YEAST GENETICS

LIFE CYCLES, CYTOLOGY, HYBRIDIZATION, VITAMIN SYNTHESIS, AND ADAPTIVE ENZYMES

CARL C. LINDEGREN

The Henry Shaw School of Botany, Washington University, St. Louis

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I. LIFE CYCLES

Common bakers' and brewers' yeast, Saccharomyces cerevisiae, exists in both haplophase and diplophase. There are two mating types in the haplophase, and haploid cells of opposite mating type copulate to produce diploid cells. Winge (76) in Copenhagen, and Kruis and Šatava (26) in Prague, showed that the standard vegetative cells of S. cerevisiae are diploid, produced by copulation of two spores or gametes derived from spores. The diploid nuclei undergo reduction at spore formation to produce four haploid ascospores. The large, ellipsoidal vegetative yeast cell is produced by the fusion of two round haploid gametes derived from ascospores. Winge established the facts of this life cycle beyond question by a classical series of observations on the germination of ascospores and fusion of haploid cells. Winge and Laustsen (78–82) in a series of notable papers, showed that colonial characteristics, fermentative ability, and cell shape are under the control of genes which segregate at the reduction division.

The Distribution of Haplophase and Diplophase

Winge was the first to distinguish clearly between haplophase and diplophase yeast cultures, and we have corroborated his observations with some slight modifications. Workers familiar with other biological material may question the propriety of speaking definitely of haplophase and diplophase in organisms where the cytological facts have not been conclusively demonstrated. I shall therefore summarize all the arguments, Winge's reinforced by ours, for distinguishing haplophase and diplophase. I should preface these rules by saying

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that over four-fifths of the cultures which one encounters are easily characterized by microscopic examination. They are either obviously haploid or diploid, as shown simply by size, shape, and aggregation of the cells. The reasons for classifying them are as follows:

a. The large vegetative cells, which we call "legitimate diploids" produce viable four-spored asci. These spores germinate to produce smaller, usually round, cells, which we call "haploid." The latter multiply vegetatively, generally maintaining their specific cell-shape and size.

b. Two of these smaller cells may fuse to produce a large "diploid" cell capable of vegetative multiplication. While the large cell is undergoing vegetative reproduction, it retains its characteristic ellipsoidal shape and size. Under certain conditions, this diploid cell can be induced to sporulate. Spores from it in turn produce haploids and the process can be repeated indefinitely.

c. The large cells which we recognize as diploids are extraordinarily stable in their genetic characteristics when they are grown under conditions in which sporulation does not occur. Transferring the cultures every forty-eight hours in broth is generally sufficient to maintain the vegetative diplophase. Colonies produced by plating out are not sectored; the plates do not show colonial variants. However, when haplophase (single ascospore) cultures of any age are plated out, a variety of colonial variants appear on the plate or the giant colonies are sectored. These facts are consistent with the view that the large cells are diploid, thus minimizing the number of spontaneous mutations which may appear, while in the haplophase most mutants become apparent and are easily discovered.

d. When the diploid cells sporulate to produce haploid cells, there is genetic evidence of a reduction division (Winge and Laustsen, 78). Genetical analysis shows that a single pair of alleles responsible for the two different mating types is segregated at this meiosis. Two a and two a type haplophase cultures are usually obtained from the four single ascospore cultures (Lindegren and Lindegren, 46). There is also genetic evidence for the segregation of a gene-pair controlling fermentation of melibiose (Lindegren, Spiegelman, and Lindegren, 50) during the meiosis that precedes spore formation. Also, evidence proving that factors controlling cell shape may be segregated in a hybrid of Saccharomyces bayanus and S. cerevisiae (Lindegren, 34) has been accumulated in addition to that previously offered by Winge and Laustsen (81) in the balanced heterozygote, Saccharomyces ludwigii.

e. Haploid cultures of a and a mating type have been paired, and the resulting diploid cultures in turn have been induced to sporulate; the haplophases have been tested and found again to fall into the a and a categories. Matings and tests for this character have been carried through four or five generations in several cases. Similarly, segregation of the gene-pair controlling melibiose fermentation has been observed; the segregants have been tested, mated, and segregation has again been observed in the succeeding generations. Pedigrees of three or four generations are available for many characters.

The above facts seem to prove conclusively that the terms haplophase and
diplophase can be used as definitely in speaking of yeasts as of organisms in which the cytological evidence is more complete. The illegitimate diploids provide an exception which is quite familiar to the mycologist. Copulations between haplophases which are usually incapable of copulating on genetical grounds were called "Durchbrechungskopulationen" by Brunsk (5). Copulations of this type frequently occur in single ascospore cultures and produce diploids which are homozygous for the a or α factors. With rare exceptions these diploids sporulate poorly, and for this reason we have not studied them intensively. A few single ascospore cultures sporulate well, and some produce large cells that are difficult to classify either as definite haplophases or illegitimate diploids. However, the general rules laid down in the preceding discussion hold very well, and exceptions are not more frequent than in the higher plants where the phenomenon of alternation of generations (diploid and haploid) is well established.

**Haploids, Diploids, and Dicaryons**

In the life cycles of other fungi, except yeasts, the only diploid nucleus is formed just preceding sporulation, and the life cycle is almost completely in the haplophase. When the equivalent of a diplophase is required for genetical stability in such structures as toadstools, a dicaryon is produced. This is a cell structure in which two genetically different haploid nuclei are paired in a single cell. Since both nuclei function in the same cytoplasm, the resultant cell is practically, although not actually, diploid for the dominant genes suppress the recessives, resulting in the expression of only normal characters, and the suppression of all mutant characters for which the dicaryon is heterozygous (fig. 1). Dicaryons or diploid nuclei are essential for organ formation because organ formation is based on the genetical stability of the building block, the cell. Dicaryons, although they do not exist in yeasts, are the usual compromise of fungi in organ formation; they give genetical stability with considerable flexibility. For example, when two dicaryons come into contact they may exchange nuclei to reconstitute new dicaryons without the necessity of first breaking down the nucleus by a meiotic mechanism. The advantage which microorganisms derive from spending most of their existence in the haplophase consists in the fact that they are able to take advantage of every mutation which occurs, since the new gene begins to function immediately and by selection it may supplant the original forms. Single mutant genes, however, cannot function in diploid cells (or in dicaryons) because the normal dominant gene suppresses the action of the mutant allele.

**Segregation, Mutation, and Recombination**

In perfect yeasts the large, ellipsoidal vegetative cells, constituting the greater part of the life cycle, are truly diploid, thus setting these organisms apart from other fungi and ascomycetes in this very exceptional character. Previously it was supposed that *S. cerevisiae* produced its spores parthenogenetically. Since haplophase vegetative cells are the rule among fungi, the large, ellipsoidal
FIG. 1. A comparison of genotype and phenotype in haploids, dicaryons, and diploids. The genes A-B, C-D, and E-F, are indicated in three chromosomes. In a haploid cell the mutation of F to f produces the new mutant f which ordinarily is distinguishable by some characteristic from its progenitor. Similarly, a mutation of B to b produces another new mutant. There are eight possible combinations of the normal, mutant f, and mutant b in dicaryons. Six are represented and they are all normal since in every case the expression of the mutant genes is suppressed by a normal allele. In diploid cells the same six combinations all appear normal for the same reason. The greater flexibility of the dicaryon over the diploid lies in the fact that sexual fusions can occur without an intervening meiosis.
vegetative cells were supposed to be haploid and spores were supposed to develop by an apogamous mechanism. Kruis and Šatava (26) were the first to observe that the spores germinated to produce what they called “reduced” forms, which later fused to develop the standard, large ellipsoidal vegetative cells. In their first joint paper, they did not give genetical significance to this phenomenon, apparently because Kruis was of the opinion that yeasts multiplied amitotically, but Šatava (65, 66) later suggested that the fused cells contained a single nucleus, and Winge and Laustsen (78, 81) showed that reduction of the nucleus to produce spores was accompanied by regular Mendelian segregation of morphological characters.

Lindegren and Lindegren (46) demonstrated that the normal so-called legitimate diploid yeast cell is heterozygous for a single pair of mating type alleles, \( a/\alpha \), and that matings occur preferentially between two gametes of opposite mating type. Two of the spores from each ascus belong to mating type \( a \), while the other two belong to mating type \( \alpha \). Each ascospore, when grown separately, produces a culture containing small, round haploid cells and hybridization occurs, producing large, dumb-bell shaped zygotes, when an \( a \) and an \( \alpha \) culture are mixed in the same broth. Copulations can be observed microscopically and large, diploid vegetative cells develop shortly thereafter.

Haploid yeast cells are much smaller and more variable than diploid cells, varying more both from culture to culture and within a single culture than diploid cells. These differences are also reflected in the colonies; the diploid colonies are larger and more uniform, while haploid cultures produce smaller colonies which are usually rough and generally show considerable variation (fig. 2).

Segregation of genes occurs when the chromosomes are segregated at the reduction division of the diploid cells just prior to sporulation. The haplophase originates by the reduction of the diplophase at spore formation, and the segregation of a heterozygote produces segregants of different genotypes. Yeasts are extraordinarily heterozygous, and a great variation of colonial forms is obtained by the isolation of single ascospore cultures. Each of the four spores formed in a single ascus is usually genetically different. The haploid segregants are usually rough-colonied; smooth-colonied diploid cells usually produce only rough-colonied haploid segregants. The segregant cultures also vary in their fermentative ability and in the size and shape of the haploid cells. The type of cell aggregation is also characteristically different. Haplophase clones generally tend to produce aggregated or agglutinated cells much more frequently than the diploid clones.

Haplophase yeasts are nearly always inferior in their fermentative ability when compared quantitatively, or even qualitatively, to the diploid parent from which they originated, and many of them have lost certain specific characteristics. For example, a single ascospore culture originating from \( S. \) cerevisiae may be unable to ferment sucrose, or maltose, or galactose, although the original cultures fermented these sugars successfully.

Mutation in the haplophase enormously increases the variation of colonial
forms, but the original segregant can generally be distinguished from the secondary mutants when the culture is plated out. At first, the mutants are usually slow-growing and produce small, round colonies, but on transfer they become adapted and stabilized and their specific colonial character becomes apparent, distinguishing them from the original segregant. *Schizosaccharomyces pombe*, which is normally unable to ferment galactose, can ferment this sugar if inoculations with cultures containing large numbers of spores are used. Mutations occurring in the haplophase are selected and propagated. Most haplophase yeasts carried in the laboratory by serial transfer become sterile, i.e., lose their ability to copulate with the opposite mating type. In spite of the wide variety of types that mutation produces, the existing genotype of any cell limits its potentialities and the range of its possible variations. This fact has been especially brought out in experiments aimed at adapting haplophases by selection. *S. cerevisiae* is unable to ferment melibiose, and prolonged exposure of haplophase cultures of *S. cerevisiae* to melibiose failed to produce any mutants capable of fermenting this sugar. A haplophase variant of *S. cerevisiae*, incapable of fermenting galactose, could not be induced through a four-month period to produce mutants capable of fermenting galactose, although this strain produced an abundance of colonial variants during that same period. Therefore, some "losses" as in the case of fertility, (see Hybridization) occur easily, while some "gains" as in the case of specific fermentative abilities, apparently do not occur at all. Continued selection and plating of colonies often lead to the appearance of stable colonial variants which seem to have lost their capacity to produce other colonial types. Some of these forms are round-celled and presumably haploid.

Segregation and mutation produce a great variety of haploid gametes. They all have the general characteristics of the species, but most copulations will produce recombinations different in a number of minor characters from the diploid from which they originated. These facts are summarized in fig. 2, showing that (1) segregation, (2) mutation, and (3) recombination, are genetic devices for producing variation in yeasts. The large, ellipsoidal cells which produce a smooth colony on solid medium are usually heterozygous for a number of characters which are segregated at the reduction division and transmitted to four different ascospores which we usually designate arbitrarily as A, B, C, and D. Each of these four spores generally produces a characteristically different colony: the original segregant. The haploid cells are usually round and much smaller than the original diploid cell. When the haploid cells are plated out, a variety of colonial forms appear, indicating that many gene mutations have occurred. When matings are made between the four clones in all combinations, the mating types are revealed. For example, in our specific case, pairing A × B and C × D fails to produce copulations, but the other four pairs all result in copulations, indicating that A and B are mating type α while C and D are mating type α. The different designations for mating type are chosen arbitrarily. Occasionally diploid cells appear in some of the single ascospore cultures, revealing that copulations had produced a diplophase homo-
Fig. 2. A diagram showing the \textit{genetical} mechanisms which produce variations in yeasts. The legitimate diploid vegetative cell produces a large, smooth colony. During spore formation, segregation occurs and four haploid spores are formed. Since the original yeast is usually heterozygous for several loci, each of the four segregants is usually genetically different and produces haploid cells which form a characteristic colony. Plating these cells out on agar results in production from each segregant of a variety of rough colonies, distinguishable morphological mutants. Three mutants are indicated in the figure but any number may be obtained depending on the persistence of the investigator. Two of the segregants, in this case from spores A and B are of sex \(a\), while the other two from spores C and D are of sex \(\alpha\). Whenever a suspension of cells from an \(a\) and an \(\alpha\) clone are mixed, copulations occur as indicated by the diagram. These produce diploid cells capable of producing viable four-spored asci and are classified as legitimate diploids. Most of these legitimate diploids may be genetically different from the original diploid genotype since many of the recombinations after segregation may be homozygous for many genes for which the original cell was heterozygous. Illegitimate diploids may be produced by copulations between different cells or mutants of an original single ascospore culture. These are characterized by smaller diploid cells and less viable, generally two-spored asci.
Fig. 3. Photograph of the ascospores of Saccharomyces cerevisiae showing two- and four-spored asci.
zygous for the mating type alleles. The illegitimate diploid cells are usually somewhat smaller than the legitimate a/a diploids.

Legitimate (a/a) diploids generally produce abundant and viable ascospores (fig. 3), while the corresponding homozygous (illegitimate, a/a, a/a) diploids generally sporulate less vigorously and produce fewer and less viable ascospores. The asci are often two-spored rather than three- or four-spored.

