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STUDIES ON THE MUTAGENIC ACTION OF CHEMICAL AND PHYSICAL AGENCIES ON YESTS

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STUDIES ON THE MUTAGENIC ACTION OF CHEMICAL AND PHYSICAL AGENCIES ON YEASTS

I. Induction of Polyploidy by Diverse Agencies

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INTRODUCTION

The cytological demonstration of an induction of autotetraploidy (Subramaniam, 1945, 1947 *a*) by treatment of the control two-chromosome brewery yeast with acenaphthene and the discovery of a naturally occurring autotetraploid (Subramaniam and Ranganathan, 1946 *a, b*; Ranganathan and Subramaniam, 1948) rendered difficult on theoretical grounds the acceptance of the published observations on the genetics of yeasts (Winge, 1944; Winge and Laustsen, 1937, 1938, 1940; Winge and Roberts, 1948; Lindegren, 1945; Lindegren and Lindegren, 1946; Spiegelman and Lindegren, 1946; Spiegelman, 1946) for the simple reason that the above

investigators had not taken into consideration the possibility of polyploidy in yeasts.

As far back as 1941, Bauch claimed the production of stable giant races by treatment with polyploidising and carcinogenic substances as well as with phytohormones. His suggestion that these may be polyploids of different degrees was based on the ascending grade of increase in cell volume shown by the various giant races isolated by him. Contrary to expectations, this suggestion of Bauch (1941 *a, b*; 1942) was ignored by all the investigators on yeasts, who subscribing to the belief that yeasts can only be either " haploid " or " diploid ", have offered novel theories to explain what they claim to be an unusual genetical behaviour of the yeasts investigated by them (Lindegren and Lindegren, 1946; Spiegelman, 1946). It would be realised that acceptance of such radical theories necessitates as a pre-requisite, proof that the so-called unusual genetical behaviour is not the result of a normal polyploid segregation. If the cause for the neglect of Bauch's suggestion is the lack of cytological data in support of his claims, it has to be emphasised that all genetic observations on yeasts (Winge and Roberts, 1948; Lindegren, 1945) are based on strains whose chromosome constitutions are unknown. If the so-called " haploids " and " diploids " could be differentiated on purely morphological grounds, one fails to understand any objection to the identification of tetraploids and octoploids on similar morphological criteria. If one has to ignore the claims of Bauch on the basis of absence of cytological data, it necessitates as a corollary, that one has to dismiss as inexact all genetic observations on yeast strains of unknown chromosome constitution.

The accidental recovery of the diploid from the autotetraploid (Duraiswami and Subramaniam, 1950) gave an experimental confirmation to the theoretical objections presented above. We could not only induce tetraploidy, but also recover later, the diploid. This discovery rendered imperative a critical analysis of the problem of haploidy in yeasts (Subramaniam, 1950 *a*). As was to be expected, such an analysis revealed the unsubstantiated nature of many of the assumptions of the earlier investigators and rendered invalid not only the major classification into haplontic and diplontic groups (Subramaniam, 1950 *b*), but also the evidence for cytoplasmic inheritance in yeasts (Subramaniam, 1950 *c*).

But even before this experimental verification of the theoretical possibilities, it was realised that any orderly advance in our knowledge of the Cytogenetics of Yeasts would be possible only if built on strong foundations. This necessitated an extensive series of investigations on the effect

of acenaphthene on yeast strains of different genic and chromosomal constitutions (Subramaniam and Krishna Murthy, 1949) as well as of different polyploidising agencies on the two chromosome control strain.

The results recorded in this paper were obtained as far back as 1947, but they had to await the solution of several connected problems. It would be admitted that a cytological demonstration of an induction of polyploidy should, necessarily be followed by a rational explanation as to the causes for the failure of the earlier investigators in this direction. Naturally this entailed a planned series of investigations aimed at gaining an idea of the effect of the various polyploidogenic chemicals on cells cultured in unlimited and limited media (Subramaniam and Sreepathi Rao, in the press) as well as the progressive changes in the population in an active culture undergoing treatment in an unlimited environment (Mitra and Subramaniam, 1949). Extensive data in these different directions indicated that the failure of the earlier investigators to identify the tetraploids was the result of a host of factors, the most important of which was the absence of valid criteria to distinguish polyploids from diploids.

CONDITIONS FOR THE PRODUCTION OF TETRAPLOIDS

The discovery of a strain of brewery yeast having only two chromosomes (Subramaniam and Ranganathan, 1945; Subramaniam, 1946; Ranganathan and Subramaniam, 1947) enabled the planning of a series of experiments to clarify the contradictions in the published literature on yeasts. Yeast is capable of utilising the aerobic and anaerobic metabolic pathways and it was considered desirable to investigate the cytology of yeast cells during aerobic growth (Subramaniam, 1946; 1948 a) as well as fermentation (Subramaniam, 1947, 1948 b). The results, though expected, were surprising. Regarding the cytological phenomena during the period of active fermentation, Guilliermond (1920) remarks : " Like all secreting cells, they present a series of cytological phenomena in connection with the secretions " (p. 46). Curiously enough, he does not appear to have investigated the nuclear behaviour during aerobic proliferation. During the aerobic phase, the cells divide mitotically. Under anaerobic conditions the cells become endopolyploid as a prelude to fermentation. It was indicated (Subramaniam, 1948 b) that when investigators failed to recognise the fundamental differences between the aerobic and anaerobic phases and that when results of investigations carried out on fermenting cells were considered comparable to those of others on aerobically growing ones, it is not at all surprising that there are glaring contradictions.

