PRODUCTION, BIOASSAY, AND PARTIAL PURIFICATION OF
EROGENS FROM TREMELLA MESENTERICA FR.

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B. Sc., University of Guelph, 1969

A THESIS SUBMITTED IN PARTIAL FULFILMENT OF
THE REQUIREMENTS FOR THE DEGREE OF
DOCTOR OF PHILOSOPHY
in the Department
of
Botany

We accept this thesis as conforming to the
required standard

THE UNIVERSITY OF BRITISH COLUMBIA
June, 1973
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Department of Botany

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A defined medium containing glucose, ammonium sulphate, thiamine, salts and microelements supports good growth of *Tremella mesenterica* Fr. 2259-7. A high concentration of microelements selectively slows growth in media containing amino acids. L-asparagine causes slow and abnormal growth.

Conjugation hormones secreted by haploid cells of each mating type of *T. mesenterica* induce the growth of conjugation tubes from cells of the other mating type. The conjugation hormones (erogens) from strain 2259-7 can be extracted from aqueous solution with n-butanol, but not with less polar solvents. They are strongly adsorbed on activated charcoal, and on the polystyrene resin Porapak. The erogens are also adsorbed on both anion and cation exchange resins, partly by non-polar forces and partly by ionic forces. Ultrafiltration indicates that the molecular weight of the erogens is less than 750. Three active materials are separated by chromatography on silica gel columns with a gradient of water in ethanol, and two active components can be separated by thin layer chromatography.

A quantitative bioassay for erogen activity has been developed, based on the fraction of cells which produce conjugation tubes. An incubation time of 12 to 18 hours, at 20°C, pH 5.5, and low cell density with a complex nitrogen source is optimal for conjugation tube production. The effect of composition of the medium on production of erogens in 2259-7 cultures has been studied, and a defined medium giving high hormone yields has been selected. The erogens can be concentrated and partially purified from cultures by foaming. Attempts to purify the erogens have been hampered by low recoveries of hormone activity. The erogens may be amino acids or short peptides with non-polar side chains.
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ACKNOWLEDGMENT

I wish to thank Dr. R. J. Bandoni for providing laboratory facilities, for suggesting and encouraging the research reported in this thesis, and for reviewing the manuscript. I also thank Dr. C. O. Person, Dr. E. B. Tregunna, and Dr. G. H. N. Towers for generously making equipment available to me. The assistance of the Biology Data Center staff in preparing the figures is gratefully acknowledged. For their willing co-operation, and for their helpful suggestions after reading the manuscript, I am indebted to the members of my committee. The National Research Council of Canada provided financial support.

I would also like to thank my wife, Shirley, for her constant encouragement, and for technical and secretarial assistance.
INTRODUCTION
Hormonal control of sexual reproduction in fungi has been frequently reviewed; for example, by Raper (1952, 1967), Köhler (1967), Machlis and Rawitscher-Kunkel (1967), Barksdale (1969), and Machlis (1966, 1972).

Machlis (1972) has discussed the terminology used to describe sexual hormones, and has proposed three new words to indicate their biological activity: an "erotactin" is a substance causing chemotactic attraction of motile gametes, an "erotropin" causes chemotropic growth of some sexual structure, and an "erogen" induces or controls the differentiation of sexual structures.

Three genera of fungi have been investigated in detail, and, within the last five years, structures have been established for hormones from each of these—sirenin, the erotactin from Allomyces, antheridiol, an erogen and erotropin from Achlya, and the trisporic acids, erogens from Mucor and related fungi. Also, an erotactin from the brown alga Ectocarpus siliculosus (Müller et al 1971), and a conjugation-initiating substance from the ciliate Blepharisma (Kubota et al 1973) have been identified.

The hormones which have been studied in the Mucorales are erogens inducing the differentiation of progametangia (zygophores) and erotropins directing the growth of compatible gametangia towards one another. The physiology of sex in the Mucorales has been reviewed by van den Ende and Stegwee (1971).

Burgeff (1924) found that if compatible strains of Mucor mucedo were grown on opposite sides of a collodion membrane, progametangia were induced and attracted to one another across the membrane. He postulated that progametangia-inducing and -attracting substances were secreted into the medium by each strain. Burgeff's experiments were repeated by Verkaik (1930) and Kehl (1937). Similar studies with similar results were performed on Phycomyces blakesleeanus by Ronsdorf (1931), and on Pilobolus crystallinus by Krafczyk (1931, 1935).
Banbury (1954) supported Burgeff's hypothesis by showing that the medium from a mated culture of *M. mucedo* would induce progametangia in an unmated plus strain, although not in the minus strain. Plempel (1957), by growing the mated cultures under vigorous aeration, produced culture filtrates that were effective on both plus and minus strains. He found that filtrates from single strain cultures were not morphogenetically active, but culture media in which mycelia of the two mating types had been grown sequentially could induce progametangia in unmated cultures of the first-grown strain. On this basis, Plempel (1963) suggested that each strain continuously produces a mating type specific "progamone". In the presence of a progamone from the opposite mating type, each strain produces a "gamone", which induces progametangia only in the opposite mating type. Plempel attempted to isolate the gamones from mated cultures of *Mucor mucedo*. The progametangium-inducing activity of a solution was bioassayed by placing the test solution in a well cut in the agar just ahead of the mycelial front of a plus or minus colony. After four hours, counts were made of the number of progametangia developed in the vicinity of the well. The number of progametangia per unit area was linearly related to the dose up to approximately one microgram of gamone per ml of test solution. Although he purified the active material extensively and obtained it in crystalline form, he was unable either to separate the plus-gamone from the minus-gamone or to assign a structure. Plempel also found that mated cultures of *Blakeslea trispora* and *Phycomyces blakesleeanus*, and, less strongly, *Rhizopus nigricans* and *Absidia spinosa* produced factors active in the *Mucor mucedo* bioassay, and indistinguishable from *M. mucedo* gamone. Recently, Ueyama (1972), has reported that a methanol extract of a mated culture of *Absidia glauca* induces gametangial initials in single strain cultures of that fungus.

Van den Ende (1967) isolated from mated cultures of *Blakeslea trispora* a material which induces progametangia in *Mucor*
mucedo, and suggested that it might be trisporic acid C. Trisporic acids A, B, and C (Fig. 1) had earlier been isolated from culture fluids of B. trispora and characterized by Caglioti et al (1967) and Cainelli, Grasselli, and Selva (1967). Detailed chemical comparison confirmed the identity of the progametangium inducing factors from B. trispora with trisporic acids B and C (van den Ende 1968). Austin, Bu'Lock, and Gooday (1969) also isolated trisporic acids B and C from Mucor mucedo. Trisporic acids B and C have been synthesized (Edwards et al 1971) and the synthetic materials were found to be biologically active in both mating types of M. mucedo.

Plempel's concept of two mating-type specific gamones has been questioned by van den Ende (1968, van den Ende and Stegwee 1971) and by Gooday (1968). Trisporic acids B and C are capable of inducing progametangia in both mating types of Mucor mucedo (van den Ende et al 1970). Reschke (1969), however, reports that the methyl esters of trisporic acids B and C were active only on the minus strain. Bu'Lock, Drake and Winstanley (1972) have confirmed this observation, and shown that minus cultures of B. trispora can hydrolyse the methyl esters to free trisporic acid much more than the plus strain. If Plempel's two gamones do exist, it seems probable that each gamone is converted to trisporic acid by the strain able to respond to it, and the trisporic acid is responsible for progametangium induction. This hypothesis explains why Plempel (1963) isolated only material active in both mating types (presumably trisporic acid) from mated cultures—the gamones were being converted to trisporic acids as fast as they were produced.

The interactions between the two mating types leading to the synthesis of trisporic acids in M. mucedo and especially B. trispora have been studied intensively. Trisporic acid production proceeds readily in mated cultures, but is negligible in single cultures of either mating type (van den Ende
Antheridiol

Fig. 1. Structure of some sexual hormones from fungi.
et al 1970). In B. trispora, a low rate of trisporic acid synthesis is found when mycelium of either mating type is incubated in culture fluid from the other mating type (Sutter 1970, Sutter, Capage, and Harrison 1971). Radioactive tracer studies have shown that precursor from the first strain is being converted to trisporic acid by the second mycelium.

Van den Ende, Werkman, and van den Briel (1972) have confirmed that each strain of B. trispora secretes small amounts of precursor material capable of inducing progametangia in the opposite mating type of M. mucida and trisporic acid synthesis in the opposite mating type of B. trispora. This low rate synthesis is not sensitive to cycloheximide. Using mycelia grown on C^{14}-glucose, they found that both plus and minus strains contribute carbon to trisporic acid synthesis in mated cultures. An increase in the amount of plus mycelium present increases the rate of trisporic synthesis by minus mycelium; the rate of synthesis by the plus mycelium depends on the intensity of synthetic activity of the minus strain. The high rate of synthesis in mated cultures was inhibited by cycloheximide or 5-fluorouracil (van den Ende et al 1970, van den Ende, Werkman and van den Briel 1972). Recently Werkman and van den Ende (1973) have clarified the role of precursor exchange between the two strains in trisporic acid biosynthesis. Labelled precursor material from single cultures of each mating type was partially purified by solvent extraction and thin layer chromatography, and shown to be converted to radioactive trisporic acid by the other mating type. One of the precursors from the plus strain may be 3-methyl-1-(2',6',6'-trimethyl-3'-oxo-cyclohexen-1'-yl)-octa-1,3-dien-7-one which Bu'Lock, Drake and Winstanley (1972) have found as a degradation product of methyl trisporate C. In addition Werkman and van den Ende (1973) developed spectrophotometric methods for measuring the amounts of plus and minus precursors present, and showed that the rate of precursor synthesis by each mating type was stimulated by trisporic acid. The stimulatory effect of trisporic acid is negated by 5-fluorouracil.
It appears, then, that the low rate of trisporic acid synthesis in culture filtrates is caused by transformation of the small amounts of precursor constitutively produced by each mating type. The high rate in mated cultures is caused by positive feedback by trisporic acid on precursor production. The sensitivity of trisporic acid stimulation of precursor formation to inhibitors of protein and RNA synthesis suggests that enzyme derepression is involved.

Besides inducing progametangia, trisporic acids stimulate carotenoid synthesis in minus cultures of _B. trispora_ (Thomas and Goodwin 1967, Thomas et al 1967, van den Ende 1968, Sutter and Rafelson 1968). This effect of trisporic acids is also inhibited by cycloheximide, and it has been suggested that trisporic acids act as derepressors of an enzyme which is rate-limiting for carotenoid synthesis. Austin, Bu'Lock and Drake (1970) have demonstrated the incorporation of radioactivity from \( \beta \)-carotene, retinyl acetate, and mevalonic acid into trisporic acids B and C. Whether or not trisporic acid is stimulating precursor synthesis through its effect on carotenoid synthesis is not yet clear. As van den Ende and Stegwee (1971) point out, the transformation of exogenous \( \beta \)-carotene to trisporic acids does not prove that \( \beta \)-carotene is the natural substrate for the biosynthesis of trisporic acid and its precursors.

Feofilova (1970) has found that \( \beta \)-ionone and acetic acid decrease trisporic acid synthesis and increase carotenoid synthesis in mated cultures of _B. trispora_. Also both insufficient and excessive aeration depress the yield of trisporic acid.

The mutual attraction of compatible zygophores (zygotropism) was also studied by Banbury (1954, 1955). He demonstrated repulsion of like progametangia and attraction of compatible progametangia between mycelia which were not in liquid contact, and concluded that the tropic stimulus was airborne. Plempel and his associates (Plempel 1960, Plempel and Dawid 1961, Plempel 1962) were unable to demonstrate diffusion of zygotropic
substances through agar despite many attempts. When cultures of the two mating types bearing zygophores were separated by a mica membrane with small holes in its aerial section, the zygophores grew toward and through the holes. Exposure to zygotropic factors increased the growth rate of the zygophores. Once again, air-borne zygotropic factors were implicated.

In Allomyces, the male gametes are vigorously motile, but the female gametes are sluggish and remain close to the female gametangia after their discharge. Machlis (1958a, b, c) has shown that the male gametes are attracted to the female gametangia because of the secretion by the female gametangia of an erotactin, sirenin. From crosses between A. arbuscula and A. macrogynus, Machlis obtained predominantly male and predominantly female hybrid strains. A female strain was used to produce sirenin and a male strain to produce male gametes for use in the bioassay. Sirenin activity was bioassayed by placing the test solution in a cup with a dialysis membrane bottom, immersed in a suspension of male gametes. The number of gametes which settled on a unit area of the membrane was measured as the response. Sirenin concentrations as low as \(10^{-10}\) molar caused noticeable attraction of the sperm. The male gametes respond to sirenin by swimming up the concentration gradient, and irreversibly remove sirenin from their environment. The original bioassay apparatus contained several sample cups, and the response to a given sirenin concentration depended on the level of hormone in the other cups to which the gametes were exposed (Carlile and Machlis 1965). Machlis (1969) has described a single sample bioassay apparatus to eliminate this interaction.

By processing 200 litres of culture fluid a week, Machlis et al (1966) succeeded in isolating 2.5 grams of sirenin. The structure (Fig. 1) was deduced by Nutting, Rapoport and Machlis (1968). Racemic sirenin was synthesized by Corey, Achiwa and Katzenellenbogen (1969), Plattner, Bhalerao, and Rapoport (1969,
Bhalerao, Plattner, and Rapoport (1970), Grieco (1969), Mori and Matsui (1969), and Corey and Achiwa (1970). The synthetic material was found to have erotactic activity on the same order of magnitude as natural sirenin. Plattner and Rapoport (1971) achieved the synthesis of the pure enantiomers d- and l-sirenin (l-sirenin is the natural product) and determined the absolute configuration of l-sirenin. Machlis (1972) reports that d-sirenin is not biologically active, and does not interfere with l-sirenin.

J. R. Raper has demonstrated an elaborate system of hormonal control in the oogamous sexual reproduction of Achlya (summarized by Raper 1952). He postulated the action of diffusible sexual hormones from his observations of the course of the sexual reaction (Raper 1939) and then provided experimental evidence for four separate hormones. The female mycelia produce hormone A, which induces antheridial hyphae in male mycelia. Male mycelia with antheridial hyphae produce hormone B, which stimulates the formation of oogonial initials on female mycelia. The oogonial initials attract the antheridial hyphae, and cause their tips to differentiate into antheridia. Raper considered that these two actions were performed by a third hormone, C, produced by the oogonial initials. After antheridia develop, they produce hormone D, which induces basal septation of the oogonia, and the formation of oospheres. Fertilization tubes from the antheridia then penetrate the oogonia, and grow to and fertilize the oospheres. Although the growth of the fertilization tubes is probably also hormonally controlled, Raper could not present any experimental evidence on this point (Raper 1940).

In a more detailed study of hormone A, a bioassay was developed, based on the number of antheridial hyphae formed in response to the hormone (Raper 1942a). Raper and Haagen-Smit (1942) attempted to isolate hormone A. From 1440 litres of culture filtrate, they obtained 0.2 mg of material which, although still impure, induced antheridial hyphae at a dilution
of $10^{-13}$. The male plants were found to produce a factor, designated A', which augmented the response to hormone A (Raper 1942b). He also found that female plants secreted a second hormone, A$^2$, insoluble in acetone, which induces antheridial branching, or increases the response to hormone A (acetone soluble). Male plants secrete hormone A$^3$, acetone soluble, which decreases the response to A or A$^2$ or A plus A' (Raper 1950a). Homothallic species of *Achlya* displayed a hormonal system similar in principle to that of heterothallic species (Raper 1950b).

Barksdale (1963a) found that strains of *Achlya* which responded to hormone A by producing antheridial hyphae removed hormone from the medium, while unresponsive strains did not. The hormone could not be recovered by extraction of the mycelium with acetone. Barksdale (1963b) also showed that polyvinyl or polystyrene plastic particles on which partially purified hormone A had been adsorbed, would attract antheridial hyphae and cause antheridia to form, in a manner similar to oogonial initials. Thus the actions attributed by Raper to hormone C can be accomplished by localized sources of hormone A. The stimulatory effect of hormone A' in the presence of A can be mimicked by adjusting the nutrient level (Barksdale 1970).

McMorris and Barksdale (1967) isolated from female plants of *Achlya bisexualis* 10 milligrams of crystalline hormone A, which they named antheridiol. Arsenault et al (1968) from spectroscopic evidence, suggested a structure (Fig. 1). Edwards et al (1969) synthesized a mixture of antheridiol and 22,23-isoantheridiol. Later they accomplished the synthesis of pure antheridiol and determined the absolute configuration (Edwards et al 1972). McMorris and collaborators have developed a synthetic method giving moderate yields of stereochemically pure antheridiol (McMorris and Seshadri 1971, McMorris, Arunchalam, and Seshadri 1972). Green et al (1971) have also isolated 23-deoxyantheridiol from a strain of *A. bisexualis*. This molecule does not appear to have any ergogenic activity.
In preliminary studies, Barksdale (1969) has found that hormone B is secreted by male plants at the end of the growth phase upon stimulation by antheridiol. This hormone can be extracted from culture fluids with methylene chloride, and shows similar chromatographic properties to antheridiol. No reports have yet appeared on the chemistry of hormones A², A³, or D.

A significant fraction of the cell wall polysaccharides in Achlya is cellulose. Antheridiol induced branching of male mycelia is correlated with a rise in endocellulase activity (Thomas and Mullins 1967, 1969) and an increase in oxygen uptake rate (Warren and Mullins 1969). The inducibility of cellulase is high in mycelia from the late log and plateau phases of growth, but low in early log mycelia. Incubation in medium from late log cultures increases inducibility of cellulase in early log cultures (Warren and Sells 1971). Branching in response to antheridiol requires an exogenous supply of carbon, nitrogen and energy. High nitrogen levels favour vegetative branching over antheridial branching (Barksdale 1970).

Besides the relatively well studied systems described above, sexual hormones have been postulated or demonstrated, but not fully characterized in several other fungi. Evidence has been found for a hormonal system similar to that operating in Achlya in two other water molds—Sapromyces reinschii (Bishop 1940) and Dictyuchus monosporus (Sherwood 1966), although these systems have not been studied in the same detail as Achlya.

Considerable attention has been paid to the possibility of hormonal control of conjugation between haploid strains of Saccharomyces cerevisiae. Levi (1956) claimed that agar on which conjugation had occurred could induce copulatory processes in cells of the minus mating type. He was unable to obtain an active culture filtrate, or to consistently demonstrate diffusion of the inducer across a membrane.
Other investigators have used cell expansion as an indicator of sexual response. Duntze, MacKay and Manney (1970) reported the partial purification of a factor, possibly an oligopeptide, from the $\alpha$-mating type which caused elongation and inhibition of budding in cells of the $\alpha$-mating type. Yanagishima (1969) isolated from each mating type a steroidal substance which induced cell expansion in the other mating type. Actinomycin D, chloramphenicol, cycloheximide, and trans-cinnamic acid inhibited the response. The action of the hormone from the $\alpha$-strain was simulated by testosterone, and estradiol showed $\alpha$-hormone activity (Yanagishima et al, 1969). Stationary phase cultures of *S. cerevisiae* can be separated by density gradient centrifugation into hormone-sensitive (pellet) and hormone-insensitive (surface) cells (Yanagishima and Shimoda, 1970). Treatment of $\alpha$ cells with $\alpha$ hormone, or testosterone, stimulated secretion of a substance, insoluble in methylene chloride, which specifically expanded $\alpha$ cells (Yanagishima, 1971). After mating $\alpha$ and $\alpha$ strains, the release of sugar and protein from the cells into the medium rapidly increased. The increase in autolytic activity preceded the appearance of conjugating cells (Shimoda and Yanagishima, 1972). It has been suggested that the cell-expanding effect of the sexual hormones is caused by activation of cell wall degrading enzymes (Yanagishima and Shimoda, 1973). The sexual agglutinability of certain $\alpha$-type strains is increased by incubation with a heat stable principle from $\alpha$ cultures (Sakai and Yanagishima, 1972).

Bistis (1956, 1957, Bistis and Raper, 1963) has uncovered a series of hormones controlling plasmogamy in the discomycete *Ascobolus stercorarius*. In this fungus, mycelia of each mating type produce both the female organs, ascogonia, and the male elements, oidia, but are self-sterile. Each strain produces a hormone which sexually activates oidia from the other mating type. Activated oidia stimulate the development of ascogonia on the compatible mycelium, and chemotropically attract the trichogyne (the apex of the ascogonium). In addition to the
oidia, pieces of the vegetative mycelium can be sexually activated to act as males (antheridia). The trichogynes are attracted to and will fuse with activated oidia or antheridia of either mating type. Finally, after plasmogamy, the ascogonium seems to stimulate hyphal branching in its vicinity, and chemotropically attract the branches to form a sheath around itself. Raper (1967) has postulated a minimum set of seven hormones to account for these observations.

In another ascomycete, Glomerella, some strains are self-fertile (homothallic) and others are self-sterile (heterothallic). Perithecia are produced along the interface between two mated mycelia in crosses in which at least one of the partners is self-fertile. Some pairs of strains interact more weakly than others. When a pair of weakly-interacting strains is mated in the presence of dialysate from a strain which crosses vigorously with one of the partners, a strong reaction is obtained (Markert 1949). McGahen and Wheeler (1951) observed that in crosses between homothallic and heterothallic strains, the perithecial initials always arise on the homothallic hyphae at or near the point where the hypha is crossed by a heterothallic hypha. Later, Driver and Wheeler (1955) showed that culture filtrates from homothallic strains stimulated the development of perithecia in the self-sterile strains. All of these results suggest that perithecial initiation is controlled by diffusible hormones, but the exact nature of the interaction between the strains is not clear.

Machlis (1966) has reviewed evidence for hormonal action in the copulation of the gametes of Synchytrium endobioticum (Chytridiales) and for chemotropic attraction of trichogynes to spermatia in Bombardia lunata.

In many of the mycelial ascomycetes and basidiomycetes, plasmogamy occurs by fusions between vegetative hyphae. Buller (1933), after studying hyphal fusions in many fungi, concluded that hyphae secreted substances which could induce lateral branching in adjacent hyphae and chemotropically guide the
branches to meet tip-to-tip. Raper (1952) suggested that all participating hyphae secrete and react to a single substance. If each hypha secreted one factor and responded to another, an infinite number of substances would need to be postulated to account for the apparent capacity of hyphae to fuse with any other hypha, sometimes of a different species. In cases where the hyphal fusion brings together sexually compatible nuclei, the branch inducing and attracting factors could be called sexual hormones.

In *Schizophyllum commune*, Ahmad and Miles (1970) have shown that chemotropic attraction of hyphal tips occurs only in compatible matings, and a greater proportion of hyphal encounters result in fusion when the two hyphae have different alleles at the A incompatibility locus than when they have the same A. When compatible strains are grown one above the other, separated by cellophane, they are activated in some manner so that subsequent matings, even between strains of the same mating type, have a high fusion frequency. The substance which diffuses between the strains, and enhances the ability of the hyphae to fuse can be considered a sexual hormone.

It seems desirable to extend Machlis's (1972) definition of the term "erogen" to include substances which cause physiological sexual activation of their target cells (as in *Ascobolus* and *Schizophyllum*) as well as those which control sexual morphogenesis.

In heterobasidiomycetes with yeast-like haploid phases, copulation of sexually compatible cells occurs by the initiation, growth, and fusion of filamentous conjugation tubes. Bauch (1925) noted that conjugation tubes were only formed in *Ustilago bromivora* when compatible strains were grown together. He suggested that each strain produces a hormone which induces conjugation tubes in the opposite mating type. Despite several attempts he was unable to obtain conjugation tube induction with cell-free culture fluids.
Bandoni (1963) analyzed the mating system of *Tremella mesenterica*, and found a modified tetrapolar pattern. The formation of conjugation tubes is controlled by a genetic locus with two alleles, A and a. In mixtures of A and a cells, conjugation tubes are produced, but in mixtures of A with A, or a with a strains, only vegetative growth ensues. Establishment of a stable dikaryon with clamp connections depend on heterozygosity at a second locus, B, with multiple alleles. For convenience in this discussion the B locus will be ignored, and mating type will refer to A or a.

Conjugation tubes are induced when strains of different mating types are grown on opposite sides of a cellophane membrane, or when cells of one mating type are inoculated on a dialysis print from the other mating type (Bandoni 1965). This is evidence for production by each mating type of diffusible erogens which specifically induce conjugation tubes in the other mating type. Bandoni showed that the hormones were produced in the absence of the other mating type, and were not destroyed by autoclaving, or by storage at room temperature for periods up to five days. Diffusible conjugation hormones have also been demonstrated in *Tremella subanomala* and *T. encephala* by Flegel (1968) and in *T. bambusina* by Brough (1970).