The Rough Colony

The roughness of colonies grown on solid medium is a useful, diagnostic character in differentiating various cultures. It apparently depends basically on the fact that the cells cohere to form a specific pattern, due to the manner in which they bud and branch. This basic pattern of aggregation is obvious in the microscopic examination of cells from broth cultures. The extreme rough-type colonies usually produce specific "rosette" aggregations. Winge (76) described the "figure eight" arrangement common in haploid cells prior to copulation. After a bud has reached full size, two new buds appear (one from the mother and one from the daughter cell) near the point of union of the daughter and mother cell, producing a "four-leaf clover" effect. Most "rosettes" appear to be a variation of this "figure eight" formation. Many cultures make what appear to be homogeneous suspensions because the "rosettes" are too small to affect the turbid appearance produced when the culture is suspended in fluid medium or grown in broth, but the extremely rough colonies cannot be easily brought into a homogeneous suspension and when extremely rough-type yeasts are grown in liquid medium, the supernatant liquid is often completely clear. This is a character much desired for wine yeasts, especially for champagne yeasts. There is a basic pattern of cohesion even in extremely smooth cultures, for nearly all colonies show some distinctive topographical structure if grown on solid medium long enough to form a giant colony. Conversely, when moderately rough colonies are sown heavily enough on agar to prevent the formation of large colonies, only smooth ones appear. On an unevenly spread plate one finds an outer fringe of extremely rough, large colonies and a central group of small, smooth ones. All belong to the same genotype but the rough character cannot come into expression until the colony attains considerable size.

We have observed several hundred different clones of rough-colonied yeasts, and although each one is distinctive and recognizable and can be duplicated and recognized when transplanted, we have not thus far discovered any exact duplicates. The range of variation is extremely great. In addition to the fundamental "rosette" or budding pattern, differences in shapes and sizes of the cells affect the colonial form. In all colonies the variation in cell size increases with age, generally in the direction of producing larger cells. As a rule, rough colonies contain more elongate cells than smooth colonies, and part of the basis for extreme roughness is possibly the maintenance of end-to-end connections after cell division, which has been described so frequently in the genus Bacillus.
All four cultures from the single ascospores isolated from a four-spored ascus originating from stable, smooth-colonied, wild-type diploid cultures of *S. cerevisiae* are usually rough-colonied. This proves that the genes differentiating rough from smooth colonies are recessive and several loci are involved. The wild-type "opposite number" alleles of the mutant genes prevent them from coming into expression in the heterozygous wild-type diplophase. Although we have dissected many asci from the same diploid cultures, practically no duplicate cultures have been found among the colonies grown from the single ascospores, which indicates that the diploid cell is heterozygous for a considerable number of mutant genes, and that many loci affect the characters lumped into the so-called "rough" class.

*The Life Cycle of Saccharomycodes ludwigii*

Copulations between yeast ascospores were observed as early as 1889 when Hansen described the genus *Saccharomycodes*. Four spores are found in each ascus, two at each end of the cell. The two paired spores always fuse (fig. 4) within the ascus and germinate to produce a diploid cell which grows vegetatively until sporulation. In this yeast the haplophase is transitory and exists only in the ascospores. Winge and Laustsen (81) studied this organism in detail and found it to be a balanced heterozygote. They showed that the haplophase could be cultivated by separating the spores from the ascus and that two spores in each ascus produced cells exhibiting normal growth, \( N \), while two carried lethal genes, \( n \), resulting in the early death of the haplophase cultures. Segregation of another pair of alleles was observed simultaneously, which produced either long, \( L \), or short, \( l \), cell growth (fig. 5). The two coherent spores are always complementary; if one is \( NL \), the other is \( nL \), or if one is \( NL \), the other is \( nl \). In each ascus the pair of coherent spores at one end is identical with the pair of coherent spores at the other end (fig. 6). Winge confirmed these observations with several pedigrees and also determined the orientation of spindles in the ascus, finding that the spindle in Meiosis I is
longitudinal and centrally located, while the spindles of Meiosis II are located rather close together and overlap. This is a variation of the mechanism in *Neurospora tetrasperma* which leads to the production of homothallic binucleate ascospores first described by Dodge (12) and analyzed further by Lindegren (36).

I have interpreted Winge and Laustsen's data according to the following scheme (fig. 6): That the two genes are not linked is indicated by the fact that the four recombination types are found in equal proportions. Therefore, both genes are on different chromosomes. However, each is so close to the spindle attachment that segregation invariably occurs at the Meiosis I without crossing over. After Meiosis I, the dyads in each nucleus will be either *NN, LL* and *nn, ll*, as in fig. 6c, or *NN, ll* and *nn, LL*, as in fig. 6C'. The fact that the spindles always overlap results in two possible final arrangements in the ascus. These are the two arrangements that invariably occur.

This analysis proves that the diploid *Saccharomyces ludwigii* contains at least four chromosomes.

II. MATING TYPES

Although Winge (76) and Kruis and Šatava (26) both observed copulations between haplophase yeast cells derived from single ascospores, they obtained no evidence that the ability to copulate was under genetical control; in fact, Winge stated specifically that "fertilization does not consist in a union of cells that differ genotypically." In the cultures which he used the phenomenon probably was not so clear-cut as with our cultures, and even with our cultures many exceptions are encountered. However, the facts which prove that a
single pair of alleles, arbitrarily designated $a/a$, control mating types are quite clear. The following exceptions are found to the rule that matings usually occur between gametes of complementary mating types: a. There are occasional rare haplophase cultures of high fertility which copulate with other strains of the same mating type or with gametes derived from the same type. (It is

![Diagram](http://mmbr.asm.org/)

**Fig. 6.** An interpretation of Winge and Laustsen's data on *Saccharomyces ludwigii* showing why the doubly heterozygous diploid always produces two coherent spores which are likewise doubly heterozygous. The genes are not linked, but are close to the centromere and segregation always occurs at meiosis I. Depending on the orientation of the spindle, either C or C' is produced. The overlapping spindles in II place two complementary genotypes together in every case.

from cultures of this type that the genus *Zygosaccharomyces* probably originated as a haplophase segregant of *S. cerevisiae*. Species of *Zygosaccharomyces* are usually rough-colonied, one of the common characteristics of haplophase segregants.) b. Some freshly isolated cultures are incapable of mating with any other gametes, and nearly all cultures, which are maintained by transfer, tend to
lose their fertility as they grow older. (Round-celled *Torulopsisidae* probably originated in this way.) c. Many single ascospore cultures produce diploid cells, but an extended analysis of pedigrees involving cultures derived from single ascospores revealed that the diplophases obtained in this manner were usually unable to produce viable ascospores, while diplophase cultures derived from four-spored asci usually produced viable four-spored asci.

Conclusive proof of genetical control of mating type specificity was obtained by isolating the four single ascospore cultures from a single ascus and pairing them in all combinations (46). This experiment was repeated many times with substantially the same results indicated in fig. 2.

This experiment cannot be carried out with every set of single ascospore cultures obtained from a four-spored ascus because: a. single ascospore cultures are often sterile; b. some cultures undergo illegitimate copulations very shortly after the ascospore germinates and diploid cells predominate in the derived cultures supplanting the haplophase; c. more rarely the derived haplophase copulates with either a or α mating types, making it difficult to draw any conclusions from the data. In almost any fresh haplophase culture from a single ascospore a few copulations may be observed, but when an active a and α mating is made, sometimes over half of the cells are copulating and it is very easy to distinguish this class from matings in which less than 1 per cent of the cells are fused. Some diplophase cultures are extraordinarily fertile while others are quite infertile. The L strain which we have studied extensively has very high fertility.

The final proof of the genetical control of mating type specificity was obtained by mating 58 haplophase cultures from the L strain and a variety of other vigorous yeasts in all combinations (34). This experiment showed that a and α alleles were generally distributed throughout the species although they were often masked by sterility factors. The α segregants of the L culture were a source of a large number of interstrain hybrids since they outcrossed rather easily with the a segregants of other strains. The La segregates mated with the a segregants from other varieties almost as easily as they did with the La segregants. It was even possible to outcross the La segregants with a cultures from a number of other strains which were apparently sterile. For example, Ba and Bα cultures failed to copulate with each other while the La cultures easily mated with Bα cultures.

**Mechanisms Insuring Cross-Fertilization**

There are a variety of mechanisms in different plants and animals for insuring cross-fertilization, but all of these differ somewhat from that found in yeasts; the symbols a/α are given to this pair of alleles to indicate their uniqueness.

a. **Self-sterility alleles**: Most hermaphroditic, flowering plants are self-sterile due to a genetic mechanism which prevents pollen shed by the flower from growing down the styles of the parent plant. A mechanism that may be fundamentally similar prevents the sperms of an individual hermaphroditic sea squirt, *Ciona*, from fertilizing eggs produced by the ovaries of the same individual.
b. Sexual Dimorphism: In higher animals and some plants sexual dimorphism insures cross-fertilization. The genetic mechanism simply operates to reduce the probability of intersexes or hermaphrodites occurring.

c. Plus-Minus Factors: This mechanism in Rhizopus is not a sexual mechanism because no unmistakable sex organs are involved and therefore it cannot be called a self-sterility mechanism. It is more precise to consider this a special case in which a single pair of alleles controls copulation.

d. Neurospora: We formerly called the alleles in Neurospora plus-minus factors (38), but later work has shown that they resemble self-sterility alleles more closely than the factors found in Rhizopus. Both plus and minus thalli contain both male and female sex organs and self-fertilization is prevented. However, since the plus and minus thalli are both haploid and the zygote is invariably heterozygous for the same pair of plus-minus alleles, this mechanism differs considerably from the standard self-sterility mechanism found in flowering plants in which a series of multiple alleles exists and a great variety of heterozygotes abound.

e. Hymenomycetes: The hymenomycete mechanism resembles the plus-minus Rhizopus mechanism rather closely since no obvious sex organs exist in these forms. It differs in that two loci are often involved and that a multiple series of alleles at these loci may further complicate the picture.

f. Mating types: The mechanism which assures cross-fertilization in the single-celled diploid Paramecium resembles the plus-minus mechanism found in fungi since no sex organs are present, but the heredity seems to be more complex. The fact that the copulating cells are diploid is a still further difference from the most closely comparable fungal mechanism.

Since no sex organs are present in Saccharomyces, Sonneborn's (67) and Jennings' (23) term "mating type" has been applied in the case of yeasts.

I have used the letters a and α to distinguish the mating type alleles because the heterozygous a/α zygote is much more vigorous than either haploid parent, and it seems possible that these alleles may control the production of complementary, essential substances. In this respect they may differ from conventional alleles which are usually indicated by large Roman letters. Mating type alleles serve the function of facilitating cross-fertilization, for although illegitimate diploids occur by copulations between two a or two α haplophase gametes, the a/α gametes are generally much more vigorous than either the illegitimate or the haplophase cultures and thus outgrow the other forms. After the reduction division has occurred, the four spores are almost invariably genetically different because of the extreme heterozygosis of the original diplophase. The fact that usually two different ascospores or gametes derived from two different ascospores are mated practically insures the production of a recombination zygote different from the original diploid from which it was derived.

The heterozygous zygotes have greater survival value than the homozygous zygotes, since the former produce viable ascospores while the ascospores produced by the latter are generally non-viable. However, some exceptional
homozygous diploids produce viable four-spored asci. A culture of this type was described by Winge and Laustsen (82) in a study of a cytoplasmic effect of inbreeding in a homozygous yeast. It may have been their knowledge of the existence of a number of strains of this type that led them to the conclusion that mating type alleles did not exist in Saccharomyces.

Cross-fertilization has considerable survival value since it insures the incorporation of a variety of genes in each zygote and makes possible the production of a number of new recombinations. However, there are many organisms, such as the close-pollinated flowers, in which cross-fertilization does not occur, and inbreeding in this case does not appear to have yielded an inferior type, although it may have made the genus somewhat less variable and therefore less plastic or adaptable than cross-pollinated forms. I have described (37) copulation in a micrococcus in which cross-fertilization did not occur. In this case the zygote was invariably homozygous since it was formed by the fusion of two genetically identical gametes, which originated from the division of a single haploid nucleus. The fusion occurred within the cell after cell division, followed by the solution of the cross wall. However, this type of copulation may give rise to variation because it may provide an opportunity for chromosomal rearrangements to occur through some aberration of the mechanism. Autogamous copulation may represent an early evolutionary type of sexual mechanism resembling the parthenogenesis or apogamy that occasionally occurs in higher forms.

I prefer to define sex as Allen does, only in terms of true male and female sex organs. When we use this definition, mating type, self-sterility alleles, and plus-minus factors take on their true significance. They are not essential to the sexual mechanism but are simply means of assuring cross-fertilization. The fact that they may occur either in the absence of sex organs, as in the Hymenomycetes, or may be superimposed on true male and female sex organs as in Neurospora, prove that they are devices distinct from the sexual mechanism.

III. HYBRIDIZATION

Winge and Laustsen (79) produced many hybrid yeasts. They mated individual ascospores by placing them side by side with a manipulator and observed them until fusion occurred.

Lindegren and Lindegren (46) developed a new technique of hybridization. Matings were made simply by mixing mass transfers of haplophase cells in a fluid medium in a test tube. This method made it possible to study the genetic characteristics of the two haplophase parents (in ascospore matings both parents are lost by the fusion which forms the diploid). The micromanipulation was eliminated, except for that involving the original separation of the ascospores.

The haplophase culture developed from one spore can be used for an indefinitely large number of matings. This is particularly important since only occasional ascospores show a high degree of compatibility in matings. Haplophase cultures of desirable strains can be preserved by lyophilization. In this
process yeast cells are suspended in a protective colloid and the water removed by evaporation. Tubes prepared in this manner are sealed and can then be stored in the dark at room temperature for many years without loss of viability of the cells. When transferred to nutrient medium they are capable of initiating new cultures. This is a helpful addition to the technique because lyophilized haplophase cultures retain their full copulative strength indefinitely. Vegetative propagation of a haplophase culture is usually accompanied by mutations which reduce mating strength and transform the original type into a sexually impotent haploid.

The advantage of alternation of generations in _S. cerevisiae_ was revealed by an extensive study of the hybrids obtained by making mixtures of mass transfers of haplophase cells. Single diploid cells or single individual colonies selected after the zygotes had been produced revealed that a considerable variety of definitely different kinds of zygotes had been formed. The hybrids were studied for growth rate or final density of growth in broth, but even with this simple classification five or six different kinds of zygotes were easily distinguishable following a single mating. The haploid cells produce a variety of mutants, and apparently matings between these mutated gametes resulted in a corresponding variety of zygotes (fig. 7). I have already pointed out that mutants produced by continued selection of cultures grown in the laboratory for a prolonged period (usually more than a year) are generally found to be sterile. However, it appears that many of the mutations in the early stages do not prejudice the capacity of the gametes for copulation. Prolonged competition and selection probably result in loss of fertility because the genes which insure fertility do not have a high survival value in competition with other rapidly growing mutants.

