INVESTIGATIONS ON THE EFFECT OF

COLCHICINE

ON YEASTS

by

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# TABLE OF CONTENTS

# INTRODUCTION

## Early Work with Colchicine and its Significance:

Several years ago it was discovered that by treating the leaves and stems or seeds of some plants with a solution of the alkaloid colchicine, artificial mutations could be induced. This was a tremendously important discovery from the point of view of the genetecist who could now speed up the processes of evolution more or less at will. It was also important to the plant breeder who could now produce new and improved economically important species. But it was especially important perhaps from the point of view of the biochemist for it is possible that the application of the colchicine technique to a study of cell division might give an insight into the chemistry of that process. It is with this latter possibility that the present work is concerned.

## Occurrence of Colchicine:

The alkaloid was first noted by Pelletier and Caventou ( 1 ) as occurring in all parts of the plant Colchicum autumnale or the autumn crocus. Colchicine also occurs in the following; Colchicum alpinum, C. arenarium, C. monatum, Gloriosa superba, Androcymbium gramineum, and Mendera bulbo-codium.

Farr and Wright ( 2 ) found that the alkaloid content of different samples of seeds (C. autumnale) may vary between

.54 and .79%. L. P. Liptak (3) reports that colchicine occurs
in highest concentrations in the inner layer of the seed coat.
E. C. Davies (4) reports that the seeds of C. autumnale yield
.75% and the corms .38% of the alkaloid. F. Chemitus (5)
claims that the ripe seeds of C. autumnale contain from .4 -
.9% of the alkaloid. Androcymbium gramineum Mac Bridge is a
colchicine containing plant of the Southern Sahara. The yield
from the seeds is .37%. The bulbs yield .29%. Mendera bulbo-
codium Ram contains 19% of colchicine in the dry finely
powdered corm.

## Extraction of Colchicine:

The alkaloid may be extracted from the seeds or corms
of Colchicum autumnale as the commonest source, by several
different methods. Recommended are the procedures employed by
E. C. Davies and J. Greir (6) and Beilstein (7). Fortunately
the alkaloid may be obtained pure from several different supply
houses. The colchicine used in this investigation was obtained
from Mallinckrodt U.S.P. Amorphous alkaloid, chloroform-free.

## Structure and Chemical Properties of Colchicine:

The alkaloid is a derivative of phenanthrene. The
structure as first suggested by Windaus and Scheele (8) is:
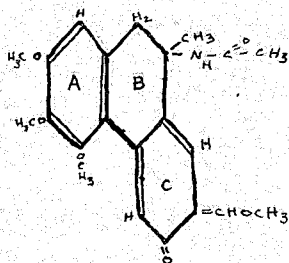
The structure was further investigated by K. Bursian ( 9 ). According to this author the structure of the "C" ring is still uncertain, and is probably
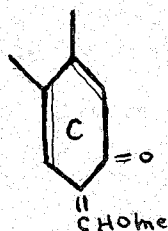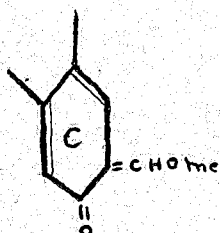
When colchicine is heated with acidified water it loses methyl alcohol and yields colchiceine.
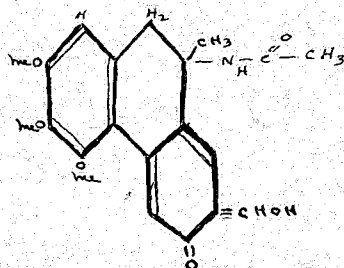
Etherification of colchiceine with methyl alcohol yields colchicine.

Pure colchicine forms nearly colorless or pale yellow inodorous plates of no sharp melting point. Dried over concentrated Sulphuric acid it softens at $142^\circ C$ and melts at $147^\circ C$. It is laevorotary. Colchicine is soluble in water to the extent of 4.54% at $20^\circ C$ and is very soluble in alcohol and chloroform. It is fairly soluble in benzene and only slightly soluble in dry ether. When one part of the alkaloid is dissolved in three parts of water (with heat) the solution deposits after some time large glittering yellowish rhombic crystals having the composition $C_{22}H_{25}NO_6 \cdot 1.5 \, H_2O$. Anhydrous colchicine is amorphous. There are two crystalline compounds of the alkaloid with chloroform $C_{22}H_{25}NO_6 \cdot CHCl_3$ colorless glittering needles

which lose $CHCl_3$ at $100^\circ C$ and $(C_{22}H_{25}NO_6)_2 \cdot CHCl_3$, fine needles arranged in rosettes.

It is precipitated by phosphotungstic acid and silicotungstic acid in hydrochloric acid solution ( 10 ). Beilstein states that colchicine gives a bright yellow color in concentrated HCl, and a violet color in concentrated $HNO_3$. An alcoholic solution of colchicine gives a garnet color with $FeCl_3$. Boyland and Huntsman (11) describe a quantitative determination of colchiceine in the presence of colchicine by a colorimetric method based on the green color given by colchiceine and $FeCl_3$ in chloroform. Under the same conditions colchicine gives no color.

## Physiological properties of Colchicine:

It is with the physiological properties of colchicine that we are most interested.

It was noticed by the Bulgarian botanist Dontcho Kostov (12) that sometimes after spraying plants with nicotine sulphate sports or mutants developed. He was led to study the effects of other alkaloids on cell division in the production of mutants, and found that among other substances colchicine was the most effective. Alpha naphthyl acetic acid is probably next most effective. He stated that acenaphthene produces less severe changes in cell division than does colchicine (12,13,14 ). Simonet and Guinochet (15) report that alpha chloro-, and alpha bromo-naphthalene have a colchicine-like action on the caryokinesis of plants.

Colchicine has the specific property of inhibiting the formation of the spindle in somatic mitosis. Perhaps it would not be out of place to review the general phenomena of mitosis. In this regard a diagram is probably most helpful.

Resting stage of nucleus    Prophases

Metaphase    Anaphase    Telophase    Resting Stage
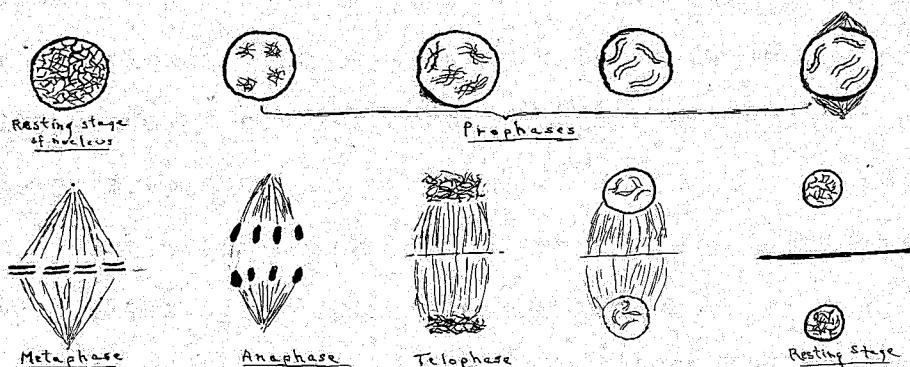
Concerning mitosis Sharp says "The outstanding and significant feature of somatic mitosis is this: each chromosome is accurately divided into two exactly equal longitudinal halves which are distributed to the two daughter nuclei. The two daughter nuclei thus receive exactly similar halves of the chromatin of the mother cell." ( 16 )

Colchicine by inhibiting the formation of the mitotic spindle at the metaphase creates conditions for chromosome doubling ( 13,14,17,18,19,20,21) that is the chromosomes split longitudinally first in readiness for ultimate division of the cell, then, when the spindle does not appear they split once more. When finally the process of cell division is resumed, as when the colchicine is removed, the daughter cells receive doubled chromosomes. The original papers outline in detail the procedures followed with plants and the results obtained. It is enough to note here that sterile hybrids may become fertile as a result of chromosome doubling for differentiation of the

cell into male and female gametes becomes possible when the chromosome sets are completed. Increase in size of pollen grains is used as an indication of polyploidy. In general the size of the cells, and correspondingly of the plants is much increased. Experiments with animals have failed to produce living polyploids. It is true that the chromosomes of the fertilized ova of rabbits have been doubled, but the embryos have never developed.

Employing chick embryo heart fibroblasts and chick iris epithelium, growing in vitro Gavrilov, Dina, and Bistram (22) showed that even in dilutions of 1:25,000,000 colchicine arrested and distorted development of the cells. The authors emphasized the fact that colchicine even in very weak solutions is a cell poison. Verne and Vilter (23) corroborated these findings, but report a higher dilution of the alkaloid required to check division of the fibroblasts. They reported that colchicine 1:4,000,000 checked the cell division in twenty minutes in vitro.

Colchicine is very toxic to the animal organism. E. Maurel (24) found that 0.005 gm/kgm of body weight is sufficient to kill a rabbit whether administered hypodermically or by stomach.

Colchicine is mildly carcinogenic, which is not surprising in that it is related to phenanthrene. It is capable of causing the production of tumours in plants and animals (25). In addition to being able to cause tumours in plants, colchicine in higher concentrations can check the growth of tumours. This effect is probably related to the inhibitory action on cell

division. By painting tumours such as those caused by
Bacillus tumefaciens on geranium and castor seed plants with
0.5% colchicine in lanolin, the growth of the tumour can be
checked. (26,27) Brushing the surfaces of indolacetic acid
tumours with 2% colchicine solutions inhibits further growth
in many instances. Under certain conditions the plant tumour
can be completely killed by colchicine preparations. In
animals (rats) the injection of colchicine solutions into
spontaneous mammary tumours renders the tumours more sensitive
to the action of X-rays ( 28 ).

## The Possible Relation of Colchicine to the Cancer Problem:

It is interesting to note that the most highly carci-
nogenic compounds are also inhibitors of cell division, but are
very much less effective in this respect than colchicine (29).
It is quite definitely a fact that the most highly carcinogenic
agents are the least inhibitory of cell division, and the least
carcinogenic are the most highly inhibitory of cell division.
On the basis of the work of other authors, the investigator has
been led to speculate that tumour genesis depends on the pro-
duction of a mutant. Since colchicine is carcinogenic, and
since it produces mutants in both plants and animals, it is
reasonable to suppose that other carcinogenic substances act in
a similar way. We are thus led to associate carcinogenicity
very closely with inhibition of cell division. It would be
expected that substances which are highly inhibitive of cell
division would be required in very small concentration to
produce the temporary inhibition of cell-division with

subsequent resumption of the process required to produce a mutant. Similarly, relatively large quantities of highly carcinogenic agents (low inhibitory power of cell division) would be required to produce this effect. Further, we would expect that the small quantity of the first substance would be eliminated rather rapidly from the animal organism, certainly more rapidly than the large quantity of the latter substance. Consider the cases of colchicine and methylcholanthrene as examples. The relatively small quantity of colchicine required to produce an inhibition of cell division would not remain localized for long due to elimination or detoxification of the alkaloid. The relatively large quantity of methyl-cholanthrene required to produce a similar effect on division of the cell would certainly take a longer time to be eliminated. It therefore seems very probably that there is more chance of producing a mutant in vivo using methylcholanthrene than there is of producing a mutant with colchicine. That is to say, on the basis of the above hypothesis methylcholanthrene would be more carcinogenic than colchicine, which it is. There is ample evidence to support the view that cancer is a mutant (30). The hypothesis also may be made to explain susceptibility variations in different tissues and strains of animals. Different tissues for example are effected differently by a fixed concentration of colchicine. This variation in susceptibility manifests itself even in the Aspergilli (31).

