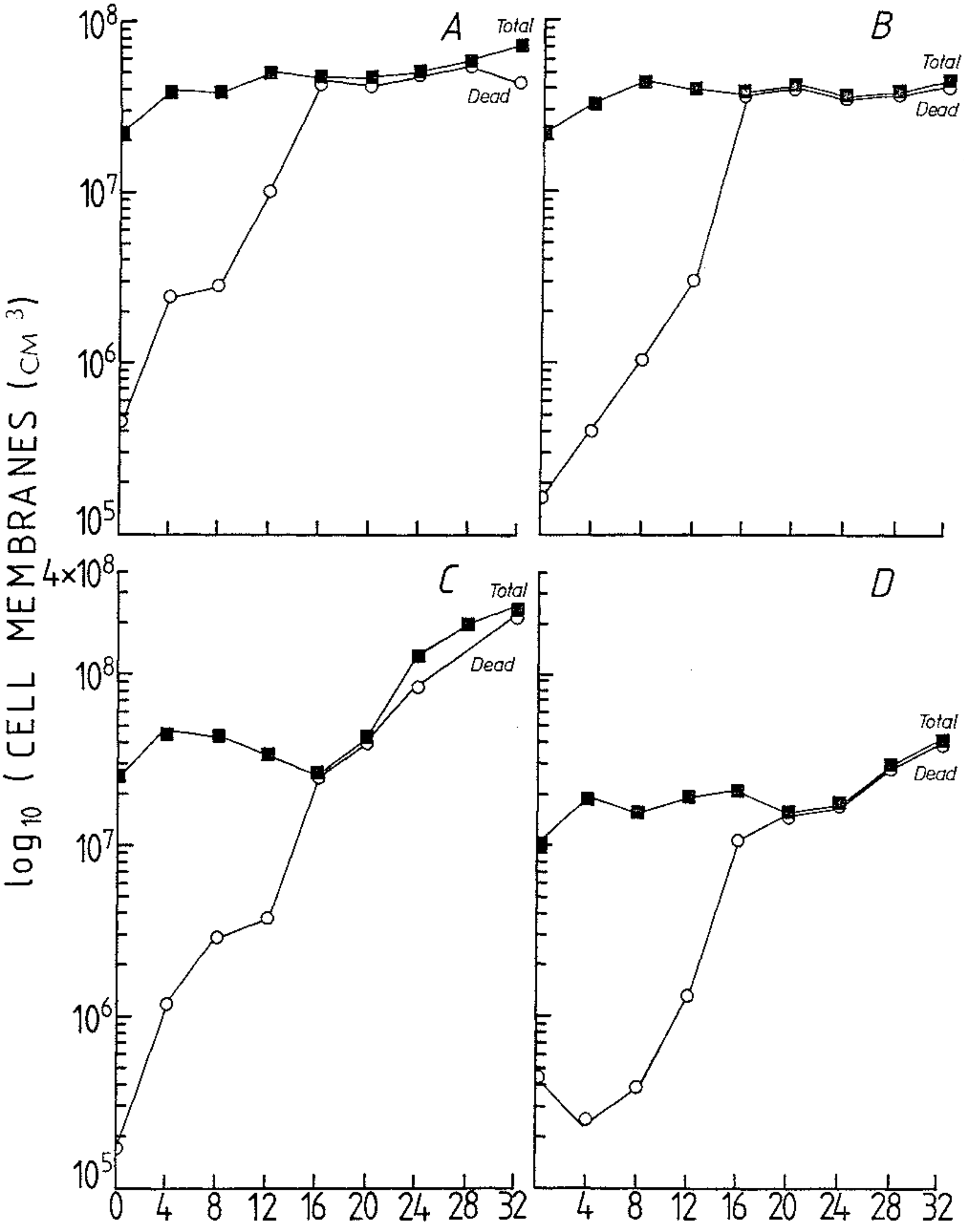
D: G.S. 2, 5

E: G.S. 4, 10, 17

F: G.S. 11

G: G.S. 14, 19, 21

H: G.S. 8


A
B
C
D
Total
Dead
10^8
10^7
10^6
10^5
4×10^8
log10 (CELL MEMBRANES (CM 3)
WEEKS
0
4
8
12
16
20
24
28
32

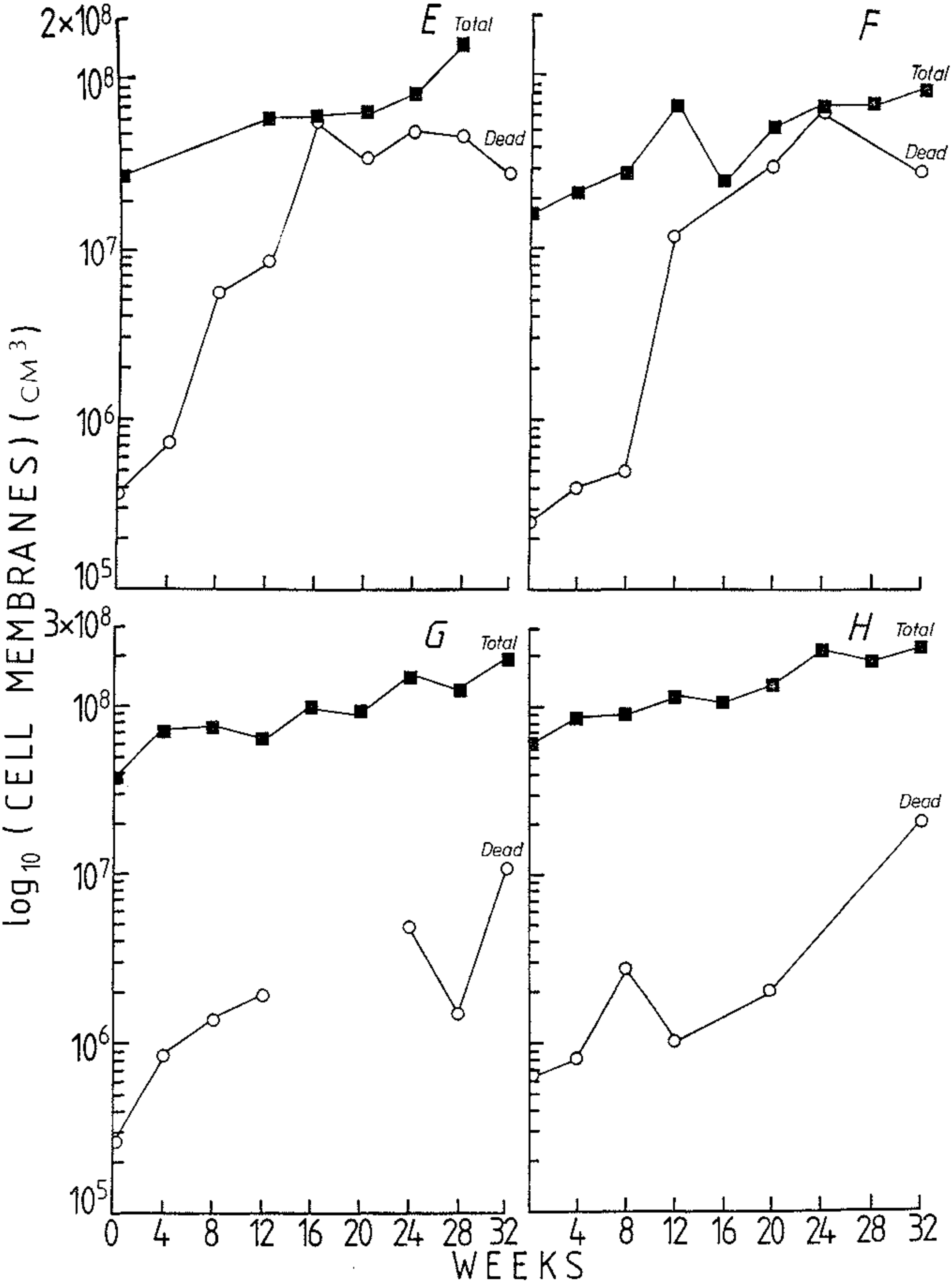
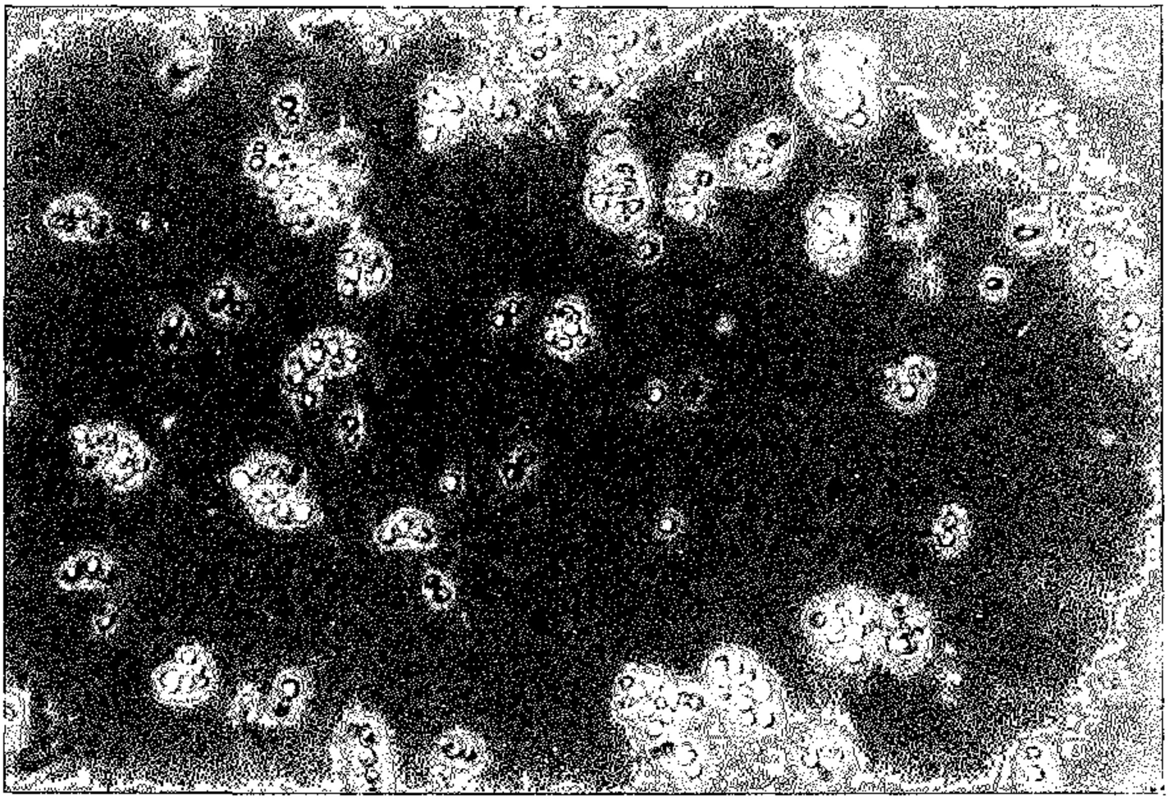

E
F
G
H
Total
Dead
2×10^8
3×10^8
10^8
10^7
10^6
10^5
log10 (CELL MEMBRANES) (CM^3)
0
4
8
12
16
20
24
28
32
WEEKS

Plate 7. Appearance of GS Strains After 8 Months in M.Y.G.P.

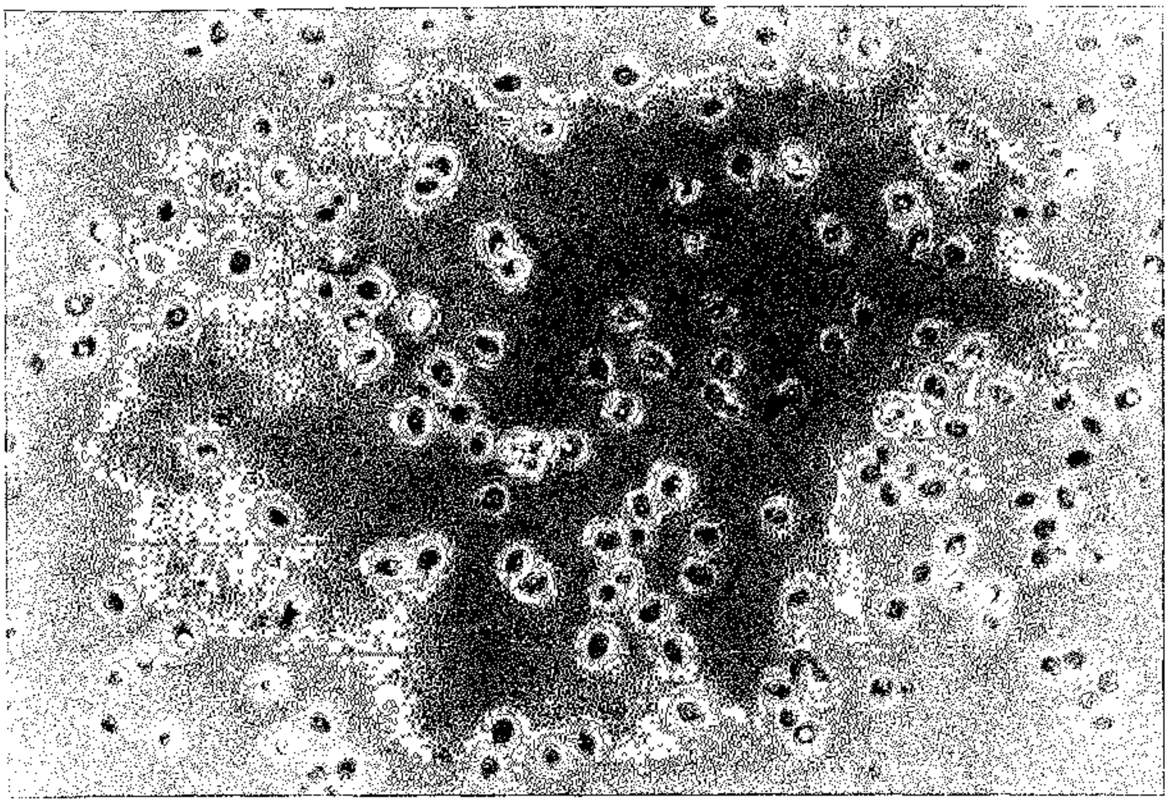
The photographs are that of a typical strain of each group shown in Figure 3.

