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SEXUAL AGGLUTINATION IN *SACCHAROMYCES CEREVISIAE*

by

KEVIN TERRANCE

A dissertation submitted to the Graduate Faculty in Biology in partial fulfillment of the requirements for the degree of Doctor of Philosophy, The City University of New York.

1983

This manuscript has been read and accepted for the Graduate Faculty in Biology in satisfaction of the dissertation requirement for the degree of Doctor of Philosophy.

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ABSTRACT

Treatment of either haploid mating type of Saccharomyes cerevisiae with the appropriate sex pheromone induced inrreased sexual agglutinability. Differences were noted between the two pheromones in the time and dose needed for maximal induction of their target cells. The action of α-factor was completely inhibited by cycloheximide, aminophylline, theophylline or tunicamycin, but only partially blocked by added cAMP. The action of a-factor was blocked by lesser concentrations of cycloheximide, but only if the cells were preincubated with the drug. a-Factor induction was only partially blocked by tunicamycin, even with a preincubation period. Overall, the results suggest asymmetry in the mechanisms of action of the two phromones.

Cells exposed to α-factor for brief times (less than 30 sec.) became committed to induction of increased agglutinability, while morphogenesis requires continuous exposure to pheromone. a-Cells in G1 or in G2 of the cell cycle responded to α-factor induction with kinetics identical to that seen in unsynchronized cells.

Constitutively agglutinable and induced a-cells exhibited agglutinability across identical ranges of pH, ionic strength and temperature. All agglutinable combinations of cells, regardless of exposure to

For my Father

PETER OCTAVE TERRANCE

pheromone, were identical in response to a wide range of inhibitors.

Mechanical disruption of $\underline{\alpha}$-cells resulted in the solubilization of a molecular species with the predicted properties of $\underline{\alpha}$-agglutinin. This species behaved homogeneously in heat inactivation experiments, and was capable of blocking all possible agglutinins on $\underline{a}$-cells.

Electrophoresis of purified $\underline{\alpha}$-agglutinin followed by staining with the new, ultrasensative silver technique (Goldman et al., 1981) revealed a total of 8 peptides, ranging from 33,000 to 120,000 daltons. This pattern persisted after two diverse purification steps. One of these steps, affinity chromatography onto lentil lectin, depended on carbohydrate residues that were only present in the 4 bands of highest molecular weight. This, along with supporting evidence, suggests a multi-subunit structure for the $\underline{\alpha}$-agglutinin.

Prologue

Saccharomyces in Paradise

While Adam and Eve,
Whom everyone knows,
Were enjoying Eden
Without any clothes,

On the skin of the apple,
Hanging on the tree,
Were the first two yeasts:
X2180-1A and X2180-1B.

Now, this pair of tiny cells,
On that fateful day,
Discovered conjugation
In a most pleasurable way.

In the exchange of factors,
α let go first.
When her peptide reached **a**,
He knew it was a flirt.

The effect of α's pheromone
On a were rather quick.
He grew more surface agglutinins,
Which made him want to stick.

Thus it was that when these cells
Just happened to collide;
They could not let each other go
And held fast side by side.

No longer could poor **a** hold back;
He let his factor go.
It took a little longer, but
On α, the effects did show.

She tightened up her lock on **a**,
Which proved a thrill for both.
Unless the pH went too high
Their bond could not be broke.

The tension grew by leaps and bounds
As cell walls softened up.
And when their cytoplasms mixed
They'd hardly had enough.

Frantically, with hearts aflame,
They drew each other in.
Instinctively, they knew that soon
Fulfillment would begin.

As organelles exchanged and mixed,
Confusion reigned inside.
Yet two large orbs moved steadily,
As if led by a guide.

Their approach was quite uncanny,
Indicative of collusion.
And so it was that yeasts learned of
The joys of nuclear fusion.

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INTRODUCTION

I. Life Cycle and Mating Type Control.

Haploid cells of the yeast Saccharomyces cerevisiae exist in two alternate mating types designated a and α. When opposite cell types are mixed, they undergo a mating reaction. Through the cellular and nuclear fusion of individual pairs of a-cells and α-cells, diploid (a/α) zygotes eventually form. Such zygotes produce diploid (a/α) buds which represent the normal vegetative state in Saccharomyces.

Under conditions of nutrient depletion, diploid cells will undergo meiosis and sporulation. Each cell yields an ascus containing four haploid spores, two of which are of mating type a, the other two being of mating type α.

Haploid yeast are capable of exhibiting either of two alternate life cycles referred to as homothallic and heterothallic. The gene controlling this trait is designated HO. Cells with the dominant (HO) allele are called homothallic and have unstable mating types. Such cells are characterized by a high probability of mating type interconversion during cell division. As a result of this phenomenon, an a-cell will divide to form two α-cells, or conversely, an α-cell will divide to form two a-cells. In such strains, a haploid cell maintained in culture will invariably give rise to cells of of both

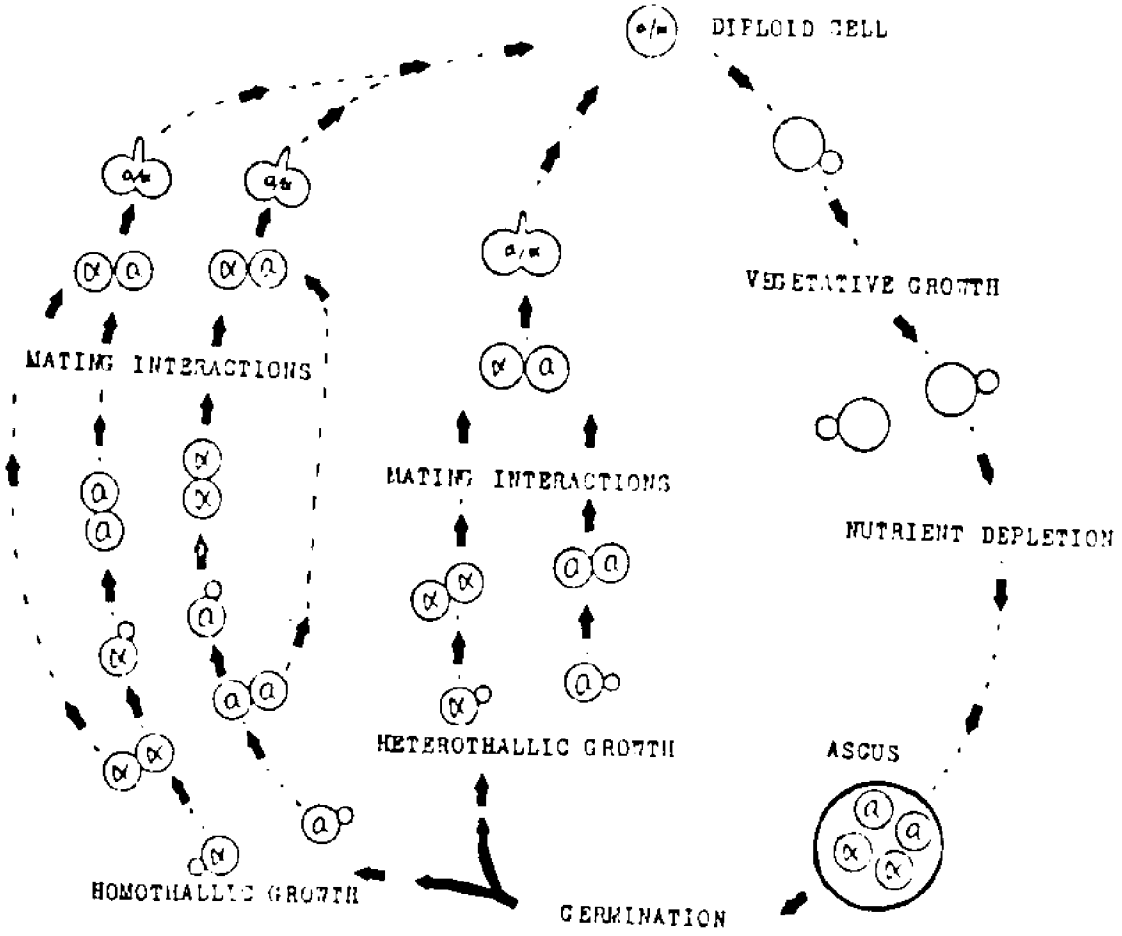
mating types, and diploidization will occur (Hicks and Herskowitz, 1976b). In heterothallic (ho) haploids, mating type is stable. Thus, pure cultures of either a-cells or α-cells may be maintained (Herskowitz and Oshima, 1981). The events of the life cycle are summarized in figure 1.

The HO locus is a device for the control of mating type stability. Mating type per se is determined at the MAT locus, which is located on chromosome III and is unlinked to HO (Thorner, 1981). The single MAT locus may be occupied by either of two alternate alleles, MAT**a** or MAT**c** (Sprague et al., 1981). An a-cell has the former allele at MAT, whereas an α-cell has the latter. In diploids, the presence of one of each allele at MAT (a/α) leads to a suppression of haploid specific functions and the expression of diploid features (e.g. sporulating ability).

MAT**α** contains at least two complementation groups, MAT**α**1 and MAT**α**2 (MacKay and Manney, 1974 a,b). MAT**α**1 mutants are deficient in a number of α-specific phenotypes, while MAT**α**2 mutants exhibit some a-specific phenotypes (Hicks and Herskowitz, 1976b; Tkacz and MacKay, 1979; Strathern et al., 1981). Mutations of MAT**a** do not seem to affect mating ability (Herskowitz and Oshima, 1981). These MAT**a**1 mutants do, however, behave abnormally in a/α diploids (Kassir and Simchen, 1976; Klar et al., 1979b). Such diploids are sporulation deficient.

An interesting feature of the mating type control

Figure 1. The life cycle of Saccharomyces cerevisiae. Diploid cells grow by budding (mitosis) until induced to undergo meiosis and sporulation. The resulting ascus contains four haploid spores, two of each mating type. Germination releases these spores. Those containing the HO gene will grow homothalically, switching will occur with high frequency, and any culture will soon contain both a and α-cells. Cells with the ho gene follow the alternate heterothallic lifestyle, in which switching is a low frequency event. In either case, when opposite mating types are mixed, interactions are initiated that ultimately lead to cell fusion and zygote formation. Zygotes produce characteristically shaped, narrow diploid buds. Such diploids will grow vegetatively until meiosis is induced.


DIPLOID CELL
VEGETATIVE GROWTH
NUTRIENT DEPLETION
ASCUS
GERMINATION
HETEROTHALLIC GROWTH
MATING INTERACTIONS
HOMOTHALLIC GROWTH
MATING INTERACTIONS

system is that mutations in MAT are healed by a mating type interconversion (switching). Thus, MAT mutants that are also HO give rise to normal cells when allowed to divide (Hicks and Herskowitz, 1977; Klar et al., 1979b; Strathern et al.,1979). The actual mechanism of switching is not a function of the HO gene itself since ho cells also exhibit healing upon switching, which they do with low frequency.

These and similar results were eventually interpreted to suggest that the yeast genome contains unexpressed copies of MATa and MATα . These are currently designated HMLa and HMRα. (These alleles flank the MAT locus left and right respectively.) Switching occurs by the insertion of either into MAT. This insertion is non-destructive to HML and HMR, but destructive to the allele residing at MAT. Thus a MAT mutant is healed by unidirectional insertion of wild type information copied from the spare flanking sequences (Strathern and Herskowitz, 1979; Herskowitz et al, 1980).

II. The Pheromones of Saccharomyces cerevisiae

A major distinction between haploids and diploids is that the former exhibit sex specific functions. Many of these functions have been shown to appear progressively as haploids grow after germination (Tohoyama and Yanagishisma, 1981). Others are expressed immediately. Conspicuous among these is the ability to mate. The

mating reaction in Saccharomyces is complex and mediated by diffusable peptide pheromones or "factors". Each mating type produces at least one such molecule, which is unique to that cell and exerts a number of effects on the opposite mating type. Thus, a-cells produce a-factor, which affects α-cells. Similarly, α-cells produce α-factor which affects a-cells. The production of specific mating factors, as well as other haploid specific functions, has been shown in homothallic as well as heterothallic strains, and is wholly controlled as a function of the information at MAT (Nakagawa and Yanagishima, 1982).

A. Effects of α-Factor

α-Factor is the more throughly studied of the two pheromones. It is a tridecapeptide of known sequence (Stotzler, et al, 1976). The best understood of the effects that this pheromone elicits in a-cells is a transient arrest in the progression through the cell cycle (Duntze et al, 1973; Bucking-Throm et al., 1973). The point at which this arrest occurs has been shown to be in very late G1 and has been designated "start" since it represents the point when cells are irreversibly destined to enter S-phase (Hartwell, 1974).

a-Cells eventually recover from α-factor induced G1 arrest. The length of the recovery period is dependent on the dose of α-factor experienced and leaves the cells

temporarily resistant to α-factor action (Chan, 1977). During recovery, α-factor is inactivated in a cell dependent reaction (Chan, 1977; Finklestein and Strauberg, 1979). This has been shown to involve specific proteolytic cleavage of α-factor, probably by cell surface bound endopeptidase (Ciejek and Thorner, 1979).

A second effect of α-factor on a-cells is the induction of a pronounced morphological change. Under prolonged exposure to α-factor (3-4 hours) and in the presence of complete medium, a-cells enlarge, and their normally round outlines become pear shaped (Levi, 1956; Duntze et al., 1970). Such cells have come to be known as shmoos. The morphological changes during shmooing are accompanied by biochemical changes at the cell surface. Localized deposition of chitin (Schekman and Brawley, 1979), mannan (Tkacz and MacKay, 1979), and acid phosphatase (Field and Schekman, 1980) occur at the shmoo tip. The overall composition of the cell wall changes. The proportion of glucan to mannan increases, the length of mannan side chains decreases (Lipke et al., 1976b), and a new immunological species appears at the cell surface (Lipke and Ballou, 1980).

A third major response of a-cells to α-factor involves cell-cell interactions. When an a-cell comes into contact with an α-cell, the two can specifically adhere to one another. This interaction is termed sexual agglutination. Early investigators (Sakai and Yanagishima, 1971; 1972; Betz et al., 1978; Doi et al.,

1979; Doi and Yoshimura, 1978) described wild type haploids as inducible agglutinators, meaning that pheromone pretreatment is necessary for agglutination. It has subsequently been shown (Campbell, 1973; Fehrenbacher et al., 1978; Terrance and Lipke, 1981) that properly grown and assayed wild-type haploids will always constitutively express significant levels of a to α binding. a-Cells do, however, show a dramatic increase in their agglutinablility after α-factor treatment.

Although much has been done in terms of characterization of the physiological responses of a-cells to α-factor, virtually nothing is known about the α-factor receptor. Attempts to measure binding of radiolabelled α-factor to a-cells have been hampered by low levels of specific interactions of α-factor with a-cells (Maness and Edelman, 1978). It has been suggested (Thorner, 1980; 1981) that this may be due to the general composition and negative charge of the yeast cell wall, which would tend to bind the positively charged α-factor molecule, to preferential degradation of the pheromone by α-cells (by, for instance, surface endopeptidases), or to the interaction being transitory in nature.

Little is known of the mechanism of α-factor action. Tanaka and Kita (1978) reacted fluorescein isothiocyanate (FITC) with the available amino groups of α-factor. They reported specific binding to a-cells, with the label entering the cells and migrating to the nucleus. However,

this seems to contradict the above mentioned experiments with radiolabelled α-factor, which failed to detect specific binding. Further, other investigators have found that reaction of the free amino groups of α-factor with either acetic anhydride (Lipke, 1976a), fluroescamine, or dansyl chloride (Maness and Edelman, 1978) completely destroys the biological activity of α-factor. Thorner (1981) has pointed out that Tanaka and Kita did not separate FITC-α-factor from unreacted pheromone and that they failed to bind FITC-α-factor to a-cells in the presence of large amounts of unlabelled pheromone. These certainly are reasons sufficient to question the results obtained.

Liao and Thorner (1980) showed that α-factor inhibits yeast adenyl cyclase in vitro. The enzyme inhibited was that present in the cell membrane, since solubilization by the detergent Triton X-100 prevented the inhibition by pheromone. Partial sequences of α-factor, which are inactive biologically, failed to inhibit adenyl cyclase (Ciejek and Thorner, 1979).

By including cAMP in the growth medium, they were able to shorten α-factor induced G1 arrest. In later studies (Liao and Thorner, 1981), inhibitors of cAMP phosphodiesterase were used to prevent G1 arrest. Thus, for this function, α-factor seems to act through the lowering of cAMP levels.

B. Effects of a-Factor

Research on a-factor has lagged compared to that on α-factor. Indeed, there was initially considerable difficulty in demonstrating a diffusable hormone from a-cells (Bucking-Throm, et al., 1973). Eventually, a molecule mediating G1 arrest in α-cells was shown to exist (Wilkinson and Pringle, 1974).

a-Factor has since been shown to have the full range of effects on α-cells including the ability to elicit shmoo formation and to increase α-cell aggutinability (Betz, Mackay and Duntze, 1977; Betz, Duntze and Manney, 1978; Terrance and Lipke, 1981). The molecule has been purified and found to be an undecapeptide (Betz and Duntze, 1979).

It is tempting to postulate that a-factor forms one symmetrical half of a simple reciprocal system, but as has been pointed out (Manney and Meade, 1977), a more cautious approach is in order. a-Factor has been notoriously elusive in attempts at its characterization and there have been reports of associated molecules (Betz, MacKay and Duntze, 1979). It has also been claimed that a-factor secretion is induced by α-factor (Strazdis and MacKay, 1982), while α-factor production is constitutive (Tanaka and Kita, 1977).

In fact, an additional molecule mediating mating interactions has already been discovered in a-cells. Known as barrier factor (Hicks and Herskowitz, 1976;

Yanagishima et al., 1977), this molecule has been shown to irreversibly inactivate α-factor. Mutant **a**-cells deficient in barrier factor are supersensitive to α-factor-induced G1 arrest (Chan and Otte, 1982 a,b). This diffusable barrier factor molecule, however, is not the same as the cell-associated endopeptidase involved in recovery from G1 arrest.

C. Significance of Pheromones in Mating

Only unbudded cells (i.e. those in G1) are competent to fuse and mate (Hartwell, 1973; Sena et al., 1973; Reid and Hartwell, 1977). A substance that causes an accumulation of unbudded cells and increased sexual agglutination in mating mixtures has obvious utility. Further, cell cycle arrest is achieved by inhibition of nuclear DNA synthesis only; protein, RNA, and mitochondrial DNA production are not diminished (Throm and Duntze, 1970; Sumrada and Cooper, 1979; Schekman and Brawley, 1979). This allows synthesis for the mating process to proceed unabated. Continued macromolecular synthesis without cell division has been postulated as the cause of shmooing (Betz et al., 1981; Thorner, 1980,1981; Strazdis and MacKay, 1982). In this view, shmooing is an artifact resulting from the disruption of the normal budding process and has no physiological significance in mating.

An attractive hypothesis is that pheromonal influences are a necessary prerequisite to mating. Hartwell (1973) conceived a mating "orgy" which seemed to demonstrate that a lag or "courtship" period was required in mating mixtures prior to zygote formation during rotation culture. Further, many mating defective mutants appear to be deficient in pheromone production (MacKay and Manney, 1974). Mating factor resistant mutants are invariably sterile (Manney and Woods, 1976) and mating factor production has been demostrated in all fertile haploid strains (Manney et al., 1981).

However, Manney and Meade (1977) modified Hartwell's mating orgy in such a way that permanent cell-cell contacts formed during courtship were discounted. The results were that uncourted cells exhibited no more of a lag than did courted cells. Therefore, a courtship period may be a necessary prelude to mating but pheromonally elicited effects may not be critical.

These same investigators attempted the definitive experiment by trying to isolate a sterile a-cell deficient in α-factor production and restoring mating ability by additon of exogenous α-factor. They found that the mutants which they selected as unable to produce α-factor usually mated normally. More rigorous assays revealed that all actually produced α-factor at reduced levels and were pleiotropic. It was consequently not possible to unambiguously correlate defects in mating with α-factor production.

III. Sexual Agglutination

A. Physiological Significance of Agglutination

The agglutination reaction is specific. It is distinguishable from "flocculation", which is a non-sexual aggregation of diploid cells. Agglutination occurs only between cells of opposite mating type. In rotation culture, agglutinated aggregates consist of about equal numbers of each cell type, regardless of the initial a/α ratio (Kawanabe et al., 1979). a-Cells and α-cells adhere to each other, but neither type to itself. Although each individual aggregate may consist of hundreds of cells, fusions are largely restricted to single a/α pairs.

As in other pheromonally mediated functions, it is likely that sexual agglutination plays an important, if not essential role. It has been noted that there is a strong correlation between agglutinablility and zygote forming ability (Sakai and Yanagishima, 1975; Kawanabe et al., 1979; Yanagishima and Yoshida, 1981).

However, wild type cells agglutinate constitutively. It is therefore unlikely that, in nature, the high level of pheromonally induced agglutination is essential. Indeed, there is a poor coincidence between the kinetics of α-factor production and response during the growth cycle. Production peaks in late exponential or early stationary phase (Duntze et al., 1973; Tanaka and Kita, 1977), whereas response to pheromone is best in mid-exponential cells.

The close proximity provided by agglutination has the obvious side effect of increased localized pheromone concentrations (Yanagishima and Yoshida, 1981). It is possible that this indirectly provides levels of pheromone that are high enough to result in G1 arrest in mixed aggregates as well as to induce increased agglutination. In this case, the latter phenomenon may be a device solely for the strengthening of intercellular attachments once they have formed.

B. Studies in Hansenula wingei

In the yeast Hansenula wingei, the mating types are designated type 5 and type 21. These correspond to a-cells and α-cells respectively. This species exhibits mating reactions similar to Saccharomyces cerevisiae (Brock, 1961). The agglutination reaction in this species has been extensively studied, and has been shown to be due to the activity of cell surface glycoproteins. These agglutination molecules (or agglutinins) are confusingly referred to as 5-factor (from type 5-cells) and 21-factor. Each agglutinin specifically adheres to its complement when incubated with the opposite cell type. If an agglutinin is functionally monovalent, it renders the cells non-agglutinable. If the molecules are multivalent, the cells become self-agglutinable. This forms the basis for assays of agglutinin activity.

The 5-factor was purified by solubilization with subtilisin, followed by gel filtration and affinity chromatography on type-21 cells (Crandall and Brock, 1968). The molecule causes self agglutination of type-21 cells from which it is judged to be multivalent. Each molecule has a molecular weigh of 9.6×10^5 daltons and contains between five and eight functional (i.e. binding) glycoprotein subunits (MW 12,000 daltons). These subunits can be released from a central glycoprotein core by dithiothreitol and behave as univalent agglutinin (Taylor, 1964; Taylor and Orton, 1968, 1970). 5-Factor is inactivated by digestion of either its protein or carbohydrate moieties (Yen and Ballou, 1974) but is heat stable (Burke et al., 1980).

The 21-factor has been solubilized with subtilisin, but has not been as well characterized (Crandall and Brock, 1968). Lability in response to moderate heating has been reported (Crandall and Brock, 1968). It does not agglutinate 5-cells, but rather inhibits their agglutinating ability. It is thus judged to be monovalent (Crandall and Brock, 1968).