We may briefly review the facts and the hypothesis:

(1) Colchicine is highly inhibitive of cell division through prevention of the formation of the mitotic spindle in

somatic mitosis.

(2) Colchicine is weakly carcinogenic in vivo.

(3) Methylcholanthrene is weakly inhibitive of cell-division.

(4) Methylcholanthrene is highly carcinogenic in vivo.

It is suggested that the property of inhibition of cell-division is a relatively constant characteristic of the compound used for any one tissue. It is suggested further that the carcinogenicity of this substance for the same tissue varies according to whether the production of a mutant takes place in vivo or in vitro. It is known that methycholanthrene has never been able to produce a tumour in vitro, but in animals, methylcholanthrene is highly carcinogenic. A factor of elimination present in vivo, but not in vitro is probably involved, as explained in more detail above.

It would be predicted on the basis of the above hypothesis that

(1) Colchicine should be more carcinogenic in vitro than in vivo. Since the factor of elimination is not present, a very small concentration (as little as 1:25,000,000) may be sufficient to cause the production of a mutant.

(2) Methylcholanthrene should be less carcinogenic in vitro than in vivo. A relatively large quantity of the substance should be required to produce a mutant. We have, then, the possibility that colchicine in vitro is more carcinogenic than methylcholanthrene. The obvious test of this hypothesis is to submit an in vitro culture of normal tissue to

the action of colchicine; to wash the tissue free of the
alkaloid using Ringer's solution, say, and to transfer the
tissue into an animal to see whether or not a tumorous tissue
has been produced.  Lacking the facilities to carry out tissue
culture methods however, it was decided to employ unicellular
organisms to study the effect of colchicine.

Only very recently has any success been secured with
the application of the colchicine technique to a study of uni-
cellular organisms.  Steinberg and Thom (31) have reported the
production of mutants in certain members of the genus
Aspergillus employing the alkaloid.  They found too that they
could secure a reversion of the mutants to the normal by
including d-lysine in the media in which the organisms were
growing.  They found it necessary to include solid excess
Calcium carbonate in the media to prevent hydrolysis of the
colchicine.  They suggest that the production of a colchicine
mutant, at least in this genus is connected with the destruction
of free amino groups on the protein molecules comprising the
nuclear material.  This claim is based on the idea that free
amino groups in protein molecules are concerned with the
presence in the molecule of lysine.

The Effect of Colchicine on Yeasts

It must be admitted that the original idea behind the
study of the effect of colchicine on the yeasts was not as
elaborate as that put forward above.  O. W. Richards (32)
working with Saccharomyces cerevisiae (strain 4360 A.T.C.)
found that inclusion of colchicine in the medium over a range
of 1:10,000,000 to 4.5:100 had no inhibitory effect on the

formation of the yeast bud. The original idea was then to try to answer the question "Why is one cell affected by colchicine while another is not?" Why for example, is the budding yeast not affected by colchicine even in 4.5% concentration when the chick embryo fibroblast is affected by 1:25,000,000?

The obvious answer is of course that if colchicine is absolutely specific for inhibiting mitosis then all cells could be grouped as

(1) Those responding to colchicine i.e. exhibiting mitosis.

(2) Those failing to respond to colchicine, i.e. exhibiting amitosis.

The chick embryo fibroblast may, therefore, be regarded as belonging to group one, and the yeast to group two. However, there is evidence to show that sporulation in the yeast is mitotic, and the possibility exists, therefore, of an organism existing in both groups.

Before going on to the next step in the development of the hypothesis it will be necessary to consider the physiology of the yeasts in more detail.

## Cytology of Yeasts.

The yeasts are unicellular fungi classed among the Ascomycetes. They are generally found living in the isolated state, but may under rare circumstances produce rudimentary mycelia.

The usual method of multiplication is by budding, but

there are a few species which multiply by transverse fission.
To be more explicit, budding consists of the appearance of a
small prominence on the yeast cell which is separated from the
mother cell by a narrow collar. The bud appears to be made up
of very dense material which has emigrated from the mother cell.
When the bud has reached a certain size the nucleus of the
mother cell begins to divide. The nucleus does not change its
position even if situated at the opposite end of the cell from
the bud. It elongates and assumes the appearance of a dumbell.
One head of the dumbell enters the bud, the other remains in
the mother cell, and the two are then separated by a constric-
tion of the narrow collar attaching the bud to the mother cell.
Guilliermond ( 33 ) states that "The nuclear division does not
offer the characteristics of karyokinesis, contrary to the
opinion of other authors (Swellengrebel and Fuhrmann). It
seems to consist simply of direct division."

It sometimes happens when a yeast is growing rapidly,
as when under optimum conditions, that the mother cell produces
several buds, and the buds themselves may begin to multiply
before separating. The genus Schizosaccharomyces is the only
genus of yeasts which does not divide by budding. This group
of organisms divides by transverse fission which consists simply
of the formation of a wall in the middle of the cell which
divides the cell into two daughter cells.

If the yeast is cultivated under favorable conditions
for growth, it divides actively until the conditions become
unfavourable either through accumulation of waste products or
from lack of food. It then ceases to divide and produces forms

which allow the organism to perpetuate itself over unfavorable
conditions. The cells may develop thick walls, and large stores
of glycogen and fats may appear. These are the durable cells
of Will and Casagrandi. The usual method of perpetuation of
the species employed by the true yeasts is that of sporulation.
A certain number of internal or endospores are formed in the
interior of each cell, the cell thus being transformed into a
sporangium called an asc. In certain species the ascospores
are formed by a sexual process which may be either iso- or
heterogamic. The asc results from the fusion of two cells.
Concerning the sexual process Guilliermond states (33)

"Two cells identical in characteristics and lying
adjacent to each other in the same colony are joined by means
of a copulation canal formed by the fusion of two little pro-
jections put out by each cell. The middle wall which separates
the two cells is rather quickly dissolved and the nuclei,
transformed thus into gametes pass down the copulation canal
where they fuse to form an egg or zygospore. Formed in this
manner by isogamic conjugation the egg soon germinates. It
increases in volume while its nucleus undergoes two successive
divisions, sometimes three which gives four or eight nuclei.
Then these become distributed about the zygospore, and sur-
rounding themselves with a zone of protoplasm form four or
eight ascospores. The zygospore is then transformed into an
asc." The commonest groups of yeasts to form spores in this
way are the Schizosaccharomyces and the Zygosaccharomyces.
In the case of heterogamy observed in the case of Zygosaccharo-
myces chevalieri copulation is carried out by two cells of

different dimensions. One is very small and represents the male gamete. It is young, while the other, the female gamete, is larger and much older. The two cells unite by a copulation canal and the contents of the male gamete pass into the female gamete in which the protoplasmic and nuclear fusion takes place. After this has taken place the female gamete separates itself by means of a wall and produces from one to four ascospores.

In the great majority of the yeasts one does not find any trace of sexuality. In this case ascospores arise by parthenogenesis. The common beer yeast, Saccharomyces cerevisiae is an example of this type. The sexual process has probably been lost through long adaptation to certain conditions, and this yeast usually forms spores to the number of four by two successive divisions of the nucleus in the single cell.

Germination of the ascospores in the preceding case involves a swelling of the spores within the ascus with a subsequent bursting of the surrounding membrane. The spores are thus released and begin to bud as single cells in the normal fashion. In yet other yeasts, the asc is formed by parthenogensis, but the ascospores conjugate two by two immediately prior to germination. Such a yeast is Saccharomyces Ludwigii.

Concerning the later work of Guilliermond ( 34 ) he definitely proposes a chromosomal basis for sporulation. Quilliermond suggests that the following types of yeast must be recognized:

(1) The haplobiontic yeasts: wherein iso-or heterogamic conjugation precedes ascus formation (Schizosaccharomyces, Zygosaccharomyces, Zygopichia, Debaromyces, Nadsonia, Nematospora).

(2)  The diplobiontic yeasts:  wherein conjugation is postponed until later in the cycle ultimately to occur between ascospores about to germinate. (S. Ludwigii, Hansenula Saturnus, and numerous other species of Saccharomyces)

"Haplobiontic" is a term applied to any plant where meiosis, or reduction division takes place as soon as the zygote germinates, the diploid phase being restricted to the zygote.  The term "diplobiontic refers to any plant where meiosis immediately precedes gamete formation, the haplophase being, therefore, restricted to the gametes."  Illustrated graphically:



Haplobiontic                    Diplobiontic

a. haplophase
b. diplophase
c. meiosis
d. mitosis
e. germination of ascospores
f. budding phase
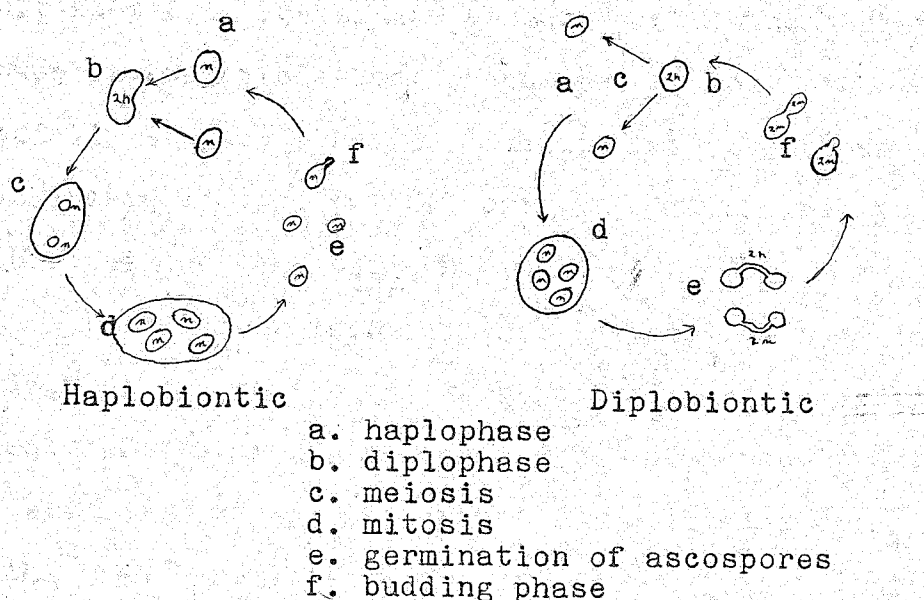
Guilliermond states further that certain aspects of the formation of ascospores seem to indicate that the nucleus divides by karyokinesis, or indirect division.  Cells which are preparing to sporulate assume a very complex structure.  Two sorts of alveoli or foam structures appear in the cytoplasm. Some are filled with metachromatic granules, other with glycogen.  The corpuscles in the former alveoli increase in

number and diminish in volume until they are quite diffuse throughout the alveoli. During this time the nucleus undergoes its first divisions. The actual mechanism is difficult to observe, according to Guilliermond, no one has yet been able to see chromosomes in a yeast nucleus, but one is able to see two small nuclei closely related to each other. Each small nucleus emigrates one to each pole of the cell. A thin thread of plasma unites them still, and the thread has been likened to the achromatic spindle. The thread soon disappears and each nucleus undergoes another division. The condensed cytoplasm or sporoplasm as it is termed, surrounds each nucleus with the result that four spores are formed. In Schizosaccharomyces octosporus the cytological phenomena of sporulation are especially easy to observe. Guilliermond has shown that a karyokinesis similar to that existing in the Ascomycetes exists in this yeast at the stage of sporulation.