A: G.S. 6
B: G.S. 9
C: G.S. 22
D: G.S. 2
E: G.S. 10
F: G.S. 11
G: G.S. 14
H: G.S. 8

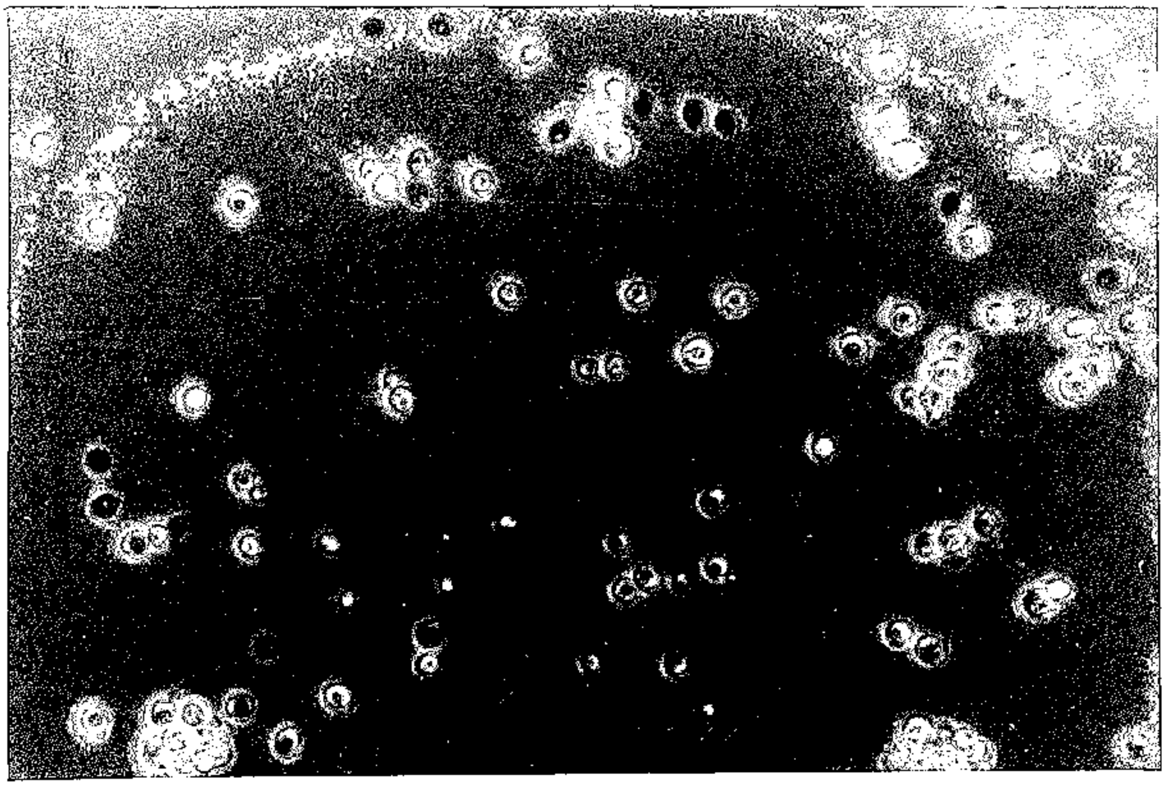
A



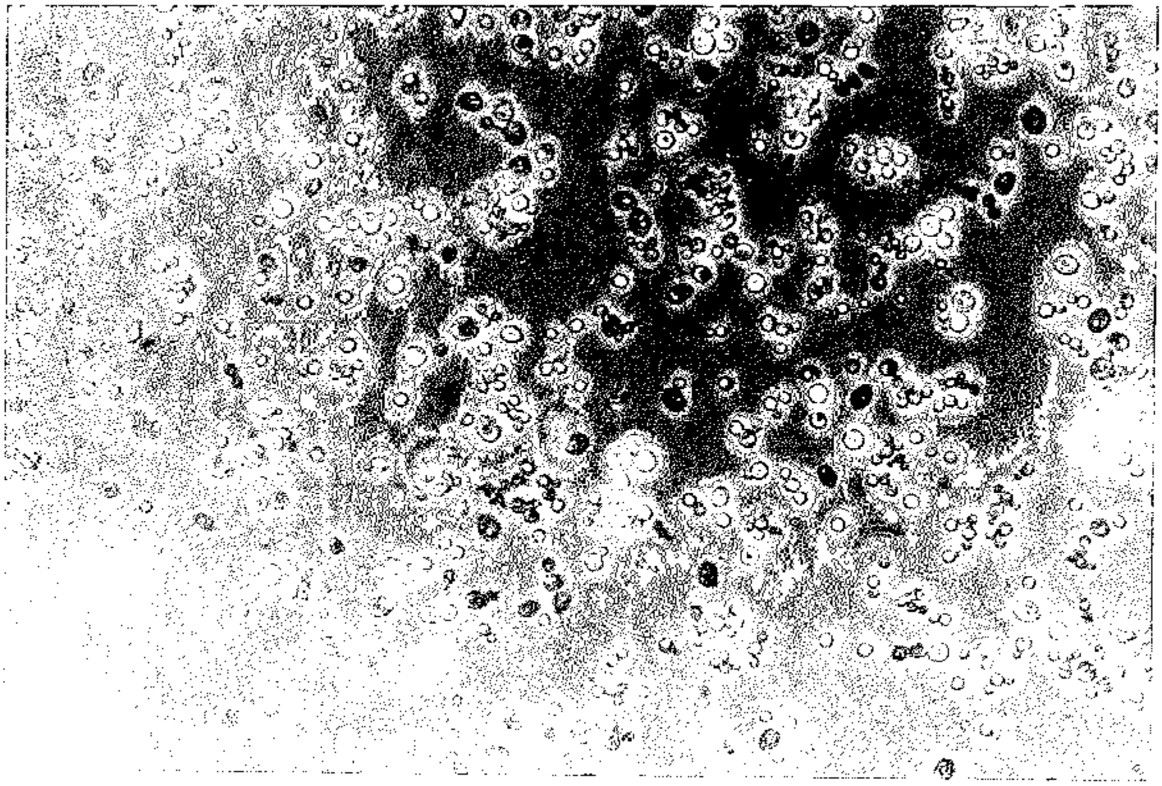
B



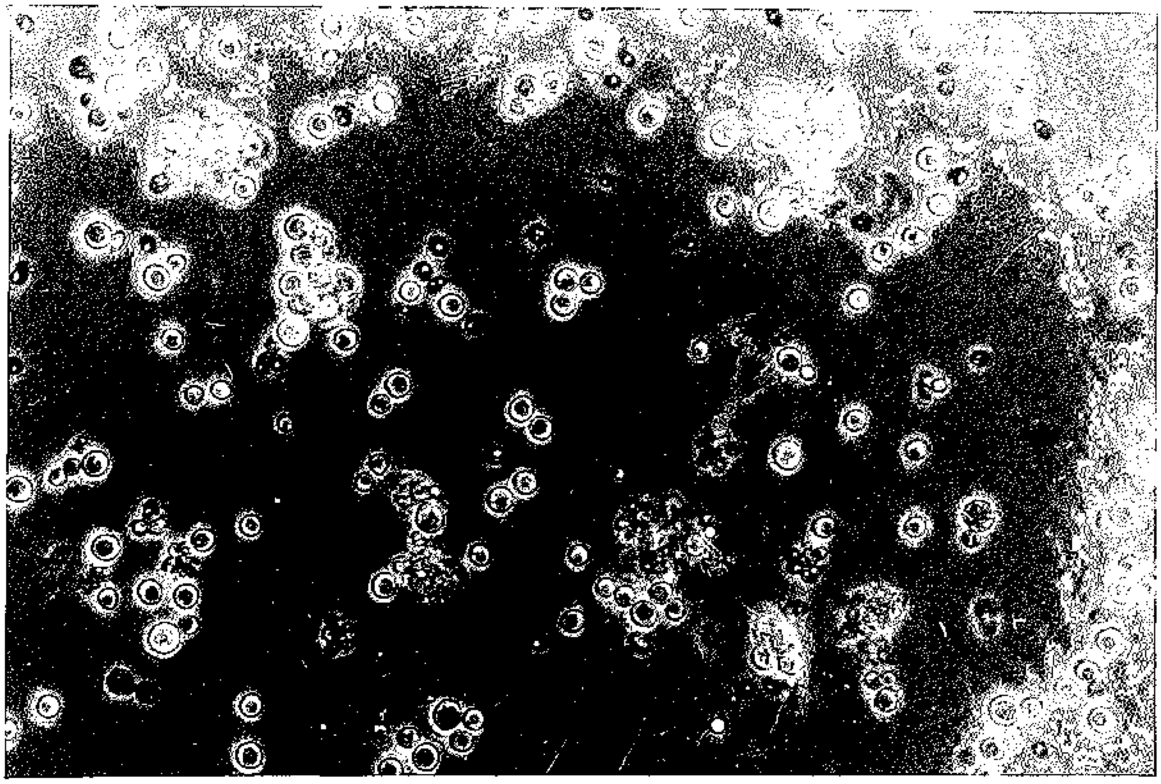
C



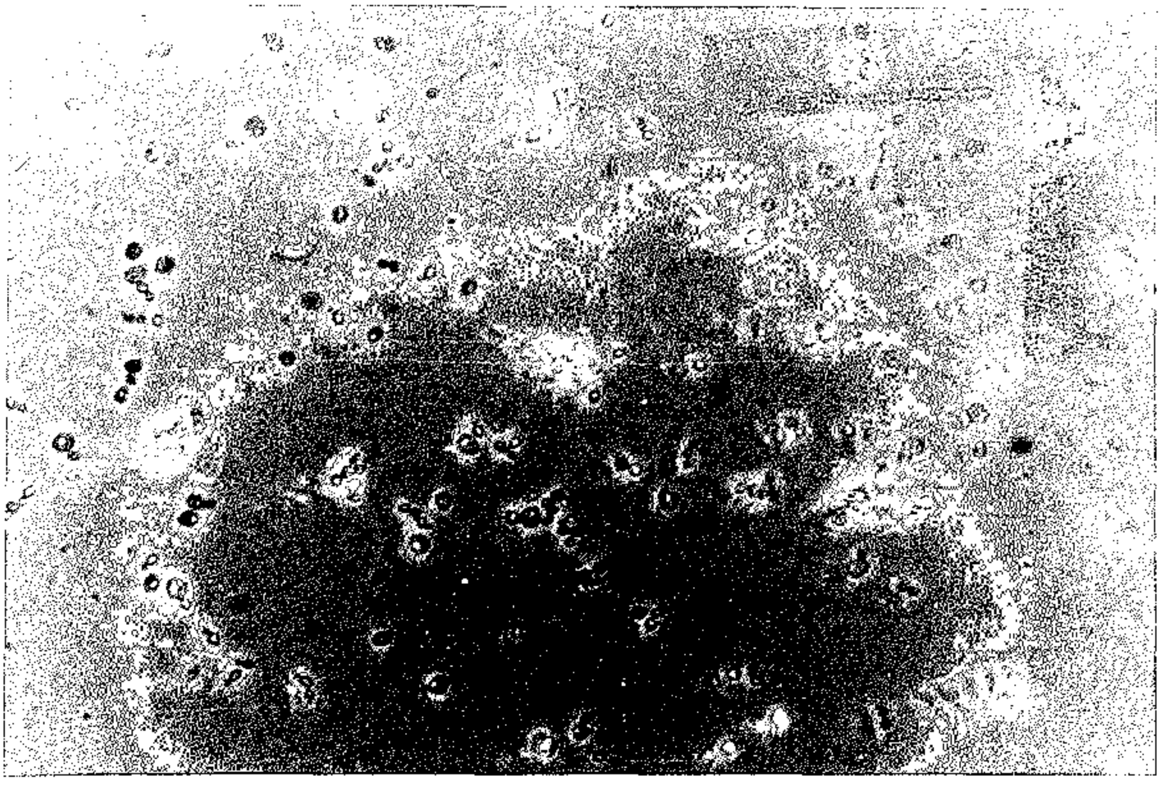
D



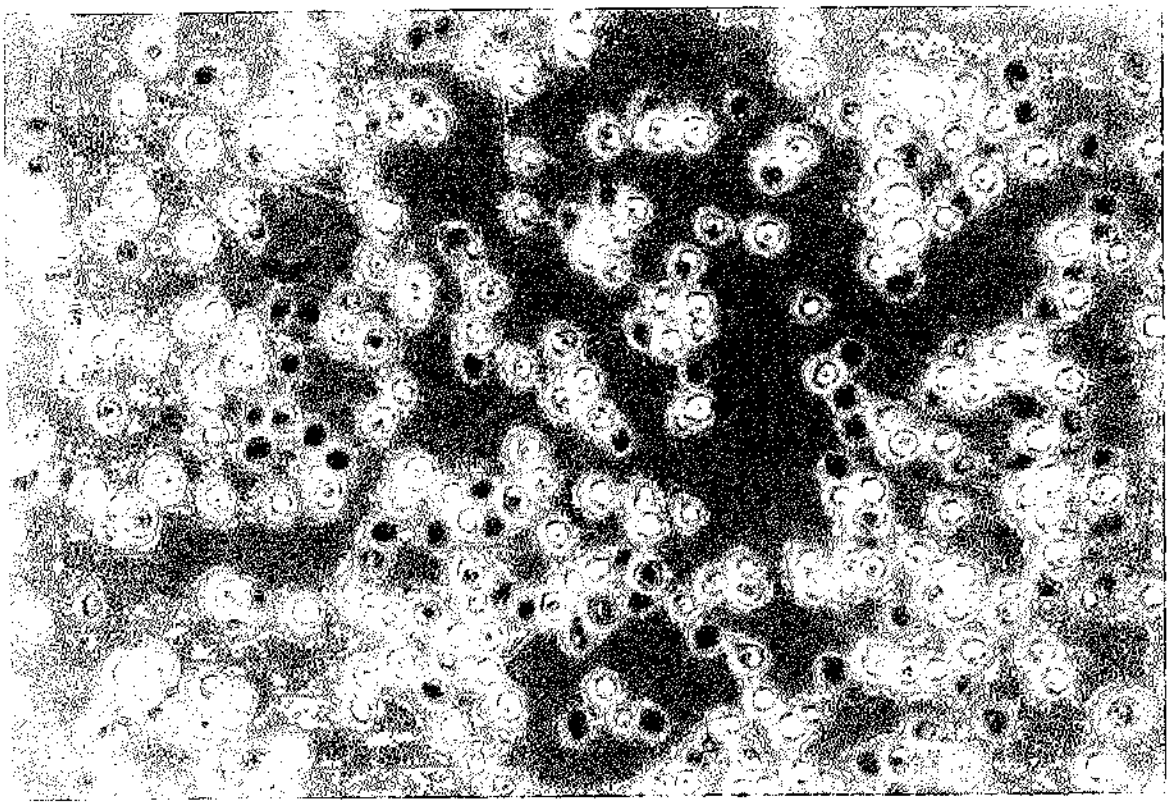
E



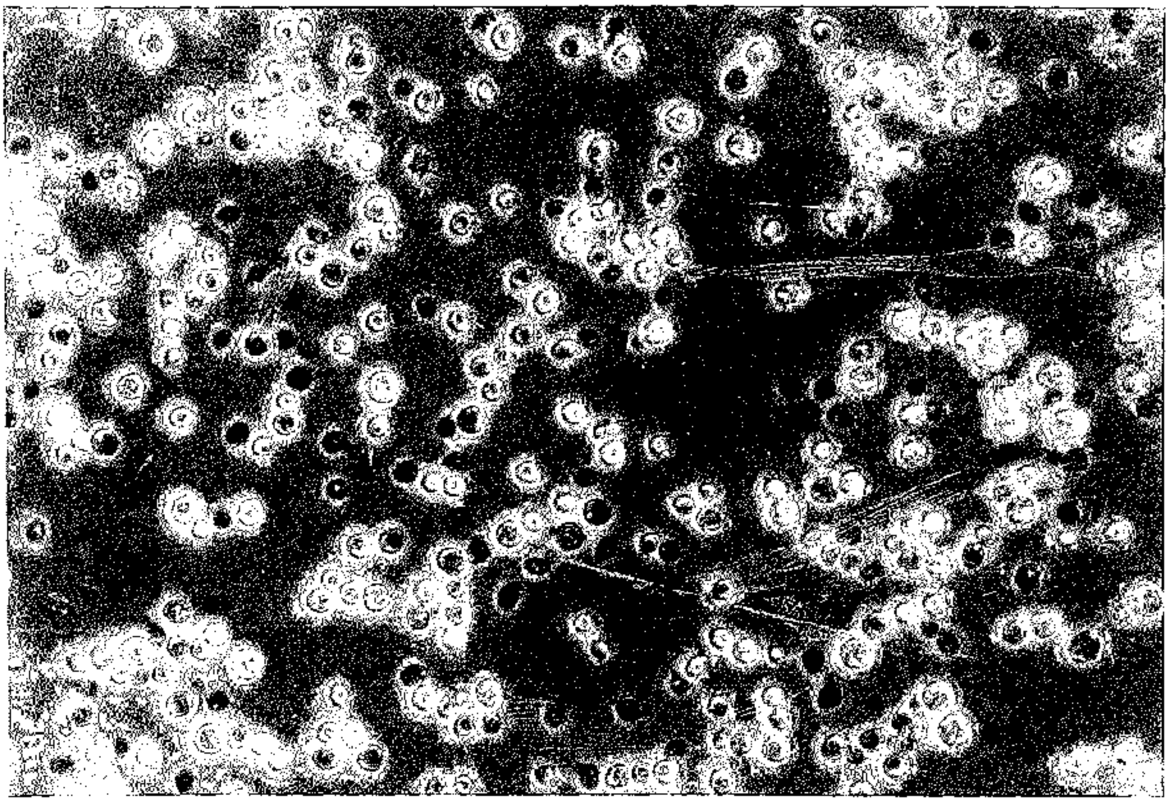
F



G



H



4.1.1 Investigation of Death and Autolysis of GS18 and GS20

The initial rapid decline in viable cells of strain GS18 in M.Y.G.P. broth (Figure 3) suggested that this strain was worth further investigation. Viable cell counts were made at four hourly intervals to establish the growth curve of GS18 (Figure 4). The growth curve of GS20 was used as a representative of the early trends of growth seen for other GS strains (Figure 4). The generation times for both strains are similar; 3.92 and 3.93 hours to double for GS18 and GS20 respectively. The calculations are shown in Appendix III.

To ascertain whether amino acid leakage was associated with the initial rapid death of GS18, and whether there was any significant difference in leakage patterns for GS18 and GS20, both strains were inoculated into minimal media lacking amino acids. The growth and death at 15°C and 30°C was followed by cell counts (Figures 5 and 6). 2 ml samples of each culture were collected at sampling times over a 90 day period and used to attempt determination of amino acid concentration changes by H.P.L.C. analysis. Detection of amino acids was attempted using infra-red detection with an 87-H column and a mobile phase of 0.1M calcium sulphate; flow rate 1 ml/minute at 85°C. The amino acid levels were less than the detectable level so the more sensitive U.V. detection was used at 210 nm. This enabled amino acid peak detection but with poor resolution of peaks even at a flow rate of 0.5 ml/minute (Figure 7). Using water as the mobile phase the same problem was encountered. An alternative method employed the carbohydrate 87C column with a mobile phase of 0.04M H_2SO_4 at a flow rate of 0.8 ml/minute at 65°C with U.V. detection at 210 nm. Using these conditions dicarboxylic acids can be detected so it was anticipated

Figure 4. Growth Curves of GS18 and GS20

Total cell numbers.

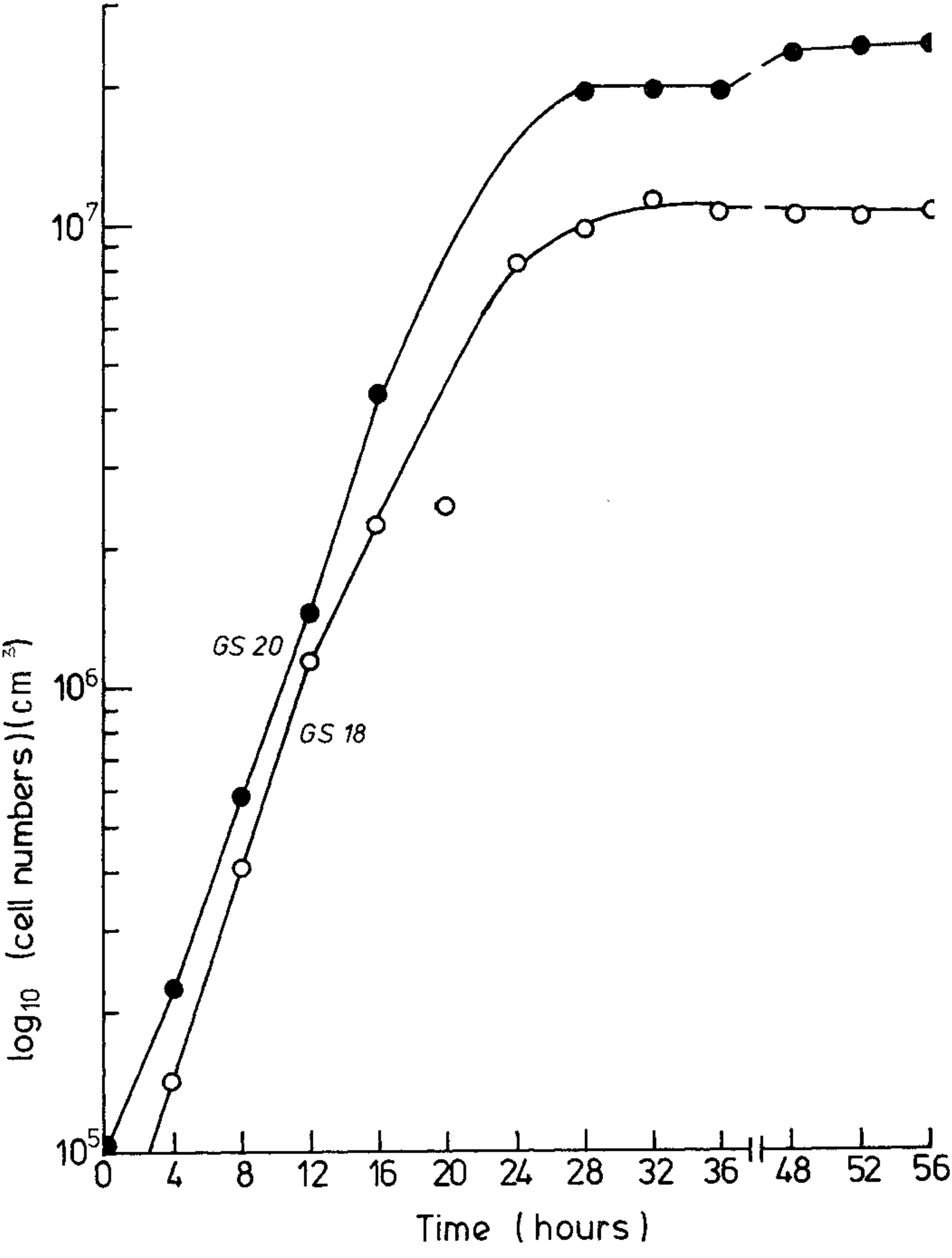

log10 (cell numbers)(cm-3)
10^7
10^6
10^5
GS 20
GS 18
0
4
8
12
16
20
24
28
32
36
48
52
56
Time (hours)

Figure 5. Growth and Death of GS18 in Minimal Media

Total and dead cell numbers are shown at 30°C (---) and 15°C (—).

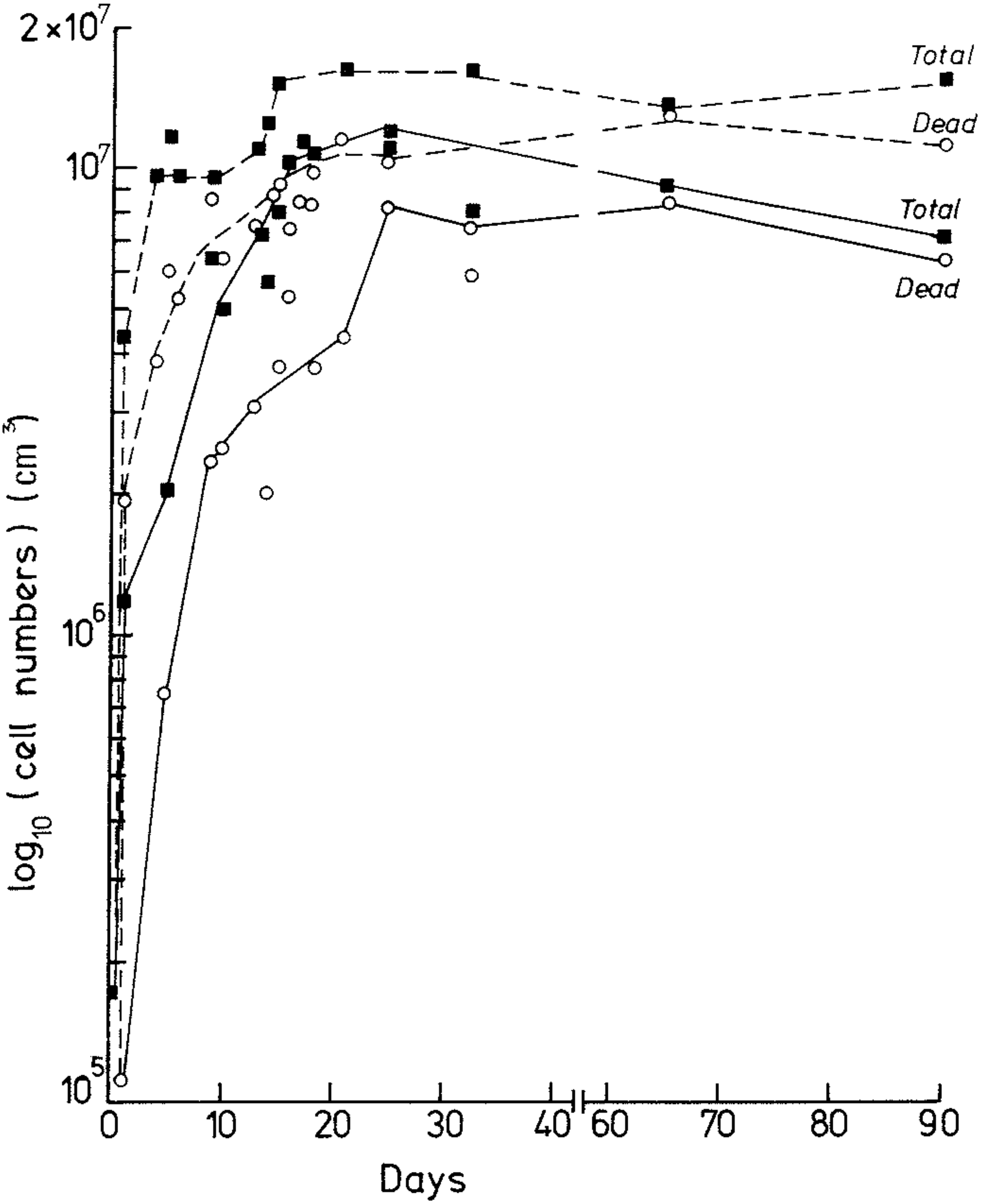
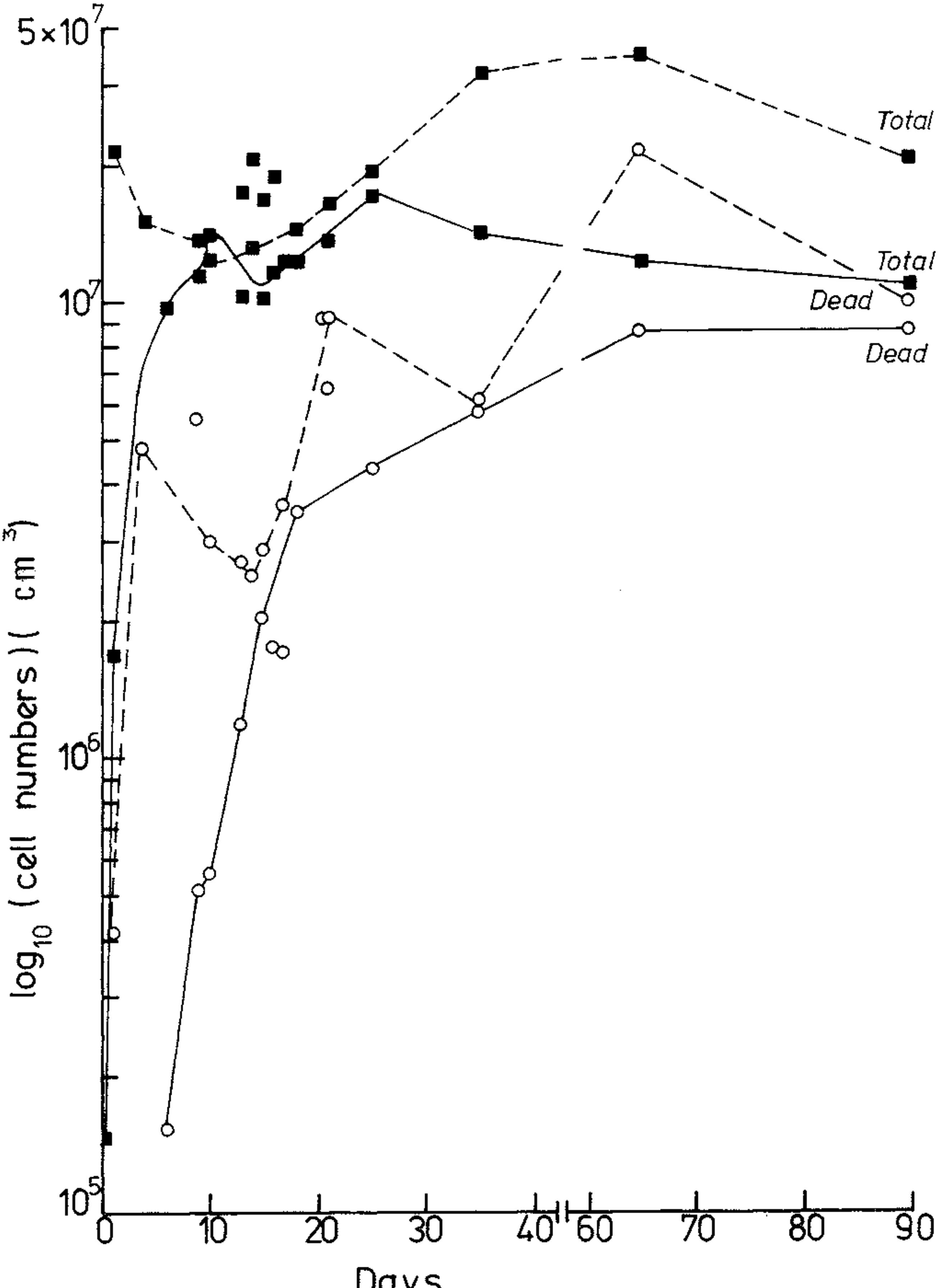

2×10^7
10^7
10^6
10^5
log10 (cell numbers) (cm^3)
Total
Dead
Total
Dead
0
10
20
30
40
60
70
80
90
Days

Figure 6. Growth and Death of GS20 in Minimal Media

Total and dead cell numbers are shown at 30°C (---) and 15°C (—).

