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FURTHER OBSERVATIONS ON REVERSE MUTATIONS IN YEASTS

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CONTENTS

1. INTRODUCTION

The recent demonstration of "Reverse Mutations in Yeasts" (Subramaniam, Ranganathan & Krishna Murthy, 1948) offered a new angle of approach to the problem of variation in yeasts. In any programme

for the planned improvement of yeasts for industry, stability of the types is an important consideration. It appears to be not an uncommon experience in brewing and distilling industries that the yeasts employed fail suddenly to give satisfactory results. The problem, therefore, is not merely one of producing an improved strain, but what is more, controlling its stability. A knowledge of the variations that occur spontaneously and those that could be induced by diverse agencies becomes a prime necessity. Naturally, investigations have to be carried out on diploid strains of known chromosomal constitution and then extended to polyploid ones, since there is a strong possibility that strains used in industry may be polyploids.

Students of Bacterial Genetics (Luria, 1947) use the term " Mutation " to " mean a permanent change affecting one or more properties of a bacterial cell and of its offspring ". Luria (1947) emphasizes: " The use of this term does not imply *a priori* identification with the process of gene mutation or with any other type of hereditary change in higher organisms " (p. 3).

In yeasts, Winge (1944) groups under the term ' Mutation ', " sudden genetic changes due to alteration in the molecular structure of a single gene or alteration in the structure of an entire chromosome " (p. 79). As would be seen, he does not visualize the possibility of the existence of polyploidy in yeasts. A clear analysis of the various changes that occur in yeasts was rendered possible in this laboratory by the intensive series of investigations on a two-chromosome brewery yeast and its spontaneous and induced mutants. The mutations recorded by Winge are in strains whose chromosome constitutions are unknown and naturally he does not attempt at any classification of the changes observed. It has been possible to classify the variants as the result of (1) duplication of the entire chromosome complement (Subramaniam & Ranganathan, 1948; Mitra & Subramaniam, 1949; Subramaniam, 1945), (2) chromosomal translocations (Subramaniam & Ranganathan, 1946; Subramaniam, 1946 *b*; Prema Bai, 1947), and (3) gene mutations (Subramaniam & Krishna Murthy, 1948; Subramaniam, Ranganathan & Krishna Murthy, 1948).

The recent recovery of the diploid from the autotetraploid, BY 3 (Duraiswami & Subramaniam, 1950) necessitated a consideration of the problem of haploidy in yeasts (Subramaniam, 1950 *a*). A critical analysis of the criteria employed for the identification of ' haploids ' revealed that the differentiation of ' haploids' from ' diploids ' is based not on cytological evidence but on such variable characters like (1) shape, (2) size and (3) mode of budding of the cells. The polymorphic character of yeast cells and the

occurrence of polyploidy in yeasts render such identifications valueless. It would be realised that the 'haploid' obtained by the direct germination of the spore of an autotetraploid should be entirely different from that obtained similarly from a diploid.

Our control two-chromosome strain (Subramaniam, 1946 *a*) sporulates with ease and reasons were adduced in a previous contribution for the probable existence of multiple alleles. The giant colonies having different allelic constitutions were shown to exhibit an ascending grade of complexity in their sculpturing. When the *Smooth* or the *Rough* types were treated with acenaphthene, autotetraploids were obtained, the giant colonies of which were highly stable from season to season and year to year (Subramaniam & Krishna Murthy, 1949). Lindegren (1945) however, considers that while the 'diploids' are highly stable and produce *Smooth* colonies, the 'haploids' produce *Rough* ones which are sectored owing to the easy facility for expression of mutant characters in such 'haploids'. That the nature of the sculpturing of the giant colony by itself cannot give an indication whether the colony is 'haploid' or 'diploid' would be evident from the fact that Winge (1944) and Winge and Laustsen (1937) illustrate diploid colonies which are *Rough*. The above confusion indicates, if anything, the necessity for caution.

There are differing explanations in published literature for the variations observed in yeasts. Variations have been interpreted as (1) 'dissociation', (2) 'saltation', (3) 'dauermodifikationen' and (4) 'mutation'. In the previous contribution (Subramaniam, Ranganathan & Krishna Murthy, 1948) reasons were adduced why the changes observed have to be considered as 'gene mutations'. The other side of the picture has also to be considered. Proof has to be adduced that these changes are neither 'dissociation' nor 'dauermodifikationen'.

After elimination of other possibilities gene mutations were identified on the basis of "reverse or back mutations". As would be appreciated, the conclusions drawn from a preliminary survey can only be tentative. Since the predominance of the different variants observed depended upon the season of the year any chance of confirmation by conventional crossing tests became a dream for the future. Crossing tests necessitate the availability of the different mutants unchanged over a period of time. The mutants could be kept unchanged during the course of investigations either by culturing under selective environmental conditions or by inhibiting mutation itself. The fact that the different mutants show different rates of growth under the same environmental conditions (Mallya & Subra-

maniam, 1949) offers the hope that by controlling the temperature, it may be possible to culture selectively the different mutants.

The results of Dickinson (1945) on *Brucella bronchiseptica* indicate that the mutational step itself could be inhibited. How far this could be applied to yeasts remains yet to be investigated. Proof for mutant genes and multiple alleles by crossing tests have, therefore, to await the discovery of methods to keep a mutant unchanged. In the present state of our knowledge one has necessarily to be satisfied with indirect proof. Confirmation of the previous evidence became, under the circumstances, a vital consideration. The observations recorded in the previous paper (Subramaniam, Ranganathan & Krishna Murthy, 1948) raised some problems of fundamental importance.

Though in bacteria, reverse mutations are of common occurrence (Luria, 1947; Braun, 1947) there was no previous record of reverse mutations in yeasts. Winge (1944) observes that remarkable variations from the original may occur as a result of the accumulation of a series of minor mutations. He considers that one cannot be sure that the later isolation of a cell from a sector showing the same sculpturing as the original type, is identical with the initial type. This view is reminiscent of Skovsted's (1943) conclusion that not only do no reverse mutations occur in *Nadsonia*, but that by 'successive' mutations origin of new species themselves may be possible. This is contrary to the view expressed by Muller (1939) that the frequency of the change is from the mutant gene towards the normal, and not from a mutant to a more extreme mutant. Muller considers it probable that mutations in the positive direction from the normal to the hypermorphic type "do occur too, perhaps with a frequency similar to that of the reverse mutations involving origination of normal itself from a hypermorphic mutant, but they could seldom be observed" (p. 264).

In view of Muller's observations, Skovsted's conclusions are novel. Reverse mutations observed in higher organisms occur also in bacteria and hence yeasts cannot be an exception to the general rule. If reverse mutations are as common in yeasts also, the "successive" mutations observed by Skovsted (1943) should be capable of an entirely different explanation.

A confirmation and extension of the previous observations on mutations in yeasts has thus an unusual significance.

2. Historical Resume

The early investigators approached the problem of variation in yeasts mainly to evaluate the causes for degeneration in industrial yeasts. Hansen

and Jorgensen held diametrically opposite views regarding the stability of the various " sports " obtained. Hansen believed that " the races prepared from industrial yeasts cannot be maintained, but disappear " (Jorgensen, 1925, p. 274). He believed that " so long as the beer yeasts are kept under brewery conditions, they display only slight alterations, which are of a transitory nature ". Jorgensen (1925) on the other hand, firmly held the view that continuous improvement was possible by progressive selection from samples of industrial yeasts and put his ideas into practice.