To sum up then, and relate this brief review of the cytology of the yeasts to the hypothesis, it seems that certain at least of the yeasts are organisms which should be ideal to work with in studying the effect of colchicine on cell division. In Schizosaccharomyces octosoporus we have an organism which apparently exhibits amitosis during transverse division, and mitosis during sporulation. In the Zygosaccharomyces and in the other haplobiontic species, and indeed in the Saccharomyces too, we have similar phenomena. In other words, we have organisms which apparently fall into both groups one and two on the basis of mitosis and amitosis, and might possibly fall into both groups on the basis of reaction to colchicine treatment.

We are now in a position to proceed to the next step in the hypothesis.

Suppose that colchicine is without effect on budding, as we might expect it to be, and as Richards has apparently proved, but interferes in some way with the phenomena of sporulation. What may we expect as a result of this interference? In the case of the haplobiontic species we might expect to find conjugation with the production of a zygospore, but since meiosis and mitosis are inhibited, we would not expect to find ascospores. In the case of diplobiontic species we might expect an inhibition of meiosis with the result that no gametes would be formed, and consequently no spores would be produced. In the case of parthenogensis without parthenogamy we might expect inhibition of sporulation.

It appears, therefore, that the haplobiontic species provide a good test group as it should be comparatively easy to study the effect of colchicine on the number of spores produced. In the case of the diplobiontic species, non-specific inhibition of sporulation, if it existed would provide confusing evidence.

Finally, let us suppose that we have found that colchicine does not affect budding, but prevents formation of ascospores in the haplobiontic species. Why does colchicine fail to act on the one hand and cause an effect on the other? Perhaps there is some fundamental chemical or physical basis for cell division. Perhaps there is some (and this is highly speculative) hormone-like substance which regulates the phenomena of division in such a way that its presence or absence spells the difference between mitosis and amitosis. Since the

phenomena of cell division are closely related to those of gelation and solution, perhaps the effect of colchicine is only of a physical nature, but it is probably safe to say that if the action of colchicine is physical, then we should not expect to find arrested development of chick embryo fibroblasts in such a small concentration as 1:25,000,000. Bhaduri suggests that colchicine appears to catalyze chemical reactions which increase the fluidity of the cytoplasm and nuclear material thus inhibiting spindle formation.( 35 )

The advantages of working with the yeasts now become obvious. The ease of manipulation of these organisms and the vast amount of literature pertaining to them are two advantages. But for the purposes of this investigation, and it is hoped, future investigations, the most important property of the yeasts is that they divide by two methods. If the cell-division regulating substance exists then we might expect to find it present at one stage and not at another, or perhaps present in smaller concentration at this latter stage. If colchicine interferes with cell division through reacting in some physical or chemical way with this substance, then it is very likely that fractionation of the yeast at the two stages, and comparison of similar fractions in their reaction toward colchicine would show in what fraction this substance exists. It is suggested that if this substance exists it would probably be found in the yeast at the sporulating stage, or immediately prior to this stage.

The work of Richards has been mentioned. He found that colchicine when weighed out and added to the medium for

S. Cerevisiae (Williams medium) over a considerable range of
concentrations was without inhibitory effect on the formation
of the yeast bud. He describes the occurrence of two cycles
in the growth of this yeast in Williams medium, which he
explains by saying that when the concentration of the unfavor-
able products of metabolism reach a certain limit, or when the
concentration of the food falls off to a certain value, there
results a selective killing of the larger buds. The addition
of food just before the end of the first cycle prevents the
retarded growth, and the population grows directly to a
maximum crop. Further, since he found that the addition of
colchicine also eliminates the period of retarded growth, he
states that colchicine must act either as a food or as a buffer
in lessening the increasingly adverse conditions existing in
the medium. He did not investigate the action of colchicine on
the sporulation of his yeast as he is interested only in
normal growth. (32)

## EXPERIMENTAL

### Test Organisms

Three attempts were made to obtain cultures of Schizosaccharomyces octosporus. Twice cultures arrived from the American Type Culture Collection, but both times they were dead on arrival. An unsuccessful attempt was made to obtain a culture of this organism from Lochead and Farrell of the Central Experimental Farm at Ottawa, but their culture had died out. They provided two other yeasts however, their cultures 138 and 58, Zygosaccharomyces priorianus, and Zygosaccharomyces barkeri respectively. (36)

Meanwhile a sample of fermenting honey was obtained through Dr. Eagles who in turn obtained it through Mr. J. Dick.

Employing aseptic precautions throughout, dilutions of 1:10, 1:100, 1:1000, and 1:10,000 of the honey in water were made.

1.0 cc of each of the above dilutions was inoculated into Petri plates by the poured plate culture method, to obtain isolated colonies of the organisms causing fermentation. The medium used was that employed by Lochead and Farrell ( 36 ). The plates were incubated at 28°C and were examined after three days. Smears were made from isolated colonies, and were stained with crystal violet for examination under the oil immersion lens of the microscope. The organisms were rather large oval budding yeasts.

According to Lochead and Farrell, of all the yeasts which they isolated from fermenting honeys, the majority were of the genus Zygosaccharomyces.

A yeast was isolated from the fermenting honey which lacking a complete classification of the organism has been called Zygosaccharomyces X. The cells are mostly oval in young culture. The size is 2.5 x 3.15 microns. In older cultures the cells may be larger, and irregularly round. The actively growing cells multiply by budding. The yeast grows rapidly in 30% glucose broth with a fine even turbidity. A surface ring is almost never formed.

The yeast forms ascospores very slowly on Plaster of Paris blocks, and not at all in Gorodovska's medium. Ascospores are, however, readily formed on 30% glucose agar by both isogamy and parthenogenesis. The number of spores formed by isogamy varies from two to four with at least one spore in each enlarged portion of the asc. Parthenogenetic asci regularly contain four ascospores. Conjugation between exactly similar cells makes its appearance on the agar in 36 hours at 30°C and ascospores may usually be observed after 48 hours under these same conditions. The spores are oval and very smooth. They average about 1.6 x 2.4 microns in size.

The giant colony on 30% glucose agar is very characteristic. It may be observed by placing a loopful of inoculum on the surface of the agar in a Petri plate and incubating at room temperature for four or five weeks. The colony is at first smooth white and round, convex raised with an entire edge. It later becomes roughened about the edge, and finally tree-like

growths appear all around the edge of the colony. The center portion is very smooth. The organism ferments the following sugars, dextrose, galactose, fructose, sucrose, maltose, raffinose. It ferments l-arabinose and mannitol only feebly, and does not ferment xylose, dulcitol, lactose, salicin, inulin, or dextrin.

The organism grows rapidly in 30% glucose broth with the production of a fine even turbidity. No agglomeration of cells occurs, that is, the cells remain quite separate and the organism is ideal for a study of rates of growth by visual counting methods using an haemocytometer, and by turbidometric methods using a modified Fisher Electrophotometer. The organism forms spores rapidly on 30% glucose agar. This yeast is, therefore, a good one to study. The other Zygosaccharomyces which were obtained are not suitable for study for several reasons. #138 forms a surface ring, and forms spores very slowly on all media tried. #58 grows slowly in broth with a granular growth. It forms spores more rapidly than #138, however. Another yeast called Zygosaccharomyces Y also isolated from fermenting honey produces a surface ring.

## The Effect of Colchicine on the Budding Yeast.

If colchicine interferes in some manner with the formation of yeast buds, then of course, the rate of growth of the yeast under defined conditions should be affected. If the process of budding is mitotic, we would expect to find a decrease in the growth rate, and an increase in the size of the cell. Richards has reported that colchicine stimulation of

yeast growth fails to reveal mitosis. While this may be true for Saccharomyces verevisiae, it is not necessarily true for a member of the Zygosaccharomyces until we have proved it so.

The first step in the Experimental was, therefore, to see what effect if any colchicine had on the budding Zygosaccharomycete. Richards employed three different methods in his work on rates of growth; that is, direct count using the haemocytometer, centrifugation of the cells into graduated centrifuge tubes, and a phototurbidometric method. During this investigation, only two methods have been used, that is, the haemocytometer method, and phototurbidometric measurements.

The Medium.

The medium used for all experimental work was based on a similar medium employed by Lochead and Farrell in their work on the osmophilic yeasts (36). They used peptone as the source of nitrogen. In an attempt to approach a synthetic medium and to eliminate the color which might be due to the peptone, asparagine was used.

| | | | |
|---|---|---|---|
| dextrose | 300. | grams | B.D.H.AnalaR |
| asparagine | 0.5 | | Difco |
| $K_2HPO_4$ | 1.0 | | Bakers' C.P. |
| $MgSO_4 \cdot 7H_2O$ | 1.0 | | Bakers' C.P. |
| $NH_4$tartrate | 0.5 | | B.D.H. |
| NaCl | 0.1 | | Bakers' C.P. |
| $CaCl_2.2H_2O$ | 0.1 | | Bakers' C.P. |
| Yeast extract | 1.0 | | S. Orla-Jensen |
| Water | 1 | litre | distilled |

For solid media 2% of agar (B.D.H.) was included and the mixture was sterilized by autoclaving at 15 lbs. steam pressure for one-half hour.

For liquid media the above medium (minus the agar of

course) was sterilized by Seitz filtration (E.K.filter). The
medium was kept in 250 cc lots in sterile 500 cc Erlenmeyer
flasks, and the flasks were sealed with Parafilm until used.
The various lots of media were checked for sterility by in-
cubating at 28°C for 48 hours prior to being stored. Any
flask which showed contamination was discarded. The medium
results in a pH of 6.8. It is almost colorless. It supports
the growth of the Zygosaccharomyces very well, especially
Zygosaccharomyces X which gives a fine even turbidity in 20
hours at 28°C.