These facts indicate that a moderately prolonged haplophase is a distinct advantage in _Saccharomyces_ because it permits competition among the new mutants that arise in the haplophase, leading to selection of the most vigorous before copulations occur. This mechanism may account for the fact that _Saccharomyces_ is the most cosmopolitan species of yeast. If the mating type strength is great, as in _Saccharomyces_, fusions occur in the ascus without selection or competition between the gametes and the plasticity of the species is limited, for in the diplophase (which constitutes almost the entire life cycle in _Saccharomyces_) mutations are not so readily selected. It is the relatively weak mating strength evidenced in _Saccharomyces_ which makes an extension of the haplophase possible. The greater vigor of the diplophase enables it to outgrow the residual haplophases. The variety of genes affecting mating strength results in the production of a large variety of sterile haploids (which are essentially "blind alleys" in terms of the continuity of the species), and many of these haploids become stabilized in the form of the genus named _Torulopsis._

**Selection and Hybridization Mutually Exclusive**

Previous to the development of a system of hybridizing yeasts all improvements resulted from selection. The selected cultures were often diploid and
because of this were relatively stable. These variant cultures were probably produced by recombinations following spore formation and were either legitimate or illegitimate diploids. Variants of this type might be picked up in a single selection. However one can carry on a continuous selection with the unstable haplophase cultures and get improvement at each step, if a sufficiently large population is tested, and if the desired character is not stabilized by the

![Diagram](http://mmbr.asm.org/)  

**Fig. 7.** A diagram indicating how segregation followed by mutation in the haplophase can produce a variety of gametes. If the original diploid cell is homozygous for \( bcd e \) and heterozygous for \( pqPQR \), the four ascospores may have the compositions indicated. In a clone isolated from a single \( abcde pqPQR \) ascospore (mating type \( a \)), a mutation of \( d \) to \( d' \) produces a mutant \( d' (abcde pqPQR) \) and a mutation of \( e \) to \( e' \) produces mutant \( c' (abcde pqPQR) \). In a clone isolated from a single \( abcde PQ pqr \) ascospore (mating type \( a \)), a mutation of \( b \) to \( b' \) produces an \( ab'cde PQR \) clone while a mutation of \( e \) to \( e' \) produces an \( abcde' PQR \) clone. When cells from the two original culture tubes are mixed, nine new recombinations may be produced.

gene complex present in the cell. Such continued selection generally involves so much mutation that the resulting culture is sterile and incapable of hybridizing. Intensive selection and hybridization are, therefore, mutually exclusive procedures. Matings can only be made between haplophase cultures that have been recently derived from single ascospore cultures. Since haploid yeast cultures become sterile when subcultured vigorously, the test
for mating type can be performed only with single-ascospore cultures obtained from freshly isolated spores. The genetical differentiation between the \(a\) and \(a\) types limits the capacity of haplophase cells for copulation, except with members of the complementary mating type.

**Generation Time**

A short generation time is an advantage in genetical studies. The following schedule reveals the minimum time intervals involved in yeast breeding.

<table>
<thead>
<tr>
<th>Day</th>
<th>Program</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>Isolation of ascospores.</td>
</tr>
<tr>
<td>1</td>
<td>Transfer of haplophase colony to agar.</td>
</tr>
<tr>
<td>2</td>
<td>Mating of haplophase with tested strains.</td>
</tr>
<tr>
<td>3</td>
<td>Observation of positive matings and transfer to pre-sporulation medium.</td>
</tr>
<tr>
<td>5</td>
<td>Transfer to gypsum.</td>
</tr>
<tr>
<td>6</td>
<td>Isolation of ascospores.</td>
</tr>
</tbody>
</table>

**IV. CULTURAL VARIABILITY AND STABILITY**

Many of the physiological and morphological characteristics of yeasts and bacteria are remarkably stable in the hands of experienced investigators. Comparable quantitative results on fermentation, infectivity, rates of growth, and a variety of other measurable characteristics are obtained regularly. Many of the workers who have had experiences of this type are inclined to minimize the importance or the frequency of variation among microorganisms. On the other hand, investigators who are interested primarily in variation encounter a surprising lack of uniformity in all the quantitative or qualitative characters which come under observation. Differences in the results obtained by investigators with different interests probably result directly from the use of different techniques in maintaining and transferring cultures. Any given clone, whether haploid or diploid, is generally quite uniform in appearance and character, provided mass transplants are consistently used. For example, the standard practice in handling yeasts in industry is to grow the cultures in broth and make transfers by pipetting most of the cells from an old broth tube to a fresh broth tube. Under these conditions, the predominant genotype is maintained by the transfer and the culture usually duplicates very closely the performance of the parent strain. Even if a rather high frequency of variation occurs, the effects of the variant forms will be swamped out by the many billions of the predominant type of cell and will affect the quantitative results only slightly. In the same way, bacteriologists accustomed to obtain duplicating results in the study of specific quantitative characteristics, use mass transplants of bacteria. Usually a full loop of cells is removed from a slant and streaked on a second slant or inoculated into broth. By contrast, students of variation streak culture plates very lightly, aiming at a large number of single colonies, or inoculate broth with single cells. Investigators using the latter techniques almost invariably uncover a great deal of variation in any microorganism. The amount of varia-
tion is multiplied in the case of yeasts when single ascospores are isolated following genetical segregation. One who is convinced that yeasts are quite stable will be amazed at the degree of variation that results when he simply takes an old culture which has sporulated well, suspends the cells in water, heats the suspension to about 60 C for five or ten minutes and then plates very lightly on agar. I believe that these two differences in technical approach are the main basis for a great deal of disagreement with regard to the amount of variation that is encountered in routine laboratory manipulations by competent workers in microbiology.

The Effect of Volume Relations on the Variability of the Haplophase

Haplophase cells multiply in the medium to produce large numbers of genetically and morphologically different forms. Selection pressure is more effective in the haplophase of single-celled microorganisms than in any other biological form. Free living cells exist in a constantly changing medium, and each new variant is tested for its survival value in competition with its predecessors. Since the population existing at any given time has been selected only with regard to its ability to compete under previously existing conditions, each new variant has a chance to outgrow the earlier forms. In a liquid medium containing a small inoculum, there is little competition at the first stage of growth because the "biological space" available to each cell is large and its by-products which function to limit growth, are quickly diluted. The available nutrients are also at a maximum. Under these conditions, a variety of forms accumulates, because there is opportunity for even the weakest to multiply. Following the early "era of good feeling" there is a phase of intense competition in which a few predominant types emerge to grow rapidly and finally come to a maximum. This prevailing type is particularly adapted to the substrate conditions during the logarithmic phase, and it succeeds in overwhelming other types by sheer weight of numbers during the latter stages of the logarithmic phase. However, in the very late stages of growth, the predominant form either is prevented from growing further or begins to die off. New conditions obtain which are especially adverse to the organisms predominating in the logarithmic phase because the peculiar complex of by-products characteristic of the predominant form is at a maximum.

The mutation rate need not be unusually high to produce many variants under these conditions. Each haplophase culture is a complex mixture of different morphological and biochemical types, and the wide range of variation makes it difficult to characterize haplophase cultures precisely. Haplophase cells generally sporulate sparsely and are less vigorous and less efficient in the utilization of carbohydrates than the corresponding diplophase. Morphological and biochemical studies lead to variable results when performed with haplophase cultures. It is possible, nevertheless, to obtain an idea of "average" biochemical potentialities of a haplophase if the culture is maintained by massive transfers which are made frequently.
There are two phases in the adaptation of a haplophase culture to an originally unfermentable carbohydrate, \( a \), the selection of a specific new mutant by a shift in the populations and \( b \), the adaptation of this mutant by exposure to the carbohydrate. The first step concerns the genes; the second concerns the cytoplasm. The haplophase complex seems to have a more or less equilibrium composition, and tests, for example, of the time of adaptation to galactose of a haploid culture yield data showing that an exposure of a few hours to several days transforms different subcultures of one originally pure single ascospore culture to a fermenting type of organism.

In the diplophase (where mutations cannot express themselves), there is no shift in population, provided sporulation is prevented, and adaptation occurs by the interaction of the carbohydrate with the cytoplasm. Diplophase cultures, under these circumstances, are either adaptable or non-adaptable, and the adaptation time can be determined to within a few minutes.

The variability of the haplophase makes it the organism of choice for the selection of variants. Haplophase yeasts are capable of a wide range of adaptation and specialization with regard to biochemical reactions, especially when freshly isolated from the ascospore. However, after several months’ selection, some cultures have been obtained with extremely specific, apparently stabilized colonial characteristics.

With organisms as variable as the haplophase yeast, it is clear that single cell isolates will produce an enormous degree of variability and that commercial production with yeasts of this type would be hazardous. If, however, an exceptionally desirable haplophase yeast is encountered, it is possible to use it in industry by growing the cells in a series of receptacles of gradually increasing size by consideration of the kinetics of competition. In spite of this high degree of variability, it is possible to utilize these haplophase cultures in industry by consideration of the relation of the volume of the available medium to the growth rate. It is important to realize that the mutation rate is beyond the control of the operator, but even if it is not possible to reduce the mutation rate, it is possible to minimize the initial “era of good feeling” by reducing the volume of available nutrients so that the forces of competition are brought into play quickly. This will minimize the period of relatively unrestrained multiplication, in which large numbers of a weaker type of cell manage to gain a foothold. When a chosen mutant has been found to have some especially desirable character, a number of cells can first be grown in a single drop. This entire drop can then be transferred while growth is in the logarithmic phase to a few milliliters of medium, bringing the forces of competition into play early in the growth cycle. The next transfer should be made by transferring the entire contents of the previous culture into a quantity of medium likewise designed to eliminate the early non-competitive phase; and each succeeding transfer should be handled in the same way. Since the increase in growth is logarithmic, a large number of serial transfers will not be required, but they must bear such a relation to the growth rate that the logarithmic phase is constantly maintained. This process can be worked out empirically once the mechanism of crowding is understood.
V. SPORES AND SPORULATION

The production of spores by \textit{S. cerevisiae} is controlled both by the genetical composition of the culture and by the nutrient on which the culture has been grown previous to transfer to gypsum. Yeasts of the proper genetical composition grown on a special pre-sporulation medium and transferred to gypsum slants usually sporulate within twenty-four hours. The percentage of cells sporulating following growth on this medium is rather high; occasionally 90 per cent of the cells form spores. Relatively slight changes in the medium cause considerable differences in the percentages of spores produced. The effect on spore production of growth on a medium containing a large amount of natural nutrients as contrasted to the effects on spore production of growth on a synthetic medium or a poor nutrient medium, suggests that sporulation is affected by a variety of accessory substances, which are either necessary in large concentrations or may not yet have been identified. Occasionally, diploid cells sporulate directly on a pre-sporulation medium, but usually it is necessary to transfer them to gypsum slants to complete the process.

Sporulation in yeasts was probably first studied by de Seynes (10) who reported that round spores found "in the surface of the water" germinated to produce elongate cells in a mixture of wine and water, but that these elongate cells produced spores again when transferred to a more dilute medium. Rees (60) found that when yeast was planted on the cut surface of various vegetables (cooked or raw) growth continued until the fourth day, when budding stopped. On the fifth day, the vacuoles in the cell disappeared and the protoplasm became coarsely granular. Spore formation occurred regularly on the sixth day. Spores also appeared on the sixth day whenever fresh yeast from beer vats or wine must was transferred to the cut surface of carrot or potato. Rees did not find spores in old lagering vats or in compressed yeasts, but washed yeasts from these sources placed in a beaker in a layer approximately 4 mm thick and protected from dust sporulated abundantly in about three weeks.

Welten (75) challenged the view that starvation was essential to sporulation. He found that on prune extract agar, yeasts sporulated well. He even doubted the necessity for oxygen, since in his experiments, colonies imbedded in the agar sporulated as well as those on the surface. He found that yeasts grown in pear extract or beer wort did not sporulate so well as those grown on prune extract agar. But sporulation occurred when the washed yeast grown on prune extract was placed on glass plates, filter paper, or sterile washed agar slopes, if a drop of prune extract were added. If no prune extract was added, no spores were produced. Welten's work proved that starvation alone is not the complete explanation of the phenomenon. He showed that acidity of the medium in which sporulation occurred was important, no spores occurring in an alkaline milieu. Welten also found that more spores were produced in concentrated than in dilute prune extract, also that a small amount of MgSO$_4$ aided sporulation. Young cells were not essential; those three to four days old sporulated better than those one to two days old.

Mrak, Phaff and Douglas (58) discovered that many yeasts sporulated well
on slopes of an agar medium containing a mixture of vegetable (cucumber, beet, potato, and carrot) extracts.

A New Medium for Inducing Sporulation

Genetical analysis requires an abundance of large, viable four-spored ascis. Lindegren and Lindegren (47) developed the following pre-sporulation medium:

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Beet (leaves) extract</td>
<td>10 ml</td>
</tr>
<tr>
<td>Beet (root) extract</td>
<td>20 ml</td>
</tr>
<tr>
<td>Apricot juice</td>
<td>35 ml</td>
</tr>
<tr>
<td>Grape juice</td>
<td>16.5 ml</td>
</tr>
<tr>
<td>Yeast (dried)</td>
<td>2 g</td>
</tr>
<tr>
<td>Glycerin</td>
<td>2.5 ml</td>
</tr>
<tr>
<td>Agar</td>
<td>3 g</td>
</tr>
<tr>
<td>CaCO₃</td>
<td>1 g</td>
</tr>
<tr>
<td>Water to a final volume of</td>
<td>100 ml</td>
</tr>
</tbody>
</table>

The mixture was steamed for ten minutes and tubed. The tubes were sterilized at 15 pounds pressure for twenty minutes and slanted. Most strains of yeast will produce spores directly on the slants if allowed to grow for a few weeks. However, if spores are needed sooner, transfer to gypsum is necessary.

Engel’s (13) gypsum block method was replaced by the method of Graham and Hastings (15). A mixture of plaster of Paris and water (100 g of each), is poured into test tubes and solidified in a slanting position. These slants are dried at 50 C for twenty-four hours and autoclaved.

About 1 ml of sterilized water is poured over a three-day growth of yeast on the pre-sporulation medium and allowed to stand 10 minutes, then a thick suspension is made by stirring the yeast cells around in the supernatant fluid. The yeast suspension is taken up in a pipette and poured over the upper part of the gypsum slant. About 3 ml of sterile water containing enough acetic acid to bring the pH to 4 is pipetted into the lower half of the gypsum slant. The inoculated gypsum slants are incubated one to two days at 25 C.

Variations in the Number of Ascospores in an Ascus

Ideally, each ascus contains four ascospores, but this ideal is not invariably attained; in fact, one much more frequently encounters two- and three-spored asci than four-spored asci, while one-spored asci abound in some cultures and on rare occasions one finds asci with more than four spores. Since the reduction division always produces four basic nuclei, less than four spores in the ascus of an ascomycete is generally interpreted as the result of disintegration of nuclei. A two-spored ascus would presumably result from the disintegration of two nuclei and the incorporation of the remaining two into ascospores. Ascospores may disintegrate as a result of competition within the ascus. An example of this type was analyzed genetically by Lindegren (35) in Neurospora. Yeasts that are capable of producing four-spored asci when properly nourished will produce almost exclusively two-spored asci on potato agar, suggesting that the
two eliminated haploid cells are capable of developing only when they are fed on a rich, natural medium.