C. Studies in Saccharomyces cerevisiae

In Saccharomyces cerevisiae, both constitutive and induced agglutinability are observed. As previously mentioned, a number of studies have differentiated between "constitutively" and "inducibly" agglutinable strains,

even in wild type cells (Sakai and Yanagishima, 1971, 1972; Betz et al., Doi et al., 1979) Mutant genes conferring constitutive agglutinibility (Doi and Yoshimura, 1978) and inducibility (Yanagishima and Nakagawa, 1980; Nakagawa and Yanagishima, 1981) have been reported.

Kawanabe et al. (1979) described a "random assembly" of a-α cell pairs which occurs before the formation of true cell aggregates. The latter phenomenon is distinguishable in their system by its preferential inhibition by Concanavalin A at 400-500 ugm/ml. Fehrenbacher et al., (1978) reported differences in the pH maxima for constitutive and induced agglutination, suggesting that two sets of molecules may be involved.

By analogy to H. wingei, one would expect the agglutinins of Saccharomyces cerevisiae to be cell surface glycoproteins that are complementarily adhesive and whose properties mimic those of the former species. Predictably, a-cells are rendered non-agglutinable by dithiothreitol (Burke et al., 1980). Although such treatment would be expected to release α-cell-inactivating fragments of a-agglutinin, this has not been observed (N. Wagner, unpublished). Also, a-agglutinin isolated by a number of different methods and investigators (Shimoda and Yanagishima, 1975; Yoshida et al., 1976; Wagner, unpublished) does not agglutinate α-cells and, therefore, is probably monovalent.

α-Cells are not rendered non-agglutinable by treatment with reducing agents (Burke et al., 1980). Fehrenbacher et al. (1978) made an initial report that α-agglutinin agglutinated a-cells. This indicated multivalence. Later investigators (see below) have failed to confirm this.

Yanagishima and collegues have reported most of the work to date on the purification of agglutinins from Saccharomyces. Their initial efforts to solubilize the molecules were by treatment of mechanically isolated cell walls with glusulase, a crude enzyme preparation from snail gut (Shimoda and Yanagishima, 1975; Shimoda et al., 1975). The resulting agglutinins were both functionally monovalent and inactivated by proteolitic enzymes. However, they were of very high molecular weight (~10 daltons). This prompted the conclusion that the larger part of the molecules was made up of non-functional cell wall components.

More recently, these same investigators have used brief autoclaving (120° C, 5 min.) of cells to release agglutinins from the cell wall (Yoshida et al., 1976) Hagiya et al., 1977). When this treatment was immediately followed by chilling, haploid cells released activities which are sex-specific inhibitors of agglutinability. After solubilization the following sequence was followed: (1) acid precipitation, (2) DEAE-cellulose chromatography, (3) ultrafiltration, (4) gel filtration, (5) Con-A-Sepharose affinity chromatography,

and (6) isoelectrofocusing. The substances obtained migrated as single bands in polyacrylamide gel electrophoresis (Yanagishima and Yoshida, 1981).

The a-agglutinin isolated by this method is monovalent. Its molecular weight, estimated by gel filtration, is 23,000. It is a glycoprotein, containing approximately 61% carbohydrate.

The α-agglutinin obtained is a functionally monovalent glycoprotein of about 130,000 daltons. It contains about 47% carbohydrate.

These two molecules (in a partially purified state) have been shown to form a complex in solution , as evidenced by a disappearence of biological activities. These activities could be recovered by raising the pH beyond the range for the agglutination reaction, followed by chromatography on DEAE-cellulose (Yanagishima and Yoshida, 1981).

Although these investigations have produced purified molecular species with the observed properties of agglutinins, the many possible effects on these entities of autoclaving at 120° C cannot be controlled. The use of boiled tester cells, which were used by these investigators in their agglutination assays, further complicates interpretation.

IV. Summary of Research Objectives

The questions that were addressed experimentally were the following:

1) What pheromone-triggered biochemical syntheses are a necessary prelude to the expression of the pheromonally induced increased agglutinability?

2) Are the agglutinin molecules induced by pheromone identical to those constitutively present?

3) Is it possible to solubilize agglutinin molecules by a method less harsh than extensive enzymatic digestion or autoclaving? Are the properties of a molecule so obtained different from those described by other investigators?

Materials

The following reagents were obtained from Sigma Chemical Company, St. Louis, Mo.: cycloheximide, p-chloromercuribenzoic acid (PCMB), actinomicin-D, Trizma Base (TRIS), chloramphenicol (CP), theophylline, aminophylline, dithiothreitol (DTT), adenosine 3'5'-cyclic monophosphate (cAMP), glass beads (450 -500 μm), bovine serum albumin (BSA), α-methyl-mannoside, amberlite XAD-2, amberlite GC-50, hexyl agarose, DEAE Sephadex A-25 and Schiff's reagent.

Ultrogel AcA-34 was obtained from LKB Instruments, Hicksville, N.Y. The agarose bound hydrocarbon series was from Miles-Yeda, Kiryat Weizman, Rehovat, Israel. Agarose bound agglutinin from Lens culinaris was from Accurate Chemical, Westbury, N.Y. . Acrylamide, bis-acrylamide, tetramethylethylenediamine (TEMED) and Bio-Gel A.5m were purchased from Bio-Rad Laboratories, Richmond, Ca.. Sodium dodecyl sulfate (SDS) and Coomassie brilliant blue were obtained from Pierce Chemical Company, Rockford, Ill.. Synthetic g-factor was the generous gift of Dr. Fred Naider of the College of Staten Island. All other materials were of reagent grade and from commercial suppliers.

Protein in solution was determinded by the binding of Coomassie brilliant blue (Bio-Rad Laboratories) and

carbohydrate was determined by the phenol-sulfuric method (Dubois et al., 1956). The assay for β-fructofuransidase (EC 3.2.1.26, invertase) was as described by Smith and Ballou (1974).

Methods

I. Strains and Growth Conditions

Saccharomyces cerevisiae wild-type haploid strains X2180-1A (a) and X2180-1B (α), obtained from the Yeast Genetics Stock Center (Berkeley. Calif.), were used exclusively. Cells were routinely cultured in a minimal medium (YNB) consisting of 2.2 g yeast nitrogen base (Difco Labs., Detroit Michigan) , 4.5 g ammonium sulfate, and 20 g glucose per liter. For certain puposes, cells were grown in a richer medium (YNBP), identical in composition to YNB but supplemented with 1.0 g/liter peptone (Difco Labs.). Cells were incubated on a rotary shaker at 30° C. Cell number was determined by optical density at 660 nm in either a Bausch and Lomb Spectronic 20 or 21. These readings were calibrated by hemocytometer counts.

II. Partial Purification and Assay of the Sex Pheromones

A. α-Factor

α-Factor was purified by two different methods: that of Duntze et al. (1973) as modified by Lipke (1976,a); and that of Strazdis and MacKay (1982). In both these methods, α-factor was adsorbed from the culture medium. In the former method, α-factor in conditioned medium from cultures of X2180-1B was adsorbed onto a column of

amberlite GC-50. The procedure was as reported except that it was carried out at 4°C and α-factor was stored and used in the pyridine acetate buffer with which it was eluted.

Strazdis and MacKay (1982) found that improved yields of α-factor could be obtained by incubating adsorbing resin (in this case, XAD-2 beads) within the growing culture of α-cells. Whether this results from increased α-factor production due to its continual removal or to protection from degradation, has not been determined. I modified their procedure slightly by replacing the 40°C water jacketed column with elution by ice-cold methanol. These methanol fractions were then concentrated by rotary evaporation, and assayed for activity. In my experiments, this procedure gave yields of α-factor that were 5-10 fold higher than that obtained by the method of Duntze et al.

Activity of α-factor was assayed by its ability to diffuse from wells in agar plates and induce morphogenesis in a-cells (Duntze et al., 1973). In later purifications, a similar assay was developed in which serial dilutions of α-factor were made in the wells of a titer plate. This assay has been described for a-factor (Terrance and Lipke, 1981). In either of these assays, the minimal concentration of α-factor needed to induce morphogenesis was designated as 1 unit/ml.

B. a-Factor

a-Factor was purified by the method of Strazdis and MacKay (1982). This method is essentially identical to that for the preparation of α-factor and the same modifications were used. Either methanol or propanol was used as elutant and eluted material was concentrated by rotary evaportion at 50° C under reduced pressure. When evaporated to dryness, a small amount of dimethylsulfoxide was added to redissolve the residue. a-Factor was assayed by its ability to induce morphogenesis in α-cells. The assay used was identical to the titer-plate method for α-factor described above except that 0.2 mg/ml BSA was added to all dilutions in order to stabilize a-factor activity (Terrance and Lipke, 1981). As with α-factor, 1 unit/ml a-factor was defined as the minimal concentration needed to induce morphogenesis.

III. Agglutination Assay

Haploid a-cells and α-cells were grown separately in minimal medium (YNB) to a density of between 2.2×10^6 and 1.4×10^7 cells/ml. The cells were harvested by centrifugation and washed once in saline-phosphate buffer (0.14 M sodium chloride, 0.01 M sodium phosphate, pH 6.0).

Cells to be induced with pheromone were then resuspended in fresh YNB (for α-factor induction) or in YNB containing 0.2 mg/ml bovine serum albumen (for a-

factor induction). The appropriate pheromone was then added and the cells incubated on a rotary shaker at 30° C.

Maximal induction of agglutinability occurred following treatment with α-factor at a concentration of 1 unit/ml for 25 minutes. For a-factor, maximal agglutination followed treatment at 0.1 unit/ml for 70 minutes. These were chosen as standard conditions. Cells that were not induced were maintained on ice during the induction period. Afterwards, all cells were harvested by centrifigation and resuspended in an equal volume of 0.1 M sodium acetate buffer (pH 5). This buffer contained 10 µg/ml cycloheximide, which inhibited any further induction of agglutinability.

Samples (1 ml) of each cell type were added to 13 x 100 mm test tubes. Control tubes received 2 ml. of a single cell type. The volume of each tube was brought to 3 ml with 0.1 M sodium acetate buffer (pH 5) with 10 µg/ml cycloheximide. All tubes were vortexed and then the cells were compacted by gentle centrifugation (200 x g for 5 minutes) in order to force cells into aggregates. This is essentially identical to the assay of Hartwell (1980), the major modification was that compacted cells were subjected to controlled resuspension by stirring with a 7 x 90 mm stainless steel paddle at 1,000 rpm for 4 seconds. A stop maintained the paddle at a constant distance from the bottom of the tube and a constant speed control unit (Cole-Parmer Instrument Co., model 4420) assured

reproducible results. The resuspended mixtures were allowed to settle for 20 minutes. The test tubes were then used as cuvettes in a Bausch and Lomb Spectonic model 20 or 21. Optical density at 660 nm was determined. Individual samples were run in duplicate or triplicate tubes. High levels of agglutination resulted in more and larger aggregates; thus, more settling and a lower average optical density.

The degree of agglutinability was quantitatively expressed as an agglutination index (A.I.). This quantity was defined as:

$$A.I. = \frac{1 - (2 \times 1)^{a+\alpha}}{D^{a} + D^{\alpha}}$$

where D is the mean optical density of the tubes containing the cell types indicated by the superscripts. The A.I. varies from 0 to 1, with higher indicies indicating greater degrees of agglutination.

Thus, the A.I. is the ratio of the amount of settling in tubes containing both a-cells and α-cells and tubes containing one cell type. This ratio therefore reflects the degree of a to c adhesion and ignores any non-sexual (i.e. a to a or α to α) pairings.

Microscopic observations of the supernatants from agglutinated mixtures revealed that suspended particles consisted mostly of single cells. Plating these cells gave rise to clones that mostly (~ 95%) agglutinated normally. These were mostly cells that, by chance did not

enter aggregates, rather than non-agglutinable cells. Therefore, agglutination index reflected the degree of agglutinability of the entire cell population and not the proportion of non-agglutinable cells.

IV. Solubilization and Purification of α-agglutinin

A. Assay of α-Agglutinin Activity

A biologically active agglutinin molecule from α-cells should be able to specifically adhere to agglutinins on a-cells. Such treatment should render the a-cells less agglutinable when subsequently mixed with α-cells. Activity of α-agglutinin was thus assayed by inhibition of a-cell agglutinability. Aliquots of each elution fraction were combined with 2×10^7 pheromonally induced a-cells in a total volume of 2 ml of 0.1 M sodium acetate buffer (pH 5.0) with 10 µg/ml cycloheximide. Incubation was carried out on a rotary shaker for 90 minutes at 30° C. The incubated a-cells were then resuspended in the same volume of fresh buffer of identical composition. α-Cells were then added, and the agglutination assay carried out as described above. One unit of agglutinin activity was defined as the amount needed to lower the A.I. of induced a-cells by 0.1 as compared to control.

B. Reversal of Binding

A species that destroys or otherwise inactivates agglutinin on a-cell surfaces will mimic α-agglutinin in the assay described above. Such inactivations will probably be permanent, especially when the cells are inhibited by cycloheximide, since this drug prevents the expression of new molecules. True α-agglutinin activity, on the other hand, should be reversible under conditons that reverse agglutination. During purification, active fractions were required to meet this criterion of reversibility.

Either high urea concentration, or high pH was used for reversal. Agglutinated mixtures were washed three times in sodium acetate buffer containing 8 M urea and 10 µg/ml cycloheximide. This was followed by three washes in buffer without urea. The mixtures were then reagglutinated and measured as before. When high pH was used as the reversal agent, 0.05 M Tris (pH 9.0 containing cycloheximide) was used in place of the urea containing buffer. As an alternative to washing, a 30 minute incubation in 0.05 M Tris (pH 9.0 with cycloheximide) was followed by a 30 minute incubation in sodium acetate (pH 5.0). The percent reversibility was defined as:

$$\% \text{ reversibility} = \frac{D - D^{r}}{D - D^{cr}} \times 100$$

where D is the average optical density of agglutinated mixtures of α-agglutinin treated a-cells, D^r is the average optical density of these tubes after the reversal treatment, and D^{cr} is the average optical density of the control tubes after reversal.

C. Partial Purification of α-Agglutinin

Two schemes were used for the solubilization and isolation of α-agglutinin. This first scheme was utilized to obtain the agglutinin used in part III of the results section.

Midlog cultures of X2180-1B (α-cells) grown in YNB were harvested and washed once in 0.1 M sodium acetate buffer (pH 5.0). The cells were resuspended at an optical density (660 nm) of 30 to 100 and the suspension brought to 5 mM EDTA and dithiothreitol (DTT). To this mixture was added 3 g/ml glass beads (450 - 500 μm). Homogenization was carried out at 4° C in a Sorval Omnimixer set at the highest speed. Blending for 4 to 6 minutes was usually sufficient to disrupt 80 - 90% of the cells, as judged by observation by phase contrast microscopy. Glass beads and cellular debris were cleared from this extract by centrifugation at 120 x g for 5 minutes and then at 28,000 x g for 30 minutes in a Sorvall SS-34 rotor. The supernatant was applied to a 4 x 80 cm column of Ultragel AcA-34 at 4°C. In later preparations, a similar column of BioGel A.5m was used. The column was

eluted with 0.1 M sodium acetate buffer (pH 5.0). The results of one such run are shown (Figure 2). Three active fractions were obtained. Region I (peak I) was specific in its ability to inhibit a-cells and was 100% reversible by either urea or high pH. It was maximally active at pH 5 to 7. Thus this material met all of the criteria for the α-agglutinin.

Region II (peak II) exhibited specificity for a-cells, but was of intermediate (20% - 60%) reversibility. This material was discarded.

The material in region III (peak III) specifically inactivated a-cells; it had no effect on α-cells. It was very different from α-agglutinin in that it was completely inactive at pH 7.0, and was always 100% irreversible in its effects. Treatment of a-cells with peak III did not release a-agglutinin activity into the medium.

The inactivation of a-cells by peak III material was progressive (Figure 3) even under standard assay conditions in which the cells were poisoned by cycloheximide. Peak III is therefore probably not inducing the metabolism of agglutinins by the cells themselves since induction and implementaion of a cell-mediated reduction of agglutinins ia likely to require protein synthesis at some point. An enzymatic inactivation of a-agglutinins through destruction seems the most likely mechanism of action.

Figure 2. Gel filtration of α-cell extract on AcA-34. A crude extract from α-cells was layered onto a 4x80 cm. column at 4 C. Elution was with 0.1M sodium acetate(pH5) and 10 ml fractions were collected: (●) activity,(▲) protein.

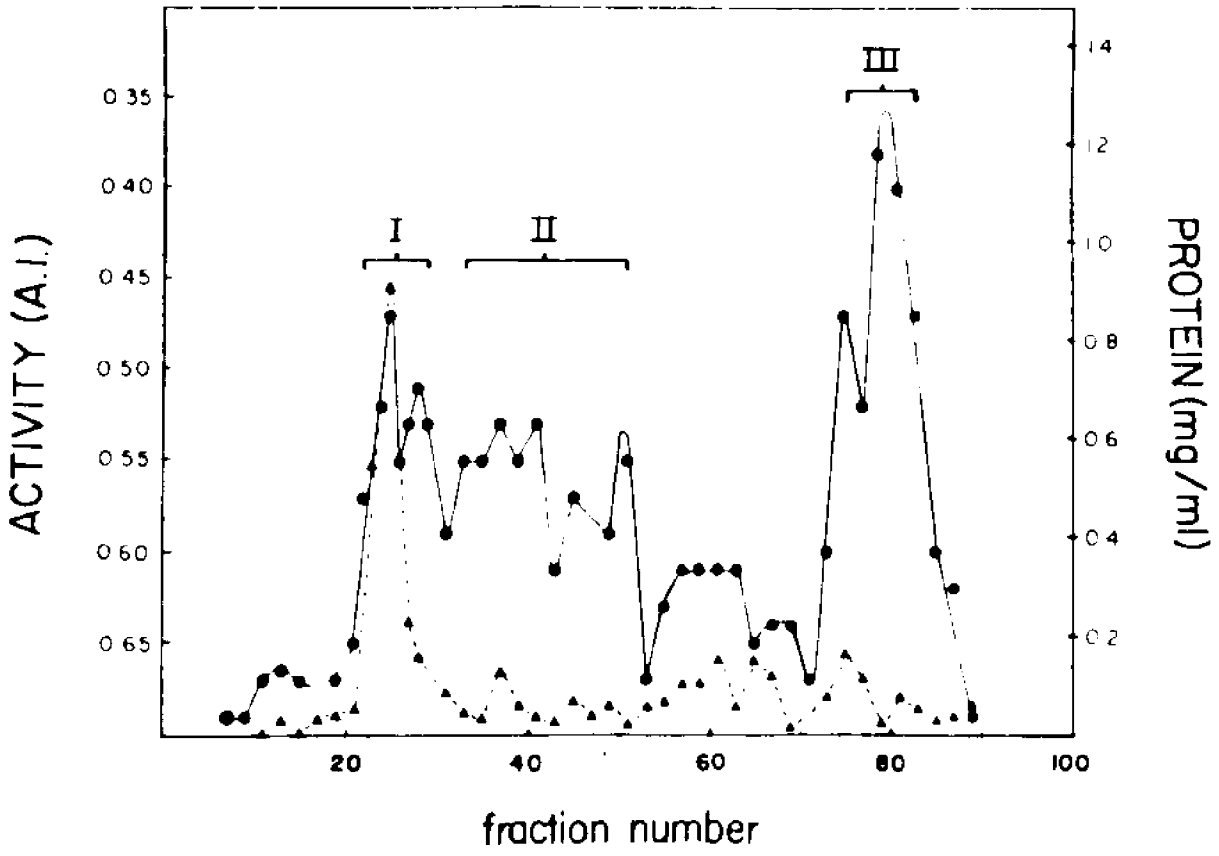
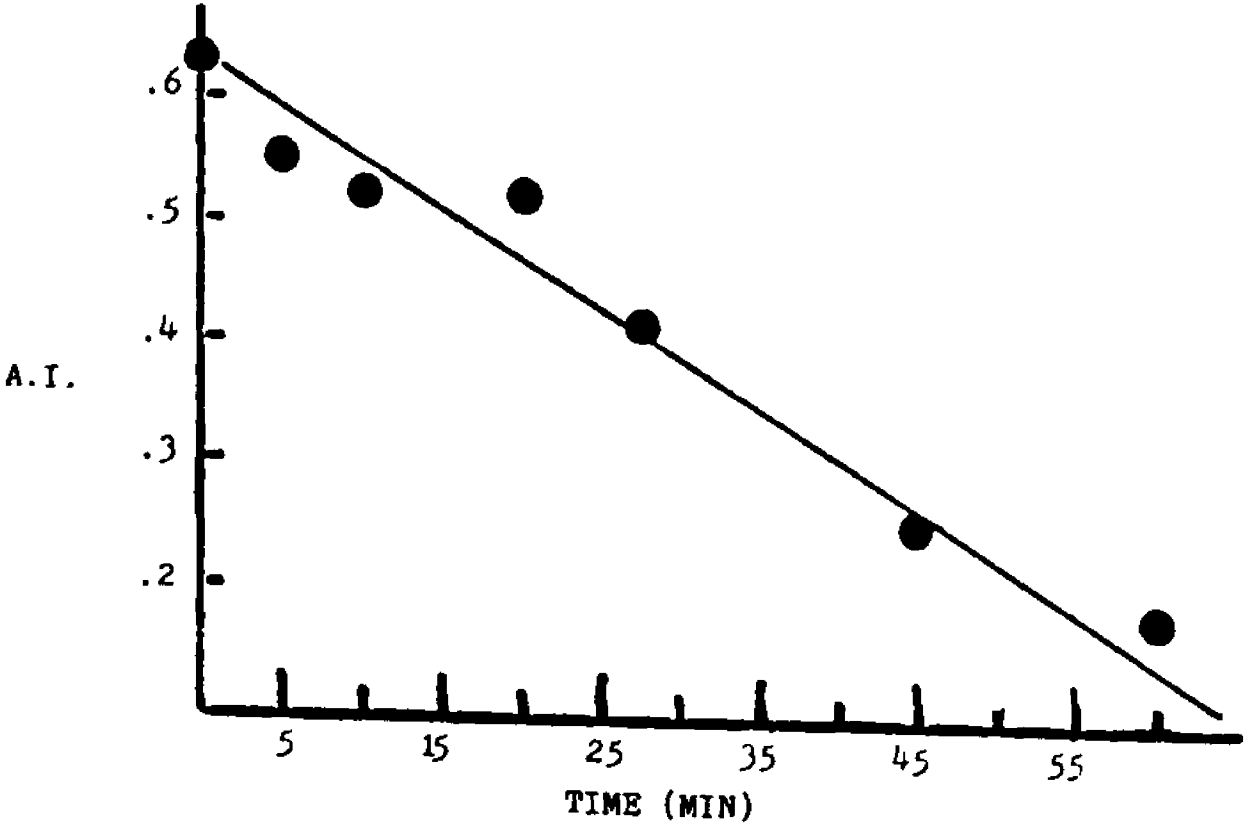

ACTIVITY (A.I.)
0.35
0.40
0.45
0.50
0.55
0.60
0.65
I
II
III
1.4
1.2
1.0
0.8
0.6
0.4
0.2
PROTEIN (mg/ml)
20
40
60
80
100
fraction number

Figure 3. Time course of inactivation of a-cells by peak III material. a-Cells were incubated with peak III material in 0.1M sodium acetate(pH5.0) for the times indicated. These cells were then washed once in fresh buffer and assayed for agglutinability with α-cells under standard conditions.