Similar studies in *Rhodosporidium toruloides* showed that conjugation tubes are produced when the two compatible strains are grown separated by cellophane. However, no hormone activity could be found in dialysis prints or cell-free culture fluid of either single or mated cultures (I. Reid, unpublished data). Apparently the conjugation hormones in this species are either very unstable, or produced only when both mating types are present, and then efficiently scavenged from the medium by the reacting strain. The morphological similarity of conjugation in other species of *Rhodosporidium*, and in the genera *Sporobolomyces*, *Leucosporidium*, *Tilletia*, and *Ustilago* to conjugation in *Tremella* suggests that conjugation hormones are acting in these fungi as well (R. J. Bandoni, pers. comm.).
Flegel (1968) showed in *T. mesenterica* that hormone must be continuously present to maintain conjugation tube growth; if hormone is removed, the cells revert to budding. He suggested that the conjugation hormones were acting as inducers or repressors of enzymes involved with cell wall metabolism.

Bandoni (1965) observed that compatible conjugation tubes of *T. mesenterica* usually met tip-to-tip—changing growth direction if necessary to accomplish this. He suggested that the tips are chemotropically attracted to each other over a range of about 15 microns. Similar chemotropism of conjugation tubes in *T. bambusina* is reported by Brough (1970). Raper (1967) has questioned whether the chemotropic attraction was due to a separate erotropic hormone, or to a high concentration of the erogenic conjugation hormone, in analogy with hormone A in *Achlya*.

J. A. Berry and T. W. Flegel began studies on the isolation and characterization of the erogen from *Tremella mesenterica* strain 2259-6. Although they did not pursue this study very far, they felt that the hormone might be a peptide (Berry and Flegel, unpublished data). R. J. Bandoni undertook preliminary studies on the hormone from strain 2259-7 of the other mating type, with inconclusive results (Bandoni, pers. comm.).

The objective of the present study was to isolate and chemically characterize the erogen from strain 2259-7 of *Tremella mesenterica* Fr. This goal has not been reached, but some information about the chemical nature of the hormone has been accumulated, and methods for producing, assaying, and partially purifying the erogenic activity have been developed.
GENERAL MATERIALS AND METHODS
I. Cultures

The organisms used in this study were two sexually compatible, haploid strains of *Tremella mesenterica* Fr. isolated and identified by Dr. R. J. Bandoni. Culture RJB # 2259-7 (UBC # 559-7), mating type AB\(_{III}\) (Bandoni 1963) was used to produce conjugation hormones, and RJB # 2259-6 (UBC # 559-6), mating type aB\(_{II}\), to assay hormone activity.

2259-6 was included by Bandoni (1963) in his original study on conjugation in *T. mesenterica*, and also in his demonstration of conjugation hormones (Bandoni 1965). Both 2259-6 and 2259-7 were studied by Flegel (1968) in his investigation of conjugation in *Tremella*.

Stock cultures were maintained on Malt Yeast Peptone (MYP) slants at 4°C, and were transferred at least once every six months.

All cultures were grown at 20°C, unless otherwise specified.

II. Media

Chemicals used in media were obtained from Mallinckrodt Chemical Works, Fisher Scientific Company, The Nichols Chemical Co., and J. T. Baker Chemical Co., and were of reagent grade. Vitamin-free salt-free casein hydrolysate, and thiamine hydrochloride were purchased from Nutritional Biochemicals Corp. Bacto Soytone, Bacto Malt Extract, and Bacto Yeast Extract came from Difco Laboratories. Agar was obtained from Difco, or K & S Laboratories, Vancouver, or Nutritional Biochemicals Corporation.

Water was glass distilled once and stored in a polyethylene carboy.

The composition of certain frequently used media are listed in Appendix A. Recipes for other media are given with the account of the associated experiment.
CHAPTER ONE

THE NUTRITION AND GROWTH OF TREMELLA MESENTERICA 2259-7
Materials and Methods

I. Nitrogen source

A basal glucose-salts-thiamine medium (BM) was prepared according to the recipe:

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose</td>
<td>6.0 gm</td>
</tr>
<tr>
<td>KH$_2$PO$_4$</td>
<td>1.0 gm</td>
</tr>
<tr>
<td>MgSO$_4$·7H$_2$O</td>
<td>0.5 gm</td>
</tr>
<tr>
<td>CaCl$_2$·2H$_2$O</td>
<td>0.1 gm</td>
</tr>
<tr>
<td>NaCl</td>
<td>0.1 gm</td>
</tr>
<tr>
<td>Microelement stock solution</td>
<td>1.3 ml</td>
</tr>
<tr>
<td>Thiamine</td>
<td>100 μg</td>
</tr>
<tr>
<td>Distilled water</td>
<td>1 litre</td>
</tr>
</tbody>
</table>

Separate 100 ml lots of basal medium were supplemented with 0.2 gm of one of the following nitrogen sources: potassium nitrate, ammonium nitrate, L-asparagine, vitamin-free, salt-free casein hydrolysate, Bacto Soytone. One lot was supplemented with 0.2 gm of asparagine plus 0.2 gm of soytone. The media were dispensed in 50 ml portions into 250 ml Erlenmeyer flasks. The flasks were capped with a double thickness of aluminum foil, and autoclaved for 15 minutes at 15 psi pressure.

Each flask was inoculated with 2.0 ml of a suspension of 2259-7 cells which had been centrifuged from a two day old culture in Growth Medium #3 (GM3) and washed with two 100 ml portions of sterile distilled water.

To determine the relation between turbidity and amount of cells present, 1/5, 2/5, 3/5, 4/5 and then 1/25, 2/25, 3/25, and 4/25 dilutions of the inoculum suspension were prepared and their turbidity measured. The cells in a sample of the 1/25 dilution were counted in a haemacytometer.

The inoculated flasks were placed on a reciprocating shaker operating at 100 strokes per minute with a travel of 3.5 cm, in a 20°C incubator room. After 24 hours, a 2 ml
sample was taken from each flask with a sterile pipette, and the flasks were returned to the shaker. The samples were diluted with 2 ml of distilled water, and their absorbance measured at a wavelength of 560 nanometers in a Bausch and Lomb Spectronic 20 Spectrophotometer using distilled water as a blank.

Samples were taken at 24-hour intervals until 216 hours after inoculation, and their turbidity measured as above.

This experiment was repeated with the following modifications. Only 0.13 ml of the microelement stock solution was added per litre of basal medium. Ammonium sulphate at 2 gm/l was included as an eighth treatment. Each lot of medium was adjusted to pH 4.5 with 0.1 N hydrochloric acid after adding the nitrogen source. The inoculum consisted of washed cells from a stationary phase culture of 2259-7 in Gluc-AmS medium. Samples were taken daily from each flask, and diluted 1 to 10 with distilled water before measuring their turbidity at 640 nm. After 7 days, the pH of each culture was measured with a Radiometer pH meter model 28.

II. Vitamins

The growth of 2259-7 in synthetic media containing thiamine as the only added vitamin demonstrated that no other vitamins are required by this strain under the conditions employed. To test the necessity of thiamine, a medium similar to the basal medium plus casein hydrolysate described above, but omitting thiamine, was prepared. In this and subsequent experiments only 0.13 ml of microelement solution was added per litre of basal medium. Two flasks containing 50 ml of this medium were supplemented with 5 micrograms each of thiamine hydrochloride, and along with two vitamin-free flasks, inoculated with 1 ml each from a 3-day culture of 2259-7 in GM3. After four days on the shaker, growth in the flasks was assessed visually, and a sample from each flask checked microscopically for contamination.
III. A growth curve for 2259-7

A more precise growth curve for 2259-7 was determined using a Coulter Counter.

Three 200 ml portions of GM3 in one litre Erlenmeyer flasks capped with Bellco stainless steel flask closures were inoculated with 2.0 ml each from a 3-day culture of 2259-7 in GM3, and incubated as described above. Two ml samples were taken from each flask after six hours and daily thereafter for eight days.

The samples were diluted with 0.9% sodium chloride to bring the cell concentration between 40,000 and 100,000 cells per millilitre. The saline diluent provided the electrolyte needed by the Coulter Counter. Blank counts were carried out on the dilution fluid each day and subtracted from the sample counts. Dilutions used ranged from 1 to 20 to 1 to 2000.

Cell counts were made on a Coulter Counter Model B fitted with a 100 micron aperture. The amplification was set at 1/2, aperture current at 1/2, the lower threshold at 7.5 and the upper threshold at infinity. From each sample, duplicate dilutions were prepared, and four 0.5 ml portions of each duplicate were counted. The reported values have been corrected for background and coincidence, and averaged.

IV. Effect of sodium acetate on pH drift

Growth of 2259-7 on the medium containing ammonium was accompanied by a drop in pH. The possibility of stabilizing the culture pH by including sodium acetate in the medium was therefore tested.

Basal medium containing 2 grams of ammonium sulphate and 20 milligrams of bromocresol green per litre was prepared. Five aliquots of this medium were supplemented with 0, 1, 2, 3 or 4 grams of sodium acetate trihydrate per litre. The pH of each medium was adjusted with 5 N hydrochloric acid until the colour of the indicator matched the green of the medium without sodium acetate (pH 4.5). The media were then dispensed in two 20 ml
replicates into 125 ml Erlenmeyer flasks, capped with foil, and autoclaved. After cooling, each flask was inoculated with 1.0 ml of a 5 day old shake culture of 2259-7 grown in medium containing 1 gm/l of sodium acetate trihydrate. After five days incubation on the shaker at 20°C, the colour of the indicator in each flask was noted.

The experiment was repeated, substituting bromocresol purple for bromocresol green in the media with 3 or 4 gm/l of sodium acetate.

In a third experiment, a series with 0, 2.2, 2.4, 2.6 or 2.8 grams of sodium acetate per litre of BM plus ammonium sulphate and bromocresol purple was prepared. These media were adjusted to an initial pH of 5.5, autoclaved and incubated as above.
Results

I. Nitrogen source

A volume of .04 microlitres in the haemacytometer contained 603 cells. This corresponds to $1.5 \times 10^7$ cells per millilitre. The relation of the absorbance at 560 nm to the concentration of cells is presented in Fig. 2. A plot of the $A_{560}$ against the logarithm of the cell concentration is show in Fig. 3. Growth curves, in terms of culture turbidity, for 2259-7 in BM plus various nitrogen sources, are illustrated in Fig. 4. At the end of the experiment, microscopic examination did not reveal any contamination of the cultures. In the media containing asparagine, casein hydrolysate, or asparagine plus soytone, some of the cells were extra-ordinarily large, spherical, and vacuolate. Doubling times were calculated from the sections of the growth curves where growth was approximately exponential (Table I).

Table I. Doubling times for *Tremella mesenterica* 2259-7 in basal medium (high microelements) plus various nitrogen sources at 20°C.

<table>
<thead>
<tr>
<th>Nitrogen Source</th>
<th>Doubling time (hours)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Potassium nitrate</td>
<td>$&gt;216$</td>
</tr>
<tr>
<td>Ammonium nitrate</td>
<td>12 1/2</td>
</tr>
<tr>
<td>Asparagine</td>
<td>59</td>
</tr>
<tr>
<td>Casein hydrolysate</td>
<td>47</td>
</tr>
<tr>
<td>Soytone</td>
<td>27</td>
</tr>
<tr>
<td>Soytone plus asparagine</td>
<td>49</td>
</tr>
</tbody>
</table>

A standard curve of absorbance at 640 nm against concentration of 2259-7 cells is presented in Fig. 5. The growth curves obtained in the second experiment are shown in Fig. 6. Doubling times calculated from the exponential phase of growth, and the final pH of the cultures with each nitrogen source are listed in Table II.

The cells grown with casein hydrolysate and especially asparagine showed the "swollen" morphology noticed in the first experiment.
Fig. 2. The relation of turbidity at 560 nm to concentration of *T. mesenterica* 2259-7 cells.
Fig. 3. Regression of turbidity on the logarithm of cell concentration.
Fig. 4. Growth curves for *T. mesenterica* 2259-7 in BM with high microelements plus various nitrogen sources at 20°C.
Fig. 5. Relation of turbidity at 640 nm to concentration of *T. mesenterica* 2259-7 cells.
Fig. 6. Growth curves for \( \textit{L. mesenteroides} \) 2293-7 in BM with low microelements plus various nitrogen sources at 20°C.

**Figure 6**

**Absorbance at 640 nm**

- **Abscissa:** Time from inoculation (hours)
- **Ordinate:** Absorbance

- **Basal medium**
- **Potassium nitrate**
- **Ammonium nitrate**
- **Ammonium sulphate**
- **Aspartagine**
- **Soytome**
- **Soytome hydrolysate**
- **Casein hydrolysate**

**Legend:**
- Xs indicate data points.
Table II. Doubling times and final pH's for cultures of 2259-7 grown in basal medium (low microelements) plus various nitrogen sources at 20°C.

<table>
<thead>
<tr>
<th>Nitrogen Source</th>
<th>Doubling time (hours)</th>
<th>Final pH</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>---</td>
<td>4.1</td>
</tr>
<tr>
<td>Potassium nitrate</td>
<td>4.1</td>
<td>4.1</td>
</tr>
<tr>
<td>Ammonium nitrate</td>
<td>13.3</td>
<td>2.3</td>
</tr>
<tr>
<td>Ammonium sulphate</td>
<td>12.8</td>
<td>2.4</td>
</tr>
<tr>
<td>Asparagine</td>
<td>38</td>
<td>4.7</td>
</tr>
<tr>
<td>Casein hydrolysate</td>
<td>15</td>
<td>3.9</td>
</tr>
<tr>
<td>Soytone</td>
<td>14.7</td>
<td>4.0</td>
</tr>
<tr>
<td>Soytone plus asparagaine</td>
<td>30</td>
<td>4.4</td>
</tr>
</tbody>
</table>

II. Vitamins

After four days, the flasks with added thiamine were very turbid; the flasks without thiamine were almost clear. Under the microscope, the cultures showed only the yeast cells typical of Tremella.

III. Growth curve

The number of cells of 2259-7 per millilitre is plotted on a logarithmic scale against time in Fig. 7. In the exponential phase of growth, the doubling time was 9 1/4 hours.

IV. Effect of sodium acetate on pH drift

After 5 days incubation, the indicator in the cultures with 0, 1, or 2 gm/l of sodium acetate trihydrate had turned yellow (pH below 4.0). In the cultures with 3 or 4 gm/l of sodium acetate trihydrate, the indicator was blue (pH above 5.5). In the second experiment, the bromocresol green in the cultures containing 0, 1, or 2 gm/l of sodium acetate turned yellow. The bromocresol purple in the flasks with 3 or 4 gm/l of sodium acetate trihydrate was purple, indicating a pH above 7.

In the third experiment, cultures without sodium acetate turned yellow as before; flasks with 2.2 gm/l and 2.4 gm/l were greenish yellow and greenish purple respectively; flasks with 2.6 gm/l or 2.8 gm/l were purple.
Fig. 7. A growth curve for T. mesenterica 2259-7 in GM3 at 20°C.
Discussion

The turbidity of microbial cultures depends on both the number and size of the suspended cells, and therefore, is more closely related to the concentration of cellular dry weight in the culture than to cell numbers (Mallette 1969). The relation between turbidity ($A_{560}$) and concentration of the cells in 2259-7 cultures in the first experiment (Fig. 2) was markedly non-linear. A plot of the turbidity against the logarithm of cell concentration (Fig. 3) is more nearly a straight line, particularly when the absorbance is greater than 0.4. In this range, a doubling of cell concentration corresponds to an increase in absorbance of 0.326.

A plot of culture turbidity against time (Fig. 4) is therefore equivalent to a plot of the logarithm of cell concentration against time (at least in the upper two-thirds of the graph). Exponential growth of cell dry weight should result in a linear increase in turbidity. Examination of Fig. 4 shows that each growth curve does include a linear portion. The slopes of these straight sections were used to calculate doubling times.

In the second experiment, the culture samples were diluted ten times before measuring the turbidity, so that the turbidity would be proportional to cell concentration (Fig. 5). Logarithms of the turbidity readings were used to calculate doubling times.

In both experiments, the basal medium without added nitrogen supported a small increase in culture turbidity. The nitrogen used for this growth may have been introduced with the inoculum, or may have come from impurities in the chemicals used or contamination of the glassware. In the first experiment, but not in the second, slightly more growth was found in the medium with potassium nitrate than in the basal medium. These data indicate that nitrate can be used only very slowly, if at all, by *Tremella mesenterica* 2259-7. The pH of these media
before autoclaving was 4.5. At the end of the experiment, the measured pH was 4.1. This pH drop may have been caused by the inoculum, which was grown in Gluc-AmS and had a pH of 2.3 before washing.

In the second experiment, growth on ammonium nitrate and ammonium sulphate showed similar initial rates, slightly faster than on soytone and casein hydrolysate. A higher peak turbidity was reached in ammonium sulphate medium than in ammonium nitrate medium, possibly because \((\text{NH}_4)_2\text{SO}_4\) contains more ammonium than \(\text{NH}_4\text{WO}_3\). In media containing ammonium, the culture pH fell drastically. The growth rates in ammonium nitrate medium in the first and second experiments were comparable. However, in the first experiment, growth on soytone was much slower than on ammonium nitrate, and growth on casein hydrolysate was still slower. The differences in growth rates between these two experiments may have been either effects of pH, or of the microelement concentration. In the first experiment, the media containing soytone and casein hydrolysate had initial pH's of 6.5. In the second experiment the initial pH of all media was adjusted to 4.5. Flegel (1968) has shown that the optimum pH for growth of \(2259-7\) in a medium containing glucose, soytone and yeast extract is about 4.7, with progressively slower growth at pH's 5.7 and 7.2. In the first experiment, because of an arithmetic error, the concentration of microelements was ten times as high as intended. The importance of microelements in determining the growth rate is suggested by the doubling time of 9 1/4 hours in GM3 (pH 6.5, no added microelements). In both experiments the growth rates in media with asparagine or soytone plus asparagine are slower than in media with casein hydrolysate or soytone. Also the fraction of cells with "swollen" morphology is high in media containing asparagine. Asparagine appears to interfere with the normal metabolism of \text{Tremella} in some way leading to slow growth and large cells. A high concentration of microelements enhances the inhibition of growth by asparagine.
It is interesting that the high concentration of microelements increases doubling times in media containing amino acids, but not in media containing ammonium. Possibly the amino acids complex heavy metal ions, and thus increase their rate of entry into the cells.

In this study, no special precautions were taken to rid the medium or glassware of trace amounts of vitamins. Therefore, I cannot claim that thiamine is the only vitamin required by $T. \text{mesenterica}$ 2259-7. However, the clear response to omission of thiamine from the medium suggests that the methods used were capable of detecting major vitamin requirements. Also, 2259-7 has been subcultured on synthetic media containing thiamine as the only added vitamin for at least 20 transfers, without decrease in growth rate. Therefore, vitamin carry over in the inoculum is not obscuring any vitamin requirements.

Flegel (1968) reports, without further comment, that no nitrogen source simpler than casein hydrolysate supported growth and conjugation in $T. \text{mesenterica}$ 2259-6 and 2259-7. The results presented here refute the part of his statement dealing with growth. Brough (1970) found that $T. \text{bambusina}$ grew well on ammonium as the sole nitrogen source, but not on nitrate. He also found that thiamine was required by that fungus. Therefore the nitrogen and vitamin nutrition seems to be similar in these two species.

Growth of fungi on media containing ammonium salts of strong acids results in a decrease in the pH of the medium, often to inhibitory levels. This pH drop can be prevented by including a neutral salt of a readily metabolized organic acid in the medium (Nicholas 1965). This is the case in $T. \text{mesenterica}$—sodium acetate trihydrate at about 2.3 grams per litre maintains the culture pH close to 5.5. Smaller amounts of sodium acetate allow the pH to drop and larger amounts cause the pH to rise as growth proceeds.

No explicit explanation of the effect of ammonium and organic acids on the culture pH have appeared in the liter-
ature. It is known, however, that in many fungi the cell membrane is freely permeable to the uncharged species, NH$_3$, (ammonia), and to undissociated organic acids, but impermeable to NH$_4^+$ or to ionized carboxylic acids (Burnett 1968). NH$_3$ is present in the medium because of the equilibrium:

\[ \text{NH}_4^+ = \text{NH}_3 + \text{H}^+ \]

Uptake of NH$_3$ by the cells drives this equilibrium to the right, resulting in an accumulation of hydrogen ion in the external medium, and a drop in pH. Organic acids exist in equilibrium between the undissociated and ionized forms:

\[ \text{RCOOH} = \text{RCOO}^- + \text{H}^+ \]

Uptake of undissociated acid by the cells drives this equilibrium to the left, and thus removes hydrogen ion from the external medium, and raises the pH.

The pH change in a culture containing both ammonium and organic acid will depend on the relative quantity of each taken up by the cells. Since the cell membrane is crossed by diffusion, the rate of uptake will depend on the concentration of the uncharged species, which is a function of pH. A decrease in culture pH decreases the concentration of NH$_3$ and increases the concentration of RCOOH. Therefore, more RCOOH and less NH$_3$ will be taken up by the cells, and the pH will tend to rise. As the pH rises, the concentration of NH$_3$ increases and the concentration of RCOOH decreases. More NH$_3$ and less RCOOH will be taken up, and the pH will tend to fall. Thus changes in pH will be self-correcting. The exact pH maintained will depend on the ratio between the concentration of ammonium (NH$_4^+$ plus NH$_3$) and the concentration of organic acid (RCOOH plus RCOO$^-$). This model correctly predicts that too little organic acid salt will allow the pH to drop, and too much will cause the pH to rise. Active transport of H$^+$ across the cell membrane, for example in exchange for K$^+$ (Rothstein 1965) will complicate the predictions of this model.
CHAPTER TWO

PRELIMINARY STUDIES ON THE EROGEN
Materials and Methods

I. A qualitative bioassay

For preliminary studies on the chemical behaviour of the erogen, a bioassay that would reveal the presence or absence of hormone activity was required.

First, an assay procedure originated by R. J. Bandoni was used. Petri plates of Conjugation Medium (CjM) solidified with 1.5% agar were streaked lightly with 2259-6, and incubated at 20°C. After 24 hours, blocks approximately 1 cm by 1 cm were cut from the plates, and deposited cell side up in plastic petri plates, 5 cm in diameter. To each assay dish, 4.0 ml of sterile liquid CjM, along with an appropriate volume of test solution (usually one to ten drops) was added. The assays were incubated overnight at 20°C, and then the cells on the agar blocks were examined under the microscope for the presence of conjugation tubes.

Later, an assay requiring less of the test solution and eliminating the agar blocks was developed. One ml of an appropriate dilution of the test solution in CjM or 0.5% soytone was pipetted into a 16 X 125 mm test tube, capped with a stainless steel or plastic culture tube closure, and autoclaved for 15 minutes at 15 psi pressure. After cooling, the assay tubes were inoculated with one drop from a sterile Pasteur pipette of a two to four day old shake culture of 2259-6 in Gs medium. The tubes were mixed by gentle shaking, and incubated overnight (12 to 18 hours) at 20°C. The next morning, a drop from each assay tube was examined under the microscope for the presence of conjugation tubes. A subjective impression of the intensity of the reaction, based on the proportion of cells bearing conjugation tubes, the length of the tubes, and the number of tubes per yeast cell, was used to roughly estimate the relative amounts of hormone in the test solutions.

Similar results were obtained from the two assay methods.
II. Production of hormone

Conjugation hormone for study was produced by growing 2259-7 in shake culture at 20°C. Media used at various times included MYP, CjM, and GM3. Inoculum was obtained either from shake cultures in the exponential or early stationary phases of growth, or by scraping cells from the surface of Petri plate cultures on MYP. The cultures were allowed to grow into stationary phase and the cells were removed by centrifugation. The supernatant was either used directly or treated with an equal volume of acetone to precipitate polysaccharide and residual cells.

III. Extraction with organic solvents

The supernatant from a culture of 2259-7 in GM3 was divided into three portions. The pH of the first portion was found to be approximately 6 by measurement with pHdrion indicator paper. The second portion was adjusted to pH 2 with concentrated hydrochloric acid, and the third portion to pH 10 with concentrated ammonium hydroxide. Ten ml samples of the culture supernatant at each pH were shaken with ten ml of one of n-butanol, ethyl acetate, diethyl ether, chloroform, benzene, methylene chloride, or petroleum ether (65-110). Persistent emulsions formed in some of the extractions, and in these cases separation of the phases was hastened by centrifugation. The solvent extracts and the aqueous raffinates were separated, and evaporated to dryness in vacuo (Buchler rotary evaporator, water bath temperature 40°C). Each residue was redissolved in 10 ml of CjM, and bioassayed, along with a sample of the original culture supernatant.

To estimate the partition coefficient of the erogens between water and n-butanol, a counter current distribution was carried out.