These observations were made at a time when knowledge of heredity even in higher organisms was yet in its infancy and when the selective action of the environment was only dimly realized. The earlier observations, therefore, are capable of being interpreted in ways not conceived of by the original investigators.

i. *Evanescent Changes*

Variations are of different types. They may be evanescent or permanent, spontaneous or induced. It was realised early that the morphology of the yeast cell was highly variable and determined by environmental conditions. The shape of the cells in old cultures was entirely different from that in young ones and cultures grown at different temperatures may contain cells having different shapes. Cultures of *S. carlsbergensis* which were normal in appearance at 27° C. gave elongated mycelial types when grown at 7° C. Hansen could isolate from *S. carlsbergensis* cells of *S. cerevisiæ*, and *S. Pastorianus* types and preserve them in that condition for six months. Guilliermond (1920) concludes: " The cells in yeasts are not constant in shape and may, depending on the circumstances, take on variable forms temporary or permanent. In a word, they are polymorphic " (p. 179). In spite of this normal variability, it is considered that each species may possess a large number of cells having a predominant shape.

ii. *Long lasting Changes*

In the bottom yeast, *S. carlsbergensis*, Hansen found (Guilliermond, 1920; Jorgensen, 1925) oval and sausage-shaped cells when grown on gelatin media. Either of these cell-forms produced on growth in wort the other type and repeatedly purified cultures were composed of a mixture of the two types of cells. This observation of Hansen probably indicates a much more deep-seated change than the usual variability observed under normal conditions. Typical mycelial forms were isolated from cultures of *Sch. pombe* and *Sch. mellacei* by Guilliermond and Lepeschkin. These mycelial types retained their characteristic morphology over a long period of time.

Hansen (Guilliermond, 1920) produced a similar change, even though of a transitory nature, by growing *S. carlsbergensis* at 7° C. The above strain which produced normal cells at a temperature of 27° C. gave rise to colonies of elongated mycelial cells when kept at 7° C.

An entirely different type of change is the appearance of asporogenous varieties from sporogenous ones. It is stated that in pure cultures of *S. ludwigii*, a certain percentage of cells show a marked variation in their spore-forming ability. Hansen was able to isolate three distinct types of cells. One was asporogenous, while another was capable of vigorous spore formation. Between these two, was a strain which had almost lost its capacity for spore formation. Asporogenous types having a similar origin are recorded in a number of species like *S. bailii*, *P. hyalospora*, *P. farinosa*, *S. multisporus* and *Sch. octosporus* (Guilliermond, 1920).

There is an optimum temperature for sporulation and Hansen produced asporogenous variants by cultivating sporogenous ones at temperatures above the optimum. Such asporogenous varieties could be made to regain their spore forming ability by repeated transfers through wort. This was sudden when the asporogenous strain was passed through 10% dextrose solution.

Physiological changes have also been induced. Hansen cultivated *S. carlsbergensis* at 32° C. and used as inoculum for the next fermentation samples from flasks in which fermentation was completed. At the end of the 9th transfer he got a variety which gave about 2% less alcohol than at the beginning of the experiment. The top yeast, *S. cerevisiæ*, when cultured on yeast water-gelatin gave rise to types yielding 3% more of alcohol than the original strain (Jorgensen, 1925).

Temperature was observed to produce an entirely different effect also. Hansen (1906) could obtain from the bottom yeast, *S. turbidans*, top fermenting ones by keeping lightly inoculated flasks at 0·5° C. for three to five months. This result was not entirely due to the exposure to the low temperature, for, he observed under normal conditions, the appearance of a small number of top yeasts in typical bottom fermenting cultures. The change, however, was not reversible. While bottom yeasts gave rise to top ones, top yeasts never gave rise to bottom ones.

Even at that early stage it was observed that apart from the mode of fermentation, top or bottom, these had slightly differing physiological characters also. The rate of respiration, the ratio between the fermentative capacity and the respiratory rate and even the energy values for metabolism were found to be different (Trautwein & Wassermann, 1930).

The earlier workers only dimly realized that some of the changes observed by them might be classed as mutations. Most of Hansen's work on variations in yeasts was carried out before the implications of the "Mutation Theorie" published by de Vries in 1900 were understood. Jorgensen and Hansen considered it possible that the transformation of a bottom yeast into a top one may be a mutation. Periodically one comes across attempts to revive the old concepts. The tendency to interpret the various changes observed in yeasts under different environmental conditions as purely of an adaptive nature persists till the present day. This attitude is not surprising since similar ideas were prevalent in the allied field of Bacteriology which has remained until recent years the stronghold of Lamarckism.

Dissociation in Bacteria

(*a*) *What is Dissociation*?—From early times bacteriologists have been observing variation in bacterial cultures. One of the important aspects of the phenomenon of variation was described as "Dissociation" (de Kruif, 1921), which has received considerable attention. By dissociation is meant the appearance in pure cultures, of variants differing in several characters from the original type. These variants were relatively stable for several generations and could produce either new types or revert to the original condition. The use of the term "dissociation" appears thus to be an innocuous one, under which could be lumped different changes produced by entirely different mechanisms.

(*b*) *Conditions Producing Dissociation.*—When cultures grown on solid media are plated, the colonies obtained are almost uniform in appearance. But when the culture is grown in a liquid medium for some time and then plated, either, colonies differing in their morphological characteristics appear, or, the same colony may show variant sectors.

These variants have been classified into different categories depending upon their morphology. The types occurring in most species have been roughly divided into the following major ones:—(*a*) the *Smooth* or the *S*-type, (*b*) the *Rough* or the *R*-type, (*c*) the *Mucoid* or the *M*-type and (*d*) the *Gonidial* or the *G*-type.

The production of variants could be accelerated by the addition of various substances to the medium. The transformation of one type into another results in morphological as well as physiological changes. The type specific surface antigen present in the *Smooth* form is absent in the *Rough* type and this produces in turn a loss of virulence and an altered sensitivity to bacteriophage.

Explanations offered for Bacterial Dissociation

(*c*) *Mutation vs. Adaptation.*—Innumerable attempts have been made to explain the underlying causes for dissociation. The old idea that such variations are merely adaptive has persisted until recent years. Bacteria have been assumed to have a unique flexibility enabling them to alter their characteristics in response to the varying environmental conditions. This is not surprising since very little was known regarding the genetics of bacteria. Recent work has shown (Luria, 1947) that resistance of staphylococci to penicillin is acquired " by a series of successive mutations, each producing further resistance " (p. 14). A highly resistant strain does not appear all of a sudden and the increasingly resistant strains obtained when cultured in the presence of penicillin are quite likely to give the impression that the organism is adapting itself to the altered environmental conditions.

(*d*) *Dauermodifikationen.*—The explanation that environmentally induced modifications could persist for variable lengths of time after removal from the specific environment was propounded by Jollos as a result of his investigations on Ciliates and was extended to interpret bacterial variation. Instead of going into the work on bacteria justifying such an explanation, it would be desirable to understand the evidences on which Jollos based his concept. " By the term Dauermodifikationen, Jollos means temporarily inherited alterations producible by environmental means, the degree of effect or the duration of its persistence often being increased by repeated or long lasting environmental treatment " (Sonneborn, 1947, p. 308). The changes induced persist through innumerable divisions, but they all revert back to the original condition. This reversion may be by a series of steps or it may be sudden and changes in environmental conditions or fertilization have been known to accelerate the reversion.

Stocks of Paramecia were exposed to a temperature of 31° C. for three years and Jollos found that individuals in the culture showed an accelerated fission rate, a decreased resistance to As_2O_3 and an increase in the lethal temperature. These new characters were retained when cultured at room temperature, but conjugation or autogamy led to their disappearance. Even in those cases where the characters persisted after the first fertilization they were eventually lost after the second or the third. Some of the lines which lost the characters regained them after another fertilization eventually to lose them again.

In another series of experiments he cultured *P. aurelia* in media containing $CaCl_2$ or $Ca(NO_3)_2$. The animals so exposed for varying periods of time had a lower fission rate even in a calcium-free medium. This charac-

teristic was lost after successive autogamies and could be hastened by quick transfers as well as by stimulating conjugation.