A.I.
.6
.5
.4
.3
.2
5
15
25
35
45
55
TIME (MIN)

D. Purification of α-Agglutinin

This scheme was used to obtain the agglutinin used in part IV of the results section. 100 liter cultures of X2180-1B were grown in YNBP medium in a Magnaferm Fermentor (New Brunswick Scientific, New Brunswick, N.J.). Growth was at 30° C and cells were harvested at 3.4×10^7 cells/ml. Packed cells were divided into blocks (approximately 70g each) and stored frozen. For extraction, one frozen block of cells was thawed and suspended in 150 ml of 0.1 M sodium acetate buffer containing 0.03% Triton X-100 and 1μM p-chloromercuribenzoic acid (PCMB). The inclusion of detergent and protease inhibitor was found to stabilize agglutinin activity. This mixture was transferred to the chamber of a Bead-Beater blender (Biospec Products, Bartlesville, Ok.) and the remaining space in the chamber filled with glass beads. The chamber was submerged in an ice bath and homogenized for 4 x 1.5 minutes with 1 minute cooling periods after each homogenization. This was usually sufficient to obtain 90% disrupted cells.

The resulting extract was cleared by twice centrifuging at 28,000 x g for 30 minutes at 4°C. The supernatant was maintained at 4°C and brought to 50% saturation with ammonium sulfate. This mixture was then cleared by centrifugation at 28,000 x g for 10 minutes. The pellet was discarded and the supernatant brought to 100% saturation with ammonium sulfate. This was

centrifuged as above, the supernatant discarded, and the pellet placed in a dialysis bag and dialyzed vs. 4 liters of 0.1 M sodium acetate (pH 5.0) containing 0.03% Triton X-100 and 1uM PCMB. Dialysis was carried out at 4°C over a period of 36-48 hours with at least 2 changes of buffer. The dialysate was centrifuged at 28,000 x g for 10 minutes and applied to a 1.8 x 6.5 cm column of DEAE-Sephadex A-25 equilibrated in 0.1 M sodium acetate (pH 5.0) with 0.03% Triton and 1 μM PCMB at 4°C. The column was washed wth 20 ml of this buffer and then eluted in a stepwise manner with 20 ml of buffer containing 50 mM, 100mM, and 500 mM NaCl.

Reversible activity was limited to the active 50 mM and 100 mM fraction. These were pooled and dialyzed vs. 4 liters of 0.1 sodium acetate with Triton and PCMB in the cold overnight. The resulting dialysate was brought to 1mM in CaCl and MnCl and applied to a 1.2 x 0.9 cm column of agarose-bound lectin from Lens culinaris (lentil lectin) at room temperature. The column was washed with 10 ml of Ca++ and Mn++ containing buffer and eluted stepwise with two aliquots of 10ml each of this same buffer containing 1M NaCl and 1M α-methyl mannoside. Active fractions from this step were pooled and dialyzed (4°C) vs. 0.01 M sodium acetate (pH 5.0) with 0.003% Triton X-100 and 0.1 uM PCMB. This dialysate was alternately used as is in hydrophobic chromatography or frozen (-80°C) and lyophilized for use in polyacrylamide

gel electrophoresis. The purification of α-agglutinin is summarized in figure 4.

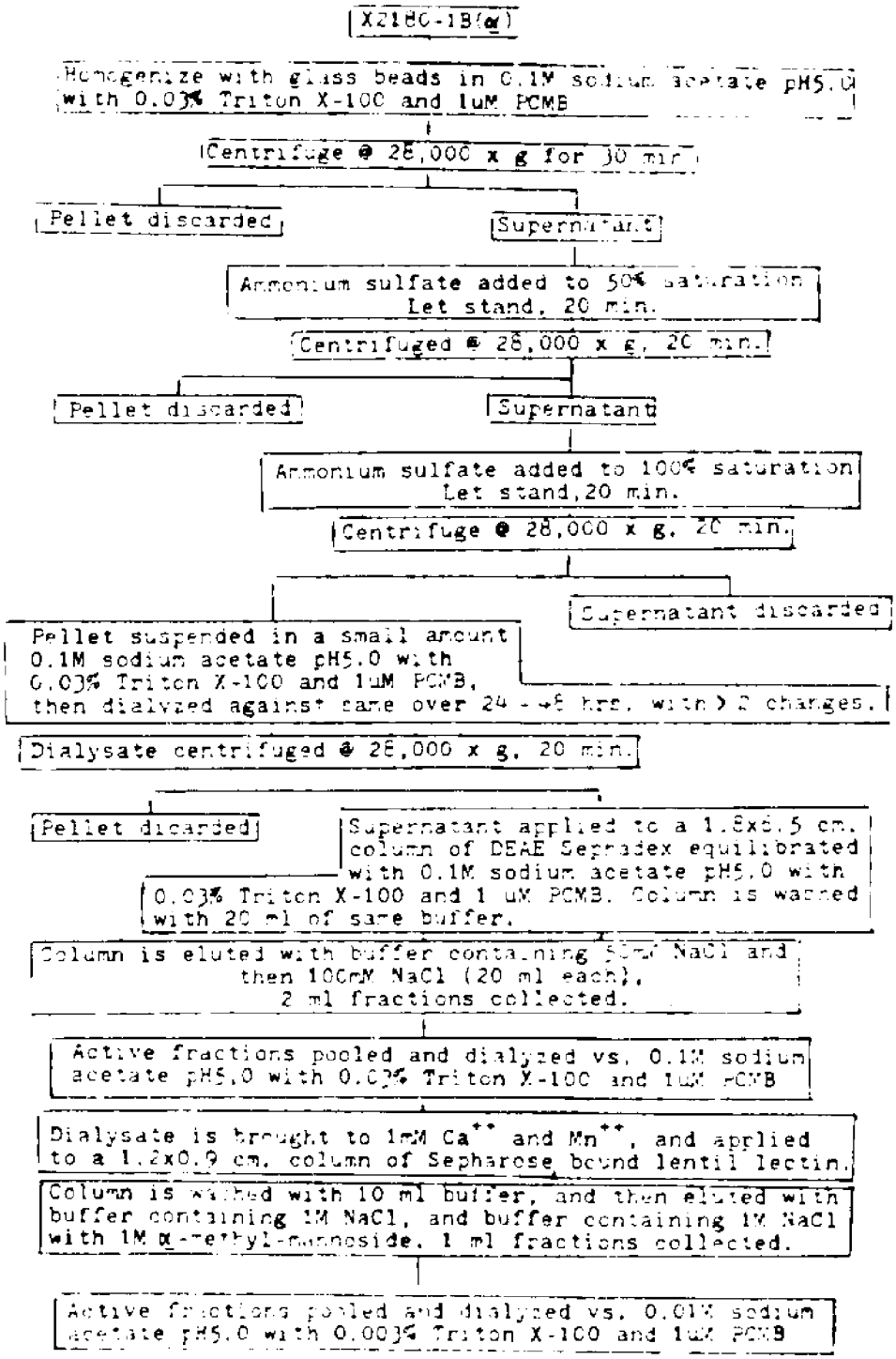
E. Hydrophobic Affinity Chromatography

A 1.2 x 0.9 cm column of agarose bound hexane was equilibrated in 0.01 M sodium acetate (pH 5.0) with 0.003% Triton X-100 and 1μM PCMB. An active pool from lectin affinity chromatography was dialyzed into this same buffer (as described above) and applied to the column at room temperature. The column was washed with 3 ml buffer and then eluted with, 3 ml each, buffer containing NaCl at: 100 mM, 200 mM, 500 mM and 1000 mM. Most activity eluted with 100 mM NaCl. This material was dialyzed vs distilled water (4°C), frozen and lyophilized in preparation for polyacrylamide gel electrophoresis.

F. Polyacrylamide Gel Electrophoresis

The procedure was a modification of that of Laemmli (1970). Slab gels (130 x 160 x 0.625 mm) were used. A solution of 7.5% acrylamide, 0.037% BIS, 0.1% SDS, and 0.003% TEMED in 0.6M Tris buffer (pH 8.8) was prepared for the running gel. Polymerization was initiated by the addition of 0.01% ammonium persulfate, freshly prepared. A layer of either 0.1% SDS or water saturated butanol was carefully applied to the gel after pouring to avoid evaporation during polymerization. A stacking gel

Figure 4. Flow chart for purification of α-agglutinin.


X2180-1B(α)
Homogenize with glass beads in 0.1M sodium acetate pH5.0 with 0.03% Triton X-100 and 1uM PCMB
Centrifuge @ 28,000 x g for 30 min
Pellet discarded
Supernatant
Ammonium sulfate added to 50% saturation
Let stand, 20 min.
Centrifuged @ 28,000 x g, 20 min.
Pellet discarded
Supernatant
Ammonium sulfate added to 100% saturation
Let stand, 20 min.
Centrifuge @ 28,000 x g, 20 min.
Supernatant discarded
Pellet suspended in a small amount 0.1M sodium acetate pH5.0 with 0.03% Triton X-100 and 1uM PCMB, then dialyzed against same over 24 - 48 hrs. with > 2 changes.
Dialysate centrifuged @ 28,000 x g, 20 min.
Pellet discarded
Supernatant applied to a 1.8x6.5 cm. column of DEAE Sepradex equilibrated with 0.1M sodium acetate pH5.0 with 0.03% Triton X-100 and 1 uM PCMB. Column is washed with 20 ml of same buffer.
Column is eluted with buffer containing 50mM NaCl and then 100mM NaCl (20 ml each), 2 ml fractions collected.
Active fractions pooled and dialyzed vs. 0.1M sodium acetate pH5.0 with 0.03% Triton X-100 and 1uM PCMB
Dialysate is brought to 1mM Ca++ and Mn++, and applied to a 1.2x0.9 cm. column of Sepharose bound lentil lectin.
Column is washed with 10 ml buffer, and then eluted with buffer containing 1M NaCl, and buffer containing 1M NaCl with 1M α-methyl-mannoside. 1 ml fractions collected.
Active fractions pooled and dialyzed vs. 0.01M sodium acetate pH5.0 with 0.003% Triton X-100 and 1uM PCMB

solution was prepared that was essentially identical to the running gel except that it was 4% acrylamide with the BIS proportionately reduced. After removal of the layer of the layer of SDS or butanol, the surface of the running gel was washed twice with the stacking gel solution. The remainder was then initiated with ammonium persulfate, poured and a 10 well (50µl/well) comb inserted. The running buffer was 0.025M Tris-glycine (pH 8.8) with 0.1% SDS. The samples were brought to 0.06 M Tris (pH 8.8), 1.0% SDS, 0.1% DTT, and 5% (by volume) gycerol. Gels were run at 10 mA overnight.

Gels were simultaneously fixed and stained with Coomassie blue by soaking in a solution of 50% methanol, 10% acetic acid and 0.1% Coomasie overnight at room temperature or for 1 hour at 60°C. Destaining was in 10% acetic acid, 10% isopropanol under the same conditions.

Proteins present in the later stages of purification failed to stain by this method. The new and highly sensitive silver staining procedure (Goldman, et al., 1981), however, did reveal protein bands.

Gels were fixed in a solution of 50% methanol, 17% acetic acid for 30 minutes, and washed (3 x 10 minutes) in 10% ethanol, 5% acetic acid. Gels were washed in acidic dichromate (0.0034M potassium dichromate, 0.0032M nitric acid) for 5 minutes, and washed extensively in distilled water until clear. The distilled water was replaced by a silver nitrate solution (0.012 M silver nitrate in distilled water) and the gel exposed to bright flourescent

light by placing it in a clear glass tray on a light box for 30 minutes. After washing once in 0.28 M sodium carbonate, fresh sodium carbonate with 1.65% formalin was added. The gel was agitated gently and bands soon appeared. When color development was sufficient, extensive washing in distilled water stopped the reaction. In my hands, this technique was at least 10 fold more sensitive than Coomassie staining, routinely detecting bands containing 0.1 µg or less protein.

Carbohydrate was detected in gels by the periodic acid Schiff method of staining (PAS; Fairbanks, et al., 1971). After fixation overnight in 40% ethanol and 5% acetic acid, gels were placed in periodate solution (0.7% sodium periodate in 5% acetic acid) for 2 - 3 hours. Gels were then washed in sodium metabisulfite (0.2% in 5% acetic acid) until clear, after which they were placed in Schiff's reagent. Pink-colored bands developed after a few minutes and intensified after soaking overnight. These gels were permanently stored in Schiffs reagent.

Results

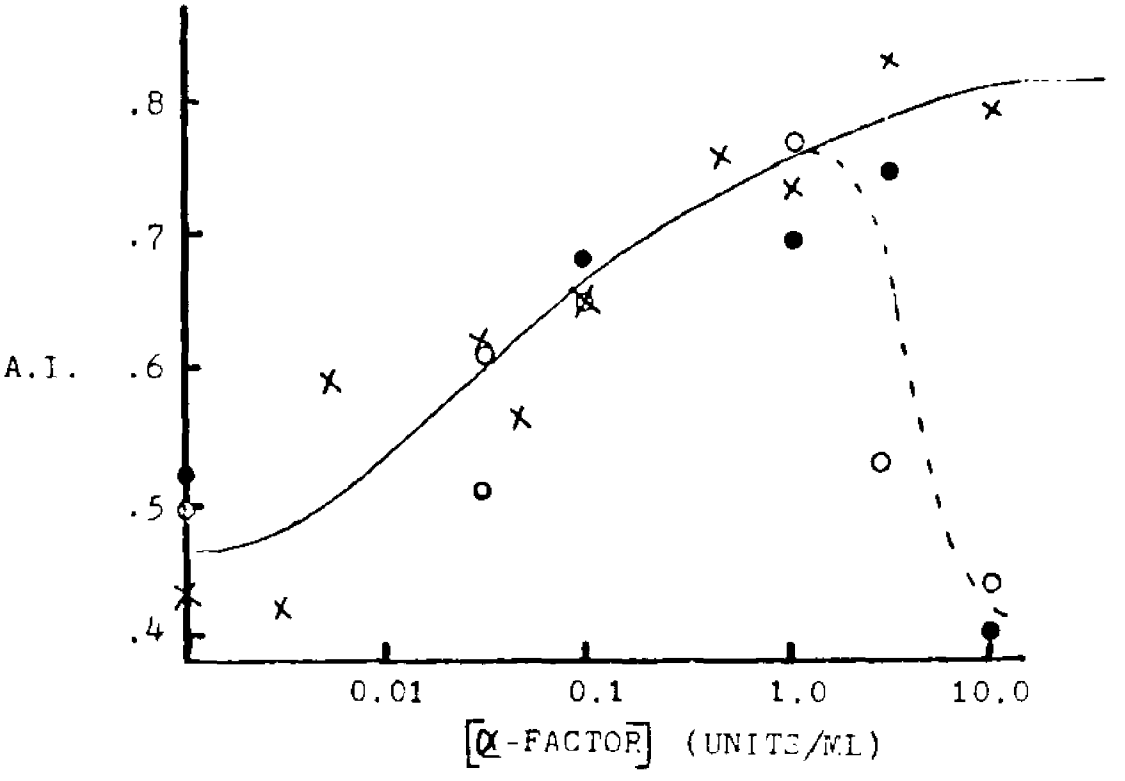
I. Pheromone Induction of Agglutinability

A. α-Factor induces increased a-cell agglutinability

The sex pheromones isolated were specific; α-factor affected a-cells and a-factor affected α-cells. Neither altered the agglutinability of the cell type from which it was derived or induced agglutinability in diploid cells.

The dose response for α-factor induction of a-cells is shown (figure 5). Pheromone, prepared by the method of Duntze et al. (1973) (closed circles), induced maximum agglutinability at a concentration of 1 unit/ml. Higher concentrations resulted in lowered agglutinability. a-Cells treated at 10 units/ml actually agglutinated less than control (uninduced) cells. I felt that it was important to investigate the cause of this inhibition, since much higher (20 - 50 units/ml) doses of partially pure α-factor have frequently been used in experiments to induce G1 arrest. To demonstrate that this was not due to the effects of pyridine added with the pheromone, a sample of α-factor was repeatedly evaporated under reduced pressure, with additions of distilled water. This treatment was sufficient to remove most of the pyridine in that sample. When used to induce a-cells, however, the results were the same (open circles). Further, pyridine added with lower concentration of α-factor failed to exhibit this inhibitory effect. When pheromone was

Figure 5. Dose-response for α-factor induction of a-cell agglutinability. a-Cells were incubated in minimal medium (YNB) with the indicated concentration of α-factor for 25 min., Then assayed for agglutinability under standard conditions. Shown is the data for: α-factor prepared by the method of Duntze et al.(1970)(●); the same α-factor with the pyridine removed by repeated evaporation (O); and α-factor prepared by the method of Strandis and MacKay(1982)(X).


A.I.
.8
.7
.6
.5
.4
0.01
0.1
1.0
10.0
[α-FACTOR] (UNITS/ML)

isolated by the method of Strazdis and MacKay (1982), however, dosage was not critical; high concentrations of pheromone resulted in maximal induction ((x) figure 5).

In this assay system, α-factor induction was rapid; maximal results were seen within 20 - 30 minutes after exposure to pheromone (figure 6). In this experiment, a-cells were incubated with α-factor in minimal medium. At the times indicated, the reaction was stopped by the addition of 10 µg/ml cycloheximide. The results indicated a 10 minute cycloheximide-sensitive lag period, during which α-factor induction was not expressed, followed by a rapid expression of increased agglutinability.

B. a-Factor induces increased α-cell agglutinability

The dose response for a-factor induction is shown in figure 7. α-Cells were incubated with a-factor in YNB supplemented with 0.2 mg/ml BSA in order to stabilize a-factor activity. This pheromone induced increased agglutinability when the treated α-cells were tested against either uninduced a-cells (●) or α-factor induced a-cells (■). a-Factor induction was maximal at a lower concentration of pheromone ($1 \times 10^{-3} - 5 \times 10^{-2}$ units/ml) compared to α-factor induction (1 unit/ml). Since the concentrations of pheromones are measured by their ability to shmoo target cells, this means that the agglutination inducing ability of a-factor was greater relative to its

Figure 6. Time course for α-factor induction of a-cell agglutinability. a-Cells were induced with 1unit/ml α-factor in minimal medium(YNB). At the times indicated, induction was stopped by the addition of 10μg/ml cycloheximide. Cells were then agglutinated under standard conditions.

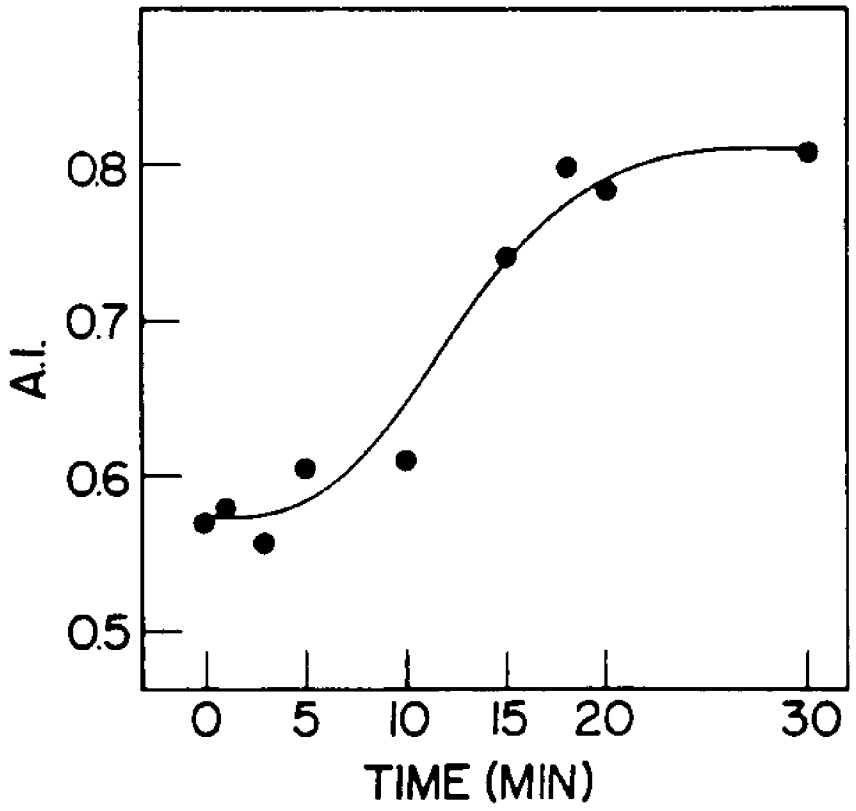
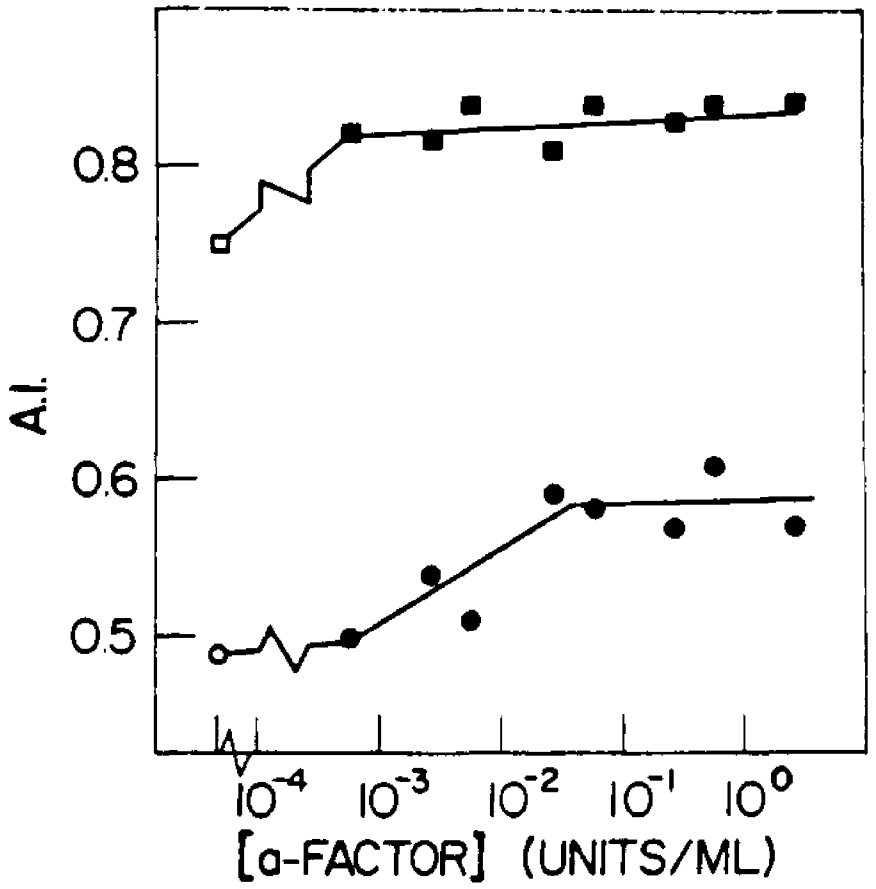

0.8
0.7
0.6
0.5
A.I.
0
5
10
15
20
30
TIME (MIN)

Figure 7. Dose-response for α-factor induction of **a**-cell agglutinability. **a**-Cells were incubated with the indicated concentration of α-factor in minimal medium(YNB) supplemented with 0.2mg/ml bovine serum albumin(BSA). The time of incubation was 75 min. Induction was terminated by the addition of 10μg/ml cycloheximide and placing the cells in an ice bath. Cells were then agglutinated under standard conditions, using either uninduced α-cells (●), or induced α-cells (■) as testers.