A sample of erogens for the distribution was obtained by extracting 150 ml of the supernatant from a culture of 2259-7 in GM3 with four 50 ml portions of n-butanol (saturated with water). The butanol phases were centrifuged briefly to remove
droplets of the aqueous phase, and then evaporated to dryness in vacuo. The extract was dissolved in 2.0 ml of water-saturated n-butanol. After the phases separated, the butanol phase was transferred as completely as possible with a Pasteur pipette into a second tube. Two ml of water (saturated with butanol) was added to the second tube and 2.0 ml of n-butanol (saturated with water) was added to the aqueous phase in the first tube. Both tubes were then shaken. After the phases separated, the butanol phase in the second tube was added to 2.0 ml of water in a third tube. The butanol phase from the first tube was transferred to the second tube, and 2.0 ml of butanol were added to the first tube. This procedure was repeated until ten tubes were in use. For each tube, a filter paper disc of the type used for antibiotic assays (Schleicher and Schuell, Inc.) was saturated with the butanol phase and dried in a stream of warm air. A suspension of 2259-6 cells was evenly spread over the surface of GS medium solidified with 1.5% agar in a Petri plate. The paper discs were placed on top of this lawn of cells, one disc in the center and four equally spaced around the edge of each plate. After overnight incubation at 20°C, the cells adjacent to the paper discs were examined under the microscope for conjugation tubes.

IV. Adsorption on cation exchange resin

Two hundred millilitres of supernatant from a culture of 2259-7 in GM3 were run through a bed of 20 ml of Amberlite IR-120 strong acid cation exchange resin, hydrogen form (Mallinckrodt Chemical Works), at a flow rate of 200 ml/hour. A sample of the effluent was neutralized with 10% sodium bicarbonate solution and saved for assay. The resin was washed with 50 ml of distilled water and then treated with 100 ml of 10% aqueous ammonia. The ammonia eluate was evaporated to dryness in vacuo, dissolved in 5 ml of distilled water, and re-evaporated. The eluted material was finally dissolved in 10 ml of distilled water, and diluted 1 to 10 in GM3 for bioassay. The resin was regenerated by running through 200 ml of distilled water, 100 ml of 2 N sulphuric acid, and another 200 ml of distilled water.
In another experiment, culture supernatant was run through the column, and the resin washed as above. A concentrated ammonium acetate buffer, pH 7, was prepared by mixing 100 ml of glacial acetic acid with 100 ml of concentrated (30% NH₃) ammonium hydroxide. Quarter-strength and half-strength buffers were prepared by dilution from the concentrated solution. One hundred ml of quarter-strength ammonium acetate was run though the column, followed by 100 ml of the half-strength buffer. Finally 100 ml of the full strength ammonium acetate solution was passed through the column. The three eluates were freeze-dried to remove ammonium acetate. The residues were dissolved in 10 ml of distilled water, and diluted 1 to 4 with GM3 for bioassay.

In a third experiment, after treating the resin with culture supernatant and washing, the column was eluted with a pyridine acetate solution (25 ml glacial acetic acid, 10 ml pyridine, 65 ml distilled water; approx. 1.75 N, pH 5). The pyridine acetate eluate was freeze-dried. The eluted material was dissolved in 10 ml of distilled water and diluted 1 to 10 with GM3 for bioassay.

V. Adsorption on anion exchange resin

The supernatant from 200 ml of a 3-day culture of 2259-7 in GM3 was adjusted to pH 7 with a 10% solution of sodium bicarbonate, and then run through a 20 ml bed of Duolite A-4 weak base anion exchange resin, chloride form (Diamond Shamrock Chemical Co.). Samples of the original culture supernatant and the column effluent were taken for bioassay. The resin was washed with 50 ml of distilled water, and then with 100 ml of 1 N acetic acid. The acetic acid changed the colour of the resin from grey to yellow. Just before the yellow front reached the bottom of the column, a fresh receiver was put in place. The acetic acid eluate was evaporated *in vacuo*, dissolved in 10 ml of distilled water, and re-evaporated. The residue was dissolved in 10 ml of distilled water, and diluted 1 to 10 with GM3 for bioassay.
The column was regenerated by washing with 200 ml of distilled water, 100 ml of 2 N potassium hydroxide, 200 ml of distilled water, 100 ml of 2 N hydrochloric acid, and 200 ml of distilled water.

VI. Adsorption on activated charcoal

One gram of Norit A decolourizing charcoal (British Drug Houses, Canada Ltd.) was mixed with 400 ml of supernatant from a culture of 2259-7 in GM3. The charcoal suspension was agitated on a reciprocating shaker at 20°C for half an hour to ensure that adsorption equilibrium was reached. Then one gram of Hyflo Super Cel filtering aid was added to the suspension, and the charcoal filtered out on a Buchner funnel which had been coated with one gram of Hyflo Super Cel. The filter cake was washed with two 10 ml rinses of distilled water, and then suspended in 50 ml of methanol. This suspension was shaken for 15 minutes, and centrifuged. The methanol supernatant was decanted and the charcoal suspended in 50 ml of absolute ethanol. After shaking for 15 minutes, the suspension was centrifuged, the ethanol decanted, and the charcoal suspended in 50 ml of acetone. After the charcoal had been centrifuged from the acetone, it was treated with a pyridine acetate solution (10 ml of pyridine, 25 ml of glacial acetic acid, made to 50 ml with distilled water). The methanol, ethanol, and acetone eluates were evaporated to dryness in vacuo, and the residues dissolved in 5 ml of distilled water. The pyridine acetate eluate was freeze-dried, and then dissolved in 5 ml of distilled water. Samples of the original culture supernatant, and the filtrate from the charcoal adsorption step were bioassayed at their original concentrations. The solutions of the materials eluted with methanol, ethanol, acetone and pyridine acetate were diluted 1 to 20 with GM3 for bioassay.

On another occasion, 20 ml of culture supernatant was treated with 0.5 gm of Norit A. After half an hour of shaking, the charcoal was sedimented by centrifugation. The supernatant was decanted and the charcoal washed with 20 ml of distilled
water. The charcoal was then washed with 20 ml of acetone-water (1 to 1), 20 ml of acetone-water (3 to 1), and 20 ml of acetone. The eluates were filtered to remove suspended carbon, evaporated to dryness in vacuo, and dissolved in 2 ml of GM3 for bioassay. The original culture supernatant, and the supernatant from the charcoal treatment were also bioassayed.

Granular coconut charcoal (Fisher Scientific Co.) was tested for ability to adsorb the hormone. Fifty ml of culture supernatant were shaken for 15 minutes with 0.1 gm of coconut charcoal. The charcoal was centrifuged out, and the supernatant after sampling, treated with another 0.1 gm of coconut charcoal. This charcoal was also centrifuged down, and the supernatant treated with a third 0.1 gm of charcoal. Samples of the original spent culture medium, and the supernatant from the first, second, and third coconut charcoal treatments were bioassayed.

One gram of coconut charcoal was wet with 10 ml of acetone, and, after the charcoal had settled, the acetone was decanted. The charcoal was washed twice with 40 ml of distilled water. Fifty ml of culture supernatant were treated as above with three 0.1 gm portions of the acetone washed charcoal, and the supernatants bioassayed.

Deactivated charcoal was prepared by a modification of the method of Asatoor and Dalgliesh (1956). A suspension of 10 gm of Norit A in a solution of 0.4 gm of stearic acid, U.S.P., in 50 ml of ethanol was stirred for half an hour, and then slowly diluted with 450 ml of distilled water. The charcoal was collected by filtration, washed with distilled water, and air dried.

Forty ml portions of 2259-7 culture supernatant were shaken with 0.1 gm of Norit A, 0.1 gm of deactivated charcoal, or 0.5 gm of deactivated charcoal. After 30 minutes the suspensions were centrifuged and samples of the supernatants taken for bioassay. The charcoal sediments were washed with 40 ml each of distilled water and then suspended in 20 ml each of
10% aqueous phenol. After 15 minutes these suspensions were centrifuged and the supernatants were decanted and evaporated to dryness in vacuo. These eluates were each dissolved in 5 ml of distilled water, and diluted 1 to 4 with 0.5% soytone for bioassay.

VII. Adsorption on Porapak

One gram of Porapak QS, 120-150 mesh (Waters Associates, distributed in Canada by Chromatographic Specialties, Belleville, Ontario) was suspended in acetone, and poured into a miniature chromatography column made from a Pasteur pipette fitted with a glass wool plug. The acetone was allowed to drain off, and the resin was washed with 50 ml of distilled water. Ninety ml of supernatant from a culture of 2259-7 in Gluc-AmS medium were passed through the column at a flow rate of approximately 2 ml per minute. Samples of the original culture medium and the effluent from the column were supplemented with 0.05 ml of 10% (w/v) soytone per ml, neutralized with 0.1 N potassium hydroxide, and bioassayed. The column was washed with 10 ml of distilled water, and then eluted with 10 ml of 50% acetone (5 ml water plus 5 ml acetone), 10 ml of 75% acetone, and 10 ml of acetone. For bioassay, 3 drops of each eluate solution was added to 1 ml of 0.5% soytone.

A similar experiment was carried out using Porapak Q.

VIII. Chromatography on Sephadex G-10

Thirty grams of Sephadex G-10 (Pharmacia) were soaked for three hours in distilled water and then poured into a glass chromatography column (2 cm internal diameter). The stopcock at the bottom of the column was opened, so that water was flowing through the column while the gel settled. To calibrate the column, a solution containing 0.1 gm of soluble starch (Difco Laboratories) and 0.1 gm of sodium chloride per ml was prepared. When the water level in the Sephadex column reached the gel surface, 1 ml of the starch-salt solution was
applied to the top of the column and washed in with a small volume of distilled water. Distilled water was passed through the column at 0.4 ml per minute and the effluent was collected in twenty 10 minute fractions. A 0.5 ml sample of each fraction was treated with a drop of Melzer's reagent (iodine 1.5 gm, potassium iodide .5 gm, chloral 100 gm, water 100 ml) to detect starch, and a separate 0.5 ml sample was treated with a drop of 0.1 N silver nitrate to detect chloride ion.

A sample of conjugation hormone was prepared by extraction of culture supernatant with n-butanol. The butanol extract was evaporated to dryness in vacuo, and dissolved in water. Two ml of this solution were applied to the top of the Sephadex G-10 column, and eluted with distilled water at a flow rate of 0.4 ml per minute. The effluent was collected in forty-five 5 minute fractions. A 0.5 ml sample from each fraction was mixed with 0.1 ml of 10% soytone and 1.5 ml of distilled water, and bioassayed.

IX. Ultrafiltration

Two hundred ml of acetone were added with stirring to 200 ml of culture supernatant, and the precipitate which formed was removed by filtration. The filtrate was evaporated to dryness in vacuo and redissolved in 15 ml of distilled water. This solution was filtered through a Pellicon type PSWP membrane filter (Millipore Ltd.), 25 mm in diameter, under suction from a water aspirator. The Pellicon filter partially retains solutes with a molecular weight greater than 750 and completely retains materials with a molecular weight greater than 1250 (Millipore Application Report AR-21). Filtration of the 15 ml sample took one and a half hours. The solution was washed through the filter with two 1 ml portions of distilled water. The filter apparatus was dismantled, and the materials retained on the filter were washed into a beaker with a stream of water from a wash bottle. The solution of retained material was made up to 15 ml with distilled water. The filtrate and the solution of retained materials were diluted 1 to 10 with GM3 for bioassay.
X. Chromatography on columns of silica gel

Fifty grams of silica gel, 60 to 120 mesh (BDH Chemicals Ltd.), were slurried in absolute ethanol and poured into a chromatography tube, 2 cm in diameter. The silica gel was allowed to settle, and the solvent drained until the top of the bed was just dry. 0.2 grams of freeze-dried n-butanol extract from 2259-7 culture supernatant was ground in a mortar with 2 ml of absolute ethanol. This suspension was pipetted onto the top of the column, and the solvent allowed to sink into the column, followed by washings from the mortar. The column was eluted with 100 ml of absolute ethanol at a flow rate of 1.95 ml per minute. The effluent was collected in 2 minute fractions. After 100 ml of ethanol had passed into the column, elution was continued at the same flow rate with 100 ml of 87.5% ethanol (87.5 ml absolute ethanol and 12.5 ml distilled water), followed by 100 ml of 75% ethanol and finally 100 ml of 50% ethanol. One hundred fractions were collected.

From each even-numbered fraction starting with 8, six drops were adsorbed on a one-half inch diameter paper disc. The discs were dried and placed on a plate of GS agar spread with 2259-6 cells, six discs per plate. After overnight incubation at 20°C, the presence or absence of conjugation tubes on the cells close to each paper disc was noted.

Another column containing 50 gm of silica gel in absolute ethanol was prepared as above. 0.3 gram of hormone preparation was ground with 2 ml of absolute ethanol and transferred to the top of the column. The column was eluted with a convex gradient of water in ethanol. To generate the gradient, 400 ml of absolute ethanol were placed in a 500 ml Erlenmeyer flask on a magnetic stirrer. The flask was fitted with a rubber stopper bearing inlet and outlet tubes. The inlet tube was connected to a reservoir containing 50% ethanol. The outlet tube extended nearly to the bottom of the mixing flask, and was connected with polyethylene tubing to the top of the chroma-
ography tube. Solvent was fed to the column at 1 ml per minute, and the column effluent was collected in 5 minute fractions. The even-numbered fractions from 6 to 134 were bioassayed by the paper disc method as above.

XI. Paper chromatography

On various occasions during this study, the behaviour of the hormone in paper chromatography has been examined. Chromatography was carried out on strips of Whatman #1 chromatography paper 2 cm by 14 cm. Sufficient hormone solution to allow detection of the hormone after chromatography was streaked along a line 2 cm from one end of the strips. The bottom ends of the strips were cut to a point. Each strip was suspended, from a paper clip stuck into a cork, inside a 25 X 150 mm test tube so that the pointed bottom end of the strip was immersed in a pool of solvent at the bottom of the tube. Compositions of the solvents used are listed in Table III. After the solvent had risen approximately 10 cm above the start line, the strips were removed from the tubes and dried in a stream of cool air. The parts of the strips between the start line and the solvent front were cut perpendicular to the long axis into ten equal sections. The sections were either placed on plates of GS agar spread with 2259-6 cells, or dropped into test tubes containing 1 ml of GS medium. The test tubes were then autoclaved and inoculated with 2259-6. After overnight incubation, the assays were examined to see which sections induced conjugation tubes, and Rf values for the erogens were calculated.

XII. Effect of cyclic-3',5'-adenosine monophosphate

The possibility that cyclic-3',5'-AMP mediates the response to the erogens was examined.

A 1 mM solution of cyclic-3',5'-adenosine monophosphate (Nutritional Biochemicals Corp.) was sterilized by filtration through a sterile Gelman membrane filter with 0.22 μ pores into a sterile flask. The sterile solution was added aseptically
to 5.0 ml portions of GS medium in 50 ml Erlenmeyer flasks to give final cyclic AMP concentrations of $1 \times 10^{-4}$, $5 \times 10^{-5}$, $1 \times 10^{-5}$, $5 \times 10^{-6}$, and $1 \times 10^{-6}$ M. The flasks were inoculated with *T. mesenterica* 2259-6, and incubated on the shaker at 20°C. Samples were taken from each flask daily for six days, and examined under the microscope.

Caffeine is known to inhibit cyclic AMP phosphodiesterases (Nair 1966) and therefore it might cause accumulation of cyclic AMP inside the cells. Caffeine was added to 20 ml portions of GS medium in 125 ml Erlenmeyer flasks at concentrations of 0, 1, 2, 4, 8, and 16 mM. After autoclaving, each flask was inoculated with 1 ml of a 2259-6 culture and incubated on the shaker at 20°C. Daily samples from each flask were examined microscopically. After six days, a sample from each flask was diluted 1 to 10 with distilled water and its absorbance measured at 540 nm.
Results

I. Extraction with organic solvents

The material extracted from water by ethyl acetate, diethyl ether, chloroform, benzene, methylene chloride, or petroleum ether at any of the pH's tried showed no hormone activity. The raffinates from these extractions had erogenic activity comparable to the original culture supernatant. The butanol extract at all pH's showed hormone activity, slightly weaker than the original supernatant. The raffinate from the butanol extractions displayed low hormone activity. No difference was detected in the amount of hormone activity extracted by n-butanol at acid, neutral or alkaline pH.

In the bioassay of the fractions from the counter current distribution, conjugation tubes were found around the discs corresponding to tubes 4 to 10. The most intense response was found in samples 7 and 8. This distribution of hormone activity among the tubes corresponds to a partition coefficient of about 2.3 for the erogens between water and n-butanol.

II. Adsorption on cation exchange resin

The sample of culture supernatant applied to the cation exchange column showed high erogenic activity. Neither the culture supernatant after passage through the ion exchange column nor the material eluted with 10% ammonia induced conjugation tubes in the assay cells.

Each of the eluting buffers tested washed brown pigment off the column. The appearance of colour in the effluent was a convenient marker for the breakthrough point of each buffer.

The material eluted from the column with quarter strength ammonium acetate showed a low level of hormone activity. More erogenic activity was eluted with the half strength and full strength ammonium acetate solutions. In none of the three eluate fractions was the activity as high as in the original culture supernatant.
The material eluted from the cation exchange resin with pyridine acetate showed hormone activity comparable to the original culture supernatant.

III. Adsorption on anion exchange resin

The culture supernatant showed high erogen activity before, but none after passing through the anion exchange column. The acetic acid eluate displayed high hormone activity.

IV. Adsorption on activated charcoal

In the first experiment, the original culture supernatant showed moderate erogenic activity and the filtrate after charcoal treatment had low hormone activity. The material eluted with methanol, ethanol, and acetone was inactive, but the pyridine acetate eluate was highly erogenic.

In the second experiment, the original culture supernatant had high hormone activity. The supernatant from the charcoal adsorption, and the materials eluted with 50%, 75% and 100% acetone were all inactive.

The culture supernatant used with the coconut charcoal showed high hormone activity, and so did the supernatants from the first, second, and third treatments with coconut charcoal. Hormone activity was not noticeably less in the treated samples than in the original. The coconut charcoal which had been washed with acetone also did not reduce the hormone activity of the supernatants.

Norit A at 0.1 gm per 40 ml and deactivated charcoal at 0.1 and 0.5 gm per 40 ml completely removed erogenic activity from the culture supernatant. The materials eluted by 10% phenol from any of the charcoal samples did not show hormone activity.

V. Adsorption on Porapak

The original culture medium was highly erogenic, but no hormone activity was found in the effluent from the Porapak QS
column. After the medium had passed through the Porapak column, a band of yellow pigment was visible at the top of the resin. When 50% acetone was passed through the column, the yellow colour moved down the column with the acetone front, and numerous air bubbles appeared in the column behind the front. The material eluted with 50% acetone had high erogenic activity, and some activity was present in the 75% and 100% acetone eluates as well.

Porapak Q gave results similar to those obtained with Porapak QS, except that very little additional hormone was eluted with 75% acetone and none with pure acetone.

VI. Chromatography on Sephadex G-10

In the calibration run of the Sephadex G-10 column, starch was found in the fraction eluted from the column after 80 to 100 minutes, and chloride in the fraction from 120 to 170 minutes after the sample was applied. In the run with a hormone sample at the same flow rate, erogenic activity was found in the fractions eluted from 200 to 225 minutes after the sample was applied.

VII. Ultrafiltration

The ultrafiltrate showed high hormone activity, and the retained materials washed from the filter were completely inactive. Apparently the erogens are not retained by a Pellicon PSWP membrane filter (molecular weight cutoff approximately 1000).

VIII. Chromatography on silica gel columns

A band of dark brown pigment travelled through the column, apparently unretarded, with the absolute ethanol eluant. This pigment was collected in fractions 8 to 10. When the eluant was changed to 87.5% ethanol, more pigment moved down the column, and was collected in fractions 35 to 37. The 75% ethanol eluted more pigment which emerged in fractions 63 to 70, and the 50% ethanol eluted pigment in fractions 87 to 92.
Conjugation tubes were found around the paper discs treated with samples from fractions 8 to 12, and 34 to 100. The highest response was to fractions 8 and 10, 36 and 38, 60 to 76, and 88 to 100. The active material in fractions 8 to 12 was designated hormone I, in fractions 33 to 53 hormone II, and in fractions 54 to 100 hormone III.

In the second run, the unretarded brown pigment emerged from the column in fraction 6. Erogenic activity was found in fractions 6 to 18, 42 to 62, and 80 to 134. The active material in fractions 6 to 18 was identified with hormone I, that in fractions 42 to 62 with hormone II, and that in fractions 80 to 134 with hormone III.

IX. Paper chromatography

The solvents used and the Rf of the erogenic activity in each solvent are listed in Table III. The Rf values obtained were repeatable.

X. Effect of cyclic-3',5'-adenosine monophosphate

The cells treated with cyclic AMP at concentrations up to $10^{-4}$ M did not show any morphological differences from the cells grown without cyclic AMP.

Conjugation tubes did not appear on any of the cells treated with caffeine. Some of the cells, particularly in the flask with 2 mM caffeine, were elongated, but remained distinguishable from conjugation tubes. Concentrations of caffeine above 2 mM inhibited the growth of the cells. The turbidities of one-tenth dilutions of the cultures at each caffeine concentration after six days are presented in Table IV.
Table III. The Rf values for the erogen from *Tremella mesenterica* 2259-7 obtained on paper chromatography in various solvents.

<table>
<thead>
<tr>
<th>Solvent Composition (v/v/v)</th>
<th>Rf of Erogen Activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>n-butanol, acetic acid, water</td>
<td></td>
</tr>
<tr>
<td>(8/1/1)</td>
<td>0.3-.5</td>
</tr>
<tr>
<td>(12/3/5)</td>
<td>0.4-.6</td>
</tr>
<tr>
<td>(4/1/5, upper phase)</td>
<td>0.35-.4</td>
</tr>
<tr>
<td>n-butanol, pyridine, water</td>
<td></td>
</tr>
<tr>
<td>(8/1/1)</td>
<td>0.1-.3</td>
</tr>
<tr>
<td>(1/1/1)</td>
<td>0.5-.8</td>
</tr>
<tr>
<td>n-butanol (saturated with water)</td>
<td>0.2</td>
</tr>
<tr>
<td>chloroform, methanol, water</td>
<td></td>
</tr>
<tr>
<td>(4/1/1)</td>
<td>0.8-1.0</td>
</tr>
<tr>
<td>(4/2/5, lower phase)</td>
<td>0</td>
</tr>
<tr>
<td>chloroform, acetic acid, water</td>
<td></td>
</tr>
<tr>
<td>(1/1/1)</td>
<td>0.7-.9</td>
</tr>
<tr>
<td>(2/1/2)</td>
<td>0-.15</td>
</tr>
<tr>
<td>(4/1/5, lower phase)</td>
<td>0</td>
</tr>
<tr>
<td>(5/4/1)</td>
<td>0.35-.5</td>
</tr>
<tr>
<td>benzene, acetic acid, water</td>
<td>0</td>
</tr>
<tr>
<td>(4/3/1)</td>
<td>0-.2, .3-.6 (two zones)</td>
</tr>
<tr>
<td>benzene, acetic acid, acetone, water</td>
<td></td>
</tr>
<tr>
<td>(10/6/10/3)</td>
<td>0-.2, .3-.6 (two zones)</td>
</tr>
<tr>
<td>ethyl acetate, ethanol, water</td>
<td></td>
</tr>
<tr>
<td>(4/1/5, upper phase)</td>
<td>0</td>
</tr>
<tr>
<td>(4/2/5, upper phase)</td>
<td>0-.5</td>
</tr>
<tr>
<td>(4/1/2)</td>
<td>0-.1, .3-.5 (two zones)</td>
</tr>
<tr>
<td>phenol, water</td>
<td>0.9-.1.0</td>
</tr>
<tr>
<td>(4/1, w/w)</td>
<td>0</td>
</tr>
<tr>
<td>methanol</td>
<td>0</td>
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<td>ethanol</td>
<td>0</td>
</tr>
<tr>
<td>ethanol, water</td>
<td></td>
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<tr>
<td>(13/1)</td>
<td>0.8-.1.0</td>
</tr>
<tr>
<td>acetone, water</td>
<td>0.95-.1.0</td>
</tr>
<tr>
<td>distilled water</td>
<td>0-.1, .2-.6 (two zones)</td>
</tr>
<tr>
<td>2% aqueous acetic acid</td>
<td>0.5-.8</td>
</tr>
<tr>
<td>0.1 N hydrochloric acid</td>
<td>0-.1, .7-.9 (two zones)</td>
</tr>
<tr>
<td>0.1 N ammonia</td>
<td>0-.1, .6-.9 (two zones)</td>
</tr>
</tbody>
</table>
Table IV. Turbidities of one-tenth dilutions of *T. mesenterica* cultures grown for six days in GS medium plus various concentrations of caffeine.