Dauermodifications were also claimed by Jollos by addition of antisera to culture media. In dilutions ranging from 1 : 500 to 1 : 100 the antisera first immobilized and then killed the Paramecia. He obtained resistant strains by gradually increasing the concentration of the antiserum in the medium. After repeated exposures certain lines resistant to concentrations of antisera as high as 1 : 25 were isolated. This resistance, like the other changes induced by him, was only temporary, since the resistant strains became sensitive even after the first autogamy.

Sonneborn (1947) states that demonstration of "the physical basis and mechanisms involved in so-called Dauermodifikationen" has to await a clear knowledge of the macronuclear cytogenetics. "Until that occurs, however, and particularly in view of the recurrent spectre of selected spontaneous mutations, alternative interpretations of cases presumed to be Dauermodifikationen will generally be sought by geneticists" (p. 314).

When that is the opinion regarding the experiments on which the concept of Dauermodifikationen was originally postulated, the validity of the application of the concept to other groups could just be imagined.

(*e*) *Cyclogenic Concept.*—An entirely different explanation for the dissociation phenomenon was elaborated by Hadley. Even from 1870 it was known that filamentous bacteria could give rise to shorter bodies and that these can revert to the filamentous condition in a suitable environment. According to the cyclogenic concept the various morphological (*S*, *R*, and *M*) types are "states in an orderly life-cycle in which the expression of each stage is dependent on the environmental conditions" (Braun, 1947, p. 80). Hadley believed that "each culture phase represents a stage in the development of the bacterial individual, whose span of life extends from the gonidium (or similar reproductive entity) to the reproductively mature rough-phase culture. On these grounds, the bacterial individuum should not be conceived of as a single cell, but as the entire range of successive culture development from gonidium to the mature form" (Braun, 1947, p. 80).

(*f*) *Mutation and Selection.*—At the first touch of an organized genetical analysis of these variations, it became apparent that the evidences for adaptation, dauermodification and the cyclogenic concept could be interpreted in terms of mutation and selection. The earlier workers little suspected that the environment instead of producing adaptive changes could act selectively on spontaneously occurring mutations.

Evidence for the spontaneous, undirected nature of the mutational changes in bacteria have been offered by Luria and Delbruck (1943), Demerec (1945), Ryan (1946), Braun (1947) and others. It has been shown that the resistant mutants observed to occur in media containing antibiotics also occur in normal media. The change to the resistant condition is, therefore, neither the result of adaptation nor a directed mutation since such mutants could be observed only in media containing the antibiotics. In such cases the selective action of the environment is only too obvious.

Dissociation in Yeasts

In the literature on yeasts, one comes across inevitable though faint echoes of the various interpretations for bacterial variation. An accidental observation seems to have suggested to Fabian and McCullough (1934) that variation in yeasts may be capable of an explanation in terms of the phenomena of dissociation in bacteria. They observed forms resembling diplococci while trying to revive a dried up agar slant culture of *Saccharomyces cerevisiæ* Saaz. When repeatedly cultured in wort these gave rise to normal cells characteristic of the particular strain. In order to determine whether the above transformation could be reproduced, they carried out a series of experiments on five different strains of yeasts. Changes were induced in the normal *Smooth* forms by desiccation or exposure to abnormal temperatures as well as ageing or serial transfers in media containing lithium chloride, brilliant green or high concentrations of alcohol. Upon such treatment the *Smooth* forms gave rise to the *Rough* and then to the *Gonidial* types. According to them the *Smooth* forms possessed the morphological and physiological characteristics described for the species. The *Rough* form, though sporogenic like the *Smooth* type, differed from the latter in the possession of elongated cells and produced dull, rugose, wrinkled colonies. These had identical physiological characteristics as the *Smooth* type, but formed a scum whenever growth was possible. The *gonidial* type, on the other hand, had entirely different characteristics. It was asporogenic and produced not alcohol but an acid after fermentation. The cells were small in size and after adaptation produced a thin, dull, spreading colony.

The transformation of the *S* and *R* forms to the *G* type was either gradual or sudden. When it was sudden, a new type of "transitional", highly refractile cells which could not be cultured, appeared. These gave rise to the *Gonidial* cells by multiple budding. Just as *R* and *G* forms could be obtained after suitable treatment of the *Smooth* type, the process could be reversed, and the original form could be recovered by repeated and quick transfers through wort. On the basis of the above results they suggest that

such transformation into the *Gonidial* type might be responsible for the production of defective beer or unsatisfactory yields of alcohol under industrial conditions.

Wickerham and Fabian (1936) extended these observations to *Saccharomyces aceris-sacchari* and *Pichia alcoholophila* and conclude that " dissociation in yeasts is a gradual process and that none of the various stages arise spontaneously. There is a gradual transition from one type of the cell or colony to another " (p. 171).

Though Fabian and McCullough (1934) explained the variations in yeasts as " dissociation " they never veered to the cyclogenic concept. Punkari and Henrici (1933, 1935) investigated the spontaneous changes occurring in the chromogenous, asporogenous, *Torula pulcherrima*. Variants appeared as sectors or secondary colonies which differed in texture or colour and sometimes in shape; the variations observed were independent and not linked. They emphasize: " We cannot find in these observations anything to support a theory of sex or life-cycles as responsible for the observed variations " (p. 136). Mutations as well as differentiation conditioned by metabolic gradients are considered to be the probable causes and they believed a correct sorting out of the underlying sources for the variations a difficult task.

Bauch (1941, 1942) obtained stable strains of yeasts with gradually increasing cell sizes by treatment with polyploidizing and carcinogenic substances as well as with synthetic phytohormones. He suggested that these might be different grades of polyploids. Without offering any proof for the existence of endopolyploidy in yeasts, Levan and Sandwall (1943) suggested that since Bauch " produced races with larger cell volume than the original strain, it does not seem to be improbable that a doubling of the chromosomes has occurred, rather by the endomitotic mechanism than by C-mitosis " (p. 176). Later, in the same year, Levan and Ostergren (1943) offer a different interpretation. They suggest that some of the giant forms may be dauermodifications. These speculations have only a historical interest. A criticism of Levan and Sandwall's contribution has already been given elsewhere (Subramaniam & Krishna Murthy, 1949).

Mutations in Yeasts

Winge (1944) interprets the spontaneous variations observed by him in giant colonies as mutations. These could easily be distinguished from variations caused by genetic segregations following sporulation. Examination of cultures left standing for months or years showed varying numbers of sectors when grown as giant colonies and owing to the frequency with

which mutations occur, he doubted whether it would be possible to keep a strain unchanged for a long period of time. The various mutations may bring about gradual changes and he sounds a pessimistic note as to whether isolation of a sector having an identical appearance as the starting type, after the lapse of a few years could really be considered as strictly comparable with the starting type.

This possibility of continuous change in a culture appears to support the conclusions reached by Skovsted (1943) from his experiments on *Nadsonia*. The reversal of the changes observed to be very common by Punkari and Henrici (1933, 1935) are said not to occur in *Nadsonia*. Each mutant was found to produce a limited number of variants and each of these produced in turn a further set of mutants. The above phenomenon, which he considers could be designated as " Successive Mutations ", is supposed to have considerable evolutionary significance. A new species could originate by a simple step mutation, " if only one had the patience of waiting long enough ". He claims that some of the new mutants of *Nadsonia* are capable of being described as new species.

This rather surprising conclusion raises such fundamental problems as what constitutes a species and whether such quick evolution of species could be observed in the laboratory. The matter is dealt with fully in the discussion.

3. Material and Methods

Being a continuation of the observations recorded in the previous paper (Subramaniam, Ranganathan & Krishna Murthy, 1948), the methods for making the media as well as their composition were strictly adhered to. Observations were limited to the three strains of the two chromosome control isolated originally on different dates, in order to study whether the changes are parallel in all the strains and whether they synchronize in their time of occurrence.