Some illegitimate cultures produce large numbers of viable one-spored asci, while in these cultures the spores from asci with more than one spore are non-viable. Asci from a culture of this type were analyzed by Lindegren and Lindegren (47). The one-spored asci were of special interest because they were found to germinate directly to produce diploid cells. The diploid cells were indistinguishable from the original culture, proving that spore formation occurred without reduction. Therefore, large, one-spored asci contain spores with a diploid nucleus that has not undergone reduction (possibly by monaster formation); a diploid nucleus is simply enclosed in a spore wall. The general low viability of the haploid spores from two-spored asci originating from homozygous diploid cells may result from the aberrations of the reduction division in homozygous diploids, suggesting the possibility that homozygosis is usually prejudicial to a regular meiosis.

_Cytoplasmic Degeneration in Homozygous Strains_

Winge and Laustsen (82) studied a strain of homozygous _S. cerevisiae_ which produced viable four-spored asci. Diploid cells were produced either by fusion of two of the gametes after multiplication of the haplophases, or by a fusion of the first cell budded from the ascospore with the original ascospore. Diploid forms produced by the fusion of two cells were able to perpetuate the homozygous race and produced asci containing four haploid, viable ascospores. An alternative mechanism for producing the diploid cells involved the direct germination of a diploid (zygote) cell from a haploid ascospore (an ascospore from a four-spored ascus). The latter case was interpreted as follows: a nuclear division occurred within the ascospore in the absence of a division of the cytoplasm; this resulted in the production of a diploid nucleus carried in a cytoplasm whose constituents (which apparently divide normally at each cell division) had not been able to reproduce themselves (fig. 8). The resultant deficiency was indicated by the fact that the ascospores of these diploid cells were of low viability. In contrast the ascospores of the homozygous strain from which they had been derived were highly viable. This constitutes proof of a mechanism of cytoplasmic inheritance in which a set of plastogenes divide regularly at each nuclear division. If this division were irregular, one would anticipate the damage which the cell had suffered could be repaired in time.

We have evidence supporting Winge and Laustsen's work. We have observed that haploid ascospores (spores isolated from four-spored asci) which germinate directly as diploid cells, usually produce only a small colony which soon ceases to grow and apparently dies since transfers from the colony do not develop.

**VI. SPECIATION IN YEASTS**

It has often been pointed out that there is no satisfactory definition of a species. This applies even more specifically in yeasts and fungi than in higher
forms. Winge and Laustsen, and Šatava, have suggested that *Torulopsis* and *Zygosaccharomyces* must have been derived from some species of *Saccharomyces* and can probably be looked upon as derivatives of the parent species. It is not known at present which species of *Saccharomyces* gave rise to them. A great variety of other genera probably also take their origin from *Saccharomyces*.

The original genus produces four viable ascospores and is therefore a perfect form. The haploid ascospores produce gametes with relatively weak copulative strength and the mating type alleles are modified by a number of sterility genes. This results in an extraordinarily plastic species. Segregation of the genes at spore formation produces a variety of haploid genotypes which compete among themselves by vegetative reproduction previous to copulation. Mutations can occur in the gametes produced from the ascospores during the period of vegetative reproduction before copulation, and selection acts to eliminate the less vigorous or the poorly adapted gametes. This increases the variety of forms which become adapted to specific environmental niches. The weak copulative strength delays copulation until the variety of gametes has been multiplied by segregation and mutation. The mating type alleles insure cross-fertilization and the production of heterozygous diploids. Copulations occur between the predominant forms in greatest abundance enabling the best adapted to take over the living space in which they originated. At the same time, copulations between less well adapted forms, which may be able to achieve eminence subsequently in another environment, are not completely excluded.

Besides legitimate diploids, other clearly differentiated forms may also be produced by the mechanism: *a*. If the mating type alleles become linked to a gene-pair containing one lethal and one normal allele, selection will increase the copulative strength. Finally, a balanced heterozygote, heterozygous for both the mating type alleles and the lethal-normal gene-pair, will develop in a manner similar to that found in *Saccharomycodes ludwigii*. *b*. Stabilized haplophases

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**Fig. 8.** A diagram copied from Winge and Laustsen (82) giving a hypothetical explanation suggesting how a diploid cell produced by the fusion of two homozygous gametes, after the cell division has permitted chondriosomes to divide (upper row), may differ from a diploid cell produced by the fusion of two nuclei which have been produced by a mechanism in which nuclear division has occurred but chondriosomal division has been suppressed.
also will arise from *Saccharomyces* by segregation of a haplophase incapable of copulation, but otherwise well adapted, resulting in forms like *Torulopsis*. In addition, segregation of genes for high fertility, readily capable of producing illegitimate zygotes, and able to sporulate easily, will lead to the development of *Zygosaccharomyces* as an offshoot from the main genus *Saccharomyces*. In fact Winge and Laustsen were able to produce an intergeneric hybrid between *Saccharomyces* and *Zygosaccharomyces*, indicating that these genera are closely related. The evidence at present available disproves Guilliermond's contention that these genera are distantly related. It is notable that the *Zygosaccharomyces* abound in media of high sugar concentrations, to which they seem extraordinarily well adapted, while *Torulopsis* is adapted to dilute, well-aerated media. Both of these genera are weaker fermentative variants of the original actively fermentative *Saccharomyces*.

*Schizosaccharomyces* is easily differentiated from *Saccharomyces* and falls into an entirely different category. Its spores stain blue with iodine, while those of *Saccharomyces* stain yellow. Its cells divide by a regular binary transverse fission which is distinct from the unique mechanism of budding characteristic of *Saccharomyces*. The cultures of *Schizosaccharomyces* available in our laboratory are homothallic and homozygous, i.e., haploid cells are first produced in profusion, those arising from a single ascospore fuse to produce a diploid from which four- or eight-spored ascii are readily obtained. There is no evidence for the existence of mating type alleles. All single ascospore cultures produce an abundance of ascii containing viable ascospores. This seems to be somewhat at variance with the yeasts originally described by Beijerinck (3, 4). He obtained many non-sporulating cultures, a fact suggesting that some stabilized haplophases may also stem from *Schizosaccharomyces*. In maintaining *Schizosaccharomyces* in the laboratory, the selection of sporulating forms would naturally occur, because this is the criterion of the species and concentration on this character may have resulted in the isolation and perfection of a homothallic strain.

**The Phylogenetical Significance of Biochemical Criteria**

Morphological characters are probably preferable to biochemical or fermentative characters in tracing phylogenies because many of the haplophase segregants from powerfully fermenting strains are singularly weak in fermentative ability as was shown by Lindegren, Spiegelman and Lindegren (51). The diplophase *S. cerevisiae* can ferment glucose, levulose, mannose, galactose, sucrose, and maltose, while many haplophases originating from this species have been obtained which had lost the ability to ferment one or more of these sugars, although they were generally able to oxidize them. Some of these segregants were unable to ferment even glucose although they could utilize it by oxidation. The loss of ability to ferment is a very common character resulting from either segregation or mutation.

*Saccharomyces fragilis* is differentiated from *S. cerevisiae* by the fact that the former ferments lactose but not maltose, while the reverse is true with *S. cere-
visiae. The ability to ferment either lactose or maltose is generally a mutually exclusive character; only Brettanomyces can ferment both. These data suggest that the same gene controls both fermentations. If this be true, the lactose fermenters may arise from maltose fermenters by a single mutation in the haplophase. The species of S. fragilis which we have studied have kidney-shaped weakly viable spores. S. fragilis may have originated from single ascospore cultures from some perfect Saccharomyces developing in milk. Mutation at the maltose locus to a gene capable of initiating the fermentation of lactose could make it possible for the culture to multiply and predominate in milk. An illegitimate copulation between the haplophase in the milk might have resulted in the diplophase of S. fragilis. Zygosaccharomyces lactis probably arose in a similar manner, except that in this case a highly copulative haplophase occupies the predominant portion of the life cycle.

VII. CYTOLOGY

The yeast cell contains more identifiable organelles than many other plant cells, but there has been little agreement on the question of which of these organelles correspond to the conventional chromosomes and nuclei of higher plants. The observations of Henneberg (22) and Wager and Peniston (74) are, in my opinion, the most complete, and my own observations follow these authorities closely. Wager and Peniston's interpretation was limited by contemporary concepts of cell structure, but their drawings reveal an organization easily understandable in terms of modern concepts of the nucleus. They show that the yeast nucleus has a structure similar to that described by Harper (21) for the ascomycete, Phyllactinia. Attached to one side of the nuclear vacuole is a smaller body which is not ordinarily visible in the living cell. This structure is visible in iodine-potassium iodide preparations (fig 9) and corresponds to the centriole in Phyllactinia. The nuclei of many fungi, especially the hymenomycetes, have a notably eccentric appearance due to the fact that the centriole, a large body with a strong affinity for hematoxylin, is attached to the apparently empty nuclear vacuole. Wager and Peniston described the chromosomes polarized to the centriole, exactly as they are in the higher ascomycetes. Guilliermond (16, 17, 18) probably mistook the centriole for the nucleus because it divides at each mitosis, shows internal structure, and retains hematoxylin rather firmly. However, this body is much too dense to be a nucleus. The nucleus is invariably a structure containing a dilute nuclear sap surrounded by a membrane. The chromosomes are suspended in the dilute nuclear sap which stains poorly or not at all, in contrast to the cytoplasm which often stains very heavily. The body attached to the nuclear vacuole in yeasts is an extremely dense, heavily staining body, thus excluding the possibility that it is the nucleus.

Volutin, or metachromatin, was originally defined by Meyer (57) as a substance which stained dense blue with methylene blue, but did not destain with 1 per cent sulfuric acid. There does not seem to be a general agreement on the identity of volutin in yeast cells. Wager and Peniston called particles in the
Fig 9. A photograph of yeast cells stained with iodine-potassium iodide showing the balled-up chromosomes inside the nuclear vacuole and the sharply outlined, dense hemispherical centriole attached to the vacuole.
cytoplasm which stained red with methylene blue, volutin, and disagreed sharply with Guilliermond on the distribution of volutin and chromatin in the yeast cell. Henneberg seems to have followed Meyer's technique closely and states that volutin is found inside the vacuole. He points out that volutin bodies inside the vacuole appear in a variety of forms. Sometimes they appear as long, slender threads; sometimes they are short, stocky, almost cylindrical bodies; sometimes they appear as six to twelve slender threads radiating from the centriole; and sometimes they exist as a rather large number (forty to fifty) of discrete dots. He did not consider them to be chromosomes; as a matter of fact, he agreed with Guilliermond in considering the centriole to be the nucleus. Wager and Peniston also found what they called "chromatin" in the vacuole in the form of about fifty small, discrete bodies.

Badian (2) developed an exceedingly effective stain for bacteria and fungi. He killed the cells with osmic vapors, stained with methylene blue, and destained with eosin. He studied mitosis and meiosis in S. cerevisiae, and stated that the cells contained two chromosomes which divided by longitudinal splitting. However, his figures show that the so-called chromosomes always pull apart finally by thinning out at the middle and the final separation is by a crude, transverse fission. Furthermore, he stated that the haploid chromosomes fuse end to end to form the diplophase, rather than associating to form a pair of chromosomes according to the usual method. If his conception is correct, the number of chromosomes in haplophase and in diplophase would be the same.

Volutin Chromosomes

The structure which I have called the centriole contains two rod-shaped bodies which stain well with aceto-orcein and divide by a crude transverse fission (fig. 10). They are the only bodies in the cell which take aceto-orcein and they are the bodies described by Badian as the chromosomes. Badian stated that these structures took the Feulgen stain and this has been confirmed (Nagel and Carson, unpublished personal communication). Badian showed that these structures fuse end to end at copulation. Harper proved that fusion of the nuclei in Phyllactinia is initiated by fusion of the centrioles, although Harper's techniques did not reveal any internal structure in the Phyllactinia centriole. It seems probable that the rod-shaped bodies which Badian observed in fusion are components of the centriole, rather than chromosomes. If the fusion of the nuclei in yeasts were initiated by end to end fusion of the centriolar bodies, the anomaly described by Badian in which a diploid chromosome is supposedly produced by the end to end fusion of two haploid chromosomes would be obviated. However, this would mean that in yeasts the centriolar bodies are Feulgen-positive and stain with both aceto-orcein and aceto-carmine while the chromosomes are Feulgen-negative, and do not stain with either aceto-orcein or aceto-carmine, but give a positive test for volutin.

It may be difficult to accept the view that the conventional chromatin in yeasts is present in the centriole rather than in the chromosomes, while the
chromosomes are composed of volutin or metachromatin. However, Henneberg's description of the stages through which the volutin passes inside the vacuole corresponds rather well with the stages through which chromosomes normally pass, and Wager and Peniston's careful description of the threadlike structures carrying chromomeres polarized to the centriole corresponds precisely with the modern concept of chromosomes and chromomeres.

We may conclude that the nucleus in yeasts is a compound structure containing the dense hemispherical centriole intimately attached to the nuclear
vacuole. The vacuole is usually flattened on one side and otherwise is almost a perfect sphere. The flattened side of the nucleus is the area of attachment to the centriole.

When standard cytological techniques are applied to yeasts, a great deal of shrinkage and distortion of the yeast cell occurs, often accompanied by the disappearance of the vacuole. It is only rarely that the vacuole remains intact when the cell is stained with aceto-orcein. An effective technique for observing yeasts is to suspend the cells in water and allow water-soluble stains to diffuse slowly between the slide and cover slip. The cells are observed as they take up the dye and before they become completely overstained. This procedure does not involve so much shrinkage of the cell, which is an especially important consideration when one deals with small cells. I have used this technique with 0.01 per cent methylene blue. At the edge of the slide or on the border of a bubble, the chromosomes in living cells take on a deep blue color and appear as small, irregular, paired bodies usually free and in rapid Brownian movement. The important addition which this observation makes to those of the earlier workers is that these bodies in the vacuole are paired. This is an important common characteristic of chromosomes. Methylene blue stain of the living chromosomes is evanescent, depending apparently on the oxidation potential within the cell. Shortly after they take up stain the chromosomes disappear presumably because the oxidized dye is reduced to the leuco base. This indicates that methylene blue passes through the reduced cytoplasm as the leuco-base and becomes oxidized on contact with the surface of the chromosomes. The chromosomes tend to ball up into small, tightly-wound bodies that cease their Brownian movement and attach themselves to the inner face of the nuclear membrane. I have observed the long threads retract toward a single point of attachment at the side of the nuclear vacuole. The phenomenon has somewhat the appearance of a deliquescent crystal. Eventually, one finds from one to six large, lenticular blue-black masses pressed to the inside of the nuclear vacuole. Since the chromosomes are paired this represents the haploid number. In old methylene blue or aniline blue lacto-phenol preparations, the cells contain from one to six clearly defined, blue bodies inside the nuclear vacuole produced by the attachment of the chromosomes to the wall of the vacuole. These facts indicate that the dancing body, frequently described in the vacuole of the yeast cell, is composed of balled-up chromosomes. In iodine-potassium iodide preparations, single spherical masses occur in the vacuole of almost every cell (fig. 9). Under the influence of this fixative the chromosomes seem always to round up into a single dancing body. Iodine-potassium iodide has the advantage of revealing the centriole with great clarity although it does not show the internal centriolar bodies.

