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GENETIC AND BIOCHEMICAL STUDIES OF SUCROSE FERMENTATION

IN THE YEAST SACCHAROMYCES CEREVISIAE

by

Richard A. Hackel

**A Dissertation submitted to the Graduate Faculty in Biology in
partial fulfillment of the requirements for the degree of Doctor of
Philosophy, The City University of New York.**

(1977)

Richard Hackel

This manuscript has been read and accepted for the Executive Committee in Biology in satisfaction of the dissertation requirement for the degree of Doctor of Philosophy.

April 8, 1977

Date

Naam A. Khan
Chairman of Examining Committee
Professor N. Khan

April 11, 1977

Date

Louis Moriber
Executive Officer
Professor L. G. Moriber

N. R. Eaton
Prof. N. Eaton

Brooklyn College
Institution

M. Himes
Prof. M. Himes

Brooklyn College
Institution

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Institution

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ABSTRACT

Genetic and Biochemical Studies of Sucrose Fermentation in the yeast Saccharomyces cerevisiae

by: Richard A. Hackel

Advisor: Professor N.A. Khan

Fermentation of the B-fructosides sucrose and raffinose by the yeast Saccharomyces cerevisiae has been reported to be dependent on the presence of any one of at least five unlinked SUC genes, which determine the presence of invertase (B-fructofuranoside fructo-hydrolase, E. C. 3. 1. 2. 26). The object of the present study was to determine if the SUC genes were regulatory or structural genes for invertase, and to determine if other genetic factors directly or indirectly affect the level of expression of the invertase structural genes.

Sucrose nonfermenting (suc) mutants were induced in yeast strains bearing SUC1, SUC2 or SUC3, and had the following properties: (1) All suc mutants failed to ferment raffinose, although some fermented sucrose slowly while others failed to ferment sucrose at all. (2) The suc mutants were not complemented in crosses to segregational sucrose nonfermenters (suc0); suc mutants crossed to one another failed to complement. (3) The suc mutants were recessive in crosses to SUC fermenters; these diploids were able to ferment both sucrose and raffinose. (4) Tetrad analysis of suc mutant x parental SUC diploids revealed that the suc mutants segregated as single gene mutations which were allelic to the SUC gene carried by the parental strain. (5) The suc mutants lacked both external and internal invertases present in the

parental SUC strains, as shown by Sephadex column chromatography and SDS-polyacrylamide gel electrophoresis. (6) Crude extracts of derepressed cultures of suc mutant x SUC diploids had about one-half the invertase specific activity of parental SUC x SUC diploids. Single-gene mutations resulted in the loss of both invertase activities and the inability to ferment both sucrose and raffinose in these suc mutants.

"Revertants" of various suc3 mutants, obtained spontaneously or induced with MNNG or EMS, were able to ferment sucrose and raffinose. These "revertants" were the result of dominant mutations at genes unlinked to SUC3; these sucrose fermenters were de novo fermenters arising from nonfunctional genes other than suc3 mutants. All de novo sucrose fermenters had invertase activity; the level of invertase could be greater or less than that reported for the parental SUC3 strain. One de novo fermenter, SR3 derived from suc3-94, had a lower invertase activity, grew more slowly on raffinose and its invertase had a lower affinity for sucrose than the invertase of the parental SUC3 or other SUC genes. This revertant may have an altered invertase and thus could be a mutant at a structural gene.

Reversion studies of suc1-D10 yielded one spontaneous revertant, one revertant induced with EMS (D10-ER1) and one revertant induced with nitrous acid (D10-NAR1). Revertant D10-ER1 was dominant and was allelic to SUC1. Revertant D10-NAR1 was recessive and was probably the result of an unlinked "suppressor" mutation. These revertants had less invertase than the parental SUC1 strain and appeared to have only the external invertase.

Induced invertase hyperproducers were derived from the SUC3 MAL3

strain EK-6B by selection for their ability to grow on raffinose plus 2-deoxy-D-glucose (2DG). These mutants, designated DGR, produced higher levels of invertase (α -glucosidase levels were also elevated) under conditions normally repressing invertase synthesis in the parent. Invertases of mutants DGR2 and DGR3 were indistinguishable from that of EK-6B with respect to their K_m 's for sucrose and thermal stabilities. Genetic studies revealed that DGR2 and DGR3 (as well as other DGR mutants) were recessive. DGR2 and DGR3 were different alleles of a gene unlinked to SUC3. This gene, unlinked to SUC3, affects the level of expression of invertase determined by the SUC3 gene.

These genetic and biochemical studies pointed toward the determination of both forms of invertase (external and internal) by SUC1, SUC2 and SUC3, which agreed with previous biochemical studies of SUC2 invertases. That the SUC genes are probably structural rather than regulatory genes was suggested by (1) gene dosage effects of SUC genes, (2) the apparent altered invertase of strain SR3 derived from suc3-94, and (3) the presence of external but not internal invertase in a number of revertants derived from suc1-D10, one of which (D10-ER1) was shown to be allelic to SUC1. The biosynthetic relationship of the two forms of invertase is discussed, as is the role of other genes (DGR mutants) in controlling directly or indirectly the level of expression of invertase structural genes.

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CHAPTER I

INTRODUCTION

Yeasts of the genus Saccharomyces have provided biochemists with a long-standing problem: determining the structure and mechanism of action of invertase, the enzyme which hydrolyzes the sugars sucrose and raffinose (for reviews, see Myrback, 1960; Lampen, 1971).

Geneticists, on the other hand, have been provided with the problems of determining the roles of various genes in the fermentation of these sugars (Winge and Roberts, 1952; Mortimer and Hawthorne, 1969), and how yeast cells control the synthesis of invertase (Lampen, Kuo, Cane and Tkacz, 1972; Gascón and Ottolenghi, Meyer and Matile, 1974).

The seminal work of Jacob and Monod (1961), culminating many years of study of the regulation of lactose utilization in the bacterium Escherichia coli, opened new vistas of research on the genetic control of the synthesis of enzymes in prokaryotic organisms (recently summarized in Beckwith and Zipser, 1970). Eukaryotic microorganisms, particularly yeasts, have also been investigated in order to broaden our understanding of gene regulation (for reviews see Gross, 1969; Hartwell, 1970). Because of their eukaryotic nature, ease of handling which makes them similar to bacteria, alternating haploid-diploid phases in their life cycle and the ready recovery of all four haploid products of meiosis (Hartwell, 1970), yeasts of the genus Saccharomyces, particularly S. cerevisiae, have provided a model system which can give insight into gene function in higher organisms.

The genetic control of the utilization of specific carbohydrates

has long been studied in various microbial systems. Approaches similar to those of Jacob and Monod have broadened our understanding of the diversity and unity in the means bacteria employ in using different carbohydrates (for recent reviews see Englesberg and Wilcox, 1974). Arabinose (Englesberg et al., 1969) and maltose (Hofnung, 1974) are two of the sugars whose use has also been studied in bacteria.

Baker's yeast has been the major lower eukaryote used to study the genetic control of the utilization of specific carbohydrates. Regulation of galactose utilization in S. cerevisiae is one of the better characterized systems in that organism (Douglas and Hawthorne, 1966). A number of structural genes are known to code for proteins essential for galactose utilization: galactose carrier protein (GAL2), galactokinase (GAL1), hexosyl phosphate uridyltransferase (GAL7), UDP-D-glucose-4-epimerase (GAL10) and phosphoglucomutase (GAL5) (for reviews see Mortimer and Hawthorne, 1969; Hartwell, 1970; Barnett, 1976). GAL1, GAL7 and GAL10 are closely linked (Douglas and Hawthorne, 1964). At least three regulatory genes have been shown to affect expression of these galactose structural genes. The GAL1 gene (GAL80) is a repressor gene; recessive mutants constitutively synthesize the enzymes controlled by GAL1, GAL7 and GAL10 (Douglas and Hawthorne, 1966). GAL4 has an operator gene (GAL81) (Douglas and Hawthorne, 1966); mutations in GAL81 allow constitutive synthesis of the enzymes controlled by the GAL4 gene product. GAL4, which is unlinked to GAL1, GAL7 and GAL10, has been shown to exert positive control on these structural genes (Klar and Halvorson, 1974). The system for galactose utilization has provided one of the best

characterized systems for genetic regulation in yeast.

In Saccharomyces, fermentation of maltose is a complex process; many genes are involved (Zimmermann, Khan and Eaton, 1973; for a recent review see Barnett, 1976). While the precise roles of various maltose genes in the utilization of maltose is unclear, several of the known "classical" maltose (MAL) genes have a regulatory function: MAL4 (Khan, Zimmermann and Eaton, 1973); MAL6 (ten Berge, Zoutswelle and van de Poll, 1973); MAL2 (Zimmermann and Eaton, 1974). The relationship(s) of those MAL genes and other genes (Zimmermann, Khan and Eaton, 1973; Khan, 1975) to the induction of maltase and to maltose fermentation is complex and still not well understood. The identification of the actual structural gene(s) for maltase has proved difficult; these genes still remain elusive.

The genetic control of sucrose utilization in Saccharomyces has not often been the subject of study. While genetic studies on maltose utilization have been quite extensive (only a few of the many papers were referred to in the previous paragraph), the genetics of sucrose and raffinose utilization has been largely neglected. There has been some early work (the classical paper of Winge and Roberts, 1952) on the sucrose fermentation genes, and one reference to nonfermenting yeast strains lacking invertase (Gascón, Neumann and Lampen, 1968).

Genes for Sucrose Fermentation in Saccharomyces

Saccharomyces strains can ferment the sugars sucrose and raffinose if they bear any one of at least five "classical" sucrose fermentation (SUC) genes; these genes constitute a polymeric gene

system (Winge and Roberts, 1952). These authors reported that these genes were dominant. Genetic studies of these SUC genes have been mostly confined to their enumeration and mapping on various chromosomes; SUC1 is on chromosome VII where it is tightly linked to MAL1. SUC2 has been mapped on chromosome IX. On the other hand the exact position of the tightly linked SUC3 MAL3 combination has not been determined, neither have SUC4 nor SUC5 (Winge and Roberts, 1952; Mortimer and Hawthorne, 1969). The relationship of these SUC genes to invertase was discussed by Winge and Roberts (1952). The most recent review of sugar utilization by yeasts identified all five SUC genes as structural genes for invertase (see Table IX of Barnett, 1976).

Yeast Invertase

The invertase (B-D-fructofuranoside fructohydrolase, E. C. 3. 2. 1. 26) activity of S. cerevisiae and related species has been intensively studied for over one hundred years; past and current information on yeast invertase has been reviewed (Myrback, 1960; Lampen, 1971). In addition to its commercial and industrial importance, invertase has played a key role in theoretical enzymology; Michaelis and Menten (1913) used sucrose hydrolysis by yeast invertase to demonstrate the relationship of substrate concentration to enzyme activity, which allowed them to discuss enzyme-substrate interactions (discussed in Dixon and Webb, 1964).

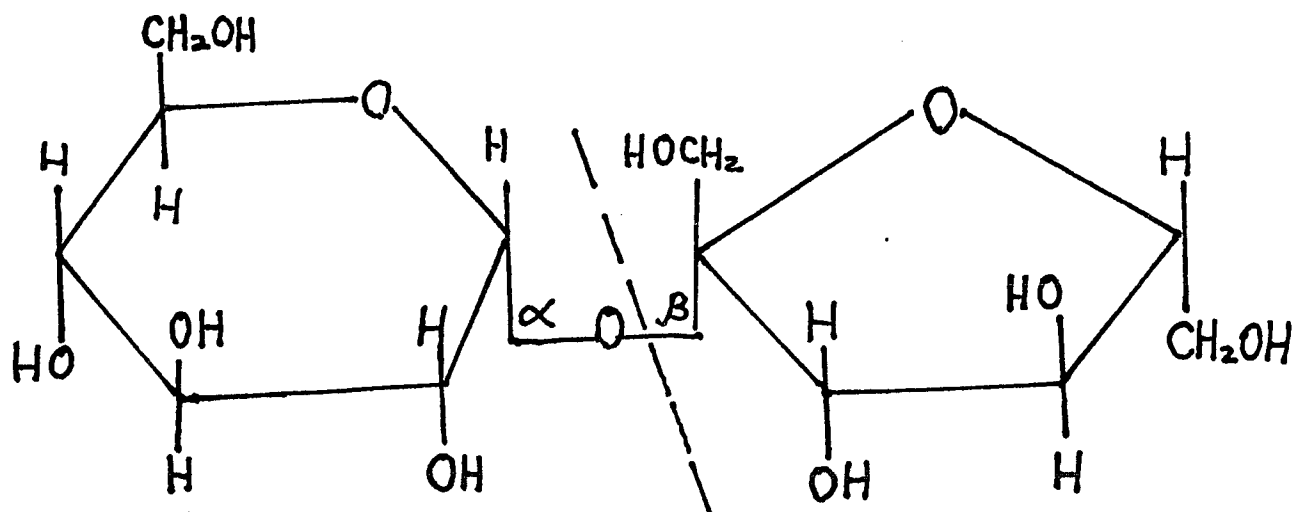
Yeast invertase cleaves the B-fructofuranoside linkage of a number of oligosaccharides, the most important being sucrose and raffinose (Fig. 1). Since sucrose and raffinose are very abundant

in the plant material that forms the natural habitat of Saccharomyces species (Suomalainen and Oura, 1971), invertase is very important in the physiology of these microorganisms.