The only change in approach was to allow the cultures to grow initially on agar slants for 20–25 days. If mutations occur in the streak cultures there would be competition with the progressive decrease in nutriment available in the slant. If that is so, the nature of the population in young and old agar slants ought to be different.

A loop of material from a 20–25 day old culture was first inoculated into a fresh agar slant and after a two-day growth, a loop from the latter was inoculated into a wort tube. This procedure was adopted to give the mutants unlimited opportunity for proliferation and to get vigorously growing cells. Giant colony inoculations were carried out with material from

such 16–18 hour wort cultures. The colonies were grown singly in petri dishes at room temperature inside a sterile chamber.

Description of Strains and Types of Sculpturing

For the sake of clarity the control purified on 12–10–'45, 19–12–'45 and 16–11–'45 are referred to as Strain I, Strain II and Strain III respectively.

The classification of the different types of sculpturing given in the earlier contribution is presented below for ready reference as well as to avoid confusion.

Smooth I has a wavy or serrated margin with radial folds and with or without faint concentric striations.

Smooth II is similar to the above but has pronounced concentric striations especially in the outer half of the colony.

Smooth III has innumerable prominent radial and concentric striations in the outer half of the colony giving it a lace-like texture.

Rough I is similar to the *Smooth III* with the sculpturing limited to the outer half of the colony, but differs from it in having a rough texture owing to the presence of minute powdery granulations on the surface.

Rough II resembles *Rough I* but with the difference that the entire surface of the colony has rough sculpturing.

4. Observations

In the previous contribution, recovery of the starting *Smooth* type and a mutation back to the *Rough* condition were demonstrated in Photos 15 and 16. That was in December 1947. The investigations were continued and the three different strains of the control culture had the appearance illustrated in Photos 1, 2, and 3, in March 1948. The sculpturing is not identical. In Photo 1, the colony which appears to be of the *Rough I* type shows sectors presumably of the *Rough II* category. In Photo 2, the colony is a mixture and shows in the centre, granules superimposed on a lace-like texture, reminding one of the *Rough II* condition. In the periphery, however, the sculpturing resembles those of the varieties *Smooth I* and *II*. The lace-like condition (*Smooth III*) with a rough centre and sectors is illustrated in Photo 3. This mixed nature of the sculpturing renders it difficult to ascertain the time when the mutations in the different directions have taken place. That the period under question may be conducive to the establishment of particular types of mutants is illustrated by the shift from the *Rough II* to the *Smooth I* and *II* conditions in the above photographs. Two other colonies made during the same month are shown in Photos 4 and 5. Except

a small *Smooth* sector, the colony shown in Photo 4 could be classified as belonging to *Rough I.* The *Rough II* cells present in Photo 1 of the same strain have either mutated or been eliminated.

In Strain *III*, the change in sculpturing has proceeded a step further (Photo 5). The presence of granulations in the centre indicates that the *Rough* cells are persisting. This is confirmed by the two narrow *Rough I* sectors. The fact that it is a mixture of types suggests two modes of origin of the sectors. There is a typical *Smooth II* sector starting from the centre, indicating that cells of that type were present in the culture at the time of inoculation. Mutations are occurring in the giant colony itself as could be seen from the small *Smooth I* sectors at the periphery. The general type of sculpturing is reminiscent of the *Smooth III* without the characteristic delicacy. The sculpturing resembles that illustrated in Photo 15, Pl. III, of the earlier contribution, and both are sectored. The lack of delicacy in sculpturing seems in all probability to be the result of the mixed nature of the population. The period under consideration (March, 1948) is one during which mutations are occurring and the population is shifting towards the *Smooth II* condition.

Photographs 6, 7 and 8 illustrate colonies grown during the months of May and June. All of them are mixtures of *Rough I* and *II* cells. The general impression that one obtains is that the *Rough I* cells are predominating. In the month of July the *Smooth* types appear once again. Photo 9 shows a *Smooth II* colony with a *Rough* sector. Though the general type of sculpturing is that of *Smooth II*, the periphery resembles the *Smooth III.* Typical *Smooth II* and *Smooth III* colonies obtained in two of the strains at the identical period are illustrated in Photos 10 and 11 respectively. These photographs indicate that during the month of July there is a fluctuation in the predominance of the various mutants in the different strains. In August, the sculpturing of the Strains I and II (Photos 12 and 13) was identical as that in July (Photos 9 and 10). The colony of Strain III showed a change (Photo 14). The pure *Smooth III* condition of July (Photo 11) had disappeared. It (Photo 14) was predominantly of the *Smooth II* type with a few *Smooth III* sectors. The mutation to the *Smooth III* condition appears to have occurred during the final stages of growth of the colony. The tendency for the predominance of the *Smooth II* cells appears slightly later in Strain I. A colony of the above strain grown in September belonging to the pure *Smooth II* type is illustrated in Photo 15.

A new allele appeared in the other two strains in September (Photos 16 and 17). Photo 17 reminds one of the colony illustrated as Photo 13 in

Pl. III of the earlier contribution (Subramaniam, Ranganathan & Krishna Murthy, 1948) as well as Fig. 1 *f* of Subramaniam & Ranganathan (1949). As would be shown in later photographs, this allele appears to occur in the heterozygous condition. The probable change resulting in the production of such a colony may be as follows:—

Lace ——→ Mutation ——→ Smooth

SMOOTH III

Lace ——→ Mutation ——→ Rim

This allele appears to occur in a different combination in Photo 16. In August, this strain gave an almost *Smooth II* type of colony (Photo 13). The following change may have resulted in the production of the sculpturing shown in Photo 16.

Lace ——————————→ Lace

SMOOTH II

Smooth ——→ Mutation ——→ Rim

The presence of *Smooth I* sectors in the same colony which do not extend to the centre suggests that the *Lace* and *Rim* alleles have mutated to the *Smooth* condition during the growth of the colony.

The timing of the predominance of particular mutants which was almost synchronous during the preceding months, disappeared at the end of September. Strains I and II developed into *Rough I* colonies (Photos 18 and 19) while Strain III gave a pure *Smooth* colony (Photo 20). In October the Strain I gave *Rough II* (Photo 21) and *Rough I* colonies (Photo 22) while the Strain II was of the *Smooth II* type (Photo 23) with a *Smooth III* sector.

Two stages of development of the *Rough* colony with a *Rim* are illustrated in Photos 24 and 25. The growing giant colony of Strain I was photographed after 11 and 20 days respectively. The only change observable after 11 days (Photo 24) is an increase in the width of the *Rim* (Photo 25). The small *Smooth I* and *II* sectors in the colony remain almost unchanged.

A colony inoculated three days later than those illustrated in Photos 24 and 25 showed a predominantly *Smooth I* condition with *Smooth II*, *Smooth III* and *Rough I* sectors (Photo 26). The period in question, therefore, seems to be one conducive to the establishment of different types of mutants. It has been emphasized in a recent contribution (Subramaniam, 1950) that the *Rough* colonies pass through the various less complicated types of sculpturing during their development. The final *Rough II* type of expression is merely the culmination of a process of development in which

Smooth I, II, III and *Rough I* appear as orderly, but abbreviated stages. This suggestion that the alleles determine not only the nature but also the time of expression finds support in Photos 27 and 28. The colony in Photo 27 is comparable to that in Photo 16, but it is only an intermediate stage and not the final expression. In cells homozygous for the *Lace* allele, the development would be stopped the moment the lace-like sculpturing appears. In the *Rough II*, however, it would proceed further and a *Rough* texture would be superimposed on the *Lace* one. This is what happens when the *Rim* allele occurs in combination with the *Rough* gene. While Photo 16 gives the final expression, Photo 27 is only an intermediate stage. After eight days of further growth, the same colony had become completely *Rough*, except for the sectors and is comparable to that illustrated in Photos 24 and 25.