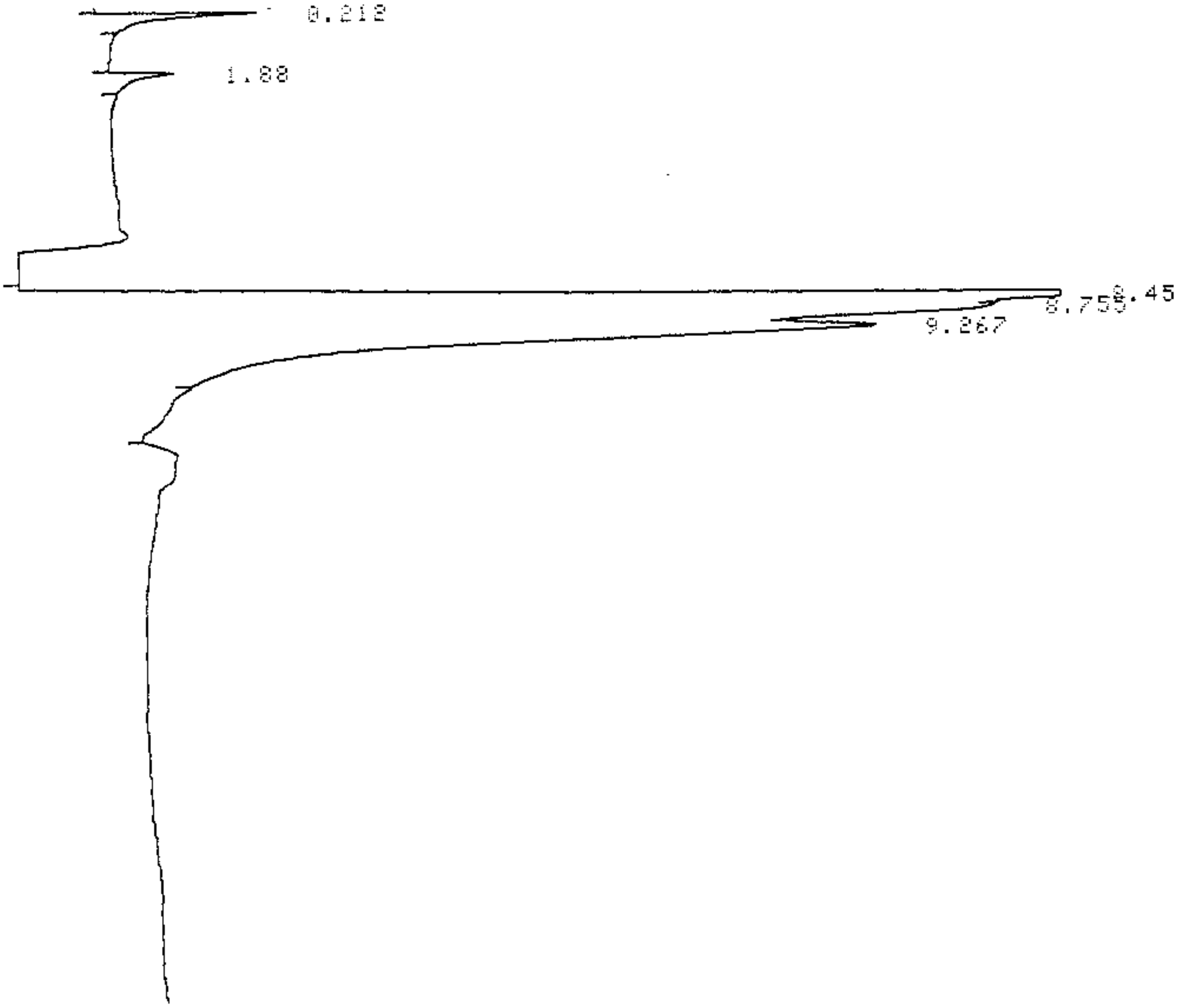

5×10^7
10^7
10^6
10^5
log10 (cell numbers)(cm^-3)
0
10
20
30
40
60
70
80
90
Days
Total
Total
Dead
Dead

that both aspartic acid and glutamic acid would be detected. Well resolved peaks for both acids were obtained with retention times of 4.658 and 4.667 minutes for aspartic acid and glutamic acid respectively (Figure 8). However an unidentified peak was found at 4.678 minutes when a sample of the minimal media used in the experiment was analyzed (Figure 9).

The area of the 4.693 minute peak of a day 25 sample of GS20 incubated at 30°C was 4.96120 (Figure 10). This represented an area increase of 4.25269 from the level of the unidentified peak in the original media.

Figure 7. Amino Acid Detection by H.P.L.C.; 87-H Column

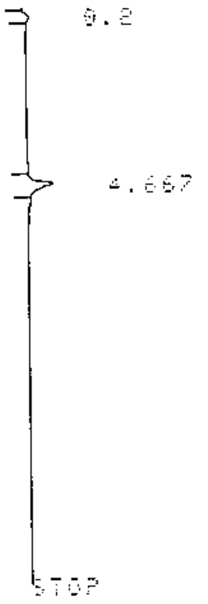
```
    6.TEST
TESTING  50 sec

     SLOPE     1186.56
     PRINT LEVEL
-557.2
     EPLOT
INJ
INJ
START
```



```
CHROMATOPAC   C-R3A                        FILE          6
SAMPLE NO    0                             METHOD          21
REPORT NO    2695

PKNO        TIME        AREA  MK   IDNO      CONC         NAME

   1       0.212       28578                   1.716
   2       1.88        13960                   0.8383
   3       8.45       704187   E              42.2838
   4       8.755      377830  V               22.6873
   5       9.267      540828  V               32.4747
                    ---------           -------------

        TOTAL     1665382               100
PRINT LEVEL
 1400
```

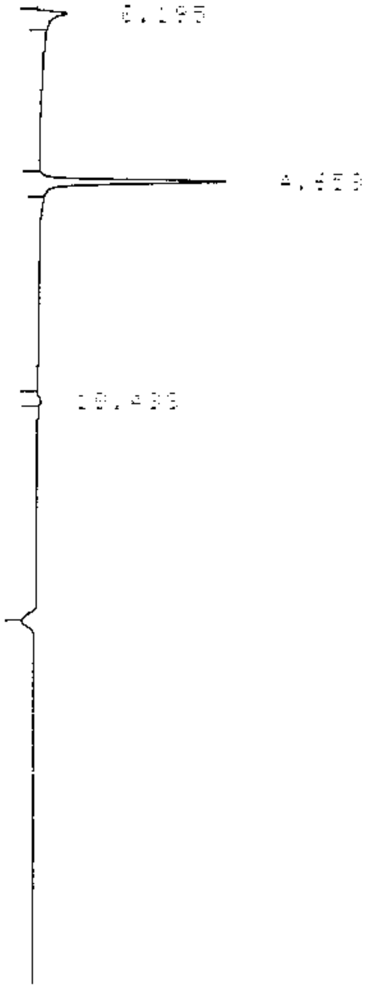
Figure 8. Detection of Glutamic and Aspartic Acids by H.P.L.C. Analysis; 87-C Column

```
END
 I.SMPL     36.
 F.SMPL     36.
INJ
START
```



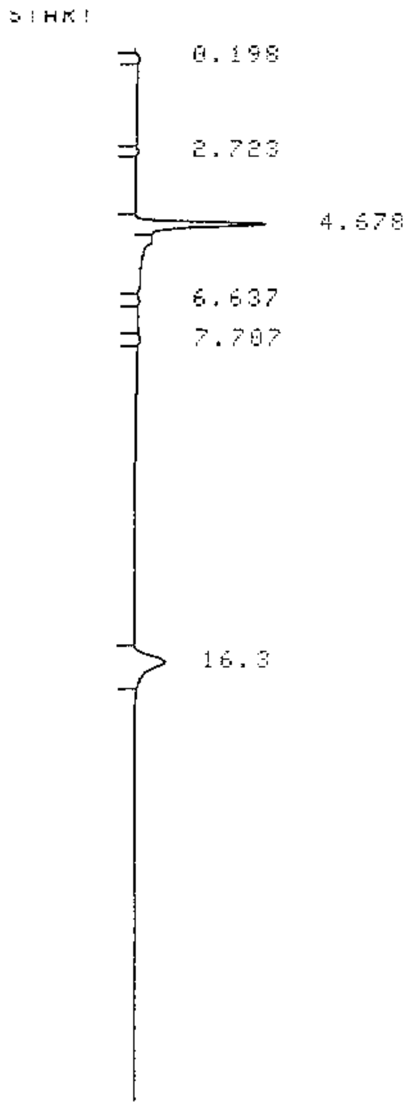
GLUTAMIC ACID

```
CHROMATOPAC   C-R3A                        FILE          6
SAMPLE NO    0                             METHOD          21
REPORT NO    2877

PKNO       TIME        AREA  MK  IDNO      CONC         NAME

   1      0.2         2447                 11.0514
   2      4,667      19692                 88.9486
                  ---------            -------------
        TOTAL        22138                100
DEGAS
```



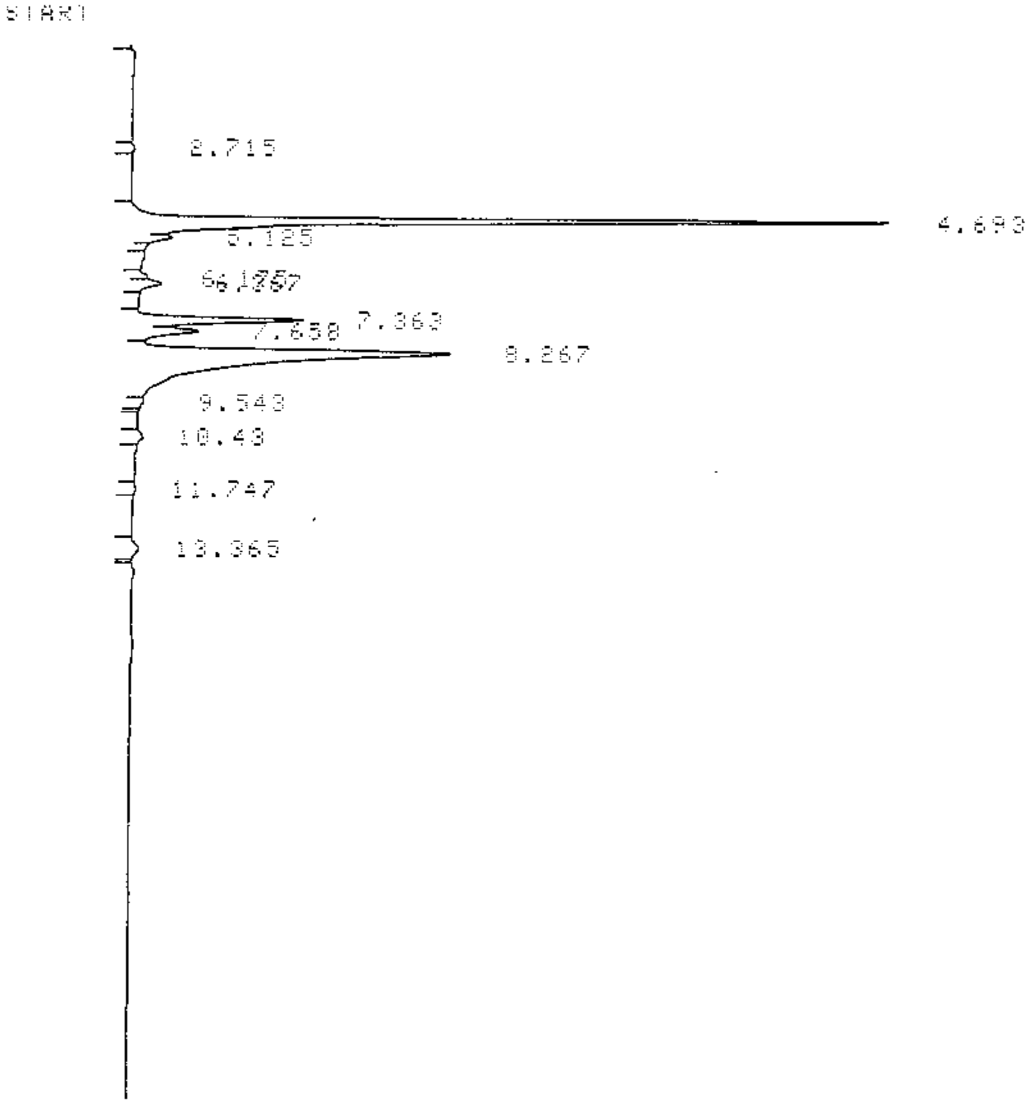
ASPARTIC ACID

Figure 9. H.P.L.C. Analysis of Minimal Media; 87-C Column



```
CHROMATOPAC   C-R3A                     FILE        6
SAMPLE NO   0                           METHOD        21
REPORT NO   2875

PKNO       TIME       AREA  MK  IDNO      CONC        NAME

   1      0.198       2031                  1.5938
   2      2.723       1758                  1.3797
   3      4.678      70851                 55.5926
   4      6.637       1509                  1.184
   5      7.707       2248                  1.7639
   6     16.3        49049                 38.4859
                 ---------          -------------
         TOTAL      127448                100
-----
```

Figure 10. H.P.L.C. Analysis of Day 25 GS20 Sample



```
CHROMATOPAC   C-R3A                      FILE          6
SAMPLE NO    0                           METHOD          21
REPORT NO    2876

PKNO       TIME        AREA  MK  IDNO       CONC         NAME

   1      2.715        1742                  0.147
   2      4.693      496120 S               41.8669
   3      5.125        5000 T                0.4219
   4      6.175        4445                  0.3751
   5      6.367       14478  V               1.2218
   6      7.363      114847                  9.6918
   7      7.658       49057  V               4.1398
   8      8.267      484398 SV              40.8777
   9     10.43         4484                  0.3784
  10     11.747        1654                  0.1396
  11     13.365        8770                  0.7401
                  ---------           -------------
        TOTAL     1184994               100
```

4.1.2 The Genetical Basis of Autolysis

To determine whether the autolytic character is genetically based, GS10(α) and GS15(α) were mated with GS11(a). Strains GS10 and GS15 had high numbers of dead cells associated with relatively constant total cell concentrations after twelve weeks in M.Y.G.P. broth (Figure 3). These two strains are of the same mating type and they cannot be mated so GS11 was selected for cross breeding as it died more rapidly than other 'a' mating type strains. Eight zygotes from each cross were selected and induction of sporulation attempted. Verification of the diploid status of each zygote was obtained by the production of red colonies on magdela red plates (Cutz et al, 1974). In conjunction, control haploid and diploid S. cerevisiae strains were streaked on magdela red agar to identify the colour reactions. After many recyclings over a five month period no sporulation occurred, consequently this line of investigation was terminated.

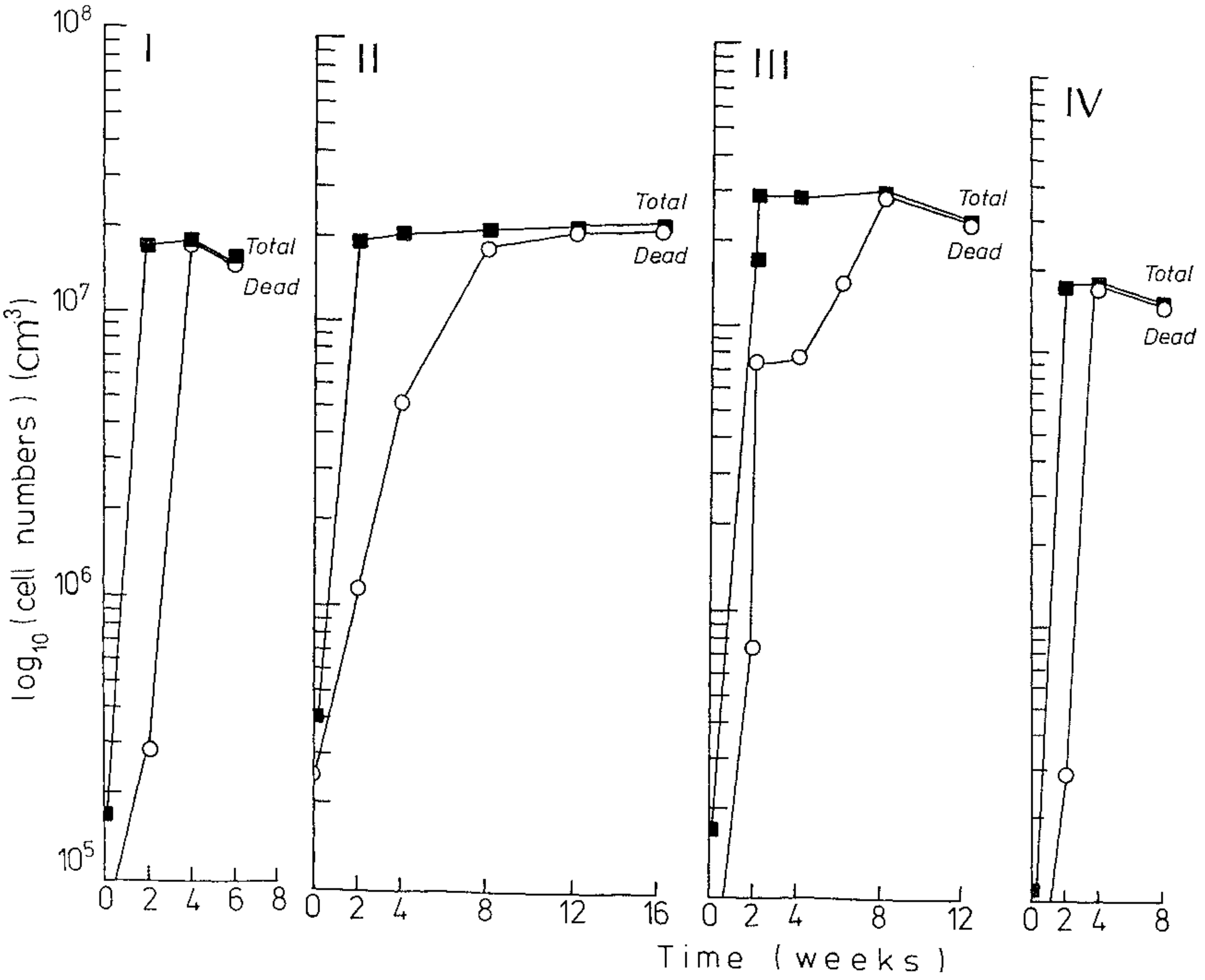
4.1.3 Effect of Ethanol on Autolysis

The 22 GS strains were acclimatized to growth in 12% (v/v) ethanol in order to compare the death of yeast in wine with death in synthetic media. Once acclimatized, each strain was inoculated into wine containing 80 p.p.m. total SO_2 and 6.9% ethanol by weight. The growth and death of cells was followed at 15°C using hemacytometer counts and methylene blue staining. When no viable cells were found using this method, viability was determined by plate counting. The six patterns of death observed are shown in Figure 11 and the actual cell numbers are in Appendix I. The typical appearance of cells after 20 weeks in wine is shown in Plate 8.