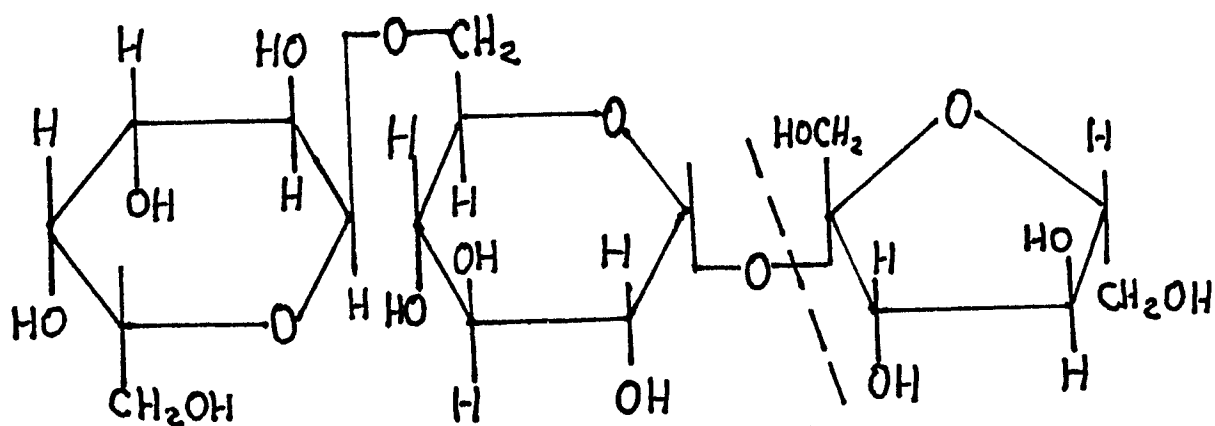
Yeast invertase also has fructose-transferring activity (Myrback, 1960). Fructose can be transferred to primary alcohols, as well as to various monosaccharides (glucose, fructose, etc.). Trisaccharides are the largest oligosaccharides that can be formed. A recent report described the enzymatic synthesis of N-p-nitrophenyl-B-D-fructofuranosylamine using yeast invertase (Baseer and Shall, 1971). This compound was a chromogenic substrate for invertase, the first to be described. Such a compound could be of immense use in genetic studies on invertase and the control of its synthesis.

As can be seen in Fig. 1, yeast invertase cleaves the B-fructosyl linkage on the side of the glycosidic oxygen closest to fructose (Koshland's work described in Lampen, 1971). The nature of the "afructan" group affects the rate of hydrolysis of this B-fructoside linkage (Myrback, 1960). Yeast invertase still elicits considerable interest mechanistically. The two known forms of invertase, external and internal, are reported to have the same mechanism of action (Baseer and Shall, 1971; Waheed and Shall, 1971). These workers reported that there is a histidine residue at the active site; a methionyl residue was also implicated in the enzyme's activity. A more recent paper reported the finding that several tryptophanyl residues in the external invertase were important to its catalytic activity (Leskovac et al., 1975). The inactivation of yeast external invertase with conduritol-B-epoxide, which is structurally related to sucrose, resulted from this compound binding irreversibly to the

Fig. 1. Hydrolysis of B-fructosides sucrose and raffinose by yeast invertase.



SUCROSE, α -D-glucopyranosyl- β -D-fructofuranoside



RAFFINOSE,

α -D-galactopyranosyl-(1 $\rightarrow$ 6)- α -D-glucopyranosyl-(1 $\rightarrow$ 2)- β -D-fructofuranoside

active site (Braun, 1976). Braun considered it likely that a carboxylate group was important as part of the active site.

The drawback with most of these biochemical studies, however, is that genetically undefined yeast strains were used (a point which has also been discussed by Ottolenghi, 1971b). Winge and Roberts (1952) pointed out the relationship between possession of any one of these SUC genes and the presence of invertase (see also Mortimer and Hawthorne, 1969). Both previous to their studies and afterwards, yeast invertase was obtained from yeast strains which were not genetically defined at least as far as particular SUC genes were concerned; often they were polyploid and had several different SUC genes (Lampen, 1971). Some groups have used genetically defined yeast strains; SUC2 has been the SUC gene most commonly studied (Friis and Ottolenghi, 1959; Lampen et al., 1967; Gascón and Ottolenghi, 1972).

It has been stated (Jennings, 1974) that yeast, like other fungi, must hydrolyze the B-fructosides sucrose and raffinose extracellularly if these sugars are to be utilized. Sucrose, however, has been reported to be utilized by yeast strains lacking SUC genes but bearing the constitutive MAL4 gene (Khan, Zimmermann and Eaton, 1973) which controls production of an internal enzyme. Yeast invertase has been located extracellularly, that is, in the periplasmic space (Arnold, 1972) or in the cell wall (Friis and Ottolenghi, 1959). This aspect of yeast invertase has been reviewed (see Myrback, 1960; Lampen, 1971). Invertase activity in intact yeast cells showed the same pH activity curve and sensitivity to inhibitors as invertase in solution (see Myrback, 1960). Using yeast strains bearing known SUC genes,

Friis and Ottolenghi (1959) showed that the major portion of invertase was located external to the cell membrane, since conversion of yeast cells into spheroplasts with snail gut juice released invertase into solution. Furthermore they showed that such spheroplasts could not hydrolyze sucrose, thus demonstrating the role of invertase in making B-fructosides available for use by the yeast cells.

A number of problems have been posed by the relationship between the SUC genes and invertase. Are the invertases controlled by the various SUC genes different enzymatically, and thus presumably different in their amino acid sequence? What is the biosynthetic relationship of the two forms of invertase to each other and to the SUC genes determining them?

Each of the five SUC genes studied produced invertases which did not differ discernibly from one another in most properties (Ottolenghi, 1971b). There were, however, slight differences in K_m s for sucrose and in sensitivity to inhibitors. The SUC2 invertase was the only one sensitive to o-dianisidine, while the invertase of all five SUC genes were sensitive to aniline (Ottolenghi, 1971b). A recent catalog of known genes from Saccharomyces listed invertases specified by the SUC genes as being the products of those genes, i.e. the SUC genes were the structural genes for invertase (Plishchke et al., 1975).

It has also been reported that each SUC gene controlled the presence of both internal and external forms of invertase (Ottolenghi, 1971a; Gascón, Neumann and Lampen, 1968). Ottolenghi reported that the relative proportions of the two invertases were controlled by the environmental glucose concentration. He hypothesized that the SUC

genes probably arose from a common ancestral gene coding for invertase or controlling its synthesis.

Lampen's group has done considerable work on characterizing the two forms of invertase determined by the SUC2 gene (Neumann and Lampen, 1967; Gascón and Lampen, 1968; Gascón, Neumann and Lampen, 1968). They were greatly aided in this by having available an ultraviolet-radiation induced mutant bearing the SUC2 gene which made higher than normal amounts of invertase (Montencourt, 1968).

The external invertase of Saccharomyces is a glycosylated enzyme (a glycoprotein with 50% mannan and 3% glucosamine by weight) with a molecular weight of about 270,000 daltons; it is located mostly in the cell wall (Neumann and Lampen, 1967). The internal invertase from the same strains has been found to have a molecular weight of about 135,000 daltons, lacks any covalently linked carbohydrate and has similar kinetic constants to those of the external invertase (Gascón and Lampen, 1968). There were differences, however, in their amino acid composition and in some enzymatic properties; while both forms of invertase, when purified, have optimal activity in the range pH 3.5 to 5.5, the external invertase is more stable at acid pH (3 to 7.5) than the internal invertase which is stable at pH 6 to 9; the external invertase contains some cysteine or cystine residues which the internal form lacked. The external invertase appeared to be richer in tyrosine, methionine than the internal; but the internal invertase may have more glycine, alanine, serine, histidine and lysine than the external invertase (Gascón, Neumann and Lampen, 1968). The similarities and differences that various workers have found in the two forms of invertase have raised the question: how are these two

invertases related?

A number of lines of evidence was interpreted by some workers to suggest that the two invertases have a precursor-product relationship. Moreno et al. (1975) reported that there is a step-wise addition of carbohydrate to the precursor of external invertase, as judged by a series of external invertases of decreasing molecular weight eluting from Sephadex G-200. Cycloheximide and 2-deoxy-D-glucose were reported to inhibit the synthesis of both external and internal invertases in protoplasts (Liras and Gascón, 1971) of the diploid strain 303-67 homozygous for SUC2 and the induced invertase hyper-producer FH4C derived from it (Montencourt, 1968). Liras and Gascón found that these inhibitors reduced the amounts of both forms of invertase to the same extent even though these authors considered cycloheximide to inhibit translation and 2-deoxy-D-glucose to inhibit glycosylation of the external invertase protein, and therefore its secretion. Further evidence supporting the precursor-product relationship was the finding that a vacuolar fraction prepared from SUC2 strains (Beteta and Gascón, 1971), when applied to Sephadex G-200 columns, had both internal and invertases. It was not conclusively proven in any of these cases, however, that the internal invertase was actually glycosylated to form the external invertase; the nonglycosylated external invertase may not be identical to the internal invertase.

That the internal invertase may not be the precursor of external invertase derived in large measure from Lampen and co-workers, who used the same SUC2 strains as the groups of workers in the previous paragraph (Moreno et al., 1975; Liras and Gascón, 1971; Beteta and Gascón, 1971). A comparative study of the two invertases (Gascón,

Neumann and Lampen, 1968) indicated differences in amino acid composition and in some enzymatic properties, despite similar enzymatic mechanisms of action being proposed for both (Baseer and Shall, 1971a). Baseer and Shall hypothesized that the mechanism of action of both forms of invertase involved acid catalysis by an imidazolium cation at the active site leading to an electron deficient intermediate stabilized by a carboxylate anion also at the active site; this was based on similar results for the two invertases in studies using various inhibitors and sulfhydryl reagents. Physiological studies on invertase biosynthesis have shown that radioactively labelled amino acids were incorporated into the two forms of yeast invertase differently (Lampen, Kuo, Cano and Tkacz, 1972). Protoplasts, prepared from strain 1016 which was an invertase hyperproducer also derived from the SUC2 strain 303-67 (Montencourt, 1968), incorporated label more effectively into the larger invertase (external) than into the smaller (internal) invertase. Chasing with unlabelled amino acids resulted in more label disappearing from the external invertase than from the internal invertase. It was concluded by these workers that the external invertase was not made from the bulk of the small invertase.

A more recent study has shown that when secretion of the external invertase stopped, a large invertase activity accumulated intracellularly; there was no change in the small internal invertase activity suggesting that the latter was not a precursor of the (large) external invertase (Meyer and Matile, 1974). Because of the lack of evidence clearly showing which view of the relationship between the two invertases is correct, the question still remains: are the two forms of yeast invertase biosynthetically related, or are they

distinct proteins? This is pertinent to whether some or all of the SUC genes control the presence of both invertase activities either as regulatory genes or as structural genes for the polypeptide(s) which may compose invertase.

Control of Invertase Biosynthesis in *Saccharomyces*

Invertase biosynthesis in *Saccharomyces cerevisiae* and related species is not inducible but is subject to repression and derepression by the glucose concentration in the medium (Gascón, Neumann and Lampen, 1968; Gascón and Ottolenghi, 1972). It thus differs from the α -glucosidase maltase (Khan and Eaton, 1967) which is subject to induction by maltose, as well as to glucose repression (Zimmermann and Eaton, 1974).

That invertase production in yeast is related to the glucose concentration in the external medium has been known for fifty years (Willstatter et al., 1925). Yeast slowly fed sucrose increased their invertase content (de Ley and Vandamme, 1950). Growth of yeast on raffinose, for which yeast invertase has a lower affinity than for sucrose (Km of 150 millimolar raffinose versus Km of 25 millimolar sucrose, Gascón, Neumann and Lampen, 1968) was reported to result in even higher levels of invertase (Davies, 1953). Davies also reported that hexoses in the medium repressed invertase formation: various intermediates in glucose metabolism (acetaldehyde and acetate; malic and succinic acids) were also reported to repress invertase biosynthesis (Dodyk and Rothstein, 1964)

The production of invertases by a SUC2 strain was found to be

inversely proportional to the glucose concentration in the medium (Gascón and Ottolenghi, 1972). A glucose concentration of 0.05% was found to allow maximal invertase synthesis in cells grown in continuous culture. The external glucose concentration appeared to affect the formation of the two forms of invertase differently (Friis and Ottolenghi, 1959; Gascón, Neumann and Lampen, 1968); the external invertase increased proportionally much more than the internal invertase when the cells were released from glucose repression (Ottolenghi, 1971; Gascón and Lampen, 1968).

It has been reported that ultraviolet-induced mutations in a diploid SUC2 yeast strain (303-67) gave rise to various apparently haploid strains (DNA content was reported to be about one-half that of the parent) which, among other changes, showed hyperproduction of invertases in the presence of glucose concentrations which normally repressed invertase biosynthesis (Montencourt, 1968; Montencourt, Kuo and Lampen, 1973). The hyperproducer strains differed from the parental strain in showing extensive flocculation, a long lag in growth on fructose or mannose, lack of sporulation when crossed to other haploids and the inability to grow on hexose-breakdown products as the sole sources of carbon (Montencourt, 1968). Genetic analysis of these mutants (FH4C, etc.), however, was not possible since diploids containing FH4C were unable to sporulate.

The way in which the external glucose concentration affects the level of invertase or other enzymes in yeast cells is not understood, but could conceivably operate in a manner like that reported for various prokaryotes (Magasanik, 1970). The external glucose concentration may affect the level of some small modulating molecule,

such as cyclic AMP, which interacts with various protein and nucleic acid species and could affect the transcription or translation of invertase messenger RNA. The relationship between the intracellular concentration of cyclic AMP and genetic regulation has been the subject of a number of reviews (positive control by Englesberg and Wilcox, 1974; cyclic AMP by Rickenberg, 1971; glucose repression by Magasanik, 1970). Cyclic adenosine-3',5'-monophosphate (cyclic AMP) has been implicated in hexose repression (carbon catabolite repression) both in prokaryotes and in yeast (Schlanderer and Dellweg, 1974; Wheeler et al., 1974).

The hexose repression resistant mutants obtained in Lampen's laboratory (Montencourt, 1968) appeared to make considerably more invertase than the parental strain, although there was only a small difference in cyclic AMP levels (Montencourt, Kuo and Lampen, 1973). It has been reported that cyclic AMP levels in cells of the fission yeast Schizosaccharomyces pombe were inversely proportional to the glucose concentration in the medium (Schlanderer and Dellweg, 1974). These authors reported that a partially catabolite repression resistant mutant had higher levels of cyclic AMP than the parent strain, when both were exposed to the same glucose concentration. Invertase and α -glucosidase activity in their strains was found to increase proportionally as the cyclic AMP concentration increased. These authors concluded that changes in the levels of cyclic AMP with growth conditions affected the rate of enzyme formation.

How carbohydrates affect adenylate cyclase activity in Saccharomyces cerevisiae has been studied by Wheeler et al. (1974), who found a negative modulation of adenylate cyclase activity (which

synthesizes cyclic AMP from adenosine triphosphate) by carbohydrates. Glucose was most inhibitory; sucrose was least. The authors considered the sugar concentrations used in their experiments to be physiological and similar to those reported to influence adenylate cyclase in Escherichia coli. They hypothesized that this could be one possible control mechanism of carbon catabolite repression.