These changes in the population conditioned by temperature fluctuations become much more pronounced in Photos 29 and 30. In Photo 29 a *Smooth I* colony shows *Smooth II* and *Rough I* sectors. But the same culture after a few days gave on inoculation a *Rough I* colony (Photo 30), which was slightly different in appearance from the *Rough I* illustrated as Photo 3 in Pl. I of the earlier paper. The possibility was indicated that even *Rough I* colonies can have two different genic constitutions. *Rough I A* was conceived to have a probable genic constitution *Rough/Lace*, while *Rough I* was supposed to be *Rough/Smooth* (Subramaniam, Ranganathan & Krishna Murthy, 1948, p. 50). Photo 30 apparently indicates the *Rough I A* condition. This type of sculpturing has been recorded previously (Photo 1, Prema Bai & Subramaniam, 1947).

Mutations in different directions reminiscent of Photo 4, Pl. I of the earlier paper is seen in the colony illustrated in Photo 31. The sectors do not extend to the centre and hence the mutations should have occurred during the growth of the colony. The various types of sculpturing excepting the *Rough II*, which occurs as a streak, may be observed in the photographs. As happened in the year 1947, there was a tendency for the *Smooth I* cells to completely eliminate the others during this period. That this tendency is not confined to any particular strain is illustrated by Photos 32, 33 and 34. The almost completely *Smooth I* condition is illustrated in Photo 34.

5. Discussion

i. *Need for a Clarification*

The recovery of the identical types of mutants in succeeding years, while disproving the conclusions of Winge (1944) and Skovsted (1943) questioning such a possibility, emphasizes at the same time that the phenomena of variations in yeasts are in no way different from those observed in higher

organisms. It is being slowly established that there is no reason to consider the cytological behaviour of yeasts as unique (Subramaniam, 1946 *a*, 1948 *b*). Evidences presented in this paper and the theoretical considerations advanced elsewhere (Subramaniam, 1950 *a*) suggest that they are not unique in their genetical behaviour also. The experiments carried out were under standardized conditions, the only variable factor being the temperature. It is known that the environment plays a dominant role in the establishment of the different types of mutants. Some unpublished observations in this laboratory on the control of the temperature indicate that a specific mutant could be obtained when cultured at 30° C.

There are very delicate populational problems arising in studies on variations in yeasts. It is not merely a question of the rate of mutation, but includes also the ability of the mutant to establish itself in competition with the original type. The different alleles postulated from the recorded observations may arise at identical rates. But they find expression only under particular environmental conditions. This observation is nothing new since the viability and the growth rate of the mutants may vary under identical conditions. Braun (1947) has shown that the apparent cycles in bacteria depend on the above factors. He remarks: "Thus, merely by spontaneous appearance of mutants differing in growth rates and viability an apparently successive change from $S \rightarrow R \rightarrow M$ can be produced under constant environmental conditions" (p. 95). He suggests that the term "Dissociation" is misleading and superfluous.

The cyclical change and the variations in the population recorded in this as well as the previous contribution indicate that yeast may form ideal material for a study of population genetics (Dobzhansky, 1947). But before that could be attempted, some of the older invalidated concepts which occasionally find support, have to be finally laid to rest.

ii. *Adaptation and Dauermodification*

The concept of adaptation as an explanation for bacterial dissociation was criticised by Mayer (Braun, 1947). He pointed out that diverse agencies could produce identical types and what is more, under identical environmental conditions widely different types as well as reversion to the original condition, could occur.

In the earlier contribution it was shown that the establishment of a mutant may be conditioned by its growth rate and viability. This suggestion was made on the basis of the shape of the sectors in giant colonies. Later and more exact investigations using a photoelectric turbidometer

indicated (Mallya & Subramaniam, 1949) that the mutants have differe growth rates. On the basis of this differential growth rate it was suggested that the establishment of particular types at the laboratory temperature is governed by their growth rates and that the same mutant may have different growth rates under different temperature conditions.

Adaptation as an explanation for the cyclical changes observed cannot, therefore, be applied to the observations recorded in this paper. A further confirmation was obtained by exposure of the *Rough* type of cells to ultra-violet irradiation (Subramaniam & Krishna Murthy, 1948). The *Smooth I* type was obtained from a purely *Rough I* culture.

The dauermodification concept presented by Jollos was based on his work on Ciliates. The genetics of Ciliates offers even to-day some puzzles. Any generalization and extension of concepts based on observations on these organisms can follow only after a satisfactory solution of the above puzzles. Recently a possible cytogenetic interpretation for the so-called " Dauer-modifikationen " in Ciliates has been offered on the basis of the endopoly-ploidy of the macronucleus (Subramaniam, 1950 *b*). It is the macronucleus that controls the physiological activities of the organism and it has been surmised to be compound by Sonneborn (1947), while Subramaniam (1947) considers it to be endopolyploid. It matters little which of these explana-tions is accepted. But the fundamental point which emerges is that the genes in Ciliates become functional only when duplicated above a basic number (Mitra & Subramaniam, 1949). The macronucleus takes its origin from the micronucleus after autogamy or fertilisation. While for any character, there are only a pair of loci in the micronucleus, this is not the case in the macronucleus. In contrast to the macronucleus, where for a mutation to find expression, it has to occur at the identical locus in all the chromosomes, in the case of the micronucleus, a gene mutation would find expression the moment a new macronucleus originates from it. A muta-tion in the macronucleus would, therefore, manifest itself only gradually (see Sonneborn, 1947, p. 301), while in the micronucleus it would find imme-diate expression as soon as a macronucleus originates from it. This simple interpretation offers an explanation for the so-called dauermodification in Ciliates, according to the traditional mutation concept. The intriguing observations on the loss of dauermodification immediately after autogamy or fertilization could be explained in a very simple manner. A reverse mutation in the micronucleus would necessarily find expression only after the formation of a new macronucleus.

On the above basis, it was emphasized by Subramaniam (1949 *b*) that it was neither desirable nor necessary to extend the dauermodification concept

to certain variations in other groups. The halting speculation that some of the changes in yeasts may be dauermodifications by Levan and Ostergren (1943) deserves no serious consideration.

iii. *Dissociation*

The phenomena of " dissociation " observed by Fabian and McCullough (1934) appear to belong to two distinct categories. They classified the types as *Smooth*, *Rough* and *Gonidial*. While the first two were sporogenic, the *Gonidial* type was asporogenous. Recent investigations on the effect of high concentrations of alcohol on our control, BY 1 and the autotetraploid, BY 3 (Duraiswami & Subramaniam, 1950) indicated that while the transformation of the *S* into the *R* type may be a gene mutation, the *Gonidial* types observed belong to an entirely different category. Investigations in this laboratory indicate that the *Gonidial* types are ' haploids '. The reversion of the *R* to the *S* form and the *G* to the *R* or *S* form have necessarily to be by entirely different mechanisms. The *S* to *R* transformation is merely the result of a gene mutation, while the reversion of the *G* into *R* or *S* form is caused by a chromosomal doubling. It may be remembered that Fabian and McCullough record transformation of the *Smooth* into the *Rough* type and the subsequent transformation of the *Rough* into the *Gonidial* type under appropriate treatments. The special media employed for such transformation appear to have two entirely different effects on the cells exposed. Initially they offer a selective environment for the establishment of the *R* type. This appears to be only transitory since the *Gonidial* types predominate in the later stages of treatment. Therefore, the primary gene mutation may be to the *Rough* type and it is from these *Rough* types that ' haploids ' arise. It is reasonable, therefore, to expect that when a chromosomal doubling occurs in such a haploid it would give rise to the *Rough* type and it is only a back gene mutation that would give origin to the original *Smooth* type again. Since mutation do occur in the haploid stage a reversal to the *Smooth* condition through the *Rough* phase cannot be universal.