Figure 11. Growth and Death of GS Strains of Wine

Each graph represents the pattern of death seen for the named strains:

I G.S. 4, 11, 13, 16
II G.S. 6, 10, 15
III G.S. 1, 3, 8, 14
IV G.S. 2, 12, 22
V G.S. 19, 20
VI G.S. 17
VII GW8021


I
II
III
IV
10^8
10^7
10^6
10^5
$\log_{10}$ (cell numbers) (cm^3)
Total
Dead
Total
Dead
Total
Dead
Total
Dead
0 2 4 6 8
0 2 4 8 12 16
0 2 4 8 12
0 2 4 8
Time (weeks)

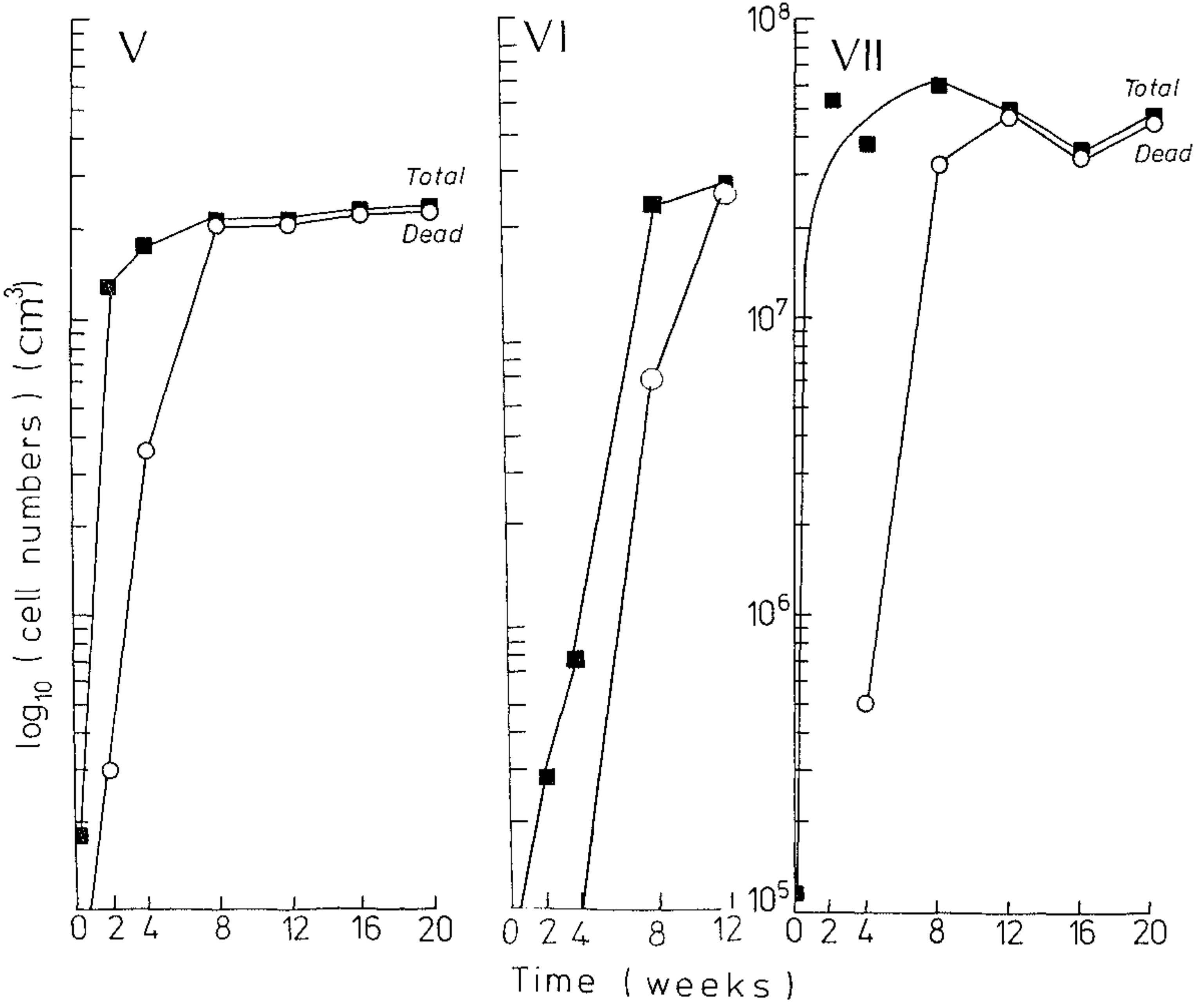
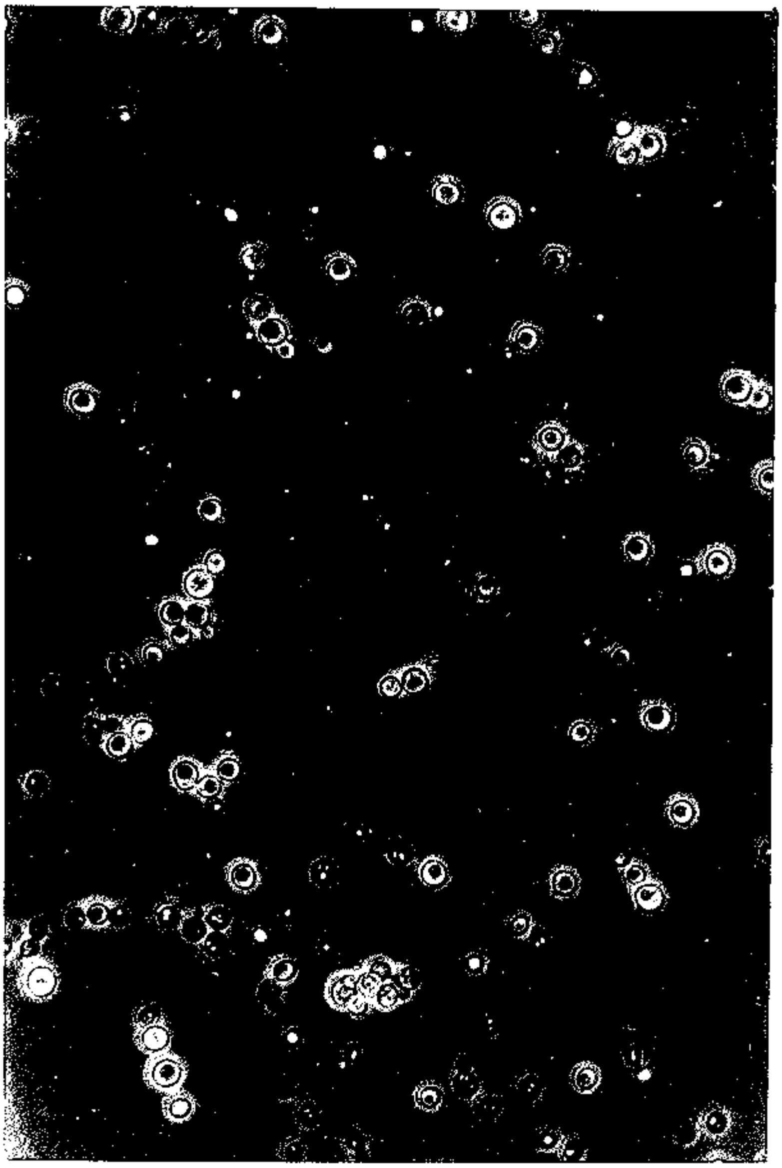

V
VI
VII
Total
Dead
Total
Dead
log₁₀ (cell numbers) (cm³)
10⁸
10⁷
10⁶
10⁵
0 2 4 8 12 16 20
0 2 4 8 12
0 2 4 8 12 16 20
Time (weeks)

Plate 8. Typical Appearance of a GS After 4 Months in Wine

16



4.2 KILLER

To ensure that pure cultures had been established, the phenotypes of the four standard laboratory killer strains were tested for killing reactions by plate assay. Plate 9 shows K_0 lawn with zones of inhibition caused by toxin released from the K_1, K_2 and K_3 tester colonies. The same effect can be seen when concentrated toxin is added to a well (Plate 10). The standard killer reactions obtained in this way are shown in Table 8.

4.2.1 Curing of a K_1 Strain

Cycloheximide can be used to cure a killer yeast of killer activity. Twelve single colonies from four clones isolated from a plate containing cycloheximide were selected. These colonies were tested three times for killing activity using the plate assay system (Table 9). In conjunction twelve single colonies from two untreated control clones were examined. Cured strain 3 was accepted as fully cured since toxin production was not detected in any colony tested.

4.2.2 Toxin Production and Standardization

The extracellular protein from a K_1 culture concentrated 25 fold by ultrafiltration was determined by U.V. spectrophotometry from a standard curve (Figure 12). The average total protein of five concentrations was 43 mg/ml. By subtracting the extracellular protein concentration of the cured K_1 strain it was estimated that the killer protein made up 4% or 1.39 mg/l of the total protein.

Plate 9. Plate Assay of Killing Reaction

Plate 10. Plate Assay of Toxin Concentrate

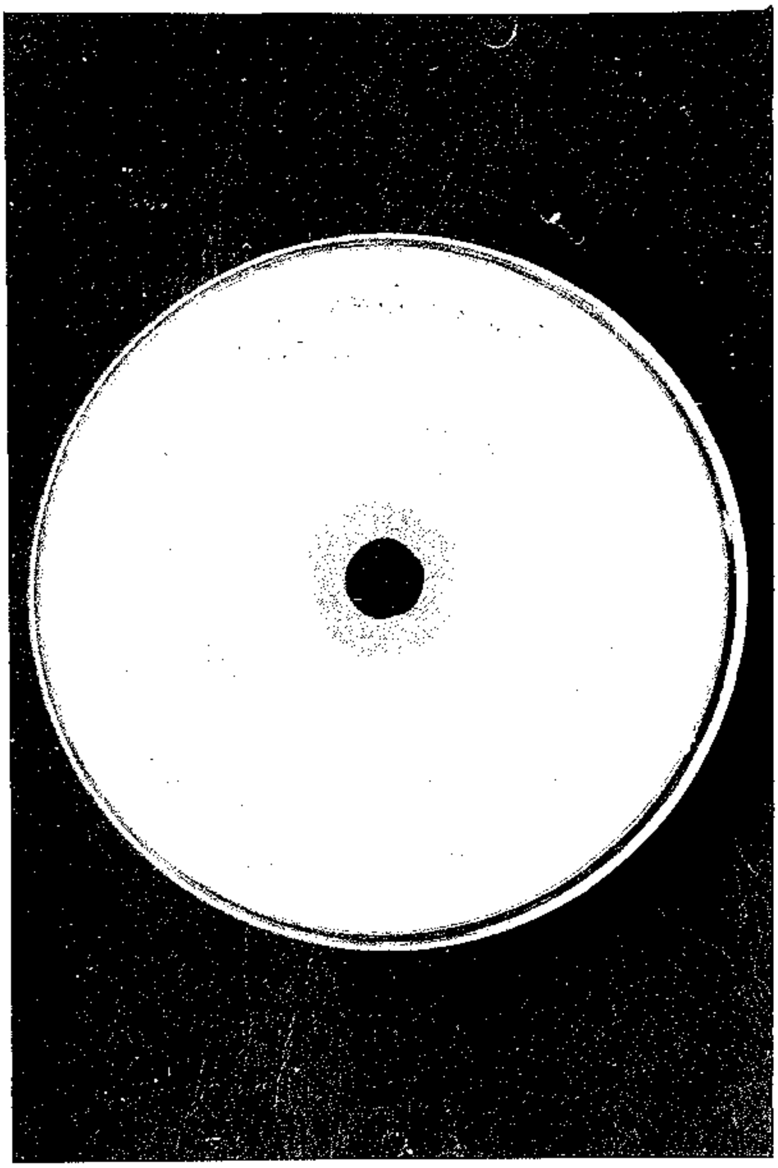


Table 8. Standard Killing Reactions

Killing reaction scored using the plate assay system; a zone of inhibition indicating killing (+); absence of inhibition (-).

Tester Strain	Lawn of			
	K_0	K_1	K_2	K_3
K_0	-	-	-	-
K_1	+	-	+	+
K_2	+	+	-	+
K_3	+	+	-	-

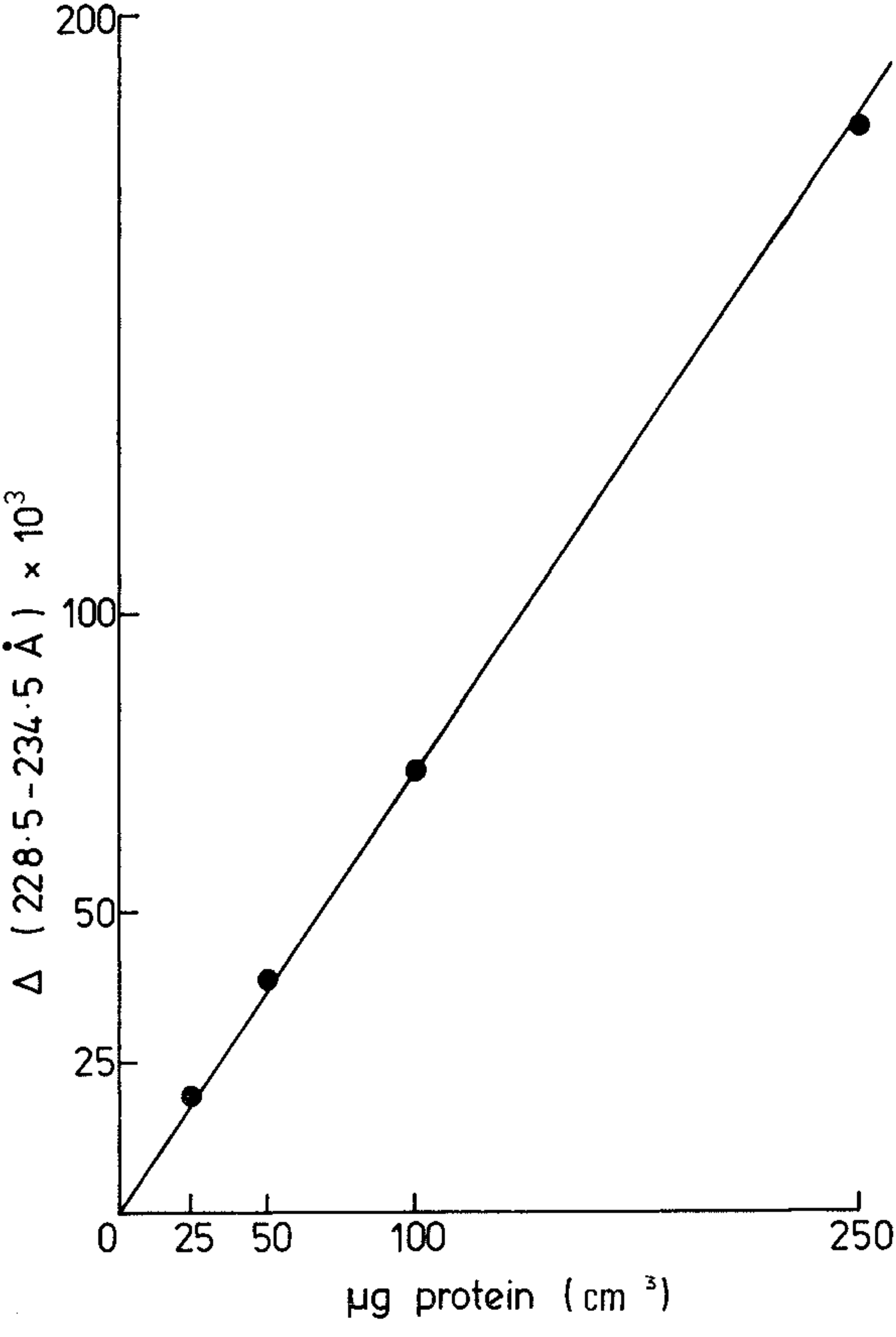
Table 9. Killing Reactions of Cycloheximide Cured K_1 Strains

The table shows the results of 3 consecutive killer plate assays (I, II, III). In each assay 12 single colonies originating from 4 cycloheximide treated clones (cycloheximide 1, 2, 3, 4) and 2 untreated K_1 clones (controls 1 and 2) were tested for toxin production. Toxin production by all colonies (+), some colonies (+/-) or absence of killing activity (-) are recorded.

	Killer Assay		
	I	II	III
control 1	+	+	+
control 2	+	+	+
cycloheximide 1	-	+/-	-
cycloheximide 2	-	+/-	-
cycloheximide 3	-	-	-
cycloheximide 4	-	-	+/-

Figure 12. Standard Curve for Protein Determination

The relationship between protein concentration and change of absorbance of bovine serum albumin is shown. Measurements at 2285 and 2345 A. Each point is the average of 5 separate determinations.


200
100
50
25
0
25
50
100
250
Δ (228·5 - 234·5 Å) × 10³
μg protein (cm ³)

4.2.3 Standardization of Optimum Killing Conditions

The killing effect of different dilutions of the killer protein concentrate was determined at three temperatures, 15, 20 and 30°C (Figure 13), and the effect of pH at each temperature was examined (Figure 14). The optimum dilution for killing activity of a 1:1 mix of K_0 cells to toxin was used in all subsequent experiments with incubation at 30°C in media buffered to pH 4.8. To standardize experiments, a 1:1 control was run to obtain base line optimum activity. Control cultures containing K_0 cells but no toxin were run for each condition studied, and the false a.k.u. obtained due to natural death were used to calculate c.a.k.u.

Toxin activity against a four day old stationary culture was compared with activity against a sixteen hour experimental culture to confirm that killing is more effective when cells are actively growing. Activities of 5.8×10^6 c.a.k.u. and 1.25×10^7 c.a.k.u. for four day and sixteen hour cultures respectively, were obtained. The experiment was repeated with the addition of a protease inhibitor, (P.M.S.F.) to determine whether this result could be due to proteases released by stationary phase cells. Similar results were obtained (5.4×10^6 c.a.k.u. and 2.0×10^7 c.a.k.u.) against stationary and exponential cultures respectively. In all subsequent killing experiments 16 hour old exponential phase cultures were used.

Figure 13. Effect of Dilution and Temperature on Killing Activity of K_1 Toxin Concentrate

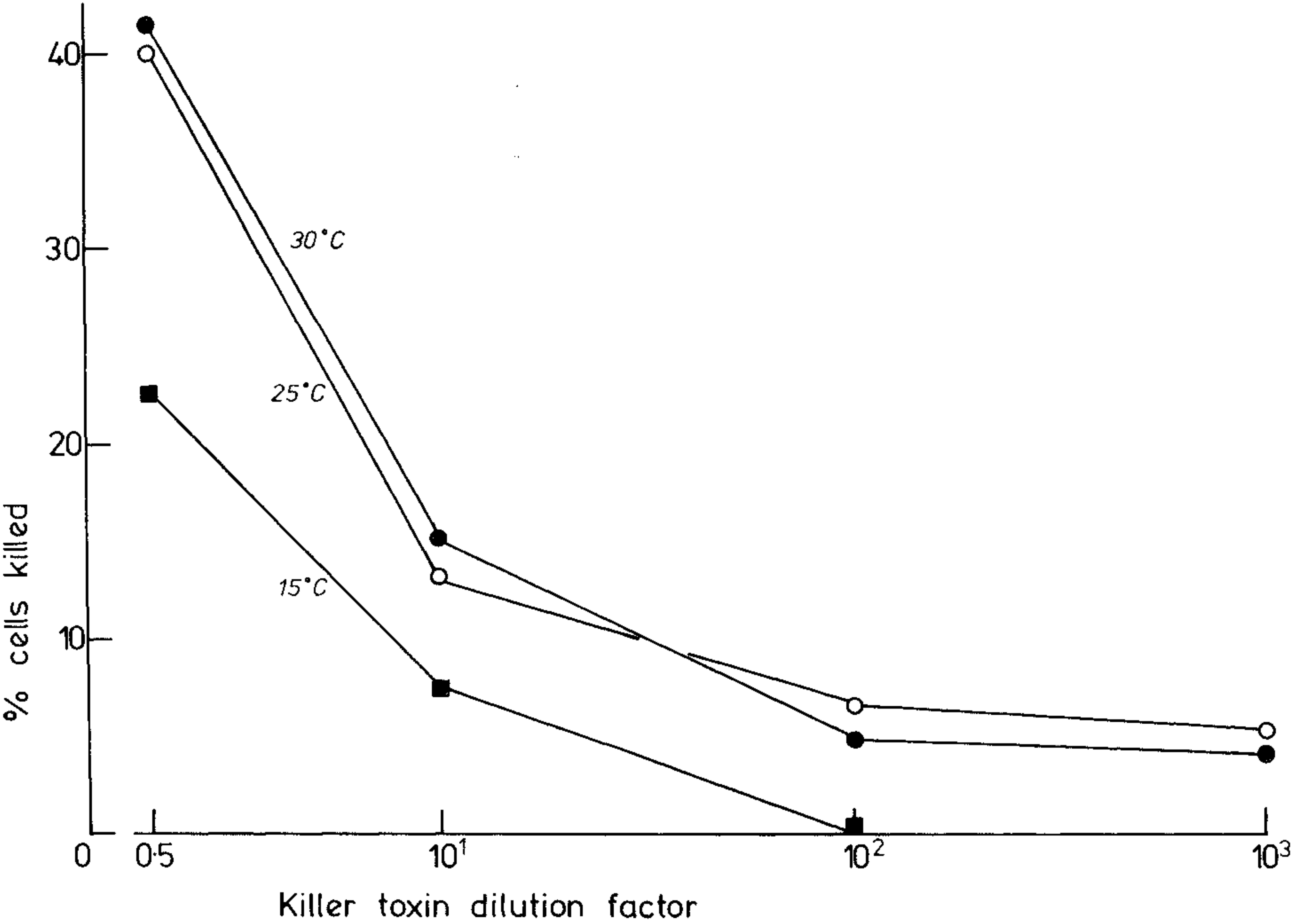
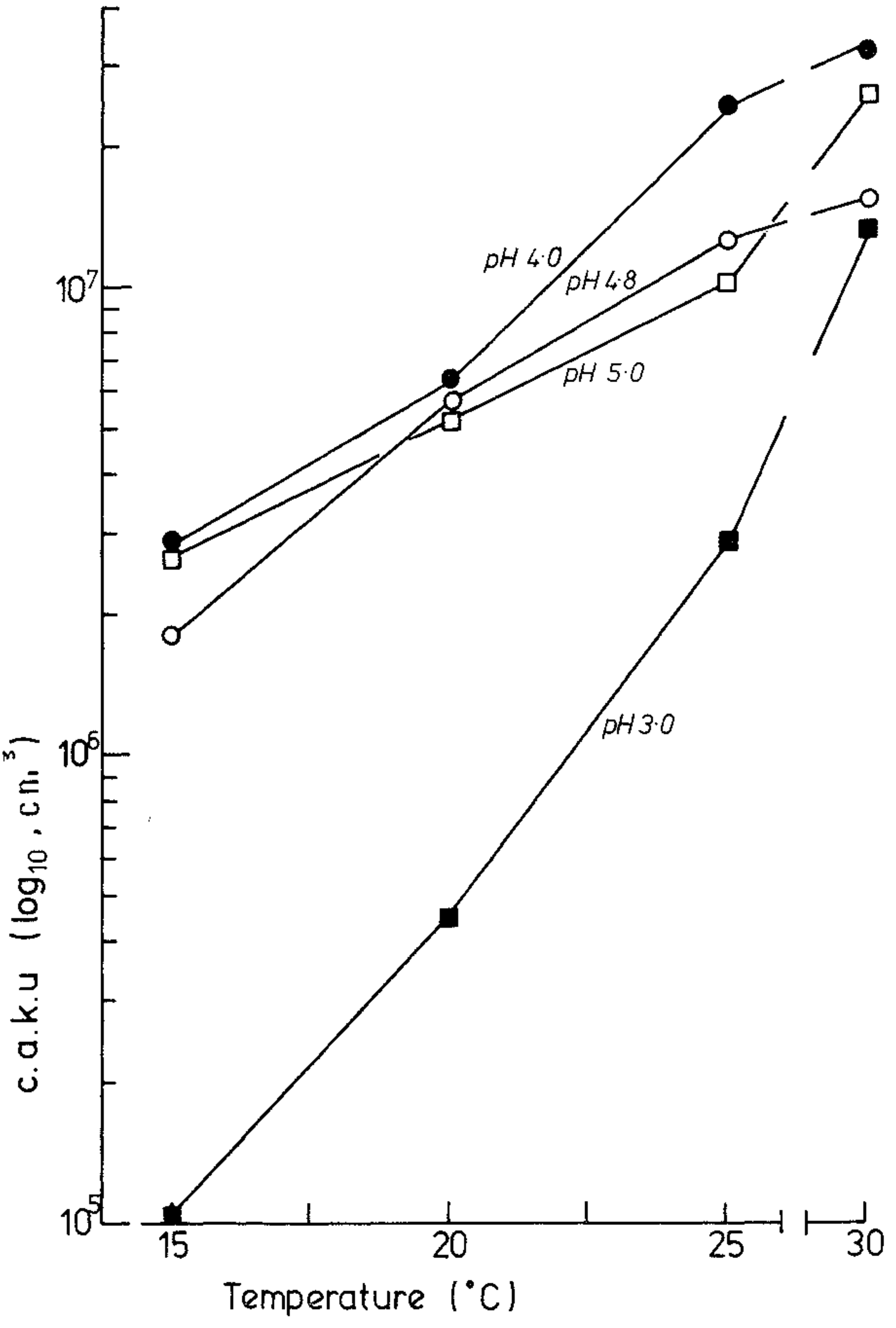