<table>
<thead>
<tr>
<th>Caffeine concentration (mM)</th>
<th>Absorbance at 540 nm</th>
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</thead>
<tbody>
<tr>
<td>0</td>
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<tr>
<td>1</td>
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<td>2</td>
<td>0.19</td>
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<tr>
<td>4</td>
<td>0.046</td>
</tr>
<tr>
<td>8</td>
<td>0.011</td>
</tr>
<tr>
<td>16</td>
<td>0.007</td>
</tr>
</tbody>
</table>
Discussion

The erogenic substances from \textit{T. mesenterica} 2259-7 can be extracted from aqueous solution with \text{n-butanol}, but not with less polar solvents. This shows that the \text{Tremella} hormone is more polar than the sexual hormones which have been isolated from other fungi. Sirenin and antheridiol were extracted from culture media with methylene chloride (Machlis et al 1966, McMorris and Barksdale 1967); trisporic acids can be extracted with ether (van den Ende et al 1970). On the other hand, the fact that the erogens partition into \text{n-butanol} shows that they are less polar than such metabolites as sugars and the common amino acids. Since pH has little effect on the amount of hormone extracted with organic solvents, the erogens are not simply acidic or basic compounds.

The adsorption of the erogens on cation and anion exchange resins is presumptive evidence that the hormone molecules carry positive and negative charges at appropriate pH's. The hormones could not be eluted from the cation exchange resin by raising the pH with 10% ammonia, and were only partially recovered with high concentrations of ammonium acetate. These results suggest that the hormones were binding to the polystyrene backbone of the resin, as well as being attracted to the ionic sites. The success of pyridine acetate in eluting the erogens supports this interpretation. The ability of pyridine to disrupt non-ionic binding to ion exchange resins has been used in a scheme for the preparative separation of peptide mixtures (Schroeder et al 1962). The ready elution of the hormones from the anion exchange resin with 1 N acetic acid may have been partly caused by the solvent effect of the acetic acid. Further experiments on the relative importance of ionic and non-ionic binding to ion exchange resins are described in Chapter 6.

The strong adsorption of the erogens on activated charcoal and on the neutral polystyrene resin Porapak is consistent with its behaviour on the cation exchange resin. Adsorption on charcoal is due to non-polar binding forces and is usually
strongest for aromatic substances (Asatoor and Dalgliesh 1956). The tenacity with which the erogens bind to activated charcoal is remarkable—aromatic amino acids and small peptides which have been adsorbed on charcoal can usually be eluted with mixtures of water and ethanol or acetone. Asatoor and Dalgliesh (1956) have recommended deactivation of charcoal with stearic acid and elution with 10% phenol to improve recoveries of aromatic substances. My results indicate that deactivated charcoal retains a high capacity to adsorb erogens, and that 10% phenol is ineffective as an eluant for the hormones. The lack of adsorption on coconut charcoal suggests that the adsorption may depend on some special surface property of the brand of charcoal used.

Porapak is a co-polymer of styrene and ethylvinylbenzene crosslinked with divinylbenzene. The two types used, Porapak Q and QS, differ in the ratio of the components. Adsorption on Porapak Q has been investigated by Niederwieser as a method for recovering non-polar amino acids and peptides (1971) and indoles (Niederwieser and Giliberti 1971) from aqueous solution. He found for \( \alpha \)-amino acids with hydrocarbon side chains, that the degree of adsorption increased with the number of carbon atoms in the side chain. Aromatic amino acids were bound less strongly than the corresponding saturated, straight chain compounds. Peptides were adsorbed more strongly than their constituent amino acids. I have found that Porapak Q is as effective as Norit A charcoal in removing conjugation hormones from culture supernatants, but the hormones can easily be eluted from the Porapak with 50% acetone.

It was hoped that chromatography on Sephadex G-10 would indicate the molecular weight of the erogens. However, with distilled water as solvent, the hormone activity is eluted later than sodium chloride. This high retention volume indicates that the hormones are being adsorbed on the gel, and no valid conclusions about molecular weight can be drawn. Adsorption on Sephadex gel has frequently been noted, especially for
aromatic substances (Wolf 1969). The complete lack of retention of erogen activity by the Pellicon PSWP membrane filter suggests that the hormone has a molecular weight less than 750.

Chromatography on silica gel columns has shown that there are at least three distinct hormones—each of which is independently capable of inducing conjugation tubes in cells of the assay strain. These components have different polarities, and can be recognized by the minimum concentration of water in ethanol which will elute them from the silica gel column. The results obtained by using a gradient of water concentration eliminate the possibility that the peaks of hormone activity observed in the first experiment were the result of discontinuities in the strength of the eluting solvent. It is not clear whether the mechanism of this separation is actually adsorption or partition on the silica gel, or differential solubility of the hormones in ethanol-water mixtures. Not all of the initial hormone preparation is soluble in the absolute ethanol used as starting solvent. As elution proceeds, the bulk of undissolved material at the top of the column gradually diminishes. Possibly the main phenomenon is differential solubility with separation enhanced by adsorption or partition on the silica gel.

It is probable that the three hormone components are variants of a basic structure. Perhaps the chemical structures necessary for erogenic activity occupy only part of the hormone molecule, and the rest of the molecule can be varied without destroying biological activity. Alternatively, some or all of the hormone components may not be erogenically active in their present form, but can readily be converted into an active form by the responding fungus. The production of three conjugation hormones by _T. mesenterica_ 2259-7 can be compared to the production by _Blakeslea trispora_ and _Mucor mucedo_ of tri-sporic acids A, B, and C. All of these have zygogenic activity, but differ in the level of oxidation at the penultimate carbon
of the side chain (Bu'Lock, Drake and Winstanley 1972). Production of several distinct compounds with the same biological activity is common among fungi—for example, the penicillins (Abraham and Newton 1967).

The behaviour of the erogens in paper chromatography is consistent with their solvent partition characteristics. The solvent must contain a minimum amount of water to cause migration of the erogens. In the n-butanol-acetic acid-water and n-butanol-pyridine-water solvent systems the Rf values for the erogens are similar to those for the aromatic amino acids (Smith 1958), but the Rf for the erogens in 80% phenol is anomalously high. In some solvent systems the erogenic activity is resolved into two zones, supporting the evidence from silica gel chromatography for multiple forms of the erogen.

Cyclic-3',5'-adenosine monophosphate has been implicated as an intracellular mediator in the responses to hormones of higher animals (Robison, Butcher, and Sutherland 1971), and in higher plants (Pollard 1970, Salomon and Mascarenhas 1971, Wood, Lin and Braun 1972). It has also been found to serve as the chemotactic agent aerasin in the cellular slime mold, Dictyostelium discoideum (Barkley 1969).

The experiments reported here do not provide any evidence for the involvement of cyclic AMP in conjugation tube production in Tremella mesenterica, but they do not rigorously exclude the possibility. Exogenous cyclic AMP may have been inactive because it could not penetrate the cells, or it may have been rapidly destroyed by hydrolytic enzymes. The toxicity of caffeine show that it was able to enter the cells, but the toxicity may have interfered with conjugation tube production in some way not related to cyclic AMP metabolism. As well, Tremella phosphodiesterases may not be sensitive to caffeine.

In summary, the adsorption of the erogens on both cation and anion exchange resins suggests that the molecules contain both weakly acidic and weakly basic groups. Strong adsorption on activated charcoal and Porapak indicate that the erogen
molecules include non-polar portions capable of hydrophobic bonding. Because the erogens are adsorbed on Sephadex, and very strongly held on activated charcoal, the non-polar portions may include aromatic rings. The solvent partition behaviour indicates that the whole molecules have medium polarity, as expected from summation of the non-polar and ionic portions. Ultrafiltration suggests a molecular weight below 750. These properties could be expected from an amino acid with a large non-polar side chain, or from a small peptide containing amino acids with non-polar side chains.
CHAPTER THREE

QUANTITATIVE BIOASSAY OF THE EROGENS
Materials and Methods

I. Relation of conjugation tube length to erogen concentration

A sample of erogens was prepared by extracting the supernatant from a culture of 2259-7 in GM3 with n-butanol. The butanol extract was evaporated to dryness in vacuo, and dissolved in a mixture of 10 ml of distilled water and 10 ml of ethyl acetate. After vigorous shaking, the water-ethyl acetate mixture was decanted and centrifuged to separate the phases. The ethyl acetate phase was discarded, and the aqueous phase was freeze-dried.

22.2 mg of the freeze-dried extract was dissolved in 10.0 ml of GS medium. This stock solution was diluted with GS medium to give solutions containing 55.5, 111, 222, 444, 666, 888, 1110, and 2220 micrograms of hormone preparation per ml. Each dilution was dispensed in one ml amounts into triplicate test tubes. The tubes were autoclaved for 10 minutes, and then inoculated with 2 drops per tube from a 23-hour culture of 2259-6 in GS. The tubes were incubated at 20°C. After 22 hours, 1 drop from each tube was pipetted onto a microscope slide. The slides were dried in an oven at 105°C, cooled to room temperature, stained with 1% Phloxine-3% potassium hydroxide-glycerol (1/1/1), and covered with glass cover slips. In each sample, the lengths of 20 randomly selected conjugation tubes were measured, using a Filar eyepiece micrometer, at X1000 magnification (oil immersion objective).

II. A bioassay based on conjugation tube length

The linear dependence of the mean logarithm of conjugation tube length on the logarithm of erogen concentration was used as the basis for a quantitative bioassay procedure.

The freeze-dried hormone preparation described in section I was used as a standard. For an assay, 6.0 mg of the standard hormone preparation is dissolved in 6.0 ml of GS medium. The standard solution is divided into two 3 ml portions in 16 mm
test tubes. One-third and one-ninth dilutions are prepared by withdrawing 1.0 ml from the standard solution, and adding it to 2.0 ml of fresh GS in a second test tube. After thorough mixing, 1.0 ml is withdrawn from this tube, and mixed with 2.0 ml of GS in a third tube. One ml from the third tube is withdrawn and discarded. A duplicate standard dilution series is prepared from the second portion of standard hormone solution. One or more test preparations are dissolved at an appropriate concentration in GS medium and duplicate dilutions series set up as above. The assay tubes are autoclaved at 15 psi pressure for 10 minutes on a dry goods cycle, and, after cooling to room temperature, inoculated with two drops per tube from a 2 day shake culture of 2259-6 in GS. After incubation at 20°C for about 18 hours, a drop from each assay tube is placed on a microscope slide, dried, and stained. The length of 25 randomly selected conjugation tubes in each sample is measured as above. For cells bearing more than one conjugation tube, the sum of the lengths of each tube is recorded.

The data thus obtained are analyzed by the method of Finney (1964). Regression lines for average In tube length against log dose are fitted for each preparation with the constraint that the lines must be parallel. From the horizontal distance between the sample and standard regression lines, the potency of the test preparation relative to the standard is calculated. The variation of the tube length within each dose is used to estimate experimental error, test the assumption that the regression lines are linear and parallel, and calculate confidence limits for potency ratios.

III. Relation of fraction of cells bearing conjugation tubes to erogen concentration

Erogens were prepared by growing 2259-7 in Gluc-AmS medium. The culture supernatants were mixed with 0.5 gm of Norit A activated charcoal per litre, and left for an hour with occasional stirring. The charcoal was recovered by centrifugation,
and washed with distilled water. Conjugation hormones were eluted from the charcoal with a pyridine acetate solution (50 ml of glacial acetic acid, 20 ml of pyridine, and 50 ml of distilled water). The eluate was evaporated to dryness in vacuo, redissolved in a small volume of water, re-evaporated, and then dissolved in distilled water and freeze-dried. The freeze-dried material was stored in a tightly capped vial in the freezer section of a refrigerator.

A sample of the erogen preparation was dissolved in a 0.5% solution of soytone, pH 5, at a concentration of 1.0 mg/ml. Nine serial dilutions in 0.5% soytone, in which each dilution had a hormone concentration 2/3 as great as the preceding one, were prepared. Two ml of each dilution in 16 mm test tubes were autoclaved 10 minutes on a dry goods cycle and inoculated with 2 drops each of a 2 day shake culture of 2259-6 in GS. After 15 hours at 20°C, a sample from each dilution was examined in a haemacytometer under the X400 power of the microscope. The total number of cells, and the number of cells with one or more conjugation tubes longer than one cell diameter were counted in 80 small squares.

IV. A bioassay based on fraction of cells with conjugation tubes

The standard hormone preparation used is the same as that described in section III. One unit of conjugation hormone is defined as the amount of conjugation hormone in 0.2 mg of the standard preparation. Standard stock solutions containing 10 mg of standard preparation per ml of 50% aqueous acetone are stored in the freezer between uses.

For an assay, .04 ml of standard stock solution is placed in the bottom of a 16 mm test tube, and mixed with 2.0 ml of 0.5% soytone, pH 5.5, to give an erogen concentration of 1 unit per ml. From this solution, serial dilutions of 1/2, 1/4 and 1/8 in 0.5% soytone are prepared in 16 mm test tubes. One ml from the 1/8 dilution is discarded so that each test tube contains one ml.
If the test solution is expected to contain less than 10 units of erogen per ml, a sample is supplemented with $1/20^{th}$ volume of 10% (w/v) solution of soytone. The pH of the test sample is adjusted to 5.5 with 0.1 N hydrochloric acid or potassium hydroxide, using bromocresol green or bromocresol purple as an indicator. The test sample is diluted with 0.5% soytone to give an expected potency of approximately 1 unit per ml. Two ml of the diluted test solution are pipetted into a test tube, and $1/2$, $1/4$, and $1/8$ dilutions prepared as for the standard. From one to fifteen test solutions can be assayed simultaneously. The assay tubes are capped, autoclaved for ten minutes on a dry goods cycle and, after cooling, inoculated with one drop each from a 24 hour shake culture of 2259-6 in GS. The tubes are incubated at 20°C overnight (13 to 20 hours) and then placed in a refrigerator at 4°C.

From each tube, a sample is mounted in a haemacytometer, and the number of total cells, and cells with conjugation tubes longer than one cell diameter are counted. Usually a total of 100 to 150 cells is counted for each sample. All of the samples in one day's bioassay are counted within as short a period as possible.

The statistical analysis of the bioassay data is patterned after Finney (1971). For each dose, the fraction of cells with conjugation tubes is transformed to its logit ($\text{logit of } p = \ln(p/(1-p))$). Weighted regression lines for logit of response against the logarithm of dose are fitted separately for the standard series and for each test preparation, and then a set of regression lines constrained to be parallel for all preparations is calculated. The deviations of the data from the separate regression lines for each preparation are tested to see if they are greater than expected from random sampling. The increase in deviation when parallel lines are fitted is used to test the hypothesis that the lines really are parallel. The erogenic potency of each test preparation is estimated from the horizontal distance between the regression line
for the test preparation and the standard line, and 95% confidence intervals for the potency ratio are calculated. A computer program which performs these calculations is described in Appendix C.
Results

I. Relation of conjugation tube length to erogen concentration

a. Data

The length measurements of the conjugation tubes at various concentrations of the conjugation hormones are listed in Appendix B. The mean tube length for each sample is plotted against erogen concentration in Fig. 8.

b. Statistical analysis

Inspection of the data in Appendix B shows that the variability of the tube length is higher at high erogen doses than at low doses. The variance of the tube length in each sample is plotted against the average tube length in the same sample on logarithmic scales in Fig. 9. The regression of log variance on log mean is significant at the .01% level, and has a slope of 2.13. A slope of approximately two means that the variance is proportional to the square of the mean. For valid statistical analysis the variance of the data should be independent of the mean. This can be accomplished for data such as the present by transforming each measurement to its natural logarithm (Steel and Torrie 1960). The means of the natural logarithms of conjugation tube lengths for each sample are plotted against erogen dose in Fig. 10. A straight line, more suitable for assay purposes, is obtained by plotting the average of ln tube length against the logarithm of hormone concentration (Fig. 11).

The regression of average ln tube length on log hormone concentration is significant at the .01% level.
Fig. 8. Relation of mean conjugation tube length to dose of erogen. Bars represent 95% confidence intervals for the mean at each dose.
Fig. 9. Dependence of variance on mean for conjugation tube length measurements.
Fig. 10. Relation of average transformed conjugation tube length to dose of erogen.
Fig. 11. Linear regression of average transformed conjugation tube length on the logarithm of estrogen dose.
II. A bioassay based on conjugation tube length

The bioassay method based on conjugation tube length was employed a few times, but measurement of tube lengths was found to be too tedious and time-consuming for routine use. Confidence intervals ranged from ±25% to ±70% of the relative potency. This assay method has been replaced by a second procedure based on the fraction of cells bearing conjugation tubes.

III. Relation of fraction of cells bearing conjugation tubes to erogen concentration

The total number of 2259-6 cells in 80 small squares averaged 150. The fraction of cells bearing conjugation tubes is plotted against hormone concentration in Fig. 12a. The logit (Finney 1964) of the fraction of cells responding is plotted against the logarithm of hormone concentration in Fig. 12b.

IV. Bioassay based on fraction of cells with conjugation tubes

The bioassay based on fraction of cells producing conjugation tubes has been used extensively, and has provided most of the data for the last two chapters of this thesis.

For each assay performed, the slope of the regression lines and the dilution of the standard which causes 50% of the cells to produce conjugation tubes (ED50) have been charted (Fig. 13). Both the slope and the standard ED50 show day-to-day variations which are significantly greater than their errors of estimation. The correlation between the slope and the standard ED50 is essentially zero.

The data frequently show greater deviations from the regression lines than can be explained by random sampling. Two types of deviation can be recognized. In some cases, the dispersion of the data points about the line is increased, but the basic relation remains linear. The ratio of the actual deviation to the deviation expected from random sampling is calculated as a heterogeneity factor. The frequency distribution of the heterogeneity factor is summarized in Table V.
Fig. 12a. Relation of fraction of cells with conjugation tubes to dose of erogen.

b. Linear regression of transformed response on the logarithm of erogen dose.
Fig. 13. Variation in the slope and intercept of the bioassay standard curve.
In other cases, the response at high doses is lower than expected from extrapolation of the response at lower doses. This effect can be seen in Fig. 12.

Table V. Frequency distribution of the heterogeneity factor for the conjugation hormone bioassay.

<table>
<thead>
<tr>
<th>Value of heterogeneity factor</th>
<th>Frequency</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.00 - 1.49</td>
<td>29</td>
</tr>
<tr>
<td>1.50 - 2.49</td>
<td>9</td>
</tr>
<tr>
<td>2.50 - 3.49</td>
<td>7</td>
</tr>
<tr>
<td>3.50 - 4.49</td>
<td>4</td>
</tr>
<tr>
<td>4.50 - 5.49</td>
<td>3</td>
</tr>
<tr>
<td>5.50 - 6.49</td>
<td>4</td>
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<td>1</td>
</tr>
<tr>
<td>7.50 - 8.49</td>
<td>2</td>
</tr>
<tr>
<td>8.50 - 9.49</td>
<td>0</td>
</tr>
<tr>
<td>9.50 - 10.49</td>
<td>1</td>
</tr>
</tbody>
</table>
Discussion

The theory and practice of statistical methods in biological assay have been described by Finney (1964). Because of biological variability, a standard curve determined on one occasion may not apply at some other time. Fig. 13 confirms that both the slope of the dose-response regression lines and the concentration of standard required to produce a particular response vary greatly from one assay to another. For this reason it is necessary to include a standard dilution series in each assay. Measuring the response to the test preparation at several dilutions increases the precision with which the slope of the dose-response curve can be calculated, and allows tests for curvature and non-parallelism of the test line. Units of biological activity should be defined as a certain weight of a stable standard preparation, rather than in terms of the amount needed to produce a specified response.

Bioassay procedures which have been used in previous studies of fungal sexual hormones do not completely meet these requirements. In the sirenin bioassay, several doses of standard preparation are tested along with one dose of the unknown. The potency of the unknown is determined by graphical interpolation (Carlile and Machlis 1965). In the bioassay for hormone A in Achlya developed by Raper (1942a), several dose levels of the unknown are tested, but only one concentration of standard is used to "calibrate" the mycelia. One unit of hormone is defined as the amount per c.c. required to produce an average of 10 antheridial branches in the terminal 3 mm of the test hyphae. Barksdale (1963a) has modified this assay. She tests several dilutions of the unknown, but notes only the lowest dilution which causes at least 25% of the hyphae to produce antheridial branches. Three dilutions of a standard solution are assayed simultaneously, apparently as a check that the sensitivity of the hyphae has not changed drastically. This assay method is only capable of measuring the potency of the unknown preparation to within a factor of two.
The description of the bioassay for "gamones" in Mucor mucedo is sketchy, but it appears that only one dose of an unknown preparation was tested, and the response was compared to a standard curve determined previously. Bu'Lock, Drake and Winstanley (1972) used a more sophisticated procedure to determine the zygogenic activity of several compounds related to the trisporic acids. Several concentrations of each compound were assayed, and the slope of the dose-response lines was taken as a measure of relative biological activity. This is a good design, and would allow the calculation of confidence limits.

Despite the deficiencies noted above, these assays seem to have served their purpose satisfactorily and have enabled the isolation of the active materials. Possibly the variability of the responses was low enough to make unnecessary the safeguards built into more careful assay designs. In Tremella, my experience has shown that the complete procedure is generally required for reliable results. However, when an approximate estimate of the relative erogen concentration in many samples, such as chromatographic fractions, is desired, a reduced version of the bioassay is adequate. (See Chapter Six, sections II, IV, and V.)

The average length of conjugation tubes after 22 hours incubation shows a strong dependence on the dose of conjugation hormone over the range tested (Fig. 8). However, the lengths of individual tubes at each dose are highly variable (coefficient of variation 0.4 to 0.5), and as might be expected, the variability at short average tube lengths is less than at long average tube lengths. The variance of tube lengths at each dose is approximately proportional to the square of the mean tube length (Fig. 9). Such a dependence of variance on mean violates the basic assumptions of common statistical techniques such as the analysis of variance, and the tube length measurements were transformed to their natural logarithms to make the variance at each dose approximately constant. Comparison of Fig. 10 to Fig. 8 shows that this transformation accentuates the curvature of the dose-response curve, but does not alter its basic shape. For biological assays it is convenient to have a function of dose to which the response is linearly related. Fig. 11 shows that the logarithm of the dose is such a function.
This preliminary information about the relation of conjugation tube length to hormone dose was used to design an assay. Since the regression of transformed response on the logarithm of dose is to be used in the computations, the doses should be equally spaced on the logarithmic scale. A serial dilution series accomplishes this. The dose-response curve was found to be linear over a dose range of at least 40 times. Four serial dilutions at a dilution ratio of 1 to 3, covering a dose range of 27 times, were used to ensure that the responses would be on the linear portion of the curve even if small errors were made in the choice of doses.

The conjugation tubes at high erogen concentration could be longer because of earlier initiation, faster growth, or growth sustained for a longer time than the conjugation tubes at low erogen concentrations, or because of a combination of these factors. This point is discussed further in Chapter Four.

The tube length bioassay was developed first because measurements of tube length give more information per cell than simply determining the presence or absence of a conjugation tube. In practice, the variability of the tube lengths and the labour required to measure large numbers of tube lengths have overcome this theoretical advantage.

Data about the fraction of cells producing conjugation tubes is of the type called "quantal", because each cell is placed into one of two categories--with conjugation tube or without conjugation tube. Quantal data is important in the assay of fungicides and insecticides, where the two categories are dead and alive. A technique known as probit analysis has been developed by Finney (1971) for quantal data. I have applied his methods to the Tremella conjugation tube counts, but the probit transformation has been replaced by the logistic transformation, because an explicit formula for the latter is available. The logit of the fraction responding plotted against the logarithm of the erogen concentration closely follows a
straight line except at extreme high and low doses. At low
erogen doses, the conjugation tubes formed are short, and it is
sometimes difficult to decide whether or not a particular cell
has a conjugation tube. For this reason, the error in estimating
the fraction of cells with tubes is larger at low doses than
at medium or high doses. Therefore the deviation from the
straight line at low dose is not a cause for concern. At the
highest dose tested the response decreased probably because of
inhibition by other components of the hormone preparation.

Dose-response curves similar to the one presented here
for the Tremella erogens have frequently been found in toxicology
(Finney 1971) and pharmacology (Ariens, Simonis and van Rossum
1964). Two types of models have been proposed to explain the
shape of these curves. In the first model each cell has a
response threshold, and at a given dose, only those cells whose
threshold is lower than the dose will react. If the frequency
distribution of the threshold is log normal, it can be shown that
the probit (or logit) of the fraction responding will be a linear
function of the logarithm of the dose (Finney 1971).

An alternative approach is the receptor theory. The admin­
istered biologically active molecules are postulated to attach
to receptor sites inside the cells in order to produce a response.
As the concentration of the active material increases, the
fraction of receptor sites which are occupied also increases
according to the Langmuir adsorption isotherm. If the response
is assumed to be proportional to the number of receptor sites
occupied, this model also predicts a linear regression of the
logit of the fraction responding on the logarithm of dose (Ariens,
Simonis, and van Rossum 1964).