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These cytological investigations gave the clue that the effect of poly-ploidogens themselves on a strain may differ according to the physiological condition of the cells undergoing treatment. In an unlimited medium where aerobic growth is going on at a rapid rate, acenaphthene can induce a chromosomal duplication and the tetraploids so produced may be capable of isolation. In a fermenting culture, on the other hand, most of the endo-polyploid cells have no genetic future at all. As in the case of the macro-nucleus of the Ciliates, endopolyploid nuclei may be capable of a few or several multiplications, but their final fate is death and disintegration. But even in fermenting cultures a very small percentage of normally dividing cells occur. Treatment of a fermenting culture with acenaphthene, there-fore, may produce entirely different effects on the few mitotically dividing cells and on the endopolyploid cells. Isolation of stable strains from endo-polyploid cells which have no genetic future, appears to be an impossibility. The few mitotically dividing cells even if they have become tetraploid are likely to be missed in a fermenting culture, where endopolyploid cells pre-dominate.

Most of the investigators have not cared to make this simple but vital distinction. The plan on which most of the experiments have been carried out appears to be to inoculate into a medium, containing the chemical to be tested, a certain number of yeast cells and examine the changes in their morphology or number at periodic intervals. If a few cells are introduced in wort, they multiply for some time and when the oxygen tension in the medium becomes insufficient, there is a gradual transformation to the anaerobic type of metabolism. It is during this period that the cells become endopolyploid. After the shift from the aerobic to the anaerobic phase, while the total number of cells may go on increasing, the percentage of viable cells would show a progressive diminution in number with the ageing of the culture. Similar changes should be occurring in a culture in a limited medium containing the various chemicals used for testing. Recent investigations indicate that the effect of polyploidogens (Mitra and Subramaniam, 1949) is not an all or none reaction. If we consider that a few cells in the inoculum introduced into the medium containing the poly-ploidogen do become tetraploid during the early stages of treatment, these would of necessity become endopolyploid, when conditions in the culture become unfavourable for aerobic growth, with the result that it may not be possible to recover them.

These considerations emphasise the necessity for conducting the treat-ment during active vegetative multiplication. This would be possible only if the medium is changed repeatedly thus preventing a change in the culture

to the fermentative phase. To evaluate the differences between the effect of polyploidogens on the metabolism of the cells from that on their genic make-up, it becomes imperative to isolate types after treatment and then investigate their behaviour in normal media. Most of the investigators (Richards, 1938; Levan and Sandwall, 1943. Levan, 1947; Skovsted, 1948) excepting Bauch (1941 *a, b*; 1942) and Thaysen and Morris (1943) have not extended their investigations in the above direction. When that is the case, any claim that various chemical agencies *do* or *do not* induce polyploidy has necessarily to be invalid.

It was on the basis of the above considerations that Subramaniam carried out the treatment of the control two chromosome strain with acenaphthene under strictly aerobic conditions. It does not appear surprising that since the control was a diploid, it was possible to isolate stable tetraploids. In the preliminary communication (Subramaniam, 1945) it was suggested that by extending the duration of treatment, even octoploids could be obtained. It is true that in a culture undergoing treatment, octoploid chromosome pictures could be observed. But these like many other aberrant types were unstable (Subramaniam, 1947). Even after treatment for one year, only tetraploids could be isolated.

CRITERIA FOR THE IDENTIFICATION OF TETRAPLOIDS

When the possibility of inducing tetraploidy with acenaphthene was established, theoretical considerations suggested that a cold shock should produce the same effect. Cytological investigations being laborious, an alternative simple method to identify tetraploids had to be devised. The changes in the giant colony characteristics of the two chromosome diploid, and the autotetraploid derived from it, were followed from season to season. The behaviour of the two strains were entirely different. While the tetraploid produced a stable smooth colony without any sculpturing (Subramaniam and Ranganathan, 1948; Subramaniam and Krishna Murthy, 1949; Duraiswami and Subramaniam, 1950), the control showed a variety of types (Subramaniam, Ranganathan and Krishna Murthy, 1948) whose predominance varies from season to season. The stable character of the tetraploid colony indicated that the giant colony characteristics could be employed to identify autotetraploids produced by cold shock. Subramaniam and Ranganathan (1948) indicated the possibility that the giant colony characteristics could be correlated with the chromosomal constitution. It has to be borne in mind that this correlation applied only to our strains. A generalisation would be possible only if parallel cytological investigations indicate that those strains producing smooth, stable (*cf.*, "Diploids" of

Lindegren, 1945) colonies indistinguishable from that of our tetraploid, have also an identical chromosome constitution.

The very interesting cytological behaviour of the induced autotetraploid, which resembles the condition already recorded in a distillery yeast (Ranganathan and Subramaniam, 1948) necessitated a prolonged series of investigations for the past two years to map out the behaviour of the products of abnormal mitoses. During the above period, a discontinuous series of giant colonies of the autotetraploid was kept under observation. The stable and uniform nature of the colonies during the different months of the year, substantiated by simultaneous cytological investigations, indicated that the giant colony characteristics could be used as valid criteria for identification. A series of studies on the physiological changes accompanying chromosomal alterations gave other interesting results. Observations on the acenaphthene induced tetraploid indicated that while it had an identical nutritional requirement as the diploid (Prema Bai, 1947) its rate of growth (Prema Bai and Subramaniam, 1947) and fermentation (Mitra, 1948) were entirely different. The nicotinic acid content was also shown to be different in the two strains (Duraiswami and Subramaniam, 1948). Just as there was a remarkable similarity between the giant colonies of strains isolated after treatment with acenaphthene and after culturing in the ice-room, their nutritional requirements and growth rate were also found to be similar (Prema Bai Mallya, unpublished). This indicated the validity of the use of the giant colony characteristics for the identification of auto-tetraploids. Confirmation was obtained by investigations on growth and fermentation rates (Mitra, unpublished), cytology and giant colony characteristics (Mitra and Subramaniam, 1949) of a tetraploid isolated after treatment with chrysene.