This work

As stated earlier in this Introduction, there have been few genetic investigations of the relationship of the SUC genes of Saccharomyces to invertase formation and to sucrose and raffinose fermentation, despite considerable biochemical interest in this enzyme. This thesis presents a genetic and biochemical study of the role of the SUC genes in the formation of yeast invertases. The relationship of SUC1, SUC2 and SUC3 to invertase and to the control of its synthesis is investigated. The role of additional genetic factors modifying the expression of the SUC genes is also studied.

In the present study a number of suc mutants induced in strains bearing particular SUC genes were unable to ferment both raffinose and sucrose. These suc mutants were uniformly found to be allelic to those SUC genes and lack both forms of invertase (external and internal) present in the parental SUC strains. Genetic and biochemical studies of these suc mutants and revertants derived from them suggested that these mutations were most likely to be at the structural genes for invertase, preventing the formation of functional invertase. Furthermore, a gene dosage effect of the SUC genes and their suc

mutants in diploid strains also pointed to the SUC genes probably being structural genes. Mutations at several genes unlinked to SUC3 were found to affect the level of expression of invertase structural genes, causing hyperproduction of invertase. It was concluded that the expression of the SUC genes, which could best be considered to be structural genes for the two forms of invertase, was influenced directly or indirectly by the products of other genes.

CHAPTER II

MATERIALS AND METHODS

Yeast Strains. The properties of the various yeast strains used in this study are described in Table 1. The strains bearing the SUC genes ferment sucrose within one to two days and ferment raffinose one-third within five days at 30°C. It is assumed that the fructose moiety of raffinose cleaved by invertase is used, and the melibiose also produced is not utilized further since these strains fail to utilize melibiose. In strain EK-6B SUC3 and MAL3 are tightly linked; in strain 4059-358D, SUC1 and MAL1 are also tightly linked.

The various segregational sucrose nonfermenters lack active classical SUC genes and are designated suc0. Most lack classical maltose fermenting (MAL) genes and are designated mal0.

Culture Media. Media for general growth purposes and for fermentation tests were used as described below. To make solidified media 1.5% bacto-agar (Difco) was added.

YEPD contained 1% yeast extract (Difco), 2% bacto-peptone (Difco) and 2% glucose (usually Difco dextrose).

NAD was modified nutrient agar plus dextrose. It contained 23 gm of nutrient agar (Difco) per liter, 1% yeast extract and 2% glucose.

Fermentation tests were performed in test tubes containing 5 ml of media plus a small inverted (Durham) tube. All media contained 1% yeast extract and 2% peptone. Raffinose tubes contained 2% raffinose (Difco). Sucrose tubes contained 2% sucrose and bromthymol blue (Difco); 5 ml of a 1.5% bromthymol blue solution (50% water/50% ethanol) was added per liter of medium. Maltose tubes contained 2%

TABLE 1: Yeast Strains

Strain Designation	Genotype	Comments (Source)
<u>Sucrose (SUC) gene strains</u>		
EK-6B	a SUC3 MAL3 ade2 ade8 leu2	ferments sucrose in one day, raffinose in three (N. A. Khan)
M6-1D 1A	Δ SUC2 mal0 ade2 his2 leu1	same as above, nuclear petite (F. K. Zimmermann)
4059-358D	Δ SUC1 MAL1 MGL adel ade2 leu1 CUP	same as EK-6B (S. Fogel via N. A. Khan)
1355-12B	a SUC4 mal0 gal0 adel leu1 ura	same as EK-6B (J. Friis via F. K. Zimmermann)
2080-8C	a SUC5 mal0 ade6	same as EK-6B, cells clump (J. Friis via F. K. Zimmermann)
<u>Segregational sucrose (suc0) nonfermenters</u>		
AZ-5A	Δ suc0 MAL1 trp1	F. K. Zimmermann
Z1-2D	Δ suc0 mal0 leu1 lys1	N. A. Khan
Z7-17D	Δ suc0 mal0 trp1	F. K. Zimmermann
EZ-5B	a suc0 mal0 trp1	F. K. Zimmermann
<u>Mating type testers</u>		
a tester	a SUC ural	L. Melnick, J. Blamire
tester	Δ SUC lys1	J. Blamire
AZ-5A	Δ suc0 MAL1 trp1	F. K. Zimmermann
AZ-5A S1	Δ SUC MAL1 trp1	UV-induced <u>de novo</u> sucrose fermenting mutant derived from AZ-5A

maltose (Difco) and bromcresol purple (Difco); 5 ml of 1.5% bromcresol purple stock solution were added per liter of medium.

Growth of various yeast strains on different sugars was studied in the above media omitting the indicator dye in each case.

Synthetic minimal (SM) medium contained, per liter: 6.7 gm yeast nitrogen base without amino acids (Difco) and 20 gm glucose.

Complete synthetic (CS) medium was based on that of Wickerham (1946). The following were added to SM:

Constituent	Concentration of stock solution (%)	volume (ml) added per liter	final concentration (mg/liter)
L-leucine	1	6	60
L-threonine	6	5	300
L-arginine-HCl	1	4	40
adenine sulfate	1	2	20
L-tryptophan	1	2	20
L-histidine-HCl	1	1	10
L-methionine	1	1	10
uracil	1	1	10
L-lysine-HCl	2	1	20

To test for nutritional requirements, single constituents of CS medium were omitted to make a drop-out series.

To study growth characteristics on defined media with a sugar other than glucose, SM or CS was prepared without glucose, and the desired sugar (raffinose, sucrose, etc.) was sterilized separately and then added.

Sporulation-potassium acetate (KAC) medium (McLary et al., 1959) contained 0.98% potassium acetate, 0.25% yeast extract and 0.1% glucose.

Media for selection and identification of mutants.

SUC-EMB (sucrose-eosin-methylene blue) agar contained 1% yeast extract, 2% peptone, 2% sucrose, 0.1% glucose and 1.5% agar. After autoclaving the following were added per liter of medium: 20 ml of 1% eosin (in 50% ethanol) and 6 ml of 1% methylene blue (in 50% ethanol).

SUC-BTB agar contained 0.5% yeast extract, 1% peptone, 2% sucrose (Difco), 0.04% bromthymol blue and 1.5% agar. The sucrose was sterilized separately by autoclaving or filter-sterilizing before being added to the rest of the components.

SUC or RAF agar contained 0.5% yeast extract, 1% peptone, 2% sugar and 1.5% agar. The sugars were always sterilized separately; raffinose was always filter-sterilized.

Mutagenesis

The various SUC gene-bearing strains listed in Table 1 (except the SUC4 and SUC5 strains) served as parents from which mutants defective in their ability to ferment sucrose and raffinose were obtained. Strain EK-6B was mutagenized with either ultraviolet radiation or with ethylmethane sulfonate (EMS). The doses used gave approximately 50% survival (Zimmermann, Khan and Eaton, 1973). For induction of sucrose nonfermenting mutants in 4059-358D (SUC1) and M6-1D 1A (SUC2), only EMS was used.

Identification of sucrose nonfermenting (suc) mutants on selection media.

Raffinose agar and SUC-BTB agar media proved to be the most satisfactory media for the isolation of suc mutants. Nonfermenters grew more poorly on raffinose agar medium than fermenters, and non-

fermenters were different in color and texture. On SUC-BTB agar medium nonfermenters also grew poorly, and the colonies were greenish in color. Sucrose fermenters on the other hand formed large colonies on this medium and changed the color from green to yellow-orange.

EMS Mutagenesis. The procedure for EMS mutagenesis was a modification of the method of Sherman et al. (1971). Cells of the strain to be mutagenized were grown overnight in 5 ml of YEPD. The cells were harvested and washed twice with sterile distilled water. The final pellet was resuspended in 2.1 ml of sterile 0.1 M sodium phosphate buffer, pH 7.0. After removing 0.1 ml of this suspension for a viable count, 0.05 ml of EMS (Eastman Kodak lot number 701B) was added. The suspension was mixed in a vortex mixer and incubated in a roller drum at 30°C for 30 minutes. An aliquot of 0.2 ml of the treated cell suspension was transferred to 8 ml of sterile 5% sodium thiosulfate (Fisher A. C. S. grade) which stopped the reaction. The mutagenized cells were diluted and plated onto SUC-EMB agar plates which were incubated at 25°C for 16 hours to fix the mutations (Zimmermann, Schwaier and von Laer, 1966); the plates were then incubated at 35°C. After five days potential sucrose nonfermenting mutants were picked from the plates. Sucrose nonfermenting colonies were not easily distinguished from respiratory deficient "petite" mutants able to use sucrose slowly and inefficiently. All potential sucrose nonfermenters, however, were screened on glycerol-containing agar plates to distinguish sucrose nonfermenting mutants from the more abundant "petite" mutants.

In some experiments, after EMS mutagenesis, the survivors were inoculated into YEPD broth and incubated overnight at 30°C to fix the mutations. Then the cells were diluted and plated onto 10 to 20 YEPD

or NAD plates and incubated at 30°C. After 3 to 5 days these plates were replica-plated onto the SUC-BTB or RAF agar plates. The latter were incubated at 35°C for 3 to 5 days, and then examined for possible sucrose nonfermenters.

Spontaneous Revertants. Spontaneous revertants were obtained by spreading 1 to 2×10^7 cells of a suc mutant on an agar plate containing either sucrose or raffinose as the sugar. These plates were then incubated at 25°C for one to two weeks. Revertants were then purified and tested.

Induced Revertants. To obtain induced revertants, a few crystals of N-methyl-N'-nitro-N-nitrosoguanidine (MNNG, Sigma Chemical Company, lot 120C-0920) were placed in the middle of a raffinose or sucrose plate spread with 1 to 2×10^7 cells; this was a modification of the method of Fink and Lowenstein (1969). The plates were then incubated at 25°C for one to two weeks, when small colonies appeared.

Revertants were also obtained by treating nonfermenting cells with either EMS or MNNG, washing with sterile distilled water and then plating to a density of 1 to 2×10^7 cells per sucrose or raffinose agar plate. These were incubated at 25°C.

Induction of Invertase Hyperproducer Mutants. To obtain mutants which had invertase activity considerably higher than the parental SUC-gene bearing strain, a procedure employing the glucose analog, 2-deoxy-D-glucose (2-DG) was used (Zimmermann, F. K., personal communication; a procedure for obtaining repression-resistant mutants in Saccharomyces carlsbergensis which made very high levels of invertase was recently described by Toda (1976) who also used 2-DG). It was reported by Zimmermann that 2-DG blocks the utilization of maltose,

and perhaps raffinose, by yeast strains capable of using them. Mutants resistant to 2-DG will grow on raffinose in the presence of this glucose analog. Cells of strain EK-6B were grown on YEP 10% glucose overnight, which repressed invertase synthesis (Gascon and Ottolenghi, 1972). These cells were then harvested, washed twice in sterile, distilled water and spread onto the surface of a number of raffinose plates. After the plates had dried, a central well was cut into each plate; 0.2 ml of 25 mg 2-DG/ml was added. On a number of the plates, a few crystals of MNNG were placed, and the plates were incubated at 30°C. A control plate without MNNG was also prepared. After one or two days growth at 30°C, all plates showed a broad zone of inhibition of growth around the central well. Only the plates spread with mutagenized cells showed resistant colonies within the zone of inhibition. These resistant colonies were purified and studied.

Genetic Analysis

Genetic Crosses. Crosses between haploid strains of opposite mating type were carried out by mixing the cells in a tube of sterile YEPD broth; diploids were selected on the appropriate selection media. Sporulation of diploids was induced on potassium acetate (KAC) agar. Asci were dissected according to the method of Johnson and Mortimer (1958).

Random Spore Analysis. When crosses gave poor spore viability, or a specific genotype was required, random spore analysis was performed. Asci were digested in 0.05 ml of a 1:10 dilution of Glusulase (Endo

Laboratories) in sterile distilled water for two and one half hours at room temperature. Then one ml of sterile water was added, the suspension was vigorously mixed on a vortex mixer, appropriately diluted and then plated onto YEPD agar or NAD plates. These plates were incubated at 30°C for several days. Haploid colonies were identified by their growth on complete synthetic agar and failure to grow on synthetic minimal agar.

Enzymatic Assays

Invertase. Invertase activity was assayed by following the release of glucose from sucrose. The procedure employed was a modification of the method of Ottolenghi (1971b). The reaction mixture contained 0.1 M sodium acetate buffer, pH 4.5, 0.15 M sucrose and 0.1 ml of either crude extract or whole cells in a total volume of one ml. The reaction, started by adding the extract or whole cells, was run at 30°C. At appropriate time intervals 0.2 ml aliquots were removed and pipetted into 0.5 ml of 0.2 M Tris-HCl buffer, pH 8.2, and then boiled for three minutes to stop invertase activity. Glucose was assayed by adding 0.7 ml of the glucose oxidase-peroxidase-o-dianisidine reagent (Lloyd and Whalen, 1969), and the reaction mixtures were incubated for 30 minutes at 37°C. The reaction was stopped by adding 1.4 ml of 5 M HCl. Absorbance was read at a wavelength of 525 nm, and compared to a standard curve for D-glucose. The assay was linear from 0 to 60 micrograms of D-glucose in 0.7 ml of solution. Invertase activity was expressed as micromoles (or nanomoles) of sucrose hydrolyzed per minute.

Alpha-Glucosidase. α -Glucosidase activity (maltase, etc.) was assayed

using the chromogenic substrate p-nitrophenyl- α -D-glucopyranoside (PNPG) as described by Halvorson and Ellias (1958) and Khan and Eaton (1967). The α -glucosidase activity was expressed as the increase in absorbance at 400 nm per minute, due to the release of p-nitrophenol from PNPG.

Preparation of Crude Extracts. Yeast strains were grown on appropriate culture media and were harvested in early stationary growth phase, unless otherwise specified. The cells were washed twice with distilled water and the final cell pellet was either used immediately or stored at -20°C . The method of preparing crude extracts was a modification of that of Neumann and Lampen (1968). Cells to be broken were suspended in 0.01 M disodium ethylenediaminetetraacetate (disodium EDTA) buffer, pH 7.0, at 4°C . The suspension was pipetted into an Eaton press (Eaton, 1962) which had been precooled on dry ice for one hour. The frozen cells were broken by being forced through a small opening at a pressure of about 15,000 pounds per square inch. The thawed, crushed cells were then centrifuged at $27,000 \times g$ for 15 minutes. The supernatant was decanted off and the pellet was resuspended in a volume of 0.01 M potassium phosphate buffer, pH 6.5, equal to the initial volume of 0.01 M disodium EDTA buffer, pH 7.0. This was then centrifuged at $27,000 \times g$ for 15 minutes. The supernatant was added to the first and used immediately or stored at -20°C .