0.8
0.7
0.6
0.5
A.I.
10^{-4}
10^{-3}
10^{-2}
10^{-1}
10^{0}
[α-FACTOR] (UNITS/ML)

ability to induce morphogenesis.

Agglutinability of α-cells increased steadily, with no lag, from the time of exposure to a-factor, whether tested against uninduced or induced a-cells (figure 8). Maximal induction was at about 90 minutes in both cases. After this time, agglutinability began to fall towards control levels. Thus, for the observation of the maximal effect, time was critical. Since a-factor induction did not stop immediately, even upon the addition of cycloheximide (see figure 10) a standard induction time of 70 minutes was chosen.

C. Effects of antibiotics on pheromone induction of agglutinability.

Cycloheximide has previously been reported to inhibit pheromone induction . Under the induction and assay conditions used here, cycloheximide completely inhibited α-factor induction at 10μg/ml when included in the induction medium. The drug had no effect on the constitutive agglutinability of a-cells (figure 9).

Cycloheximide also inhibited a-factor induction. Although this effect was evident at lower concentrations of the drug (1μg/ml), a preincubation period before the addition of pheromone, was necessary in order to hold the agglutination index (A.I.) to control levels (figure 10). When cycloheximide was added at the time of pheromone addition, only partial blockage of induction was observed.

Figure 1. Time course for α-factor induction of α-cell agglutinability. α-Cells were incubated in minimal medium (YNB) supplemented with 0.1 mg/ml BSA. At the times indicated, induction was halted by the addition of 10 μg/ml cycloheximide and icing. Cells were then agglutinated under standard conditions using either uninduced α-cells (●), or induced α-cells (■) as testers.

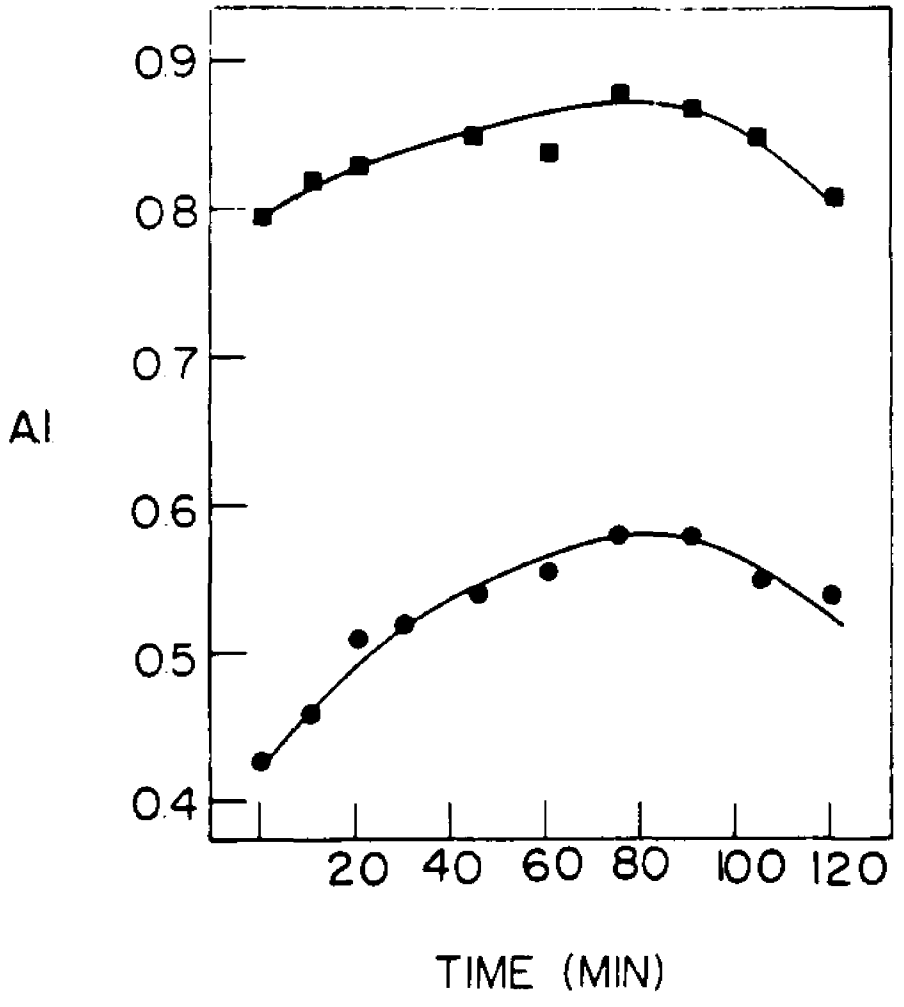

0.9
0.8
0.7
0.6
0.5
0.4
AI
20 40 60 80 100 120
TIME (MIN)

Figure 9. Inhibition of α-factor induction by cycloheximide. a-Cells were induced with α-factor under standard conditions in the presence of the indicated concentration of cycloheximide (■). a-Cells were also incubated in the presence of cycloheximide without α-factor (●).

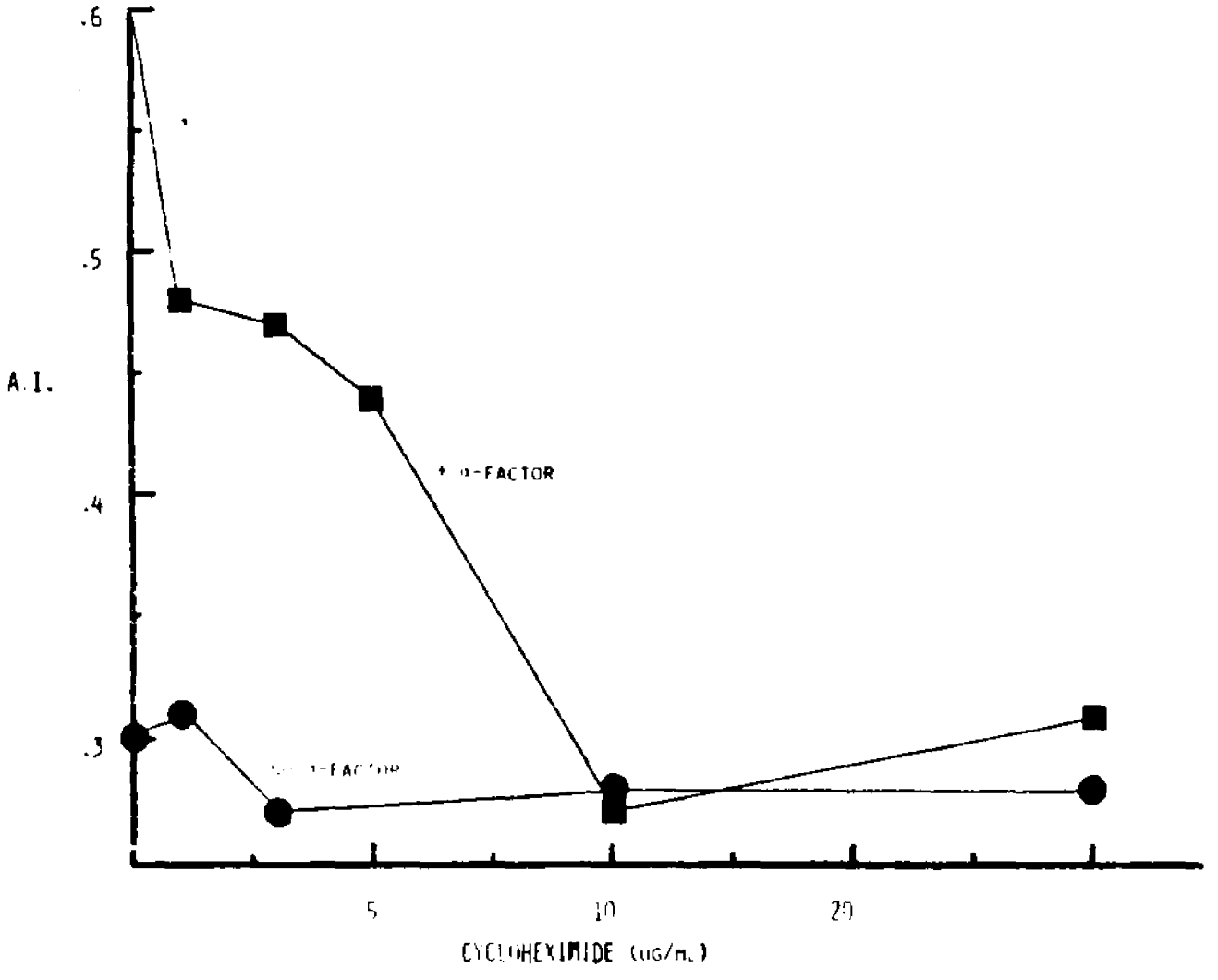
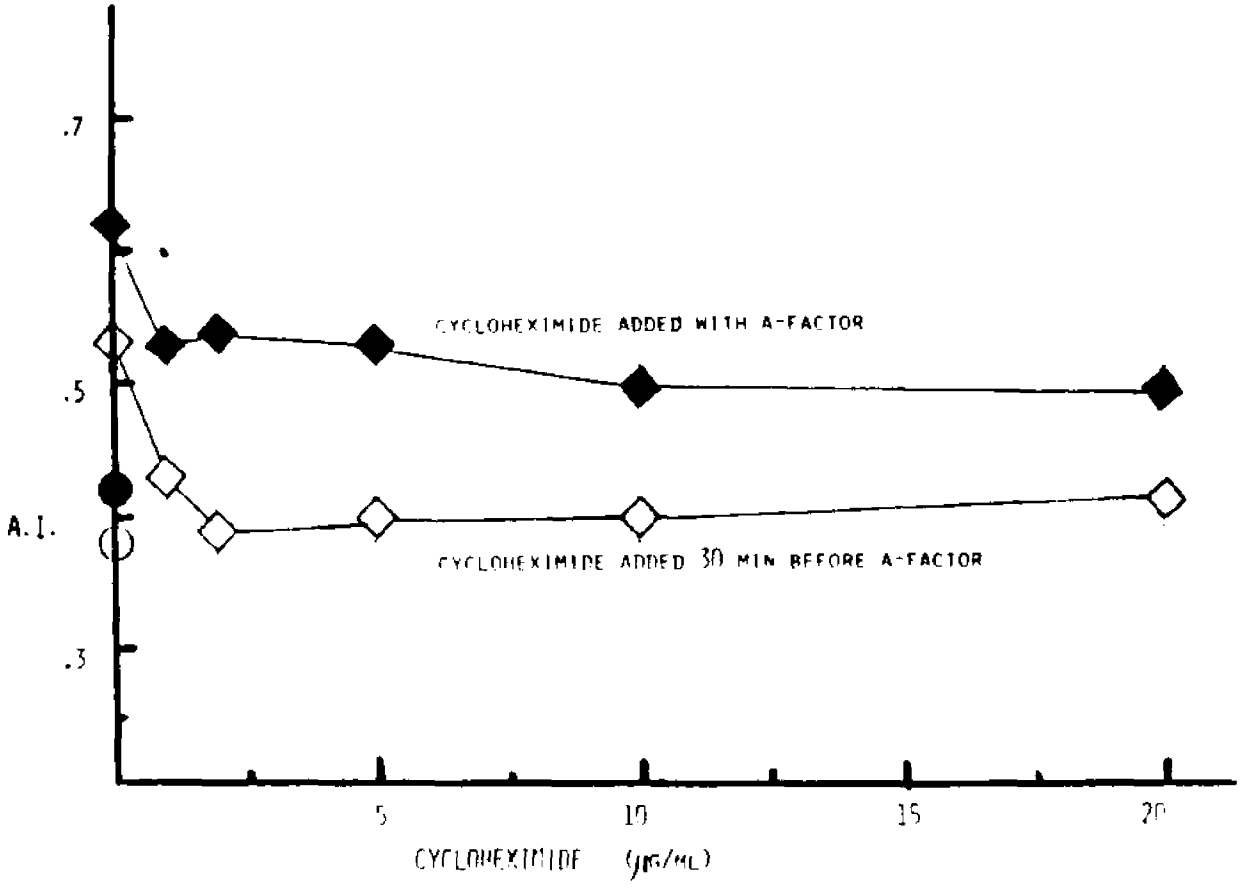

.6
.5
.4
.3
A.I.
+ α-FACTOR
5
10
20
CYCLOHEXIMIDE (uG/mL)

Figure 16. Inhibition of a-factor induction by cycloheximide. α-Cells were induced by a-factor under standard conditions in the presence of the indicated concentration of cycloheximide. The drug was added either 30 minutes before a-factor (◇), or at the same time as a-factor (◆). Agglutination was then carried out as usual with uninduced a-cells.


.7
.5
.3
A.I.
CYCLOHEXIMIDE ADDED WITH A-FACTOR
CYCLOHEXIMIDE ADDED 30 MIN BEFORE A-FACTOR
5
10
15
20
CYCLOHEXIMIDE (μG/ML)

Pheromone induction may require ribosomal protein synthesis. These experiments, however, do not indicate whether or not the critical peptide or peptides synthesized include the actual agglutination molecule. Since agglutinins have been postulated to be glycoproteins on the basis of analogy with yeasts of the genus Hansenula and reports of glycoprotein agglutinins from Saccharomyces (see introduction) a reagent that specifically blocks glycoprotein synthesis may also inhibit induction. Tunicamycin, an antibotic originally isolated from tunicates, is just such a reagent. It inhibits the formation of N-glycosidic protein-carbohydrate linkages by blocking the transfer of N-acetylhexosamine-1-phosphate to dolichol monophosphate (Mahony and Dustin, 1979) and is effective in yeasts (Kuo and Lampen, 1974).

When a-cells were induced in the presence of tunicamycin, their increase in agglutinability was inhibited. At concentrations of 50 µg/ml tunicamycin, induction was totally blocked (table I).

This drug, on the other hand, had little effect on a-factor induction. In view of the results with cycloheximide, in which a preincubation was necessary to inhibit induction, this experiment was performed with and without such incubation (table II). At concentrations of tunicamycin twice as high as those needed to completely inhibit α-factor induction, a-factor induction was only partially inhibited.

TABLE I

Effect of Tunicamycin on α-Factor Induction

Concentration of Tunicamycin (μg/ml)	A.I.[a]
0	.68
1	.69
2	.74
5	.63
10	.59
20	.44
50	.35

a. Agglutination indices are for a-cells induced in the presence of the concentration of tunicamycin indicated. The A.I. for uninduced a-cells was .38.

TABLE II

Effect of Tunicamycin on a-Factor Induction

Concentration of Tunicamycin (μg/ml)	A.I. Without Preincubation[a]	A.I. With Preincubation[b]
0	.90	n.d.
10	.86	.90
50	.80	.82
100	n.d.	.82

a. $\underline{\alpha}$-cells were induced in the presence of the indicated concentration of tunicamycin, then agglutinated with induced a-cells.

b. $\underline{\alpha}$-cells were incubated with the indicated concentration of tunicamycin for 30 min. before the addition of pheromone.

n.d. Data not available.

The agglutination index for $\underline{\alpha}$-cells not induced was .75.

Actinomysin D is a well known antibiotic that binds to DNA blocking the action of RNA polymerases. Since pheromone induction requires complete medium and since α-factor is known to inhibit selectivly the initiation of DNA synthesis only, RNA synthesis may be a part of the response to α-factor. Specifically, the necessary ribosomal protein synthesis or syntheses may be triggered by the sudden availability of α-factor induced mRNA. As can be seen (table III), actinomycin D up to 50 µg/ml during induction did not block the effects of α-factor. This, and the experiments reported hereafter, were not repeated for a-factor induction due to the inability to consistently isolate this very hydrophobic and tempermental molecule.

Calleja et al. (1981) have studied the "fission" yeast Schizosaccharomyces pombe, so-called because, instead of an asymmetric budding, cells divide equally (or fission) into two daughter cells. In this genus, haploid cells undergo an agglutination referred to as flocculation. This terminology is somewhat unfortunate, since this is a sexual process that is analogous to sexual agglutination in Saccharomyces. (The term flocculation in Saccharomyces is reserved for a non-sexual aggregation of diploid cells commonly utilized by brewers to help clear their product after fermentation.) The so-called "flocculation", or sexual agglutination, in Schizosaccharomyces requires cells of both haploid mating

TABLE III

Effect of Actinomycin-D on α-Factor Induction

Concentration of Actinomycin-D (μg/ml)[a]	A.I.
0	.81
1	.81
3	.80
5	.82
10	.87
30	.86
50	.81

a. a-cells were incubated with the indicated concentration of actinomycin-D for 45 min. before the addition of pheromone.

types. It differs form the agglutination reaction in Saccharomyces in that: it is not inhibited by salts or proteolytic enzymes, it is induced by oxygenation rather than by pheromones, and it is believed to involve hydrogen bonding (Calleja et al., 1981). An intriguing aspect of this system, however, is that in order for the induction of agglutinability to take place, mitochondrial function is essential. Consequently, chloramphenicol blocks induction in this genus (Calleja, 1973).

To test for this possibility in Saccharomyces, a culture of a-cells was grown in YNB to the usual density used in agglutination experiments. This culture was then diluted twofold with fresh YNB and divided into halves. One half was grown to the original density in plain YNB, the other was brought to 2 mg/ml chloramphenicol for the final doubling.

Both cultures were then divided into parts that were uninduced, induced under standard conditions, and induced in the presence of chloramphenicol. The results are shown in Table IV. As can be seen, chloramphenicol inhibition of mitochondria during the latter stages of growth inhibited neither uninduced (constitutive) nor induced agglutination. Indeed somewhat higher A.I.'s were oberved for those cells either grown and/or induced in the presence of the drug.

TABLE IV

Effect of Chloramphenicol on α-Factor Induction

Treatment of a-cells				A.I.
Uninduced	Induced Without CP	CP During[a] Induction	CP During[b] Growth	
+	-	-	-	.23
-	+	-	-	.66
+	-	-	+	.28
-	+	-	+	.79
-	-	+	+	.82

a. Cells were induced in the presence of 2.0mg/ml chloramphenicol.

b. Cells were grown during their final doubling time in the presence of 2.0mg/ml chloramphenicol.

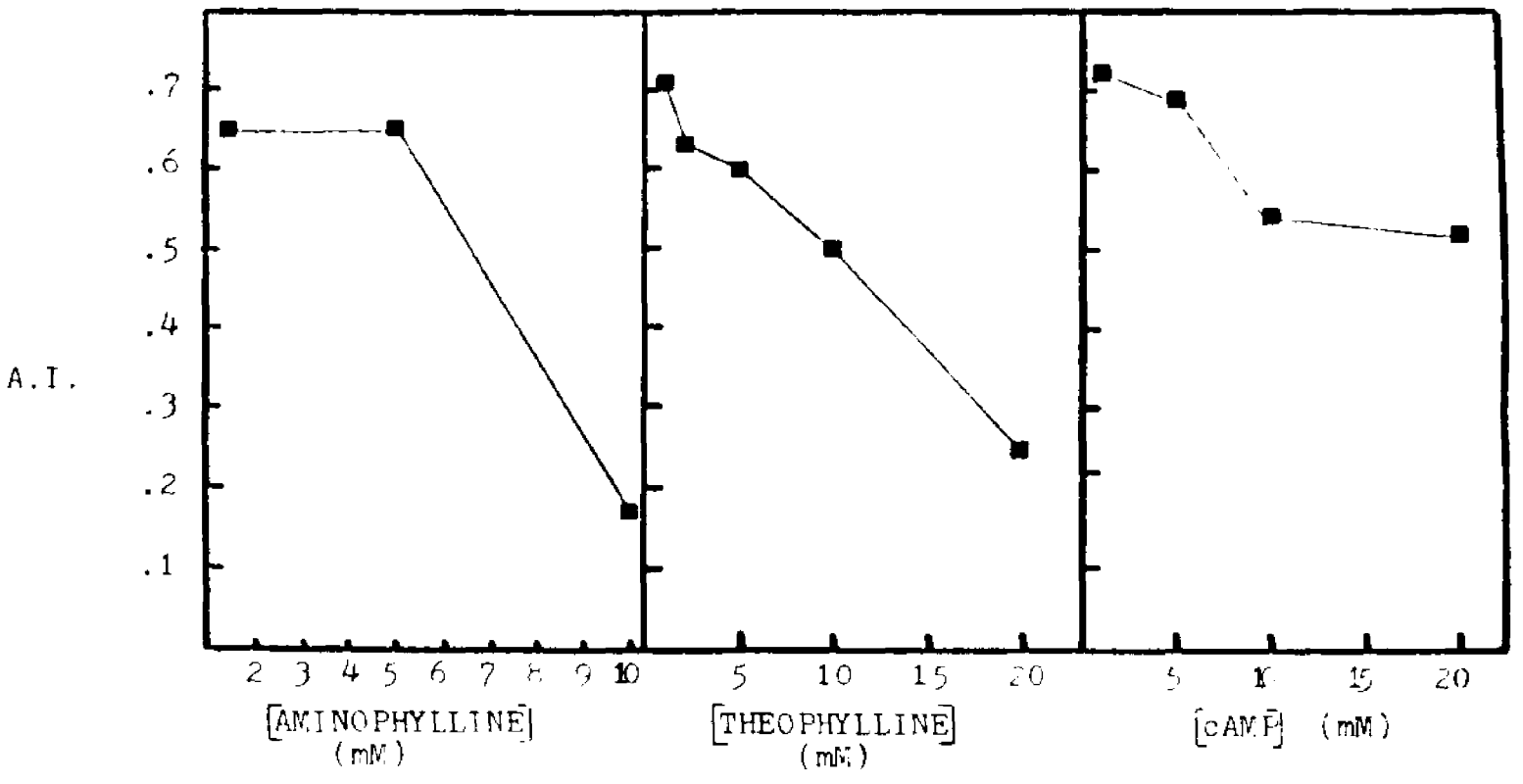
II. Some Aspects of the Mechanism of Action of α-Factor in Induction.

Although little is known of the mechanism of α-factor action, the lowering of cAMP levels has been implicated as an intermediate step in α-factor's effect of cell cycle arrest (Liao and Thorner, 1980; Ciejeck and Thorner, 1979; see also the introduction). These investigators identified several substances as specific inhibitors of yeast membrane-bound 3'5'-phosphate (cAMP) phosphodiesterase, and reported that they, or the addition of exogenous cAMP, could block α-factor-induced cell cycle arrest.

α-Factor, however, exerts three major effects on a-cells: cell cycle arrest, morphogenesis, and increased agglutinability. I tested the effect of two inhibitors (aminophylline and theophylline) as well as exogenous cAMP on the ability of α-factor to induce agglutinability. The results are shown in figure 11.

Liao and Thorner reported that 1mM aminophylline, 5mM theophylline or 5mM cAMP blocked cell cycle arrest. In this experiment 10mM aminophylline or 20mM theophylline totally blocked induction of agglutinability. 20mM cAMP, however, resulted in only partial inhibition. To make certain that the relatively fast-acting α-factor (see figure 6; see also figure 13) did not complete its function before these reagents took effect, a

Figure 11. Effects of aminophylline, theophylline and cyclic AMP on α-factor induction. a-Cells were preincubated for 5 min. with the indicated substances in YNB, after which 1unit/ml α-factor was added for the standard induction period. Cells were then agglutinated as usual.