## The Incubator.

Two incubators were used. The first was an air
incubator. The temperature of a large asbestos lined box was
kept constant by the use of a sensitive mercury expansion
thermostat and relay. Heating was by a carbon filament lamp.

The other incubator was a constant temperature water
bath. Heating was by a 125 watt knife heater. The temperature
was controlled by an easily constructed thermostat, and a relay
was used. Details of the construction of the thermostat appear
below.
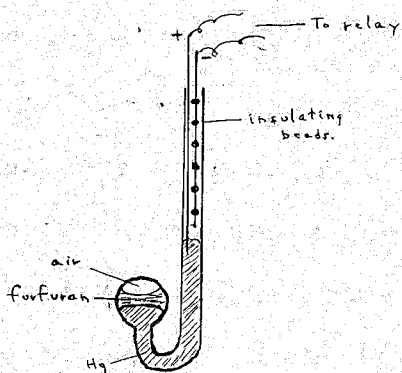


This apparatus controlled
the temperature of the water
bath to within 0.05°C of the
required temperature.

## Preparation of the Colchicine Solutions.

Colchicine was dissolved in water or saline as the case might be. The solutions were sterilized by filtration through a Seitz E. K. filter. The filters were always washed with a large volume of the solvent before sterilization.

## The Haemocytometer Method.

It was early realized that one factor which would have to be eliminated was that of the hydrolysis of colchicine. If colchicine is included in the medium in which the yeasts are growing, there is certain to be some hydrolysis of the alkaloid. Boyland and Huntsman ( 11 ) state that colchicine is 20% hydrolyzed in 24 hours at $37°C$ in 1/50 N HCl. We might expect that Richards' results were affected to some degree at least by the above fact, since the pH of the medium fell off to about 3.5 or thereabouts. The effective concentration of colchicine was thus reduced. However, he should certainly have found an effect over the range employed, had such an effect existed. In an attempt to get around the difficulty outlined above, it was decided to determine a normal rate of growth in the medium, then remove the cells after finishing a run and soak them in 1% colchicine in an isotonic saline solution for about one generation time of the organism. A control culture would be treated similarly except that the cells would be suspended in saline only. The rates of growth would then be compared. It would be expected that if the yeast bud forms by mitosis then the inhibition of budding during treatment

with colchicine with subsequent reinoculation into the medium
should produce a mutant, and the rate of growth should be
different. The determination of normal rate of growth would
serve as an additional control.

## Details of the Method.

Since the errors of the haemocytometer method are
many, its use must be made the subject of careful control and
standardization of procedure. To minimize errors in sampling
20 cc of medium was used in 125 cc Erlenmeyer flasks. This
provided for easy and thorough shaking to suspend all the
growth evenly throughout the culture, and to break up any small
colonies of cells. It is very necessary to have a uniform
suspension of single organisms as it is extremely difficult to
count a colony of cells.

The medium was inoculated with 0.2 cc of a 36 hour
broth culture of Zygosaccharomyces X. The organism had pre-
viously been plated out so as to secure a pure culture.
Several test runs had shown that the best time to inoculate was
on the afternoon of the day preceding that on which the counting
was to be done. Nineteen to twenty-three hours was considered
most satisfactory. The original population of the culture
after inoculation was usually about 100,000 organisms per cc,
as shown by direct count.

The next day the counts were started. Every hour
over a period of 6 hours the flask was removed from the
incubator, and the contents were well shaken, care being taken
not to wet the cotton plug. A small sample was withdrawn with

a Pasteur pipette and the cells were counted at 600X using the
haemocytometer under the microscope. The flask was placed at
28°C again immediately after sampling. The time and count were
recorded each time, and curves were drawn with populations as
ordinates, and times as abscissae.

The control and treated rates of growth were
obtained as follows: 10 cc of a 36 hour broth culture of the
yeast were thrown down by centrifugation. The supernatant was
removed and the cells were washed three times by suspending in
isotonic saline and recentrifuging. The cells were then
suspended for $1\frac{1}{4}$ hours in 1% colchicine (Richards found that 1%
colchicine gave the most stimulatory effect when included in
the medium with S. cerevisiae). For the control the cells were
merely soaked in isotonic saline for the same length of time.
The cells were then freed of colchicine by three washings in
saline as before. The cells were finally suspended in saline
and were inoculated into 20 cc of medium as in the determination
of normal rates of growth. All times were carefully recorded
and an attempt was made to carry out the controls in as closely
similar a way as possible to the tests.

The results of a typical series appear below.

1. Normal Growth Rate:

| Time | : | Count (cells per cc.) |
|---|---|---|
| Inoculated               0: | | 375.000 |
| 19 hrs. 6 min: | | 7,880,000 |
| 1 hr.  8 min: | | 12,470,000 |
| 1 hr. 35 min: | | 21,047,000 |
| 1 hr. 16 min: | | 28,525,000 |

2.  Times taken for treatment of Test and Control.

| Procedure | | Test | Control |
| --- | --- | --- | --- |
| | | Intervals in Minutes. | |
| First Wash. | ( Saline | 0.0 | 0.0 |
| | ( Centrifuge started | 2.0 | 2.0 |
| | (      stopped | 6.0 | 6.0 |
| | ( Saline removed | 1.0 | 1.0 |
| Second Wash | ( Saline added | 2.0 | 2.0 |
| | ( Centrifuge started | 1.0 | 1.0 |
| | (      stopped | 5.0 | 5.0 |
| | ( Saline removed | 1.0 | 1.0 |
| Treat-ment | ( Saline plus colchicine added ( Test) | 2.0 | |
| | ( Saline added ( Control) | | 1.0 |
| | ( Centrifuge started | 58.0 | 60.0 |
| | (      stopped | 5.0 | 4.0 |
| | ( Saline plus colchicine removed ( Test ) | 2.0 | |
| | ( Saline removed ( Control ) | | 2.0 |
| Third Wash | ( Saline added | 1.0 | 1.0 |
| | ( Centrifuge started | 1.0 | 1.0 |
| | (      stopped | 6.0 | 6.0 |
| | ( Saline removed | 1.0 | 1.0 |
| Fourth Wash | ( Saline added | 1.0 | 1.0 |
| | ( Centrifuge started | 2.0 | 2.0 |
| | (      stopped | 6.0 | 6.0 |
| | ( Saline removed | 6.0 | 6.0 |
| Total | | 109.0 | 109.0 |

3.  Treated Growth Rate

| Time | Count (Cells per cc) |
| --- | --- |
| 0 | $12.48 \times 10^6$ |
| 22 min | 12.89 |
| 38 min | 10.1 |
| 25 min | 11.1 |
| 35 min | 19.9 |
| 42 min | 22.59 |
| 66 min | 27.64 |

POPULATION PER CC

5x10⁵

120x10⁶

115x10⁶

110x10⁶

5x10⁶

Treated

Normal

Control

0    30    60    90    120    150    180    210

Time in Minutes
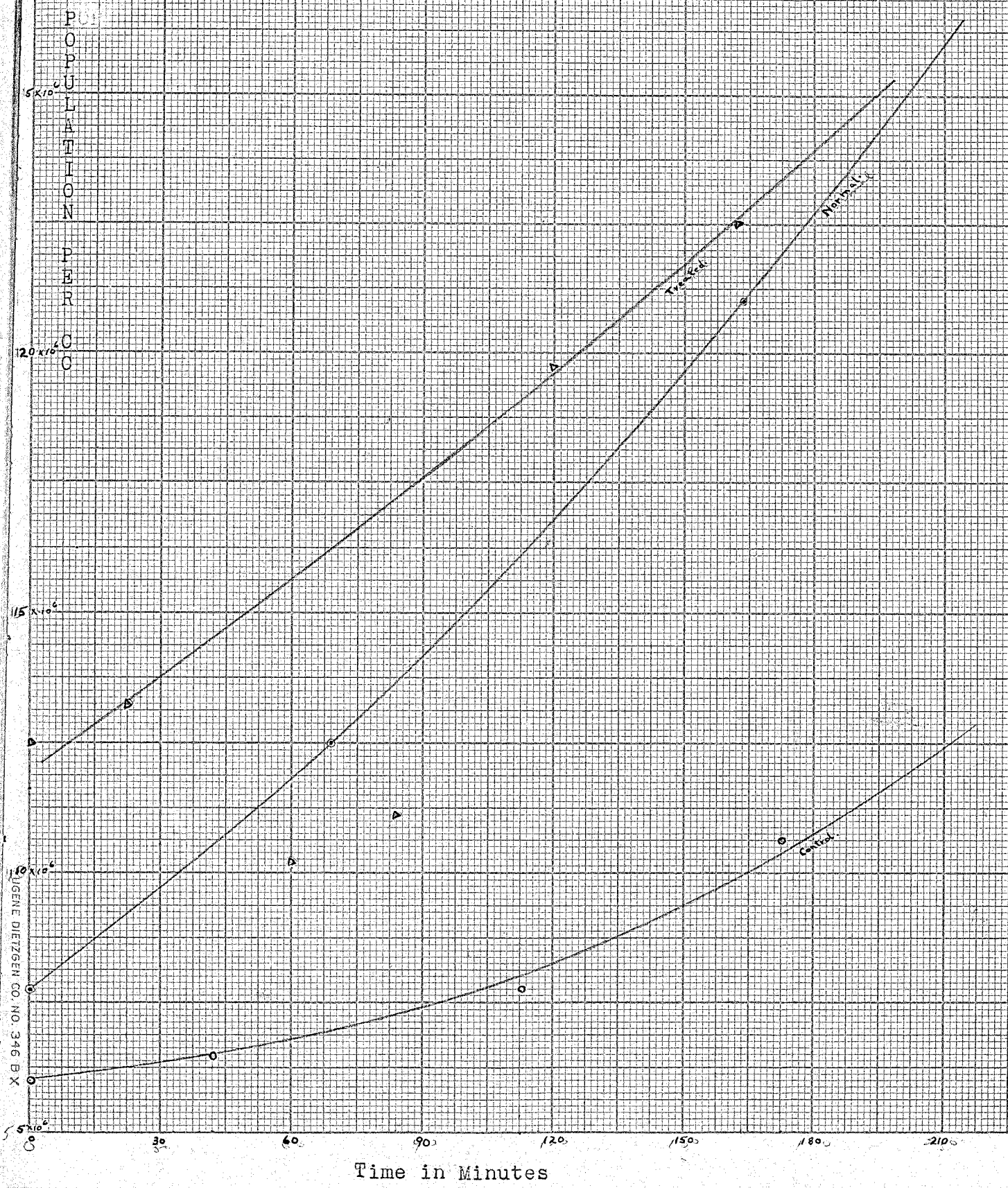
## 4. Control Growth Rate

| Time | Count (cells per cc) |
|---|---|
| 0 | $6.0 \times 10^6$ |
| 42 min. | 6.75 |
| 40 min. | 7.75 |
| 60 min. | 10.66 |
| 53 min. | 11.45 |
| 62 min. | 16.14 |

The generation time of Zygosaccharomyces X in the medium was determined from trial runs. This time is that taken for any given count to be doubled, and should remain relatively constant over the phase of logarithmic growth. For Zygo X in the 30% glucose medium at 28°C under the defined conditions outlined above, it is about two hours. It will be observed that the time of treatment in each, of the test and the control, was about one-half the generation time of the yeast.