An alternate method of crude extract preparation (N. R. Eaton, personal communication) involved harvesting and washing 5 or 10 ml aliquots of cultures as above, and resuspending the final pellet in 0.5 ml of 0.01M disodium EDTA buffer, pH 7.0. The cells were then broken in a Braun MSK homogenizer using acid-washed glass beads with

a diameter of 0.5 to 1 mm. The cells were subjected to four one-minute intervals of shaking with 15-second bursts of compressed carbon dioxide gas in between. The broken cells were centrifuged for four minutes in a Beckman model 152 Microfuge, at 4°C. Supernatants were pipetted off and used immediately or stored at -20°C.

Protein Determination. Protein was determined by the microbiuret method (Zamenhof, 1957) using bovine serum albumin as a standard.

Gel Filtration of Invertases. Separation of invertases and α -glucosidase activities was performed on Sephadex G-150 or G-200 using a modification of the method of Khan and Eaton (1967).

SDS-Polyacrylamide Gel Electrophoresis of Invertases

The method of Maurer (1968) had been used (Meyer and Matile, 1965) for separating and quantitating the invertase activities of yeast, using 5.35% (w/v) SDS-polyacrylamide gels. The following solutions were prepared separately and stored at 4°C.:

- Stock Solution A: 100 ml distilled water
30 gm acrylamide (Canalco)
0.8 gm bis-acrylamide (Canalco)
- Stock Solution B: 100 ml distilled water
0.14 gm ammonium persulfate (Canalco)
- Stock Solution C: 100 ml distilled water
6.85 gm Tris (tris(hydroxymethyl)aminomethane)
48 ml 1 N HCl
0.46 ml Temed (N, N, N', N'-tetramethyl ethylene diamine) (Eastman Kodak)
pH 7.5
- Stock Solution D: 100 ml distilled water
5 gm sodium dodecylsulfate (SDS)

To prepare 5.35% (w/v) SDS-polyacrylamide gels, the above solutions were added in the following proportions: A 1.5; B 4.0;

C 1.0; D 0.4 and distilled water 1.1. The solution B was added last. After allowing the gels to polymerize, 0.05 ml of crude extract and 0.005 ml of 5% SDS were added to the tops of the gels; 0.005 ml of 0.01% bromphenol blue solution was added to serve as a tracking dye. These solutions were stabilized by adding Sephadex G-25 beads. Electrophoresis was carried out at 4°C in a Shandon electrophoresis apparatus, using a constant current (2 milliamperes per gel) for 4 to 5 hours, provided by a Vokam power unit (model SAE 2761 Shandon Southern). The electrode buffer contained per liter: 5.52 gm Veronal salts buffer (LKB) and 1.0 gm Tris; the pH was adjusted to 7.0 with HCl.

To visualize invertase activities in the gels, a modification of the method of Gabriel (Gabriel and Wang, 1969; Gabriel, 1971) was employed (N. R. Eaton, personal communication). After electrophoresis the gels were removed from the tubes, and invertase activity was located by incubating at 37°C in 5 ml of the following solution: 0.05 M sucrose in 0.1 M sodium acetate buffer, pH 4.5, containing 0.1% sodium tetrazolium salts (TTC, 2, 3, 5-triphenyl tetrazolium chloride, Fisher Certified). To stop the reaction, 0.5 ml of 10 N NaOH was added to the gel-containing test tubes, and the tubes were placed in a boiling water bath for three minutes. After the tubes were allowed to cool, the red solutions were decanted off and the distance of the red formazan bands was compared to the distance of the tracking dye bromphenol blue from the starting (cathodal) end of each gel. The ratio of the former to the latter is called the Rf (Meyer and Matile, 1975).

CHAPTER III

RESULTS: INDUCED SUCROSE NONFERMENTING MUTANTS IN A STRAIN OF YEAST BEARING THE SUC3 GENE

Isolation and Characterization of Mutants Affecting Sucrose and Raffinose Utilization.

In order to clarify the genetic control of invertase function in Saccharomyces cerevisiae, sucrose and raffinose nonfermenting (suc) mutants were isolated from the sucrose and raffinose fermenting strain EK-6B bearing the SUC3 gene.

In the first series of experiments, nine suc mutants defective in sucrose utilization were isolated from the parental strain EK-6B, following mutagenesis by either ultraviolet irradiation or ethyl methane sulfonate. They fall into three phenotypic classes as shown in Table 2:

1. unable to ferment either sucrose or raffinose.
2. unable to ferment raffinose but able to ferment sucrose slowly.
3. unable to ferment raffinose, sucrose or maltose (one mutant).

All mutants except the single mutant in class 3 (suc220) fermented glucose and maltose as rapidly as the parental strain EK-6B.

The suc mutants were compared with EK-6B in their ability to utilize various carbon sources in YEP-based culture media (Fig. 2). Most suc mutants were similar to EK-6B in the extent and rate of utilization of glucose or maltose. Mutant suc34, however, was also respiratory deficient being unable to utilize glycerol; as a result it

TABLE 2

Phenotypes of SUC3 Parental Strain EK-6B and Sucrose Non-fermenting
(suc) Mutants Derived From It

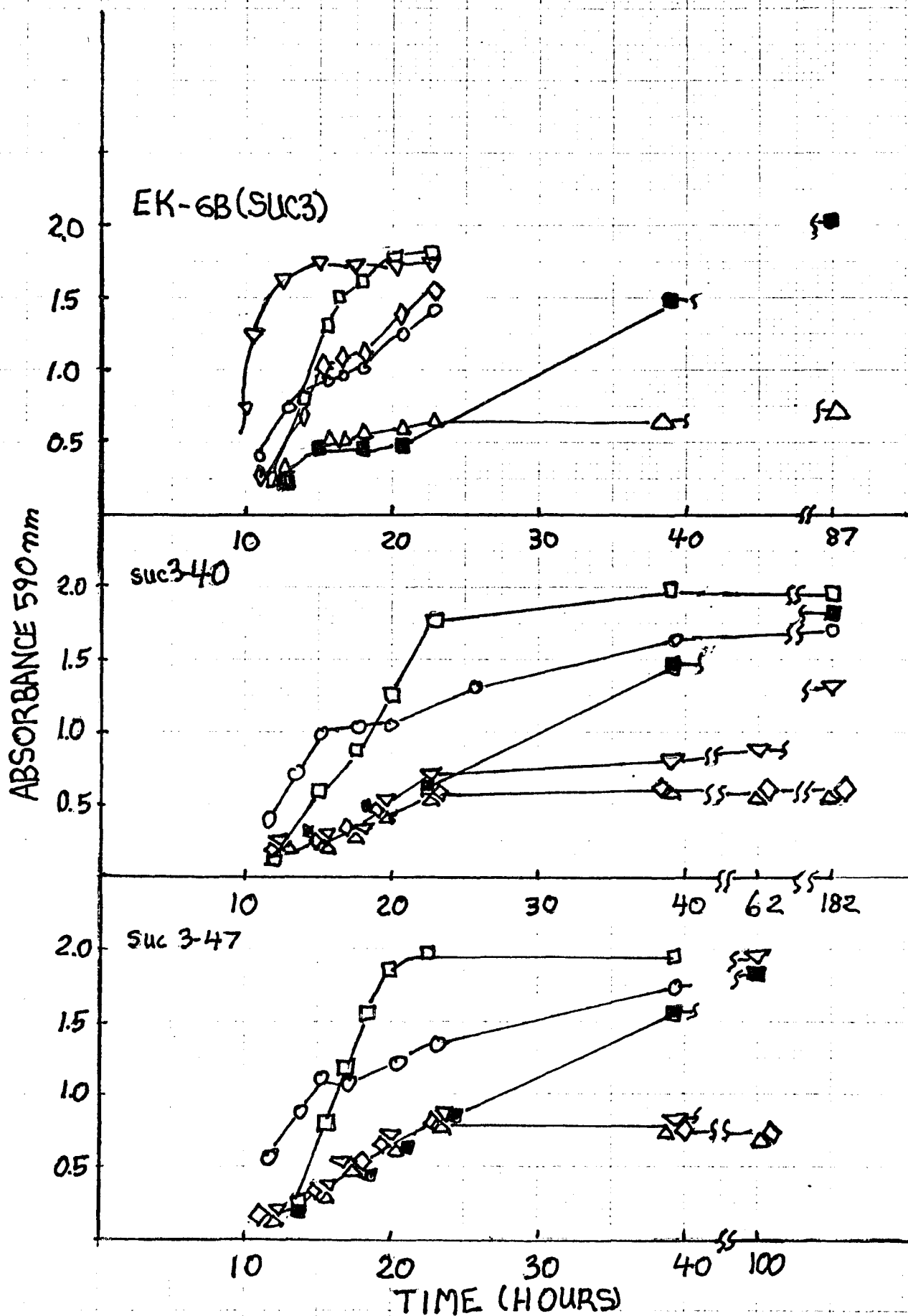
Strain	Fermentation at 30°C	
	Raffinose	Sucrose
EK-6B (SUC3)	+ (2-3)	+ (1)
Class 1		
suc34	-	-
suc94	-	-
suc236	-	-
Class 2		
suc2	-	+ (5)
suc3	-	+ (5)
suc20	-	+ (5)
suc40	-	+ (5)
suc47	-	+ (5)
Class 3		
suc220	-	-

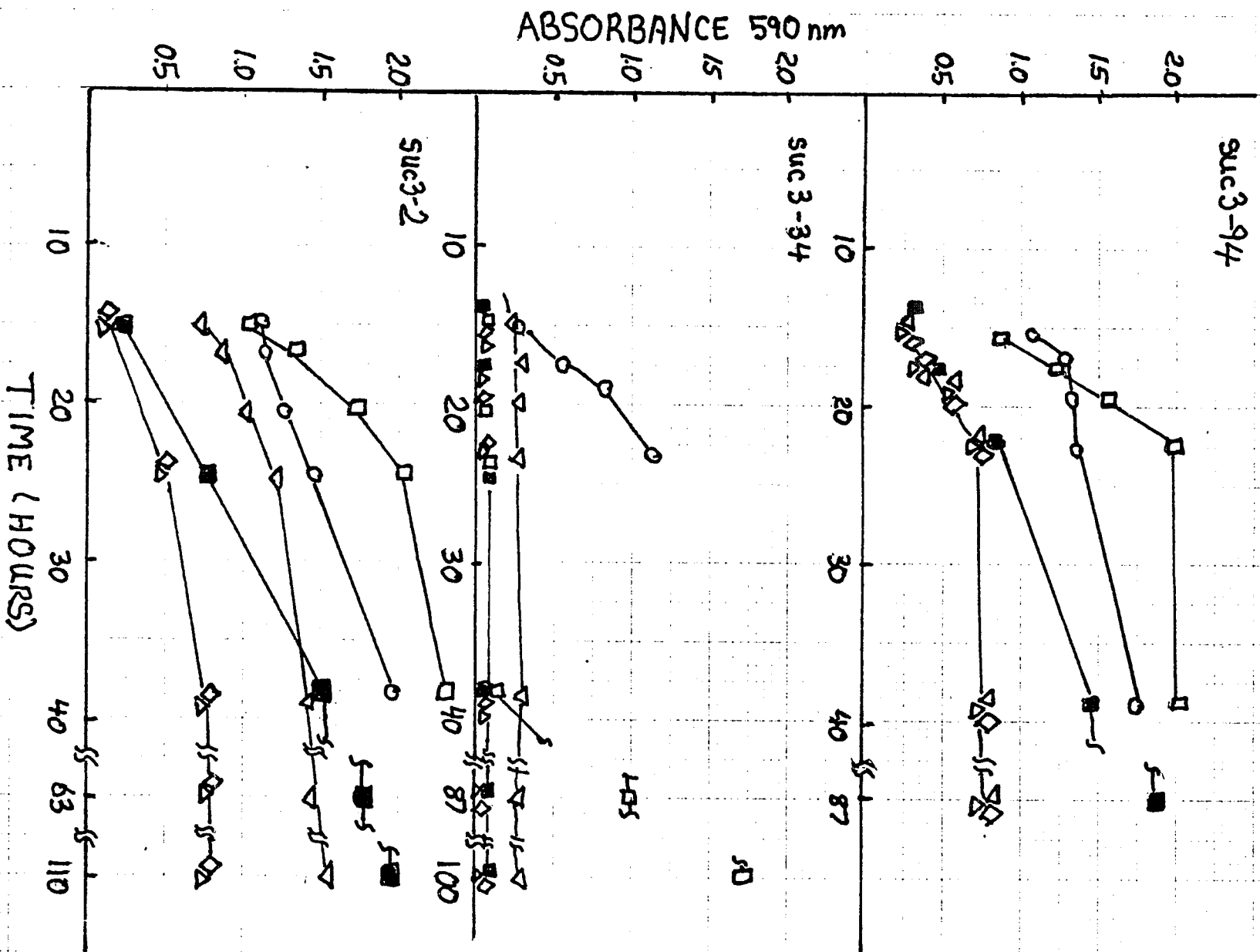
All mutants except suc236 were obtained by UV mutagenesis from EK-6B. Mutant suc236 was obtained using EMS. Inability to ferment a sugar is indicated by a minus (-) sign. Ability to ferment a sugar producing acid and gas is indicated by a plus (+) sign. The number of days required for fermentation is indicated in parentheses. All strains except suc220 are able to ferment maltose in 2 days at 30°C. All strains except suc34 are respiratory competent.