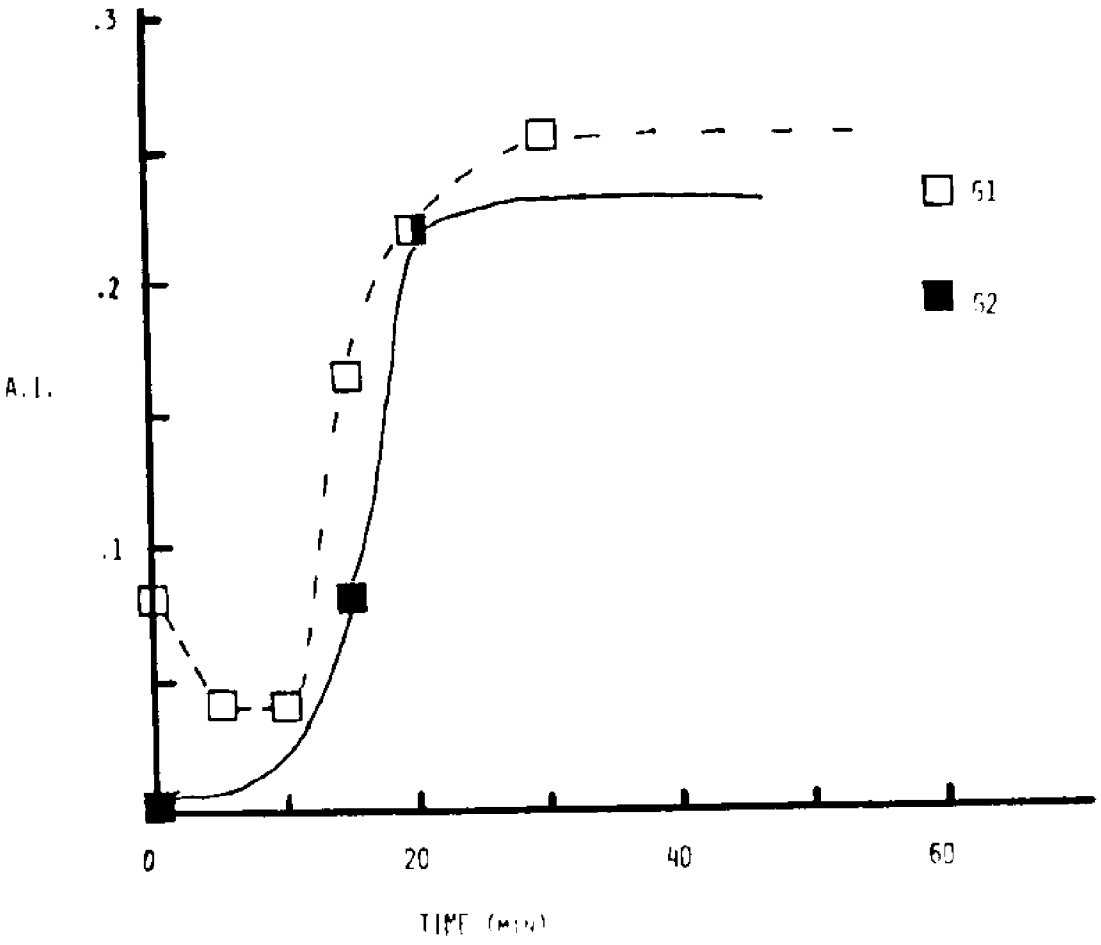

A.I.
.7
.6
.5
.4
.3
.2
.1
2 3 4 5 6 7 8 9 10
[AMINOPHYLLINE] (mM)
5 10 15 20
[THEOPHYLLINE] (mM)
5 10 15 20
[cAMP] (mM)

preincubation period (5 minutes) was allowed before the addition of pheromone. Thus it seems that cAMP can interfere with but not block α-factor induction.

Since α-factor arrests cells in G1 preparatory to mating, it is a reasonable speculation that only cells in G1 agglutinate or respond to α-factor. Cell cycle studies are facilitated in yeast by the fact that they exhibit distinct morphological markers during different stages: unbudded in G1; bud emergence at S; large buds in G2. To test the possibility of differential α-factor action during the cell cycle, the following experiment was performed in cooperation with Dr. V.L. MacKay of the Waksman Institute for Microbiology of Rutgers University. Cultures of a-cells were grown as usual for agglutination assay. After harvesting, cells were resuspended as a thick slurry and layered carefully onto 8 to 35% sorbitol gradients and centrifuged. The cells sedimented according the their size and shape; thus, it was possible to isolate small unbudded (G1) cells by carefully drawing off the upper layers with a bent needle syringe. Fractions containing 80% or more small, unbudded cells were pooled as G1.

Cells so isolated were washed once with distilled water and resuspended in fresh medium (YNB). These cells were induced with α-factor under standard conditions to obtain the G1 curve (figure 12). Alternately, these cells were re-cultured for 100 minutes until they synchronously

Figure 1[illegible]. α-Fact[illegible] [illegible] G1 and G2 cells. Populations of synchronous cells were prepared by orbital gradient centrifugation as described in the text. Each such population isolated was induced and agglutinated as usual.


.3
.2
.1
A.I.
0
20
40
60
TIME (min)
G1
G2

entered G2 as evidenced by carrying large buds. These cells were then induced under standard conditions to obtain the G2 curve in figure 12. In both cases, induction occured with kinetics that were identical to that for unsynchronized cells (figure 6).

The fact that a given effect of $\underline{\alpha}$-factor appears over a certain period of time does not necessarily mean that exposure to pheromone is needed for the entire period. I therefore compared $\underline{\alpha}$-factor induced morphogenesis, which requires a period of 4 hours to be fully expressed (Lipke et al., 1976), to increased agglutinability, which is complete in 20 - 30 minutes (figure 6).

Cells were prepared as usual for induction. Multiple, small induction cultures were prepared. At intervals after the addition of pheromone (1 unit/ ml) a culture was quickly harvested by suction filtration onto a Millipore filter and washed with YNB without pheromone. The cells were then transferred to fresh YNB for the remainder of a normal induction period. Thus, all cells were in nutrient medium for 25 minutes, but were in pheromone containing medium for a variable part of 25 minutes.

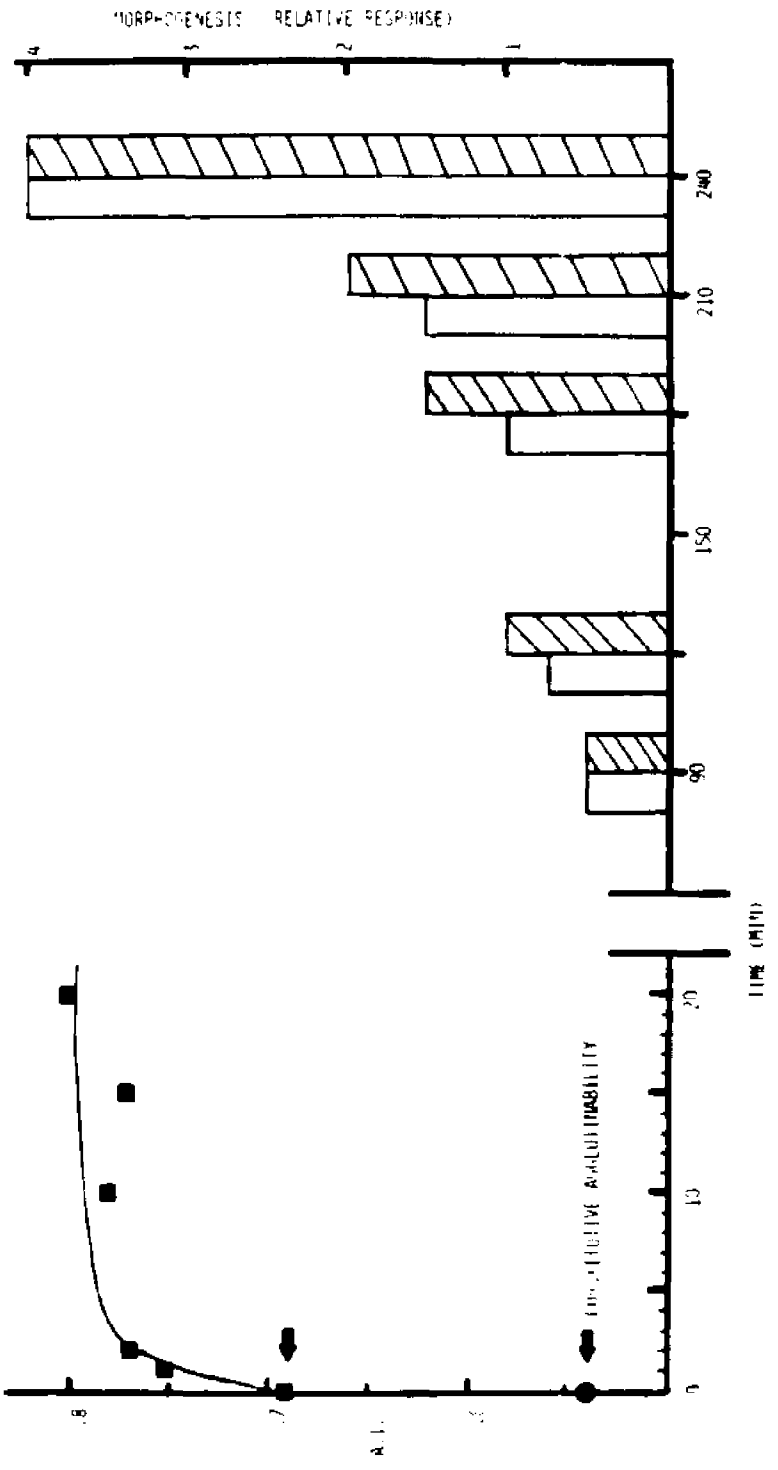
A similar procedure was followed with $\underline{a}$-cells induced to undergo morphogensis. Here, conditions designed to induce morphogenesis in all cells were used: 20 units/ml $\underline{\alpha}$-factor and 4×10^5 cells/ml. The time intervals between sacrifices of cultures was longer and cells were incubated for a full 4 hour period.

The results of this experiment are shown in figure 13. The left hand portion shows the agglutinability of the cells tested. Those from which the α-factor was removed immediately show 67% of the total inductive effect. Due to the time needed to filter and wash the cells, these were probably in contact with α-factor rich medium for some 20-30 seconds. It is nonetheless clear that cells need not remain in α-factor containing medium more than a few minutes for the effect to be fully and irreversibly initiated.

It is possible that α-factor is irreversibly binding to its receptor or being internalized in the cells. If either of these are part of the mechanism of its action, and if they occur quickly, the above result would be obtained. It is also possible that α-factor pulls an irreversible "trigger", such that once the cells have experienced α-factor, for even a brief amount of time, the presence of the pheromone is no longer needed.

This latter hypothesis is supported by the results of the morphogenesis part of the experiment (figure 13, right half). Here, morphological response to pheromone was graded as follows: budding inhibition, +1; cell enlargement , +1 to +2; appearence of small, pear-shaped cells (commas), +3; and appearence of large, pear-shaped cells (shmoos), +4. All cells that were washed prior to 90 minutes showed no obvious response to pheromone. Indeed, only the morphological changes that were normally

Figure 15. Time-course of initiation of α-factor effects. At the times indicated during a standard induction (■) the pheromone containing induction medium was rapidly removed by suction filtration, and the cells immediately washed in fresh medium without pheromone. They were then transferred to fresh medium, without pheromone, for the remainder of a normal induction period. These cells were then agglutinated as usual. a-Cells were also incubated with 20units/ml α-factor (bar graph). At the indicated times, cells were removed from the induction medium, washed, and transferred to fresh medium as described above. At the end of a normal period for morphogenesis (240 min.), microscopic observations of shmoos were made. Open bars represent the experiment done with biologically derived α-factor; striped bars represent the experiment done with synthetic trideca-peptide α-factor.


MORPHOGENESIS (RELATIVE RESPONSE)
4
3
2
1
240
210
150
90
30
15
0
TIME (MIN)

present at the time of washing were evident at 4 hours. This result indicated that α-factor must be continually present in the medium to induce morphological changes and suggests that it can be removed by washing, since the expression of its effects can be stopped. This experiment was repeated with synthetic tridecapeptide α-factor, which gave qualitatively identical results (figure 13).

Induction of increased agglutinability presumably occurs through the elevated expression of agglutination molecules (agglutinins) at the cells surface (Terrance and Lipke, 1981). In the Materials and Methods section is described a substance (peak III) that was isolated as a by-product of the purification of the α-cell agglutination molecule (α-agglutinin). This substance was specifically destructive to a-agglutinin as judged by its ability to specifically and permanently inactivate a-cells (figure 3).

The availability of such a reagent allowed the possibility of testing the regulation of expression of agglutinability in response to α-factor. a-Cells were digested with peak III material by incubation with this material in 0.1 M sodium acetate buffer (pH 5.0) for 90 minutes. Cells were subjected to various series of digestions and α-factor inductions. These experiments are illustrated in Table V. As can be seen, digestion with peak III material eliminated the agglutinability of either uninduced or induced a-cells. If cells were digested, then induced, the cells aggutinated at a level that was

essentially equivalent to cells that were induced without digestion. This result was unexpected, since these former cells increased their agglutinability from almost zero A.I., whereas the latter cells increased their agglutinability from a strong constitutive base level. It would seem that α-factor induction increases agglutinability to a certain high level, regardless of the initial concentration of agglutinins on the cell surface.

More information is to be gained from the experiment in which cells were sequentially induced, digested, and then reinduced. Here, the second induction brought the agglutinability to levels that were actually slightly higher than undigested induced cells. This further confirms the previous conclusion. It shows that a cell can respond twice to α-factor but only if it "needs" to (i.e. it lacks functional cell-surface agglutinins) since second doses of α-factor given to normally induced cells have no effect.

III. Demonstration of a Single Interacting System in *Saccharomyces* Agglutination

The agglutination system of *Hansenula wingei* has been characterized on the molecular level. In this yeast, a single pair of complimentary molecules, one on each mating type, interact to cause sexual agglutination (see Introduction). The existence of both constitutive and induced agglutination in *Saccharomyces cerevisiae* suggests

TABLE V

Regulation of Cell-Surface Expression of a-Agglutinin

Treatment of a-cells	A.I.
Uninduced	.31
Induced[a]	.79
Uninduced, digested[b]	.07
Induced, then digested	.15
Digested, then induced	.70
Induced, digested, then reinduced	.63

a. All inductions were carried out under standard conditions.

b. Cells were digested by incubation with peakIII material (described in the text) in pH5.0 buffer for 90 min.

the possibility of the existence of two different adhesive mechanisms. This hypothesis is further indicated by the existence of genes conferring constitutive and inducible agglutinability (Doi and Yoshimura, 1978; Yanagishima and Nakagawa, 1980; Nakagawa and Yanagishima, 1981) and by the early report of differences in the pH maxima for constitutive and induced agglutination (Fehrenbacher et al., 1978). It would also explain the initial "random assembly" vs. the "true cell aggregates" described by Kawanabe et al. (1979; see Introduction also).

If two or more sets of complementary molecules are involved in Saccharomyces, then it might be possible to affect each set differentially. Specifically, conditions may exist in which inhibition of agglutination exhibits a bi-phasic or multi-phasic character. Further, if one of the different adhesive mechanisms is preferentially expressed through pheromone induction, the behavior of different combinations of induced and uninduced cells should vary.

A. Similarity of conditions for agglutination of both uninduced and induced a-cells.

This problem was initially addressed by examining the conditions under which uninduced a-cells and induced a-cells would agglutinate. The results are shown in figures 14 - 16.

The agglutination reaction showed a broad pH maxima (pH 5 - 7). This value was identical for both induced and uninduced a-cells (figure 14). Similarly, both types of a-cells agglutinated across the same range of ionic strengths when either sodium chloride or sodium acetate was the salt used (figure 15). Finally, agglutination required the same minimal temperature (12° C, figure 16) regardless of the pheromone treatment of a-cells.

The induction of a-cells by α-factor leads to a greater increase in the A.I than does the induction of α-cells by a-factor (see figures 5 - 8). It would seem therefore, that the comparison of uninduced a-cell to induced a-cell agglutination is most likely to reveal differences in conditions.

B. Inhibiton of Agglutination in All Agglutinable Combinations of Cells.

There are, nonetheless, four different agglutinable combinations of cells: neither cell type induced; α-cells only induced; a-cells only induced; and both cell types induced. These combinations are listed in the inverse order of their relative agglutinability. This order, incidentally, is always the same. It is possible that a second adhesive mechanism comes into play only with a-factor induction of α-cells, and/or when cells are mixed in some particular combination.

Figure 14. Dependence of agglutination on pH. Haploids were agglutinated under standard conditions, except for differences in the agglutination buffer: (○,□), agglutination in 0.1M sodium acetate buffer; (●,■), agglutination in 0.1M tris-hydrochloride; (□,■), cell mixtures in which the a-cells were induced with α-factor; (○,●), cell mixtures in which the a- cells were uninduced.

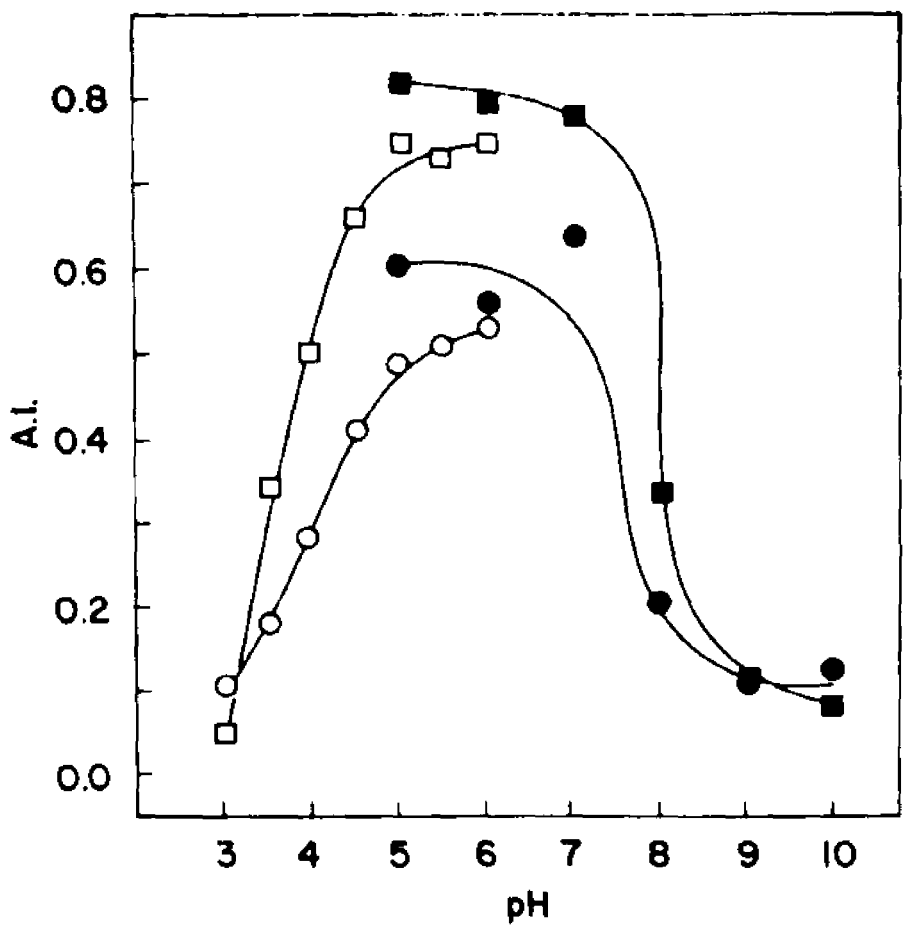

0.8
0.6
0.4
0.2
0.0
A.I.
3
4
5
6
7
8
9
10
pH

Figure 15. Dependence of agglutination on ionic strength. Uninduced (○,●), and induced (□,■) _g_-cells were agglutinated in sodium acetate buffer(pH5.0) at the ionic strength indicated. In the curves marked by open symbols, the ionic strength was altered by adjusting the concentration of sodium acetate in the standard agglutination buffer. In the curves marked by closed symbols, the ionic strength was adjusted by the addition of sodium chloride to the standard agglutination buffer.