Essentially, the threshold distribution model assumes that
the effective dose in each cell is the same, and the sensitivity
of the cells to that dose is variable, whereas the receptor
model assumes that the effective dose (number of occupied
receptors) per cell is variable, and the sensitivity of the
cells to a given effective dose is constant. These two concepts
are not mutually exclusive—variation in the number of occupied receptors per cell could be teamed with an independent variation in the number of occupied receptors per cell necessary to produce a response.

Those assays in which deviations from the regression lines are greater than can be explained by random sampling require special attention. When the basic dose-response relation remains linear, the heterogeneity factor can be used to take into account the increased estimation error. Significant heterogeneity indicates non-uniformity of conditions among the assay tubes. In the *Tremella* assay, inadequate mixing of the assay solutions may be a major cause of heterogeneity. When assays were performed in shaken flasks to provide continuous mixing, the cells became entangled by their conjugation tubes into large clumps, and accurate counting was impossible. Under the static incubation conditions now employed, most of the cells settle to the bottom of the tubes. Conjugation tube production is not noticeably less in static than in shaken media, but the concentrations of cells at the bottom of the assay tube could alter the local environment in a non-reproducible way and thus effect the response to the erogens.

The second case, in which the responses at high doses are anomalously low, leading to curvature of the dose-response regression, occurs most frequently in two situations—when relatively impure preparations such as crude culture supernatant are assayed, or when the doses of the unknown are too high. These low responses are probably caused by inhibitory substances present in the crude preparations, or because the response is limited by some factor other than hormone concentration. The procedure adopted here has been to examine the data, and to omit from the analysis the results from the one or two highest doses of a preparation if the responses to these doses are not substantially higher than the response at the next lower dose. Rejection of data after examination carries the danger of introducing bias into the results, but the alternatives are
even less appealing. To include all the doses in the analysis would produce misleading results, because the data would not conform to the assumed linear regression. To reject the entire assay would waste the information available from the lower doses.

The design of assays based on quantal responses has been discussed by Finney (1964). He recommends a minimum of three doses of each preparation, to allow tests of linearity and parallelism of the dose-response regressions. For the Tremella erogen bioassay, four doses were used. The fourth dose, by providing an extra independent datum, reduces the sensitivity of the assay to heterogeneity, and also reduces the severity of rejecting the results from one or two of the higher doses. For an assay with four doses of each preparation, Finney recommends choosing the doses so that the expected fractions responding are about 0.15, 0.35, 0.65, and 0.85 to give highest precision. Because, as mentioned above, the error in counting the number of cells with conjugation tubes is high at low response rates, somewhat higher doses were used in the Tremella bioassay, and response rates below 0.25 are not normally encountered. A ratio of 1/2 between successive doses gives the proper spacing.

Crude erogen preparations from T. mesenterica 2259-7 contain at least three active components. Dose-response relations for mixtures of biologically active materials have been described by Finney (1971). The dose-response curves observed for the erogen preparations from 2259-7 have always been linear or convex, suggesting that the components are showing "simple similar action". In other words, all three components are stimulating the cells through the same mechanism. More complex types of interactions between the components should lead to dose-response curves which are concave (Finney 1971).
CHAPTER FOUR

THE RESPONSE TO THE EROGENS
Materials and Methods

I. Time course of conjugation tube initiation and growth

Two 5.0 ml portions of 0.5% soytone, pH 5.0, in 25 ml Erlenmeyer flasks were supplemented with 1.0 unit/ml and 0.2 units/ml, respectively, of the standard erogen preparation. After autoclaving and cooling, the flasks were inoculated with five drops each of a two day old culture of 2259-6 in GS. A sample was taken aseptically from each flask, and the flasks were placed on a shaker at 20°C. Samples were taken from each flask after 2, 4, 5, 6, 7, 8, 10, 12, 14, and 24 hours. One drop from each sample was mounted in a haemacytometer, and the number of cells with and without conjugation tubes was counted. Another drop of each sample was dried and stained on a microscope slide. The lengths of 35 randomly selected conjugation tubes were measured in each of the stained slides from the 8, 10, 12, 14, and 24 hour samples.

II. Distribution of number of conjugation tubes per cell

The supernatant from one litre of a stationary phase culture of 2259-7 in Gluc-AmS medium was passed through a bed of 10 grams of Porapak Q at a flow rate of 300 ml/hour. The Porapak was washed with 100 ml of distilled water, and the erogens were eluted with 100 ml of 50% aqueous acetone. The eluate was concentrated in vacuo until the acetone had evaporated, and then was freeze-dried. One mg of the freeze-dried powder was dissolved in 1.0 ml of distilled water. This solution was diluted 1 to 50 with a 0.5% solution of soytone, pH 5.5, and from this primary dilution 1/2, 1/4, 1/8, 1/16, 1/32, 1/64, and 1/128 secondary dilutions in 0.5% soytone were prepared. These dilutions were autoclaved, inoculated and incubated as for a bioassay. After seventeen hours, the fraction of cells producing conjugation tubes at each dilution was measured, and stained slides were prepared from the 1/2, 1/8, and 1/32 secondary dilutions. Comparison with a standard dilution
series assayed simultaneously indicated an erogen concentration of 20.5 units/ml in the primary dilution. On the stained slides, approximately 200 cells were examined at each dose, and the numbers with 0, 1, 2, 3, ..., 8 conjugation tube growing points originating from one yeast cell were counted.

III. Effect of temperature

Five test tubes containing 1.0 ml of 0.5% soytone, pH 5.0, with 1.0 unit of standard erogen per ml and five similar tubes with 0.25 units/ml were prepared. After autoclaving each tube was inoculated with one drop from a one day old culture of 2259-6 in GS. One tube with 1 unit/ml and one tube with 0.25 units/ml were incubated at each of 10°C, 15°C, 20°C, 25°C and 28°C. After 18 hours, the fraction of the cells with conjugation tubes at each dose-temperature combination was determined. Stained slides were also prepared, and the lengths of 25 randomly selected conjugation tubes from each treatment were measured.

IV. Effect of pH

Aliquots of 0.5% soytone were adjusted to pH 3.0, 4.0, 5.0, 6.0, 7.0, and 8.0 with 0.1 N hydrochloric acid or potassium hydroxide. One ml portions of the medium at each pH in test tubes were supplemented with 1.0 or 0.25 units of the standard erogen preparation, and autoclaved. The tubes were inoculated with 1 drop from a one day old culture of 2259-6 in GS, and incubated at 20°C. After 17 hours, the fraction of cells with conjugation tubes in each test tube was determined.

V. Effect of cell concentration

Six ml of 0.5% soytone, pH 5.5, containing 1.0 unit of the standard erogen preparation per ml were distributed into six test tubes. After autoclaving, one tube was inoculated with 0.01 ml of a three day old culture of 2259-6 in GS. The other tubes were inoculated with 0.02, 0.04, 0.06, 0.08 and 0.10 ml of the culture. Sterile GS was added to each tube to bring the total volume to 1.1 ml. The tubes were incubated at
20°C for 18 hours. The total number of cells, and the number of cells with conjugation tubes in a known volume from each test tube were counted in a haemacytometer. Stained slides were prepared, and the lengths of 25 conjugation tubes in each sample were measured.

VI. Effect of nitrogen source

A basal medium similar to the BM of Chapter One, but containing 10 gm of D-glucose, and 0.13 ml of microelement stock solution per litre was prepared. Aliquots of this basal medium were used directly or supplemented with one gram of ammonium sulphate per litre, or five grams of casein hydrolysate or soytone per litre. The pH of each medium was adjusted to 5.5 with 0.1 N hydrochloric acid or potassium hydroxide. A sample of conjugation hormones eluted from Porapak was added to aliquots of each medium at concentrations of 1.0 unit/ml and 0.25 units/ml. Duplicate 2 ml portions of each medium at both erogen concentrations were pipetted into test tubes and autoclaved. Inoculum was prepared by centrifuging, in a sterile centrifuge tube, the cells from a two day old culture of 2259-6 in GS, washing the cells with a volume of sterile distilled water equal to the original culture volume, and resuspending the cells in another volume of sterile distilled water. One drop of the washed cell suspension was added to each test tube, and the tubes were incubated at 20°C for 18 hours. Samples from each test tube were mounted in the haemacytometer, and the cells with conjugation tubes and without conjugation tubes in 400 small squares were counted.
Results

I. Time course of conjugation tube initiation and growth

The fraction of cells with recognizable conjugation tubes is plotted against time in Fig. 14 for both erogen concentrations. On the same graph, the average length of the conjugation tubes in the 8, 10, 12, 14 and 24 hour samples are shown. Histograms of the frequency distributions of tube length at each time are presented in Fig. 15 for the low erogen concentration, and in Fig 16 for the high erogen concentration. Tube lengths are given in eyepiece units, which equal 1.76 μ.

II. Distribution of number of conjugation tubes per cell

The frequency distributions of the number of conjugation tubes per cell are shown in Fig. 17. The average number of tubes per cell is plotted against erogen dose in Fig. 18. The regression was significant at the 10% level.

III. Effect of temperature

The fraction of cells with conjugation tubes, and the average conjugation tube length at each dose are plotted against incubation temperature in Fig. 19 and Fig. 20 respectively.

IV. Effect of pH

The fraction of cells producing conjugation tubes at each dose of erogen is plotted against the pH in Fig. 21. The lengths of the conjugation tubes produced at each pH varied in a similar manner to the fraction of cells responding.

V. Effect of cell concentration

Table VI lists the counts of total cells, and cells with conjugation tubes, along with the volumes in which the cells were counted. Confidence limits for the concentration of cells in each test tube were calculated by assuming a Poisson distribution for the total cell counts. The fraction of cells with conjugation tubes, and the average conjugation tube length are plotted against the total cell concentration in Fig. 22.
Fig. 14. Time course of conjugation tube initiation and growth in response to erogen. Upper curves 1.0 unit/ml, lower curves 0.2 unit/ml. Bars represent 95% confidence intervals.
Fig. 15. Frequency distributions of conjugation tube lengths at various times after inoculation of 2259-6 cells into media containing 0.2 units of erogens per ml.
Fig. 16. Frequency distributions of conjugation tube lengths at various times after inoculation of 2259-6 cells into media containing 1.0 unit of erogens per ml.
Fig. 17. Frequency distribution of the number of conjugation tubes per cell, at three erogen concentrations.
Fig. 18. Regression of average number of conjugation tubes per cell on the erogen concentration.
Fig. 19. The fraction of cells responding to the ergogens at two concentrations as a function of temperature. Upper curve—1.0 unit/ml; lower curve 0.25 units/ml. Bars represent 95% confidence intervals.
Fig. 20. The average length of conjugation tubes produced in response to the concentrations of erogens as a function of temperature. Upper curve—1.0 unit/ml; lower curve—0.25 unit/ml. Bars represent 95% confidence intervals.
Fig. 21. The fraction of cells responding to the erogens at two concentrations as a function of pH. Upper curve—1.0 unit/ml; lower curve—0.25 units/ml. Bars represent 95% confidence intervals.
Fig. 22. Dependence of fraction of cells with conjugation tubes, and average length of conjugation tubes on concentration of cells.
Table VI. Cell counts from the experiment on the effect of cell concentration.

<table>
<thead>
<tr>
<th>Volume of inoculum in ml</th>
<th>Number of cells with conjugation tubes</th>
<th>Total number of cells</th>
<th>Volume in which cells were counted nanolitres</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.01</td>
<td>162</td>
<td>186</td>
<td>100</td>
</tr>
<tr>
<td>0.02</td>
<td>275</td>
<td>324</td>
<td>100</td>
</tr>
<tr>
<td>0.04</td>
<td>201</td>
<td>331</td>
<td>40</td>
</tr>
<tr>
<td>0.06</td>
<td>299</td>
<td>622</td>
<td>40</td>
</tr>
<tr>
<td>0.08</td>
<td>208</td>
<td>458</td>
<td>40</td>
</tr>
<tr>
<td>0.10</td>
<td>176</td>
<td>373</td>
<td>20</td>
</tr>
</tbody>
</table>

VI. Effect of nitrogen source

The numbers of cells with conjugation tubes, and the total numbers of cells counted in each replicate of the erogen dose-nitrogen source combinations are given in Table VII.

Table VII. The number of cells with conjugation tubes/the total number of cells counted at two doses of erogen with various nitrogen sources.

<table>
<thead>
<tr>
<th>Nitrogen source</th>
<th>0.25 units of erogen/ml</th>
<th>1.0 unit of erogen/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>0/280, 0/500</td>
<td>0/485, 0/400</td>
</tr>
<tr>
<td>Ammonium sulphate</td>
<td>22/851, 34/910</td>
<td>43/300, 68/680</td>
</tr>
<tr>
<td>Casein hydrolysate</td>
<td>136/445, 133/670</td>
<td>260/274, 172/330</td>
</tr>
<tr>
<td>Soytone</td>
<td>350/851, 252/610</td>
<td>359/443, 312/400</td>
</tr>
</tbody>
</table>

The fractions were transformed to the arcsines of their square roots (Steel and Torrie 1960) and the data was analyzed as a 4 X 2 factorial experiment with 2 replications. The analysis of variance is presented in Table VIII.

The contrast between the fraction of cells producing conjugation tubes with soytone and casein hydrolysate as nitrogen sources was significant at the 2.5% level.
Table VIII. Analysis of variance for effect of nitrogen source on conjugation tube production.

<table>
<thead>
<tr>
<th>Source of variation</th>
<th>Sum of squares</th>
<th>Degrees of freedom</th>
<th>Mean square</th>
<th>F</th>
<th>Probability of a larger F</th>
</tr>
</thead>
<tbody>
<tr>
<td>Main effect of nitrogen source</td>
<td>1.942</td>
<td>3</td>
<td>0.6474</td>
<td>84.7</td>
<td>0.000003</td>
</tr>
<tr>
<td>Main effect of ergon concentration</td>
<td>0.2781</td>
<td>1</td>
<td>0.2781</td>
<td>36.4</td>
<td>0.0003</td>
</tr>
<tr>
<td>Interaction</td>
<td>0.1013</td>
<td>3</td>
<td>0.03378</td>
<td>4.42</td>
<td>0.041</td>
</tr>
<tr>
<td>Error</td>
<td>0.06112</td>
<td>8</td>
<td>0.00764</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Discussion

The effects of some environmental variables on production of conjugation tubes in response to the erogens were examined to determine optimum conditions for the bioassay.

First, the kinetics of conjugation tube initiation and growth at 20°C were studied. Recognizable conjugation tubes were first observed 4 hours after exposure of the 2259-6 cells to the erogens. Extrapolation of the curves in Fig. 13 suggests a lag of slightly more than 3 hours before any conjugation tubes were initiated. In the medium containing 1.0 unit of erogens per ml, the fraction of cells with conjugation tubes increased rapidly until 7 hours after inoculation, and then more slowly until 14 hours. In the medium containing 0.2 units of erogens per ml, the proportion of cells with conjugation tubes rose more slowly. After 24 hours, at both erogen concentrations, the fraction of cells with conjugation tubes is lower than at 14 hours. This decrease is probably caused by the formation of new yeast cells, either by budding from the original yeast cells, or by reversion of conjugation tube growing points to budding. Flegel (1968) has shown that conjugation hormones must be continuously present to maintain conjugation tube growth; if cells with growing conjugation tubes are switched to hormone-free medium, budding occurs at the growing point of the conjugation tubes. I have frequently observed buds at the apices of conjugation tubes, especially at incubation times longer than 18 hours, and at low initial erogen concentrations. Bandoni (1965) has suggested that the cells use up the conjugation hormones as they respond. Reversion of the conjugation tubes to budding at long incubation times supports this view. A similar phenomenon has been found in other fungi reacting to sexual hormones—sperm of Allomyces remove sirenin from their medium (Carlile and Machlis 1965) and male mycelia of Achlya take up antheridiol irreversibly (Barksdale 1963a).
In Flegel's hormone pulsing experiment (1968), conjugation tubes were found 2 hours after exposure of the cells to the hormone. He does not specify the strain, or the concentration of the hormone used. He also carried out a time lapse photographic study of conjugation tube production in mixed cultures of 2259-6 and 2259-7. The earliest conjugation tube recorded was initiated 90 minutes after mixing of the two strains, but was not recognizable at a tube before 3 hours. This early tube has a constriction at its point of attachment to the yeast cell. Such a basal constriction is characteristic of buds, but not of conjugation tubes. Perhaps this structure was initiated as a bud, and later transformed into a conjugation tube.

By 8 hours, the average conjugation tube length at 1 unit of erogens per ml is twice as great as at 0.2 unit per ml. At 0.2 units/ml a large fraction of the tubes are shorter than 1.5 epu (recently initiated), but at 1 unit/ml very few of the conjugation tubes are in this length range. Therefore, the greater average length of the tubes at high erogen dose is at least partly caused by earlier initiation. As time progresses, the frequency distributions for both hormone concentrations shift to longer lengths, and broaden. By 12 hours, few recently initiated tubes are found in the medium with 0.2 units/ml, and by 14 hours, the distribution of tube lengths is similar to the 8 hour distribution at the higher hormone concentration. The average length of the conjugation tubes increases faster at high than at low hormone concentration, suggesting that the average growth rate of the tubes is faster at high hormone concentration. The average tube length increases from 14 hours to 24 hours, in contrast to the fraction of cells with conjugation tubes. This does not necessarily mean that growth of established conjugation tubes continued while new tubes were no longer being initiated. These data do not tell whether the average length was still increasing at 24 hours, or stopped at some earlier time. The distributions of tube lengths at 24 hours are very broad—there are some tubes as short as 2 epu,
and as long as 13 epu at 0.2 units/ml, and as long as 18 epu at 1 unit/ml. The short tubes probably stopped growing at an early time, the extremely long tubes continued growing until the end of the incubation period, and the medium length tubes stopped growing at some intermediate time. The data presented here are not extensive enough to permit a clear separation of the effects of initiation time, growth rate, and growth stopping time on the average length of conjugation tubes at various hormone doses, but suggest that all three factors are important.

The best incubation time for a bioassay is long enough to ensure that all cells which are going to react have done so, and that the conjugation tubes are long enough to be easily recognized, but not so long as to allow accumulation of new yeast cells. Incubation times between 12 and 18 hours should meet these criteria.

The response time for *Tremella* is longer than for *Allomyces*, where bioassays are read after 40 minutes (Machlis 1958a), *Achlya*, where bioassays are read after 2 hours (Barksdale 1963a), and *Mucor* where bioassays are read after 4 hours (Plempel 1963).

As the concentration of erogens is increased, the number of points on the surface of the yeast cells at which conjugation tubes are produced, and the branching of the tubes, increase. These effects cause an increase in the average number of conjugation tube growing points per cell (Fig. 17). The linearity of the regression of average number of conjugation tube growing points per cell on concentration of erogens over the range tested (Fig. 18) is striking, and suggests a third bioassay method. This possibility has not been investigated.

Among the temperatures tested, the largest fraction of cells produced conjugation tubes at 15°C, with 20°C almost as good. However, the conjugation tubes formed at 20°C were longer than those formed at 15°C and at other temperatures. Therefore 20°C was selected as the standard temperature for the bioassay. Longer incubation might have allowed a higher response at 10°C, but this would not be of practical interest for the bioassay.
At an erogen dose of 1 unit/ml, the fraction of cells responding was almost constant over the pH range from 5 to 7, but decreased at pH 4, and was zero at pH's 3 and 8. At a dose of 0.25 units/ml, the response at pH 6 was higher than at other pH's tested. A pH of 5.5 was selected for the bio-assay. Flegel (1968) found maximum conjugation tube formation in mixed cultures of 2259-6 and 2259-7 at pH 4.7, no tubes at pH 7.2, but some short branched tubes at pH 8. He used different buffer systems at different pH's, so that the effects of pH are confounded with possible specific effects of the buffer salts. In the present study, the amino acids and peptides of soytone were used as buffers at all pH's. The discrepancies between Flegel's results and mine may be caused by the difference in buffer systems, or by the difference between mixed cultures and pure cultures with added partially purified erogens.

At high concentrations of 2259-6 cells, a smaller fraction of the cells produce conjugation tubes, and the tubes are shorter than at lower cell concentrations. There are at least two possible explanations for this effect. The 2259-6 cells could produce a self-inhibitor. The concentration of this inhibitor would be higher at high cell concentrations, and thus decrease the response. Alternatively, the cells could compete for the erogen molecules. At high cell concentrations, each cell would receive a smaller share of the erogen dose, and therefore respond less strongly. To allow near maximal response the amount of inoculum added to the assay tubes is designed to give a density of 250 cells per hundred picolitres.

If no nitrogen source is included in the assay medium, no conjugation tubes are formed. Ammonium sulphate is a poor nitrogen source for conjugation tube production by 2259-6, in contrast to its ready utilization for growth and erogen production by 2259-7. Conjugation tube production is better in casein hydrolysate medium, and significantly greater in soytone medium. It appears therefore that amino acids stimulate
the response to the erogens. The superiority of soytone to casein hydrolysate may arise from more favourable proportions of amino acids, or from some other stimulatory substances. The importance of amino acids suggests that protein synthesis is involved in the response to the erogens. Flegel (1968) found that cycloheximide, which inhibits protein synthesis in some fungi, did not inhibit conjugation tube production. It only slightly decreased the growth rate of the cells, suggesting that it was not penetrating the cells very effectively.

Time limitations have prevented investigation of many other factors which might influence the response to the erogens, and also of the interactions between various factors.
CHAPTER FIVE

PRODUCTION OF THE EROGENS
Materials and Methods

I. Time course of erogen production

For a pilot study of the kinetics of erogen production during the growth of 2259-7 cultures, two 200 ml portions of Gluc-AmS medium in 1 litre flasks were inoculated with 2.0 ml from a 4-day culture of 2259-7 in GS. The flasks were incubated on a shaker at 20°C. Every 12 hours, samples of approximately 5 ml were taken aseptically from each flask. The turbidity at 640 nm of the samples was determined in a Bausch and Lomb Spectronic 20 after dilution with distilled water to keep the absorbance below 0.5. The pH of each sample was measured using a Radiometer pH meter model 28. Samples taken at odd multiples of 12 hours after inoculation were stored overnight in a freezer. Each day, the thawed samples from the previous evening, and the samples from that morning were centrifuged for 5 minutes at full speed in a clinical centrifuge (International Equipment Co., Model HN) to sediment the cells. Two ml portions of each sample supernatant were supplemented with 0.1 ml of 10% soytone, and their pH adjusted to 5.5 with 0.1 N potassium hydroxide. From these samples, 1/2, 1/4, and 1/8 dilutions in 0.5% soytone, pH 5.5, were prepared, and the bioassay was carried out as usual. Sampling of the cultures was continued until 168 hours after inoculation.

The information from the pilot study was used to plan a second experiment. Three 1 litre Erlenmeyer flasks containing 200 ml each of Gluc-AmS were inoculated with 3.0 ml each of a 7 day old culture of 2259-7 in Gluc-AmS. The flasks were incubated on a shaker at 20°C. Five ml samples were taken from each flask immediately after inoculation, and at 12 hour intervals until 120 hours after inoculation, and then at 144 and 168 hours. The pH and turbidity of the samples were measured, and the samples prepared for bioassay as above. The samples taken at 24, 36 and 48 hours were bioassayed at their original concentration. The samples taken at 58 hours were diluted 1 to 2;
the samples taken at 72 hours were diluted 1 to 4; the 84 and 96 hour samples were diluted 1 to 8; and the 108, 120, 144 and 168 hour samples were diluted 1 to 16 with 0.5% soytone, pH 5.5, before bioassaying.

To determine the ratio between turbidity and concentration of cell dry weight, 2259-7 cells from a stationary phase culture in Gluc-AmS were washed three times with distilled water, and then resuspended in distilled water. A sample of this suspension was diluted 1 to 50 with distilled water, and its turbidity measured at 640 nm. Forty ml of the suspension were pipetted into a dry, weighed Petri dish. The dish was dried overnight in an oven at 105°C, and cooled to room temperature in a desiccator before weighing.

II. Effect of nitrogen source on erogen production

Portions of the basal medium of Chapter Four, section VI were supplemented with 1 gm of ammonium sulphate, or 1 gm of ammonium sulphate plus 2 grams of sodium acetate trihydrate or 1.33 gm of vitamin-free, salt-free casein hydrolysate, or 2 gm of soytone per litre. Three 50 ml replicates of each medium in 250 ml Erlenmeyer flasks were prepared and autoclaved for 15 minutes on a dry goods cycle. Each flask was inoculated with 1.0 ml of a 4 day old culture of 2259-7 in GS, and incubated on a shaker at 20°C. After seven days the cultures were centrifuged for 10 minutes at full speed in a clinical centrifuge (International Equipment Co., Model HN, angle head). The supernatants were decanted, diluted 1 to 4 with 0.5% soytone, pH 5.5, and bioassayed.