% cells killed
40
30
20
10
0
0·5
10^1
10^2
10^3
Killer toxin dilution factor
30°C
25°C
15°C

Figure 14. Effect of pH on Killing Activity at Different Temperatures


pH 4·0
pH 4·8
pH 5·0
pH 3·0
10^7
10^6
10^5
c.a.k.u (log₁₀, cm⁻³)
15
20
25
30
Temperature (°C)

4.2.4 Effect of Media on Killing

The effect of various media on killing activity and subsequent regeneration is shown (Figure 15). Resuspension of K_0 cells in fresh media prior to toxin addition enhances killing activity. In all subsequent experiments, control tubes were resuspended in the same media as used for assay tubes, in order to reduce experimental differences due to media.

4.2.5 Bioassay for Detection of Amino Acid Release by Toxin Killed Cells

The killer phenotypes of laboratory auxotrophs were examined using the killer plate assay system (Table 10). Two single amino acid requiring K_0 strains, arg^- and lys^- were selected for the killing bioassay. The minimum level of amino acid supplementation needed for growth was determined by spectrophotometry at 650 nm in minimal media containing various amounts of the required amino acid (Figures 16 and 17). The minimum concentration of amino acid supplementation needed for growth was defined as that concentration which supported an exponential growth phase, and was determined as 0.4 mg/l arginine and 4 mg/l lysine for arg^- and lys^- auxotrophs.

Standardized patterns of killing and regeneration in minimal media, buffered to pH 4.8 of a K_0 strain as determined by haemocytometer counts were obtained (Figure 18). Regeneration of surviving viable cells gave a total cell number slightly below the yield obtained for a control culture without toxin. The pattern of killing of the arg auxotroph (Figure 19) was similar, (1×10^6 surviving cells) but regeneration gave a yield greater than the control. A similar pattern was seen for killing of the auxotroph lys (Figure 20). Each auxotroph was grown in minimal media supplemented with various concentrations of amino acids to determine the amount of each amino acid needed to support the regeneration seen (Figures 21, 22 and 23).

Figure 15. Effect of Media on Killing

○ Resuspended phosphate/citrate buffer
● Resuspended saline
□ Resuspended M.Y.G.P. broth
■ M.Y.G.P. broth

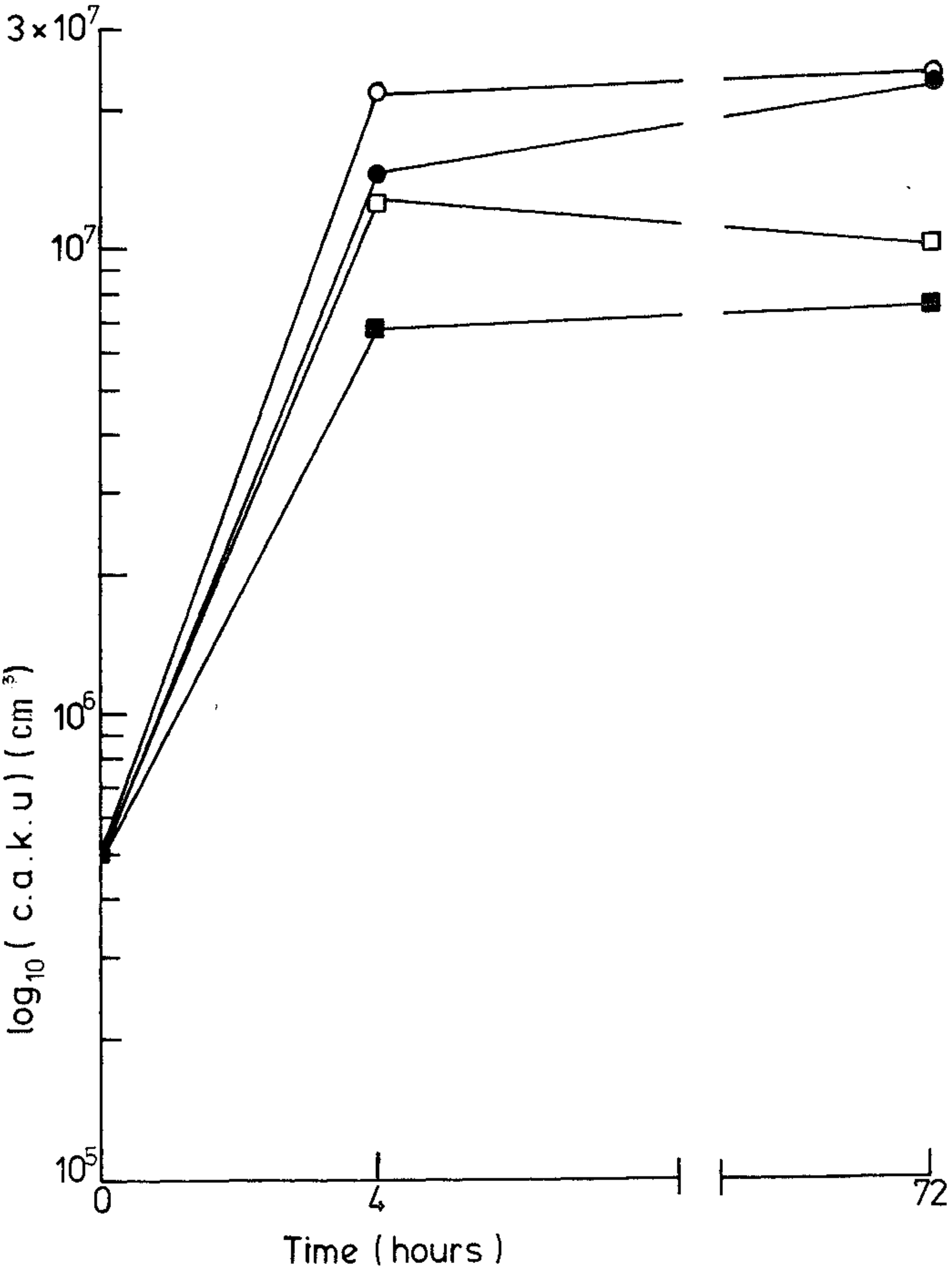

3 × 10^7
10^7
10^6
10^5
log10 (c.a.k.u) (cm^-3)
0
4
72
Time (hours)

Table 10. Killing Reactions of Auxotrophs

K indicates a positive killing reaction.

Tester Strain	Lawn of				Lawn of	Tester Strain				Phenotype
	K_0	K_1	K_2	K_3		K_0	K_1	K_2	K_3	
arg$^-$	-	-	-	-	arg$^-$	-	K	K	K	K_0
ser 1a	K	-	-	-	ser 1a	-	-	K	K	K_1
thr 1	-	-	-	-	thr 1	-	-	-	-	N
ade 1a	-	-	-	-	ade 1a	-	-	-	-	N
AH22	-	-	-	-	AH22	-	-	-	-	N
lys$^-$	-	-	-	-	lys$^-$	-	K	K	K	K_0
met 3a	-	-	-	-	met 3a	-	-	-	-	N
ura 2	-	-	-	-	ura 2	-	-	-	-	N
his$^-$	K	-	-	-	his$^-$	-	-	K	K	K_1
thr 4	-	-	-	-	thr 4	-	-	-	-	N
thr 4a	-	-	-	-	thr 4a	-	-	-	-	N
ser1 leu1 thr	K	-	-	-	ser1 leu1 thr	-	-	K	K	K_1

Figure 16. Growth Curves of arg$^-$ at Various Concentrations of Amino Acid Supplementation

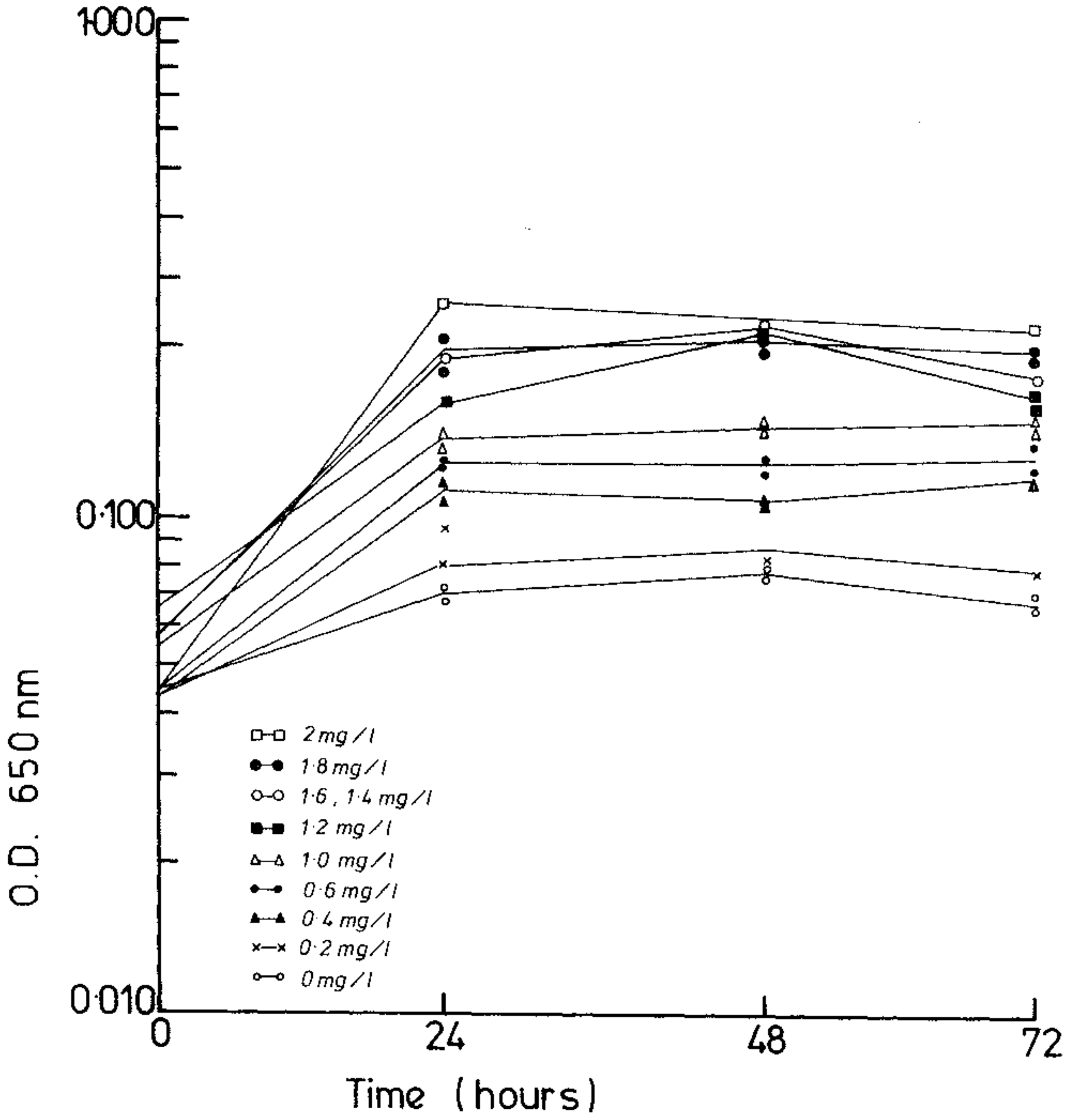

1·000
0·100
0·010
O.D. 650 nm
0
24
48
72
Time (hours)
2 mg/l
1·8 mg/l
1·6, 1·4 mg/l
1·2 mg/l
1·0 mg/l
0·6 mg/l
0·4 mg/l
0·2 mg/l
0 mg/l

Figure 17. Growth Curve of lys$^-$

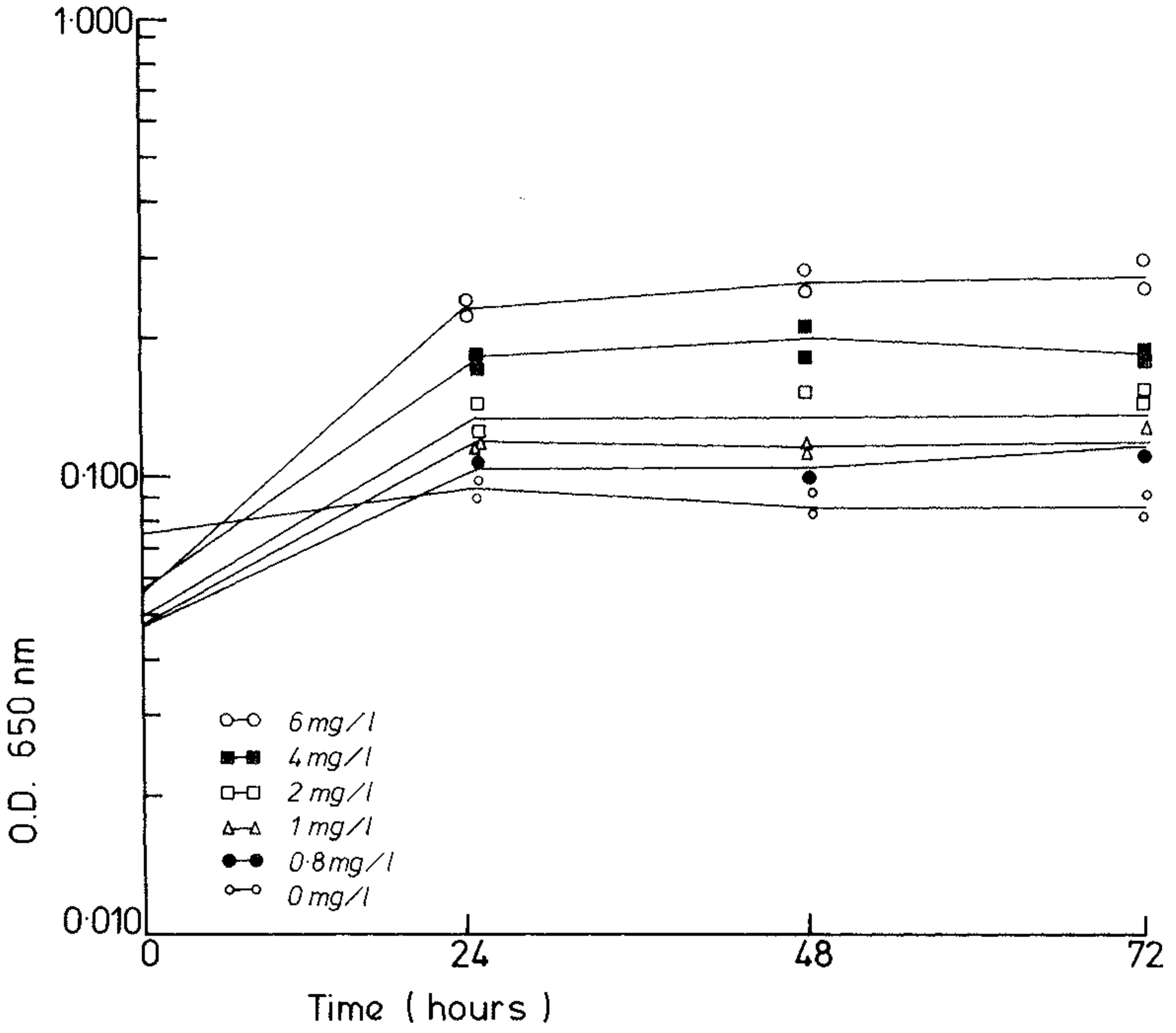

1·000
0·100
0·010
O.D. 650 nm
0
24
48
72
Time (hours)
6 mg/l
4 mg/l
2 mg/l
1 mg/l
0·8 mg/l
0 mg/l

Figure 18. Killing and Regeneration of K_0 in Minimal Media

Total (■), viable (□) and dead (○) cell numbers are shown for a toxin treated culture. Total cell numbers (—●—) are given for untreated control culture. All points are the average of 2 sample tubes.