Toluidine blue used as a vital stain is the best stain that I have found for the chromosomes (figs. 11 and 12). It does not seem to cause the chromosomes to "ball up" nearly so frequently as is the case with the other dyes and is not evanescent like methylene blue. Usually about six bodies are observed in each vacuole. In especially good preparations of diploids each of the six bodies
can be seen to be composed of a pair of chromosomes. In the germinating ascospore there are six single chromosomes. I have concluded that there are

twelve somatically paired, Feulgen-negative chromosomes in the diploid cells of *S. cerevisiae*, and that the haploid number is six.
When a small drop of aniline blue in lacto-phenol is placed near the edge of a wet mount and allowed to diffuse between the slide and the cover slip, one can observe long, slender, delicately beaded, threadlike strands, vibrating in the nucleoplasm of some vacuoles. These structures do not take the dye but seem merely to change their refractive index (possibly due to action of the acid or the phenol), so that they become observable. Sometimes one larger, thicker strand, possibly produced by the coalescence of several strands, may be seen. Even the slender strands seem to be relatively rigid, bending something like a very slender, but rather long, thin steel wire. The chromosomes in this condition are only visible momentarily and soon disappear, but they resemble Wager and Peniston's figures closely enough to constitute confirmation of their observations.

These observations indicate that the chromosomes in the living cell vibrate in the nuclear sap. After one has observed the phenomenon in cells in which the refractive index of the chromosomes makes them visible, suggestions of the movement are visible in other yeasts such as Torulopsis utilis, Saccharomyces ludwigii, and Schizosaccharomyces octosporus. The motion may be visible in unstained material and may continue after flooding with Lugol's iodine solution which stains the glycogen brown and often brings the chromosomes into higher relief. The vibration of the chromosomes should greatly facilitate the exchange of materials between the nuclear sap and the cytoplasm.

VIII. BUDDING

The ability of yeast cells to reproduce by budding has distinguished them from other fungi as well as from other organisms and the observations presented here show that the mechanism is quite unique. The nuclear vacuole puts out a slender tube which forms and enters a small protuberance on the cell
wall (fig. 13). The bud and an enlargement at the end of the vacuolar tube (the bud-vacuole) grow simultaneously.

When a cell buds both the nuclear vacuole and the centriole divide. The first step is the formation of the long, slender tube leading from the vacuole to the periphery of the cell. This phenomenon can be observed only in cells containing enough glycogen so that the iodine stain delimits the vacuole and its tube as a clear space in the surrounding reddish brown cytoplasm. Observation is facilitated by the use of a Wratten 45 filter which converts the reddish brown color of the cytoplasm to blue-black and reduces the chromatic aberration of the lens system. The canal from the vacuole may originate any place on the surface of the vacuole, but usually appears at a point near the attachment of the vacuole and the centriole. The bud is always produced near the centriole and when the canal emerges at the opposite side of the vacuole, the long, slender channel extends all the way from the most distant part of the cell through the cytoplasm and finally produces the bud near the centriole. A bulb is produced at the end of this canal to form the bud-vacuole. Occasionally, the opening between bud and mother cell is too small to permit the contents of the mother-vacuole to enter the bud-vacuole and the canal is distended at this point like the oesophagus of an ostrich swallowing an orange. During this period the centriole is a hemispherical, solid, unyielding structure that is not deformed by movements of bodies near it. After the bud-vacuole is formed, the centriole divides. Sometimes in the Lugol's solution, it is seen as two bodies, each of which divides by stretching out and thinning out at the center. After the division of the centriole is completed and the establishment of contact of bud-vacuole and bud-centriole has been attained, the interconnecting canal between the mother- and the bud-vacuole disappears.

As soon as the bud approaches the size of the parent cell, the nuclear apparatus in the bud- and the mother-cell reorients itself so that the centriole in each cell is distal to the bud partition.

IX. STRUCTURE OF THE COLONY

Knowledge of life cycles and variations in bacteria has developed almost exclusively from the study of isolated individual cells, without consideration of the structural organization of the colony. Legroux and Magrou (27) studied the structure of colonies of Vibrio cholerae and of a number of other bacteria.
They discovered that rod-shaped variants of Vibrio appear in perpendicularly arranged packets on the exterior of the colonies, held together by a transparent substance apparently exuded from the cells. They assumed that this substance gave the rough colonies their rigidity. The rod-shaped organisms in the outer layer of the colony were made up of a central region and an exterior. The central region underwent division into two or four rounded particles, often of unequal size. This phenomenon was found in Vibrio, in the typhoid bacillus, in the diphtheria bacillus, and in the tubercle bacillus.

Pisova (59) found that, when a yeast colony grows on agar, a pseudo-mycelial growth of long fibrous cells penetrates the agar, especially at a high sugar concentration. After a few days or weeks, the surface cells begin to autolyze, continuing until an outer layer of autolyzed cells is formed. Lindegren and Hamilton (41) repeated Pisova’s work. After yeast colonies had been grown on malt-yeast agar, portions of the agar, containing colonies, were cut out and dropped into Flemming’s solution. The material was imbedded in paraffin. Sections were made and stained with a variety of dyes. Direct smears were also made by cutting the fresh colony in half vertically with a razor and pressing the exposed section gently against the slide.

In a section of the yeast colony, the outer layer of autolyzed cells stains very lightly, and the inner central mass of vegetative cells with their dense plastids is much darker. A pseudo-mycelium of yeast cells penetrates the agar and is thickest and deepest at the edges of the colony, apparently where oxygen is most abundant. The thin peripheral film of cells at the edge of the colony spreads over the surface of the agar. At the points of origin of penetration of the agar the growth of cells is very abundant. This may be due to the channeling of the substrate nutrient into these regions along the cracks made by the pseudo-mycelium. The central vegetative cells are extremely small, indicating that cell division continued after the nutrients became less readily available and competition resulted in a decrease in cell size. A few cells in the outer autolyzed layer produce asci. Autolysis apparently occurs early in the history of the colony, at least before competition reduces cell size. The autolyzed layer contains the only asci found, suggesting that autolysis supplies essential nutrients on which sporulation depends.

In some of the contact smears the autolyzed cells were not so shrunken as those obtained by the paraffin method. The walls seemed relatively intact, but there were no stainable cell contents. The “ghost” cells were larger than the densely stained cells in the vegetative section of the colony. These autolyzed cells, which apparently serve as sources of nutrients for the sporogenous cells, have a parallel in the paraphyses found in pyrenomycetes and discomycetes which also act as nurse cells. In some regions of the autolyzed layer, small clusters of round, apparently haploid, cells were found, suggesting that some spores may germinate in the layer. If copulations occur this could be a source of recombinations producing new genotypes.

The striking parallelism between the structure of yeast colonies and those of bacteria, as shown by Legroux and Magrou, suggests that the life-cycles may be
similarly parallel. Yeasts possess a nuclear mechanism, and the vegetative cells undergo meiosis and sporulation in the outer layer of the colony. In bacterial colonies a similar division of the cell contents into two or four bodies occurs in the corresponding layer. The analogy suggests strongly that the granulation of the bacterial cell is the result of a reduction division similar to that known to occur in yeasts. The bacterial cells in the outer layer of the colonies may be homologous to the ascospores of yeasts.

Harper (21) pointed out that the ascomycetes are differentiated from all other living organisms by the capacity for "free cell" formation. This is a unique type of spore formation in which the spore is cut out of the cytoplasm by the astral rays originating from the centriole. Usually four or eight spores are produced in a single ascus with the production of a certain amount of residual cytoplasm called the epiplasm. In all other types of cell formation, cells are usually cut out of a syncytium by cleavage without residual cytoplasm. In the Bacillaceae, the spores are formed as free cells and an epiplasm is produced. Since this characteristic distinguishes the ascomycetes from all other living forms, this fact automatically includes the Bacillaceae in the ascomycetes.

The Coccaceae, however, are distinct from the Bacillaceae for I have shown that in Micrococcus ochraceus (37) the autogamous copulation is followed by a reduction division in which the spore mother cell produces the tetrad by cleavage, rather than by free cell formation. In this form the chromosomes were demonstrated as strings of chromomerones (gene-strings) which synapsed chromomerone to chromomerone and finally underwent reduction after forming a typical reticulate nucleus.

X. DORMANCY

Diploid cells which have been grown on pre-sporulation agar for a week or more (but have not yet sporulated) become filled with stored fat and carbohydrate and, as a result of these reserve accumulations, become dormant. When tested in a Warburg apparatus, they are unable to give off CO₂ or consume O₂. Accessory substances may be involved in the induction of dormancy. Dormancy can only be broken by presenting the cells with a nutrient containing sufficient vitamins, carbohydrate, and a nitrogen source to insure continued growth. Vegetative cells which have grown on rich natural substrates, such as ripened fruits, fill with reserves and are probably dormant. They may germinate from dormancy by a simple vegetative procedure, or the diploid dormant cells may sporulate when placed on gypsum. In the latter case, conditions are unfavorable for continued vegetative growth just as they would be in a sandy soil. These observations suggest that the vegetative cells grown on ripe fruit become loaded with reserves and turn dormant. If they fall on another fruit or into a rich sugary nutrient, they germinate and grow vegetatively. At the end of the season when the fruits finally fall on the soil, sporulation occurs and the spores germinate the following spring.

It has long been known that yeasts store both fats and carbohydrates and the principal conditions controlling the storage of these reserve materials have been
fairly well worked out. It was not known, however, that cells containing abundant accumulations of reserve materials are in a state of dormancy; they are unable to take up O₂ to give off CO₂ or to bud, either in a phosphate buffer solution or in a buffer-glucose solution in the Warburg apparatus, but they begin to grow when they are brought into a complete nutrient medium. The mechanism has a high survival value, since it prevents cells from "wasting" their reserves, since growth can only begin under conditions in which continued or considerable growth is possible.

Meissner (56) studied the appearance and disappearance of glycogen in the yeast cell and showed that cells filled with glycogen produce more than the theoretical amount of CO₂ in fermenting a sugar substrate. He also found that glycogen accumulates in the cell and attains a maximum at the end of the principal fermentation when it begins to disappear from the cell, even before all the sugar is consumed. He designated glycogen as a temporary reserve used by the cell through an endogenous diastatic enzyme. He pointed out that the deposition of an insoluble carbohydrate inside a semi-permeable membrane enables the cell to take in soluble carbohydrate continuously by osmosis.

Wager and Peniston (74) studied the same question with cytological techniques. They found that glycogen was deposited in the cell in the form of small granules which coalesced to form a solid mass of glycogen almost completely filling the cell.

McAnally and Smedley-Maclean (53, 54, 55) and Smedley-Maclean and Hoffert (63, 64) showed that both carbohydrates and fats accumulated in the cell as a result of continued feeding with sugars. They found that phosphates increased the deposition of both reserves and that maltose seemed to increase the carbohydrate reserve. In addition, they pointed out that an excess of oxygen favored storage of fat.

Henneberg (22) also pointed out that the presence of phosphates favored the deposition of glycogen, while chlorides seemed to inhibit its deposition. He found that the maximal protein content of the cell was 67 per cent but that yeasts containing stored reserves might contain only 22 per cent protein. He stated that the amount of protein in a cell stands in inverse relation to the amount of glycogen (and presumably fat). He pointed out that the yeast cells collected directly from fruits are generally rich in glycogen and furthermore that yeasts kept in moist condition on filter paper live longer if they contain large amounts of glycogen. Some apiculate and lactose-fermenting yeasts were unable to store glycogen.

Lindegren (39) observed that the vegetative budding yeast cell, in the logarithmic growth phase (fig. 14a), which contains a very large centrally located vacuole and stains a light golden yellow with iodine, contains a few tiny fat globules. If these cells are placed under conditions of relatively low oxygen tension and supplied with an abundance of sugar, after budding has ceased, granular deposits of glycogen appear in the cytoplasm, which finally deform the vacuole and diminish its size (fig. 14g, h). If the cells are well-aerated and supplied with an abundance of sugar, fat globules appear and increase in size
Fig. 14.  
a. Budding vegetative yeast cells at the logarithmic stage of growth showing a single vacuole characteristic of this condition.
b. Budding yeast cell in the lag phase showing the apparently multiple vacuole resulting from deformations of the single vacuole by interference of reserve material.
c. Dormant vegetative yeast cells loaded with fat and glycogen, grown on pre-sporulation agar; the dark color is glycogen stained with iodine.
d. Germinating dormant cells from pre-sporulation agar, showing the vacuole breaking through the enclosing net-work of fat and glycogen.
e. Cells loaded with fat by growth in aerated sugar solution.
f. Cell of the type shown in e germinating.
g. Cell grown in sugar under conditions of reduced oxygen tension loaded with glycogen, a few fat globules surrounding vacuole.
h. Cell of the type shown in g stained with iodine, revealing the deformation of the vacuole by the reserve materials.
and number and also tend finally to obscure the vacuole, although it retains its spherical form (fig. 14e).

**Granular Glycogen**

The granular carbohydrate reserve in yeasts stains dark reddish brown with Lugol's iodine-potassium iodide solution. In many cells one can observe twenty or more small isolated granules. Occasionally, these granules are linked by connecting bands. Hundreds of small glycogen granules can be seen occasionally in some cells in addition to fifteen or twenty larger ones. These observations suggest that, in most of the cells containing a solid mass of dark-staining glycogen, the distribution is similarly non-homogeneous with the basic granular structure obscured by overstaining.

Unstained cells containing glycogen can be recognized by the high refractive index of the cytoplasm (fig. 14g). The nuclear vacuole in a glycogen-containing cell is often concealed by the glycogen. The visible vacuoles often appear to be multiple, but critical observation shows that the small vacuoles are all interconnected with each other by fine canals and are merely separate compartments of one major vacuole. This is consistent with the view that the vacuole is the nucleus and that each yeast cell contains only a single vacuole. In many unstained glycogen-containing cells no vacuole is visible, but staining with Lugol's solution always reveals the vacuole either compressed into the middle of the cell by a surrounding sheath of glycogen, or at one pole of the cell (fig. 14h). The vacuole in a glycogen-containing cell is usually much reduced in size.