There is certainly an obvious risk in depending on giant colony characteristics alone. The naturally occurring tetraploid *distillery* yeast (Ranganathan and Subramaniam, 1948) produced on inoculation a giant colony indistinguishable from that of the tetraploid *brewery* yeast. Active cells of the above strain were treated for an year with acenaphthene and one of the strains isolated after such treatment was found to be identical in appearance as at the commencement of the experiment. The fact, however, that a top yeast could be isolated after such long treatment indicated that acenaphthene should have had some action (Subramaniam and Krishna Murthy, 1949). Owing to pressure of other work, the cytology of the new strain of distillery yeast so isolated could not be carried out. Unlike in the case of the control two chromosome brewery strain, culturing for a year in media incorporated with acenaphthene may have induced octoploidy.

The above facts emphasise that if one depends solely on morphological characters like the sculpturing of a giant colony, the only conclusion that could be drawn is that even such long treatment has produced no change at all. But as would be realised, such an assertion could be possible only if it is proved cytologically, that tetraploids and octoploids produce entirely different types of colonies.

Proof that tetraploidy could be induced by acenaphthene and cold shock does not entitle one to believe that all polyploidogens can induce a chromosomal duplication in yeast. It is well known that certain plants differ in their reaction to the various agencies producing tetraploidy. In this paper evidence is presented that a chromosomal duplication in yeast is possible by such diverse agencies as camphor, colchicine and Ultra-violet irradiation.

MATERIAL AND METHODS

It would have become evident from the critical evaluation of the factors affecting the production and isolation of types with duplicated chromosomes, that success in the above direction depends on treating aerobically growing cells of known chromosome constitution (Subramaniam and Krishna Murthy, 1949). A simple method was devised to enable treatment of actively dividing cells. Richards (1928) found that if fresh medium was added to a culture at 3-hour intervals, there is unlimited proliferation. If such addition was carried out after discarding the spent medium, he found that there was an increase not only in the volume of the cells, but even in their number.

The above observation of Richards (1928) suggested a modification to suit our needs. The dormancy, if any, in cells of the stock agar slants of the diploid two chromosome control could be broken by streaking on fresh slants. If a loop of cells from such 24-hour streak cultures is inoculated into sterile wort tubes (Subramaniam, 1948 a) there is a good crop at the end of 24 hours. To obtain optimum conditions for unlimited proliferation, instead of the periodical replacement of the medium at short intervals, it was found sufficient to add fresh wort to the 24-hour cultures after discarding most of the contents subsequent to a thorough shaking. The small number of cells remaining in the culture tubes gave a similar crop every day. It has already been shown (Subramaniam, 1948 b) that addition of fresh wort to fermenting cultures provides a stimulus even to highly endopolyploid cells, which in trying to divide, usually become necrotic and later disintegrate. Even if some of the cells in 24-hour wort cultures have become endopolyploid, they automatically get eliminated on addition of fresh wort. It was a simple matter, therefore, to observe the effect of colchicine and camphor on cells

showing unlimited proliferation. Five drops of a 4% aqueous solution of colchicine or a concentrated solution of camphor in absolute alcohol were added to the cultures immediately after the daily change of wort. Necessarily, the above procedures have to be carried out inside a sterile chamber. Being interested in the qualitative nature of the changes produced, the optimum amount of colchicine and camphor to be added was determined experimentally. The important consideration kept in view in selecting a suitable concentration of the chemicals in the medium was that a fairly good crop should be obtained every 24 hours.

The experiment was commenced on the 26th of August 1947 and was continued till the end of September. On termination of the treatment, the cultures were grown for about 20 hours in ordinary wort and were plated by serial dilution on 1st October. A fortnight later, four colonies were picked out from the colchicine and three from the camphor series. These were given the arbitrary numbers, BY Col. 1–4 and BY Cam. 1–3 respectively.

The isolated strains were cultured in wort till the 31st of October 1947, on which day giant colony inoculations were carried out. The mode of handling the material for giant colony investigations has already been given elsewhere (Subramaniam and Ranganathan, 1948). The colonies were grown at room temperature.

OBSERVATIONS

Effect of Colchicine

The seasonal variations in the population of the mutants in the cultures of the control BY 1, grown at room temperature during the year 1947 has already been published elsewhere (Subramaniam, Ranganathan and Krishna Murthy, 1948). Immediately after the commencement of the treatment, a colony of the control was grown and is illustrated as Photo 1 (Photo 1, Prema Bai and Subramaniam, 1947). This colony is of the type, *Rough I A* described by Subramaniam, Ranganathan and Krishna Murthy (1948) and shows a tetraploid sector. From the nature of the sector it appears as if a duplication of the chromosome complement has occurred during the growth of the giant colony. The giant colonies of the strains BY Col. 1, 3 and 4 are illustrated as Photos 2, 3 and 4. These colonies are strictly comparable to those of the acenaphthene induced autotetraploid BY 3 (*cf.*, Photo 2, Subramaniam and Ranganathan, 1948; Photos 2, 7 and 8, Subramaniam and Krishna Murthy, 1949; Photos 1, 2, 3, 11 and 12, Duraiswami and Subramaniam, 1950). Curiously enough, the colony of BY Col. 2 resembled the colony of the control BY 1, illustrated in Photo 1 suggesting that it may

be an aberrant type in which a chromosomal duplication had not occurred. Induction of tetraploidy is thus unrelated to mere inhibition of mitosis. Neither is it an all or none reaction. Colchicine should induce a specific gene mutation in order that the doubled complement of chromosomes can function harmoniously (Subramaniam and Sreepathi Rao, 1950).