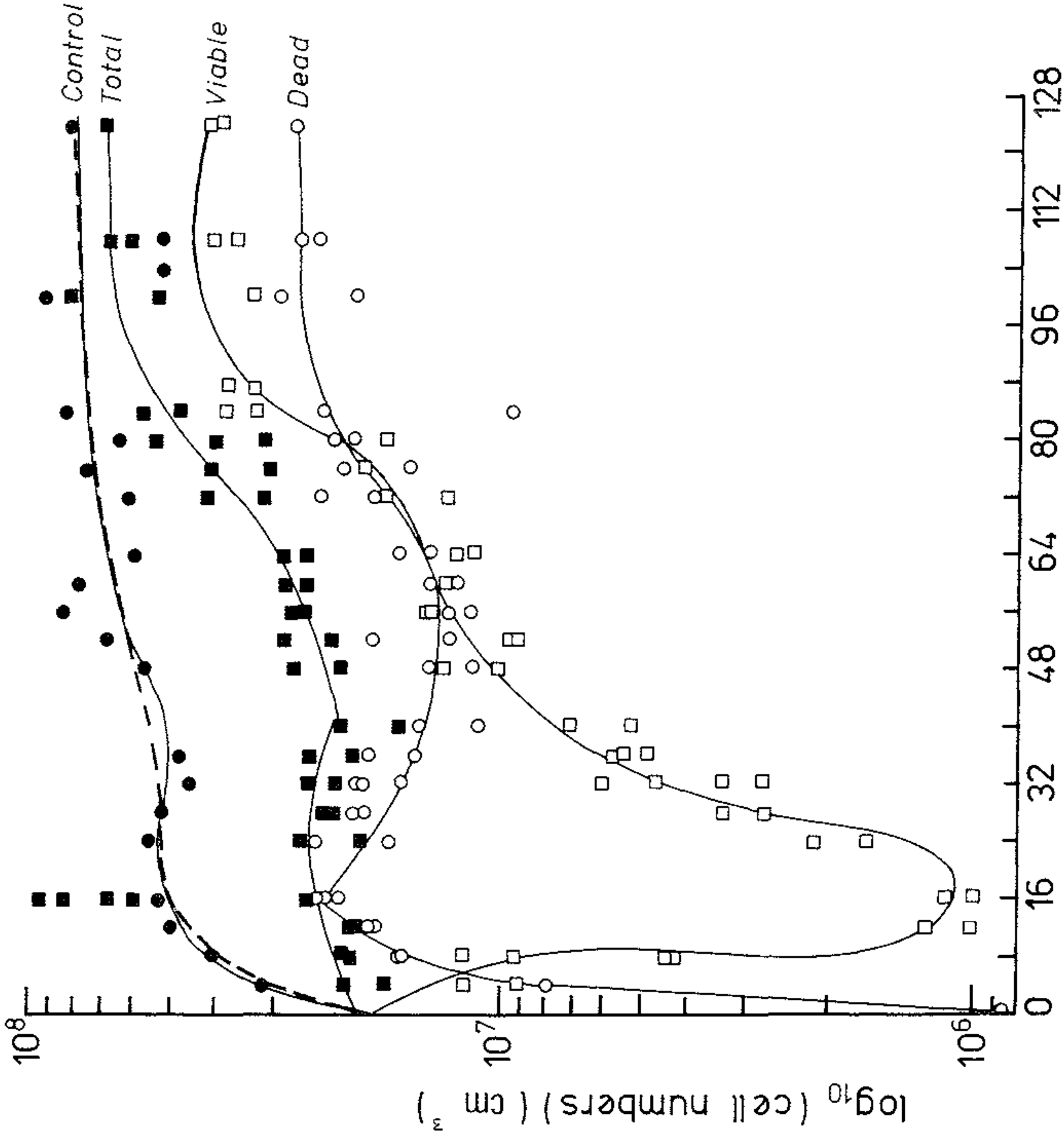

Control
Total
Viable
Dead
Time (hours)
0
16
32
48
64
80
96
112
128
$\log_{10}$ (cell numbers) (cm^{-3})
10^8
10^7
10^6

Figure 19. Killing and Regeneration of arg$^-$

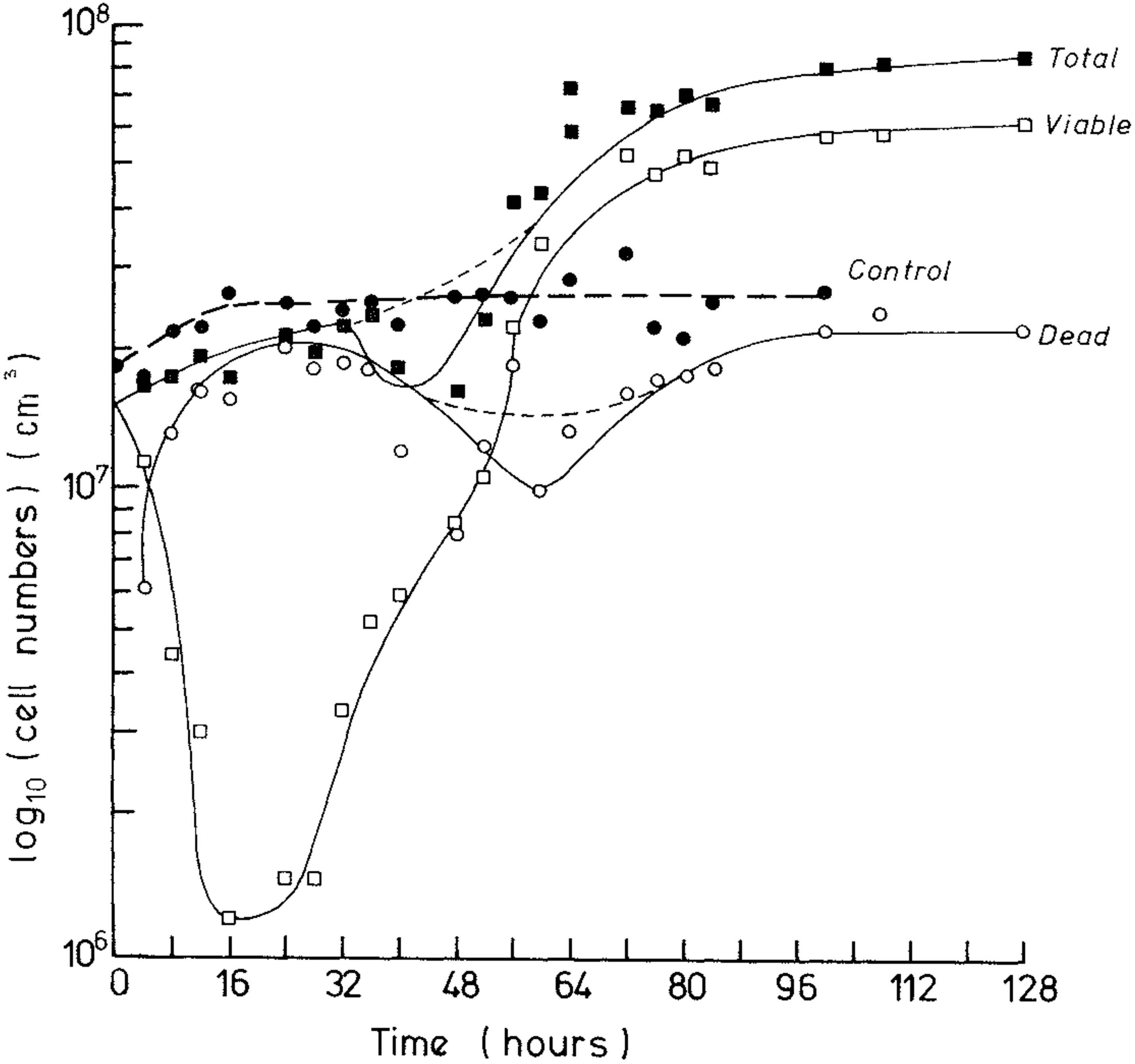

10^8
10^7
10^6
log10 (cell numbers) (cm^-3)
0
16
32
48
64
80
96
112
128
Time (hours)
Total
Viable
Control
Dead

Figure 20. Killing and Regeneration of lys$^-$

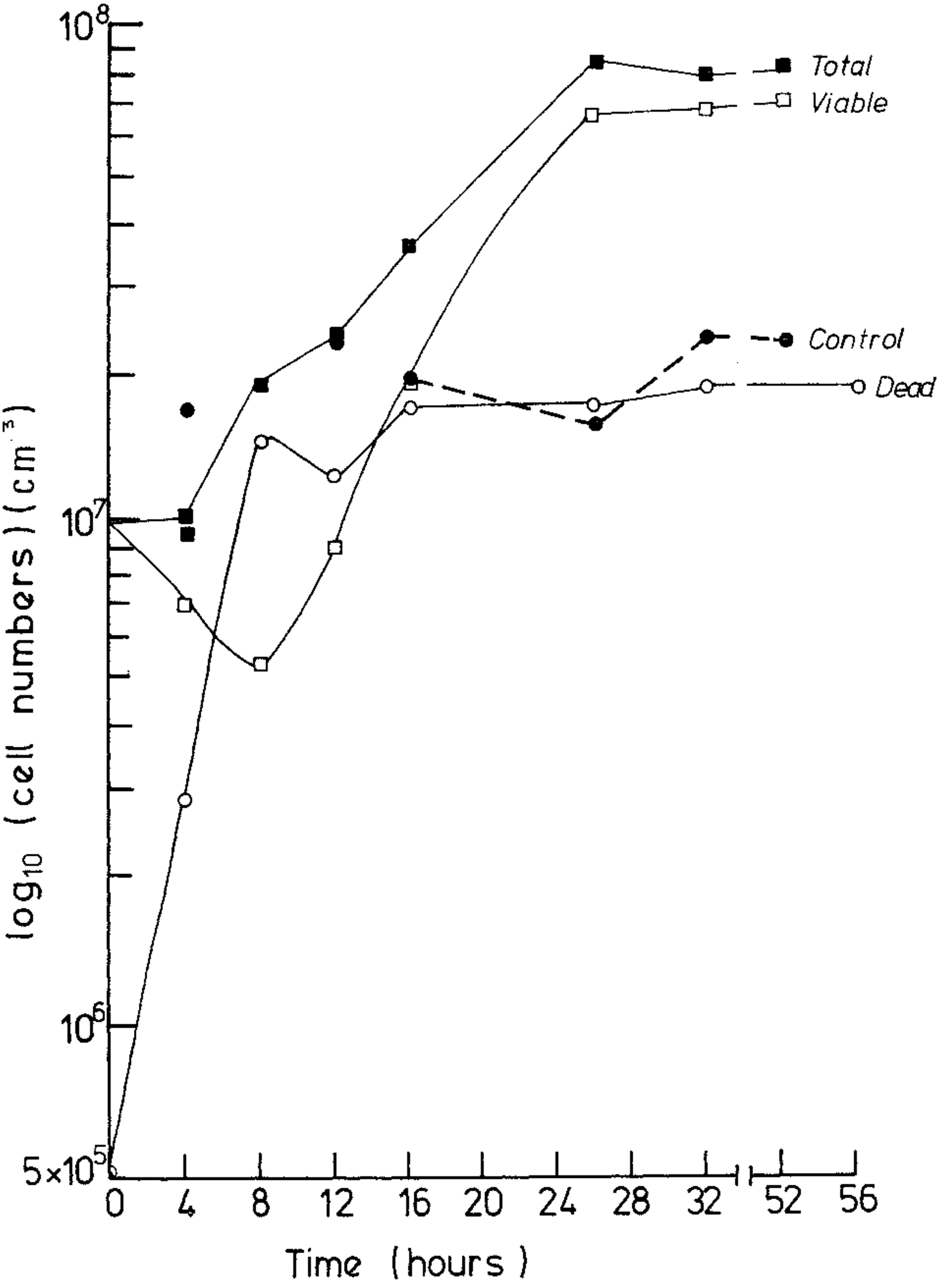

Total
Viable
Control
Dead
10^8
10^7
10^6
5×10^5
log10 (cell numbers) (cm-3)
0
4
8
12
16
20
24
28
32
52
56
Time (hours)

Figure 21. Cell Yield of arg$^-$ in Media Supplemented with Various Concentrations of Arginine

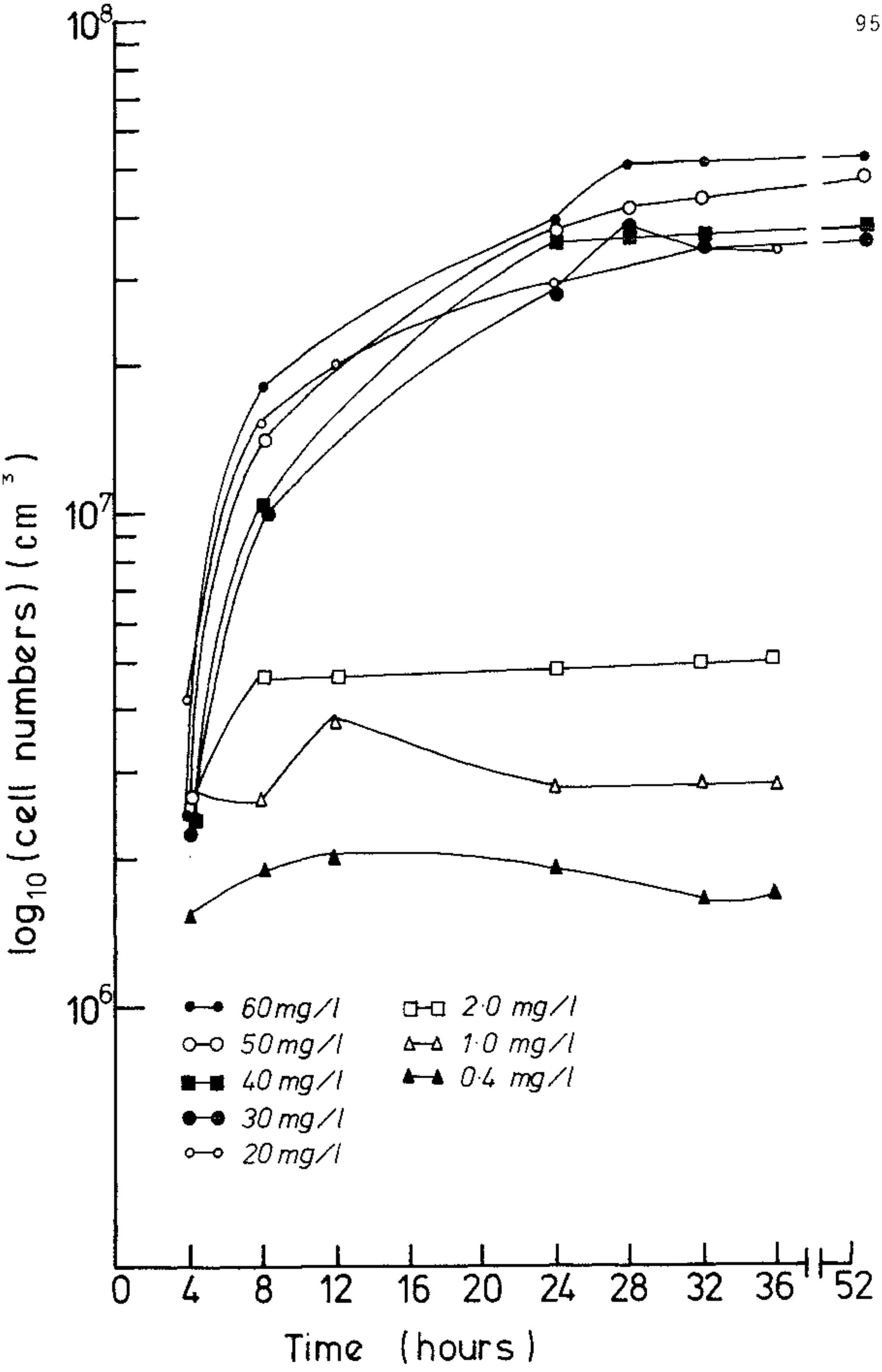

10^8
10^7
10^6
$\log_{10}$ (cell numbers) (cm^{-3})
0
4
8
12
16
20
24
28
32
36
52
Time (hours)
60 mg/l
50 mg/l
40 mg/l
30 mg/l
20 mg/l
2·0 mg/l
1·0 mg/l
0·4 mg/l

Figure 22. Cell Yield of arg$^-$ in Double Strength M.Y.G.P. Broth

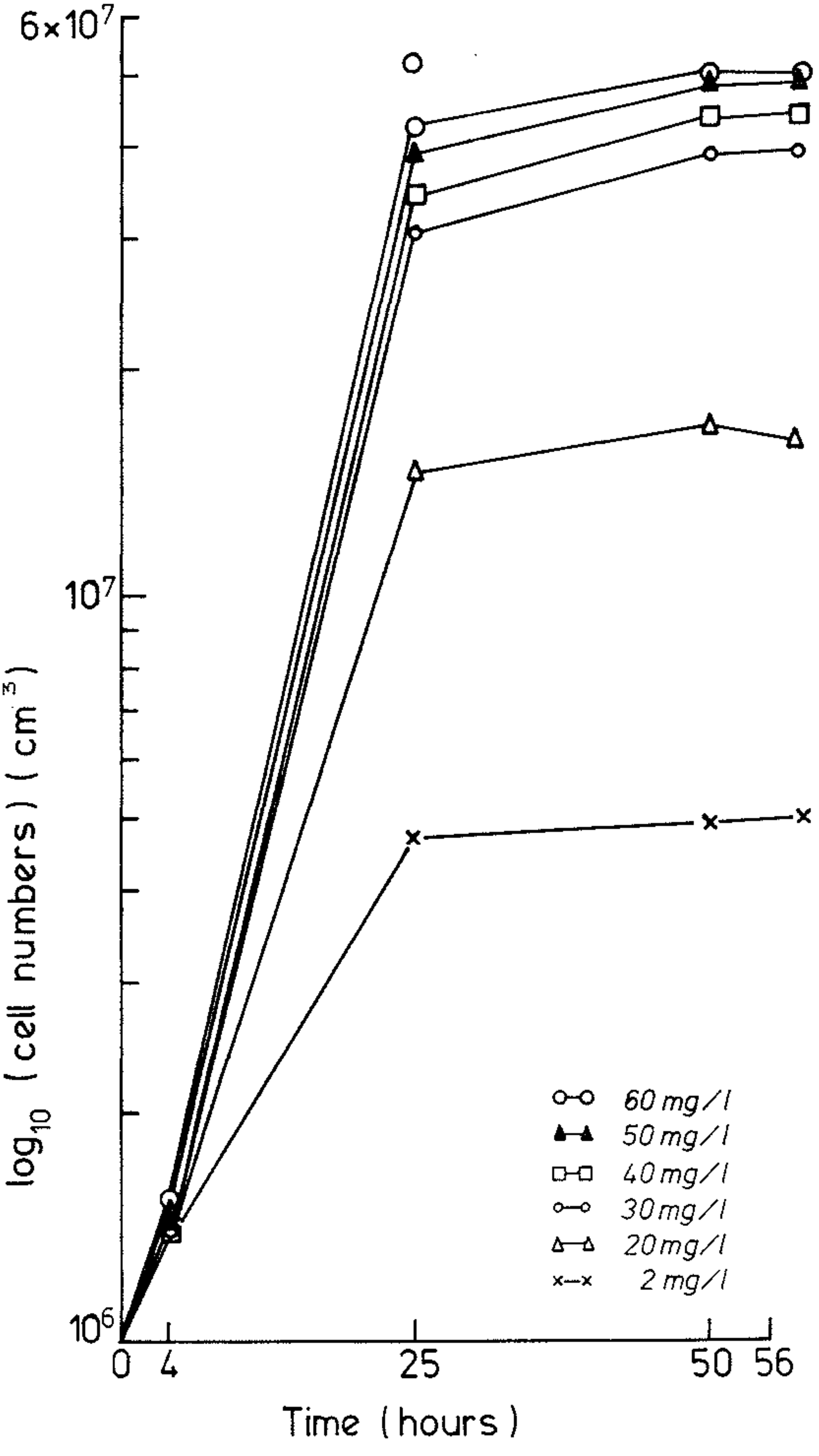
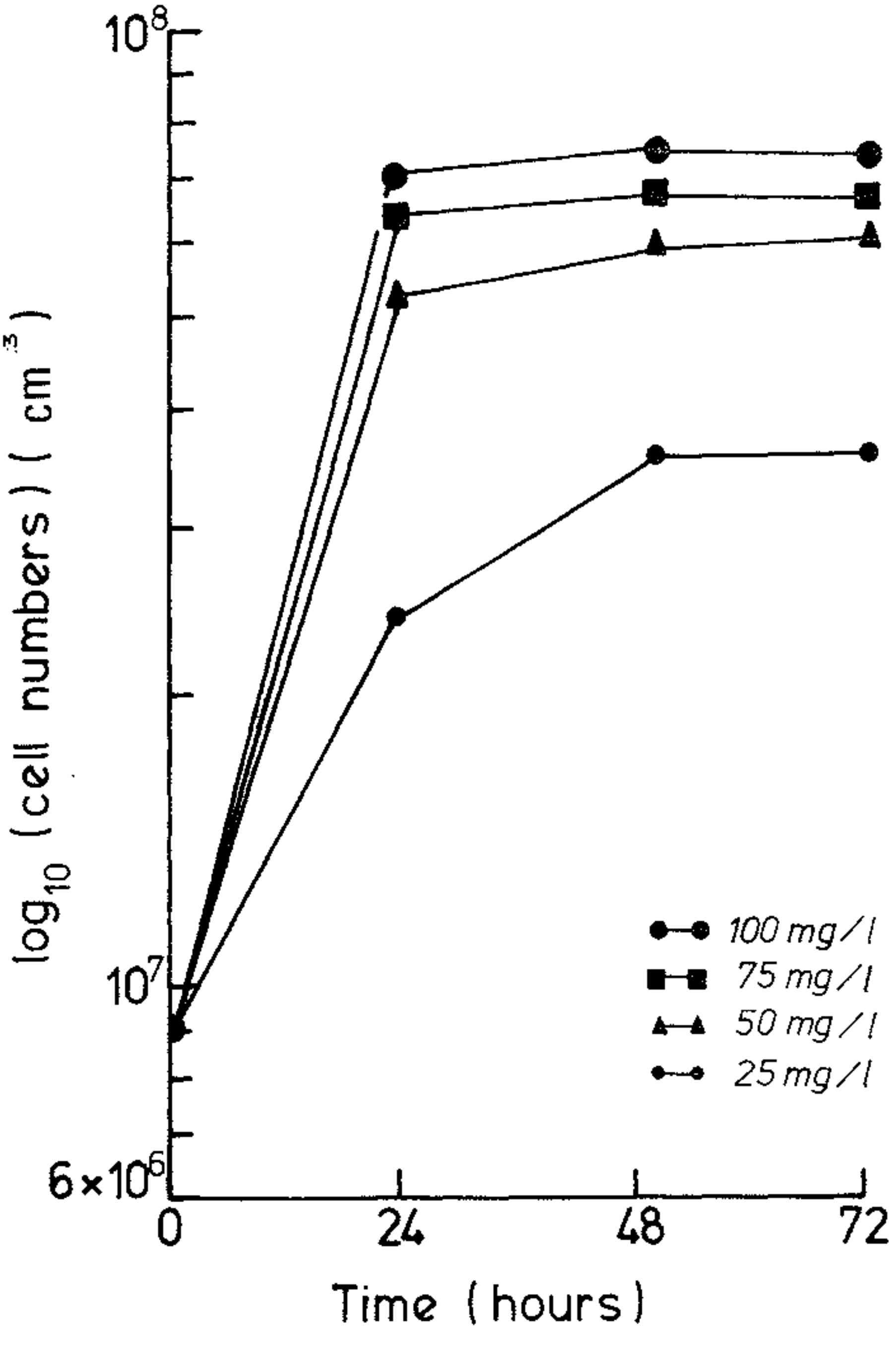

6×10^7
10^7
10^6
log10 (cell numbers) (cm^-3)
0
4
25
50
56
Time (hours)
○—○ 60 mg/l
▲—▲ 50 mg/l
□—□ 40 mg/l
○—○ 30 mg/l
△—△ 20 mg/l
×—× 2 mg/l

Figure 23. Cell Yield of lys$^-$ in Media Supplemented with Various Concentrations of Lysine


10^8
10^7
6×10^6
$\log_{10}$ (cell numbers) (cm^{-3})
0
24
48
72
Time (hours)
100 mg/l
75 mg/l
50 mg/l
25 mg/l

4.2.6 The Killing Process

Different concentrations of cells were treated with a standard amount of toxin. Regardless of the cell concentration 97% to 99% of the sensitive cells were killed (Table 11). Surviving cells were grown in fresh media and treated with toxin for a second time and an identical percentage kill was observed. 99% of the cells were killed when fresh toxin was added to a treated cell pellet.