It seemed necessary that the saline used should have the same osmotic pressure as the medium to eliminate any effects which might be due to changes in osmotic pressure. By calculation the concentration of sodium chloride required was 4.0%. That this concentration of sodium chloride is isotonic was proven by washing the cells in the saline solution, and leaving them suspended for twenty-four hours. Microscopic examination showed no exploded cells.

## Discussion of Results

The results would indicate that colchicine treatment

of the cells in this fashion was without effect. It is very likely that multiplication ceases as soon as the cells are removed from the medium. It might be expected, therefore, that colchicine would be without effect. It is difficult to suggest an alternative method, however, for treating the yeast at the budding stage in a neutral medium. It was thought that by adding excess solid calcium carbonate to the medium containing colchicine that the pH might be kept near the neutral point. This technique was found to be worthless in this case, however, as the particles of calcium carbonate made a visual count of the yeast population very difficult.

It might now be profitable to consider the difficulties of the haemocytometer count method, and rates of growth measurements in general.

It is obvious that growth rates are affected by many factors. It might be surmised that the rate of growth of a defined organism in a defined medium at a defined temperature over a definite population range would be constant. In the case of facultative anaerobes such as the yeasts, however, additional factors come into play. The quantity of medium employed, the type of medium, and the size and shape of the container all affect the growth rate. The yeasts grow faster in the presence of oxygen than they do in its absence, that is, the growth rate of yeast is affected by the oxygen tension in the medium. The oxygen tension in turn varies directly as the surface area exposed, and indirectly as the depth of the medium. Here lies the explanation of the importance of fixing the quantity of medium to be employed, and the shape and size

of the container. It is well known that growth rates of micro-organisms increase with increased amounts of inoculum in a synthetic medium. This effect is probably due to the fact that organisms such as the yeasts require certain accessory factors for growth, for example, the so-called Bios fractions. The yeasts are able to manufacture these substances since the various Bios fractions may be extracted from their cells, but apparently they do not synthesize enough to meet their requirements. If no bio-activator fractions are included in the medium, therefore, the growth of the cells is slow. As soon as some of the older cells die and autolyze, their contents are released to the medium and the growth rate becomes faster. This explains why the growth rate is faster when a large inoculum is used. We might suppose, therefore, that a more constant rate of growth could be obtained if the activator fractions could be included in the medium. This is the reason for the addition of yeast extract to the medium. The yeast extract was prepared some years ago by S. Orla-Jensen and was obtained from Dr. Eagles. The necessity of including the preparation in the medium was proven as follows.

A sample of medium containing no yeast extract, and another sample with the addition of yeast extract (as in the complete medium on page (22) were inoculated with Zygosaccharo-myces X. Visible growth occurred in nineteen hours in the medium plus yeast extract. No growth appeared in the medium minus yeast extract in two weeks. Identical amounts of inoculum were used.

A complete series, i.e. one normal rate of growth,

one treated rate of growth, and one control rate of growth
required six days to complete. The method is laborious, but
the counts are quite accurate as compared to one another. One
square was counted twice with the following results:

| Number of cells | Number of cells |
|:---:|:---:|
| 65 | 64 |
| 67 | 68 |
| 90 | 92 |
| 92 | 89 |
| 89 | 83 |
| 61 | 58 |
| 77 | 77 |
| 84 | 86 |
| Average 78.1 | Average 77.1 |

$$\text{Count } \tfrac{1}{4} \times 78.1 \times 10^6 \qquad \text{Count } \tfrac{1}{4} \times 77.1 \times 10^6$$

$$= 19.5 \times 10^6 \qquad\qquad = 19.3 \times 10^6$$

In the hands of a practiced person the method gives
reproducible results, but the personal error is nevertheless
large. No two persons would be likely to arrive at the same
count on the same sample. The range of population covered
without making dilutions is small. If dilutions are made, new
errors are introduced. The method has so many disadvantages
that it was decided to discard it.

## The Phototurbidometric Method.

A Fisher Electrophotometer No. 7-089 was modified
for use in phototurbidometric measurements of growth rates.
The instrument contains a lamp and photocell. The e.m.f.
developed by the photocell varies directly as the intensity of
the light falling on it. The instrument employs the potentio-
metric principle, that is, the e.m.f. developed by the photo-
cell is balanced by a graduated slide-wire using a very

sensitive galvanometer. The instrument is made for use with glass cells as receptacles for the standards and unknowns. In the pit for the glass boxes a space $5\frac{1}{2}$ cm by $5\frac{1}{2}$ cm was available so a block of wood was cut $5\frac{1}{2}$ cm on an edge. A 5/8" hole was drilled through it so that the light beam from the lamp would pass through the hole on to the center of the photo-cell. Another 5/8" hole was drilled perpendicular to the first in another face of the block, but not extending through the block. This hole was to hold the culture tubes. The block was painted black.

The instrument was standardized by inserting a tube of distilled water in the perpendicular hole in the wood-block. A black cardboard cover with a hole to coincide with that in the wooden block was placed over the pit. Since the readings with the instrument were to be relative values only, it was easy to eliminate errors which might arise from using ordinary culture tubes. A mark was put on the tube and another was placed on the cardboard cover. When the two marks were placed to coincide, then for consecutive readings on the same tube the errors due to irregularities and defects in the glass cancelled each other. It was only necessary to plot time against scale readings to obtain growth curves.

Trial runs had shown that the best quantity of medium to use in the tubes (each of which was carefully selected for size and freedom from flaws) was 5 cc. The best time for in-oculation was about thirteen hours before counting. The cultures were incubated at 28°C in the water bath.

It was decided to run a series of growth rate

determinations of Zygosaccharomyces X in the medium plus colchicine. A run of 30 tubes was set up as follows.

Test 1 - 10     5 cc medium plus 0.52 cc 1% colchicine, i.e. a dilution of 1:1000

Test 11 - 20    5 cc medium plus 1.04 cc 1% colchicine, i.e. a dilution of 1:500

Control 1 - 5   5 cc medium plus 0.52 cc 4% saline

Control 6 - 10  5 cc medium plus 1.04 cc 4% saline

The above tubes were inoculated with two loopfuls of a two day old pure culture of Zygosaccharomyces at 7:45 P.M. of the day preceding that on which the readings were to be taken. The cultures were incubated at 28°C in the water bath. To aid in identifying the tubes each was numbered with a wax pencil, and the tubes were arranged in three rows of ten each. The plugs of the tubes in the first row were painted red, those in the second row, blue, and the third were left white. The next morning the measurements were started and were repeated on all thirty tubes every two hours for twenty-four hours. The instrument was calibrated against the tube of distilled water by adjusting the scale and galvanometer readings to zero. The culture tube was then removed from the incubator, the outside of the tube was wiped clean and dry using a clean soft cloth, and the contents of the tube were shaken vigorously, care being taken not to wet the plug. The tube was then inserted in the instrument and the new scale reading was determined. The calibration was then checked again before returning the culture to the incubator.

## Colchicine Tests. - see page 33

$C_1$, $C_2$, etc. refer to Test 1, Test 2, etc.

"T" refers to Time in hours as in ships' time.

"R" refers to Instrument reading.

| $C_1$ | | $C_2$ | | $C_3$ | | $C_4$ | | $C_5$ | |
|---|---|---|---|---|---|---|---|---|---|
| T | R | T | R | T | R | T | R | T | R |
| 915 | 9.0 | 916 | 9.5 | 917 | 8.9 | 918 | 11.2 | 919 | 8.6 |
| 1107 | 9.3 | 1107½ | 9.6 | 1108 | 10.0 | 1108½ | 12.7 | 1109½ | 10.1 |
| 1312 | 11.1 | 1313½ | 10.0 | 1315 | 11.1 | 1315½ | 12.3 | 1316 | 10.7 |
| 1548 | 16.1 | 1551 | 14.5 | 1552½ | 14.9 | 1554½ | 14.8 | 1555½ | 14.8 |
| 1824 | 19.6 | 1826 | 15.4 | 1827½ | 18.8 | 1828 | 17.0 | 1829 | 17.0 |
| 2024 | 24.4 | 2026 | 20.1 | 2026½ | 24.1 | 2027½ | 23.4 | 2028 | 20.9 |
| 2211 | 29.2 | 2211½ | 24.0 | 2212 | 27.9 | 2212½ | 26.3 | 2213 | 26.7 |
| 2406 | 34.5 | 2407½ | 28.9 | 2410 | 33.2 | 2410½ | 29.7 | 2411 | 32.1 |
| 151½ | 40.2 | 152½ | 33.0 | 153 | 38.7 | 154 | 34.2 | 154½ | 38.7 |
| 330 | 45.7 | 332 | 38.0 | 333 | 43.5 | 334 | 39.7 | 334½ | 43.0 |
| 500 | 51.2 | 501 | 44.1 | 502½ | 49.1 | 503½ | 45.5 | 504 | 49.6 |
| 833 | 57.6 | 834 | 52.5 | 835 | 58.0 | 835½ | 56.0 | 836½ | 58.6 |

| $C_6$ | | $C_7$ | | $C_8$ | | $C_9$ | | $C_{10}$ | |
|---|---|---|---|---|---|---|---|---|---|
| T | R | T | R | T | R | T | R | T | R |
| 921 | 8.1 | 921½ | 7.0 | 925½ | 8.5 | 926 | 8.2 | 928 | 6.1 |
| 1110 | 8.5 | 1112 | 8.0 | 1113½ | 9.0 | 1114½ | 9.0 | 1115 | 6.1 |
| 1317 | 8.9 | 1319 | 8.5 | 1324 | 9.6 | 1325 | 9.5 | 1326 | 6.4 |
| 1556½ | 14.1 | 1557 | 11.4 | 1600 | 12.7 | 1601 | 12.5 | 1602 | 8.0 |
| 1831 | 17.0 | 1833 | 16.8 | 1834 | 14.3 | 1834½ | 13.9 | 1835 | 9.9 |
| 2029 | 21.9 | 2030 | 22.7 | 2033 | 19.5 | 2034 | 17.5 | 2037 | 16.6 |
| 2213½ | 26.9 | 2216½ | 24.2 | 2217 | 23.4 | 2217½ | 21.2 | 2218 | 15.1 |
| 2411½ | 32.5 | 2415 | 28.1 | 2416½ | 26.5 | 2417 | 24.5 | 2418 | 19.0 |
| 1550 | 37.7 | 158½ | 33.5 | 159 | 30.7 | 200.0 | 29.4 | 200½ | 22.8 |
| 335 | 44.3 | 338 | 38.4 | 338½ | 35.9 | 339½ | 34.7 | 341 | 27.5 |
| 505 | 49.5 | 507½ | 44.9 | 508 | 41.0 | 509 | 39.9 | 509½ | 32.3 |
| 838 | 58.0 | 840½ | 54.8 | 841 | 52.2 | 842 | 53.8 | 842½ | 44.3 |