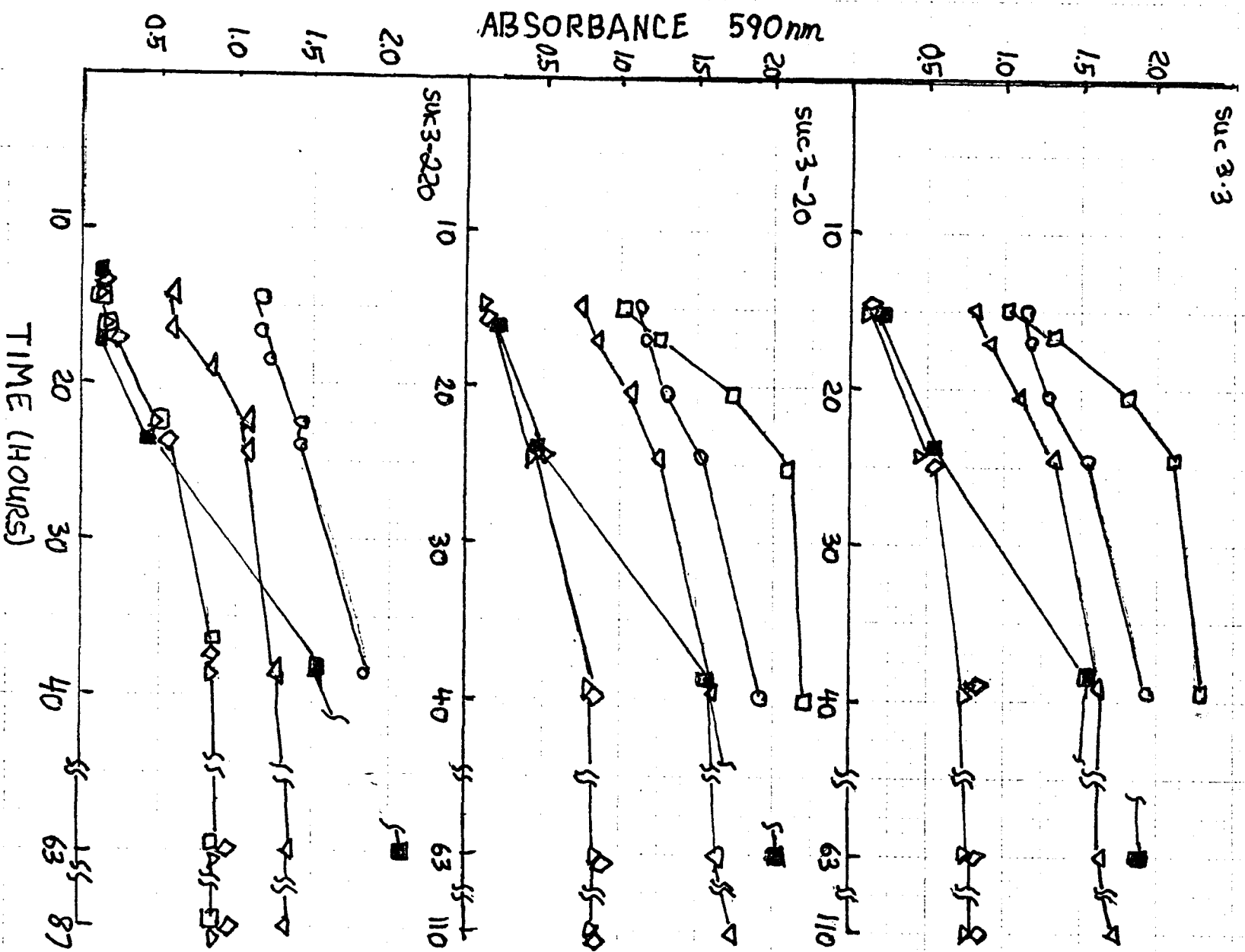
Fig. 2. Growth of EK-6B (SUC3 MAL3) and various suc mutants derived from it on various carbon sources. To either 10 ml or 50 ml of YEP-based media supplemented with different carbon sources was added an appropriate volume of inoculum to give an initial cell density of 1 to 2 x 10⁵ cells per ml. Cultures were incubated at 30°C, the 50 ml cultures (in 125 ml cotton-plugged Erlenmeyer flasks) in a New Brunswick gyratory shaker incubator; the 10 ml cultures (in 15 x 150 mm glass test tubes with metal closures) in a roller drum. The growth of the various cultures was followed by reading the absorbance at 590 nm at appropriate times using a Beckmann DU spectrophotometer.

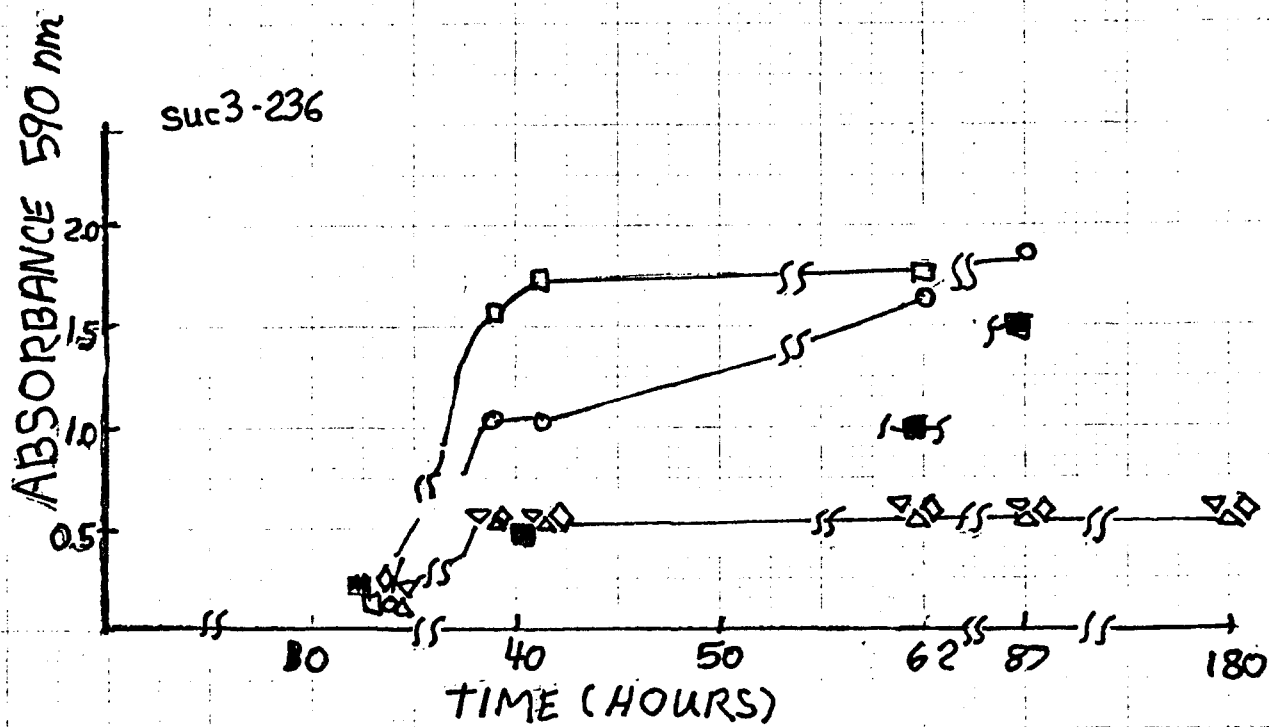
Legend

- △ YEP alone
- YEP 0.5% glucose
- YEP 2% maltose
- ▽ YEP 2% sucrose
- ◇ YEP 2% raffinose
- YEP 3% glycerol









used all fermentable substrates such as glucose more slowly and inefficiently compared with the respiratory competent parent (the mutation responsible for this respiratory deficient condition was found to be unlinked to the suc34 mutation responsible for the inability to ferment sucrose and raffinose; results not presented). Mutant suc220 was unable to utilize maltose. Mutant suc236 showed a long lag period in the utilization of these sugars (glucose and maltose) but their use was ultimately comparable in extent to that of the parent as measured by the cultures' absorbance at 590 nm.

The suc mutants could be grouped in regard to their ability to utilize sucrose and raffinose. Raffinose was apparently not utilized since the absorbance at 590 nm was the same for these cultures as it was for the YEP controls. All mutants used sucrose except those of Class 1 (suc94, suc34 and suc236) although at rates slower than that shown by EK-6B. Mutant suc40 showed a slow increase in absorbance at 590 nm, reaching a value of 1.4 after 180 hours at 30°C. Mutant suc47, on the other hand, showed a lag on sucrose initially but by 40 hours there was considerable growth on this sugar, as shown in a steady increase in absorbance over that of the YEP control. All suc mutants were nonetheless at least somewhat retarded in their growth on sucrose, as compared with the parental SUC3 strain EK-6B which was entering stationary growth phase by 20 hours.

There were various possible explanations for the ability of suc mutants in Class II to grow on sucrose albeit slowly. Perhaps spontaneous revertants appeared during growth of these suc mutants on sucrose-containing media, and these were responsible for the subsequent ability to grow on sucrose. This possibility was explored

by growing mutants such as suc40 and suc47 on YEP 2% sucrose and taking viable counts at different culture ages; at the same time the number of revertants able to utilize sucrose was examined by plating on sucrose and raffinose agar media. If spontaneous revertants were appearing, the proportion of colonies showing a restored ability to utilize sucrose and raffinose would be expected to increase with culture age. This, however, was not observed. The frequency of sucrose fermenters was between 10^{-8} and 10^{-7} , which was probably not enough to explain the slow sucrose fermentation of those Class II suc mutants. Another possibility, that slow sucrose fermenting suc mutants have an altered invertase which can hydrolyze sucrose very slowly, is also unlikely to be correct. This will be considered in a later section of this Chapter.

One interesting, although not new, observation (N. R. Eaton, personal communication) was the great difference in the extent of growth on liquid YEP-based media with and without carbon sources shown by respiratory competent and respiratory deficient yeast strains. Mutant suc34 grew very poorly on YEP, whereas the other suc mutants and EK-6B showed substantial growth. These differences in growth rendered the determination of fermentative abilities based on the amount of growth on agar-containing media very difficult since a respiratory deficient, but sucrose fermenting, yeast strain might grow no better on sucrose or raffinose agar plates than a sucrose non-fermenter which was respiratory competent. Criteria for distinguishing sucrose fermenting from nonfermenting colonies were thus not definitive. Initial mutant screening and later tetrad analysis were rendered more difficult. Use of fermentation tubes was virtually mandatory for

tetrad analysis where carbon dioxide production versus nonproduction gave an indication of whether a particular yeast strain fermented a sugar.

Genetic Analysis

Failure of these suc mutants to utilize raffinose and sucrose as efficiently as the parental SUC3 strain could have resulted from a mutation in the structural gene(s) for invertase or in a control gene. In order to determine the nature of the genetic block among the suc mutants, the mutants and the parental strain were crossed with segregational sucrose nonfermenters (suc0) and diploids were isolated. When parental strain EK-6B was crossed to Z1-2D (suc0 mal0), diploids of this cross fermented raffinose in three days, sucrose in one day and maltose in two days at 30°C. Diploids isolated from crosses of the suc mutants to Z1-2D failed to ferment raffinose, even after 27 days at 30°C. All of the diploids fermented maltose within two to three days, except the cross involving suc220 which required ten days. Sucrose was fermented slowly by diploids derived from mutants suc3, 20, 40, 220 and 236; there was a lag of 4 to 27 days. Diploids derived from mutants suc2, 47, 94 and 34 did not ferment sucrose even after 27 days. It was concluded that the segregational sucrose nonfermenter Z1-2D had genetic lesions allelic to those of the suc mutants since diploids did not show complementation, namely fermentation of both sucrose and raffinose.

Diploids isolated from crosses of the parental strain EK-6B to either AZ-5A or Z1-2D were sporulated and dissected (Table 3).

TABLE 3
Crosses of Sucrose Nonfermenting Mutants Derived from EK-6B, and EK-6B
with Segregational Sucrose Nonfermenters

Cross	Tetrad Types				
	suc	SUC	SUC MAL	SUC MAL	SUC mal
	suc	SUC	SUC MAL	SUC mal	SUC mal
	suc	suc	suc mal	suc MAL	suc MAL
	suc	suc	suc mal	suc mal	suc MAL
	PD		TT		NPD
EK-6B x Z1-2D ^a	0	80	79	1	0
EK-6B x AZ-5A	0	7	-	-	-
suc2 x Z7-17D	13	0	-	-	-
suc3 x Z7-17D	10	0	-	-	-
suc20 x Z7-17D	7	0	-	-	-
suc94 x AZ-5A	3	0	-	-	-
suc220 x AZ-5A ^b	-	-	-	-	-

Ascospores able to ferment sucrose and raffinose are denoted by SUC; unable to ferment both sugars or able to ferment only sucrose slowly, by suc. PD means parental ditype tetra, TT tetratype, and NPD nonparental ditype.

^a In the cross EK-6B x Z1-2D of 24 3-spore asci obtained, 14 were 1 SUC MAL:2 suc mal and 10 were 2 SUC MAL:1 suc mal.

^b For the cross suc220 x AZ-5A no complete tetrads were obtained. In this cross all nine 3-spore asci obtained were 0 SUC:3 suc.

Tetrads from these crosses gave a consistent pattern of two ascospores able to ferment both raffinose and sucrose to two ascospores unable to ferment both sugars. That MAL3 is tightly linked to SUC3 was indicated by the fact that of 80 tetrads dissected in the cross EK-6B x Z1-2D, 79 were parental ditype and one was a tetratype for maltose and sucrose fermentation. Diploids of suc mutants crossed to various segregational sucrose nonfermenters gave tetrads showing only 0 SUC:4 suc segregation pattern for sucrose and raffinose fermentation; maltose fermentation segregated 2 MAL:2 mal. These results suggested that the ability to ferment both raffinose and sucrose was controlled by the same gene (SUC3) and that a single-gene difference was responsible for these segregation patterns. These results reconfirmed earlier reports that the SUC3 gene allowed fermentation of both sucrose and raffinose, and was closely linked to MAL3 (Winge and Roberts, 1952; Mortimer and Hawthorne, 1969; N.A. Khan, unpublished results). The tetrad analysis data shown in Table 3 suggested that the lesion in the segregational sucrose nonfermenters could not complement those of the mutational nonfermenters probably because they were at the same gene, i.e. allelic.