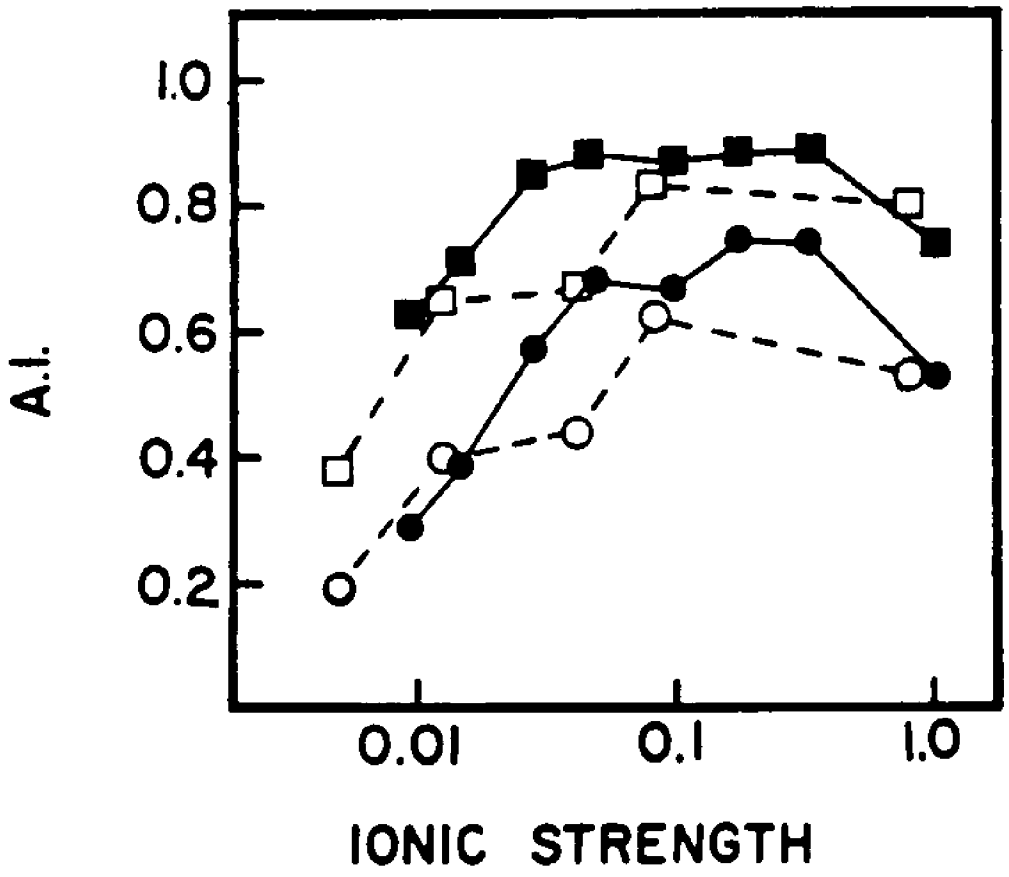
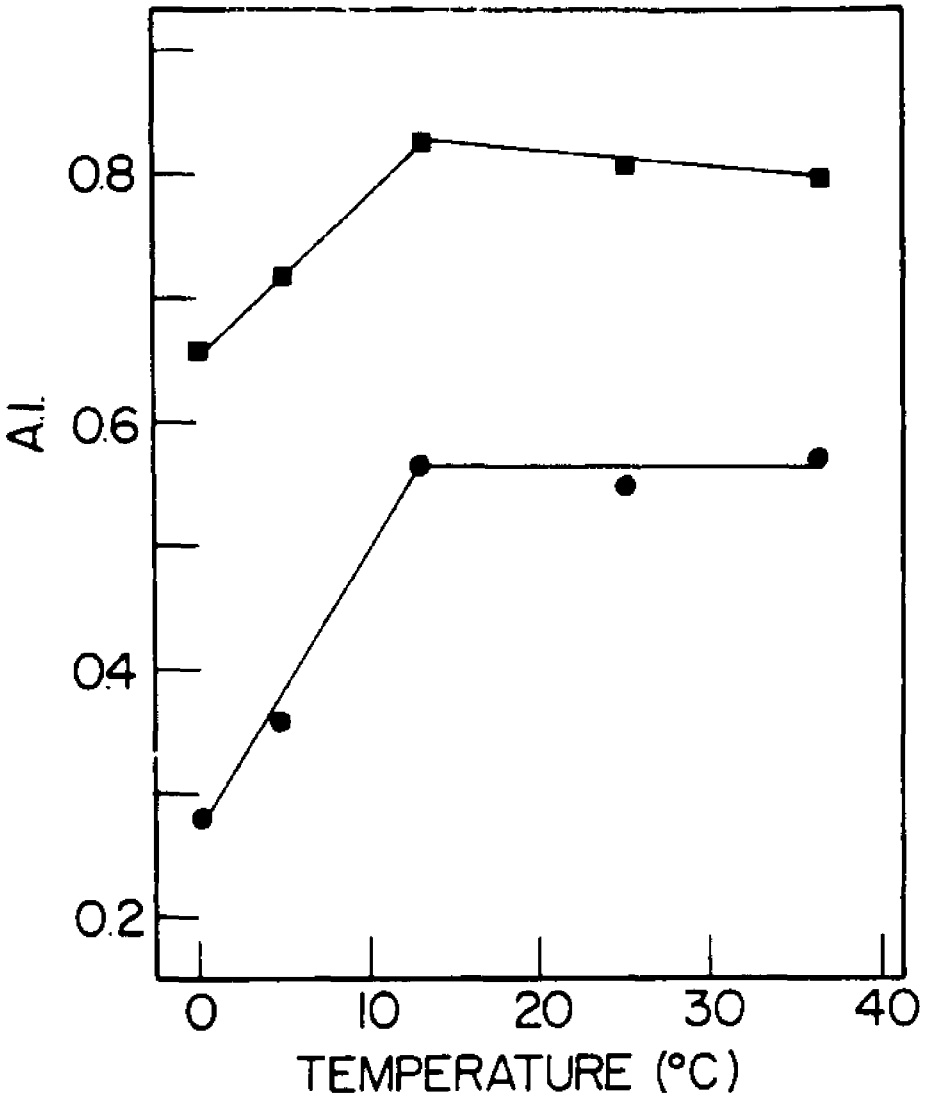

1.0
0.8
0.6
0.4
0.2
A.I.
0.01
0.1
1.0
IONIC STRENGTH

Figure 16. Dependence of agglutination on temperature. Uninduced (●), and induced (■) g-cells were agglutinated at the indicated temperature.


A.I.
0.8
0.6
0.4
0.2
0
10
20
30
40
TEMPERATURE (°C)

I investigated this possibility by inhibiting agglutination in all four agglutinable combinations. A simple way to do this is to agglutinate the cells by gentle centrifugation as usual, and then resuspend them at a range of speeds above and below the standard. The results are shown in figure 17. The standard resuspension speed was 1000 rpm. This was chosen because it yielded high levels of agglutinability while maintaining maximum differences between uninduced and induced a-cells. At higher speeds (more shear force), the A.I. of all cell combinations was reduced proportionately. None showed a clear-cut difference in resistance to shear.

Another strategy was to include chemical inhibitors in the agglutination buffer or to treat cells with potential inactivating reagents before agglutination. The results of these experiments are shown in Table VI. As can be seen, NaCl at 1M and 5M , 0.3M KI, 0.01% SDS, 1% Tween-80 and 1M pyridine all inhibited each combination of cells to about the same extent. EDTA had no effect, nor did added di-valent cation (data not shown). DTT treatment inactivated a-cells but not α-cells. This was expected from analogy to Hansenula wingei S-cells. In no case was agglutination preferentially inhibited in one or two agglutinable combinations. These inhibitons were reversible at the concentrations of inhibitors used. The inclusion of cycloheximide in both agglutination and reversal media precluded cell-mediated alterations in agglutinability. Thus, only the in vitro, physical

Figure 17. Inhibition of agglutination by mechanical shear. Cells were agglutinated by gentle centrifugation as usual, then resuspended with the stirrer set at the speeds indicated. All four agglutinable combinations of cells were tested: neither cell type induced (●); α-cells only induced (◆); a-cells only induced (■); both cell types induced (▲).

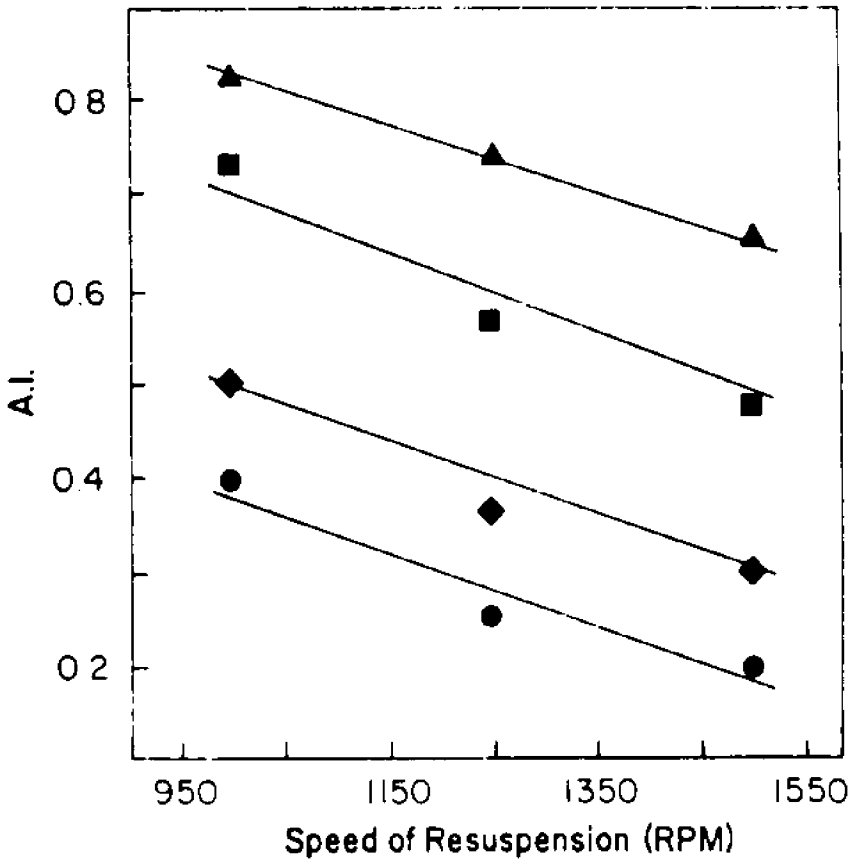

0.8
0.6
0.4
0.2
A.I.
950
1150
1350
1550
Speed of Resuspension (RPM)

TABLE VI

Effect of Potential Inhibitors on Agglutination

Inhibitor[a]	Percentage of Control A.I.[b]			
	Neither Induced	α-cells Induced	a-cells Induced	Both Induced
1M NaCl	9[illegible]	7[illegible]	89	83
5M NaCl	2[illegible]	26	18	28
0.3M KI	90	88	94	97
0.01% SDS	61	87	90	93
1% Tween-80	95	97	99	96
1M Pyridine	44	57	56	71
20mM EDTA[c]	106	101	104	99
50mM DTT:				
a-cells pretreated[d]	45	31	26	23
α-cells pretreated[d]	95	88	99	103

a. Inhibitors were included in the agglutination buffer unless otherwise noted.

b. Control A.I. was obtained by agglutinating cells under standard conditions.

c. Agglutination was carried out at pH 7.0.

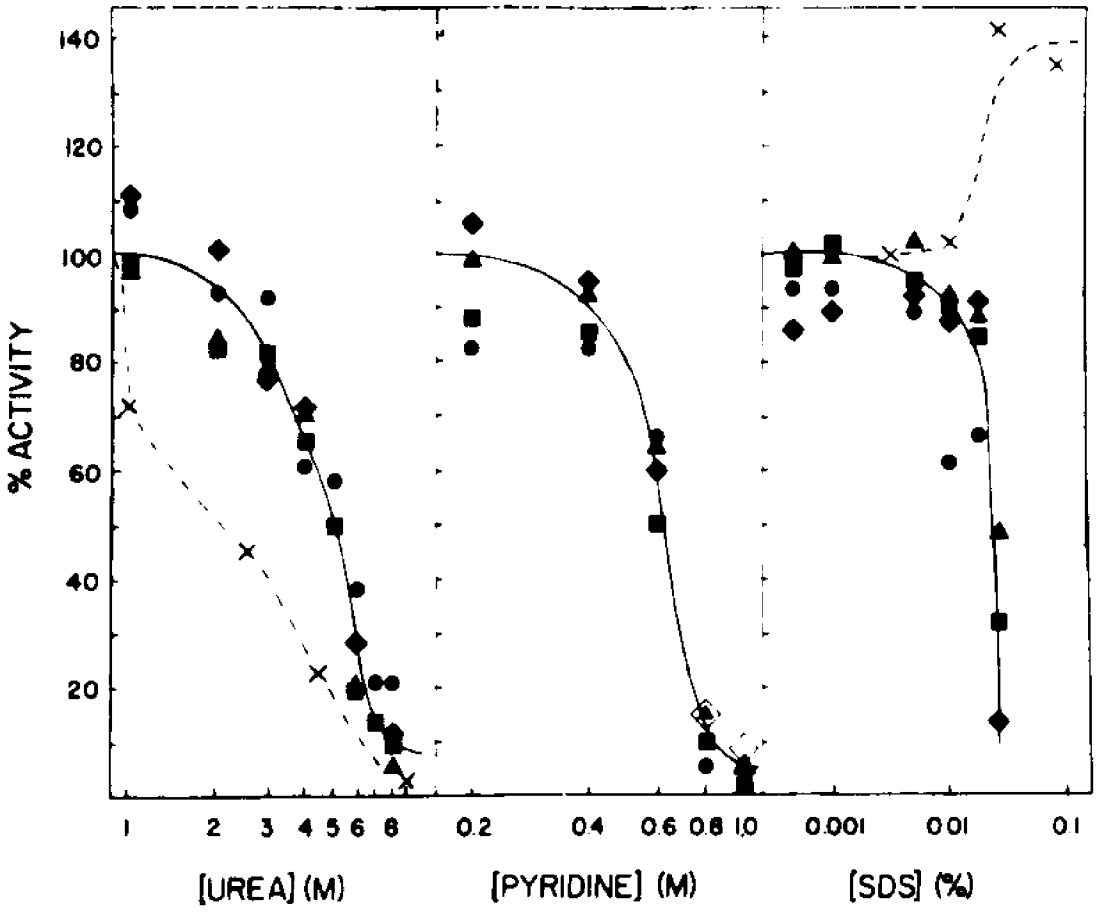
d. Cells were pretreated with dithiothreitol for 30 min., washed once with buffer at pH5.0, and then agglutinated under standard conditions.

interaction between agglutinins was being affected.

The inhibition experiments were carried further by performing complete titrations of agglutination in all combinations of cells in the presence of cycloheximide. Three inhibitors were chosen for this experiment: urea, pyridine, and SDS. The effects of these inhibitors were reversible at the concentrations used. Therefore, their action was probably via interference with the actual agglutination mechanism. The results are shown in figure 18. All the titration curves showed sharp inflection points which were identical for all cell combinations.

To demonstrate that these inhibitors were affecting the agglutination mechanism differentially from other cell-surface functions, invertase, a cell wall associated enzyme, was assayed under the same conditions. For this experiment, a-cells and α-cells were grown in minimal medium with sucrose as the carbon source. Invertase activity was measured in these cells by the appearence of reducing sugar (Smith and Ballou, 1974) in the presence of the indicated concentrations of inhibitors (broken curves, figure 18). Invertase was clearly more susceptible than agglutinin to the presence of urea and less susceptible to SDS. The increase to above 100% in the latter case may be due to permeabilization of the cells and consequent assay of both extracellular and intracellular enzymatic activity.

Figure 1. Titration of agglutination by three inhibitors. Cells were agglutinated in the presence of the indicated concentrations of urea, pyridine, or sodium dodecyl sulfate (SDS). All four agglutinable combinations of cells were tested: neither cell type induced (●); α-cells only induced (◆); μ-cells only induced (■); both cell types induced (▲).


% ACTIVITY
140
120
100
80
60
40
20
1
2
3
4
5
6
8
[UREA] (M)
0.2
0.4
0.6
0.8
1.0
[PYRIDINE] (M)
0.001
0.01
0.1
[SDS] (%)

C. Experiments with Partially Purified α-Agglutinin.

The above evidence suggests a single interacting system in Saccharomyces, but is largely circumstantial in nature. More direct evidence can be obtained by examination of the agglutinin molecules themselves.

α-Agglutinin was partially purified by gel filtration of crude α-cell extracts on AcA-34 as described in the Methods section. The eluted material (peak I) exhibited the properies of a monovalent α-agglutinin. It had no effect on α-cells and did not render a-cells agglutinable with each other. It did, however, inactivate a-cells in terms of their ability to agglutinate with α-cells. Agglutinin activity was removed from solution by incubation with a large excess of a-cells, but not by a similar incubation with α-cells (Table VII). Repeated attempts to recover activity from a-cells by washing in buffer at high pH or at high concentration of salt or detergent were unsuccessful (see discussion section), even though the a-cells recovered their agglutinability.

The kinetics of inactivation of a-cells by α-agglutinin was progressive (figure 19) and was identical for both uninduced and induced cells. The agglutinin was active across a range of pH (5 - 7) that was identical to that for agglutination (see figure 14).

Dose-response to α-agglutinin was linear at low concentrations. Of interest is the fact that a-cells were completely inactivated at sufficiently high concentrations

TABLE VII

Effect of Incubating $\underline{\alpha}$-Agglutinin Activity with $\underline{a}$-Cells

Substance Tested	Percentage $\underline{\alpha}$-Agglutinin Activity Remaining
$\underline{\alpha}$-agglutinin, before incubation[a]	100
$\underline{\alpha}$-agglutinin, incubated without cells	77
Supernatant from $\underline{\alpha}$-agglutinin incubated with $\underline{\alpha}$-cells	80
Supernatant from $\underline{\alpha}$-agglutinin incubated with $\underline{a}$-cells	0

a. All incubations were at pH5.0 and 30°C. for 90 min.

Figure 14. Kinetics of g-cell inactivation by X-agglutinin. Uninduced (●) and induced (■) g-cells were incubated with 3.7 units X-agglutinin/tube in sodium acetate buffer for the times indicated before agglutination.

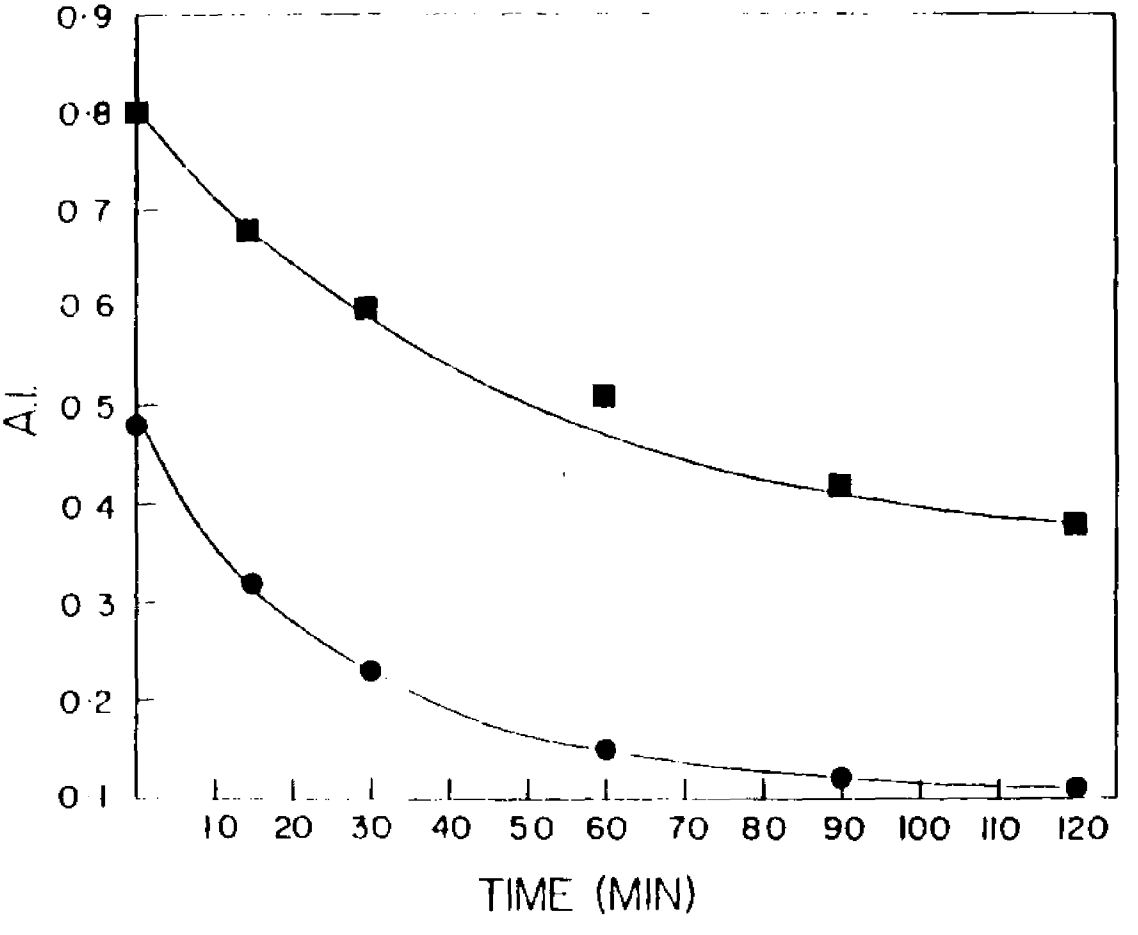
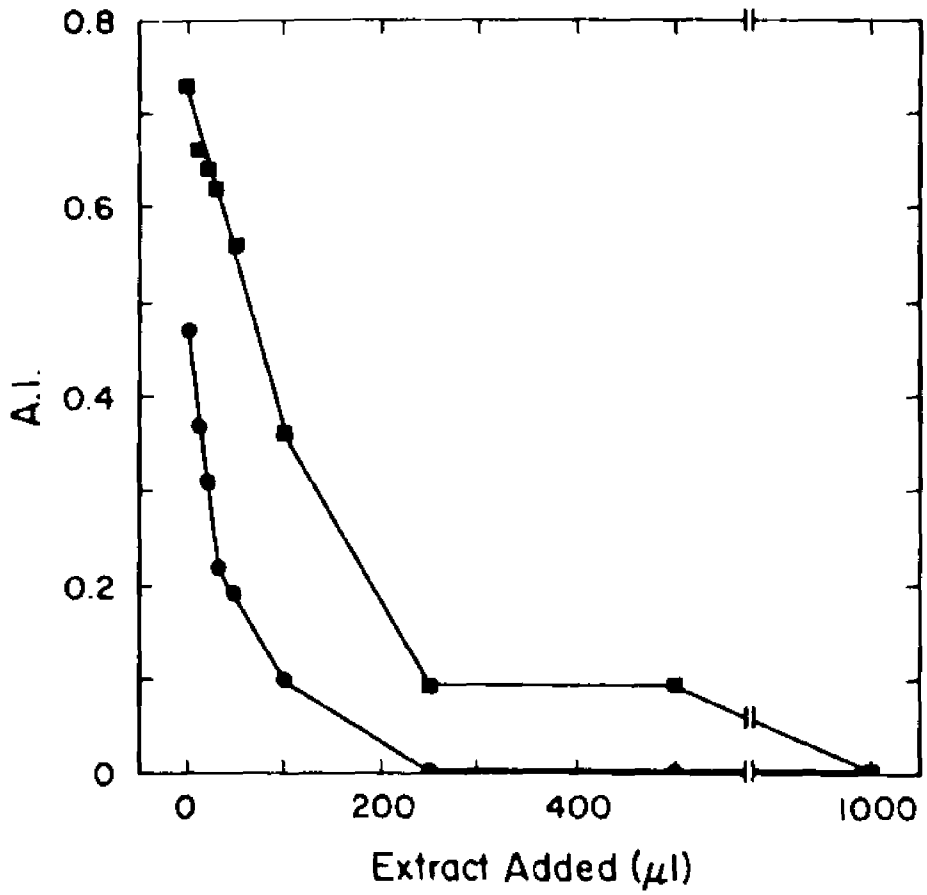

0·9
0·8
0 7
0 6
0 5
0 4
0 3
0·2
0 1
A.I.
10 20 30 40 50 60 70 80 90 100 110 120
TIME (MIN)

Figure 7. Dose-response of a-cells to α-agglutinin. α-Agglutinin was concentrated 10 fold by dialysis against polyethylene glycol. The indicated amounts of this concentrate were incubated with uninduced (●), and induced (■) a-cells in 0.1M sodium acetate (pH 5.0) for 1½ hrs.


0.8
0.6
0.4
0.2
0
A.I.
0
200
400
1000
Extract Added (μl)

of $\underline{\alpha}$-agglutinin regardless of their state of pheromone induction (figure 20). Such $\underline{a}$-cells were totally inactivated even when tested against induced $\underline{\alpha}$-cells (data not shown).

All of this indicates that the $\underline{\alpha}$-agglutinin can block all $\underline{a}$-cell binding sites. To show that the $\underline{\alpha}$-agglutinin isolated was a single molecular species, the kinetics of its inactivation by heat was investigated. Intact cells are highly resistant to heat (Table VIII). Both uninduced and induced $\underline{a}$-cells, as well as $\underline{\alpha}$-cells show only marginal reductions in A.I. after being maintained at 55° C for 30 minutes in pH 5 buffer.

Solubilized $\underline{\alpha}$-agglutinin, on the other hand, was quite heat labile. Heating for 10 minutes at 55°C destroyed all such activity (figure 21). At 50°C, the kinetics of this inactivation was log-linear regardless of the state of induction of the tester cells (figure 22).

IV. Purification of $\underline{\alpha}$-Agglutinin

Attempts to purify the agglutinin isolated by gel filtration of crude cell extract were only partially successful. The gel filtration medium required frequent replacement, which proved to be prohibitively expensive. Contaminating proteins could be removed from crude by

TABLE VIII

Heat Sensitivity of Intact Cells

a-Cells		α-Cells	
Induced	Time Heated[a] (min)	Time Heated[a,b] (min)	A.I.
-	0	0	.53
-	2	0	.47
-	5	0	.51
-	10	0	.56
-	20	0	.51
-	30	0	.49
-	0	2	.42
-	0	5	.41
-	0	10	.43
-	0	20	.42
-	0	30	.35
+	0	0	.86
+	2	0	.82
+	5	0	.86
+	10	0	.82
+	20	0	.87
+	30	0	.73
+	0	2	.83
+	0	5	.80
+	0	10	.79
+	0	20	.80
+	0	30	.77

a. Cells were heated to 55°C. for the times indicated.

b. All α-cells were uninduced.