Stability is an important consideration in producing types suitable for industry. Continuous observations in this laboratory (Subramaniam and Krishna Murthy, 1949; Duraiswami and Subramaniam, 1950) have been consistently substantiating the theoretical possibility that autotetraploids should be highly stable. This view finds additional confirmation in Photos 5, 6 and 7 of the giant colonies of the strains, BY Col. 1, 3 and 4 grown an year later than those illustrated in Photos 2, 3 and 4. It would be apparent that these show little variation. The radial striations absent in Photos 2, 3 and 4 appear pronounced in Photo 6. It has already been indicated (Subramaniam and Krishna Murthy, 1949) that there is a waxing and waning of the prominence of the radial striations in the colonies of the autotetraploid, BY 3, during the different seasons and the treatment of the *Rough I* colony with acenaphthene produced an autotetraploid with such radial striations. Naturally, it was suggested that mutations to the different alleles may occur at the loci in the autotetraploids also.

BY Col. 2 when inoculated gave a *Rough I* colony (Photo 8) in November 1948. A careful examination of the sculpturing reveals a slight difference from that of the control illustrated as Photo 1. The *Smooth I* sector occurring in this colony (Photo 8) has a relatively inferior growth rate than the rest of the cells in the colony.

At the commencement of the experiment the control, BY 1, was of the *Rough I A* type. When diploid cells having such a genic combination were treated with acenaphthene they gave rise to autotetraploids in which radial striations were evident. Exposure to colchicine, however, gave rise to colonies which did not have faint striations. The probability suggests itself, that colchicine may have induced a gene mutation before a chromosomal duplication.

Effect of Camphor

The above possibility that polyploidogens may induce gene mutations prior to a doubling of the chromosomes is exemplified by the photographs of the strains, BY Cam. 1, 2 and 3 (Photos 9, 10 and 11). While BY Cam. 1 developed into a colony showing the radial striations (Photo 9), BY Cam. 2 and 3 (Photos 10, 11) gave absolutely smooth colonies. Judging from the

results obtained with acenaphthene (*cf.*, Photos 2, 7, 8 and 9, Subramaniam and Krishna Murthy, 1949) while BY Cam. 1 justifies the expectations. BY Cam. 2 and 3 give added evidence to the probability that gene mutations should have preceded chromosomal duplication.

Eight months later, giant colony inoculations were carried out to test the stability of the above three strains. There was no change in the sculpturing of BY Cam. 1 (Photo 12). Actually, the radial striations are relatively more prominent in Photo 12 than in Photo 9. Colonies of the other two strains, BY Cam. 2 and 3 (Photos 13 and 14) showed faint radial striations and resembled the colonies illustrated in Photos 10 and 11. The typical tetraploid colonies conforming to the description of such presented originally by Subramaniam and Ranganathan (1948) led to an extension of the observations to verify whether the colonies BY Cam. 2 and 3 do produce during some seasons a type of sculpturing characteristic of BY Cam. 1. Giant colonies grown in the month of November are illustrated in Photos 15 and 16. Both the strains. BY Cam. 1 and 2 show little change during the interval.

Effect of the Composition of the Medium on Giant Colony Expression

Ragi Malt was used for culturing and studying some of the new strains produced by ultra-violet irradiation. Being available in the form of a concentrated extract it was thought that the use of the above may simplify labour. Actually, this complicated issues by altering the expression of the different genic combinations of the control and raised questions, such as whether ragi malt altered only the genic expression or whether it formed a selective environment for particular mutants. The evidence obtained in an extended series of investigations suggested that it affected only the expression. Two tetraploids were isolated after ultra-violet irradiation. But they were grown on ragi malt agar. How far the medium affects the expression of the tetraploids was investigated in the latter part of 1948. BY Cam. 1–3 were grown simultaneously on barley and ragi malt media. Those grown on barley malt agar are illustrated as Photos 12, 13 and 14, while those on ragi malt are presented as Photos 17, 18 and 19. A comparison of the different strains grown in the two media indicates that while BY Cam. 1 and 2 show an identical type of sculpturing in both, there is a slight difference between those of the strain, BY Cam. 3. The radial striations are more prominent in ragi malt agar. The slight differences between the colonies of BY Cam. 3 in the two media seem to be the result of causes yet to be evaluated. They do not appear to be the effect of the composition of the medium. This fact is exemplified by Photo 20 of the strain BY Cam. 2

grown on ragi malt agar at the same time as the colony illustrated in Photo 15. While the latter shows faint radial striations, that grown on ragi malt is absolutely smooth justifying the suggestion that minor differences in the sculpturing are determined by purely fortuitous conditions.

The effect of the medium was tested for the colchicine strains also. Simultaneous inoculations of the different strains were carried out on barley and ragi malt agar. The strains BY Col. 1 and 3 produced identical colonies in the two media (Photos 5, 6 and Photos 21 and 22). BY Col. 2, on the other hand, showed a remarkable difference. The prominent rough sculpturing observed in barley malt media (Photo 8) is absent in ragi malt agar (Photos 23 and 24).