When sucrose nonfermenting mutants were crossed to strains bearing the SUC3 gene, all of the diploids isolated fermented both raffinose and sucrose as rapidly as the diploids isolated from the cross of EK-6B to the same SUC3 strain. Thus all suc mutants were recessive to SUC3. These crosses were sporulated. As shown in Table 4 in every case tetrad analysis revealed a 2 SUC:2 suc segregation pattern for raffinose and sucrose fermentation. This again suggested a single-gene difference between the SUC3 and suc mutants in

TABLE 4

Crosses Between Sucrose Nonfermenting Mutants Derived from EK-6B and a
SUC3-bearing Strain

Mutant Isolation Number	Tetrad Types				
	SUC MAL	SUC	SUC	SUC	suc
	SUC MAL	SUC	SUC	suc	suc
	suc MAL	SUC	SUC	suc	suc
	suc MAL	suc	SUC	suc	suc
40	9	0	0	0	0
47	7	0	0	0	0
94	11	0	0	0	0
2	6	0	0	0	0
3	10	0	0	0	0
20	9	0	0	0	0
34	9	0	0	0	0

All mutants except suc34 were crossed to the SUC3-bearing strain ZEKAZ-20D derived from the cross EK-6B x AZ-5A. Mutant suc34 was crossed with HZ1-27D, a SUC3 strain derived from the cross EK-6B x Z1-2D. All complete tetrads examined were parental ditype (2 SUC MAL:2 suc MAL); the two SUC ascospores fermented both sucrose and raffinose; the two suc ascospores failed to ferment both of these sugars.

each cross.

In order to determine if the suc mutants were allelic to SUC3, or at least closely linked, the suc mutants (suc3 MAL3) were crossed to a SUC3 mal0 recombinant R. S. 5-1 (which was derived from the SUC3 mal0 ascospore of the single tetratype tetrad of the cross EK-6B x Z1-2D in Table 4). If all of the tetrads dissected proved to be parental ditype (2 suc3 MAL3:2 SUC3 mal0), this would strongly support the idea that the suc3 mutants were allelic to SUC3. The results presented in Table 5 indicated that all of the suc mutants were either allelic to or closely linked to SUC3 since only parental ditype tetrads were recovered from these crosses.

Complementation between the suc mutants was studied by crossing the suc mutants amongst each other. None of the suc mutants complemented any of the others. Thus all of the suc mutants were probably allelic to each other, and presumably to SUC3. On the basis of the above genetic studies, the mutational sucrose nonfermenters derived from the SUC3 strain should be designated suc3-; for instance, suc94 would be suc3-94.

Enzymatic and Biochemical Studies

The parental SUC3 MAL3 strain EK-6B grown under either derepressing (initial glucose concentration 0.5%, low) or repressing (initial glucose concentration 10%, high) conditions produces two types of invertase (see Fig. 7 and 8, for example): external and internal, as had previously been reported for other yeast strains (Gascón and Ottolenghi, 1972). EK-6B grown in culture media with an initial

TABLE 5

Allelism Tests for Sucrose Nonfermenting Mutants from EK-6B Crossed to
a SUC3 mal10 Recombinant

Mutant Isolation Number	Tetrad Type		
	Parental Ditype	Nonparental Ditype	Tetratype
	SUC mal SUC mal suc MAL suc MAL	SUC MAL SUC MAL suc mal suc mal	SUC mal SUC MAL suc MAL suc mal
40	14	0	0
47	9	0	0
94	15	0	0
236	8	0	0
2	9	0	0
3	15	0	0
20	7	0	0
34	13	0	0
220	- ^a	-	-

The above mutants (suc3 MAL3) were crossed to a SUC3 mal10 random spore (R. S. 5-1, α lys1) derived from a cross between HZ1-113B (a ade2 leu2 SUC3 mal10) and Z1-2D. HZ1-113D was derived from a tetratype tetrad of the cross EK-6B x Z1-2D. While not presented, 3-spore asci contained ascospores which were either suc3 MAL3 or SUC3 mal10.

^a Spore viability was too low to recover complete tetrads.

glucose concentration of 0.5% was assumed to lead to derepression of invertase biosynthesis, whereas an initial glucose concentration of 10% was assumed to cause repression of invertase formation when cultures grown in these sugar concentrations were harvested during early stationary growth phase.

Invertase Activity in Strain EK-6B

In crude extracts of derepressed cells of strain EK-6B, invertase activity was proportional to the protein concentration (Fig. 3). The specific activity was independent of protein concentration. This was important since invertase activity had to be measured in different strains growing under different culture conditions at widely varying protein concentrations. The release of glucose from sucrose catalyzed by invertase from strain EK-6B was linear with time (Fig. 4).

Effect of Culture Age on Invertase and α -Glucosidase Activities in Strain EK-6B grown on Glucose and Sucrose

It was necessary to determine variation of invertase and α -glucosidase levels as functions of culture age in strain EK-6B if comparisons of enzyme levels between EK-6B and the suc3 mutants derived from it, as well as diploid constructed from these strains, were to have any validity.

Enzyme levels in crude extracts of cells of EK-6B grown on YEP 0.5% glucose (initial concentration) were examined. Invertase and α -glucosidase levels were measured at various times during exponential and stationary growth phases (as measured by the absorbance at 590 nm). Invertase and α -glucosidase levels were dependent on the glucose concentration in the medium as was previously reported (Gascón and Ottolenghi, 1972). Both enzyme activities were low during mid-

Fig. 3. Proportionality between sucrose hydrolysis by invertase and protein concentration. A crude extract of strain EK-6B (SUC3) grown on YEP 0.5% glucose for 20 hours at 30°C. Invertase activity was measured in 0.05 M sucrose in 0.1 M sodium acetate buffer, pH 4.5 at 30°C. The extract was diluted in 0.025 M potassium phosphate plus 0.001 M disodium EDTA, pH 7.0, and aliquots of these dilutions were added to each reaction mixture. Glucose released was assayed as described in "Materials and Methods".

Legend

- μ mole sucrose hydrolyzed per 4 min per 0.1 ml
crude extract
- △ μ mole sucrose hydrolyzed per min per mg
protein

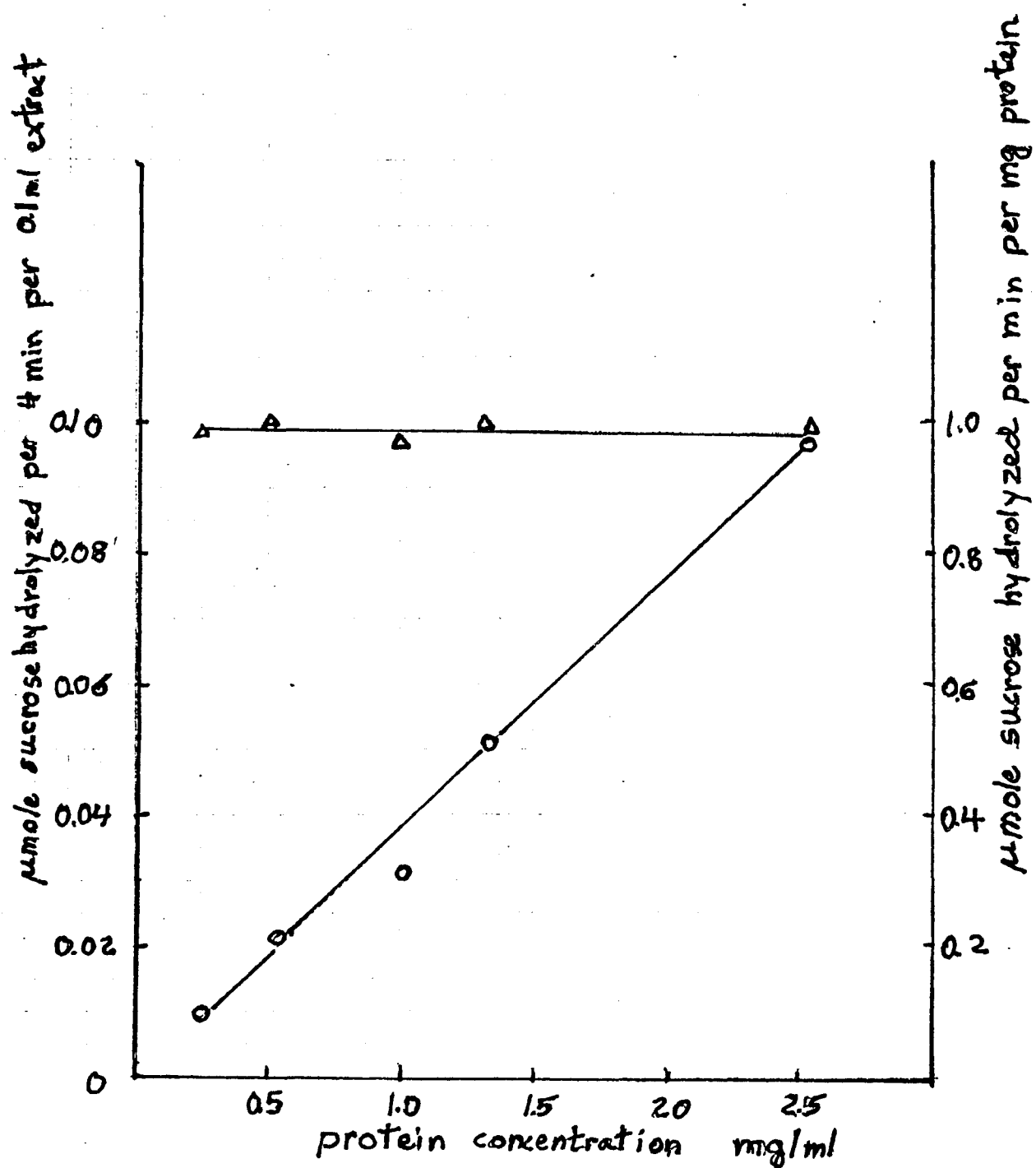
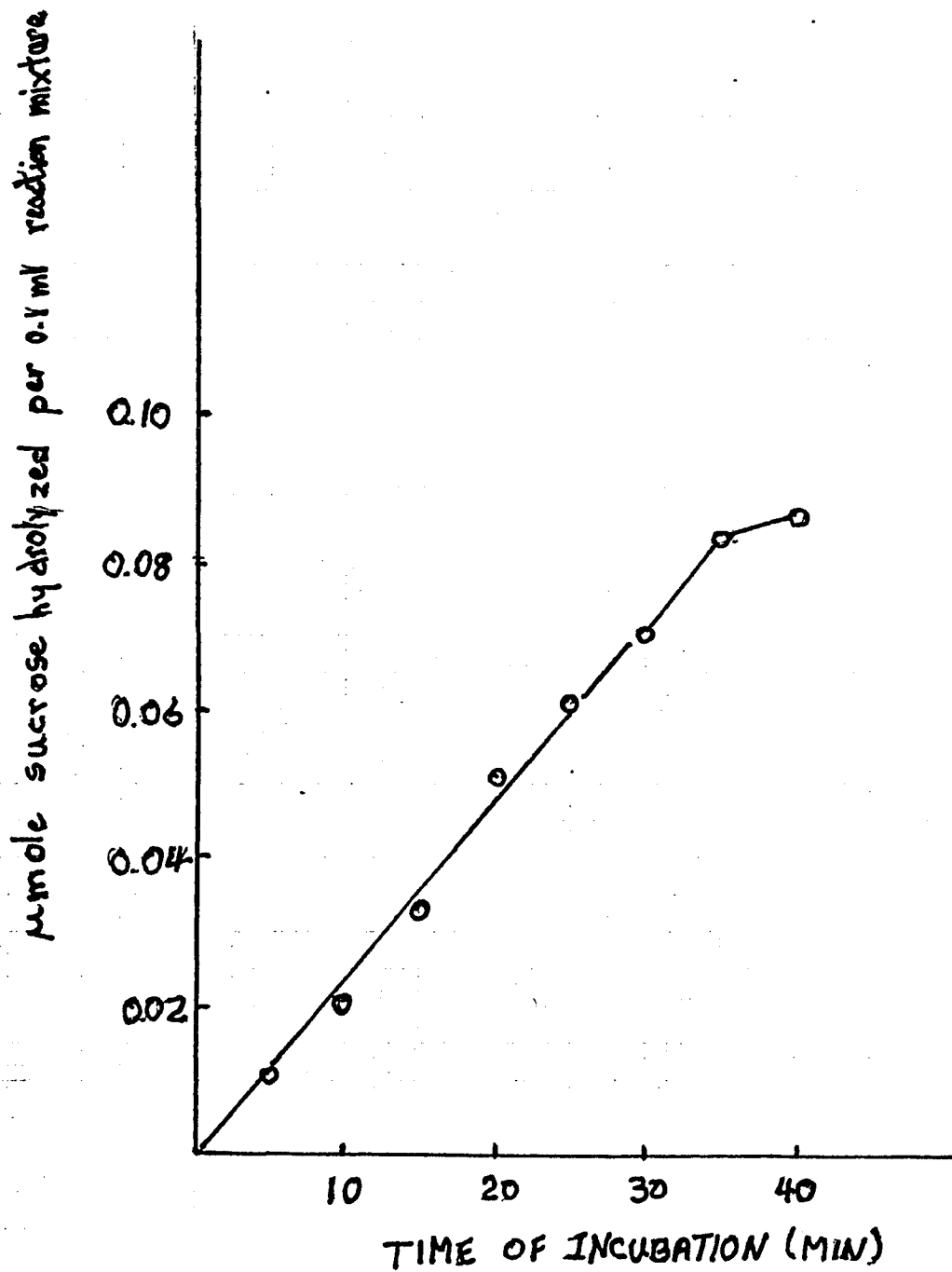


Fig. 4. Release of glucose from sucrose by EK-6B invertase is proportional to the incubation time. See Fig. 3 for preparation of crude extract and invertase assay conditions. A 1:20 dilution of crude extract (0.26 mg protein per ml) was used.



exponential growth phase, about 11 hours (Fig. 5). These values probably represented at least partially repressed levels for these enzymes. As the culture exhausted glucose and growth consequently slowed, both invertase and α -glucosidase activities in crude extracts reached maximal values between 13 and 19 hours. By 42 hours (late stationary growth phase) enzyme levels fell to values approximating those of mid-exponential growth phase. These results suggested that harvesting cultures at about 16 to 20 hours (late exponential - early stationary growth phases) would give maximal levels of invertase and α -glucosidase. Results of this experiment on the kinetics of enzyme formation showed an increase in both activities, although α -glucosidase rose about 20-fold, while invertase rose only three-fold.