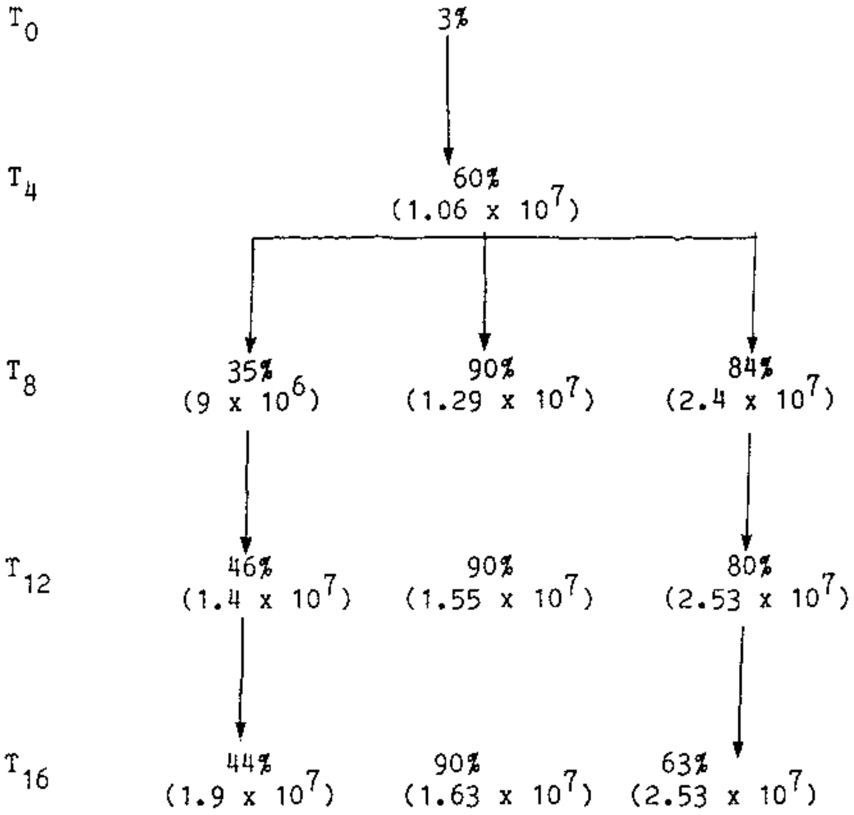
The supernatant of a four hour killing experiment was examined for residual toxin activity. The viability of the cell pellet obtained was followed for a further eight hours. The results suggested that 93% of treated cells were destined to die after a four hour incubation with toxin while enough activity was left in the supernatant to kill 50% more cells (Figure 24). When cells were removed after a four hour kill and plated for viability the number of killed cells equates with the number of methylene blue stained cells by sixteen hours.

Table 11. Effect of Cell Concentration on Killing

(Cell Number) (cm^3)			% cells killed
initial	survivors	killed	
1.60×10^6	7.50×10^3	1.59×10^6	99
8.65×10^4	3.45×10^2	8.62×10^4	99
3.25×10^3	3.20×10^1	3.23×10^3	99
9.15×10^2	2.00×10^1	8.95×10^2	98
1.5×10^1	0.5	1.45×10^1	97

Figure 24. Examination of Toxin Activity Remaining After a 4 Hour Incubation with K_0 Cells

The figure shows the % of dead cells each time and in brackets the actual number of dead cells.


T_0
3%
T_4
60%
(1.06×10^7)
T_8
35%
(9×10^6)
90%
(1.29×10^7)
84%
(2.4×10^7)
T_{12}
46%
(1.4×10^7)
90%
(1.55×10^7)
80%
(2.53×10^7)
T_{16}
44%
(1.9×10^7)
90%
(1.63×10^7)
63%
(2.53×10^7)

4.2.7 Effect of Ethanol on Killing Activity

A K_0 strain acclimatized to grow in the presence of ethanol was used to examine the effect of ethanol on killing. The normal killing procedures were used, with the addition of 0, 2, 4, 6, 8 or 10% (v/v) ethanol into each assay tube. The killing process was inhibited by concentrations of ethanol greater than 2% at all temperatures studied (Table 12).

Toxin was treated with the same concentrations of ethanol to determine whether ethanol denatured killer protein. Removal of ethanol by dialysis with phosphate-citrate buffer resulted in dilution of toxin to such an extent that this method was impractical. Ultrafiltration was used as an alternative method for removal of ethanol. Toxin without ethanol and treated in this way retained the initial activity, while at each percentage ethanol examined, toxin activity was totally lost.

The effect of ethanol on fluidity of the cell membrane was examined by treating K_0, K_1 and K_2 cells with 0.1% cetyltrimethyl ammonium bromide (C.E.A.B.). The results suggested that membrane solubilization did not alter the resistance or susceptibility of cells to toxin (Table 13).

Table 12. Effect of Ethanol on the Killing Reaction

Figures shown are % of total cells killed by toxin; no killing activity (-).

Temperature	% Ethanol						
(^{o}C)	0	1	2	3	4	5	6
30	67	55.4	4.0	-	-	-	-
25	32	9.0	3.0	-	-	-	-
20	13	10.5	3.5	-	-	-	-
15	12.5	2	2.5	-	-	-	-
5	7	-	-	-	-	-	-

Table 13. Effect of Membrane Solubilization on the Killing Reaction

The activity of toxin against K_0, K_1 and K_2 cell suspensions which had been treated with : 0.1% of 100% ethanol; 0.1% C.E.A.B. dissolved in 100% ethanol; and a toxin treated control.

	Killer Phenotype		
Treatment	K_0	K_1	K_2
Control	2.53×10^7	NK	2.09×10^7
Ethanol	3.64×10^7	NK	2.37×10^7
CEAB	3.07×10^7	NK	1.64×10^7

DISCUSSION

5.1 AUTOLYSIS

The role of autolysis of yeast is of importance during champagne ageing for the achieving of desirable organoleptic quality (Joslyn, 1955; Hajslova et al 1980; Molnar et al 1981; Feuillat et al 1982). An increased understanding of the autolytic process is essential in the development of strains which could be used to reduce ageing time. This study examines cell death in wine and synthetic media to extend the knowledge of cellular changes occurring during death and autolysis of yeast.

5.1.1 METHYLENE BLUE STAINING TO DIFFERENTIATE VIABLE AND NON VIABLE YEAST CELLS

Dead cells stain with methylene blue, giving clear distinction between dead and viable cells by light microscopy. It was observed that initial cellular shrinkage due to cytoplasmic loss did not affect staining but resulted in clumping of cells. Stain definition decreased as cellular components were lost. Stain was no longer retained by the cells when ghosting occurred. Those cells appeared dark and granular under phase contrast microscopy, while viable cells remained refractile.

Methylene blue enters cells through sites on the cell membrane, possible using the citrart carrier, and is decolourized by enzymes within cells. Apparently it is the cellular components that are stained, and the loss of these components associated with ghosting, results in the loss of the staining effect. Hence this method could not be used to differentiate viable and dead cell numbers in ageing populations. This situation was observed in some strains after 12 weeks in MYGP broth and thereafter plate counting was used to determine viability.

5.1.2 AUTOLYSIS IN NUTRIENT DEFICIENT MEDIUM, BASE WINE AND DISTILLED WATER

The results of initial comparison of selected strains in nutrient deficient media, base wine and distilled water (Tables 5, 6, and 7) were distorted due to the presence of residual nutrients and the unkown effect of ethanol in the wine.

In base wine, all five strains examined were dead after 53 days, with the most rapid loss of viability in GS3 and GS5 (Table 6). By contrast, in distilled water all five strains retained viability and total cell number at a constant level (Table 7). In supposedly nutrient deficient media, GS1, GS2, and GS3 showed slow steady growth while GS5 and GS6 were dead by 53 days (Table 5).

The general trend was of sygmoidal growth in the presence of nutrients, and stationary phase replacement in nutrient deficient media. Strains GS3 and GS5 were the most ethanol sensitive. The conclusion drawn from the investigatory experiment is that the rate of death and autolysis was determined by environment conditions as well as being strain specific, which agrees with the observations of Stateya et al (1981).

5.1.3 GROWTH AND AUTOLYSIS IN MYGP BROTH

An eight month study of twenty two strains in MYGP broth was designed to examine strain variation. The strains were divided into groups according to mortality trends. Eight distinct patterns emerged (Figure 3; Plate 8).

Strains in groups 'A' and 'B' took sixteen weeks for a high proportion to die. These strains have been divided into two groups: 'A' strains, exhibiting a second burst of growth after twenty three weeks; 'B' strains, in which the total and dead cell populations remained constant after sixteen weeks. Viable cells in group 'B' appeared to be in stage III described by Lafourcade (1954) as 'living cells incapable of reproducing'.

In group C the pre-sixteen week death pattern resembled that of 'A' and 'B', with a linear increase of dead cells. This pattern continued for the next sixteen weeks but the increases in dead cells was matched by an increasing total cell population. Examination of actual cell numbers of these strains showed that a burst of growth occurred at twenty four weeks which increased the total cell number, but by thirty two weeks the whole population was dead (Appendix I). A similar, but delayed, pattern began to emerge in group 'D' between the twenty fourth and twenty eighth weeks. Increasing total and dead populations suggested that the rate of cell death matched the rate of cell replication. Group 'C' and 'D' patterns indicated that intracellular products released by autolysing cells were being used to support new growth.

Groups 'E' and 'F' represent strains in which dead cell counts peaked at sixteen and twenty weeks respectively and then showed decreasing numbers of dead cells and an associated increase of viable cells. The occurrence of cell lysis would explain the loss of dead cells and the cellular components released into the media would be used to support growth and replication of viable cells.

Group 'G' strains had populations whose total cell counts slowly increased at a relatively constant rate. Dead cell numbers were low, but at weeks sixteen and twenty the total population was viable as verified by plate counts repeated twice in duplicate. This sudden and total disappearance of dead cells can only be accounted for by lysis of the entire dead cell population. A possible explanation could be a trigger effect by a substance released at lysis which induces lysis of other dead cells. It would be interesting to follow these populations over a longer time span to see if a cyclic pattern of lysis occurs, and to examine changes in media composition. If this theory of a lysis trigger were true, the potential exists to lyse a whole population of dead cells resulting in a high concentration of autolysate.

GS8, the only strain represented in group 'H' had high viability with the dead cell population increasing slowly over a thirty two week period.

The strain variations observed provide a basis for selection of strains with death patterns desirable for champagne ageing, in which autolytic products are of importance to organoleptic quality. Intitial vigorous growth, followed by accelerated death and autolysis, with the maximum retention of autolytic products is desirable in champagne making to ensure complete fermentation followed by cellular component release. Strains of group 'B' fulfilled these requirements while the high dead population of group 'C' strains may also have potential to produce high concentrations of autolysate. The potential exists for natural lysis of a total dead population within sixteen weeks if the lysis trigger of a group 'G' strain was incorporated into a group 'B' or 'C' strain.

5.1.4 INVESTIGATION OF DEATH AND AUTOLYSIS OF GS18 AND GS20

After one week in MYGP broth strain GS18 showed a high mortality rate; 1.9 x 10 dead cells out of a total population of 6.65 x 10 (Figure 3). To understand the growth pattern of this strain, cell counts were taken every four hours (Figure 4). Strain GS20 was used to represent a low mortality strain typical of other GS strains (Figure 4). GS18 and GS20 had similar generation times (4 hours). As both strains were supplied with the same nutrient supply, the discrepancy in the final population size of 2.4 fold suggested that the metabolism of GS18 was less efficient than GS20 (Appendix III). This factor in combination with the high mortality rate suggested that GS18 may be an aneuploid with a genetical defect in one or more metabolic pathways.

The relationship between amino acid leakage and death and autolysis was examined in strains GS18 and GS20 by growth at 15°C and 30° C in minimal media. Under these conditions, second phase growth was probably a consequence of cellular leakage from dead or autolysing cells. A decrease in the dead cell population, coupled with an increase in total population, was suggestive of autolysis. This pattern was observed for GS18 at 30° C with the dead population decreasing between days two to twelve, and twenty to thirty five, while the total population size increased (Figure 5). At 15°C by day sixty three the number of dead cells became constant with a corresponding gradual decrease of total cells indicating a loss of dead cells and replacement by viable cells dying. Loss of dead cells at 30° C was more pronounced after sixty days. By contrast, GS20 at 15 C showed evidence of loss of dead cells after sixty five days (Figure 6). Thus temperature and genetical factors contribute to cell death, autolysis and regeneration from cell autolysate.

Amino acid sampling at each sample time would have enabled a clearer insight into cellular leakage. The problem of poor resolution with I.R. was described in the results (Section 4.1.11). The unidentified peak observed at 4.678 minutes with U.V. detection could be caused by any of the minor components present in minimal media (Figure 9). It could not be assumed that the size of this peak would remain constant for the duration of the experiment. This H.P.L.C. procedure could not be used to determine amino acid leakage because the retention time of the unidentified peak was within 5% of the aspartic and glutamic acid peaks (Figure 8). The area increase of 4.25269 of the unidentified peak by the day 25 sample showed that the compound concentration increased. At the time of this sample, the GS20 growth phase was complete and 50% of the population was dead (Figure 10). The unknown compound could be the result of excretion during growth or could be associated with death and autolysis (Joslyn 1955; Jund et al 1977; Molnar et al 1980; Latov 1984).

Amino acids could be detected using a reverse phase exchange column with gradient elution followed by U.V. detection of dansyl derivatives. However this equipment was not available and amino acid release during autolysis could not be followed by H.P.L.C. because of peak interference.

5.1.5 THE GENETICAL BASIS OF AUTOLYSIS

The chromosome complement of the GS strains was assumed to be haploid or aneuploid because of the triploid status of the parent strain GW8021. Mating of GS10(α) and GS15(α) with GS11(a) was successful, but all attempts to induce sporulation failed, which suggested that GS11 or GS10 and GS15 contain defective sporulation genes. These strains were selected after twelve weeks incubation in MYGP broth on the basis of initial rapid death. In future breeding programmes using GS strains, this problem might reoccur so whenever possible, more strains should be incorporated into the breeding programme.

5.1.6 EFFECT OF ETHANOL ON AUTOLYSIS

A study was made to determine the effect of ethanol on cell death and autolysis. A requirement was to acclimatize strains to grow in the presence of ethanol. GS5, GS9 and GS21 proved to be ethanol sensitive, while GS7 and GS18 showed retarded growth, consequently these strains were not used in the ethanol study. However, the remaining strains were acclimatized to grow in 12% ethanol.

After eight weeks in wine at 15 C six patterns of mortality were distinguished (Figure II; Appendix II). Strains in classes I to V took eight weeks for the total population to die but were separated into different classes according to the rate of growth and death. Class I strains showed initial vigorous growth followed by rapid death to give a 74% mortality rate within four weeks. In contrast, 10.5% of group II cells were dead in the same period followed by accelerated death between four and eight weeks. Growth of class III strains matched group I as did the initial death rate.

These strains were distinguished by the lag phase for death observed between weeks two and four, Class IV strain all showed weak growth paralleled by rapid death.

Strains of classes V and VI took twelve weeks for the total population to die, with the latter showing slower growth.

Growth of GS17, the only strain in class IV, was very slow, indicating poor adaptation to growth in ethanol.

GW8021, the parent strain, had a vigorous fermentation with viability decreasing after two weeks, 4% of the population was still viable after twenty weeks.

Vigorous fermentation in wine followed by a high mortality rate made the strain of classes I, II and III candidates for further investigation.

5.1.7 POSSIBLE LINES OF INVESTIGATION AS A CONSEQUENCE OF THIS WORK

Knowledge of what compounds are leaked at the time of cell death and during autolysis would provide an insight into selection of strains for rapid autolysis. The patterns of cell death appear to be strain dependent which suggests that the autolysis may also be genetically determined (Kluka 1953; Stateya et al 1981). The behaviour of strains in MYGP broth, wine and loss of methylene blue differentiation need to be taken into account in the development of improved strains. Table 14 analyses strain behaviour and can be used for the selection of strains. It is suggested that group 'B' and class I, II and III have potential for further development.

Strains GS3, GS13 and GS16 were all group 'B' strains, i.e. they had a high mortality rate and did not show regeneration from

Table 14. Comparison of Strain Performance

s = sensitive

+ = loss of stain differentiation

± = 50% loss of stain differentiation

G.S Strain No.	Mating Type	MYGP Group	Wine Class	Loss of Stain Differentiation
1	a	A	III	-
2	a	D	IV	-
3	α	B	III	+
4	α	E	I	-
5	α	D	S	±
6	a	A	II	+
7	a	B	S	+
8	a	H	III	-
9	a	B	S	+
10	α	E	II	±
11	a	F	I	-
12	a	B	IV	+
13	a	B	I	+
14	a	G	III	-
15	α	A	II	-
16	a	B	I	+
17	a	E	VI	-
18	α	C	S	+
19	a	G	V	-
20	a	C	V	+
21	α	G	S	-
22	a	C	IV	-

autolytic products; but did show loss of strain differentiation; all fermented strongly in ethanol and had total population death after eight weeks (Classes I and III). To see if these desirable qualities can be improved, GS13(a) and GS16(a) could be crossed with GS(α), sporulated, and the haploid spore clones examined for death patterns and autolytic behaviour.

Group 'G' strains GS(α) and GS(a) exhibited the phenomenon of disappearance of dead cells (Molnar et al 1980; Perez-Leblic et al 1980). If this 'cell lysis' factor could be incorporated into group 'B' class I, II or III strain, the result would be a vigorously fermenting, ethanol tolerant strain which shows rapid death in wine, incorporated with spontaneous cell lysis. Possible crosses to see if these factors can be incorporated in one cell are; GS14(α) x GS16(a) or GS16(a); GS19(a) x GS5(α). Alternatively, fermentation using a mixed population could be studied to see if the lysis trigger can cause lysis of dead cells of another strain; this could be true if the trigger is of a molecular nature rather than a genetical trigger.

5.2 YEAST KILLER TOXIN INDUCED CELL DEATH AND LYSIS

Yeast cell death and autolysis is a critical step in the production of sparkling wines by the 'methode champenoise' process for the development of characteristic flavour and bouquet. 'Charmat' process, or tank secondary fermentation, lacks these organoleptic characters. Killer yeast activity has the potential to induce cell death and accelerate autolysis. The effects of the principal environmental factors present in winemaking on killer activity have been examined.

5.2.1 TOXIN PRODUCTION AND CURING OF A K1 STRAIN

Killer toxin was concentrated by ultrafiltration and maintained as a stable preparation in 20% glycerol (Palfree et al 1975; Ouchi et al 1978). This stock preparation permitted the addition of known amounts of killer toxin in each investigation and eliminated a possible source of variation. A killer yeast strain was cured of toxin producing ability by cycloheximide treatment (Fink et al 1972; Table 9). The quantity of killer protein obtained by the ultrafiltration procedure was estimated by comparing the amounts of extracellular protein produced by K1 killer and K1 cured strains (Figure 12). Four percent of the total protein was estimated to be killer protein, rather less than the 10% found by Wickner (1981).

5.2.2 EFFECT OF GROWTH PHASE AND MEDIA ON KILLER TOXIN ACTIVITY

The activity of the killer toxin varies with the phase of growth of the sensitive culture. Activity is highest during the exponential growth phase and lowest during the stationary phase of growth (Woods et al 1968). These observations were confirmed in this study. Killer activity was enhanced when killer sensitive (KO) stationary phase cells were suspended in fresh medium prior to toxin addition (Figure 15). Stationary phase KO cells retained in their initial growth medium were the most resistant to killer toxin. However the greatest sensitivity to killer toxin was detected in stationary phase KC cells resuspended in nutrient deficient medium. These observations suggest that the stationary phase was caused by the accumulation of inhibitory metabolic products in the growth medium. Their removal, achieved by resuspension in the nutrient deficient medium, permitted growth until reserve metabollites were consumed.

The reduction of killer toxin activity observed with stationary phase cells could be explained by denaturation by proteases excreted from autolysing cells, since the killer toxin is a protein. However, the addition of a protease inhibitor did not increase the activity of the killer toxin. These observations support the suggestion that killer toxin is energy dependent, possibly through a requirement for energy for binding of the toxin to the cell wall (Middlebeek et al 1980; Hutchings et al 1983).

5.2.3 STANDARDIZATION OF OPTIMUM KILLING CONDITIONS

Examination of pH and temperature interactions on killing were examined (pH 3.4, 4.8, and 5; at 15, 20, 25, and 30°C). Increasing the temperature up to 30°C increased killer activity and increased the sensitivity of toxin to pH (Figure 14). Optimum kill (measured as corrected active killer units) was found at 30 C for all pH levels examined; a pH of 4.0 at 30°C gave optimum kill. These results were unexpected and in contradiction to published results on killer pH sensitivity (Young and Yagüi 1978). The experiment was repeated and the same results obtained, which suggests that the pH range for activity is not as narrow as is believed (Palfree and Bussey 1979). For all subsequent experiments pH 4.8 was used to standardize results obtained with published killer papers.