Effect of Ultra-Violet Irradiation

In a previous contribution evidence was adduced (Photo 1 c, Subramaniam and Ranganathan, 1949) to show that ultra-violet irradiation induced, among other things, a duplication of the chromosomes. That photograph is included here for elucidation (Photo 25). The colony was grown on barley malt agar from material of the control, BY 1, after irradiation for 3 hours. It shows a tetraploid sector and the other regions of the colony are completely rough. Material from both the regions of the colony was inoculated into agar slants and numbered as BYU 8 and BYU 9. In September 1947, the strain BYU 8 derived from the tetraploid sector in Photo 25 developed into the characteristic tetraploid colony with no trace even of the radial striations (Photo 26). In January 1948 it retained its identical characteristics (Photo 27). BYU 9 was tested only in December 1947 and it was surprising to find that it had also become tetraploid (Photo 28). Since the changes in the strain were not closely followed, it is difficult to evaluate the causes for the chromosomal duplication. The possibility that it may be the result of a delayed action of irradiation is being systematically investigated. A colony of the same strain photographed a month later (Photo 29) was identical in its characteristics.

DISCUSSION

The invalidity of the criteria employed by the previous investigators for the identification of polyploids

The genetics of yeasts is yet in its infancy but in order to keep abreast of the phenomenal advances in our knowledge of heredity in higher organisms, investigators on yeasts have proceeded on a series of assumptions. The obvious, but hard path is to plan a series of investigations on orthodox lines following the trails blazed by students of Cytology and Genetics of

higher plants and animals. Such a procedure would necessarily slow down
the progress of our knowledge of Cytogenetics of Yeasts, but it would enable
one to avoid the remarkable contradictions found in the published literature
(Subramaniam, 1950 a, b, c). Any attempt to alter accepted genetic con-
cepts on the basis of investigations on yeasts (Lindegren and Lindegren,
1946; Spiegelman, 1946) necessitates as a pre-requisite that such investiga-
tions should be supported by experimentally proved facts. When facts are
replaced by assumptions, the sequence is naturally confusing. When one
tries to proceed on orthodox lines, the glaring fact emerges that many of
the assumptions lack experimental justification. One such is the implied
belief that polyploidy does not occur in yeasts. This, of course, is not the
result of any extended series of cytological investigations. It is based on
the assumption that the so-called " haploids " and " diploids " can be
differentiated on purely morphological criteria (Subramaniam, 1950 a).
The various investigators do not seem to be aware that there is lack of
agreement among themselves as regards the criteria to be employed. A
perusal of the table given below would make it obvious that " haploids "
are distinguished from " diploids " by Lindegren and by Winge purely by
cell size, shape and mode of budding. Using these criteria, it would have
been manifestly impossible for them to differentiate diploid from polyploid
cultures. According to them, all yeasts have to be either " haploids " or
" diploids " and if some of the cultures employed by them were really
polyploids, these would have been classified as " diploids ". Since hybridi-
sation is possible in yeasts, both auto- and allopolyploids should occur. It
follows, therefore, that " real haploids " and " polyhaploids " should have
been included among their so-called " haploids ". Diploids and polyploids
may exhibit marked differences in their reaction to polyploidogens. When
their classification into the " haploids " and " diploids " is itself delight-

		Haploids		Diploids	
		Winge	Lindegren	Winge	Lindegren
Origin	..	Spore germina-tion	Spore germina-tion	Spore, cell or nuclear fusion	Spore, cell nuclear fusionof
Size	..	Small	Small	Large	Large
Shape	..	Round	Round	Oval	Ellipsoidal
Mode of budding	..	Proximal budding resulting in 'short shoot' growth	?	Distal budding resulting in 'long shoot' growth	?
Spore formation	..	Nil	A few sporulate	Present	Present
Giant Colony	..	?	Rough sectored	Rough or smooth	Smooth — not sectored

fully vague, the dearth of criteria to identify " real polyploids " has led to considerable confusion in the evaluation of the reactions of the strains employed by them to the various chemical agencies (Bauch, 1941, *a*, *b*, 1942; Levan and Sandwall, 1943; Skovsted, 1948). One can go further and suggest that even if in their experiments, the chemical agencies employed had really induced a chromosomal duplication, these would have been completely missed by the investigators. Levan (1947) concedes: " Yeast types with in all probability polyploid chromosome number have originated on several occasions in the work of other authors " (p. 509). It has been our experience (Subramaniam, 1947) that after a single duplication of the chromosomes, acenaphthene ceases to have any further effect on the cells. Some cytological pictures observed by Levan (1947) seem to have suggested such a possibility. He remarks: " Since it is known that the yeast has the ability of rapidly acquiring a certain resistance towards *cf*-treatments, especially if the concentration of the active substance is immediately above the threshold value, it is perhaps not absolutely inconceivable that one mitosis may become narcotised, but that the cell after that again takes command over its mitotic mechanism, the subsequent mitoses being normal " (p. 466). The investigations carried out in this laboratory (Subramaniam, 1947) enabled a critical evaluation of the work of Levan and Sandwall (1943) and it was indicated (Subramaniam and Krishna Murthy, 1949) that even if acenaphthene or colchicine had induced a doubling of the chromosomes in their experiments, the prolongation of the lag phase due to the inhibition of cell division would be compensated by the shorter generation time of the new strain. Naturally cell counts made at the end of 20 and 48 hours would give no indication of the changes in the population which had taken place in such cultures.