| $C_{11}$ | | $C_{12}$ | | $C_{13}$ | | $C_{14}$ | | $C_{15}$ | |
|---|---|---|---|---|---|---|---|---|---|
| T | R | T | R | T | R | T | R | T | R |
| 930 | 4.6 | 931 | 7.0 | 933 | 7.7 | 934 | 8.6 | 934½ | 7.0 |
| 1116 | 5.4 | 1117½ | 7.5 | 1119½ | 7.3 | 1123 | 8.6 | 1123½ | 7.7 |
| 1328 | 6.1 | 1329½ | 8.6 | 1332 | 7.8 | 1334 | 9.6 | 1334½ | 8.6 |
| 1605 | 9.1 | 1607 | 12.9 | 1609 | 10.0 | 1611 | 12.2 | 1611½ | 11.2 |
| 1836 | 11.6 | 1836½ | 15.3 | 1839 | 12.7 | 1840 | 14.6 | 1840½ | 12.0 |
| 2038 | 12.4 | 2039 | 14.5 | 2040 | 20.3 | 2043 | 15.4 | 2044 | 14.1 |
| 2218½ | 18.5 | 2219 | 24.4 | 2223½ | 18.5 | 2225 | 21.2 | 2226½ | 16.9 |
| 2419 | 23.0 | 2420 | 31.0 | 2423 | 23.5 | 2424 | 26.9 | 2425 | 21.8 |
| 201½ | 36.4 | 202 | 28.1 | 205 | 28.5 | 206½ | 31.1 | 207 | 25.9 |
| 341½ | 42.3 | 342½ | 33.6 | 345 | 34.6 | 346½ | 36.5 | 347 | 31.1 |
| 510 | 47.5 | 511 | 38.6 | 513½ | 39.3 | 514 | 42.3 | 515 | 36.5 |
| 843½ | 57.1 | 844 | 50.6 | 846½ | 49.1 | 847 | 52.9 | 848 | 47.5 |

| $C_{16}$ | | $C_{17}$ | | $C_{18}$ | | $C_{19}$ | | $C_{20}$ | |
|---|---|---|---|---|---|---|---|---|---|
| T | R | T | R | T | R | T | R | T | R |
| 935 | 8.5 | 936 | 9.6 | 938½ | 7.2 | 945 | 8.5 | 946 | 8.7 |
| 1124 | 9.5 | 1125 | 11.3 | 1126 | 8.0 | 1128 | 8.7 | 1130 | 9.6 |
| 1336 | 10.7 | 1337½ | 14.0 | 1338 | 8.6 | 1341 | 9.6 | 1343½ | 12.7 |
| 1612 | 12.6 | 1613 | 20.3 | 1614 | 12.0 | 1614½ | 12.3 | 1618 | 17.1 |
| 1841½ | 14.8 | 1842½ | 27.6 | 1843 | 15.9 | 1845½ | 16.6 | 1847 | 23.5 |
| 2045 | 17.5 | 2048 | 34.9 | 2049½ | 18.7 | 2025½ | 20.9 | 2054 | 28.0 |
| 2227 | 21.2 | 2228 | 38.4 | 2229 | 24.1 | 2232 | 23.3 | 2232½ | 32.5 |
| 2426 | 26.3 | 2426½ | 45.5 | 2427 | 28.9 | 2430½ | 28.5 | 2431 | 40.0 |
| 208 | 31.1 | 209 | 49.9 | 210 | 34.7 | 212 | 34.9 | 213 | 45.3 |
| 348 | 35.7 | 348½ | 54.1 | 349 | 39.5 | 351½ | 40.9 | 352 | 49.9 |
| 515½ | 41.2 | 516 | 57.5 | 517 | 44.3 | 519 | 45.3 | 520 | 53.2 |
| 849 | 54.0 | 850½ | 65.0 | 851 | 57.6 | 855 | 51.3 | 855½ | 61.9 |

Controls  -  see page 33

$K_1$, $K_2$, etc. refer to Control 1, Control 2, etc.

| $K_1$ | | $K_2$ | | $K_3$ | | $K_4$ | | $K_5$ | |
| --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
| T | R | T | R | T | R | T | R | T | R |
| 947 | 8.0 | 948 | 12.7 | 949 | 9.5 | 950 | 7.6 | 950½ | 8.3 |
| 1131 | 8.1 | 1131½ | 12.7 | 1132 | 10.3 | 1133 | 9.1 | 1134½ | 9.0 |
| 1347 | 9.7 | 1348 | 14.6 | 1348½ | 11.5 | 1349 | 11.4 | 1353 | 11.3 |
| 1620 | 12.5 | 1620½ | 18.1 | 1621 | 15.2 | 1622 | 14.4 | 1623 | 15.1 |
| 1848 | 16.0 | 1849 | 22.4 | 1850½ | 19.0 | 1852 | 18.5 | 1856 | 20.3 |
| 2055 | 20.5 | 2055½ | 27.6 | 2056 | 24.5 | 2057 | 23.5 | 2100 | 26.1 |
| 2233 | 24.0 | 2234 | 31.3 | 2235 | 27.5 | 2236 | 27.5 | 2238 | 31.9 |
| 2432 | 29.6 | 2433 | 38.0 | 2433½ | 33.4 | 2434 | 34.0 | 2437 | 37.9 |
| 214 | 35.2 | 215 | 43.1 | 216 | 39.6 | 216½ | 38.6 | 219 | 42.5 |
| 353 | 41.5 | 353½ | 49.2 | 354 | 45.5 | 355 | 47.5 | 357 | 49.8 |
| 521 | 45.7 | 521½ | 52.6 | 522 | 49.5 | 522½ | 49.3 | 525 | 52.3 |
| 856½ | 55.4 | 857 | 62.5 | 858 | 58.4 | 859 | 59.3 | 903 | 60.6 |

| $K_6$ | | $K_7$ | | $K_8$ | | $K_9$ | | $K_{10}$ | |
| --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
| T | R | T | R | T | R | T | R | T | R |
| 951½ | 8.5 | 952 | 4.7 | 954 | 5.5 | 957 | 8.5 | 959 | 4.2 |
| 1136 | 8.3 | 1137 | 4.7 | 1139 | 6.0 | 1140 | 9.0 | 1141 | 4.5 |
| 1356 | 10.3 | 1358 | 6.1 | 1400 | 6.7 | 1402 | 10.9 | 1404½ | 6.1 |
| 1625 | 13.3 | 1626 | 8.0 | 1627 | 9.0 | 1628 | 14.0 | 1629 | 7.8 |
| 1858 | 17.0 | 1859 | 10.6 | 1900 | 12.1 | 1901 | 18.6 | 1901½ | 12.4 |
| 2101 | 22.0 | 2102 | 12.8 | 2103 | 15.2 | 2104 | 22.5 | 2105 | 16.5 |
| 2239 | 25.7 | 2240 | 16.0 | 2241 | 18.5 | 2241½ | 27.1 | 2242 | 19.5 |
| 2437½ | 31.7 | 2438½ | 21.0 | 2439 | 24.0 | 2440 | 32.4 | 2440½ | 24.3 |
| 220½ | 35.1 | 221 | 28.1 | 222 | 25.9 | 223 | 38.1 | 223½ | 29.5 |
| 358 | 41.6 | 359 | 34.0 | 359½ | 30.7 | 400 | 43.1 | 400½ | 35.7 |
| 525½ | 46.6 | 526 | 36.1 | 526½ | 38.4 | 527 | 46.9 | 528 | 41.1 |
| 904 | 54.9 | 904½ | 47.8 | 905 | 51.1 | 906 | 56.8 | 906½ | 51.2 |