The experiments of Fabian and McCullough (1934) were carried out on strains whose chromosome constitutions were unknown. The investigations in this laboratory, on the other hand, were carried out on strains whose chromosome constitutions were known. Culturing in media containing high concentrations of alcohol (Duraiswami & Subramaniam, 1950) produced a reversion to the diploid condition in the case of the autotetraploid and to the haploid one, in the case of the control two-chromosome diploid. While the diploid recovered from the autotetraploid was stable and showed changes in the nature of sculpturing of the giant colonies comparable to that recorded in the present paper, the haploid was unstable. It reverted

to the diploid condition and its giant colonies showed identical variations in sculpturing, indicating changes in populations of the gene mutants, as in the diploid control and the diploid recovered from the autotetraploid.

The intensive work on the cytology and genetics of the control and the autotetraploid for the past few years enabled a rational correlation of some cryptic scattered observations with those of Satava (Winge & Laustsen, 1937) recorded several years previously. The "reduced forms" described by Satava are comparable to the *Gonidial* forms of Fabian and McCullough. A detailed discussion of this similarity may be found in the paper by Duraiswami and Subramaniam (1950).

iv. *The Problem of Haploidy*

The above very interesting observations raise the problem of Haploidy in Yeasts. If the chromosome constitutions of the diploid and the tetraploid yeasts employed for investigations in this laboratory were unknown, it would have been impossible to classify the "reduced forms" recorded by several investigators. As would have become apparent, the diploid recovered from the tetraploid and the unstable haploid, exhibit entirely different giant colony characteristics. Previous investigators had not taken into consideration the possibility of polyploidy in yeasts. Demonstration of induction of tetraploidy in this laboratory (Subramaniam, 1945, 1947 *a*) necessitated a realignment of the older criteria employed for distinguishing haploid yeasts from diploid ones. In a recent publication (Subramaniam, 1950 *a*) the purely morphological criteria employed by Winge and Lindegren were critically evaluated and the disagreement between the workers regarding the characters to be employed for such differentiation was pointed out. It is rather surprising that haploids are identified on such highly variable characters as size, shape and mode of division. It has been emphasized that for any advance in our knowledge, identifications should be based on cytological and not on morphological data.

The above conclusion was inevitable in view of the fact that auto and allopolyploids could occur in yeasts and hence there are chances of confusing real haploids with polyhaploids if, as at present, identifications are wholly based on morphological grounds. The unsatisfactory nature of the criteria on which haploids are differentiated from diploids, renders the classification of yeasts into 'haplontic' and 'diplontic' questionable. The so-called haplontic yeasts are identified on a series of assumptions unsupported by any experimental evidence. "We have to accept without any valid evidence that: (1) the vegetative cells are haploid, (2) that under particular conditions they have a tendency to fuse, (3) that there is an immediate reduction division, (4) that the spores have only the haploid chromosome complement

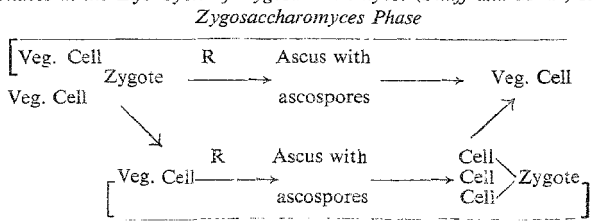
and (5) that these germinate directly" (Subramaniam, 1950 *c*). A simple alternative interpretation is offered by Subramaniam. All the so-called haplontic yeasts differ from the diplontic ones only in the timing of their meiosis. Unlike what occurs in *Saccharomyces*, if we consider that a reduction division occurs in the vegetative condition, then, the gametes formed should show a tendency for fusion. It is after such a fusion of vegetative cells that the spores are formed and the spores, therefore, should have the diploid, instead of the haploid, complement of chromosomes and hence should be capable of direct germination.

v. *Is Nadsonia a Haploid?*

The assumption that in haplontic yeasts meiosis occurs after the fusion of vegetative cells and prior to spore formation leads to some absurdities. Guilliermond (1920) describes parthenogenesis in *Schizosaccharomyces pombe* and *Schizosaccharomyces mellacei*. In the above species two cells come together, but each of them form spores without fusion. If the original fusing cells are haploid and if a reduction division precedes spore formation, then, the spores should have sub-haploid number of chromosomes! Or, we have to assume that meiosis does not take place at all.

Similarly, it is stated that *Zygosaccharomyces* species could be made to show the life-cycles characteristic of diplontic *Saccharomyces* species and *vice versa*, by altering the environment. Lodder (Phaff and Mrak, 1948) interprets such changes on the basis of haploidy and diploidy. It is considered that *Saccharomyces macedoniensis* is the diploid phase of *Zygosaccharomyces marxianus*. This conclusion is supported by Phaff and Mrak (1948). They assume that during the *Zygosaccharomyces* phase, the haploid vegetative cells fuse, the fusion being followed immediately by a reduction division prior to sporulation. According to Phaff and Mrak (1948, p. 266) two vegetative haploid cells fuse to give rise to a zygote. A meiosis occurring in the zygote results in the formation of haploid ascospores.

Phases in the Life Cycle of Zygosaccharomyces (Phaff and Mrak, 1948)



From the chart given above, it would appear that the reduction division which immediately follows zygote formation is postponed to a later stage in Saccharomyces Phase. This change, in order to become intelligible, necessitates the assumptions that (1) a chromosomal duplication is possible and (2) that gene mutation altering the time of reduction division should have taken place. Thus, the transformation of a haplontic into a diplontic type should be the result of chromosomal as well as gene mutations. A reversion from the *Saccharomyces* to the *Zygosaccharomyces* phase presents insuperable difficulties. A reversion of the gene mutation may be possible, but a reversion from diploidy to haploidy is not such an easy matter. It may be virtually impossible. Phaff and Mrak (1948) quote Saito's observations that the substrate determines whether the asci are formed before or after conjugation. If the transformation could be so easily achieved, it must merely be a gene mutation determining the time of meiosis. On the above argument, Subramaniam (1950 *c*) suggested that both the *Saccharomyces* and *Zygosaccharomyces* should really be diploids differing only in the timing of their meiosis. This character may after all be governed by a single locus.

This argument could naturally be extended to the production of iso- or anisogametes. If all yeasts are diploid or polyploid in the vegetative phase and if the difference between them consists merely in the timing of meiosis, it follows that the products may be iso- or anisogamous. This interpretation takes us from *Zygo-* and *Schizosaccharomyces* to *Debaryomyces* and *Nadsonia*, where the gametes are unequal in size.

The above considerations make it questionable whether *Nadsonia* is really a haploid. It has to be distinctly remembered that it is purely on the observed fact that cells of different sizes fuse in order to give rise to the zygote, and not on cytological grounds, that *Nadsonia* is assumed to be a haploid. The validity of the claim that a reduction division ought to take place before spore formation, unsupported as it is by cytological evidence, could therefore be just imagined.

vi. *Haploidy and the Species Concept*

The new interpretation regarding the behaviour of the so-called haplontic and diplontic yeasts cuts at the very root of even the broad classification of yeasts into major groups. This is further complicated by Skovsted's (1943) claim that new species may arise by " step mutations ", if one has the patience to study a strain over a period of time. If we assume that speciation is such a rapid process, the strain identified as *Nadsonia richteri* Kostka

by Skovsted cannot be identical with that isolated by Kostka. Innumerabl mutations should have occurred during subsequent culturing after the isola tion, and these should naturally have been followed by the phenomena o population pressure and selection. Skovsted claims that under particula conditions each type produces a number of mutations and that each of thes mutants produces in turn a fresh number of mutations. This statement if taken at its face value, and judged in conjunction with his observatior that no back mutations occur will, therefore, lead to the belief that the *Nadsonia* investigated by him can have no relation to the one originally described by Kostka.

Our recovery of all the types of mutants described in the previous paper (Subramaniam, Ranganathan & Krishna Murthy, 1948) suggests that Skovsted's conclusions are untenable. The probability that *Nadsonia* may be a diploid, throws doubt on Skovsted's conclusions based on the assumption that it is a haploid. The moment one admits that *Nadsonia* is a diploid, there is immediate necessity for interpretation based on two mutant loci and multiple alleles.