A rational explanation of the confusing cytological pictures observed by Levan (1947) in the course of his investigations is possible. In a series of contributions (Subramaniam, 1946; 1947 *a*; 1947 *b*; 1948 *a*; 1948 *b*; Ranganathan and Subramaniam, 1948) it has been emphasised that the cytological pictures observed in cells during active vegetative multiplication under aerobic conditions are entirely different from those in active fermentation. The necessity for such a vital distinction has not even been realised by Levan. He states: " All treatments described in the preceding chapters have been made on yeast grown under optimal conditions. At the time of inoculation the yeast had been in vigorous fermentation, and immediately before it has been inoculated several times over on fresh wort. It has for a long period had good nutrition conditions " (p. 498). No wonder then, that he found the fixation and staining of both treated and untreated cells

" always whimsical " (p. 464). The necessity for the study of the cytology of yeast during different phases of activity does not appear to have suggested itself and even his observations on untreated cells are based on fermenting ones. The cytological pictures presented by Levan are those of endopolyploid cells and hence show a remarkable similarity to those published by Subramaniam (1948 b). Fermenting cultures were shown to contain small percentages of vegetatively multiplying cells showing the diploid chromosome number (Subramaniam, 1948 h, p. 331, and Figs. 2, 3, 4 and 5). Without investigating the cytological behaviour of the strain during the active aerobic phase, when what apparently were diploid chromosome pictures were observed in fermenting cultures, Levan dismisses them as the result of a fusion of the chromosomes. " After the anaphase separation the two daughter groups of chromosomes tend to be strongly coloured, and each of them may form one or two distinct bodies that often give the impression of a chromosome number of two or four " (p. 464). At a particular stage during the mitotic cycle of both the diploid and the tetraploid, the chromatin appears as a fused mass (Fig. 35, Subramaniam, 1946; Figs. 19-30, Ranganathan and Subramaniam, 1948). While similar but uncorrelated observations recorded by Levan confirm our results, the fact that all his investigations are limited to actively fermenting cultures renders his attempts at generalisation valueless. Under the circumstances how far his conclusions that the " low chromosome number earlier published for yeast (e.g., Badian, 1937; Sinoto and Yuasa, 1941) may have been influenced by such fusions " and that the " chromosome number is higher than has been generally assumed, ten being the minimum number " (p. 464), are valid could just be imagined. Subramaniam's paper on " Endopolyloidy in Yeasts " was awaiting publication at the time Levan's (1947) paper appeared. Appearance of vagabond chromosomes and formation of micronuclei are common phenomena in fermenting cells. Similar pictures presented by Levan from cultures which are obviously fermenting, cannot, therefore, be attributed to the various chemicals added to the media.

It was clearly emphasised in the section on the conditions necessary for the production of tetraploids, that treatment has to be carried out on cells in active aerobic vegetative multiplication and that if cells inoculated into media containing the chemicals are allowed to ferment, even those in which a chromosomal duplication had occurred would become endopolyploid and may be lost for ever. It does not strike one as strange that neither Levan and Sandwall (1943) nor Levan (1947) were able to isolate stable polyploid strains. Their method of approach precluded any such possibility.

Polyploidy and Genetic Analysis

The yeast employed by Levan (1947) is stated to be highly stable as its use by the Stockholm Breweries would indicate. It may in all probability be a polyploid. Similar may be the case with the strains used by Lindegren and Lindegren (1946) as well as Winge and Roberts (1948). Proceeding on the experimental demonstration of polyploidy in this laboratory (Subramaniam, 1945, 1947 *a*; Subramaniam and Ranganathan, 1948), it is perhaps reasonable to consider here the indirect evidence for polyploidy in yeasts.

The hybrid obtained by crossing *S. mandschuricus* (melibiase +) with a Saccharomyces Sp. (melibiase —) produced melibiase + (Winge and Laustsen, 1939) and in the asci of the hybrid there was "an unquestionable predominance of melibiase + spores, namely 9 + to 1 —." "The fermentation behaviour of the spore zygotes from this hybrid was also examined. Through simple Mendelian inheritance it should be expected that the spore zygotes (arising through copulation of two spores) should show the numerical ratio of 3 + : 1 —, but of 26 spore zygotes not a single minus was found. The explanation of the inheritance behaviour was therefore uncertain" (Winge and Roberts, 1948, p. 271). *Is it because the hybrid was not a diploid* ?

Lindegren (1945) after trying to justify the use of morphological characters for distinguishing the so-called "haploids" from "diploids" observes: "A few single ascospore cultures sporulate well and some produce large cells that are difficult to classify either as definite haplophases or illegitimate diploids" (p. 110). Even according to the criteria employed by him it appears that some of the sporulating ascospore cultures have to be identified as definite haplophases. There is an unproved but widely accepted assumption in yeast literature that meiosis precedes sporulation. If we conclude that his so-called "haplophases" are really "haploid", meiosis, if regular, can only produce spores having sub-haploid number of chromosomes. If on the other hand, conclusions drawn from observations on haploid plants could be extended to yeasts, then the spores formed should possess the unreduced vegetative haploid complement of chromosomes.

Lindegren (1945) has not apparently realised the difficulties in accepting his conclusions at their face value on the basis of the criteria employed by him to differentiate haploids from diploids. He says that the illegitimate diploid derived from the hybrid between *S. bayanus* and *S. cerevisiæ* yielded a curious segregation. "Three asci were dissected from this illegitimate diploid, and each ascus yielded two small and two large colony cultures" (Lindegren, 1945 *b*, p. 120). This 2:2 segregation in a diploidised culture which ought to be homozygous renders it questionable whether (1) a

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diploidisation had occurred at all and (2) whether the spore had the haploid chromosome complement. If we assume that his Hybrid IV was tetraploid, the spores cannot have the haploid constitution, and may show segregations observed by Lindegren. Instead of trying to explain the curious behaviour on the basis of polyploid segregation, he assumes without any evidence—not even for diploidisation—that (1) mutations should have occurred in the single ascospore cultures and (2) copulation between the mutated and non-mutated cells should have occurred, thus rendering possible the curious segregation.