Variation in invertase and α -glucosidase levels with culture age was examined when strain EK-6B was grown on YEP 2% sucrose (Fig. 6). Invertase specific activity showed little variation in crude extracts of cells harvested at mid exponential growth phase (12 hours) through early stationary phase (20 hours). α -Glucosidase activity, on the other hand, was relatively low during exponential growth phase and increased only in late exponential and early stationary phases. It was concluded that the best time to harvest cultures grown on sucrose in order to maximize these enzyme activities was between 16 and 20 hours. As α -glucosidase levels tend to fluctuate dramatically during this part of the growth cycle (N. R. Eaton, personal communication), α -glucosidase levels may thus vary greatly in different strains harvested at the same time, but not necessarily at the same growth stage.

The major difference in the kinetics of invertase formation in

Fig. 5. Invertase and α -glucosidase activities as functions of culture age of EK-6B grown on YEP 0.5% glucose. To 200 ml of YEP 0.5% glucose in a cotton-plugged 500 ml Erlenmeyer flask was added 0.2 ml of a 24 hour old YEPD broth culture of strain EK-6B. The culture was incubated in a New Brunswick gyratory shaker at 150 rpm at 30°C. Aliquots of 40 ml were removed at the indicated times and treated as described in "Materials and Methods".

Legend

- Δ Invertase specific activity in crude extract
- $\circ$ α -Glucosidase specific activity in crude extract
- $\square$ Absorbance at 590 nm measured in a Beckman DU spectrophotometer with a Gilford Absorbance Indicator

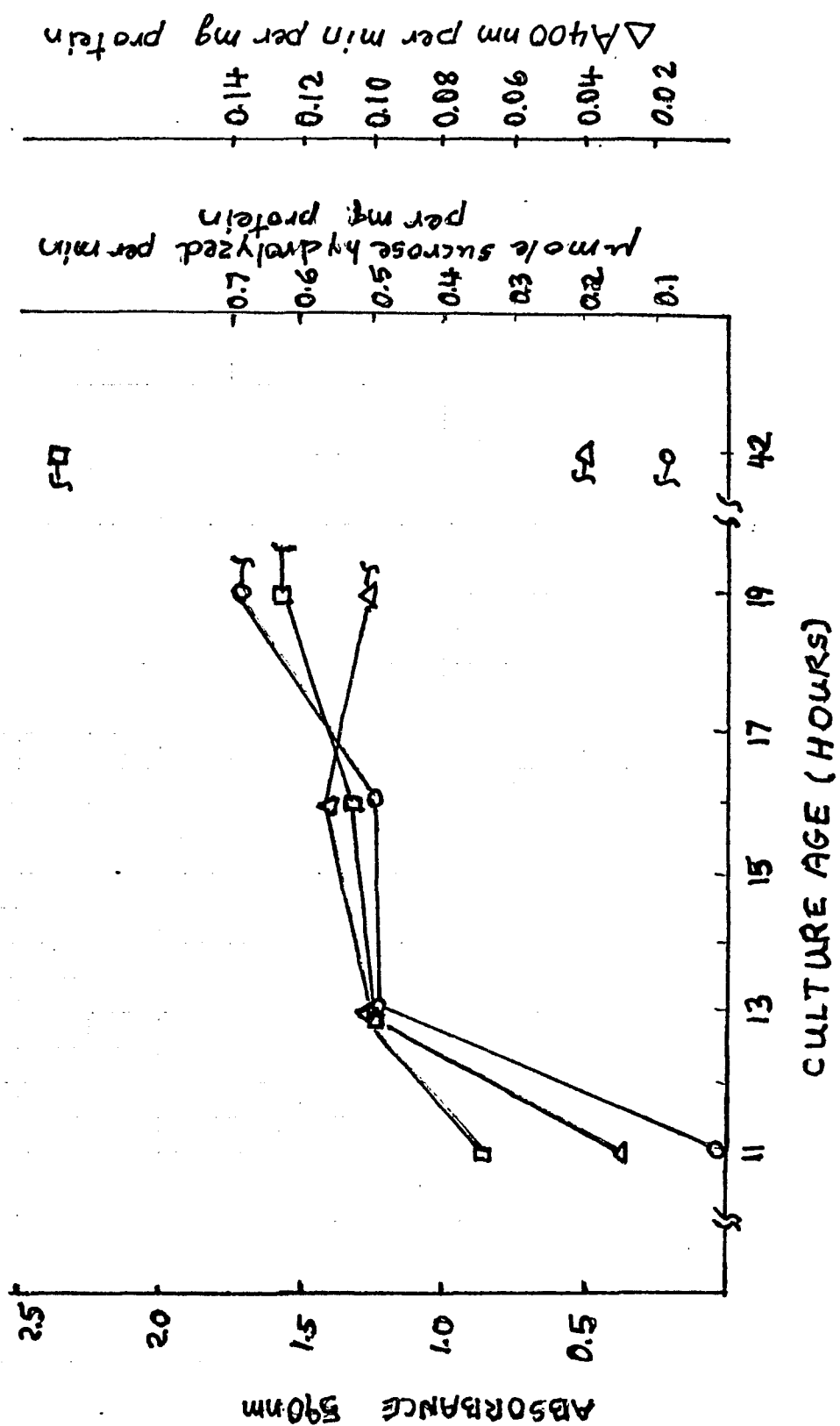
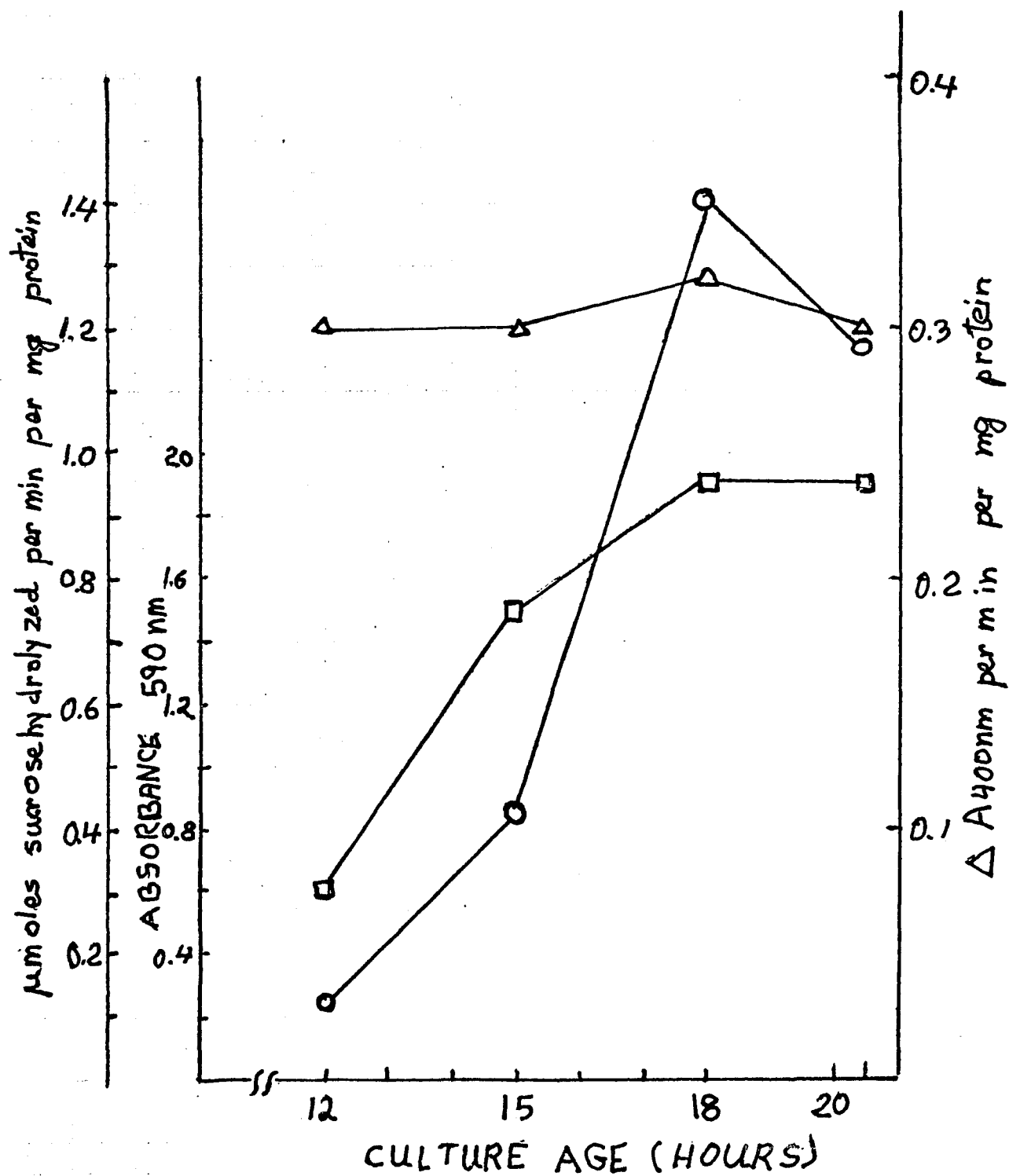


Fig. 6. Invertase and α -Glucosidase activities as functions of culture age in EK-6B grown on YEP 2% sucrose. Eight liters of YEP 2% sucrose equilibrated at 30°C in a 10-liter capacity Virtis Fermenter (model 40-300) were inoculated with 8 ml of a 24 hour old YEPD culture of EK-6B. The speed of the magnetic stirrer was 360 rpm. Air was delivered at 8.6 liters per min. One ml of autoclaved Antifoam A Emulsion (Sigma) was added. Aliquots of 50 ml were removed at the times indicated for determination of growth parameters and enzyme assays. Enzymes were assayed as described in "Materials and Methods".

Legend

- Absorbance at 590 nm in Beckman DU Spectrophotometer
- Δ Invertase specific activity
- α -Glucosidase specific activity



cells of EK-6B grown on glucose as compared to sucrose was that invertase levels were high on sucrose even when there was considerable sucrose present, while invertase levels in glucose-grown cells increased only as glucose was exhausted. It was concluded that sucrose did not "repress" invertase formation as much as did glucose; both sugars, however, appeared to "repress" α -glucosidase biosynthesis. The mechanism by which these sugars act to control enzyme synthesis in yeast at the molecular level is unclear.

Enzyme levels in EK-6B and suc3 Mutants Derived from it

Strain EK-6B had substantial invertase activity when grown on YEP 0.5% glucose, whereas the suc3 mutants had no detectable invertase activity (Table 6). Parental and suc3 mutant strains had similar values for α -glucosidase activity; exceptions were suc3-34 and suc3-220, which had lower specific activities.

The specific activity of invertase was also determined in heterozygous diploids (suc3/SUC3) obtained by crossing the suc3 mutants with a strain carrying the SUC3 gene (ZEKAZ-20D, see also Table 4). These results are presented in Table 7; in every case the heterozygotes had about half the specific activity of the homozygous (SUC3/SUC3) diploid. In contrast, the derepressed α -glucosidase activity was the same in homozygous and heterozygous diploids; the exception was suc3-34 x ZEKAZ-20D, which had a lower α -glucosidase activity. These results suggested that the suc mutants affected only invertase activity, but not α -glucosidase activity in the heterozygous diploids.

It has been shown that the external and internal invertases can be separated on Sephadex G-200 (Gascón, Neumann and Lampen, 1968) or

TABLE 6

Invertase and α -Glucosidase Activities in SUC3 Parental Strain EK-6B
and suc3 Mutants Grown under Derepressing Conditions

Strain	Specific Activities	
	Invertase (μ moles sucrose hydrolyzed per min. per mg protein)	α -Glucosidase (Δ A400 nm per min. per mg protein)
EK-6B (SUC3 MAL3)	0.850	0.152
suc34 (suc3 MAL3)	n. d. ^a	0.120
suc94 "	n. d.	0.149
suc236 "	n. d.	0.156
suc2 "	n. d.	0.160
suc3 "	n. d.	0.147
suc20 "	n. d.	0.159
suc40 "	n. d.	0.161
suc47 "	n. d.	0.135
suc220 "	n. d.	0.058

Crude extracts were prepared from cells grown on YEP 0.5% glucose for 20 hours at 30°C. Preparation of crude extracts and enzyme assays were performed as indicated in "Materials and Methods".

^a Invertase activity not detectable.

TABLE 7

Invertase Activity in Diploids Homozygous for SUC3 and Heterozygous
for suc Mutants (SUC3/suc3)

Diploid	Specific Activity	
	Invertase (μ moles of sucrose hydrolyzed per min per mg protein)	α -Glucosidase (Δ A400 nm per min per mg protein)
EK-6B x ZEKAZ-20D (SUC3/SUC3)	0.655	0.128
suc34 x ZEKAZ-20D (SUC3/suc3)	0.305	0.086
suc94 x " "	0.367	0.130
suc236 x " "	0.341	0.145
suc2 x " "	0.324	0.139
suc3 x " "	0.312	0.142
suc20 x " "	0.366	0.127
suc40 x " "	0.333	0.127
suc47 x " "	0.388	0.128
suc220 x " "	0.423	0.128

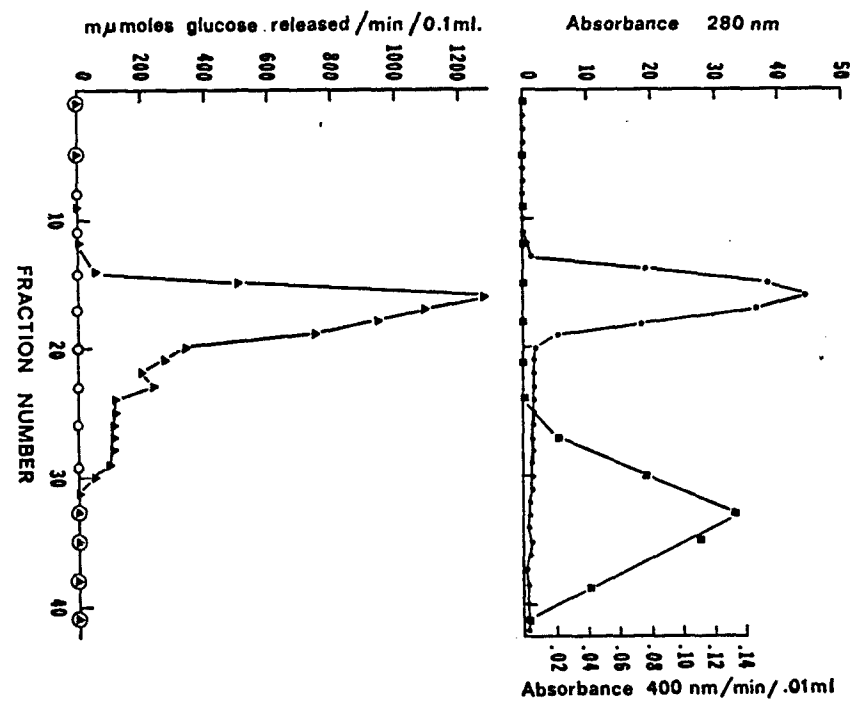
Crude extracts were prepared from cells grown on YEP 0.5% glucose for 18 hours at 30°C. Preparation of crude extracts and enzyme assays were performed as described in "Materials and Methods".