It would be interesting to remember here the considered opinion of Goldschmidt (1948) that " even in the extensively investigated organism like Drosophila, never has even the first step in the direction of a new species been accomplished, not to mention higher categories " (p. 470).

Yeasts cannot, therefore, be considered unique and once the cobweb of unsubstantiated assumptions is removed, they would be found to conform in their behaviour to higher organisms.

vii. *What is a Pure Culture?*

The observations recorded in this paper indicate that the predominance of the different types depends on (1) the rate of mutation, (2) the viability and the growth rate of the mutants and (3) the time of examination. The difference between the observations recorded in the previous paper and the present one could mainly be interpreted on the basis of the age of the agar slants from which samples were taken for giant colony inoculations. The illustrations accompanying this paper would indicate that when material from agar slants 20 to 25 days old are used for investigations, the colonies have a tendency to be of the mixed type with one of the mutants predominating. Many investigators characterise a pure culture as one arising from a single cell or a single spore. The rapidity with which yeasts proliferate necessarily results in the quick appearance of the mutants even though the frequency of mutation in yeasts may be identical with that in higher organisms. The selective action of the environment and the inherent potentialities

of the mutants themselves determine whether a particular type is capable of either predominating or completely eliminating the others in competition. When that is the case, any emphasis that a culture is pure just because it originated from a single cell or spore is unjustified as well as unwarranted. Any claim for the purity of a culture based on cell or spore isolation would remain meaningless until methods are perfected which would enable either the prevention of mutations in cells or, alternatively, would facilitate the culture of specific types by offering a selective environment.

Should knowledge of the cytology and genetics of yeasts expand on conventional orthodox lines, yeasts may form unique material for a study of the genetics of population.

6. Summary and Conclusions

1. Reverse Mutations and changes in the population of mutants observed in the two-chromosome control strain of a brewery yeast are confirmed. A survey of the literature on variations in yeasts is given and it is indicated that since many of the observations were made at a time when knowledge of heredity even in higher organisms was yet in its infancy, these are capable of being interpreted in ways not conceived of by the original investigators.

2. The tendency to interpret the various changes observed in yeasts under different environmental conditions as purely adaptive in nature necessitated a consideration of the phenomena described as dissociation and dauermodification. The various types of mutations observed in yeasts are reviewed.

3. A slight change was made in the experimental approach from that followed last year. Cultures were initially grown on agar slants for 20–25 days and then used for investigation. This was in the belief that if mutations occur in the streak cultures there would be competition, with the progressive decrease in nutriment available in the slant. Descriptions are given of the strains of the control used and the types of sculpturing observed.

4. The colonies during the different months tended to be mixtures of the various types of mutants with one or the other predominating. The changes in the three strains do not synchronise exactly, but are parallel. A new allele "*Rim*" has been shown to occur in different combinations. Evidences for such an assumption is given on the basis of the time of expression of the alleles. Mutations in different directions during the growth of a giant colony reminiscent of the observations made last year are illustrated. There

is a tendency for the *Smooth I* type to completely eliminate the other types during the cold season.

5. The establishment of the different types of mutants appear to be governed by the temperature conditions; and the same mutant may have different growth rates during the various seasons. The changes in the populations are not, therefore adaptive. Since alternative genetic interpretations on orthodox lines are available for the so-called dauermodification and dissociation, the extension of such concepts to explain variations in yeasts is neither necessary nor desirable.

6. The demonstration of polyploidy in yeasts necessitates a revision of our ideas not only as regards the occurrence of haploidy but also on classification into haplontic and diplontic yeasts. A detailed discussion of such problems is presented and it appears doubtful whether *Nadsonia* is, after all, a haploid.

7. The recovery of all types of mutants observed previously indicates that reverse mutations do occur and that yeasts conform in their behaviour to higher organisms. Any claim for the purity of a culture based on cell or spore isolation would remain meaningless until methods are perfected which would enable either the prevention of mutations in cells or, alternatively would facilitate the culture of specific types, by offering a selective environment.

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9. Description of Photographs

Photo 1. BY 1 (12-10-1945), 2·6 cms., 10 days' growth, photographed on 15-3-1948.
Photo 2 BY 1 (19-12-1945), 2·6 cms., 10 days' growth, photographed on 15-3-1948.
Photo 3 BY 1 (16-11-1945), 2·9 cms., 10 days' growth, photographed on 15-3-1948
Photo 4. BY 1 (12-10-1945), 2·5 cms., 10 days' growth, photographed on 27-3-1948.
Photo 5. BY 1 (16-11-1945), 2·7 cms., 10 days' growth, photographed on 27-3-1948.
Photo 6 BY 1 (12-10-1945), 3·2 cms., 18 days' growth, photographed on 29-5-1948.
Photo 7 BY 1 (19-12-1945), 3·6 cms., 22 days' growth, photographed on 21-6-1948.
Photo 8. BY 1 (12-10-1945), 4·0 cms., 23 days' growth, photographed on 21-6-1948.
Photo 9 BY 1 (12-10-1945), 3·5 cms., 17 days' growth, photographed on 23-7-1948.
Photo 10 BY 1 (19-12-1945), 3·6 cms., 17 days' growth, photographed on 23-7-1948.
Photo 11. BY 1 (16-11-1945), 3·2 cms., 18 days' growth, photographed on 24-7-1948.
Photo 12 BY 1 (12-10-1945), 3·7 cms., 19 days' growth, photographed on 17-8-1948.
Photo 13. BY 1 (19-12-1945), 3·3 cms., 18 days' growth, photographed on 16-8-1948.
Photo 14 BY 1 (16-11-1945), 3·2 cms., 19 days' growth, photographed on 17-8-1948.
Photo 15. BY 1 (12-10-1945) 3·7 cms. 12 days' growth, photographed on 21-9-1948.
Photo 16. BY 1 (19-12-1945), 2·7 cms., 15 days' growth, photographed on 15-9-1948.
Photo 17 BY 1 (16-11-1945), 3·0 cms., 19 days' growth, photographed on 14-9-1948.
Photo 18. BY 1 (12-10-1945), 4·2 cms., 20 days' growth, photographed on 7-10-1948.
Photo 19. BY 1 (19-12-1945), 3·5 cms., 20 days' growth, photographed on 7-10-1948.
Photo 20. BY 1 (16-11-1945), 3·3 cms., 13 days' growth, photographed on 1-10-1948.
Photo 21. BY 1 (12-10-1945), 3·4 cms., 15 days' growth, photographed on 22-10-1948.
Photo 22. BY 1 (12-10-1945), 4·0 cms., 19 days' growth, photographed on 26-10-1948.
Photo 23. BY 1 (19-12-1945), 4·4 cms., 19 days' growth, photographed on 26-10-1948.
Photo 24 BY 1 (12-10-1945), 2·8 cms., 11 days' growth, photographed on 13-11-1948.
Photo 25. BY 1 (12-10-1945), 3·5 cms., 20 days' growth, photographed on 22-11-1948.
(Later stage of the colony illustrated in Photo 24)
Photo 26. BY 1 (12-10-1945), 3·1 cms., 18 days' growth, photographed on 23-11-1948.
Photo 27 BY 1 (19-12-1945), 2·3 cms., 11 days' growth, photographed on 13-11-1948.
Photo 28. BY 1 (19-12-1945), 3·4 cms., 21 days' growth, photographed on 23-11-1948.
(Later stage of the colony illustrated in Photo 27)
Photo 29. BY 1 (12-10-1945), 2·8 cms., 18 days' growth, photographed on 13-12-1948.
Photo 30 BY 1 (12-10-1945), 3·5 cms., 21 days' growth, photographed on 25-12-1948.
Photo 31. BY 1 (19-12-1945), 3·6 cms., 21 days' growth, photographed on 25-12-1948.
Photo 32. BY 1 (16-11-1945), 3·2 cms., 23 days' growth, photographed on 6-1-1949.
Photo 33. BY 1 (16-11-1945), 3·3 cms., 22 days' growth, photographed on 12-1-1949.
Photo 34. BY 1 (12-10-1945), 2·4 cms., 15 days' growth, photographed on 18-2-1949.