When the chromosome constitution of the starting types are unknown, when the spores have not been shown to be real haploids, when there is no evidence that either mutations or copulations had occurred, and when haplophase cultures are considered by him to be capable of sporulation without diploidisation, his conclusions that the curious $2:2$ segregation suggests a similarity to the cytoplasmic deficiencies found in illegitimates by Winge and Laustsen (1940) turns out to be a speculation lacking any experimental justification (p 120).

In a previous contribution (Duraiswami and Subramaniam, 1950) it has been indicated how the so-called inbreeding degeneration observed by Winge and Laustsen is itself capable of an explanation in terms of haploidy, diploidy and polyploidy. Winge and Roberts (1948) have disputed the radical conclusions of Lindegren by investigations on identical lines. Their results on maltose fermentation suggested the probability that the hybrid was heterozygous with respect to three dominant maltase genes. They consider that each of these should be in a different chromosome pair. The observed results did not agree with the theoretical expectations. They state: " But if it is assumed that 50 per cent. crossing over takes place with respect to all three maltase genes, there should theoretically occur $2:2$, $3:1$ and $4:0$ segregations, with the frequencies of $2 \cdot 8$, $44 \cdot 4$ and $52 \cdot 8$ per cent. respectively ($\frac{1}{4}$, $5\frac{1}{4}$ and $6\frac{1}{4}$ asci), which without doubt could be the case in our material. If it is assumed that crossing over occurs in only one non-sister chromatid pair, which, however, must be considered as less probable, one will arrive at theoretical segregation ratios which show still closer agreement with those actually found " (p. 280). It need hardly be emphasised that these assumptions have not been experimentally verified. In spite of that they suggest for consideration another unsubstantiated assumption that " the segregation behaviour may be influenced by the effect of external factors upon the crossing-over process " (p. 280). In their temperature experiments they could not confirm the suggested possibility. They comment: " This is indicative of the crossing-over mechanism, *even if positive evidence*

was not obtained in the above experiments; the lack of 2:2 segregations is striking and can only be understood by assuming that crossing-over is not in accordance with probability " (p. 302).

Even accepting the suggestion of Winge and Roberts that there are three dominant maltase genes in three different chromosome pairs in the hybrid, one has to imagine that it should have at least a minimum of three pairs of chromosomes. The question naturally arises whether the hybrid is a diploid or a polyploid. Since strains (Subramaniam, 1946) having two as the diploid chromosome number are on record, one can reasonably suspect that the strains employed for hybridisation may be polyploids.

Winge (1944) considers it probable that since mutations affecting morphological characters are common, mutations affecting physiological behaviour should also be possible. He considers that such mutations affecting physiological characters may not occur at the loci determining the production of species specific enzymes. Mutations according to him cannot possibly change the characteristics of a species but the probability is presented that they may affect the quantity of the enzyme produced. This takes us naturally to the problem of quantitative inheritance which has been considered to be one of the most baffling and complex in genetics. Quantitative inheritance is usually met with in polyploids.

Stability of Tetraploids

In a previous contribution it was demonstrated that tetraploids are highly stable. The stability tests reported in this paper offer further confirmation. As has been emphasised a giant colony is not strictly comparable to tissues of higher organisms. The stable types of colonies observed give only a general indication since the major alterations in the cell size, shape and mode of budding accompanying polyploidy can alter the nature of sculpturing. Autotetraploids are usually said to offer difficulties in obtaining true breeding lines. Phenotypically uniform progeny may differ in their genotypes and hence would segregate duplex and simplex types in the immediate generation in plants.

The problem of stability in Yeasts has to be viewed from an entirely different angle. Even with the same frequency of appearance of mutations as in higher organisms, the generation time being very short, remarkable changes could be observed in yeast cultures. Moreover, in industry, yeasts are kept in the vegetative condition and even if mutations do occur, only the hypermorphic ones can necessarily get established. The angle from which stability in yeasts should be judged has therefore to be different from that in higher plants where propagation is mostly from seeds. Those

mutations which may be lethal or which in some way may lower the viability in a diploid, usually do not have such disastrous consequences in a tetraploid.

We have approached the problem with a view to produce stable industrial types. At every stage in industry, the environment would act as a selective factor. Those mutations which are inferior either in growth rate, viability or in some other important physiological character, would get automatically eliminated. The probability of a quick reversion which usually happens in a diploid is not only eliminated but there are greater chances of balance between the genes in a polyploid.

Genes and Fermentation

It was suggested in a previous contribution (Ranganathan and Subramaniam, 1950) that the genes said to initiate the production of melibiozymase or galactozymase may in all probability govern only the master reactions. It was indicated that the genes responsible for the various basic steps of fermentation should be having very low mutation frequencies. The above suggestion was based on the fact that both our diploid BY 1, and the autotetraploid, BY 3, become endopolyploid during fermentation. On this basis Mitra and Subramaniam (1949) explained the quantitative differences shown by the above two strains in their rate of fermentation. The necessity for the cell to reach a particular stage of endopolyploidy before it can become fermentative suggested the possibility that the genes controlling fermentation may be heterochromatic and may possibly belong to the category known as " polygenes " which are said to have " small, similar, supplementary effects ". The ability of the yeast to use the anaerobic metabolic pathway indicates that at least the genes governing the basic steps of fermentation of any sugar should be protected from the vagaries of the mutational phenomena.