III. Effect of medium concentration

A medium (4X) containing the components of Gluc-AmS medium at 4 times their normal concentration was prepared. Part was diluted with an equal volume of distilled water to give 2X medium, and part of this was diluted with distilled water to give regular Gluc-AmS. Duplicate 50 ml aliquots of 4X and 2X,
and triplicate 50 ml aliquots of Gluc-AmS were dispensed in 250 ml Erlenmeyer flasks. The flasks were capped with a double thickness of aluminum foil, and autoclaved for 15 minutes on a dry goods cycle. Each flask was inoculated with 1.0 ml from a culture of 2259-7 in Gluc-AmS, and incubated on a shaker at 20°C. Progress of the cultures was monitored by daily turbidity measurements on samples from one of the Gluc-AmS flasks. The turbidity reached a maximum 5 days after inoculation, and after 7 days the flasks were removed from the shaker. The turbidity at 640 nm of 1/10 dilutions of samples from each flask was measured, and 5 ml samples from each flask were centrifuged for 5 minutes at full speed in an IEC model HN clinical centrifuge. The supernatants were adjusted to pH 5.5 with 0.1 N KOH, diluted 1 to 16 with 0.5% soytone, pH 5.5, and bioassayed.

In a separate experiment four 125 ml Erlenmeyer flasks containing 25 ml each of Gluc-AmS and four flasks of half-strength Gluc-AmS (x/2) were prepared, autoclaved, inoculated with 0.5 ml per flask from a 31 hour old culture of 2259-7 in Gluc-AmS, and incubated on a shaker at 20°C. Samples were taken daily from one flask of each medium, and the turbidities measured. Peak turbidity was reached after four days. On the fifth day, the turbidity at 640 nm of 1/10 dilutions of samples from each flask was measured, and samples of the 6 undisturbed cultures were centrifuged, adjusted to pH 5.5, diluted 1 to 16 with 0.5% soytone, pH 5.5, and bioassayed. While the assay cells were being counted, the tubes containing the assays for one culture in each medium were inadvertently left at room temperature for two hours.

IV. Effect of varying the concentration of individual medium components

Aliquots of Gluc-AmS medium were supplemented with 10 gm of D-glucose (+G), or 1.0 gm of ammonium sulphate (+N), or 1.0 gm of KH₂PO₄, plus 0.5 gm of MgSO₄·7H₂O plus 0.1 gm CaCl₂·2H₂O (+S), or with all three (+GNS) per litre. Duplicate 50 ml
portions of regular Gluc-AmS, +G medium, +N medium, +S medium, and +GNS medium were dispensed into 250 ml Erlenmeyer flasks. After autoclaving each flask was inoculated with 1.0 ml from a 4 day old culture of 2259-7 in Gluc-AmS. At the same time, a third flask of Gluc-AmS was inoculated. The flasks were incubated on a shaker at 20°C and the turbidity of the culture in the third flask of Gluc-AmS was measured daily. Peak turbidity was reached four days after inoculation, and on the fifth day the flasks were sampled for turbidity readings and bioassays as described in section III.

In a separate experiment, Gluc-AmS supplemented with 100 µg of thiamine hydrochloride (+T) or 0.13 ml of the microelement stock solution (+M) per litre was prepared. Duplicate 50 ml portions of Gluc-AmS, +T medium, and +M medium in 250 ml Erlenmeyer flasks were autoclaved and inoculated, along with a third flask of Gluc-AmS, with 1.0 ml per flask from a 3 day old culture of 2259-7 in Gluc-AmS. The flasks were incubated on a shaker at 20°C, and the turbidity of the third Gluc-AmS flask was monitored. Peak turbidity was reached four days after inoculation, and on the sixth day samples were taken from each flask for turbidity measurements and bioassay.

To test the effect of lower concentrations of ammonium sulphate and microelements and their interaction on erogen yield, a 2 X 2 X 2 factorial experiment was set up. The media used were variations of Gluc-AmS. Media designated N/2 contained 0.5 gm of ammonium sulphate per litre; those designated N/4 had 0.25 gm of ammonium sulphate per litre. Similarly M/2 media had 0.065 ml of microelement stock solution and M/4 media had 0.033 ml of microelements per litre. IR media contained D-glucose, KH₂PO₄, MgSO₄•7H₂O, CaCl₂2H₂O and thiamine at the same concentration as in Gluc-AmS; 2R media contained these components at twice the concentration in Gluc-AmS. Two 26 ml portions of each of the eight combinations i.e., (N/2, M/2, IR), (N/4, M/2, IR), (N/2, M/4, IR), and so on, were prepared in 125 ml Erlenmeyer flasks. The experiment was divided into two replicates.
which were run at different times. One flask of each medium was included in each replicate. The flasks were inoculated with 0.5 ml each from a 3-day culture of 2259-7 in Gluc-AmS, and incubated on a shaker at 20°C. After five days, the cultures were sampled for turbidity measurements and bioassays as described above.

V. Large scale production of erogens

To produce amounts of the erogens sufficient for purification, four 5 gallon polypropylene bottles (Fisher Scientific Co.) were employed. Each bottle was fitted with a #13 rubber stopper, through which passed two glass tubes—an air inlet tube, 3 mm i.d., and an air outlet tube, 10 mm i.d. Both tubes were loosely packed with cotton wool. The air inlet tube was connected inside the bottle by a short length of rubber tubing to another piece of glass tubing of the same diameter which reached almost to the bottom of the bottle. The air outlet tube was bent over outside the bottle through an angle of 120°.

The bottles were loaded with 12 litres of distilled water, and then the ingredients for 12 litres of Gluc-AmS medium. The sugar and salts must be adequately dispersed before autoclaving to prevent caramelization. The rubber stoppers were wired into position, and the bottles were autoclaved for 30 minutes at 15 psi pressure on a dry goods cycle. It was usually necessary to leave the bottles cooling overnight before inoculation.

Inoculum was grown in 200 ml batches of Gluc-AmS medium in 1 litre Erlenmeyer flasks on a shaker at 20°C. The inoculum cultures were used close to the end of their log phase. Generally 10 ml from each inoculum flask was used to start fresh inoculum, and the rest was used to inoculate 1 bottle. After inoculation, the bottles were placed in a 20°C incubator room, and the air inlet tube was connected to a compressed air tap. Air was bubbled through the cultures at 1 to 2 litres per minute.
After five days the culture was passed through an eight tube continuous flow attachment in a Sorvall RC2-B centrifuge. Culture flow rate was about 300 ml/min, the centrifuge rotor speed was 15,000 rpm, and the temperature was 2 to 3°C. A sample of the supernatant was taken for bioassay, and the remainder was treated with 6.0 gm of Norit A activated charcoal. The suspension was stirred vigorously to evenly distribute the charcoal, left for two hours with occasional stirring, and then centrifuged under the same conditions as the original culture. The recovered charcoal was washed with distilled water from the centrifuge tubes onto a filter, and scraped from the filter into a 50 ml glass centrifuge tube. Erogens were eluted by suspending the charcoal in 30 ml of pyridine acetate solution (20 ml pyridine, 50 ml acetic acid, and 30 ml distilled water), leaving for 15 minutes, and then centrifuging out the charcoal. The supernatant was decanted, and the charcoal was resuspended in another 30 ml of pyridine acetate solution. A total of five portions of pyridine acetate solution were used for each lot of charcoal. The eluates were combined, evaporated to dryness in vacuo, dissolved in 10 ml of distilled water, re-evaporated, again dissolved in 10 ml of distilled water, and freeze-dried. The freeze-dried material was stored in a tightly capped vial in a freezer.

To determine the specific activity of the erogens in the original culture supernatant, 10 ml of culture supernatant were freeze-dried. 46.6 mg of the freeze-dried material was dissolved in 2.0 ml of 0.5% soytone, pH 5.5, and bioassayed.

VI. Foam

To determine if different conditions of agitation and aeration could increase the erogen yield in large scale cultures, a series of experiments using a Microferm laboratory fermenter (New Brunswick Scientific Co.) was begun. This apparatus has a culture volume of 5 litres, and facilities for controlling stirrer speed, air flow rate, and temperature. To prevent foam
from overflowing onto the floor, the air outlet was connected by plastic tubing to a Pasteur pipette held by a cotton wool plug in the neck of a 1 litre Erlenmeyer flask. The flask, Pasteur pipette, and connecting tubing were autoclaved along with the fermentor and its contents.

In one experiment, 5 litres of Gluc-AmS in the Microferm was inoculated with 50 ml of a 4 day old shake culture of 2259-7 in Gluc-AmS. The air flow rate was set at 2 litres/min, stirrer speed at 0, and temperature at 20°C. On the fourth, fifth, and sixth days after inoculation, samples of the fermentor contents were taken aseptically, centrifuged, and bioassayed.

In another experiment, the stirrer speed was set at 300 rpm, air flow at 2 litres/min, and temperature at 20°C. Samples for bioassay were taken 4, 5, and 6 days after inoculation. On the seventh day, the liquid which had collected in the foam trap was assayed.

A third Microferm culture was set up with stirring at 300 rpm and air flow at 0.5 litres/min. The inoculum was 200 ml of a 2 day old culture of 2259-7 in Gluc-AmS. After five days of incubation, approximately 500 mls of condensed foam had collected in the foam trap. The trap flask was replaced with a fresh sterile 1 litre flask, and the collected foam was bioassayed. Serial dilutions down to 1/2048 were included in the bioassay series.

To test the possibility that the poorly aerated condition of the foam trap was stimulating the cells carried over in the foam to synthesize large quantities of erogens, two 2800 ml Fernbach flasks containing 500 ml each of Gluc-AmS were prepared. Each flask was inoculated with 5.0 ml from a 3 day old shake culture of 2259-7 in Gluc-AmS, and incubated on a shaker at 20°C for three days. One of the flasks was then moved to a bench in the incubator room, and the other was left on the shaker. After a total of six days of incubation, samples from each flask were bioassayed, starting with a 1/16 dilution.
To determine what fraction of the total erogens produced by the cultures could be recovered in the collected foam, 5 litres of \((N/2, M/2, 1R)\) medium (see section IV) was prepared in the Microferm, and inoculated with 200 ml of a 3 day old culture of 2259-7 in Gluc-AmS. The stirrer speed was set at 300 rpm, and the air flow at 200 ml/min. After six days, samples were taken from the main culture volume, and from the collected foam, and centrifuged. The sample from the main volume was bioassayed at its original concentration, and the foam sample was diluted 1 to 100 with 0.5% soytone, pH 5.5, before bioassaying.

The air outlet tube on one of the 5 gallon polypropylene bottles was connected by plastic tubing to a foam trap. The bottle was loaded with 12 litres of \((N/2, M/2, 1R)\) medium, sterilized, inoculated, and incubated according to the methods of section V. After 7 days, 125 ml of condensed foam had collected. This was diluted 1/400 and bioassayed.
Results

I. Time course of erogen production

The culture pH, turbidity (measured $A_{640}$ X dilution), and the concentration of erogens in the supernatant are plotted against culture age in Fig. 23 for the pilot study, and Fig. 24 for the second experiment. The pH values did not vary more than 0.1 unit between any of the replicates.

The dry weight in 40.0 ml of the washed cell suspension was 0.3725 gm, or 9.3 mg/ml. The $A_{640}$ of the 1/50 dilution of the suspension was 0.248. Therefore, a culture turbidity of 1.0 corresponds to 0.75 mg of cell dry weight per ml.

II. Effect of nitrogen source on erogen production

The cells from the medium containing ammonium sulphate without sodium acetate formed a compact pellet during centrifugation; the cells grown in the other media formed a loosely packed pellet. When the bioassays were examined, the samples from assays of the media containing ammonium sulphate plus sodium acetate, casein hydrolysate, and soytone contained large numbers of cells without conjugation tubes, presumably 2259-7 cells which had not been removed by centrifugation. The presence of these cells made counting of the fraction of 2259-6 cells with conjugation tubes impossible. Therefore, the lengths of the conjugation tubes formed in the 1/16 dilution of each culture supernatant were compared visually. The conjugation tubes formed in response to supernatants from the cultures grown in ammonium sulphate were longer than those formed in response to the other samples. No difference was observed between the samples from ammonium sulphate plus sodium acetate, casein hydrolysate, or soytone.

III. Effect of medium concentration

The final turbidities and the erogen concentrations of the 2259-7 cultures grown in 4X, 2X, and Gluc-AmS media are
accumulation in 2259-7 cultures in glucose medium.

Fig. 23. Time course of turbidity increase, pH drop, and ergogen
Fig. 2h. Time course of turbidity increase, pH drop, and ergogen accumulation in 2259-7 cultures in Gluc-Ams medium.
shown in Table IX. The final culture turbidities increase as the concentration of the medium increases, but the yield of erogen appears to decrease. The difference in average erogen yield between media is not significantly greater than experimental error. The data from the cultures grown in Gluc-AmS and X/2 in the second experiment are included in Table IX. The bioassay results have been split into two blocks—the second block contains the assays which were left at room temperature for two hours longer than the assays in the first block. An analysis of variance for the logarithms to the base two of the erogen yields in this experiment is presented in Table X.

IV. Effect of varying the concentration of individual medium components

The final turbidities and bioassay results from the experiments on the effect of increasing the concentration of glucose, ammonium sulphate, salts, thiamine and microelements are shown in Table XI. The analysis of variance is presented in Table XII. The difference between media was highly significant in the first experiment, and on the border line of significance in the second experiment. Application of Dunnett's procedure for comparing treatment means with a control (Steel and Torrie 1960) showed that the erogen yields in the +N medium and the +GNS medium were significantly less (95% confidence) than the yield in Gluc-AmS. The yields in the +G and +S media were not significantly different from the yield in Gluc-AmS. The data from the second experiment indicate a lower yield in the +M medium than in the +T medium and Gluc-AmS.

The data from the factorial experiment is listed in Table XIII. The analysis of variance for the effects of the medium components on the final turbidity and on the logarithms of the erogen yield is shown in Table XIV. The main effects of N, R and replicates on final culture turbidity are significant. Only the main effect of N on erogen yield is significant.
Table IX. Final turbidities and erogen yields for 2259-7 cultures grown in various concentrations of Gluc-AmS medium.

<table>
<thead>
<tr>
<th>Medium</th>
<th>Final turbidity at 640 nm</th>
<th>Erogen yield (units/ml)</th>
<th>95% confidence limits</th>
</tr>
</thead>
<tbody>
<tr>
<td>First</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>experiment</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>4X</td>
<td>6.0</td>
<td>9.3</td>
<td>7.6, 11.2</td>
</tr>
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<td></td>
<td>5.7</td>
<td>4.5</td>
<td>3.6, 5.6</td>
</tr>
<tr>
<td>2X</td>
<td>5.0</td>
<td>5.9</td>
<td>4.7, 7.2</td>
</tr>
<tr>
<td></td>
<td>5.2</td>
<td>7.4</td>
<td>6.1, 8.8</td>
</tr>
<tr>
<td>Gluc-AmS</td>
<td>3.4</td>
<td>11.8</td>
<td>9.9, 14.1</td>
</tr>
<tr>
<td></td>
<td>3.3</td>
<td>10.6</td>
<td>8.8, 12.5</td>
</tr>
<tr>
<td>Second</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>experiment</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Gluc-AmS</td>
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<td>11.3 first block</td>
<td>9.5, 13.3</td>
</tr>
<tr>
<td></td>
<td>3.8</td>
<td>10.5</td>
<td>8.9, 12.3</td>
</tr>
<tr>
<td></td>
<td>3.8</td>
<td>9.0 second block</td>
<td>7.6, 10.5</td>
</tr>
<tr>
<td>X/2</td>
<td>3.0</td>
<td>9.0 first block</td>
<td>7.6, 10.5</td>
</tr>
<tr>
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<td>3.3</td>
<td>8.8</td>
<td>7.5, 10.3</td>
</tr>
<tr>
<td></td>
<td>3.1</td>
<td>6.1 second block</td>
<td>5.1, 7.1</td>
</tr>
</tbody>
</table>
Table X. Analysis of variance of logarithms to the base two of erogen yields from 2259-7 cultures grown in Gluc-AmS and X/2 media.

<table>
<thead>
<tr>
<th>Source of variation</th>
<th>Degrees of freedom</th>
<th>Sum of squares</th>
<th>Mean square</th>
<th>F</th>
<th>Probability of a larger F</th>
</tr>
</thead>
<tbody>
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<td>0.2296</td>
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<td>0.2166</td>
<td>22.0</td>
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<tr>
<td>Error</td>
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<td>0.00983</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table XI. Final turbidities and erogen yields in media with doubled concentrations of individual components.

<table>
<thead>
<tr>
<th>Medium</th>
<th>Final turbidity</th>
<th>Erogen concentration units/ml</th>
<th>95% confidence limits</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gluc-AmS</td>
<td>3.4</td>
<td>13.1</td>
<td>11.7, 14.7</td>
</tr>
<tr>
<td></td>
<td>3.4</td>
<td>14.6</td>
<td>13.0, 16.3</td>
</tr>
<tr>
<td>+G</td>
<td>4.0</td>
<td>13.6</td>
<td>12.1, 15.3</td>
</tr>
<tr>
<td></td>
<td>3.8</td>
<td>14.6</td>
<td>12.9, 16.4</td>
</tr>
<tr>
<td>+N</td>
<td>4.1</td>
<td>7.0</td>
<td>6.1, 7.9</td>
</tr>
<tr>
<td></td>
<td>4.0</td>
<td>6.8</td>
<td>5.9, 7.7</td>
</tr>
<tr>
<td>+S</td>
<td>3.4</td>
<td>13.8</td>
<td>12.2, 15.4</td>
</tr>
<tr>
<td></td>
<td>3.5</td>
<td>13.9</td>
<td>12.3, 15.7</td>
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<tr>
<td>+GNS</td>
<td>4.7</td>
<td>12.0</td>
<td>10.7, 13.5</td>
</tr>
<tr>
<td></td>
<td>4.7</td>
<td>10.8</td>
<td>9.5, 12.2</td>
</tr>
</tbody>
</table>

Second experiment

<table>
<thead>
<tr>
<th>Medium</th>
<th>Final turbidity</th>
<th>Erogen concentration units/ml</th>
<th>95% confidence limits</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gluc-AmS</td>
<td>3.5</td>
<td>18.0</td>
<td>14.6, 22.5</td>
</tr>
<tr>
<td></td>
<td>3.6</td>
<td>23.1</td>
<td>18.6, 29.0</td>
</tr>
<tr>
<td>+T</td>
<td>3.7</td>
<td>21.7</td>
<td>17.6, 27.3</td>
</tr>
<tr>
<td></td>
<td>3.5</td>
<td>19.5</td>
<td>16.2, 24.0</td>
</tr>
<tr>
<td>+M</td>
<td>3.7</td>
<td>9.8</td>
<td>8.0, 12.0</td>
</tr>
<tr>
<td></td>
<td>3.6</td>
<td>5.3</td>
<td>4.3, 6.5</td>
</tr>
</tbody>
</table>
Table XII. Analysis of variance of logarithms of erogen yields in media with doubled concentrations of individual components.

<table>
<thead>
<tr>
<th>Source of variation</th>
<th>Degrees of freedom</th>
<th>Sum of squares</th>
<th>Mean square</th>
<th>F</th>
<th>Probability of a larger F</th>
</tr>
</thead>
<tbody>
<tr>
<td>First experiment</td>
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<tr>
<td>Media</td>
<td>4</td>
<td>1.564</td>
<td>0.3909</td>
<td>67.5</td>
<td>0.0001</td>
</tr>
<tr>
<td>Error</td>
<td>5</td>
<td>0.02895</td>
<td>0.00579</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Second experiment</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Media</td>
<td>2</td>
<td>3.020</td>
<td>1.510</td>
<td>9.52</td>
<td>0.050</td>
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<tr>
<td>Error</td>
<td>3</td>
<td>0.4758</td>
<td>0.1586</td>
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Table XIII. Final turbidities and erogen yields from factorial experiment on effect of ammonium sulphate concentration, microelement concentration, and concentration of rest of medium.

<table>
<thead>
<tr>
<th>Medium</th>
<th>Final turbidity</th>
<th>Erogen yield units/ml</th>
<th>95% confidence limits</th>
</tr>
</thead>
<tbody>
<tr>
<td>N/2, M/2, 1R</td>
<td>3.09</td>
<td>11.3</td>
<td>8.9, 14.1</td>
</tr>
<tr>
<td>N/4, M/2, 1R</td>
<td>2.47</td>
<td>8.8</td>
<td>7.2, 10.7</td>
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<tr>
<td>N/2, M/4, 1R</td>
<td>3.05</td>
<td>9.5</td>
<td>7.6, 11.7</td>
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<tr>
<td>N/4, M/4, 1R</td>
<td>2.44</td>
<td>11.3</td>
<td>9.1, 13.9</td>
</tr>
<tr>
<td>N/2, M/2, 2R</td>
<td>3.46</td>
<td>12.6</td>
<td>10.1, 15.6</td>
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<tr>
<td>N/4, M/2, 2R</td>
<td>2.75</td>
<td>7.8</td>
<td>6.3, 9.5</td>
</tr>
<tr>
<td>N/2, M/4, 2R</td>
<td>3.27</td>
<td>13.3</td>
<td>10.7, 16.4</td>
</tr>
<tr>
<td>N/4, M/4, 2R</td>
<td>2.55</td>
<td>8.0</td>
<td>6.3, 9.9</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Medium</th>
<th>Final turbidity</th>
<th>Erogen yield units/ml</th>
<th>95% confidence limits</th>
</tr>
</thead>
<tbody>
<tr>
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<td>14.0</td>
<td>10.3, 19.0</td>
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<td>17.8</td>
<td>13.2, 24.1</td>
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<td>N/4, M/4, 1R</td>
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<td>7.3</td>
<td>5.4, 9.5</td>
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<tr>
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<td>15.9</td>
<td>11.5, 21.8</td>
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<td>6.0</td>
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<td>8.3</td>
<td>6.0, 10.9</td>
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</table>
Table XIV. Analysis of variance for effects of ammonium sulphate concentration, microelement concentration, and concentration of rest of medium on final turbidity and logarithm of erogen yield.

<table>
<thead>
<tr>
<th>Source of variation</th>
<th>Degrees of freedom</th>
<th>Sum of squares</th>
<th>Mean square</th>
<th>F</th>
<th>Probability of a larger F</th>
</tr>
</thead>
<tbody>
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<td>a. Turbidity</td>
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<tr>
<td>Main effects:</td>
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<tr>
<td>N</td>
<td>1</td>
<td>0.01879</td>
<td>0.01879</td>
<td>119</td>
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</tr>
<tr>
<td>M</td>
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<td>0.00001157</td>
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<tr>
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<td>0.002352</td>
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<td>0.002305</td>
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<tr>
<td>Interactions:</td>
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<td>0.000053</td>
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<td>0.58</td>
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<td>b. Erogen yields</td>
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<td>Main effects:</td>
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</table>
V. Large scale production of the erogens

After three days incubation in the large bottles, the cultures of 2259-7 began to foam. The foam would wet the air outlet filter and escape around the edges of the rubber stopper. Reducing the air flow rate did not eliminate this problem.

The erogen concentration in the culture supernatants varied between 0.25 and 0.5 units/ml. After charcoal treatment, the residual erogen concentration in the supernatant was generally below 0.05 units/ml. Five hundred and sixteen litres of 2259-7 culture supernatant were produced and processed by the methods described. The total yield of concentrated erogen was 125,000 units, a recovery of approximately 80%.

The freeze-dried culture supernatant at 23.3 mg/ml had an erogenic potency of 0.85 units/ml (95% confidence limits 0.708 and 1.005). This corresponds to a specific activity of 0.037 units/mg. The hormone concentrate prepared by elution of the charcoal had a specific activity of 5.0 units/mg. Therefore the charcoal adsorption and elution steps accomplished a purification of 135 fold.

VI. Foam

In the first experiment, the erogenic potency of the culture fluid after 4 days incubation was 3.1 units/ml (95% confidence limits 2.5 and 3.7). On the fifth day, the potency was 0.92 units/ml (95% confidence limits 0.56 and 1.3), and on the sixth day 0.37 units/ml (95% confidence limits 0.14 and 0.57). Foam started to pass over into the foam trap between the four day and five day samplings.

In the second experiment, the four day sample assayed 0.23 units/ml (95% confidence limits 0.076 and 0.38), the five day sample 0.55 units/ml (95% confidence limits 0.31 and 0.82) and the six day sample 0.33 units/ml (95% confidence limits 0.29 and 0.37). The collected, condensed foam assayed on the seventh day showed high erogenic activity down to the lowest concentration tested (1/16).
The collected foam in the third experiment had an erogen concentration of 196 units/ml (95% confidence limits, 174 and 220).