(All the giant colonies were grown on Barley Malt media; only the longest diameter of the giant colony is recorded.)

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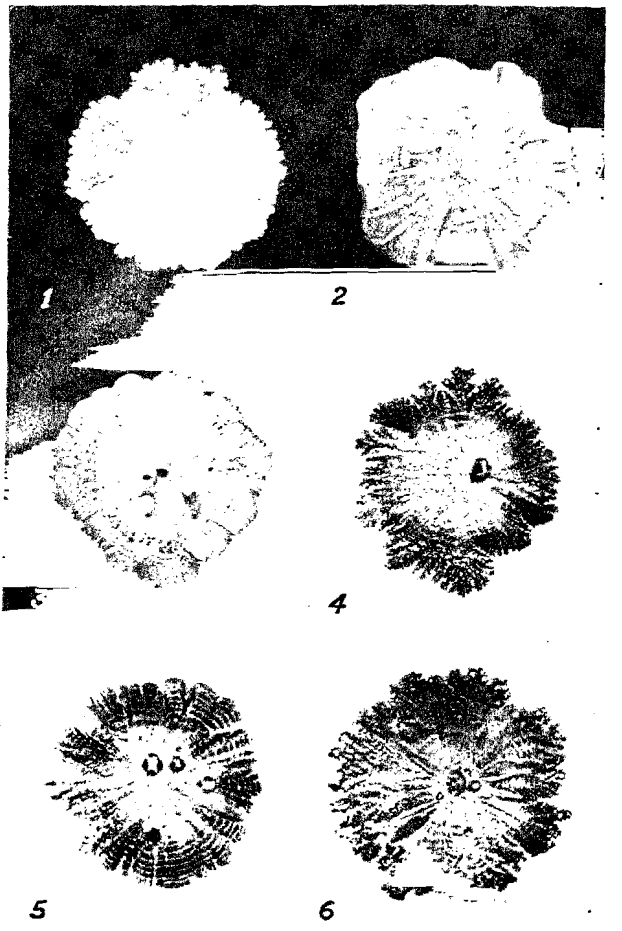
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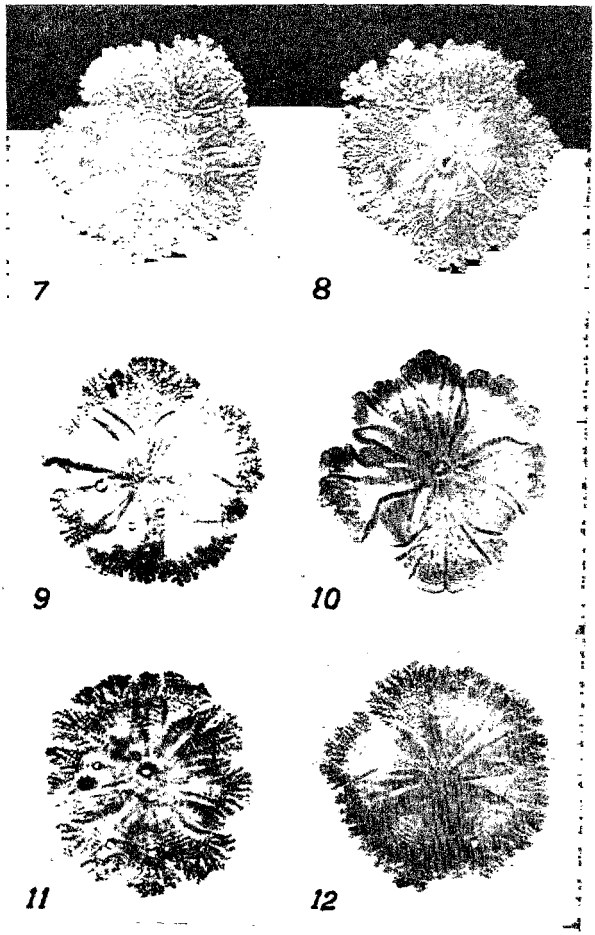
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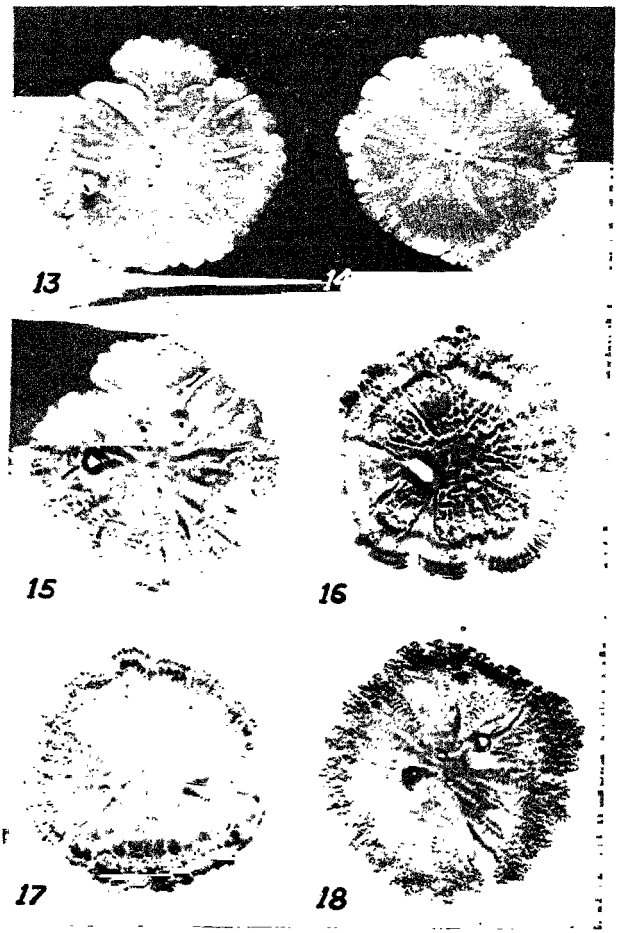
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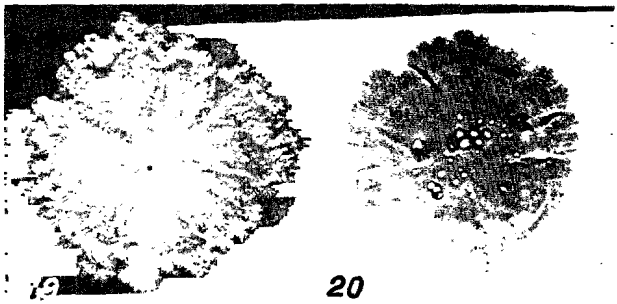

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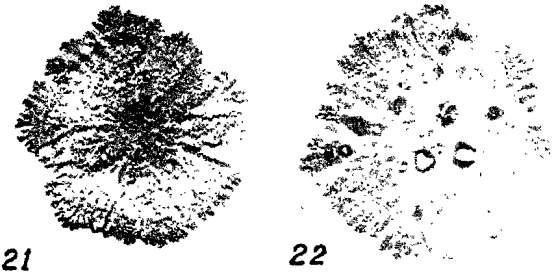

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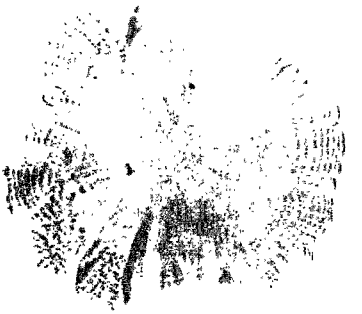

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