INSTRUMENT READING

TIME IN HOURS

$C_1$

$K_1$

vii

INSTRUMENT READING

TIME IN HOURS
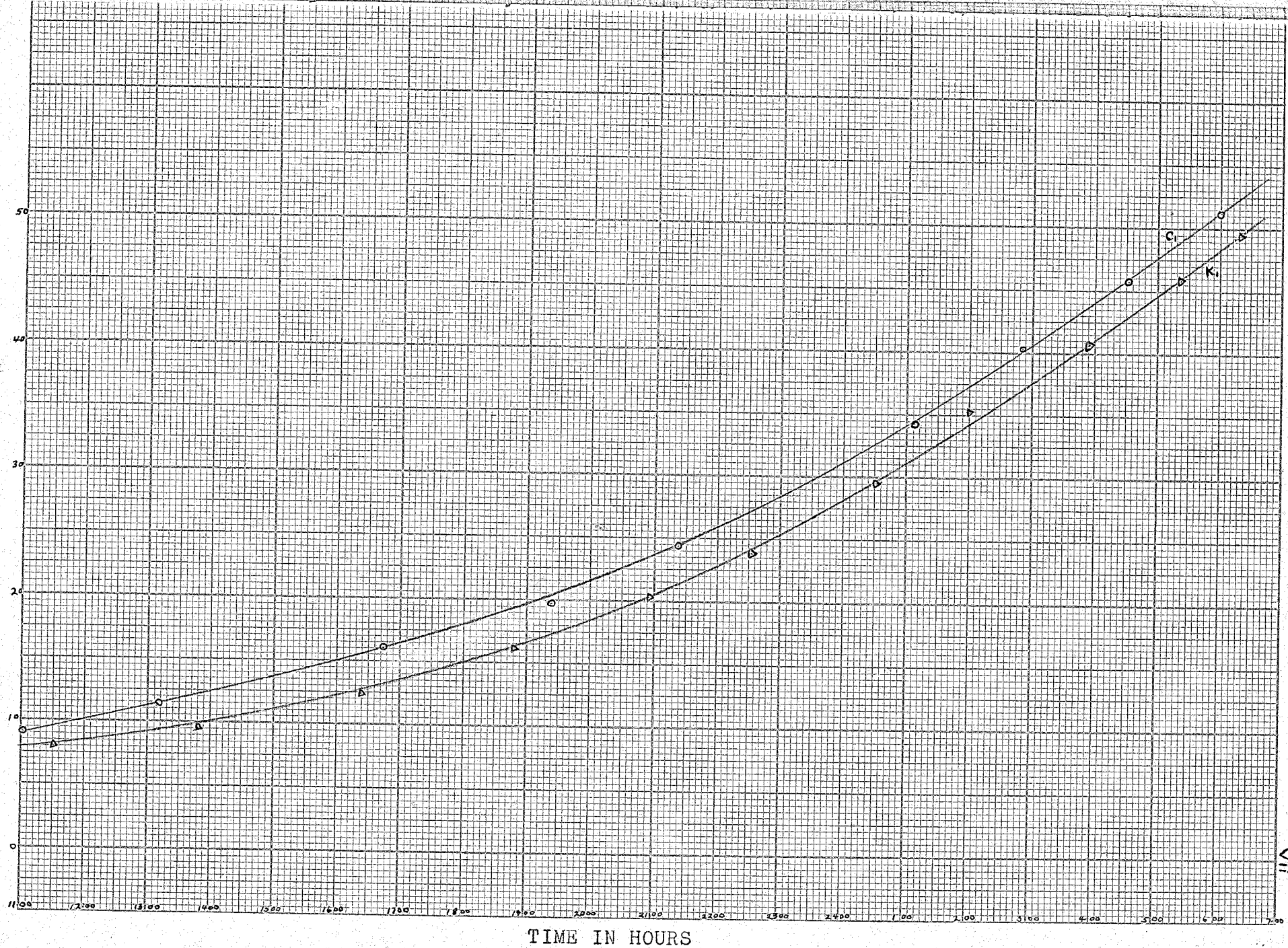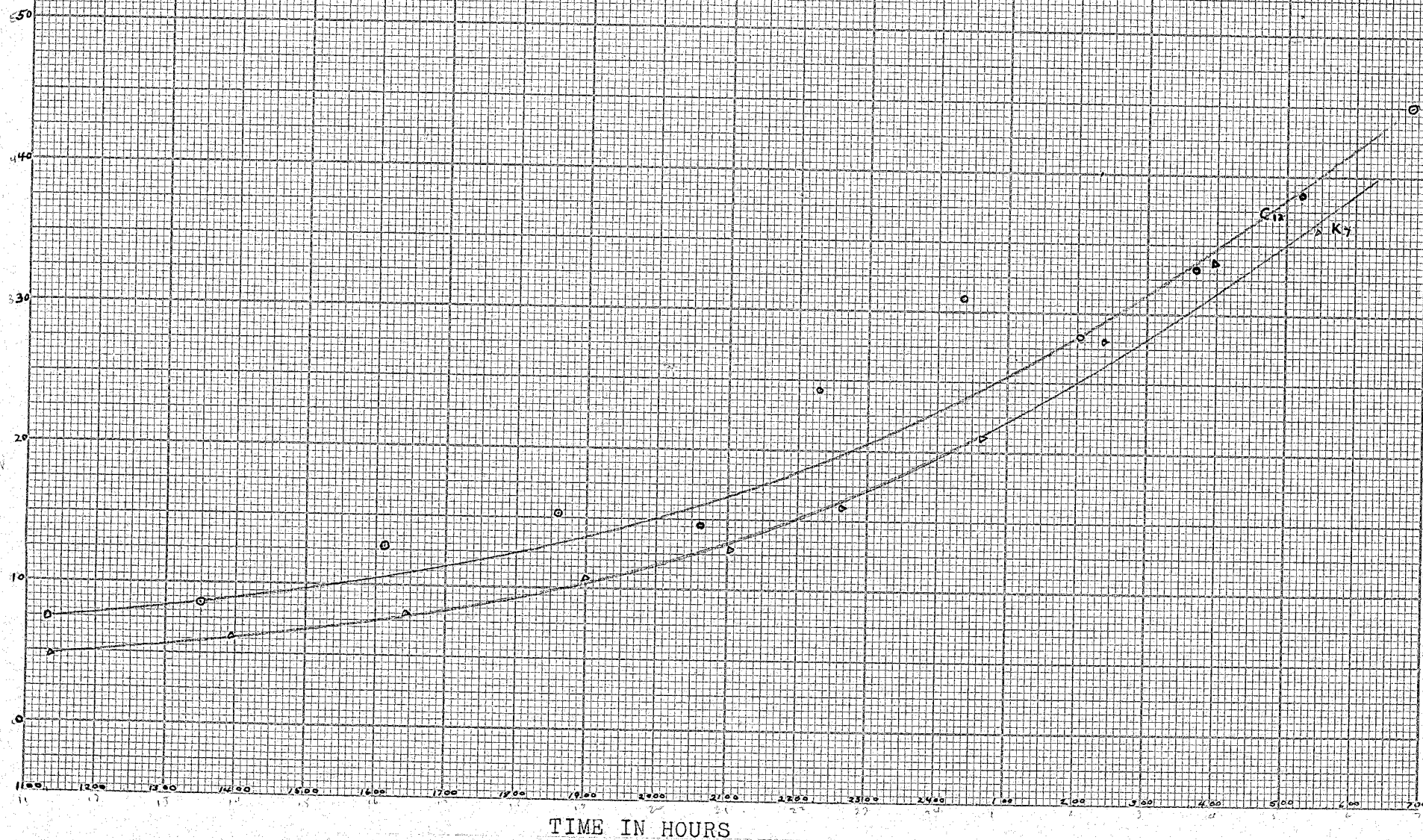
## Discussion of the Results and the Method

It appears that colchicine in dilutions of 1:1000 and 1:500 is without effect on the budding of yeast. The obvious procedure was to follow up this work with increased concentrations of colchicine. It was decided, however, not to continue along this line as it is very doubtful whether the results obtained are of any real significance in view of the fact of hydrolysis of the alkaloid in the medium.

That this method of measuring rates of growth is an excellent one is evident from an examination of the curves obtained. The method is easy to carry out, is quite accurate and is very rapid. To carry out a similar series by the haemocytometer method would have taken six months. The instrument is very sensitive, and personal error is almost completely eliminated. As with other methods of course a rigid control of all procedures must be exercised. The method of shaking for example must be repeated in the same way for each tube. The method is applicable over a wide range of populations. That range used in the above experiments is most satisfactory, however, since it includes only that portion of the curve from the end of the lag phase and beginning of the logarithmic to the point of saturation of the medium with carbon dioxide. The bubbles of carbon dioxide which appear above this point interfere with the measurements.

## Summary of the work on the Budding of Yeast.

It appears that colchicine does not interfere with the formation of the yeast bud, but this statement is made with

reservations. In the first method, in an attempt to eliminate the hydrolysis of colchicine, perhaps no real test of the effect of the alkaloid was made. It is certainly a fact that no method tried has eliminated the factor of hydrolysis, and it is almost impossible to conceive a quantitative method which is likely to get around this difficulty. Every qualitative procedure employed in these investigations has, however, indicated that colchicine is without inhibitory effect on the type of cell division characterized by budding. This will become evident as further experimental work is outlined. It is suggested that any stimulatory effect on cell-division as recorded by Richards is connected with a secondary effect on cell metabolism. Richards (32) states that the alcohol production in cultures of Saccharomyces cerevisiae increases to a maximum of six times the control culture concentration, in 1% colchicine. This fact cannot be explained on the basis of hydrolysis of colchicine alone; i.e. production of methyl alcohol, even assuming complete hydrolysis of the alkaloid. Experiments suggest that colchicine activates some enzyme in the chain of fermentative reactions responsible for Carbohydrate metabolism. It is obvious that an increased rate of metabolism of carbohydrate would result in an increased rate of liberation of energy. Pasteur produced evidence to show that the rate of growth of yeasts was increased under conditions of increased liberation of energy. (33)

## The Effect of Colchicine on Sporulation of the Yeast

It has been stated that Zygosaccharomyces does not

form spores in liquid media, forms them only very slowly on Plaster of Paris blocks, and forms them quickly and easily on agar plates. The effect of colchicine on the sporulation of Zygosaccharomyces priorianus, Zygosaccharomyces barkeri, and Zygosaccharomyces X was investigated.

Broth cultures of the above yeasts were made. After a sufficient time had elapsed to allow the cells to build up glycogen and fat stores (determined by microscopic examination) the cells were removed from the cultures by centrifugation, were washed with saline, and were thence transferred to Plaster of Paris blocks. Aseptic precautions were used throughout.

Test blocks were soaked in 1% colchicine in 4% saline.

Control blocks were soaked in 4% saline.

The plates were incubated at 28°C for three weeks or more. Frequent microscopic examinations, and frequent subcultures were made into liquid media. Evidence was obtained that sporulation was inhibited in the case of Zygosaccharomyces barkeri for after three days the colchicine blocks containing this organism were sterile whereas the control blocks were not. In more detail, the results were these; two subcultures were taken from the control block and two from the test blocks into broth and then after several days spread plates were made from the broth. The two test broths showed no growth. The control showed heavy growth. The spread plates from the test blocks were sterile - the plate from the control was not. This observation may be explained as follows. The yeast, being deprived of food must form spores to perpetuate itself or die. Since colchicine inhibits mitosis and the formation of

ascospores seems to be mitotic, then we should expect sporulation to be inhibited and the yeast would die. Some evidence was also obtained for Zygosaccharomyces priorianus, but the results were not conclusive. They may be tabulated as follows:

| Time of subculture | Control in broth | Test in broth |
|---|---|---|
| 1 week | growth | growth |
| 2 weeks | growth | no growth |
| 3 weeks | 1 growth | 1. growth |
|  | 2 no growth | 2. sl. growth |

After this time the control block was found to be contaminated with a mold. It seems likely, therefore, that chance might have been a factor in obtaining an inoculum from the blocks.

Because it was desired to work with Zygosaccharomyces X it was thought necessary to study the effect of colchicine on the sporulation of this yeast. More or less by chance it was discovered that Zygosaccharomyces X forms spores in two to three days on the 30% glucose agar at 28°C. It was decided to include colchicine in the medium over a considerable range of concentrations. Granted that hydrolysis of the colchicine was not eliminated, it should have been possible by varying the concentration from 1:2000 to saturation to cover the range of possible effect of the alkaloid if sporulation could be induced to occur quickly and at a fairly low temperature.