5.2.4 BIOASSAY FOR THE DETECTION OF AMINO ACID RELEASE BY TOXIN KILLED CELLS

A bioassay was developed to examine amino acid leakage from toxin treated cells since the stock toxin preparation contained too many impurities for H.P.L.C. identification. Twelve amino acid auxotrophs were screened for killer phenotype, and killer sensitive (KO) arginine and lysine auxotrophs identified (Table 10). The minimum level of amino acid supplementation needed to support growth was found to be 0.4 mg/l arginine, and 4.0 mg/l lysine. Lower concentrations of supplementation resulted in growth curves typical of growth being supported by internal amino acid supplies (Figures 16 and 17).

The KO stock culture used did not have any amino acid requirements and growth in minimal media was sygmoidal with a population of 8 x 10^7 cells being established sixteen hours after inoculation. Viability dropped to 10^6 cells/ml sixteen hours after treatment with toxin, followed by rapid regeneration of cells and establishment of a stationary phase culture with a total cell count at the same level as the untreated control by eighty hours (Figure 18). This pattern appeared to be typical of a toxin treated culture with no nutritional deficiencies.

Actively growing arg$^-$ and lys$^-$ cultures for killer trials were obtained by growing cultures in minimal media containing the minimal level of amino acid supplementation needed to support growth (Figures 16 and 17). Cells were removed during exponential phase, washed and resuspended in minimal media. Untreated arg$^-$ and lys$^-$ cultures in minimal media did not grow exponentially; the population remained constant at the inoculum level of 1.3 x 10^7 cells/ml, confirming that arginine and lysine were not released at growth supporting levels. However, positive killing reactions indicated that the resuspended cells were still able to supply the energy required for toxin action (Middlebeek et al 1980; Hutchings et al 1983).

Actively growing arg$^-$ cells resuspended in minimal media and treated with toxin were killed in the same way as the KO control. Regeneration of the 1.2 x 10^6 surviving cells began sixteen hours after toxin addition, and stationary phase was established by one hundred hours at a level of 6 x 10^7 cells/ml, 2.7 fold higher than the untreated arg control culture (Figure 19). The increase of arg$^-$ cell concentration in media deficient in arginine suggests that the regeneration of viable cells was due to amino acid release from toxin killed cells.

The lys$^-$ culture behaved similarly to the arg$^-$ culture, with the maximum kill being obtained after eight hours with 9 x 10^6 cells/ml surviving. Regeneration was complete and stationary phase reached after twenty four hours at a concentration of 7 x 10^7 cells/ml (Figure 20). To support this regeneration lysine must have been released from toxin killed cells.

Amino acids were not present in the killer concentrate as the ultrafiltration method used for concentration was specific for the retention of compounds containing peptide bonds, that is protein and peptides. Amino acids present in the Synthetic-B media would have been diluted out during protein concentration.

The amount of amino acid supplentation needed to support the regeneration seen was found by inoculating arg^- and lys^- at the survival concentrations (1×10^6 and 9×10^6 cells/ml respectively) into minimal media containing increasing concentrations of the required amino acid (Figures 21 and 22). To establish a population of 6×10^7 arg^- cells/ml, 80 mg/l arginine was needed, implying that 1.88×10^7 toxin killed arg^- cells in 10 ml excrete 0.8 mg of arginine, or 4.26×10^{-6} mg per cell (Appendix IV). Addition of 100 mg/l of lysine was needed to generate a population of 7×10^7 lys^- cells/ml, implying that 1×10^7 toxin killed cells in 10 ml excreting 1 mg of lysine, or 1×10^{-6} mg per cell (Appendix IV).

As a basic and an acidic amino acid have been shown to be released from toxin killed cells it can be assumed that other amino acids will also be released. Amino acid leakage may occur passively via toxin induced channels in the cell membrane that have been shown to allow leakage of potassium ions, ATP and other small metabolites (Bussey et al 1973; Bussey et al 1975; Middlebeek et al 1980).

The killing pattern observed above raises two questions: (1) Why is a total kill not achieved, and (2) why is maximum killing not obtained for sixteen hours when previous reports claim three to four hours is needed for killing? (Middlebeek et al 1980)

The explanation for the first observation could be resistance of cells to toxin action or insufficient toxin to kill all of the cells. These possibilities were examined by treating different concentrations of cells with a standard amount of toxin. Regardless of the cell concentration only 97% to 99% of the populations were killed (Figure 24), which suggested that 1% to 3% of the population may be resistant to toxin. When surviving cells were grown in fresh media and again treated with toxin, the same percentage kill was obtained. These results implied temporary resistance rather than a mutation to resistance. Those cells surviving toxin treatment may have been in stationary phase and lacked the energy needed to support toxin binding.

The time taken for killing to be complete was examined (Figure 24). Four hours after toxin addition 1.06 x 10^7 cells were dead as determined by methylene blue staining. When a sample taken at four hours was plated, the number of killed cells correlated with the number of methylene blue stained cells at sixteen hours. This suggested that by four hours cells were irreversibly set in a death pathway as determined by methylene blue staining (thus do not form colonies when plated), but that death does not occur for up to sixteen hours

5.2.5 THE EFFECT OF ETHANOL ON KILLING ACTIVITY

The addition of ethanol at concentrations above 2% was found to totally inhibit the killing reaction (Table 12). Ethanol is known to cause membrane solubilization and to denature protein (Mitchaelis et al 1982; Janoff et al 1982). One or both of these factors cause the loss of killer activity. Solubilisation of the cell membrane may confer resistance by altering toxin binding sites or may open the membrane to allow freer passage of toxin into the cell. When the toxin activity against cells treated with a membrane solubilizer, C.E.A.B., was examined, it was found that membrane solubilization did not affect the susceptibility or resistance of cells to toxin (Table 13). This observation implies that ethanol acts on the killer protein causing denaturation. This theory is supported by the observation that toxin treated with ethanol lost its ability to kill sensitive cells.

5.2.6 IMPLICATIONS OF THESE OBSERVATIONS WITH REGARD TO SPARKLING WINE PRODUCTION

Wine yeasts have been selected for their ability to ferment under unfavourable conditions. Grape juice has a high sugar content and a low pH and fermentation causes the formation of a high level of ethanol. The parameters of the secondary fermentation in the production of sparkling wines, low pH, low temperature, low nutrient level, high ethanol level and CO_2 pressure, present an even more hostile environment to the biological activity of wine yeasts. These environmental factors also limit the activity of killer yeast toxin. Evidence has been presented which confirms that pH, temperature and yeast growth phase, limit the activity of killer toxin (Woods et al 1968; Young et al 1978). In addition this study presents evidence that ethanol concentrations greater than 2% denature and inactivate killer toxin. Thus the direct use of killer wine yeast strains for the autolysis and accelerated ageing of sparkling wines does not seem practical under current winemaking conditions.

On the other hand, the bioassay system demonstrates that not only does killer toxin action result in the leakage of potassium ions, ATP and other small metabolites (Bussey et al 1973), but also the leakage of amino acids from the cell. Furthermore, the effect of ethanol on toxin activity has been a neglected area of research. As killer toxins are all structurally distinct, a survey of ethanol sensitivity may uncover an ethanol resistant toxin, e.g. k_2 or k_3 toxins may be ethanol tolerant.

Toxin activity at a wine pH of 3.5 is reduced, but enough activity is retained to enable efficient killing in grape juice (Seki et al 1985) and as demonstrated by this study at pH 3.0 at 30°C (Figure 14). It is conceivable that the pH range of killer toxin activity could be extended since the yeast Hansenula mrakii produces a killer toxin which is heat stable and a pH range of activity from 4 to 11 (Shoda 1984).

The use of both classical and modern genetic techniques could result in the breeding of wine yeast strains whose killer toxin activity was not inhibited by low pH, temperature or ethanol. If these problems cannot be overcome an alternative method of winemaking could negate these inhibitory conditions. The wine and yeast could be separated and fresh grape juice added to the yeast to induce growth. The yeast population would be killed by toxin addition and added back to the original wine. This procedure would be time consuming, but the result would be enhanced flavour and bouquet.

5.3 SUMMARY

5.3.1 AUTOLYSIS

The observations made support the definition of autolysis as the 'process of cellular degradation catalyzed by intracellular enzymes'. Associated with autolysis is the loss of membrane integrity, protoplasm and intracellular components resulting in cell shrinkage, and eventual ghosting of cells, i.e. cellular degradation occurs. Lysis of the cell wall occurred in some strains resulting in visible cellular debris. The observed differences in behaviour of strains under the same environmental and physical conditions suggests that cell wall lysis has a genetical basis and occurs only in some strains. This explains discrepancies between reports of whether or not cell wall lysis occurs during autolysis.

The rate of cell death is influenced by environmental conditions such as temperature, nutrient supply, the presence or absence of ethanol, and also has a genetical basis.

5.3.2 KILLER

These studies confirm previous reports that the activity of yeast killer toxin is pH and temperature dependent. In addition, it has been demonstrated that killer toxin activity is also affected by the presence of ethanol. Ethanol concentrations of greater than 2% activated the killer toxin, probably a a result of protein denaturation. Between 1 - 3% of killer sensitive yeast cells survive in contact with killer toxin, probably because these cells are in stationary phase and lack the energy necessary for the binding of the killer protein to the cell wall. The kinetics of cell death due to killer protein action was followed by two techniques: sample plating and methylene blue staining. A 12 hour lag period was detected between a cell becoming incapable of reproduction due to killer toxin action and this fact becoming discernable by methylene blue staining. Experimental evidence has been presented which suggests that amino acids are released during cell death and lysis due to killer toxin action. A bioassay technique was developed to estimate the amounts of arginine and lysine released by yeast cells upon lysis by killer toxin.

APPENDICIES

Appendix I: G.S. Strains in M.Y.G.P.

GS STRAIN	WEEKS								
	1	4	8	12	16	20	24	28	32
1. T	2.66	4.78	4.23	5.50	4.87	5.61	-	-	-
D	.035	11	.363	.863	4.87	-	-	-	-
2. T	1.13	2.57	2.05	2.20	2.76	2.21	2.03	3.85	3.92
D	.043	.040	.063	.188	2.76	2.21	2.03	3.85	3.92
3. T	2.55	6.07	4.06	5.53	6.07	5.34	5.40	5.93	8.40
D	.041	.030	.050	.425	6.06	5.34	5.40	5.93	8.40
4. T	1.31	3.40	3.90	4.57	4.50	5.06	7.44	10.40	11.8
D	.005	0	.063	.138	4.42	4.93	4.13	3.38	4.4
5. T	0.87	1.20	1.05	1.76	1.41	0.85	1.44	1.93	3.53
D	0.05	0.01	.013	.075	1.38	0.85	1.44	1.93	3.53
6. T	2.58	4.88	5.09	6.96	5.73	5.50	5.84	5.42	5.76
D	.054	.290	.250	.725	5.70	5.50	5.84	5.42	5.76
7. T	1.70	7.43	1.53	2.56	2.57	2.95	3.70	3.64	3.36
D	.104	.050	.138	.625	2.53	2.95	3.65	3.64	3.36
8. T	5.99	7.43	7.98	11.60	10.70	13.70	21.6	18.2	21.3
D	.064	.080	.275	.100	0	2.00	0	0	1.96
9. T	.084	1.33	1.44	1.84	1.59	2.86	1.8	1.90	2.39
D	.014	.040	.088	.150	1.59	2.86	1.81	1.90	2.39
10.T	3.55	5.07	4.83	5.63	6.87	6.27	9.42	16.30	23.0
D	.065	.180	1.58	1.35	6.86	3.90	5.42	4.50	22.9
11.T	1.59	2.17	2.81	6.93	2.56	5.23	6.87	7.14	8.63
D	.024	.040	.050	1.19	2.56	3.02	6.47	3.24	2.93
12.T	1.37	3.02	3.29	3.42	3.00	3.11	3.45	3.77	4.68
D	.003	0	.038	.188	3.10	3.11	3.45	3.77	4.68
13.T	3.16	4.76	8.98	6.03	6.03	6.73	5.70	5.97	7.59
D	.015	.020	.150	.188	5.56	6.71	5.70	5.97	7.59
14.T	3.72	7.13	8.71	8.06	10.3	8.88	11.0	9.60	1.61
D	.025	.080	.175	.223	0	0	.600	0.30	0.61
15.T	1.43	2.30	2.04	2.82	3.28	2.68	4.20	6.39	7.45
D	.010	.070	.213	1.38	3.27	2.67	2.60	2.42	2.35
16.T	3.18	3.95	3.73	4.64	4.44	3.58	3.58	3.68	4.88
D	.030	.060	.150	.675	4.44	3.58	0	3.68	4.88
17.T	3.76	6.14	1.06	7.38	6.32	7.41	6.65	15.60	1.02
D	.040	.040	.025	1.04	5.19	1.26	0	5.60	1.02
18.T	6.65	4.06	7.31	3.12	5.98	6.87	15.8	19.8	-
D	0.19	.050	.025	.313	4.98	0	0	0	-
19.T	3.83	7.12	6.26	4.76	9.51	9.42	18.9	15.2	20.9
D	.028	.090	.100	.150	0	0	.360	15.2	20.9
20.T	1.31	2.50	1.53	1.86	2.27	3.78	6.66	19.6	16.7
D	.011	0	.175	.175	2.22	3.45	0.31	19.6	16.7
21.T	2.76	4.19	5.43	7.05	7.00	6.04	14.9	18.0	32.9
D	.090	.125	.288	.738	1.09	0	0	-	12.8
22.T	4.00	6.41	7.11	4.87	3.13	4.70	19.0	20.0	27.7
D	.023	1.20	.413	.575	3.13	4.70	1.60	20.0	27.7

Total (T) and Dead (D) cell concentration per $cm^3 \times 10^7$

Appendix II: Growth and Death of GS Strains in Wine

GS Strain		Weeks					
	inoculum	2	4	8	12	16	20
1. T	$1.19x10^5$	$9.88x10^6$	$1.69x10^7$	$1.87x10^7$			
D	$1.90x10^4$	$9.48x10^6$	$1.40x10^7$	$1.87x10^7$			
2. T	$1.22x10^5$	$4.00x10^5$	$2.48x10^7$	$1.65x10^7$			
D	$2.26x10^4$	$1.95x10^5$	$1.88x10^6$	$1.65x10^7$			
3. T	$1.0x10^5$	$2.05x10^5$	$3.20x10^5$	$2.89x10^7$			
D	$4.0x10^4$	$2.00x10^5$	$3.05x10^5$	$2.84x10^7$			
4. T	$1.17x10^5$	$4.43x10^6$	$1.29x10^7$	$1.08x10^7$			
D	$1.7x10^4$	$8.0x10^4$	$1.28x10^7$	$1.08x10^7$			
6. T	$1.17x10^5$	$6.8x10^6$	$7.65x10^6$	$1.43x10^7$			
D	$1.7x10^4$	0	$1.95x10^6$	$1.43x10^7$			
7. T	$1.5x10^5$	$2.5x10^5$	$1.60x10^5$	$1.90x10^5$			
D	$5.0x10^4$	$2.25x10^5$	$1.60x10^5$	$1.90x10^5$			
8. T	$3.53x10^5$	$3.86x10^7$	$3.73x10^7$	$3.76x10^7$	$3.63x10^7$		
D	$2.46x10^5$	$1.13x10^7$	$9.8x10^6$	$3.70x10^7$	$3.59x10^7$		
10.T	$1.33x10^5$	$2.85x10^7$	$2.74x10^7$	$2.41x10^7$	$2.89x10^7$	$3.19x10^7$	$3.19x10^7$
D	0	0	$1.8x10^6$	$2.41x10^7$	$2.89x10^7$	$3.19x10^7$	$3.19x10^7$
11.T	$3.60x10^5$	$2.15x10^7$	$1.73x10^7$	$1.88x10^7$	$1.86x10^7$	$2.02x10^7$	
D	0	0	$1.71x10^7$	$1.88x10^7$	$1.86x10^7$	$2.02x10^7$	
12.T	$1.07x10^5$	$1.10x10^5$	$2.54x10^7$	$1.17x10^7$			
D	$7.0x10^3$	$3.5x10^4$	$1.50x10^6$	$1.17x10^7$			
13.T	$1.05x10^5$	$2.00x10^7$	$2.14x10^7$	$1.36x10^7$			
D	$6.4x10^3$	$4.0x10^5$	$2.14x10^7$	$1.36x10^7$			
14.T	$1.5x10^5$	$5.48x10^7$	$2.35x10^7$	$4.92x10^7$	$2.85x10^7$		
D	$4.9x10^4$	$1.28x10^7$	$8.10x10^6$	$4.83x10^7$	$2.82x10^7$		
15.T	$1.81x10^5$	$1.06x10^7$	$2.23x10^7$	$1.55x10^7$	$1.50x10^7$		
D	$7.7x10^4$	$2.0x10^5$	$9.0x10^5$	$1.55x10^7$	$1.50x10^7$		
16.T	$1.19x10^5$	$2.74x10^7$	$1.74x10^7$	$1.97x10^7$			
D	$1.9x10^4$	$4.0x10^5$	$1.74x10^7$	$1.97x10^7$			
17.T	$1.42x10^5$	$1.5x10^5$	$5.4x10^5$	$1.45x10^7$	$1.56x10^7$		
D	$3.8x10^4$	$2.5x10^4$	$6.5x10^4$	$4.60x10^6$	$1.56x10^7$		
18.T	$9.5x10^4$	$5.45x10^4$	$1.0x10^5$	$1.50x10^5$	$1.30x10^5$	$1.15x10^6$	$1.45x10^5$
D	0	$9.5x10^3$	$4.5x10^4$	$1.50x10^5$	$1.2x10^5$	$1.15x10^6$	$1.45x10^5$
19.T	$2.42x10^5$	$2.14x10^7$	$1.62x10^7$	$2.05x10^7$	$1.58x10^7$		
D	$1.41x10^5$	$8.0x10^5$	$1.5x10^6$	$1.95x10^7$	$1.51x10^7$		
20.T	$1.23x10^5$	$1.75x10^5$	$6.88x10^6$	$1.74x10^7$	$1.06x10^7$	$2.16x10^7$	$1.44x10^7$
D	0	0	$2.5x10^5$	$4.9x10^6$	$1.06x10^7$	$2.16x10^7$	$1.44x10^7$
22.T	$9.75x10^4$	$4.65x10^5$	$7.13x10^6$	$1.86x10^7$	$2.39x10^7$	$1.65x10^7$	$2.62x10^7$
	0	$5.0x10^3$	$2.5x10^5$	$1.86x10^7$	$2.39x10^7$	$1.65x10^7$	$2.62x10^7$
GW8021.T	$1.14x10^5$	$5.33x10^7$	$3.75x10^7$	$6.00x10^7$	$4.89x10^7$	$3.66x10^7$	$4.83x10^7$
D	0	0	$5.0x10^5$	$3.25x10^7$	$4.84x10^7$	$3.48x10^7$	$4.66x10^7$

Total (T) and Dead (D) cell numbers are recorded.

Appendix III: Calculation of Generation Time

The growth rate constant (k), can be determined by the equation:

$$x_t = 2^{kt}x_0 \tag{1}$$

by taking the logrithrim to the base 2 and converting logrithrims to the base 10.

$$k = \frac{\log_{10}x_t - \log_{10}x_0}{0.301t} \tag{2}$$

where:

x_0 = initial number of cells

x_t = number of cells at time t

t = length of time taken from x_0 to x_t

As k is given in doublings per hour, the generation time or the time taken for the population to double is the reciprocal.

For GS18 from Figure , 28 hours is needed for stationary phase to be reached ($t = 28$), at cell concentration of 8.27×10^6 (x_t) from an initial concentration (x_0) of 9.26×10^4

$$1/k = \frac{\log_{10}8.27 \times 10^6 - \log_{10}9.26 \times 10^4}{0.301 \times 28}$$

$1/k = 3.92$ = generation time

For GS20

$$1/k = \frac{\log_{10}1.99 \times 10^7 - \log_{10}1.05 \times 10^5}{0.301 \times 28}$$

$1/k = 3.93$ = generation time

Appendix IV: Calculation of Amino Acid Secretion by arg$^-$ and lys$^-$

The number of toxin killed cells (k) is calculated by the equation:

$k = Tm - Sm$

where: T = total number of cells at time m

S = number of surviving cells at time m

m = time at which maximum kill is achieved

For arg$^-$

$m = 16$ hours

when $m = 16$; $T = 2 \times 10^7$

$S = 1.2 \times 10^6$

$k = (2 \times 10^7) - (1.2 \times 10^6)$

$k = 1.88 \times 10^7$ cells/ml

As the sample volume was 10 ml, a total of 1.88×10^7 cells were killed.

To support the regeneration observed 80 mg/l arginine must have been excreted, or 0.8 mg arginine in the 10 ml volume, that is 1.88×10^8 cells excrete 0.8 mg arginine.

The amount of arginine released per killed cell

$= 0.8 \div 1.88 \times 10^8$

$= 4.26 \times 10^{-9}$ mg of argine

For lys$^-$

$m = 8$ hours

when $m = 8$; $T = 1 \times 10^7$

$S = 9 \times 10^6$

$k = (1 \times 10^7) - (9 \times 10^6) = 1 \times 10^6$ cells/ml

The amount of lysine released per killed cell

$= 1 \times 10^6 \div 1 \times 10^6$

$= 1 \times 10^6$ mg of lys$^-$

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