In the comparison between the shaken and static cultures, the shaken culture had an erogen concentration of 13 units/ml (95% confidence limits 7.4 and 19), and the culture which was left on the bench for the last three days of incubation had an erogen concentration of 3.5 units/ml (95% confidence limits 1.4 and 5.5).

In the experiment to determine the fraction of the erogens carried over with the foam, the volume of the collected, condensed foam, after centrifuging out the cells, was 250 ml. The erogenic potency of the main culture volume was 0.57 units/ml (95% confidence limits 0.49 and 0.64) and the potency of the foam was 170 units/ml (95% confidence limits 148 and 191). The total amount of erogens left in the main culture was therefore 5,000 ml X 0.57 units/ml = 2850 units. The total amount of erogen in the foam was 250 ml X 170 units/ml = 42,000 units, or 92% of the total.

The volume of condensed foam collected from the 12 litre culture of 2259-7 in the 5 gallon bottle was 125 ml. The concentration of erogen in the foam was 1,360 units/ml (95% confidence limits 994 and 4300). This corresponds to a total erogen yield of 170,000 units or 14.2 units/ml of the original culture volume.
Discussion

The rise in erogen concentration in shake cultures of 2259-7 in Gluc-AmS follows a curve approximately parallel to the increase in turbidity, but lagging by about one day. However, the maximum erogenic potency is reached at the same time as the peak turbidity. There may be a small decrease in potency during the time when the culture turbidity is decreasing, but the bioassay data are not precise enough to firmly establish this. In the second experiment, the samples from 84, 108 and 144 hours showed consistently lower hormone activity than the adjacent samples, indicating that some of the erogen was destroyed during overnight storage in the freezer. The kinetics of hormone production indicate that the erogens are products of primary metabolism—secondary metabolites should appear mainly after growth has stopped (Aiba, Humphrey and Millis 1965). Most of the erogen was produced after the culture pH fell below 2.5. This may be a coincidence. In subsequent experiments, erogen yields were determined one or two days after the peak turbidity of the monitored culture was reached, to ensure that all cultures had reached their maximum yield.

Ammonium sulphate was the best of the nitrogen sources tested for erogen production. Addition of sodium acetate to the medium to stabilize the pH decreased the hormone yield. Whether the higher pH of the culture with sodium acetate, or some more specific effect of acetate, was inhibiting erogen production is not clear.

Logarithms of the erogen yields rather than the absolute values were used for the analysis of variance because the erogen concentrations are estimated in the bioassay as their logarithms, and hence errors of estimation will be normally distributed on a logarithmic scale. Also the variability of the erogen yields can be expected to increase with the average yield.

Increasing the concentration of Gluc-AmS did not increase the final concentration of the erogens, and appears to have
actually decreased it, although the differences were not significantly greater than experimental error. Halving the medium concentration significantly reduced the erogen yield. In this experiment, the highly significant difference between blocks supports the division of the assay results into two blocks. Apparently the medium concentration chosen initially is close to optimal for erogen production.

When individual components of the medium were doubled in concentration, increased ammonium sulphate and increased microelements significantly reduced the erogen yields. Increases in other components did not change the yield of erogens. Further effects might be found if the salts were tested individually rather than in a group, or if the concentrations of individual components of the microelement stock solution were varied.

The highly significant effect of ammonium sulphate concentration on final turbidity of the cultures in the factorial experiment indicates that nitrogen is the main limiting nutrient for growth, at least at 0.25 gm/l. The R factor also had a significant, although smaller, effect, suggesting that one of its components is close to a limiting concentration. The significant difference between the average turbidities in the two replicates means that the second replicate was sampled at a slightly earlier phase of growth than the first replicate. This did not produce a significant difference in average erogen concentration between the two replicates. Concentration of ammonium sulphate was the only factor that had a significant effect on the yield of erogen. The concentrations of microelements and of the rest of the medium, and their interactions, were without effect. An ammonium sulphate concentration of 0.5 gm/l is near optimal for synthesis of erogens, and the (N/2, M/2, IR) medium appears to be a good choice for hormone production.

Large scale production of the erogens was begun before the quantitative bioassay procedure was developed. Therefore I did not realize at the time how abnormally low the erogen yields from the bottle cultures were.
Three methods for recovery and primary purification of the erogens from large volumes of culture media were considered—extraction with n-butanol, adsorption on ion exchange resin, and adsorption on activated charcoal. Solvent extraction was rejected because the high boiling point of n-butanol made evaporation of large volumes difficult, and made continuous extraction impractical. Adsorption on charcoal was favoured over adsorption on ion exchange resins because fewer components of the culture supernatants would compete with the erogen for binding sites on the charcoal, leading to higher recoveries and better purification. The charcoal adsorption procedure was practical, and gave 80% recovery of the erogens, with 135-fold purification. Both the recovery and the purification might have been higher if the concentration of the erogens in the culture supernatant had been greater. The erogens eluted from the charcoal would have been purer if the charcoal had been pre-washed with the pyridine acetate eluant.

When I realized that the erogen yields from the 5 gallon bottles were much lower than those from shake cultures, I first thought that differences in aeration and agitation might be responsible. Bubbling air through the 12 litre cultures might provide too low or too high a concentration of dissolved oxygen, or provide inadequate mixing of the cultures. Experiments to investigate the effect of stirring and air flow rate were begun in the Microferm, where these parameters could be easily controlled. Foam traps were included initially merely for neatness. The erogen yields from the Microferm cultures at various conditions of stirring and air flow were comparable to the yields in the 5 gallon bottles. In the first experiment described, the erogen concentration in the medium decreased rather than increased with time. The sharp drop between the first two samples coincided with the beginning of foam carry over into the foam trap. At the suggestion of Scott Redhead, in the second experiment the collected foam was assayed, and found to have high erogenic activity. The comparison between the shaken and static flask
cultures confirmed that the erogens were being transported from the main culture into the trap by the foam, rather than being synthesized by the cells after they reach the trap. In a typical culture, 92% of the total erogens produced was concentrated in the condensed foam. The foam effectively removes the erogens from the bottle cultures as well. The amount of erogens in the foam indicates an erogen yield from the bottle cultures comparable to that in shake cultures, and explains the low yields found earlier for the bottle cultures when foam was discarded.

Collection of the foam from the cultures is an excellent method for preliminary concentration and purification of the erogens. Such a high percentage of the erogen activity accumulates in the foam trap that it is practical to discard the residual hormone in the main culture.

Selective transport of solutes from aqueous solution by foaming has received attention because of its possible application to such problems as removal of detergents from waste water. As well as surface active agents, any material which is adsorbed at a surface can be removed. The evidence for both polar and non-polar sections in the erogen molecules discussed in Chapter Two suggests that the erogen molecules themselves are surface active. However, the erogens are probably not principally responsible for the foaming of 2259-7 cultures. When the culture supernatant or condensed foam are passed through a bed of Porapak Q, the effluent, which has very low erogen content, is as foamy as the original solution. The theoretical basis of foam fractionation has been investigated in order to predict optimum operating condition (Lemlich 1968). The only operating condition that can be readily varied in the concentration of erogens from 2259-7 cultures is the air flow rate. High air flow rates produce "wet" foams, and remove surface adsorbed materials most completely. Low air flow rates produce "dry" foams, which give a higher concentration of surface adsorbed substances in the condensed foam, and better separation from surface inactive materials. Clearly an intermediate flow rate giving a compromise between high recovery and high purification of the erogens is best for the Tremella cultures.
In these experiments, only the total erogen concentration has been measured. The proportions of the individual active components may vary with the age of the culture and the medium, and they may have different affinities for the foam.
CHAPTER SIX

PARTIAL PURIFICATION OF THE EROGENS
Materials and Methods

I. Instability

47.1 mg of the standard erogen preparation was dissolved in 1.0 ml of 10% ammonia. 38.8 mg was dissolved in 1.0 ml of 1 N hydrochloric acid. 40.0 mg was dissolved in 1.0 ml of 1 N potassium chloride solution. The solutions in small screw cap vials were autoclaved for 10 minutes at 15 psi steam pressure, on a dry goods cycle. After cooling, the contents of each vial was quantitatively transferred to a 10 ml volumetric flask and diluted to the mark with distilled water. 0.1 ml of each diluted solution was added to 2.0 ml of 0.5% soytone, and the pH was adjusted to 5.5 for bioassay.

II. First attempt at purification

516 litres of culture medium processed by the methods of Chapter Five, section V yielded 25 grams of erogen concentrate, with a specific activity of 5.0 units/mg.

Five grams of this concentrate were dissolved in 20 ml of distilled water, and the pH of the solution was adjusted to 6.5 by addition of 10% sodium bicarbonate solution. The neutralized solution was passed at a flow rate of 50 ml/hour through a 2 cm X 20 cm column of Duolite A-4 anion exchange resin which had been equilibrated with 0.2 M ammonium acetate buffer, pH 6.5, in a cold room at 4°C. The column was washed with 300 ml of cold distilled water, and then eluted with 350 ml of cold 1 N acetic acid. As the acetic acid progressed down the column, the resin changed colour from grey to yellow. The acetic acid eluate was collected from just before the time that the yellow acid front reached the bottom of the column until no more pigment was visible in the effluent. The eluate solution was freeze-dried. The column was regenerated by washing with 400 ml of distilled water, 200 ml of .1 N potassium hydroxide, 400 ml of distilled water, and 250 ml of 0.2 M ammonium acetate buffer, pH 6.5. In total the erogens from 20 gm of concentrate were adsorbed on and eluted from the anion exchange resin, in four
batches. The freeze-dried eluate material was combined and weighed. 5.4 mg was set aside for bioassay.

The remainder of the eluate (1.0 gm) was suspended in 100 ml of distilled water. The pH of the suspension was adjusted to approximately seven with 10% sodium bicarbonate solution, whereupon some of the dissolved material precipitated. The precipitate was removed by centrifugation, and the clear brown supernatant was decanted. The pellet was washed with two 20 ml portions of distilled water, which was added to the supernatant. This supernatant solution was extracted with 100 ml of n-butanol saturated with water, followed by five 50 ml portions of n-butanol saturated with water. The butanol extracts were centrifuged briefly to remove water droplets, and then evaporated in vacuo just to dryness. The residue was treated with 10 ml of distilled water and 20 ml of ethyl acetate. Despite vigorous swirling, some of the dried material on the walls of the flask would not dissolve. The water-ethyl acetate mixture was decanted into a centrifuge tube, followed by water washings from the flask. The undissolved material left in the flask did not completely dissolve in n-butanol saturated with water, nor in 50% acetone. The water-ethyl acetate mixture was centrifuged to separate the phases, and the ethyl acetate phase was decanted. The aqueous phase was briefly concentrated in vacuo to remove ethyl acetate, and then freeze-dried. The precipitate which had formed on neutralization of the eluate solution mostly dissolved in 20 ml of 1 N acetic acid. Undissolved material was removed by centrifugation, and the supernatant was freeze-dried.

The freeze-dried anion exchange eluate was bioassayed at a concentration of 0.036 mg/ml. The redissolved precipitate was bioassayed at 0.27 mg/ml. The n-butanol extract was bioassayed at 0.0024 mg/ml. The ethyl acetate extract was evaporated to dryness in vacuo, and redissolved in 10 ml of 50% acetone. This solution was diluted 1 to 50 for bioassay. The raffinate from the butanol extraction was also diluted 1 to 50 for bioassay.
The redissolved material from the precipitate was suspended in 100 ml of distilled water saturated with n-butanol, and extracted with five 50 ml portions of n-butanol. The butanol extracts were centrifuged, evaporated, redissolved in water-ethyl acetate, and freeze-dried as before. The two freeze-dried butanol extracts were combined and thoroughly mixed. 6.1 mg was dissolved in 6.0 ml of 20% acetone. This solution was diluted 1/5000 for bioassay. The remainder of the butanol extract was dissolved in 160 ml of 25% aqueous acetone, and diluted 1/250 for bioassay.

The butanol extracts were again freeze-dried, and the freeze-dried powder was ground in 1 ml of absolute ethanol in a mortar. Fifty grams of silica gel (BDH) was slurried in absolute ethanol, and poured into a chromatography column, 2 cm in internal diameter. The ethanol suspension of the erogen preparation was applied to the top of the column, and eluted first with 50 ml of absolute ethanol, and then with a gradient of water in ethanol, generated by the method of Chapter Two, section X. The eluant flow rate was 1 ml/min, and the effluent from the column was collected in 5 minute fractions. From each fraction, four drops were added to 1.0 ml of 0.5% soytone, pH 5.5, in a test tube for bioassay. The tubes were autoclaved for 10 minutes on a dry goods cycle, and then inoculated with 1 drop each from a 30 hour old culture of 2259-6 in (4,4) medium. After 12 hours incubation at 20°C, the fraction of cells with conjugation tubes in each assay tube was determined.

Fractions 5 to 18 were pooled as hormone I, evaporated to dryness in vacuo, dissolved in 3 ml of absolute ethanol and stored in the freezer. Fractions 41 to 76 were pooled as hormone II, evaporated to dryness in vacuo, dissolved in 3 ml of 50% aqueous ethanol and stored in the freezer. 100 ml of 50% ethanol was run through the silica gel column, and the effluent combined with fractions 77 to 84 as hormone III. This solution was evaporated to dryness in vacuo, and the residue treated with 50% ethanol. It would not completely dissolve, and the solution and as much as possible of the insoluble material were transferred to a vial and stored in the freezer.
III. Interaction of the erogens with ion exchange resins

To clarify the importance of ionic and non-polar binding in the adsorption of the erogens in ion exchange resins, the equilibrium distribution of the erogens between the resin phase and the solution phase was determined under various conditions.

Amberlite IR-120 cation exchange resin was washed with distilled water, 2 N hydrochloric acid, more distilled water, converted to the K\(^+\) form with 2 N potassium hydroxide, and washed again with distilled water. The resin was then converted to the H\(^+\) form with 2 N hydrochloric acid and washed with distilled water until the washings were neutral. Excess water was removed by filtration, and the moist resin was stored in a tightly capped jar.

Three 1.0 gram portions of the moist resin were weighed into 16 X 125 mm screw cap tubes. Two of the resin samples were converted to the K\(^+\) form by adding 15 ml of 2 N potassium hydroxide to each, and shaking vigorously. After the resin settled to the bottom, the supernatant was decanted and each resin sample was washed with three 15 ml portions of distilled water. One of the K\(^+\) resin samples was then equilibrated with .05 M KH\(_2\)PO\(_4\) by suspending the resin in 15 ml of this buffer, allowing it to settle, and decanting the buffer. The procedure was repeated three times. The second sample of K\(^+\) resin was similarly equilibrated with a 1 to 1 mixture of acetone and 0.1 M KH\(_2\)PO\(_4\). The sample of resin which had been left in the H\(^+\) form was washed with three changes of 50% acetone. After the third wash had been decanted, the tubes containing the resin samples were inverted over a paper towel for 5 minutes to drain. After draining, 5.0 ml of the solution with which the resin had been equilibrated was added to each tube. A control tube containing 5.0 ml of the .05 M KH\(_2\)PO\(_4\)-50% acetone, but no resin, was set up. To each tube, 20 units of erogens (eluted from Porapak Q) were added. The tubes were capped and placed on a reciprocating shaker, with their long axes parallel to the direction of motion, at 20\(^\circ\)C for 1.5 hours to allow the distribution of the erogens between the solution and the resin to
reach equilibrium. The resin was then allowed to settle, and the supernatants were decanted and adjusted to pH 5.5. A sample from each supernatant was diluted 1/4 with 0.5% soytone, pH 5.5, and bioassayed.

Duolite A-4 anion exchange resin was washed with distilled water, 2 N potassium hydroxide, distilled water, 1 N acetic acid, and distilled water, and air-dried. Two 0.5 gm samples of dry resin in screw cap tubes were converted to the free base form by suspending each in 15 ml of 2 N potassium hydroxide, and washing with three changes of distilled water. One resin sample was equilibrated with three 15 ml changes of 0.1 M K$_2$HPO$_4$, and the other with three 15 ml changes of 0.1 M K$_2$HPO$_4$ containing 50% acetone. The resin samples were drained as above, and then suspended in 5.0 ml of the same buffer with which they had been equilibrated. A control tube containing 5.0 ml of 0.1 M K$_2$HPO$_4$-50% acetone, but no resin, was also set up. 100 units of erogens were added to each tube. The tubes were capped and left on the shaker for 1.5 hours. Then the resin was allowed to settle, the supernatants were decanted, adjusted to pH 5.5, and diluted 1/20 for bioassay.

In a second experiment, four 0.5 gm samples of dry Duolite A-4 resin were weighed into screw cap tubes. Two of the samples were equilibrated with three changes of 0.1 M K$_2$HPO$_4$. The other two samples were converted to the free base form as above. One of the resin samples equilibrated with 0.1 M K$_2$HPO$_4$ and one of the samples in the free base form were washed with three 15 ml portions of distilled water, drained, and resuspended in 5.0 ml each of distilled water. The other resin sample of each type was washed with three 15 ml portions of 50% acetone, drained, and resuspended in 5.0 ml of 50% acetone. A control tube containing 5.0 ml of 50% acetone, but no resin, was prepared. 50 units of erogens were added to each tube, and the capped tubes were agitated on the shaker at 20°C for 3 hours. Then the resin was allowed to settle, and the supernatants were decanted, adjusted to pH 5.5, and diluted 1/10 for bioassay.
IV. Further attempts at purification

Erogens were produced in 500 ml lots of Gluc-AmS in 2800 ml Fernbach flasks, incubated on a shaker at 20°C. The turbidity of the cultures at 640 nm was measured on samples taken daily, and the cultures were harvested one day after they reached peak turbidity. The cells were removed by centrifugation for 5 minutes at full speed in a clinical centrifuge (IEC model HN, angle head). The supernatants were passed through a bed of 10 grams of Porapak Q in a chromatography tube 2 cm in internal diameter at a flow rate of 200 ml/hour. The effluent was discarded. The resin was washed with 200 ml of distilled water, and the erogens were eluted with 100 ml of 50% acetone. The eluate was concentrated in vacuo until no more acetone distilled over, and then freeze-dried. The freeze-dried eluate was stored in a tightly capped vial in a freezer. The Porapak resin was washed with 100 ml of acetone followed by 500 ml of distilled water before reuse.

100 mg of the freeze-dried eluate was suspended in 50 ml of water saturated with n-butanol, and extracted with five 50 ml portions of n-butanol saturated with water. The extracts were centrifuged to remove water droplets and then evaporated to dryness in vacuo. The extracted solids were dissolved in 8 ml of distilled water and 4 ml of ethyl acetate. The water-ethyl acetate mixture was centrifuged to separate the phases, and the aqueous phase re-extracted with a second 4 ml portion of ethyl acetate. The combined ethyl acetate extracts were made up to 50 ml with acetone, and diluted 1/50 for bioassay. The raffinate from the butanol extraction, and the aqueous solution of extracted materials were made up to 50 ml with 50% acetone, and diluted 1/50 for bioassay. The solution of materials extracted with butanol was then concentrated in vacuo to remove acetone, and freeze-dried.

At another time the 50% acetone Porapak eluates from several batches of culture supernatant were combined, concentrated in vacuo to remove acetone, and then extracted four times with two
volumes of n-butanol. The n-butanol extracts were centrifuged, and evaporated to dryness in vacuo. The residue was dissolved in 10 ml of water plus 10 ml of ethyl acetate. The water-ethyl acetate mixture was centrifuged, and the aqueous layer was made up to 50 ml with 50% acetone, and diluted 1/200 for bioassay. The acetone was then removed from the extract solution by concentration in vacuo, and the solution was freeze-dried.

The combined freeze-dried butanol extracts were ground in 1 ml of absolute ethanol in a mortar, and applied to the top of a chromatography column containing 50 gm of silica gel in absolute ethanol. The column was eluted with 100 ml of ethanol, followed by a gradient of water in ethanol generated by the method of Chapter Two section X. The flow rate was 0.25 ml/min, and 20 minute fractions were collected. For bioassay, 50 µl of each fraction was diluted with 1.0 ml of 0.5% soytone, pH 5.5, autoclaved, and inoculated with 1 drop of a one day old culture of 2259-6 in (4,4) medium. The even numbered fractions from 40 to 174 were assayed again with only 10 µl added to each assay tube. Fractions 42-174 were combined and evaporated in vacuo to dryness. The residue was dissolved in 10 ml of 75% acetone, and stored in the freezer.

The collected foam from 5 litres of 2259-7 culture in Gluc-AmS in the Microferm was centrifuged to remove cells. The supernatant was passed through a bed of 10 grams of Porapak Q on a sintered glass funnel 6 cm in diameter. The resin was washed with distilled water, and erogens were eluted with 100 ml of 50% acetone. The eluates from three 5 litre cultures were combined, and concentrated in vacuo to remove acetone. The aqueous solution (170 ml) was extracted with 100 ml of n-butanol, and then with four 50 ml portions of n-butanol. The butanol extracts were centrifuged and evaporated to dryness in vacuo. The residue was dissolved as completely as possible in 10 ml of distilled water plus 10 ml of ethyl acetate. The phases were separated by centrifugation, and the aqueous phase was made up to 50 ml with 50% acetone and diluted 1/200 for bioassay. The
raffinate from the butanol extracts (140 ml) was diluted 3/100 for bioassay. The acetone was distilled in vacuo from the extract solution and the extract was freeze-dried.

V. Thin layer chromatography

Thin layers of cellulose were prepared by spreading a slurry of 35 grams of microcrystalline cellulose (J. T. Baker Co.) in 175 ml of distilled water, mixed in an electric blender for 1 minute, in a layer 750 μ thick on 20 X 20 cm glass plates. After drying, the layers were washed by ascending development in acetone-0.1 N hydrochloric acid (1/1).

One plate was spotted 2 cm from the bottom with 25 μl of the hormone I preparation described in section II of this chapter and 10 μl of the erogens prepared by silica gel chromatography as described in section IV. The plate was developed in 0.1 N aqueous ammonia. After drying, the area between the solvent front and the origin for each preparation was divided into nine zones. The cellulose from each zone was scraped into a test tube and suspended in 1.0 ml of 0.5% soytone, pH 5.5. The tubes were autoclaved for 10 minutes on a dry goods cycle, and inoculated with 1 drop each from a one day culture of 2259-6. After 17 hours, the fraction of cells with conjugation tubes in each assay was determined. Similarly, plates spotted with samples of the two erogen preparations were developed in ethyl acetate-ethanol-0.1 N hydrochloric acid (40/40/20) and chloroform-methanol-0.1 N ammonia (40/25/2.5). The areas between the origin and the solvent front were divided into ten zones, which were scraped off and assayed as above.

Four TLC plates were streaked with 0.5 ml each of the hormone preparation described in section IV (i.e. 1150 units per plate). The start line was compressed by brief development in 50% acetone, and then the plates were developed twice in chloroform-methanol-0.1 N ammonia (40/25/2.5). The erogens were located by scraping off zones 1 cm high and 5 mm wide, and assaying the scrapings. The cellulose from Rf 0.05 to 0.50
was scraped off, powdered in a mortar, and poured into a mini-
ture chromatography column made from 3/16" glass tubing. The
erogens (designated H-1) were eluted with 50% acetone. The
cellulose from Rf 0.85 to 0.95 was similarly scraped off,
powdered, and eluted (H-2). One TLC plate was streaked with the
H-1 solution, and another with the H-2. The plates were developed
in ethyl acetate-ethanol-0.1 N hydrochloric acid (40/40/10).
Ten zones, 5 mm wide, were scraped from each plate and bioassayed.
The cellulose from Rf 0.05 to 0.30 on the H-1 plate and the
cellulose from Rf 0.35 to 0.70 on the H-2 plate were scraped
off, powdered, and eluted. The H-1 eluate was applied to the
left half, and the H-2 eluate to the right half, of a TLC plate,
and the plate was developed in n-butanol-acetic acid-water (60/
15/25). The erogen zones were located as usual. The cellulose
from Rf 0.30 to 0.40 on the H-1 side of the plate was scraped
off, powdered, and eluted. The eluate was streaked along the
origin of another TLC plate, which was developed in n-butanol-
pyridine-water (30/30/30) (the pyridine was redistilled just
before use). Zones 1 cm wide and 5 mm high were scraped from
the area from Rf 0.45 to 0.90 for assay. The cellulose from
Rf 0.64 to 0.73 was scraped off, powdered, and eluted with 50%
acetone. The eluate (2 ml) was diluted 1/800 for bioassay.

25 μl of the erogens prepared by silica gel chromatography
were spotted on a cellulose layer, sprayed with a 0.5% solution
of ninhydrin in acetone, and heated at 105°C for 10 minutes. A
plate bearing another 25 μl of erogen preparation was placed
for 5 minutes in a chromatography tank containing chlorine gas
generated by mixing 5 ml of concentrated hydrochloric acid with
5 ml of a 10% solution of potassium permanganate. After removal
from the tank, the plate was heated at 105°C for 15 minutes to
drive off chlorine, and sprayed with a solution containing 0.2%
potassium iodide and 0.1% soluble starch (Rydon and Smith 1952).