A test series was run employing a range of 1:2000 to 1:200 using the three Zygosaccharomyces on the agar. Sporulation was not inhibited. Colchicine was made up in water solution in 2% concentration and sterilized by filtration

The yeasts on Calcium carbonate-agar. ( see page 38 )
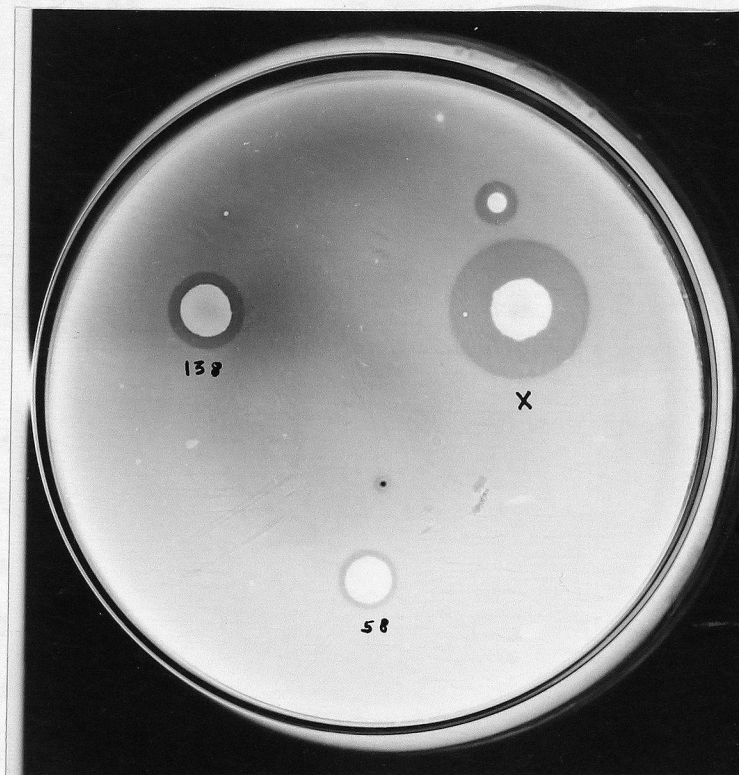
through a Seitz EK filter. The next several series were run
on the same agar to which three grams of solid calcium
carbonate had been added in an attempt to minimize the
hydrolysis of the alkaloid. A typical series was set up as
follows

| Colchicine 2% in water | Agar | Resulting dilution of colchicine |
|---|---|---|
| 0.25 cc | 9.75 cc | 1:2000 |
| 0.5 cc | 9.5 cc | 1:1000 |
| 1.0 cc | 9.0 cc | 1:500 |
| 2.0 cc | 8.0 cc | 1:250 |
| Water control | | |
| 2.0 cc | 8.0 cc | ----- |

Each plate was divided into three segments (see print) and was
inoculated with Zygosaccharomyces X, 138, and 58. Growth of the
cells was not inhibited which is an added indication that
colchicine is without inhibitory effect on the budding of yeasts.
The calcium carbonate which contributed a certain opacity to the
medium was found to have been dissolved around each colony (see
print). The zone around X was larger than that around 138 and
58 because X grows faster than 138 or 58. It was postulated
that the calcium carbonate had been dissolved by an acid
diffusing from the colony as a product of metabolism. This was
proven by growing the organisms on agar containing (a) litmus,
and (b) bromthymolblue. The presence of a red zone around the
colony on the litmus agar plate, and a broad yellow zone on
the bromthymol blue agar plate indicated the production of an
acid. Sporulation was not inhibited in any case above.

In pouring the plates the following procedure was
employed: The required amount of colchicine solution was put in

the plate and the melted agar (at about 40°C) was pipetted in. The colchicine solution and the melted agar were well mixed by rotating the plate, and the mixture was cooled quickly. Since the pH of the agar is very close to seven when calcium carbonate is added, and since the temperature of the agar falls rapidly when admitted to the cold plate, hydrolysis of the colchicine should be almost nil.

It was now decided to investigate the effect of a concentrated solution of the alkaloid on sporulation. 0.5 gm of solid colchicine was added to 10 cc of the melted $CaCO_3$ - 30% glucose agar at 40°C in a plate and the colchicine was dissolved at least to the saturation point by rotating the plate in the usual fashion. Some of the alkaloid remained undissolved after the agar had solidified. The plate was inoculated with Zygo-saccharomyces X and was incubated at 28°C. Budding was not inhibited, but sporulation was almost completely stopped. Parthenogenetic asci did not make their appearance in four days whereas in the control cultures spores appeared formed by parthenogenesis in two days. Only a few spores formed by isogamy made their appearance, and the number of spores was such as to suggest zygotes and not asci were formed. It is true that the colchicine added in this test was not sterile. Four molds made their appearance after five days but the yeast had by this time shown the desired affect. A method of preparing a sterile saturated solution of colchicine was devised. It was desired to eliminate Seitz filtration if possible as filtration always introduces the possibility of change in concentration due to absorption by the filter pad.

Colchicine was dissolved in 70% ethyl alcohol in a sterile flask. The alcohol sterilized the colchicine and was evaporated off in vacuo. Experiments are still proceeding employing this technique to prepare concentrated sterile solutions of the alkaloid and their subsequent use to determine the smallest concentration of colchicine just sufficient to inhibit sporulation.

Two other methods of application of the alkaloid to a study of sporulation were investigated. The first of these procedures was carried out as follows: Zygosaccharomyces X was inoculated on to 30% glucose agar and mass inocula were removed from the colonies at 24 and 48 hours to be transferred to water agar (2% agar in water), and to 1:250 colchicine in water agar. The plates were incubated at 28°C. Sporulation did not appear on either plate in 26 days.

The second method involved the following: a large inoculum of a two day old agar culture of Zygosaccharomyces X was removed from the plate as above and was washed twice in 4% saline. The cells were finally suspended in 4% saline in the controls, and 4% saline plus 1% colchicine in the tests. The suspensions were incubated at 28°C. Sporulation did not occur in a month.

Summary of the Work on Sporulation of Zygosaccharomyces X.

It seems that colchicine is without effect on sporulation of Zygosaccharomyces X over a range of concentration of the alkaloid from 1:2000 to 1:250. The evidence is however for an inhibition of sporulation in concentrations of 1:100 to 4.5%

and this would seem to indicate that the phenomenon of formation of ascospores in this yeast is mitotic. It is noted that a very great difference exists between the concentrations of the alkaloid required to arrest the development of chick embryo fibroblasts (1:25,000,000) and that required to check the formation of spores in the yeasts (1:100 to 4.5%). It is also obvious that the yeast cell is a relatively undifferentiated protoplasm compared to the chick embryo cell. The yeast seems to be intermediate between those cells which divide by true amitosis, and those which divide by mitosis. Perhaps the Zygosaccharomyces are not the best organisms to study in respect to the application of colchicine to a study of cellular chemistry.

Further Experimental with Suggestions for Future Work.

In the light of the observations of Steinberg and Thom on the Aspergilli ( 31 ) and those of the above investigation on the Zygosaccharomyces, it seems likely that other fungi might have been better studied. In fact yeasts other than Zygosaccharomyces X might have been studied to better advantage since it is possible that the yeasts exhibit the variation in susceptibility to colchicine treatment as did the Aspergilli studied by Steinberg and Thom. A yeast which should provide an interesting study would be Schizosaccharomyces octosporus.

The physiological conditions for sporulation of Zygosaccharomyces X should be studied in more detail in an effort to find condition where hydrolysis of colchicine would be minimal. Other compounds such as alpha naphthyl-acetic acid

should be studied and their effect compared to those of colchicine.

Richards (32) has reported that the alcohol concentrations in Williams medium plus 1% colchicine rose to a maximum six times the control value. It is true that his alcohol determinations did not allow for hydrolysis of the alkaloid, but, as stated before, this high concentration of colchicine alone. The production of alcohol according to his graphs starts before much sugar is used up, indicating the hydrolysis of colchicine with the appearance of methyl alcohol in the medium. It seems very likely that colchicine activates one of the enzymes of the zymase complex, and since a chain of reactions can only go as fast as the slowest link, colchicine probably activates one of the slowest enzyme reactions. At least it is logical to suppose that an investigation of these systems would be most profitable. Evidence was obtained from a series of experiments that colchicine has an effect on the enzyme systems of yeast. Old cultures of yeasts which had long since stopped fermenting may be brought back to active fermentation as evidenced by gas production through the introduction of small quantities of colchicine. It is obvious that excess carbohydrate persists in the medium used (30% glucose broth). That the effect of colchicine is not merely due to a possible function as a Nitrogen source was proved by introducing known excellent Nitrogen sources into old cultures of the yeasts. The N-sources used were ammonium sulphate, and ammonium tartrate. No effect was observed in either case. A series of experiments

was set up to try to learn more of the effect of colchicine on fermentation. It was proved that colchicine had no effect on a sterile filtrate of an old culture of Zygosaccharomyces X. Likewise it had no effect on a sterile filtrate plus old washed dead cells (chloroform killed) but gas was produced in cases where sterile filtrate and washed live cells were used. It appears that the method of killing the yeasts had something to do with the observed affect. Chloroform treatment altered the permeability of the cell wall.

Since a yeast growing in a shallow liquid medium is obtaining oxygen both by respiration (molecular oxygen) and fermentation (anaerobic metabolism of carbohydrate) it was decided to try to trace the effect of the alkaloid still further. Time did not allow a continuation of this work beyond outlining a method of attack and carrying out a few preliminary experiments. It is suggested however that these experiments might well be continued. It was decided to inhibit fermentation with iodoacetic acid and to study the effect of colchicine on respiration in the first instance, and to inhibit respiration by growing the yeasts anaerobically to study fermentation alone in the second instance. An apparatus was devised for a study of alcohol production in the presence of colchicine under both respiration and fermentation. Provision was made for passing sterile air through the cultures in the presence of iodoacetic acid. Any alcohol produced was refluxed by a small condenser run at $0^\circ C$ to return to the culture. After incubation for several days the mixture was distilled using a small condenser. One cc of the distillate was collected and two cc of

concentrated sulphuric acid were added. Then 0.1 N potassium dichromate was added until a permanent color was obtained. A green color due to reduction of the dichromate showed the presence of reducing substances. Trial experiments showed that the test is sensitive to 1:5000 of ethyl alcohol in water. It was found that 1:200,000 of iodoacetic acid was sufficient to inhibit formation of volatile reducing substances under conditions of aeration and in the apparatus mentioned above. This meant that less than 1:5000 of ethyl alcohol was produced in the culture. The culture showed very heavy growth under these conditions. The method of testing for reducing substances is a modification of the quantitative procedure outlined by Matiu, Popesco, and Popa ( 37 ) for a micro determination of ethyl and methyl alcohols in mixtures of the two. The qualitative test was devised as a measure of the applicability of the quantitative method to any particular determination. It is suggested that in a mixture containing colchicine where the effect of the alkaloid on the production of alcohol by res-piration or by fermentation is being measured, that the best procedure would be to precipitate the colchicine with phos-photungstic or phosphomolybdic acids. After filtration the filtrate would be distilled and an aliquot part of the distil-late would be taken for analysis by the above procedure. In this way the amount of ethyl alcohol produced by the desired reaction could be determined separate from the methyl alcohol resulting from hydrolysis of the colchicine.

# CONCLUSIONS

The work of Richards concerning the effect of colchicine on the formation of the yeast bud had been corroborated at least in part. Budding seems to be amitotic. Some doubt is to be given to results obtained by the application of the colchicine technique to the fungi in the light of the fact of partial hydrolysis of the alkaloid.

An accurate method of obtaining rates of growth of unicellular organisms by phototurbidometric methods has been devised.

Evidence has been produced that colchicine in one to 4.5% concentration has an inhibitory effect on sporulation of certain members of the genus Zygosaccharomyces.

A possible connection of the problem with the Cancer Problem has been suggested and a mechanism of tumour genesis has been postulated. It is suggested that the production of a tumour in situ by carcinogenic agents depends on the production of a mutant in an exactly similar manner to the production of a mutant by colchicine. Various lines of approach to the problem are outlined.

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