Budding is retarded or inhibited in cells containing much glycogen, and occurs only after the glycogen has begun to disappear from the cell. Growing cells during the lag phase contain enough unidentified reserve to obscure or deform the vacuole (fig. 14b). After the cell has completed one or two divisions, the refractive index drops and the vacuole reappears or loses its deformity.

Deposition of granular glycogen is irregular at high (12 per cent) concentrations of sugar and rarely fills the whole cell. The stained granules are darker than those observed at lower concentrations of sugar. Many small granules may coalesce, often forming two large polar deposits. Small granules of glycogen may be linked by arcs of glycogen. At lower sugar concentrations (4 per cent), the deposition of glycogen continues until it fills the entire cell with the exception of a small region at the one end into which the vacuole is crowded, or the vacuole may be concealed in the center of the cell inside the spherical envelope of glycogen. After the deposit has reached a maximum, the glycogen disappears on aeration, by peripheral disintegration or solution, with a decrease in the density of the glycogen mass. Finally a single large diffuse granule is found in the cell, and the vacuole has regained its original size. Deposition of glycogen does not ordinarily begin until at least half the total number of cells that are to be formed are present. Under favorable conditions, involving low oxygen tension and continued addition of sugar, all the cells may become filled with glycogen.
Stainable glycogen does not accumulate in well-aerated yeast cultures grown in 1 per cent glucose broth. The nuclear vacuole attains its maximal size under these conditions.

Glycogen is never found in the vacuole of living cells, but some dead cells contain glycogen in the vacuole. Most of the dead cells remaining in a culture which has been reactivated after glycogen deposition contain glycogen in the cytoplasm. Either glycogen tends to deposit in dead cells or dead cells are unable to metabolize their accumulation of glycogen.

**Non-Granular Glycogen**

Dark brown (granular) glycogen is deposited rather regularly in deep broth cultures in test tubes. Glycogen-free cells (which usually contain about 20 per cent of an unspecified carbohydrate) grown in aerated 1 per cent glucose broth stain golden yellow with iodine. Cells from well aerated cultures which have received additional sugar differ from both the above by staining with iodine without any dark brown granular deposit. The entire cytoplasm is light brown, suggesting general distribution of a non-granular carbohydrate throughout the cell. The vacuoles are round, centrally located and usually large, indicating that the non-granular carbohydrate does not deform the vacuole, at least in the early stages of its deposition.

**Fat**

Fat is also stored in yeast, if the culture is well aerated and well supplied with phosphate and sugar. Fat first appears as an accumulation of highly refractive droplets around the vacuole. Deposits are often polar or comprise a network of streptococcus-like threads of granules which sometimes branch and are closely appressed to the outer surface of the vacuole. In most types of *S. cerevisiae* the fat globules tend to increase in numbers and to enlarge individually as the culture becomes older and storage increases. The addition of alcohol or the application of heat causes many of the fat globules to coalesce. Dead cells usually contain coalesced fat globules, just as they often contain glycogen. Fat invariably accumulates in yeast cells in shallow Erlenmeyer cultures.

**Dormant Vegetative Cells**

After most yeasts have grown for several weeks on slants of our pre-sporulation agar, the cells contain abundant deposits of fat and glycogen, and although all the cells are alive, they are dormant (fig. 14e). Subsequent experiments were undertaken to reproduce dormancy by loading the cells with reserve materials in broth cultures, but we were unable to obtain cells with precisely the appearance of those taken from pre-sporulation agar, which appear to be much more heavily packed with larger fat granules. The vacuoles in a fat-filled cell from pre-sporulation agar are usually spherical, indicating that fat and glycogen deposition on this medium occur without deformation of the vacuole. After an hour in nutrient medium, much of the fat disappears from most of the
cells and after the second hour, half of the cells show buds and some contain a few fat globules. The vacuoles appear to be multiple or obscured in the growing cells (fig. 14d). In three hours, there is very little visible fat left in the cells, nearly all of which contains multiple or obscured vacuoles. Practically all the cells bud, showing that the culture is viable.

Manometric studies were made with the cells taken directly from the pre-sporulation slant (fig. 14c) and shaken with phosphate buffer containing 4 per cent glucose, but with no other nutrients. The Q values showed that the cells were incapable of taking up oxygen and incapable of producing CO₂ either aerobically or anaerobically over a 150-minute period. During the same period, all the cells in the nutrient broth budded. Ninety-eight per cent of the cells from the Warburg vessel showed little or no change.

Cells of a standard baking yeast, strain A, which had been grown on pre-sporulation agar, were collected from the agar surface and washed with M/15 KH₂PO₄. Three Warburg vessels were inoculated with equal amounts of dormant cells. Each vessel contained 4 per cent of glucose in solution. The first received phosphate buffer, the second received 1 per cent of corn-steep-water solids, and the third received 0.3 per cent ammonium sulfate, biotin (2 γ per liter) and pantothenic acid (200 γ per liter).

Since our culture of _S. cerevisiae_ is incapable of synthesizing biotin and pantothenic acid, these substances were added, together with ammonia, to see if they would break the dormancy of the fat-filled yeast cells. The cells suspended in sugar were dormant and gave off no CO₂ after over five hours in the Warburg apparatus, but the cultures in the other vessels fermented the sugar, the action being much more rapid in the richer nutrients. This experiment cannot always be duplicated, because cells from the pre-sporulation agar slants are not always in precisely the same condition, since some cultures sporulate directly in the slant.

**Storage of Fat and Carbohydrate**

Storage of reserves only occurs in a medium in which growth has nearly ceased. A 1 per cent glucose broth was prepared with half the standard amount of nutrient broth to insure the early cessation of growth. Fifty ml of broth in 500 ml Erlenmeyer flasks were inoculated and shaken for 48 hours. The suspension of cells from some of the Erlenmeyer flasks was placed in 8 x 1 inch tubes to favor the development of glycogen, while the remainder were kept on the shaker to favor the development of fat. Tests with Fehling’s solution were made to determine when the sugar disappeared, and sugar was added as soon as a deficiency was indicated. Phosphate was also added, since this is known to increase the deposition of both fat and glycogen. The addition of sugar was continued for four days.

Three cultures were used, two standard baking yeasts, strains U and R (_S. cerevisiae_) and a hybrid (_S. cerevisiae_ X _S. globosus_). Strain U stored both fat and carbohydrate relatively uniformly, as indicated by microscopic ex-
amination. Strain R stored carbohydrate well, but the accumulations of fat were irregular and the fat-containing culture was discarded. The hybrid stored fat in large clusters of extremely tiny granules approaching the limits of visibility. The cells appeared to be crowded with stored materials, but chemical analysis (ether extraction) revealed that only 6.16 per cent fat was present. The hybrid stored carbohydrate poorly. Nitrogen analyses were converted to protein by multiplying by the factor 6.25 (table 1). The sample was ashed, and the difference was calculated as carbohydrate.

Yeast cells may contain different amounts of carbohydrate and fat reserves. These reserves hinder the respiratory, fermentative, and budding activity of the cell. The Q values are reduced nearly to zero. After a lag, the dormant cells begin to respire, ferment, and bud. The low Q values of the nearly dormant cells are due to causes different from those responsible for the low values of cultures containing large numbers of dead cells. In the early phases of this work, cells were grown in 8 per cent sugar, peptone, yeast-extract medium and in this medium, 50 to 90 per cent of the cells died, especially if the cultures were well-aerated. The dead cells gave normal Q2O2 and Q8O2 and values ranging from 300 to 450, but with Q02 values of 0. The dead cells were unable to consume O2, although they were able to ferment. Some of our dormant cultures contained nearly 100 per cent viable dormant cells. These viable dormant cells, loaded with reserve materials, were unable to consume O2 or to evolve CO2, but this situation is obviously different from that found when the cultures contain many dead cells.

The reproducibility of results obtained by the Warburg respirometer depends upon the absence of accumulations of reserve materials in the cells. Cells should be aerated and transferred to the Warburg apparatus when a minimum number of dead cells is present and a considerable proportion is actually budding. In order to make reproducible analyses, growth must be stopped in an active phase and the cells washed with M/15 phosphate. If the cells are al-

<table>
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<th>CULTURE</th>
<th>VISIBLE DEPOSIT</th>
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<td>5.22</td>
<td>57.7</td>
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</table>
allowed to stand in a nutrient medium, they will accumulate some reserve fat or carbohydrate, or both, depending on the aeration and the concentration of sugar. Accumulated reserves are responsible for the lag in growth observed on the inoculation of the fresh medium. The lag can be completely eliminated if the cells are transferred before any storage has occurred.

Hansen (20) developed a technique of preserving yeast cultures by growing them in 10 per cent sugar broth and allowing the culture to dry down. The excess of carbohydrate caused growth to cease and storage of reserves brought the cell into dormancy. This procedure could not be used to supply dormant cells in our experiments because a very large percentage of the cells die in high concentrations of sugar and only a few attain full dormancy. Winge and Hjort (77) recovered living cells from fifty-year-old cultures prepared by Hansen.

The fact that dormant cells require a medium containing a relatively full complement of nutrients enables the cells to start growing under conditions that assure continued growth. The fact that they require specific vitamins which they are unable to synthesize may have some importance in solving the problem of inducing other fungal spores to germinate.

XI. VITAMIN SYNTHESIS

A survey by Burkholder, McVeigh, and Moyer (8) has revealed that practically all yeasts can synthesize riboflavin, but that they vary considerably in their ability or inability to synthesize the other B vitamins. Our cultures of S. cerevisiae, S. carlsbergensis, S. globosus, and S. bayanus differed from each other in the ability to grow on media deficient in different B vitamins. A study of eight bakers' yeasts collected on the market showed that all these strains of S. cerevisiae resembled each other rather closely in their vitamin requirements, even on a quantitative basis. They were all unable to synthesize biotin. Growth in the medium without pantothenic acid was always less than with all the vitamins, and the only considerable variation encountered was in response to the absence of this vitamin. Some yeasts did slightly better when niacin was omitted from the medium than when it was present, and all grew nearly as well in the absence of inositol, thiamine, and pyridoxine as they did when all six vitamins were present. Burkholder (6) has shown that other strains of the S. cerevisiae do not conform to these bakers' yeasts in their ability to grow in the absence of specific B vitamins. This suggests that the close similarity of the eight bakers' yeasts may indicate that they are all closely related.

Our culture of S. carlsbergensis differed from the baking yeasts in being able to synthesize biotin, but unable to synthesize pyridoxine. The technique was that developed by Burkholder and Moyer (7). The standard medium containing glucose and asparagine and various minerals was supplemented with the six B vitamins (exclusive of B_6). In the medium containing the six vitamins, growth was nearly complete at the end of three days. Other nutrient media were made up corresponding to the complete medium described above except
that single B vitamins were lacking. Since these B vitamins are essential to cell metabolism, it is assumed that a culture able to produce good growth in a nutrient lacking a given vitamin is able to synthesize this vitamin and that the converse is also true. The amount of inoculum was tested and shown not to carry enough vitamin to obscure the results.

A hybrid was made between *S. carlsbergensis* and one of the baking yeasts, *S. cerevisiae*. The data on the pedigree are recorded in Table 2. The turbidity readings of yeast growth in tubes from which pyridoxine, pantothenic acid, and biotin respectively, are absent, but the remaining five vitamins present are shown. A high reading indicated a large number of cells while a low reading indicated that few cells are present in the suspension. The diploid culture of *S. carlsbergensis* failed to grow in the absence of pyridoxine, but grew in the absence of both pantothenic acid and biotin, and the single surviving haploid ascospore culture likewise failed to grow in the absence of pyridoxine. The diploid culture of *S. cerevisiae* grew in the absence of both pyridoxine and pantothenic acid, but failed to grow in the absence of biotin. Two surviving ascospores which were tested, grew in the absence of pyridoxine but were unable to grow in the absence of either pantothenic acid or biotin, suggesting that the strain may be heterozygous for a gene pair controlling the synthesis of pantothenic acid. An interspecific hybrid between a pair of *S. cerevisiae* and *S. carlsbergensis*.

**Table 2**

*Turbidity readings of yeast grown in culture media lacking pyridoxine (Py.), or pantothenic acid (Pa.), or biotin (Bi.). The cultures used are from a pedigree of *S. carlsbergensis* by *S. cerevisiae*.*

<table>
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<tr>
<th>GENOTYPE</th>
<th>DIPLOID</th>
<th>ASCUS NO.</th>
<th>ASCOSPORES</th>
</tr>
</thead>
<tbody>
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<td>22 200 125</td>
<td>d*</td>
</tr>
<tr>
<td><em>S. cerevisiae</em></td>
<td>350 220 11</td>
<td>d</td>
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<td>(A)</td>
<td>22 200 125</td>
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<td>274 45 50 312 202</td>
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<td>318 212 140 100 210 140 300 235 194 345 235 110</td>
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<td>1A × <em>S. cerevisiae B</em></td>
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*d* = died.
carlsbergensis haplophase cultures grew well in the absence of all three vitamins. The twelve ascospore cultures from three ascii of the interspecific hybrid were tested for their ability to grow in the absence of the three vitamins. In spite of the fact that one parent produced a growth of 22 and the other 350, in the absence of pyridoxine, nine of the haplophase progeny from the hybrid produced about 300 units of growth. Two were better than 80 and only one resembled its S. carlsbergensis parent in being unable to grow well in the absence of pyridoxine, but it grew so poorly in the absence of both pantothenic acid and biotin that its poor growth probably resulted from some other more fundamental deficiency. The fact that the first generation segregants from the hybrid all grew fairly well in the absence of pyridoxine suggests that some cytoplasmic effect may be obscuring any gene-segregations that may have occurred. In the absence of pantothenic acid also the growth of these haplophase cultures resembled the better growing rather than the weaker parent. Only one of the twelve gave the same poor growth in the absence of pantothenic acid as its S. cerevisiae parent and this yeast was apparently weak in other respects as well. In the absence of biotin, the same absence or obscuring of the Mendelian ratio occurred. The progenies of these three matings gave irregular results suggesting that a cytoplasmic mechanism may be obscuring the Mendelian ratios. The characters may be fundamentally under genetic control, but the ratios may be obscured by cytoplasmic effects. If many genes were involved, rather than cytoplasmic obscuring of the Mendelian ratio, a greater variety of progeny would be expected. This pedigree resembles that of S. bayanus × S. cerevisiae in which the galactose-fermenting S. cerevisiae transmitted the capacity to ferment galactose to all the progeny in the pedigree (Lindegren, 34). In the backcross in which both parents were incapable of synthesizing either pantothenic acid or biotin, the fact that the progeny were generally inferior suggests that adaptations do not seriously complicate the interpretation. Leonian and Lilly (28, 29, 30) derived yeasts capable of synthesizing vitamins from cultures incapable of performing the syntheses. Their procedure probably favored the selection of mutations. In our experiments, we attempted to minimize this possibility. Interspecific crosses such as these are generally quite complex because the two genomes usually have a greatly different collection of modifiers and the expression of each gene is influenced differently than it was in the original environment.