Figure 21. Thermal lability of α-agglutinin. α-Agglutinin was maintained at the temperatures shown for 10 min. Its activity was then measured and compared to that of unheated agglutinin.

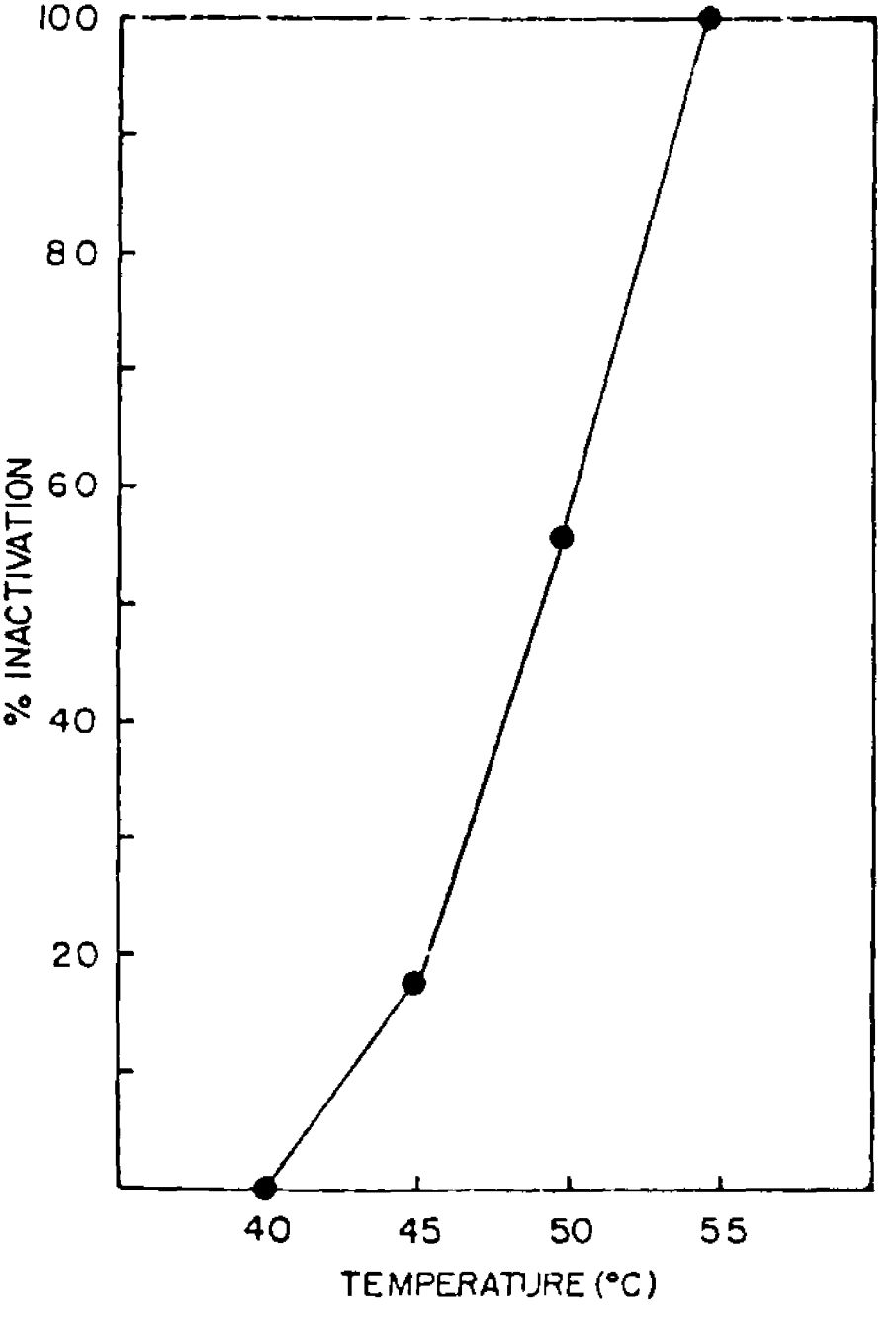
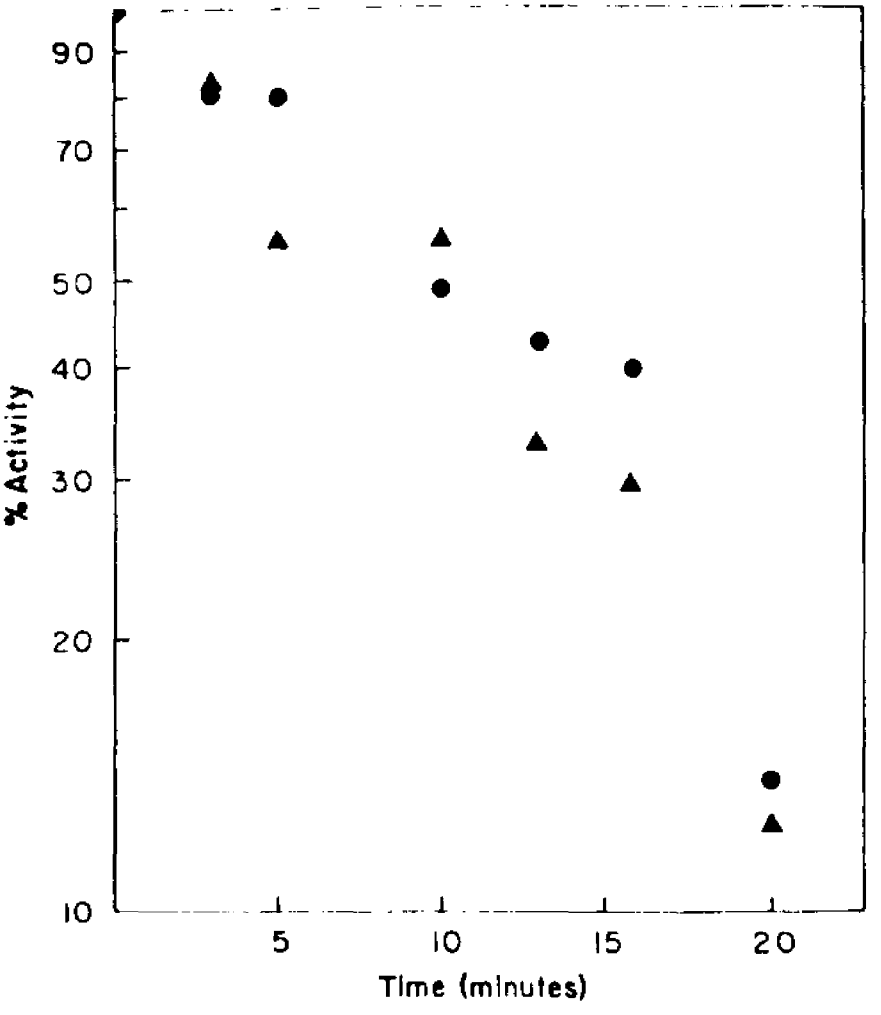

100
80
60
40
20
% INACTIVATION
40
45
50
55
TEMPERATURE (°C)

Figure 12. Kinetics of thermal lability of α-agglutinin. α-Agglutinin was heated to 56°C for the times indicated, then assayed against uninduced a-cells agglutinated with uninduced α-cells (●), and induced a-cells agglutinated with induced α-cells (▲).


90
70
50
40
30
20
10
% Activity
5
10
15
20
Time (minutes)

basic precipitation at pH 9 before chromatography, but the resulting agglutinin activity was unstable and would often inexplicably become irreversible. These problems were eventually resolved by the addition of Triton X-100 (0.03%) and PCMB (1 μM) to the isolation buffer and by replacing basic precipitation with ammonium sulfate precipitation. Dialysis of the ammonium sulfate precipitated material left a residue consisting mostly of contaminants, thus resulting in further purification. Gel filtration was abandoned due to poor yields and possible column interactions with agglutinin activity. DEAE-Sephadex at pH 5 bound all agglutinin activity, which could by eluted by 50 - 100 mM NaCl. After removal of the salt by dialysis, this material was applied to agarose-bound lentil lectin. This lectin interacts most strongly with mannose residues and bound all agglutinin activity. Concanavalin-A also bound agglutinin activity, but the interaction was much stronger. Activity could not be eluted from Con A, even by 1M α-methly-mannoside. Elution from lentil lectin was with 1M NaCl and/or 1M α-methyl-mannoside. The procedure for the purification of α-agglutinin is detailed in the Methods section. The purification is summarized in figure 4 and the results of one such purification are shown in Table IX.

The active fractions from lentil lectin were of high and constant specific activity. Polyacrylamide gel electrophoresis and subsequent silver staining of this

TABLE IX

Purification of α-Agglutinin

Fraction	Volume (ml)	Activity (U/ml)	Total Units	Specific Activity U/mg Protein	U/mg Carbohydrate	Yield (%)	Purification[b]
Crude Extract	180	350[a]	63,000[a]	5.8[a]	35[a]	-	-
Ammonium Sulfate 100% Pellet	30	400	12,000	32	200	100[b]	1[b]
DEAE-Sephadex 0.5% NaCl	20	400	8,000	2500	8900	67	78
Lentil Lectin (200 units applied)	2	140	280	19,000	50,000	94	597

a. Activity estimates in crude extract were inaccurate due to the presence of competing irreversible activities.

b. Yield and purification are based on the dialyzed ammonium sulfate pellet.

material revealed a number of protein bands (figure 23) along with diffuse staining at lower molecular weights. Periodic acid- Schiff (PAS) staining indicated that only the four bands of highest molecular weight contained carbobydrate (figure 24). These same bands are diffuse, suggesting the possibility of heterogeneity. This is a resonable expectation if the included carbohydrate moieties are variable.

α-Agglutinin purified through lentil lectin was subjected to hydrophobic affinity chromatography. The affinity of α-agglutinin activity was first determined by loading equal amounts of material purified through the DEAE-Sephadex step onto a series of small agarose columns, each containing hydrocarbons of different chain length. The results are shown in figure 25. α-Agglutinin activity did not associate with the agarose beads themselves, since 100% of loaded activity passed through the column without conjugated hydrocarbon. As the chain length increased, activity was progressively bound. All activity remained in the column when the hydrocarbon chains contained six or more carbons.

Activity could be eluted by either raising the concentration of detergent (1% Triton X-100) or salt (100 mM NaCl).

The lentil lectin purified material was then subjected to chromatography on hexyl agarose. This chain length was chosen since it was the minimum needed to bind all activity. The column was eluted with 100mM NaCl since

Figure 33. Protein bands present in purified N-agglutinin. Agglutinin purified through the lentil lectin step was lyophilized and prepared for polyacrylamide gel electrophoresis as described in the methods section. Gels were 7½% acrylamide. Protein bands were revealed by staining with silver (Goldman et al., 1981).

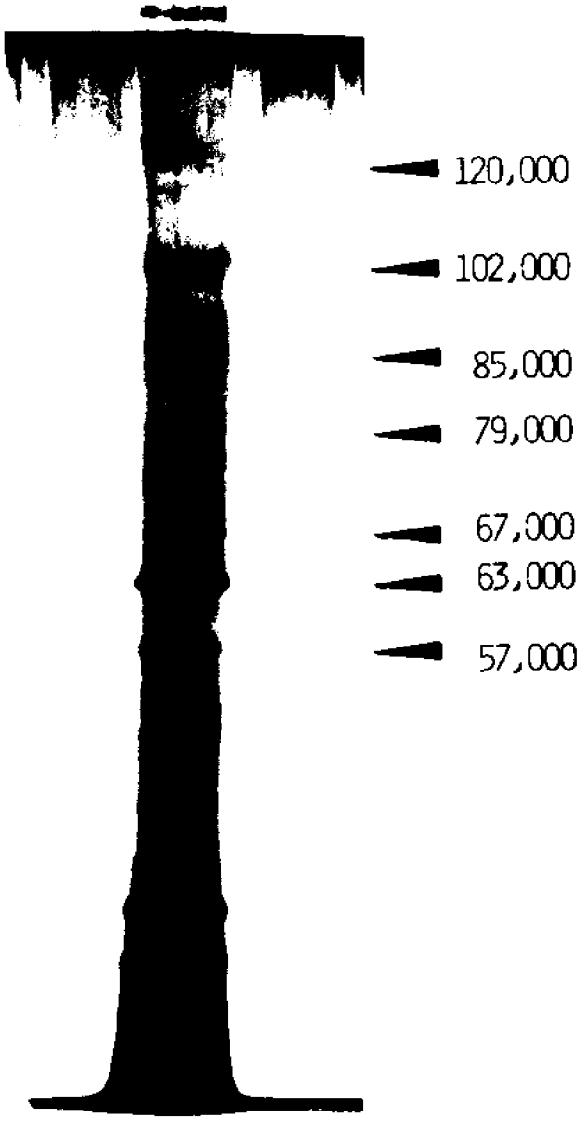
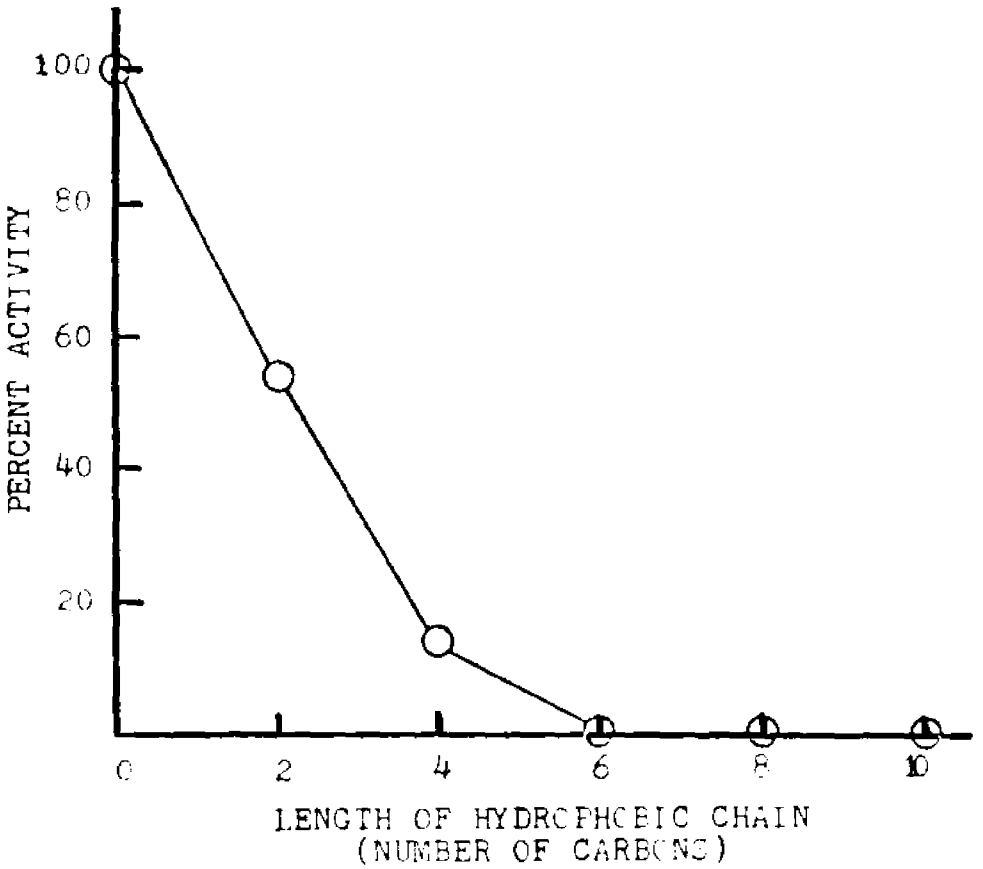

120,000
102,000
85,000
79,000
67,000
63,000
57,000

Figure 14. Carbohydrate bands present in purified α-agarotriose. The procedure was the same as in figure 13 except that the gel was stained for carbohydrate by the periodic acid-Schiff (PAS) method (Fairbanks et al., 1971).


120,000
102,000
84,000
77,000

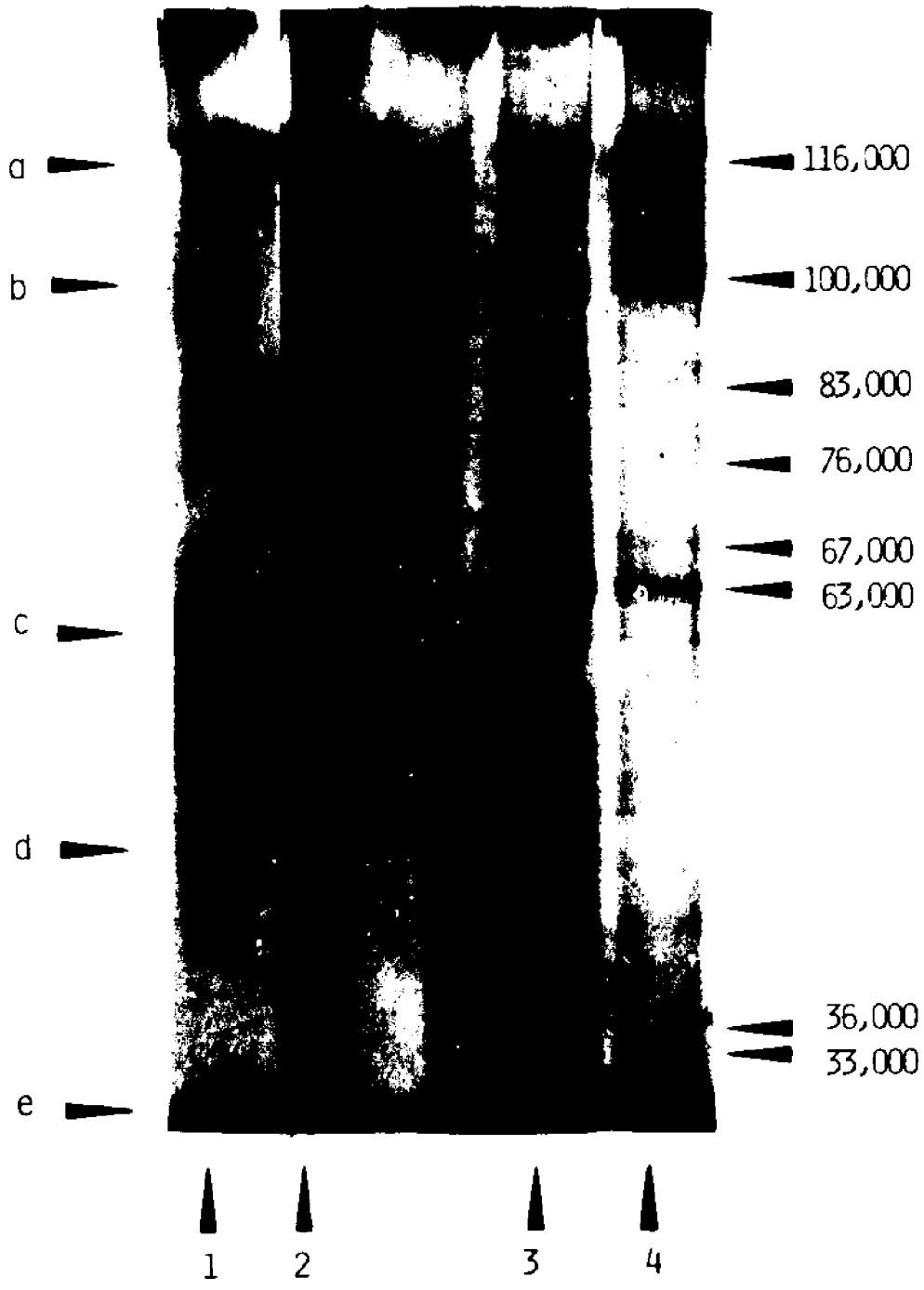
Figure 25. Hydrophobic affinity of the α-agglutinin molecule. Aliquots of α-agglutinin were applied to a series of affinity columns with the indicated hydrophobic chain lengths. The pass-through was assayed for total α-agglutinin activity, which was compared to that in the load.


100
80
60
40
20
PERCENT ACTIVITY
0
2
4
6
8
10
LENGTH OF HYDROPHOBIC CHAIN
(NUMBER OF CARBONS)

Triton X-100 was difficult to remove by dialysis and interferred with electrophoresis. The details of this procedure are described in the Methods section.

When the active pool from hexyl agarose was subjected to electrophoresis, it showed a banding pattern that was essentially identical to that obtained after lentil lectin (figure 26). The only difference was that the diffuse material of low molecular weight was removed to reveal two low molecular weight bands. Thus, there were a total of 8 protein bands, of apparent molecular weights: 116,000; 100,000; 83,000; 76,000; 67,000; 63,000; 36,000; and 33,000 daltons.

Figure 26. Protein bands present in hydrophobic affinity fractions of α-agglutinin. α-Agglutinin was subjected to polyacrylamide gel electrophoresis as described in the methods section. Lanes 1 and 2 contained the following standards: a, B-galactosidase, 116,000 daltons; b, phospherylase B, 97,400 daltons; c, bovine serum albumin, 66,000 daltons; d, ovalbumin, 45,000 daltons; e, carbonic anhydrase, 29,000 daltons. Lanes 3 and 4 contained α-agglutinin.


a
b
c
d
e
116,000
100,000
83,000
76,000
67,000
63,000
36,000
33,000
1
2
3
4

Discussion

I. The Sex Pheromones of *Saccharomyces cerevisiae*

A. Pherome induction of agglutinability.

Several of the experiments on the pheromonal induction of increased agglutinability were carried out in both cell types. A review of these results reveals distinct differences in the parameters of the two induction reactions as well as in their individual suceptibilities to inhibition. These results are summarized in Table X. Together, they suggest asymmetry in the mechanisms of action of α-factor and a-factor.

The fact that a-factor induction takes about three times as long as α-factor induction suggests that the mechanisms of induction are at least quantitatively different in a-cells and α-cells. The antibiotic experiments support this idea. Cycloheximide is a well known blocker of induction, but in this study, a difference was noted in the sensitivity of a-cells as compared to α-cells. Cycloheximide blocked the induction of a-cells when added along with the pheromone (α-factor), whereas induction of α-cells (by a-factor) was totally blocked only when the cells were preincubated with the drug.

TABLE X

Asymmetry of Pheromonal Induction of Increased Agglutinability

	α-Factor Induction	a-Factor Induction
Dose-Response	Concentrations ≥1.0 units/ml needed for maximal induction.	Concentrations from 0.001 to 0.10 units/ml needed for maximal induction.
Time Course	Induction is complete in 20 to 30 min. Cells induced 30 to 60 min. exhibit maximal induction.	Induction requires 60 to 90 min. Cells 90 to 120 min. exhibit submaximal induction.
Cycloheximide	Inhibits induction at 5 to 10 μg/ml and takes effect ≤5 min. after addition. Complete block when drug is added with pheromone.	Inhibits induction at 1 to 2 μg/ml and takes effect 30 min. after addition. Partial block of induction when added with pheromone.
Tunicamycin	Blocks induction at 50 μg/ml.	Partial block of induction at 50 to 100 μg/ml.