Indirect evidence for such a possibility was obtained in the shape of a non-fermenting purely aerobic top yeast as a mutation. The series of investigations on this strain (Subramaniam and Ranganathan, 1946; Subramaniam, 1946; Prema Bai, 1947; Mitra, 1949; Ranganathan and Subramaniam, 1950) indicate that its peculiar behaviour is the result of a chromosomal translocation.

The possibility of induction of polyploidy by diverse agencies described in this paper ought to have important repercussions on investigations on the genetics of yeasts. It suggests that much of the published work on yeast genetics may have to be re-evaluated in the light of polyploid segregation.

SUMMARY

1. Tetraploidy could be induced in yeast by diverse agencies. Treatment with polyploidogens has to be carried out on mitotically dividing cells during the aerobic phase. Under anaerobic conditions the cells become endopolyploid as a prelude to fermentation. If a few cells do become tetraploid during the early stages of treatment, these would of necessity become endopolyploid, when conditions in the culture media become unfavourable for aerobic growth. It is problematical whether these could be recovered later.

2. The correlation between chromosome constitution and giant colony characteristics confirmed by an extended series of investigations indicated the validity of the use of giant colony characteristics for the identification of tetraploids.

3. The treatment with colchicine and camphor was carried out continuously for a period of 35 days under conditions suitable for unlimited proliferation. Out of the four colonies picked up from plated colchicine treated material, three were tetraploids, and one a diploid. All the three colonies isolated after camphor treatment were tetraploids. It appears that gene mutations may occur before an actual duplication of the chromosome complement. The tetraploid colonies are highly stable and unlike in the case of diploid, their expression is identical in Ragi as well as Barley Malt agar. Tetraploid colonies isolated after ultra-violet irradiation are indistinguishable from those produced by chemical agencies.

4. It appears that the arbitrary division of yeasts into the so-called " haploids " and " diploids " by earlier students of yeast genetics has resulted in a dearth of criteria to identify the real polyploids. Owing to the above reason, even if in their experiments, the chemical agencies employed had really induced a chromosomal duplication, these would have been completely missed by the investigators.

5. The possibility of induction of polyploidy by diverse agencies, described above ought to have important repercussions on future work on the genetics of yeasts. It suggests that much of the published work on yeast genetics may have to be re-evaluated in the light of polyploid segregation.

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DESCRIPTION OF PHOTOGRAPHS

No.	BY	Type	No.	Form	cms.		day			date	location
1.	BY	1	1	Diploid	2·5	cms.	10	day	growth,	30-9-47.	Barley Malt agar.
2.	BY	Col.	1	Tetraploid	2·1	cms.	13	day	growth	3-11-47	,,
3.	BY	Col.	3	,,	2·1	,,	11	,,	.,	14-11-47	,,
4.	BY	Col.	4	,,	2·1	.,	11	,,	,,	14-11-47	,,
5.	BY	Col.	1	,,	2·9	,,	18	,,	,,	27-11-48	,,
6.	BY	Col.	3	,,	2·9	,,	18	,,	,,	27-11-48	,,
7.	BY	Col.	4	,,	3·5	,,	19	,,	,,	1-12-48	,,
8.	BY	Col.	2	Diploid	3·8	,,	22	,,	.,	1-12-48	,,
9.	BY	Cam.	1	Tetraploid	2·3	,,	24	,,	,,	14-11-47	,,
10.	BY	Cam.	2	,,	1·9	,,	14	,,	,,	14-11-47	,,
11.	BY	Cam.	3	,,	2·2	,,	13	,,	,,	3-11-47	,,
12.	BY	Cam.	1	,,	2 4	,,	22	,,	,,	25- 8-48	,,
13.	BY	Cam.	2	,,	2·6	,,	13	,,	,,	25- 8-48	,,
14.	BY	Cam.	3	,,	2·6	.,	13	,,	,,	25- 8-48	,,
15.	BY	Cam.	2	,,	2·6	,,	17	,,	,,	22-11-48	,,
16.	BY	Cam.	3	,,	2·3	,,	22	,,	,,	27-11-48	,,
17.	BY	Cam.	1	,,	2·0	,,	22	,,	,,	25- 8-48	Ragi Malt agar.
18.	BY	Cam.	2	,,	2·2	,,	21	,,	,,	3- 9-48	,,
19.	BY	Cam.	3	,,	2·5	,,	18	,,	,,	30- 8-48	,,
20.	BY	Cam.	2	,,	2·0	,,	22	,,	,,	27-11-48	,,
21.	BY	Col.	1	,,	2·4	,,	22	,,	,,	1-12-48	,,
22.	BY	Col.	3	,,	2·1	,,	22	,,	,,	1-12-48	,,
23.	BY	Col.	2	Diploid	2·6	,,	29	,,	,,	18-11-48	,,
24.	BY	Col.	2	,,	2·9	,,	28	,,	,,	7-12-48	,,
25.	BY	1 u.v	treated 3 hrs.		2·5	,,	9	,,	,,	28- 7-47	Barley Malt agar.
26.	BY	u 8	Tetraploid		2·3	,,	15	,,	,,	30- 9-47	,,
27.	BY	u 8	,,		2·0	,,	16	,,	,,	22- 1-48	Ragi Malt agar.
28.	BY	u 9	,,		2·3	,,	22	,,	,,	24-12-47	,,
29.	BY	u 9	,,		2·1	,,	22	,,	,,	22- 1-48	,,

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