S. globosus is capable of synthesizing pantothenic acid, but incapable of synthesizing thiamine. A hybrid was made between a haplophase from a homozygous pantothenic-deficient culture of S. cerevisiae (different from the one used above) and a haplophase culture of S. globosus. Several hybrids were produced by this mating, and one of them sporulated well, but only a few of the ascospores were viable. One of the hybrid-haplophases was backcrossed to the original pantothenic-deficient S. cerevisiae. The resulting diploid synthesized both pantothenic acid and thiamine efficiently. Since neither parent could synthesize both vitamins, the hybrid had obviously obtained its ability
to synthesize pantothenic acid from *S. globosus* and thiamine from *S. cerevisiae*. The hybrid was a poor synthesizer of biotin, but this was according to expectation, since neither parent possessed the ability.

**XII. ADAPTATION**

The ability of an organism to adapt itself to the environment depends upon its capacity for producing variants. Variation in yeasts depends upon both genetical and cytoplasmic mechanisms. The genetical mechanism produces variations through *a*, the operation of segregating the chromosomes together with the aberrations of this mechanism, *b*, by mutation, especially in the haplo-phase, and *c*, by matings which produce new combinations of different genotypes to form new diplophases.

*Plastogenes, Plasmagenes, and Cytogenes*

The cytoplasm is also a possible source of variation. Darlington (9) has given names to two kinds of cytoplasmic components which are found in higher plants: the plastogenes and the plasmagenes. The plastogenes are contained in plastids similar to the chloroplasts that have long been recognized as self-perpetuating cytoplasmic bodies which may vary and produce changes in the organism either independently or under the influence of the genes of their host (Rhoades, 61). Winge and Laustsen demonstrated that there are certain cytological constituents, which they assumed to be the chondriosomes, that divide regularly at each nuclear division and which may produce degeneration if they diminish in number. It is possible that Darlington would include the cytoplasmic components described by Winge and Laustsen under the category of plastogenes. Chloroplasts are typical plastogenes but they do not divide regularly at each cell division, for a deficiency in chloroplasts can be made up by a cell which has received less than a standard number simply by multiplication of the chloroplasts while the cell is in the resting condition. If we include the cytoplasmic components described by Winge and Laustsen under the heading of plastogenes, we recognize two categories of these organelles, one which multiplies independently of nuclear division, and the other which divides regularly at each nuclear division.

Plasmagenes, in Darlington's view, are related to viruses inasmuch as they are relatively independent of any definitely recognized plastids or cytoplasmic organelles, but apparently multiply more or less independently in the cytoplasm. He considers them to be the phylogenetical forerunners of viruses. I (32) have developed a hypothesis suggesting that viruses were developed from the genes of the host by partial digestion and mutation in insect vectors.

The cytogene (Lindegren, 34) is a third type of cytoplasmic component capable of producing variation 'and in effecting the adaptation of yeasts to different substrates. The cytogene is a self-perpetuating entity, initiated by the action of a gene but made specific by a specific substrate under whose influence it becomes self-perpetuating (Spiegelman, Lindegren and Lindegren, 71).
Spontaneous and Induced Mutations (?)

In the adaptation of microorganisms to a specific substrate, while they are increasing in numbers on that specific substrate, the question arises as to whether the substrate induces the change resulting in adaptation, or whether the change occurs spontaneously and the substrate acts merely to select the new variant, permitting it to outgrow the original normal form. There are, therefore, two fundamental hypotheses: a, that the microorganisms may produce a new variant spontaneously, independently of the substrate, and b, that the contact of the substrate with the microorganism induces the variation, which is transmissible to the progeny of the microorganism.

Previous to the analysis of this problem by Luria and Delbrück (52) experienced workers could not determine with the available data which mechanism functioned. For example, when Bacterium coli mutabile (a non-lactose fermenting organism) is plated on lactose agar, papilli appear on the colonies at the point of origin of lactose fermenting variants. This phenomenon was studied intensively by Lewis (31) and Stewart (72), who obtained substantially the same results, but drew precisely opposite conclusions. Lewis concluded that the variation from non-lactose fermenter to lactose fermenter is independent of the substrate and occurs spontaneously in all cultures whether or not lactose is present; while Stewart concluded that transformation from non-lactose fermenter to lactose fermenter is induced by lactose. Luria and Delbrück have shown that the question can not only be answered definitively, but that the rate at which new forms are produced can be precisely calculated. However, a more subtle mathematical approach is required than previous workers had used. (I am indebted to Dr. A. D. Hershey for explaining to me the significance of Delbrück's mathematical analysis.) They analyzed the question in which the transformation of bacteria from virus sensitivity to virus resistance was involved. The same approach can solve the question of the effect of lithium chloride, antisera, bactericidal substances, or specific substrates such as the different sugars, on the selection or induction of variations in multiplying cultures. The procedure is as follows: A number of culture tubes without the specific bactericidal substance (or the virus, or the carbohydrate substrate) are all inoculated simultaneously and allowed to grow, then finally sampled and tested for the normal form and the adapted variant. The conditions are that a, the normal and the variant forms grow about equally well in this initial non-selective nutrient medium and b, that the probability of the variation occurring is relatively small. If the variation is not induced by the substrate, adapted forms may appear either early or late as the culture grows in the absence of the substrate. Random spontaneous production of the adapted forms without competition will result in a great variation in the number of adapted forms present in the different tubes. Large variations in the number of adapted forms appearing when samples of organisms (grown in the absence of the specific inhibitor or substrate) are planted on the specific inhibitor or substrate, prove that the new variant appeared independently of the environmental condition to which it is adapted. Furthermore, the specific frequency of mutation can be
accurately calculated. Luria and Delbrück point out that their use of the term "mutation" is merely formal. On the basis of their analysis, Demerec (11) was able to show that resistance of *Staphylococcus aureus* to penicillin was the result of a number of heritable changes and that these changes were not induced by the action of penicillin, but originated spontaneously. Since this type of analysis has not yet been made in the case of *B. coli mutabile*, it is not possible to say whether this variation is spontaneous or induced. At the present time, only the two cases, *a*, resistance to penicillin by *Staphylococcus aureus* and *b*, resistance to bacteriophage of the colon bacillus have been analyzed and both have been found to occur spontaneously, independently of the environmental agent. Variation in bacteria involving changes in colony morphology are not generally referable to any substrate, although they have been correlated with transformation from virus-susceptible to virus-resistant forms, but Luria and Delbrück point out that a difficulty in analyzing this phenomenon arises from the fact that an entire colony is necessary to express the character. There is no evidence, however, that colonial characteristics can be induced by an interaction between cell and substrate; on the contrary, there is every indication that this is a character which arises spontaneously. Since often no environmental effect seems to be involved, the difficulty of analysis existing in the case of adaptation does not apply.

Werner in a forthcoming article points out that the rate of mutation may not be so precisely calculable as Luria and Delbrück's article suggests unless more precise data on growth rates are available. The mutant probably only rarely grows at precisely the same rate as the parent form, and Werner has discovered that small differences in growth rates must be considered in calculating the mutation rate.

*Induced Cytoplasmic Adaptation in Yeasts*

We were able to demonstrate the existence of an induced adaptation in yeasts in the study of an illegitimate diploid (Lindegren, 34). The culture sporulated only rarely and did not sporulate at all in broth. This fact eliminated genetical variation except for the unusual dominant mutations. When a transfer was made from malt extract agar to corn-steep-water agar, only small numbers of the cells survived and there was considerable variation in colony size. However, a second transfer to corn-steep-water agar resulted in very good growth, indicating that one transfer had sufficed to adapt the cells completely to this substrate. The adaptation was lost completely by one transfer to malt agar. The crucial test was made by showing that the cells also became adapted if allowed to stand one day in the cold room in corn-steep-water broth in the absence of cell division. Luria and Delbrück's analysis is necessary if cells are increasing in number in the medium to which they become adapted, but if adaptation of a stationary population occurs, then their method is not necessary. This fact alone proves that a heritable non-genic variation has been induced by an interaction of the substrate and cytoplasm which adapted the cells to growth in corn-steep water.
The fact that heritable, induced adaptations (which should presumably be referred to the cytoplasm) occur makes it impossible, in microorganisms without a sexual cycle, to determine whether a given heritable variation is or is not a gene mutation. The only possible technique for establishing the existence of a gene mutation is to make matings between the assumed mutant and a normal form and demonstrate that the genes controlling the expression of the characters segregate regularly at the reduction division. New variations that are physiologically equivalent to gene mutations can be induced in bacteria by radiations. In *Neurospora*, the genes responsible for synthesis of the B vitamins can be destroyed by radiation. Roepke, Libby, and Small (62) showed that the ability of bacterial cells to synthesize B vitamins can also be destroyed by radiation and have suggested on the basis of the similarity of these experiments to those of Tatum and Beadle (73) that the new types which they obtained were bacterial gene mutations. However, Lindegren and Lindegren (42) have shown that a large proportion of the heritable changes resulting from treatment of *Neurospora* with ultraviolet and X-rays are not gene mutations but some type of cytoplasmic change. (Beadle and Tatum's experiments were devised in such a manner that this type of variant would not have been discovered.) The production of cytoplasmic variants by radiation was demonstrated by mating different radiation-induced variants to normal individuals and finding that only normal progeny were obtained. Since the progeny were haploid, a gene mutation would have given a 1:1 ratio of mutant to normal. The fact that only normal progeny were obtained revealed that the genes in the radiation-induced variant were all normal, and the only possible explanation was that some constituents of the cytoplasm had been destroyed or injured, but that fusion with the cytoplasm of the untreated normal had replaced or repaired the deficiency.

**Induced Cytoplasmic Adaptation to Galactose**

Kluyver (25) showed that *S. cerevisae* is capable of fermenting galactose, but the fermentation occurs only after a definite period of exposure to the sugar. Spiegelman, Lindegren, and Hedgecock (70) found that when a suspension of diploid cells grown on glucose is washed with M/15 KH₂PO₄ and then resuspended in this solution with added purified galactose, the cell count remains constant over a long period of time. When the addition of galactose is made in a Warburg vessel, fermentation begins explosively after a period of about three hours. This action is an adaptation without the formation of new cells and under standard conditions the lag period is reproducible and is characteristic for the given strain. Some strains have longer lag periods extending up to eight hours. This phenomenon of acclimatization to galactose fermentation makes galactozymase in *S. cerevisiae* an adaptive enzyme as defined by Karström (24). Since the adaptation occurs in the absence of cell division, it is due to the interaction of substrate with the cytoplasm, although it may nevertheless be basically under genetic control, as will be indicated later.
Adaptation to Galactose by Mutation (?)

Spiegelman et al. (70) tested a haplophase culture, derived from S. cerevisiae, but incapable of fermenting galactose directly, for its capacity to adapt to galactose fermentation. It was able to adapt to the fermentation of galactose after considerable, but variable, periods of growth in galactose broth. An examination of the culture was made to determine if the starting population were homogeneous or heterogeneous. Each cell was characterized by the type of colony which it produced when grown in agar under special conditions. A cell suspension was plated on 4 per cent nutrient agar containing 4 per cent galactose and allowed to dry; then 5 per cent nutrient agar containing 4 per cent galactose was poured over the inoculated surface.

Two kinds of colonies were observed: one grew in the conventional lenticular form, appearing circular when observed from the top of the plate; the other produced an excessive amount of gas, resulting in cracks in the agar. The former utilized galactose oxidatively with an R.Q. of 1, while the latter produced CO₂ in such excessive amounts that bubbles accumulated, rupturing the agar. A large number of colonies was counted, revealing an average of about 7 per cent of fermenting type colonies with a variation in individual experiments from 2 to 15 per cent. Similar test plates using glucose were examined and only one negative colony found in 2592 colonies examined. The negative colony was examined manometrically and appeared to be a new non-fermentative variant.

The technique was checked by making combinations of suspensions of cells. The number of positive colonies appearing in the mixtures was the arithmetic mean of the percentages of positives in the two suspensions from which they originated, thus establishing the fact that local conditions in the plate had not induced fermentations.

The percentages of positives obtained from positive colonies varied from 75 to 99 per cent, indicating that there was a selection of the fermentative type and that it tended to replace the non-fermentative type. The non-fermenters used galactose slowly through aerobic oxidation and therefore their rate of division is depressed in this medium. The few fermenters present, after a lag period, start to divide rapidly since they possess the enzymatic apparatus necessary to use this sugar at a rapid rate. The number of fermenters thus increases due to two sources: first, the rapid cell division of those already present, and second, the transformation of non-fermenting to fermenting cells. This latter mechanism can, even with relatively low rates of change, be numerically significant in the early history of the populations because of the relatively large number of non-fermenters initially present. On the other hand, the number of non-fermenters present at any time can increase only by virtue of simple cell division.

A mathematical analysis by Spiegelman and Lindgren (68) revealed that when a glucose-grown haplophase culture was transferred to galactose broth the number of cells capable of producing fermenting colonies increased at a rate consistent with the hypothesis that natural selection had operated to enable the adapted to take precedence over the unadapted type. That is, the ad-
vantage which the galactose-fermenter had over the galactose-oxidizer enabled the former to replace the latter. This work was done before that of Luria and Delbrück (52) and the conclusion that the fermenters had arisen "spontaneously" rather than being "induced" by the presence of galactose was apparently unwarranted. On this point our data on the haploid form were indeterminate.

In contrast, the mathematical analysis applied to the diploid form revealed that the explosive appearance of fermenters after the three-hour adaptation period could not possibly have arisen by growth and competition, but that essentially the whole population became adapted after the lag period. Therefore, the adaptation of the diplophase involves an interaction between the genetically-adaptable protoplasm and the substrate which results in the production of galactozymase by every cell.

This presumably means that the adaptation of the non-fermenting haplophase involves two steps: 1, the transformation of a genetically non-fermenting to a fermenting type (either spontaneously or by induction) and 2, a lag period in which the protoplast of the transformed type (the new "mutant," genetically like the adaptable diploid) reacts with the substrate galactose to produce galactozymase.

The capacity of a protoplast to react with galactose to produce galactozymase is heritable, for the adaptable diplophase usually produces adaptable diplophases and the unadaptable haplophase usually produces unadaptors, a small percentage of which become transformed (either spontaneously or by induction) into adaptables. This fact, however, does not establish it as a gene mutation as has already been indicated.