Barring possible differences in the absorption and/or action of the drugs in the two mating types, the following speculation can be made regarding pheromone induction. a-Cells alone seem to require ribosomal protein synthesis throughout the induction period. The glycosylation of at least one protein is important since tunicamycin fully blocked the induction of these cells (table I). This drug too, seems only to be able to create a partial block in α-cells (table II).

α-Cells on the other hand, might contain an intracellular pool of peptides that are mobilized or otherwise utilized during induction. If these molecules are being rapidly turned over, and need to be replenished by new synthesis during the course of induction, then a preincubation with antibiotic would be needed to deplete this pool and completely block a-factor induction of agglutinability in α-cells.

The inability of actinomycin-D to inhibit induction of a-cells suggests that synthesis of new mRNA may not be involved in α-factor effects (table III). The results with chloramphenicol demonstrate that, unlike Schizosaccharomyces, mitochondrial gene expression is not involved in Saccharomyces induction (table IV).

Figure 5 illustrates that impurities isolated along with α-factor by the method of Duntze et al. (1973) act antagonistically to the pheromone in the induction of

agglutinability. At high concentrations, these inhibitory substances block or reverse the induction by α-factor, and can lower agglutinability to levels below that of untreated cells. This effect is seemingly not present at low concentrations of pheromone, since α-factor lacking inhibitors due to isolation by a different method (Strazdis and MacKay, 1982) did not show any apparent increase in inducing ability at low doses. Such an increase was expected in the absence of inhibitors. Alternately, the proposed inhibitors may antagonize both morphogenesis (which was used to establish pheromone concentration) and induction of agglutinability equally. In either of these cases, the only change that would be observed upon the elimination of inhibitors would be the loss of inhibition at high concentrations. In any event, the possibility of competing activities is important to the interpretation of many classical studies (e.g. Bucking-Throm et al., 1973) in which similarly purified α-factor was used at very high concentrations. (50 units/ml).

B. The mechanism of α-factor action.

As described in the introduction, the G1 arrest induced by α-factor can be reduced or eliminated by the addition of cAMP or specific blockers of yeast cAMP phosphodiesterase (Liao and Thorner, 1980, 1981). Two of

these inhibitors, aminophylline and theophylline, inhibit induction of agglutinability, albeit at higher concentrations than reported for G1 arrest (figure 11). This difference in concentration may be easity explained by differences in the strains used. Yet cAMP caused only partial inhibiton. Since Liao and Thorner reported that cAMP was added at the same time as pheromone, it seems certain that this reagent entered the cells readily and blocked the intracellular drop in its concentration. The lack of complete inhibition in the case of agglutinability suggests that this effect is not mediated by cAMP per se. If this is true, then the effects of aminophylline and theophylline may be due to their inhibition of another enzyme than cAMP phosphodiesterase. In this case, cAMP may interfere by competing with the intended substrate of this enzyme.

The regulation of agglutinability is obvicusly complex. Such diverse factors as the composition of the growth medium, growth temperature, and culture density all affect the competence of cells to agglutinate. Once expressed, agglutinins are stable and permanent parts of the cell-surface. This is evidenced by their persistence and slow disappearance from the cell wall after zygote formation (Tohoyama et al., 1979).

In this study, it was found that digestion of a-agglutinins does not lower the ultimate level to which cells are induced (table V). This suggests that the induction of a-agglutinin is regulated by some perception

of the numbers of molecules already expressed. Conventional feedback control seems an unlikely mechanism for a molecule that is presumably firmly embedded in the external cell wall. Nonetheless, insertion of new agglutinins is regulated by the presence of molecules already deployed.

The experiment recorded in figure 13 gave a most unexpected result: cells cease to undergo morphogenesis if washed free of α-factor containing medium; they continue to increase in agglutinability in spite of such washing. This suggests that there is a tenuous and slow external interaction with pheromone, which can be disrupted, and a second interaction, that either cannot be disrupted or is completed very quickly. This is consistent with the existence of two different α-factor receptors for morphogenesis and agglutinability, exhibiting a weak and strong binding respectively. Alternately, there may be one receptor that triggers two response mechanisms, one of which requires continual stimulation.

If two or more totally different receptors (i.e. recognizing two different portions of the α-factor molecule) exist, then it might be possible to alter one recognition site on α-factor molecule such that a single effect is abolished.

Recently, a rather comprehensive series of modified α-factor peptides have been synthesized, and their

potencies in agglutinability induction and morphogenesis have been measured (Shenbagamurthi et al., 1983; Baffi et al., *in preparation*). The fidelity of certain amino acid side groups has been found to be critical to the biological activities of α-factor, but a peptide exhibiting only one activity (e.g. the ability to induce agglutinability but not morphogenesis) has not been found.

Nonetheless, the ratio of inducing ability to morphogenizing ability can be drastically changed by altering the α-factor molecule. Moreover, modified α-factor molecules that are inactive in both biological effects can block the effects of active peptides, presumably through competition for binding sites (i.e. receptors). Yet vastly different concentrations of competing peptide are needed in order to block the two different biological effects. Thus, it is actually possible to block the increase in agglutinabiliy without blocking morphogenesis. The conditions required to achieve this result are a low concentration of competitor combined with a high concentration of active peptide. It would seem then, that the receptor through which α-factor induces increased agglutinablity may be distinguishable from that mediating morphogenesis by virtue of a drastically lower K_m . The hypothesis of high and low afffinity α-factor receptors mediating increased agglutinability and morphogenesis respectively is fully consistent with the experiment reported in figure 13. The available evidence therefore, suggests that α-factor

elicits increased agglutinability and morphogenesis through mechanisms that are different from the time of recognition at the cell surface.

C. Pheromones in the mating reaction of *Saccharomyces cerevisiae*.

It has not, as yet, been definitively demonstrated that mating pheromone production or response is essential to mating (Thorner, 1981). Yet, pheromonal interactions clearly facilitate the mating process.

Reciprocal pheromone induction of agglutinability has sometimes been described as the first step of the mating process. This, however, is unlikely. Although relatively low concentrations of pheromone are needed (0.1 - 1.0 units/ml α-factor, and 0.01-1.0 units/ml a-factor) for maximal induction, these substances appear in these concentrations only as cells enter stationary phase (Scherrer et al., 1974) Obviously then, the observation of significant concentrations of pheromone throughout the environment of a cell can occur only in the laboratory.

The fact that wild type cells exhibit constitutive agglutination suggests that this may be the first step in the mating sequence. The purpose of this intial agglutination, brought about by random encounters between cells of the opposite mating type, might be to raise local

pheromone concentrations in the vicinity of the agglutinated cells. This would allow reciprocal induction of agglutinability without the need for the accumulation of large amounts of pheromones in the medium as a whole. That this sort of induction can occur is shown by the fact that cells in an agglutinated pellet, in the absense of cycloheximide, increase in agglutinability according to the amount of time that they are allowed to remain in such intimate contact (Lipke, unpublished). Of course, it is possible that a cell-contact mediated induction is being observed in this case. The potency of the hormonal system, however, suggests that it is the primary, if not the sole system of induction.

Several of the experiments in this study support the idea of pheromonal induction through cell proximity. Cells in both G1 and G2 are competent to induce (figure 12). Since the initial random collision, and subsequent constitutive agglutination is probably a rare event in nature, it is practical for all cells to be able to induce. In this way, intercellular associations will be immediately strengthened whenever they happen to form during the cell cycle. The fact that induction is rapid, especially in the case of $\underline{\alpha}$-factor, is consistent with this line of reasoning. Induction then seems most likely as the second step in the mating process.

Once a mating pair is tightly bound, the cells are certain to remain together until they reach late G1. Since localized pheromone concentrations remain high,

arrest will occur at "start". This would then be the next logical step in the mating sequence; it makes the cells fusion competent. Cell fusion, nuclear fusion, and the appearance of the first diploid bud follow in order. The events during mating are summarized in figure 27.

II. Sexual Agglutination

A. Demonstration of a single interacting system.

When either a-cells or α-cells alone are pheromonally induced, they agglutinate better with uninduced cells of the oposite mating type. The simplest model to explain this finding is one in which pheromone treatment results in an increased number of a single type of agglutination molecule (agglutinin) at the cell surface. In this case, the complement to each agglutinin will necessarily be present on the opposite cell type (figure 28,A).

If a new species of agglutinin is expressed in response to pheromone, its complement must already be present on its uninduced mate (figure 28,C). Alternately, a new species may appear, along with an increase in the old species. In this case, only one species need be initially present on each mating type, and the second system would come into play only when both cell types are induced (figure 28,C).

By analogy to Hansenula wingei, it would be expected that sexual agglutination in Saccharomyces is due to the reciprocal interaction of a single pair of agglutinins,

Figure 27. Mating Interactions in Saccharomyces cerevisiae. (1) When haploid cells of opposite mating type are mixed, mating interactions occur. (2) Since wild type cells are constitutively agglutinable, a chance encounter between cells may result in adherence. (3) This can lead to high pheromone concentrations in the vicinity of the cells. The first result of this will be the induction of increased agglutinability in a-cells (1½-2 hrs.), and α-cells (1½-90 min.). (4) These strengthened adhesions will more securedly be maintained, additional cells will more easily be included in the aggregate, and high pheromone levels will persist. (5)at 1½-2 hrs., G1 arrest is induced, and the cells become fusion competent. (6-7) Cell and nuclear fusion soon follow. (8) Finally, at about 2½ hrs., the first diploid bud emerges.

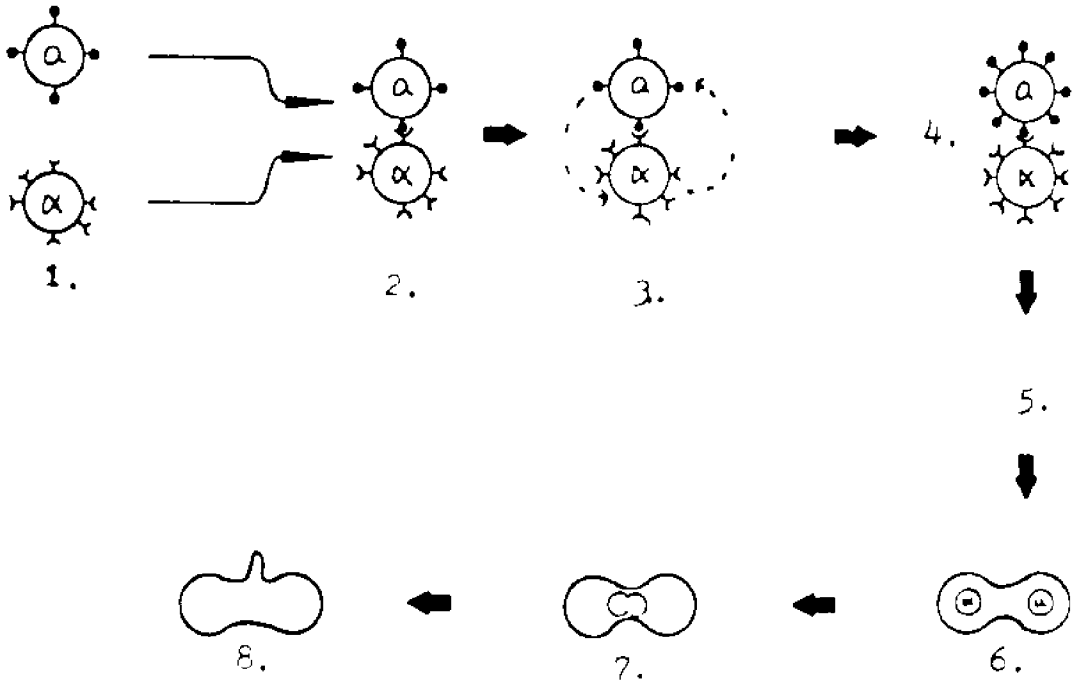
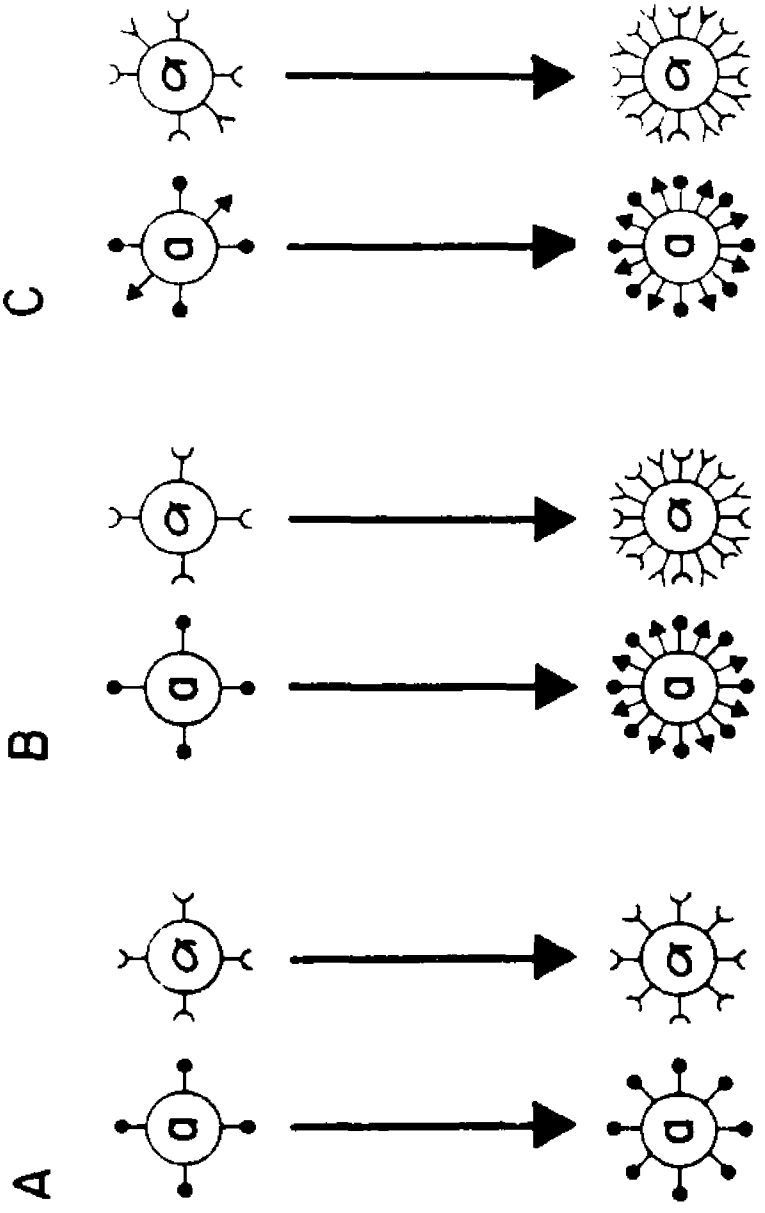

1.
2.
3.
4.
5.
6.
7.
8.

Figure 18. Model of neural cell recognition in Caenorhabditis elegans. Induction of either cell type alone results in increased aggregation. There are three models consistent with this theory that: (A) The simplest assumes that only one aggregation species is present on each cell type. Induction (denoted by the arrows) then causes an increase in the cell-surface expression of these molecules. Alternately, there may be two or more pairs of complimentary aggregins. (B) If only one pair is initially expressed, then induction must cause an increase in these original species, as well as the appearance of any new species. (C) If all aggregins are initially present, then one or all may be increased by induction.


C
B
A

one on each mating type. Fehrenbacher et al. (1978), however, reported a difference in the pH maxima for induced and non-induced a-cells. Kawanabe et al. (1979) described two types of agglutination, distinguishable by their senitivity to inhibition by Concanavalin A.

In this study, the pH differences reported previously could not be reproduced. It was found that uninduced and induced a-cells exhibited identical profiles across a wide range of pH, temperature and ionic strength. Con A was found to promote self-agglutination among both a-cells and α-cells. This makes it impossible to assess the effects of this lectin on mixed agglutination.

The proposal that only one species of agglutinin exists on each cell type rests on two types of evidence. First, all agglutinable combinations of cells are inhibited similarly by a wide range of inhibitors (table VI). This holds true for the complete titrations of agglutination with the inhibitors tested (figure 18). The effects of these latter substances were reversible at the minimum concentrations causing complete inhibition. Thus, if two or more agglutination systems are operating, the molecular interactions involved must be very similar.

The second line of evidence involves α-agglutinin. The molecule isolated from uninduced α-cells causes the progressive, dose-dependent inactivation of a-cells. It can totally inhibit a-cells, regardless of their state of pheromone induction or the state of the tester cells.

Thus, whatever is present in the α-agglutinin preparations is adequate to mask all binding sites on a-cells.

The kinetics of heat inactivation of agglutinin preparations is log-linear, indicating that a single molecular species is involved. This inactivation was measured in blocking the agglutination of induced a-cells to induced α-cells. The inactivation measured is therefore that of a single species that blocks all possible a-agglutinins. Since a single molecular species is competent to do this , the agglutination system must consist of a single pair of interacting molecules: a-agglutinin and α-agglutinin. Thus, the model for agglutination shown in figure 28A is the correct one.

B. The α-agglutinin molecule.

The agglutinins of Saccharomyces function in cell-cell interactions. Thus, their active sites at least, must be located on the external cells wall. The cell wall of yeasts consists almost wholly of two components: glucan and mannan (McMurrough and Rose, 1967). Glucan is a polymer of glucose containing both β1-6 and β1-3 linkages (Manners et al., 1973 a,b). Mannose is a glycoprotein that contains two types of polymannose chains: short chains O-linked to seryl or threonyl residues and long, branched chains attached to chitobiose, which is in turn N-linked to asparagine (Nakajima and Ballou, 1974). The side chains of the latter contain phosphodiesters, which

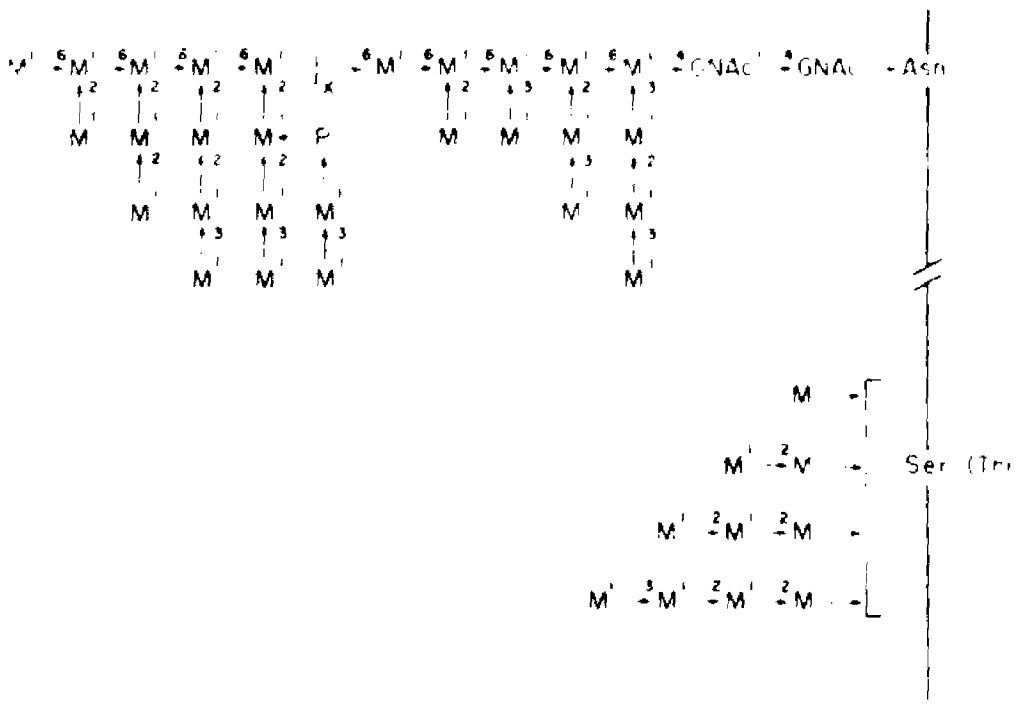
impart and overall negative charge to the cell wall. The structure of yeast mannan is illustrated in figure 29.

Specific amino acid sequences are an essential part of the active structure of agglutinins, as evidenced by the differential susceptibility of a-cells and α-cells to proteases (Shimoda et al., 1975). It is therefore likely that agglutinins are mannoproteins.

During purification, the α-agglutinin behaved accordingly. It bound to anion exchanges, and to lectins specific for mannose residues. Thus, mannan type side chains seem to be present. These were not needed for agglutinin function, however, since mnn2 mutants, which lack side chains, agglutinate normally (Lipke, unpublished).

If α-agglutinin binds to DEAE and to lentil lectin by virture of a major carbohydrate moiety, then it would be expected to stain by both the silver and periodic-acid Schiff (PAS) procedures. The fact that only the high molecular weight bands seem to contain carbohydrate was initially perplexing. It was interesting that such a variety of proteins could co-purify on a lectin specific for mannose containing carbohydrate. This was especially true in view of the fact that all of these bands resisted elution with 1M NaCl (with which the column was washed before elution). Thus, the interactions of all of these species with lentil lectin and/or each other was quite strong.

Figure 19. The structure of *Saccharomyces cerevisiae* mannan.



When α-agglutinin purified through lentil lectin was subjected to hydrophobic affinity chromatography on hexyl agarose, all of the previously observed protein bands eluted coincident with activity. The interaction between the molecule and this column is through the hexane side chains (as illustrated by figure 25). This interaction, therefore, is probably through a different portion of the molecule than is involved in DEAE or lectin affinity.

All of this suggests that the functional α-agglutinin consists of a number of peptides that are physically associated. Alternately, it is possible that many of the molecules observed are coincidentally released from α-cells during disruption and have a high affinity for the actual α-agglutinin.

The former of these hypotheses is, I believe, favored on the basis of several observations. First, α-agglutinin could not be purified by affinity chromatography on a-cells. This was an obvious technique to try, since the a-agglutinin on a-cell surfaces is a natural and readily available affinity reagent for α-agglutinin. Indeed, incubation of α-agglutinin with a large excess of a-cells caused the disappearance of solubilized agglutinin activity (table). Attempts to elute this activity by washing cells at high concentrations of salt, SDS, at high pH (9.0), or with a combination of the preceeding met with uniform failure. During such treatment, the a-cells would become competent to agglutinate once more, even under conditions of continual cycloheximide inhibition. Yet α-

agglutinin activity could not be recovered in the wash, even after extensive dialysis to remove the disruptive reagent. It is possible that any treatment capable of releasing the α-agglutinin from a-cells was also capable of disrupting its structure.

Second, soluble α-agglutinin activity is generally unstable. Although the agglutinability of intact cells could be restored by washing after treament with inhibitors, solubilized α-agglutinin was readily destroyed. The results of the a-cell affinity experiments, described above, are in accord with this. Note also the loss of activity at 30° C in table VI. The rapid inactivation at temperatures above 40° C has already been discussed, and contrasts with the heat stability of intact cells (table VIII).

Finally, the α-agglutinin behaved as a molecule of high molecular weight in gel filtration. Activity elutes very early when run on AcA-34 or Bio-Gel A.5m (figure 2). These have exclusion limits of 340 Kdal. and 500Kdal. respectively. The molecular weights of these proteins, as observed by electrophoresis, total 574 Kdal, which indicates a minimum molecular weight that is high enough to explain this behavior.

Thus, the α-agglutinin isolated by gentle methods appears to be a highly labile molecule consisting of a number of different subunits.

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