Two TLC plates were spotted with 50 μl of erogen preparation
in one corner, 2 cm from each edge. The plates were developed
first in chloroform-methanol-0.1 N ammonia (40/25/2.5), and then
at right angles in ethyl acetate-ethanol-0.1 N hydrochloric acid (40/40/15). One plate was sprayed with 0.5% ninhydrin and heated for 15 minutes. The other plate was exposed to chlorine, heated and sprayed with starch-iodide solution.
Results

I. Instability

The erogen samples which had been autoclaved in 1 N potassium hydroxide and 1 N hydrochloric acid showed no conjugation hormone activity. The sample autoclaved in 10% ammonia had 0.023 units per ml (95% confidence limits .002 and .058). The sample autoclaved in 1 N KCl had 0.15 units/ml (95% confidence limits .06 and .25). If no erogen activity had been destroyed, the potency expected for each sample was about 1 unit/ml.

II. First attempt at purification

When the hormone concentrate solution was applied to the anion exchange resin, some of the brown colour was washed straight through the column. More pigment was eluted by the 1 N acetic acid, and when the resin was regenerated with potassium hydroxide, more brown colour came off. The weight of the material eluted from the anion exchange column was 1.092 grams. The butanol extract after freeze-drying was a fluffy yellow material weighing 105 mg.

The material eluted from the anion exchange resin, the redissolved precipitate and the butanol extract all showed erogenic activity of about 1 unit/ml at the concentrations assayed. The raffinate from the butanol extraction contained a total of 1250 units. The ethyl acetate extract had very low hormone activity.

The combined butanol extracts had a specific activity of 125 units/mg, and a total activity of 30,500 units. The yield and purification at each step of the procedure are summarized in Table XV.

The fraction of cells with conjugation tubes in the assay of each fraction from the silica gel column has been converted to relative erogen concentration, and plotted against fraction number in Fig. 25.
Fig. 25. Chromatography of first erogen preparation on silica gel with a gradient of water in ethanol. Bars represent 95% confidence intervals.
Table XV. Progress of the erogen purification.

<table>
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<th>Stage</th>
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<th>Specific activity units/mg</th>
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<th>Yield (%)</th>
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</table>

III. Interaction of the erogens with ion exchange resins

The .05 M KH₂PO₄ supernatant from the cation exchange resin in the K⁺ form contained .03 (95% confidence limits .01 and .05) times as much erogen activity as the control. The .05 M KH₂PO₄-50% acetone supernatant had 1.36 (95% confidence limits 1.0 and 1.9) times as much erogen activity as the control. The 50% acetone supernatant from the H⁺ resin had no erogen activity. These data indicate that adsorption of the erogens from 0.05 M KH₂PO₄ onto the resin was almost complete, but was negligible from .05 M KH₂PO₄ containing 50% acetone. Repetition of the experiment gave similar results.

The .1 M K₂HPO₄ supernatant from the Duolite A-4 anion exchange resin contained no detectable erogen activity. The .1 M K₂HPO₄-50% acetone supernatant, however, had .86 (95% confidence limits .61 and 1.1) times as much erogen activity as the control.

In the second experiment, the two distilled water supernatants from the Duolite A-4 resin had very low erogen activity. The 50% acetone supernatant from the Duolite A-4 resin which had been equilibrated with .1 M K₂HPO₄ contained 0.14 (95% confidence limits 0.11 and 0.16) times as much erogen activity as the control. The 50% acetone supernatant from the resin in the free base form had 0.15 (95% confidence limits .11 and .18) times as much erogen activity as the control. Thus the erogens are almost completely adsorbed on the anion exchange resin from
distilled water or .1 M K₂HPO₄. From 50% acetone, 85% of the erogens are adsorbed on the resin at pH 7 or higher, but from 0.1 M K₂HPO₄-50% acetone, only 15% or less of the erogens are adsorbed.

IV. Further attempts at purification

The specific activity of the erogens prepared by Porapak Q adsorption from shake culture supernatants was 48 units/mg (95% confidence limits 43 and 54). The raffinate from the n-butanol extraction, and the ethyl acetate extract showed low erogenic activity. The n-butanol extract solution contained 1375 units of erogens. The second butanol extract contained 25,000 units of erogens. The distribution of erogenic activity among the fraction from the silica gel column is shown in Fig. 26. The erogens pooled from fractions 42 to 174 had a total activity of 23,000 units, and a specific activity of 250 units per mg (95% confidence limits 228 and 272).

The combined Porapak eluates from the foam from 15 litres of 2259-7 culture in Gluc-AmS contained 162,000 units of activity. The butanol extract had a total activity of 54,000 units, and a specific activity of 1500 units/mg (95% confidence limits 1355 and 1670). The raffinate from the butanol extraction contained only 1260 units. The specific activity of the erogens prepared by Porapak adsorption from condensed foam was 660 units/mg (95% confidence limits 596 and 724).

V. Thin layer chromatography

The distribution of erogen activity on the TLC plates after development in the three solvent systems is shown in Fig. 27 for the hormone I preparation of section II, and in Fig. 28 for the erogen preparation of section IV.

Of the original 4600 units of erogen activity, less than 100 units of H-1 were recovered after preparative TLC in the four solvent systems. The activity of H-2 after TLC in n-butanol-acetic acid-water was very low, and appeared at Rf 0.4 to 0.6.
Fig. 26. Chromatography of second erogen preparation on silica gel with a gradient of water in ethanol. Bars represent 95% confidence intervals.
Fig. 27. Thin layer chromatography of hormone I preparation.
Fig. 28. Thin layer chromatography of second erogen preparation.
The erogen preparation gave a pink colour with ninhydrin, and a bluish purple colour in the Rydon-Smith test. After two dimensional TLC, neither ninhydrin nor the Rydon-Smith test gave a colour reaction in the area where the erogen activity was expected to be.
Discussion

The complete loss of erogen activity on autoclaving in either 1 N hydrochloric acid or 1 N potassium hydroxide indicates that high temperatures and extremes of pH should be avoided in the handling of the erogens. Autoclaving in 10% ammonia destroyed 98% of the activity and autoclaving at neutral pH in 1 N KCl destroyed 85%. The high salt concentration may have increased the destruction. The loss of activity during autoclaving under the conditions of the bioassay has not been measured. Although the erogens are unstable at extreme pH and high temperature, the standard preparation has been kept in the dry state at freezer temperatures for several months without noticeable loss of activity.

In the first purification attempt, the specific activity was increased 11 fold by adsorption on and elution from Duolite A-4 anion exchange resin, but the recovery of activity was low. The five gram portions of concentrate used could have exceeded the capacity of the resin, and some of the erogens may have been lost in the column effluent. The adsorption and elution steps were carried out at 4°C, and the acetic acid eluant was removed by freeze-drying, to minimize destruction of the erogens by the acid. However, some of the activity could have been lost because of exposure to the acid. Also, the 1 N acetic acid may not have eluted all of the adsorbed erogens from the resin.

Further purification of the hormone II preparation after silica gel chromatography by cation exchange chromatography was attempted, using ammonium acetate and pyridine acetate buffers. Under all the initial conditions tried the erogen activity was only slightly retarded by the resin, and little separation was achieved. The effects of pH and organic solvents on adsorption of the erogens on ion exchange resins were studied to allow more rational design of ion exchange purification schemes. The ability of 50% acetone to elute the erogens from Porapak resins suggested that this solvent might eliminate the non-polar binding of the erogens to the polystyrene backbone of the ion exchange resins.
Comparison of the adsorption of the erogens from 0.05 M KH$_2$PO$_4$ and 0.05 M KH$_2$PO$_4$-50% acetone onto Amberlite IR-120, and from 0.1 M K$_2$HPO$_4$ and 0.1 M K$_2$HPO$_4$-50% acetone onto Duolite A-4 support this view. Adsorption from aqueous solution can be interpreted as the sum of non-polar and ionic binding, and adsorption from solutions containing 50% acetone can be interpreted as ionic attraction alone. The complete adsorption from 50% acetone onto the H$^+$ form of the resin indicates that the erogens can acquire a positive charge at sufficiently low pH. The erogen molecules may have an acidic isoelectric point. On Duolite A-4 resin, 50% acetone reduces but does not eliminate erogen adsorption. Apparently the erogens are negatively charged at pH 7 and above. The equality of adsorption on resin at pH 7 and in the free base form suggests that the isoelectric point of the erogens is well below 7. The decreased adsorption in 0.1 M K$_2$HPO$_4$-50% acetone could be caused by competition for the ion exchange sites by phosphate. In all cases where only part of the erogen activity is adsorbed, each of the active components may be bound to a different degree. Measurement of the erogen distribution between the resin phase and 50% acetone at more pH's could be used to determine the isoelectric pH of the erogens, and to check that the results obtained with anion and cation exchangers are consistent. Electrophoresis could provide an independent estimate of the sign and magnitude of the charge on the erogens at various pH's.

Extraction of the erogens from aqueous solutions with n-butanol increases the specific activity, but gives low recovery. The missing activity is not left in the raffinate. Possibly this activity is destroyed during evaporation of the butanol, or is held in the part of the residue which cannot be re-dissolved after evaporation of the butanol.

An abbreviated form of the bioassay has been used for the large number of samples arising from chromatographic separation. Only one dose of each sample is assayed, and the fraction of cells producing conjugation tubes is converted to erogen concen-
tration using values for the regression line slope and standard ED50 from a standard series assayed simultaneously. This method is less accurate than the full bioassay procedure, but allows the assay of higher numbers of samples.

Chromatography on silica gel as described in section II clearly separated two peaks of activity, with a suggestion of a third peak at the end of the chromatogram. The material in the first peak was unretarded by the column, and emerged with the void volume. There may be a small peak centered on fraction 33. The main peak appears to contain four sub-peaks. However, the response in the assays of all these fractions was near maximum, and the errors of estimation are large.

When the hormone preparation of section IV was chromatographed on silica gel, no hormone I peak appeared. All of the erogen activity was found in one broad peak, at approximately the elution volume expected for hormone II. It is possible that hormone I is an artifact, caused by some chemical change during the preparation procedures described in section II.

The relation between hormone I and the erogen peak from the silica gel chromatography of section IV was investigated by TLC. After development with 0.1 N ammonia, both erogen preparations were separated into a component with Rf about 0.1, and a second component with Rf 0.75 to 1.0. On TLC in ethyl acetate-ethanol-0.1 N hydrochloric acid (40/40/20), two components, with approximate Rf's 0.75 and 0.95 were indicated in each preparation. TLC in chloroform-methanol-0.1 N ammonia (40/25/2.5) demonstrated active components with Rf's 0.3 and 0.95, and possibly a third with Rf 0.05, in both preparations. The hormone I preparation seems to contain more of the less polar component, and the other erogen preparation has more of the more polar component.

When the more polar (H-1) and less polar (H-2) components of the second erogen preparation were separated by preparative TLC, they showed distinct Rf's in the solvent systems ethyl acetate-ethanol-0.1 N hydrochloric acid (40/40/10) and n-butanol-acetic acid-water (60/15/25). There was no separation of either
H-1 or H-2 into more components during chromatography in these solvents. The yield of erogen activity after successive chromatography in four solvent systems was very low. The erogens may have been destroyed during chromatography, or they may have been incompletely eluted from the powdered cellulose.

The colour reactions with ninhydrin and the Rydon-Smith test indicate that the erogen preparation after silica gel chromatography contains amino acids and/or peptides. The erogens were separated from the reacting materials by two dimensional chromatography. The lack of detectable colour formation in the area where the erogens were expected to be could have been caused by insufficient material.

Adsorption on Porapak Q is a rapid and effective method for primary purification of the erogens from crude culture supernatants. The erogens can be eluted in high yield under gentle conditions. Chromatography of the erogens on columns of Porapak, using an appropriate concentration of acetone in water as solvent, might provide good purification.

The purification of the erogens achieved by foaming can be seen from comparison of the specific activity of erogens prepared by Porapak adsorption from culture supernatants (48 units/mg) and from condensed foam (660 units/mg).

With present information, the best preliminary purification procedure for the erogens appears to include concentration of the erogens in the foam from the culture, centrifuging the condensed foam to remove cells, passing the supernatant through Porapak Q, eluting the erogens with 50% acetone, distilling off the acetone, and extracting the aqueous solution with n-butanol.
SUMMARY AND GENERAL DISCUSSION
Tremella mesenterica 2259-7 can use ammonium but not nitrate as the sole nitrogen source for growth. A high concentration of microelements selectively slows growth in media containing amino acids. L-asparagine, alone or in combination with other amino acids, causes slow and abnormal growth of the cells. Thiamine is the only vitamin for which this fungus shows a requirement. Sodium acetate counteracts the pH drop in cultures using ammonium as nitrogen source. A defined medium containing glucose, ammonium sulphate, thiamine, salts and microelements supports good growth of T. mesenterica 2259-7.

The erogens produced by 2259-7 can be detected by their ability to induce the growth of conjugation tubes from the cells of strain 2259-6. The erogens have a partition coefficient of 2.3 from aqueous solution into n-butanol, but are not extracted in detectable amounts by less polar organic solvents. The erogens are strongly adsorbed on activated charcoal, requiring pyridine acetate solution for desorption, and on the polystyrene resin Porapak, from which they can be eluted with 50% acetone. The erogens are also adsorbed on both cation and anion exchange resins. Part of the binding is due to non-polar forces, and part to ionic attraction. The isoelectric pH of the erogens appears to be below 4.5. The erogens are also adsorbed on Sephadex G-10, so that their molecular weight cannot be estimated by gel chromatography. However, ultrafiltration suggests a molecular weight below 750. Chromatography on columns of silica gel with a gradient of water in ethanol separated three active erogenic components. Similar chromatography of a later preparation of erogens gave only one active peak. Thin layer chromatography demonstrated at least two active components in each erogen preparation. The erogens may be amino acids or short peptides with non-polar side chains.

The concentration of erogens has been shown to affect the fraction of 2259-6 cells which produce conjugation tubes, the length of the conjugation tubes, and the number of conjugation
tubes per cell. A statistically valid and practical quantitative bioassay for the erogens has been developed, using the fraction of cells which produce conjugation tubes as the response. The unit of erogen activity is defined as the activity of 0.2 mg of a standard preparation. Optimum conditions of incubation time, temperature, pH and cell density for the bioassay have been established. Complex nitrogen sources such as soytone are best for conjugation tube production, and ammonium is poor.

Maximum erogen activity in the supernatant of 2259-7 cultures is reached at the same time as peak turbidity. Ammonium is the best nitrogen source tested for erogen production. The concentration of various components of the defined medium have been adjusted for highest erogen production. The erogens are removed from 2259-7 cultures by the foam which forms spontaneously when air is bubbled through the cultures. Foaming is a useful method for preliminary concentration and purification of the erogens in large scale production.

Attempts to purify the erogens have given low yields of the hormone activity. Future research will need to develop purification methods with high recoveries of the erogens. Then using the methods for erogen production developed in this thesis, sufficient material can be purified for chemical characterization. When purified erogens become available, studies on the mechanism by which they redirect the growth of the cells from budding to conjugation tube production can begin.

The number of distinct erogens produced by T. mesenterica 2259-7 is still unclear. Chromatography on silica gel suggests at least three, although some of these may be artifacts of the preparative process. Individual peaks of erogen activity from the silica gel column are separated into at least two active components by thin layer chromatography. The components from different peaks show similar Rf values. Monitoring the separation on a silica gel column by TLC may clarify the relation between the peaks of erogen activity and the components resolved by TLC.

Parallel studies on the conjugation hormones from the other mating type of T. mesenterica, and from other species of Tremella
would be of interest for the similarities to and differences from
the erogens of 2259-7 that they might show.
BIBLIOGRAPHY


Appendix A. Frequently used media.

### Malt Yeast Peptone (MYP)

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### Glucose Soytone Medium (GS)

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*stored in refrigerator*

### Conjugation Medium (CJM)

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Glucose-Ammonium Sulphate Medium (Gluc-AmS)

- D-Glucose: 10.0 gm
- \((\text{NH}_4\text{)}_2\text{SO}_4\): 1.0 gm
- \(\text{KH}_2\text{PO}_4\): 1.0 gm
- \(\text{MgSO}_4\cdot7\text{H}_2\text{O}\): 0.5 gm
- \(\text{CaCl}_2\cdot2\text{H}_2\text{O}\): 0.1 gm
- Thiamine·HCl: 100 µgm

**Microelement stock solution**: 0.13 ml

**Distilled water**: 1 litre
Appendix B. Measurements of conjugation tube length at various concentrations of erogen.

Doses are expressed in micrograms of hormone preparation per ml. Lengths are measured in microns.

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Appendix G. A computer program to process bioassay data.

This program has been written in Fortran IV, and run on an IBM 1130 computer at the Biology Data Center, University of British Columbia.

Input:

Data cards required are:
1. A first card, specifying the number (1-99) of assays to be processed.
2. For each assay, an initial card giving the number of preparations assayed (1-20) in I2 format, and the date (A4).
3. For each preparation a card giving the number of doses (1-5) in I1 format, a sequence number (I2), the name of the preparation (A4), and for each dose, the logarithm to the base two of the dilution (0, -1, -2, etc.) in F4.1 format, the number of cells with conjugation tubes (F4.0), and the total number of cells counted (F4.0).

Output:

The program first lists the data for each preparation along with the calculated fraction of cells with conjugation tubes (P), the logit of these fractions (Y), and the expected logits from the regression lines (Y3). The slope of the regression line and the chi-squared for the deviations of the data from the regression line are given along with the probability that the deviations are caused by random sampling.

Next comes a list of actual and expected logits from the regression with all lines parallel. The value and confidence interval for the common slope are printed, along with G, a figure of merit for the precision of the assay. An analysis of the deviations of the data from the regression lines follows with tests for deviations greater than can be explained by random sampling (heterogeneity), and non-parallelism of the regression lines. If heterogeneity is significant, a heterogeneity factor is calculated, and used to broaden the confidence intervals for
the parameter estimates. Finally, for each preparation, the ED50, with 95% confidence limits, the logarithm to the base two of relative potency \((M)\), and the potency relative to the standard, with 95% confidence limits, are listed.
DATA STT/12.7,4.3,3.2,2.8,2.6,2.5,2.4/

TRIP = 0.5*ALOG(P/(1-P))

READ(2,2)NR

FORMAT(12)

DO 100 L = 1, NR

READ(2,3)NP, DATE

FORMAT(I2, A4)

WRITE(3, 60) np, DATE

60 FORMAT(I1, A4, //)

*TREMELLA HORMONE BIOASSAY PERFORMED 7//G4 ///G1/ PREP DO

* CONTINUE

NID=1

NID=0

XBAR(ID)=SX/AND

YBAR(ID)=SY/AND

SX=5/SXY/SXX

B1=SXY/SXX

27 SHU=0

SX=0
C-ERR*...STNO*...... FORTRAN SOURCE STATEMENTS ........ IDENTFCN **COMPILER MESSAGES**

SY = 0.
SYZ = 0.
SYZ = 0.
C YBAR(ID) = B1*XBAR(ID)
DO 11 I = 1, ND
 Y4(ID) = C = B1*O(I)ID
 ZY4(ID) = I
Z = Y4(ID) +
Y/NZ = (P(ID) + O2 - 1)/(2*(1-O2)*O2)
N** = (1-O2)*O2

SN = S/N = YBAR(ID)
SX = S = YBAR(ID)
SY = S = YBAR(ID)
SXZ = S = YBAR(ID)

11 CONTINUE
 YBAR(ID) = SY/SN
 XBAR(ID) = S/SN
 SX = S = SX2/SN
 SY = S = SY2/SN
 SXY = SXY = S/SN
SX2 = SX2 + SX2/SN
SY2 = SY2 + SY2/SN
SXY = SXY + SXY/SN
SX2 = SX2 + SX2/SN
SY2 = SY2 + SY2/SN
SXY = SXY + SXY/SN

U2 = SXY/SX2
C = YBAR(ID) - B2*XBAR(ID)

IF(ABS(Y3(IDC) - Y4(IDC)) > 0.01)
101 CONTINUE
GOTO 30

20 ITER*ITER*1
30 I = I + 1
IF(ITER > 20)

40 WRITE(3,29) ITER
WRITE(3,30) FORMAT(3,12.F5.2, 12.F7.4)

1F7.4

30 CONTINUE
WRITE(3,64) ITER
WRITE(3,64) FORMAT(100X, 'NUMBER OF ITERATIONS ', ' 12/ SLOPE ', ' 13/ WITH 12/ DEGREES OF FREEDOM AND HETOGEN')

1.4)

101 CONTINUE
WRITE(3,64) ITER
WRITE(3,64) FORMAT(100X, 'NUMBER OF ITERATIONS ', ' 12/ SLOPE ', ' 13/ WITH 12/ DEGREES OF FREEDOM AND HETOGEN')

1.4)
C-ERR-036: FORTRAN SOURCE STATEMENTS

IF (ITER < 20) GO TO 20
IF (ITER < 3) GO TO 30
ELSE IF (ITER >= 20) GO TO 200

WRITE (1,1)
1 FORMAT (1X, 36X, 'COMPOSITE ANALYSIS DOES NOT CONVERGE')

GO TO 100

WRITE (1,31)
31 FORMAT (1X, 36X, 'IDM = I')

WRITE (1,46)
46 FORMAT (1X, 36X, 'CHI1 = CHI2 = CHI3')

GO TO 100

WRITE (1,59)
59 FORMAT (1X, 36X, 'CHI4 = CHI5 = CHI6 = CHI7')

GO TO 100

WRITE (1,72)
72 FORMAT (1X, 36X, 'CHI8 = CHI9 = CHI10 = CHI11')

GO TO 100

WRITE (1,85)
85 FORMAT (1X, 36X, 'CHI12 = CHI13 = CHI14')

GO TO 100

END
C-ERRORS...... FILE ........ FORTRAN SOURCE STATEMENTS .......... IDENTFCN **COMPILER MESSAGES**

1401 AND PATH= 'AF7.4/' HETEROGENEITY FACTOR = 'AF9.4/
1402 WRITE('AF7.4',HW,HP)
1403 IF(PHI=0.00009+5.0/50)
1404 WRITE(3,51)
1405 FORMAT('PREP ND CHISQ CONTRIBUTION')
1406 DO 52 I=1,NP
1407 XBAR(I)=XBAR(I)-10
1408 WRITE(3,52)
1409 FORMAT('ED50 (XBAR(I))) = XBAR(I)/ED50(I)
1410 IF(NP>100.100.57
1411 DO 58 I=2,NP
1412 CL=AM+AD-AD+T3/11.11-G1
1413 WRITE(3,58)
1414 FORMAT(A4.1X,F6.2.1X,F6.2.1X,F6.2.7F7.3.2F7.3.2F7.3)
1415 CONTINUE
1416 CALL EXIT
1417 END

FEATURES SUPPORTED
ONE WORD INTEGERS
STANDARD PRECISION
LOAD & GO
ICCS=1132
PRINTED CARD

CORE REQUIREMENTS FOR
COMMON=O
VARIABLES AND TEMPORARIES=2016
CONSTANTS AND PROGRAM=2496

/// XEQ ** COMMENCE PROGRAM EXECUTION **
### Tremella Hormone Bioassay Performed A 2

**PREP DOSE R T P Y1 Y2**

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**NUMBER OF ITERATIONS**

2

**SLOPE**

0.324

**HETEROGENEITY CHISQR**

2.41 WITH 1 DEGREES OF FREEDOM AND PROB = 0.2931

**PREP DOSE R T P Y1 Y2**

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**NUMBER OF ITERATIONS**

2

**SLOPE**

0.344

**HETEROGENEITY CHISQR**

0.21 WITH 1 DEGREES OF FREEDOM AND PROB = 0.648

**PREP DOSE R T P Y1 Y2**

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**NUMBER OF ITERATIONS**

2

**SLOPE**

0.610

**HETEROGENEITY CHISQR**

1.37 WITH 1 DEGREES OF FREEDOM AND PROB = 0.2387

**PREP DOSE R T P Y1 Y2**

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**NUMBER OF ITERATIONS**

3

**SLOPE**

0.392 WITH CONFIDENCE LIMITS 0.4014 AND 0.333

**HETEROGENEITY CHISQR**

4.013 WITH 2 DEGREES OF FREEDOM AND PROB = 0.093

**HETEROGENEITY FACTOR**

-1.000

**PARALLELISM CHISQR**

2.9972 WITH 2 DEGREES OF FREEDOM AND PROB = 0.055

**PREP ED50 CONFIDENCE LIMITS IN RELATIVE POTENCY CONFIDENCE LIMITS**

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