**Cytoplasmic Inheritance of Galactose-Fermenting Enzymes**

*S. bayanus* is incapable of fermenting galactose and a hybrid was made between it and *S. cerevisiae* (Lindegren, 34). The diploid hybrid (*bayanus* × *cerevisiae*) culture was able to ferment galactose and the sixteen haploid ascospore cultures from four asci dissected from the hybrid were also capable of fermenting galactose. One of this group of first-generation ascospore cultures was backcrossed to the original haplophase isolate of *S. bayanus* and the diploid hybrid [(*bayanus* × *cerevisiae*) × *bayanus*] was capable of fermenting galactose. Single ascospores were isolated from three ascii and all twelve cultures were capable of fermenting galactose. Subcultures obtained by plating some of these fermenting clones produced some non-fermenting cultures. In view of our experiments on melibiose fermentation (see below), this particular experiment suggests that cytoplasmic factors have been involved which were transmitted to the progeny, independently of the germplasm, obscuring any Mendelian inheritance that may have been present. *S. bayanus* produces long, cylindrical, haploid cells, while *S. cerevisiae* produces round, haploid cells. The segregation of cylindrical versus round cells in the ascus was regularly Mendelian, indicating that a basic genic inheritance existed.
Adaptation of Supposedly Unadaptable Yeasts

_Schizosaccharomyces pombe_, _Schizosaccharomyces octosporus_, and _Saccharomyces ludwigii_ are among the non-fermenters of galactose. All three yeasts were examined by Armstrong (1), who concluded that they were incapable of adaptation to galactose fermentation. In _S. ludwigii_, under ordinary conditions, the haplophase is transitory because fusion occurs within the ascus to reconstitute the diplophase almost immediately (fig. 4). In _S. octosporus_ grown on solid medium the spores fuse very shortly after they are formed to reconstitute the diplophase. In _S. pombe_, the haplophase lasts a relatively longer time. Adaptation of the species to galactose was studied by Spiegelman and Lindegren (59). Since mutations in the diplophase are not expressed unless they are dominant, it is not possible for selection mechanisms to operate to select them. If one wishes to obtain extremely variable populations of yeasts this can be accomplished by inducing the diploid cells to sporulate and maintaining the culture in the haplophase since each mutation in the haplophase usually comes into immediate expression.

The three species named above were induced to sporulate and inoculated into a medium containing 2 per cent glucose with 8 per cent galactose. In from two to six days cultures of _S. pombe_ produced fermentation in the flasks and a stable galactose fermenter was obtained from the previously unadaptable form. The glucose was necessary, apparently to insure sufficient initial growth to get a large population from which to select. _S. octosporus_ and _S. ludwigii_ were not adaptable, presumably because in them the haplophase lasts a shorter time than in _S. pombe_.

_S. cerevisiae_ and _S. carlsbergensis_ are both capable of fermenting galactose, but non-fermenting haplophase variants have been obtained from both of these. An additional non-fermenting haplophase culture was found in the examination of 2592 colonies of the galactose-fermenting haplophase of _S. cerevisiae_ described above, page 159. These three non-fermenters of galactose all used it aerobically with an R.Q. of 1. Microscopic examination revealed that they were haplophases. When streaked on agar plates, they produced a great variety of colonial mutants, indicating that they had the genetical instability characteristic of haploid segregants. However, they were all carried for five months on 8 per cent galactose without obtaining mutations to the fermentative type. This seemed to indicate that some haploids may be quite incapable of the potentiality for mutating to produce galactose fermenters, although they may at the same time be quite unstable genetically and capable of producing a wide range of morphological variants.

**Genic Balance in the Haplophase**

In the analysis of our hybridization experiments, we have found that all genes which control physiological reactions have proved to be dominant. The heterozygote is capable of synthesizing a given vitamin just as well as the homozygote, and hybrids, heterozygous for the loci that control the fermentation of
melibiose, ferment melibiose just as well as homozygotes. Therefore, mutations do not occur in the haplophase rather than in the diplophase, solely because the genes come into expression more easily in the haplophase, for dominant genes come into expression immediately in the diplophase.

The phenomenon studied by Fisher (14), Wright (81) and Haldane (19) provides a possible explanation. It has long been realized that naturally occurring genes are practically always dominant over mutant genes. The genes controlling vitamin-synthetases and carbohydrate-fermentations are well established, naturally occurring genes that have probably become dominant by natural selection. Newly mutated genes capable of inducing the fermentation of a sugar may be incapable of producing an effect in the heterozygous condition. According to Fisher's theory, this would occur if all genes are originally recessive and become dominant against a specific genetical background. He suggested that genes which have high survival value may become dominant through the selection of modifying genes in the evolution of the species. By this mechanism a gene which was originally recessive becomes a stable dominant through a number of mutations at other loci. Wright and Haldane suggest that modifiers may be relatively unimportant, but that the strength of the gene itself is increased by natural selection. If Fisher's view be correct, the situation in yeasts may be explained as follows: Yeasts are extremely heterozygous and may even be heterozygous for the fundamental modifiers that determine dominance. If the balance of modifiers that control the dominance of many wild-type genes in the diplophase be upset when the haplophase is formed, the haplophase segregant would be more plastic than the original diplophase. This may make it possible for mutations, which would ordinarily be suppressed by the complete complex of modifiers, to come into expression. The mechanism making specific genes dominant is apparently much better stabilized in the case of genes controlling physiological activities than of those affecting the relatively less important morphological and colonial characters for the former are stable and dominant, while the latter are unstable and recessive. In an interspecific heterozygote the gene controlling fermentation of a carbohydrate may be dominant simply because half of the genes in the heterozygote come from the parent species in which this gene has been evolved and therefore, half of the genome consists in part of modifiers, which act to make the fermenting gene dominant. A mutation to ability to ferment galactose might fail to bring about the fermentation even if it occurred in the haplophase simply because a set of modifiers exists capable of suppressing this fermentation.

The situation is further complicated since the apparent dominance of a fermentative mechanism may not be real, but may merely result from transfer of cytogenes. Winge and Laustsen (79) found that in every case in which a fermenter was mated by a non-fermenter, the hybrid was capable of performing the fermentation. To make certain that this is due to the dominance of the heterozygote, subsequent genetical analysis would be required. Sometimes this analysis leads to confusion as in the pedigree of S. bayanus × S. cerevisiae; in this case cytoplasmic transfer of the cytogene is a complicating factor.
Mendelian Inheritance of an Adaptive Enzyme

*S. cerevisiae* is incapable of fermenting melibiose, and its haploid segregants fail to ferment this sugar even after continued growth in broth containing melibiose. This suggests that the *S. cerevisiae* genome limits the mutational range of this species, possibly by the mechanism discussed above. *S. carlsbergensis* is capable of fermenting melibiose, as are all its haploid segregants. This is the principal character upon which *S. cerevisiae* and *S. carlsbergensis* are differentiated. On the basis of the preceding discussion, the ability to ferment a single sugar may be sufficient to define two naturally occurring yeasts which produce viable four-spored asci. Those cases in which the haplophases of one species are unable to adapt by mutation to the fermentation of a given sugar suggest that an elaborate system of modifiers may be necessary to enhance the expression of the gene in the other species. Therefore, a single apparent genetical difference may mean that an elaborate hidden complex differentiates the two species. However, as pointed out under "Speciation," fermentative differences in haplophase yeasts generally have little or no significance. Figure 15 is a pedigree describing the progenies of matings between these two species (Lindegren, Spiegelman, and Lindegren, 50). The data were obtained by growing the cultures in a broth tube containing a smaller inverted tube to collect the gas produced by fermentation. Accumulation of gas in the inverted tube is indicated by a plus sign.

Hybrid I was an interspecific hybrid (*cerevisiae* × *carlsbergensis*) made by mixing melibiose-plus and melibiose-minus haplophase cultures. Three diploid cells isolated after this mating were all capable of fermenting melibiose. Eight asci were dissected from interspecific hybrids, and all the haplophase progeny were tested for the ability to ferment melibiose. The results showed that all the haplophase cultures from three asci were melibiose +; two asci produced three + and one − culture; and one ascus produced two + and two − cultures.

This experiment was performed before we had had any experience with the cytoplasmic transfer of enzymes and we thought that each haplophase culture which was able to ferment melibiose did so by virtue of a gene. These data suggested that the haplophase of *S. carlsbergensis* carried two genes capable of inducing the fermentation of melibiose. However, a hybrid between the same *S. carlsbergensis* haplophase and a haplophase incapable of fermenting either galactose or melibiose revealed that the *S. carlsbergensis* haplophase carried only one gene controlling the fermentation of melibiose. In this latter pedigree there was a 1:1 ratio in most of the ascii for fermentation and non-fermentation of galactose as well as a 1:1 ratio for fermentation and non-fermentation of melibiose, and these factors were segregated independently. However, a few asci produced 4 spores, all of which fermented both melibiose and galactose. The latter asci were clearly those in which a great deal of cytoplasmic transfer had occurred and the more frequent 1:1 ratio of mel + to mel − can be considered the proper Mendelian ratio unobscured by cytoplasmic transfer. When transfer of the cytoplasm occurred, it included both enzymes as one would expect.
Hybrid II was produced by backcrossing a positive haplophase culture from an ascus producing four + cultures with a negative haplophase culture from S. Saccharomyces cerevisiae. Regular Mendelian segregation in the progeny shows that this haplophase carried a single gene, capable of controlling melibiose fermentation,
apparently derived from *S. carlsbergensis*. The backcross to *S. cerevisiae* cleared up the Mendelian ratio by further dilution of the *S. carlsbergensis* cytoplasm.

Hybrid IV was made by backcrossing a second positive haplophase culture from the same ascus to a negative haplophase culture from *S. cerevisiae*. In this case, regular Mendelian segregation again shows this haplophase also carried a single melibiose-fermenting gene.

Hybrid III was produced by mating the two positive cultures, each of which carried a single positive gene derived from *S. carlsbergensis*. Two of the twenty haplophase progeny failed to ferment melibiose. Actually every one should have carried the gene, so these two failures may have been due to modifying genes from *S. cerevisiae* suppressing the fermentation. The inability of *S. cerevisiae* haplophases to ferment melibiose after repeated trials suggests that such suppressors exist.

Hybrid V was made by backcrossing a negative haplophase segregating from Hybrid IV to a negative haplophase from *S. cerevisiae*. The three haplophase progeny were all negative.

Hybrid VI was made by backcrossing the same negative culture to a positive haplophase of *S. carlsbergensis*. Five of seven haplophase progeny fermented melibiose, while two failed.

This pedigree is of especial interest because the fermentation of melibiose is due to an adaptive enzyme (Karström, 24). Cells which have been adapted to ferment melibiose lose this ability when removed from the substrate and have to be readapted to use it fermentatively.

The Cyto gene

In the preceding pedigree, the first contact with melibiose occurred when the culture was transferred to a fermentation tube containing melibiose. A second series of experiments, (Spiegelman, Lindegren, and Lindegren, 71) showed that if contact with melibiose were maintained during the growth of the haplophase cultures during copulation, during growth on the pre-sporulation agar, and during spore formation, all four cultures obtained from a four-spored ascus of heterozygous diploids, such as Hybrids II and IV, were able to adapt to melibiose fermentation. (Without this continued exposure to melibiose only two of the four cultures obtained from the four spores of each ascus are able to ferment melibiose and two from each ascus are unable to ferment melibiose.) However, two of the four melibiose-fermenting cultures from each melibiose-treated ascus completely lost their ability to ferment melibiose when vigorously dissimated by shaking in phosphate solution in the absence of melibiose. This proves that melibiozymase was built up in the cytoplasm of the melibiose-fermenting gametes before copulation, maintained in the diploid hybrid and in the sporulating cell, and transmitted in the cytoplasm to each of the four spores irrespective of whether or not that particular spore carried the gene, and finally transmitted to the haplophase gametes derived from the spore even in the absence of the gene. Melibiozymase was stabilized in the geneless clones containing a large amount of *S. cerevisiae* genes and cytoplasm as long as melibiose
was present, but disappeared when the melibiose was withdrawn. The \textit{melibiozymase} was maintained in the cytoplasm solely by an interaction between \textit{melibiozymase} and \textit{melibiose}. Therefore, \textit{melibiozymase} is a self-perpetuating cytoplasmic entity which is gene-initiated, but whose quantitative level below a certain maximum depends on an interaction between \textit{melibiose} and the enzyme and is independent of the gene so far as maintenance under these conditions is concerned.

Genes initiate the production of the adaptive enzymes, but adaptation occurs only by interaction of the cytoplasm of the cells with the specific substrate; once the adaptive enzyme has been formed it is self-perpetuating in the presence of the substrate.

I propose to call adaptive enzymes of this type \textit{cytogenes}. The fact that a period of exposure to \textit{melibiose} must occur before \textit{melibiozymase} is produced suggests that the original product is a relatively non-specific substance which is transformed into \textit{melibiozymase} when it is “imprinted” by the \textit{melibiose}. The original relatively non-specific substance I propose to call the \textit{protocytogene}.

If the \textit{melibiose-plus} gene transmits a \textit{protocytogene} to the cytoplasm which becomes a specific \textit{cytogene} by being “imprinted” by the \textit{melibiose} molecule, it is possible that the same locus may be responsible for the production of other \textit{cytogenes} as well. The original gene-product which becomes specific by contact with the \textit{melibiose} molecule presumably become differently specific by contact with some other molecule. Genes are “enzyme factories,” but each gene may not necessarily be restricted to the production of a single enzyme.

Sonneborn’s (67) “killer” phenomenon can also be explained in terms of the following hypothesis based on our knowledge of \textit{cytogenes}: The \textit{kappa} substance at present is a plasmagene which is stabilized by the \textit{K} gene, but it originally was a \textit{cytogene} produced by the (thus far undiscovered and hypothetical) \textit{KAPPA} gene. It has become established as a plasmagene by the transmission of the \textit{kappa} substance from the cytoplasm of a heterozygous \textit{KAPPA/kappa} individual to an individual carrying the \textit{K} gene. The \textit{K} gene stabilized the \textit{kappa} \textit{cytogene} in a \textit{kappa/kappa} individual and the \textit{cytogene} was transformed into a plasmagene.

XIII. REFERENCES

YEAST GENETICS

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Mendelian segregation of cytogenes. In some recent experiments I made a hybrid between a clone carrying the recessive alleles for both melibiose and galactose fermentation by another with the recessive alleles but also carrying cytogenes cytoplasmically transmitted from the dominant. The cytogenes were segregated in a one to one ratio at meiosis indicating that they could be carried on a recessive allele if it were contaminated with cytogenes. This experiment shows that a locus on a chromosome is simply a passive place of attachment for cytogenes. It confirms and extends Sonneborn's concept of the duality of the gene. The gene does not generate anything except itself. The dominant gene has a greater affinity for the cytogene than the recessive. Many cytogenes reside at one locus moving into the cytoplasm when substrate appears and returning to the locus when the substrate has been transformed.