Sephadex G-100 columns (Khan, Zimmermann and Eaton, 1973). Crude extracts of cells grown under derepressing conditions were applied to a Sephadex G-150 column. As shown in Fig. 7, the parental strain EK-6B had two peaks of invertase activity, the first to be eluted with the main peak of protein being the external invertase, and the smaller second peak (actually this looks more like a tail of the major invertase peak) being the internal invertase, which came off after the major peak of protein.

That the first major peak is the external invertase and the second smaller peak is the internal is supported by the following facts: purified external and internal invertases from a SUC2 strain correspond to the respective activities in these peaks and the external invertase was the more abundant species in cells grown under derepressing conditions (Gascón, Neumann and Lampen, 1968). All SUC genes make the two forms of invertase which elute from Sephadex columns in these positions (Ottolenghi, 1971b). Partial purification of external invertase from EK-6B by ultrafiltration through an Amicon xM-150 ultrafilter results in the retention of the external invertase corresponding to that form which elutes first from Sephadex columns. Furthermore, external and internal invertase activities can be separated by polyacrylamide gel electrophoresis (see Fig. 9).

Crude extract of derepressed cells of suc3-40 was also applied to a Sephadex G-150 column and neither peak of invertase activity was detectable (see Fig. 7). In this mutant as well as the parent EK-6B, the α -glucosidase was detected as a peak of activity which eluted slightly later than the internal invertase of EK-6B; in suc3-40, the α -glucosidase peak was found in the same position. Two other suc3

Fig. 7. Gel filtration on Sepacex G-150 of crude extracts of cells of EK-6B and suc3-40 grown under derepressing conditions (YEP 0.5% glucose, 19 hours at 30°C). Aliquots of crude extract containing approximately 200 mg of protein were added to a column of Sephadex G-150 (2.5 x 30 cm) equilibrated with 0.025 M potassium phosphate plus 0.001 M disodium EDTA buffer, pH 7.0. Fractions of 3 ml were collected. The top panel represents protein concentration (left ordinate, solid circles) and α -glucosidase activity (right ordinate, solid squares) for both strains. The lower panel represents invertase activity measured by sucrose hydrolysis at pH 4.5. Invertase activity of EK-6B is represented by solid triangles; invertase activity of suc3-40 by open circles. All assays were performed as described in "Materials and Methods".



mutants, suc3-47 and suc3-94, were also found to lack both invertase peaks (results not presented). Therefore, a single point mutation in these three suc3 mutants led to the loss of both invertase activities.

Co-segregation of Invertase Activity with the SUC3 Gene for Sucrose and Raffinose Fermentation

It has already been demonstrated in this Chapter that the ability to ferment sucrose and raffinose by SUC3-bearing strains segregated 2 SUC:2 suc in crosses with segregational (suc0) and mutational (suc3) sucrose nonfermenters (Tables 3, 4, and 5). The ability to ferment sucrose and raffinose was found to segregate with the presence of invertase activity. This was found in representative tetrads from three different crosses involving SUC3 (Table 8). While invertase activity co-segregated with SUC3 gene, α -glucosidase activity was present in basal levels even in ascospore progeny which were unable to ferment maltose (for example ascospores 27B and 27C of cross EK-6B x Z1-2D).

There was a correlation, albeit a poor one quantitatively, between invertase activity in crude extracts and in intact cells. Perhaps not all of the invertase activity in the intact cells was available to react with the substrate sucrose, or the glucose released from the invertase catalyzed hydrolysis may have been transported into the cells and metabolized rendering it unavailable subsequently for glucose assay. Another possibility is that crude extracts may not always yield the same proportion of invertase compared with what was actually present. There was, however, no detectable invertase activity in any of the sucrose nonfermenting ascospores examined. This

TABLE 8

Segregation of Invertase Activity with the Ability to Ferment Sucrose
and Raffinose

Gross	Ascospore	Specific Activity			Fermentation at 30°C		
		Invertase ¹ Extract	α -Glucosidase ² Whole cells	Glucosidase ³ Extract	Raf	Suc	Mal
EK-6B x	23A	0.734	0.013	0.071	+(2)	+(1)	+(2)
AZ-5A	23B	0.932	0.004	0.165	+(6)	+(1)	+(2)
(ZEKAZ)	23C	0	0	0.109	-	-	-
	23D	0	0	0.211	-	-	-
suc40 x	1A	1.43	0.003	0.344	+(4)	+(1)	+(2)
ZEKAZ-20D (SUC3 MAL3)	1B	2.19	0.003	0.800	+(4)	+(1)	+(2)
	1C	0	0	4.2	-	+(4)	+(2)
	1D	0	0	5.5	-	+(4)	+(2)
EK-6B x	27A	0.307	0.003	0.245	+(2)	+(1)	+(2)
Z1-2D	27B	0	0	0.135	-	-	-
(HZ1)	27C	0	0	0.160	-	-	-
	27D	0.680	0.003	0.145	+(2)	+(1)	+(2)

¹ μ moles of sucrose hydrolyzed per min per mg protein

² μ moles of sucrose hydrolyzed per min per 10^6 cells

³ Δ A400 nm per min per mg protein

Tetrad 23 of cross EK-6B x AZ-5A was grown on YEP 0.5% glucose for 20 hours at 30°C. Tetrad 1 of cross suc3-40 x ZEKAZ-20D was grown on YEP 2% sucrose; ascospores 1A and 1B for 15½ hours, ascospores 1C and 1D for 20 hours at 30°C. Tetrad 27 of cross EK-6B x Z1-2D was grown on YEP 0.5% glucose for 20 hours at 30°C.

finding agreed with an earlier brief mention of the same observation (Gascon, Neumann and Lampen, 1968).

The significance of the high levels of α -glucosidase activity in ascospore 1C and 1D of the second cross (suc3-40 x ZEKAZ-20D) in Table 8 was uncertain. It may be related to the ability of mutant suc3-40 to ferment sucrose slowly. Alternatively, a mutation permitting constitutive α -glucosidase synthesis may be responsible, analogous to the constitutive MAL4 gene (see Khan, Zimmermann and Eaton, 1973).

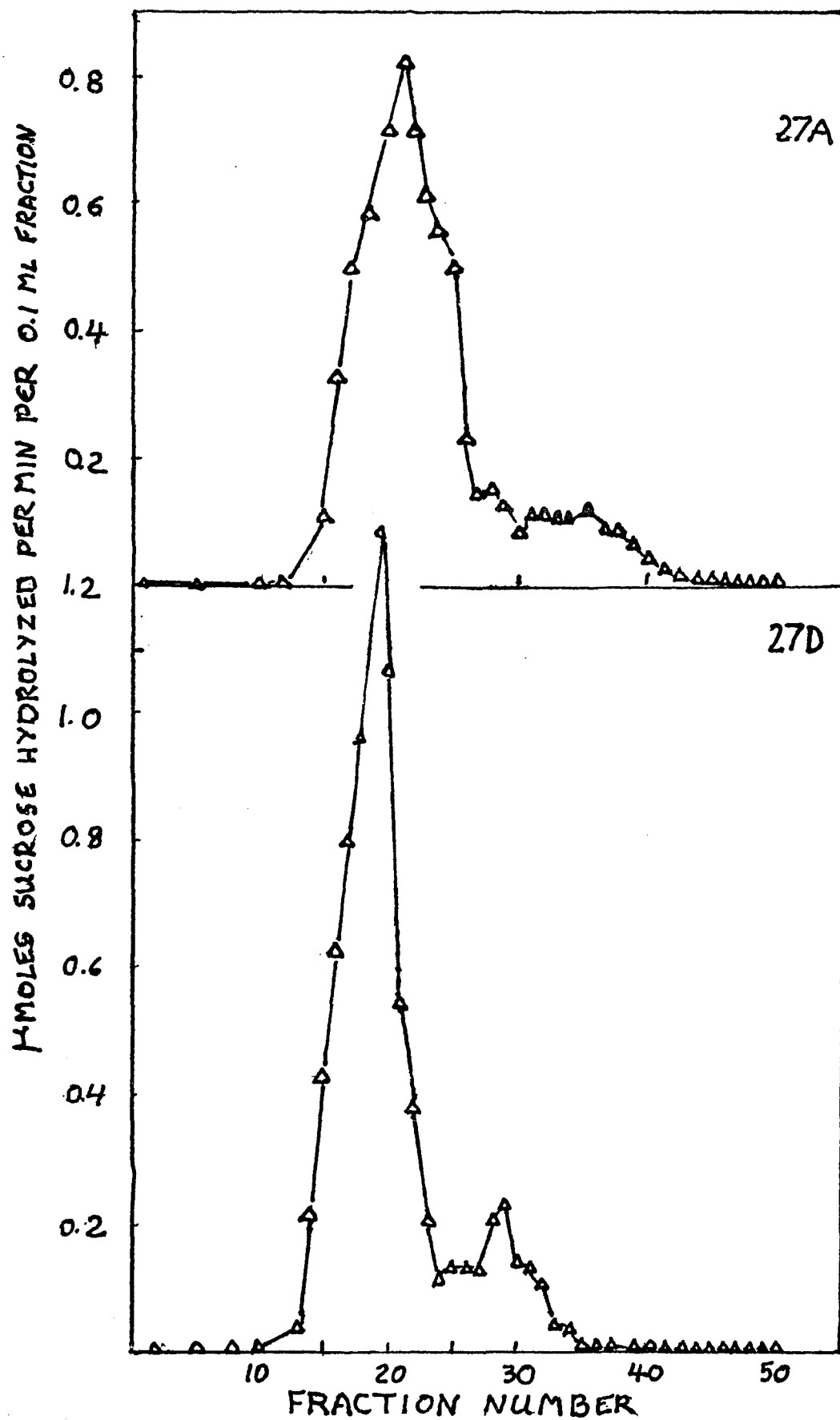
The Presence of Both Invertase Activities Determined by SUC3

Tetrad 27 of cross HZ1 (EK-6B x Z1-2D, Table 8) was used to investigate whether both invertase activities segregated together. As shown in Fig. 8, sucrose fermenting ascospores 27A and 27D from this cross have both forms of invertase: the major peak eluting first being the external, and the second smaller peak being the internal invertase. Since the cells used to make the crude extracts that were fractionated were grown under derepressing conditions, the external invertase was the more abundant species, as was reported earlier with different strains (Gascón, Neumann and Lampen, 1968).

Invertase and α -Glucosidase Activities in SUC3 mal0 and suc0 MAL3 Reciprocal Recombinants

Tetrad 113 from the cross HZ1 was the only tetratype tetrad in that cross (Table 5). It was found that the ascospore HZ1-113B (SUC3 mal0 recombinant) was able to ferment sucrose and raffinose and unable to ferment maltose; the reciprocal recombinant ascospore HZ1-113D (suc0 MAL3) was unable to ferment sucrose and raffinose but fermented maltose. It was found that the SUC3 mal0 recombinant had

Fig. 8. Presence of both invertase activities in the two sucrose fermenting, invertase-positive ascospores of tetrad 27 of the cross EK-6B x Z1-2D (HZ1). Crude extracts were prepared from 1 liter cultures of cells grown in YEP 0.5% glucose for 20 hours at 30°C as described in "Materials and Methods". Aliquots of crude extracts containing 200 mg protein (ascospore 27A) and 224 mg of protein (ascospore 27D) were layered separately onto a Sephadex G-150 column (2.5 x 30 cm). Fractions of 3 ml were collected. The elution buffer was 0.025 M potassium phosphate plus 0.0001 M disodium EDTA, pH 7.0. Invertase activity is indicated by open triangles. The upper panel is ascospore 27A; lower, ascospore 27D.



substantial invertase activity whether grown on sucrose or maltose (where this strain grew very poorly). The reciprocal suc0 MAL3 strain had no detectable invertase activity (Table 9). The suc0 MAL3 ascospore (HZ1-113D) in fact did not grow at all on CS 2% sucrose liquid medium, whereas HZ1-113B (SUC3 mal0) reached stationary phase in CS 2% sucrose in 15 hours. As expected, the SUC3 mal0 ascospore had lower α -glucosidase activity than the suc0 MAL3 ascospore. This illustrated the important difference between sucrose and maltose nonfermenters: the former always appeared to lack detectable invertase activity, while the latter had at least low levels of α -glucosidase activity.

SDS-Polyacrylamide Gel Electrophoresis of Invertase

Further evidence that the two peaks of invertase activity seen in Sephadex gel filtration were the two forms of invertase reported by various groups (Gascon, Neumann and Lampen, 1968; Gascón and Ottolenghi, 1972) came from SDS-polyacrylamide gel electrophoresis of crude extracts containing invertase from various yeast strains. The method here used qualitatively, was that of Meyer and Matile (1974), who used it to study the quantitative changes in yeast invertase (Meyer and Matile referred to the SUC2 invertases as being isoenzymes). It was found in the present study that the thickness and intensity of the bands of invertase activity was related to the amount of invertase present as well as to the duration of the invertase assay of a particular gel.

The gels obtained for invertase that are presented in Fig. 9 indicated that the segregational sucrose nonfermenter Z1-2D lacked both bands of invertase activity present in three sucrose fermenting

TABLE 9

Invertase and α -Glucosidase Activities in HZ1-113B (SUC3 mal10) and
ZH1-113D (suc0 MAL3)

Ascospore	Culture Conditions (age of culture in hours)	Specific Activity ^a	
		Invertase	α -Glucosidase
HZ1-113B (SUC3 mal10)	YEP 2% sucrose (23½)	1.417	0.087
	YEP 2% maltose (48)	2.229	1.89
	CS 2% sucrose (15)	1.48	0.069
	CS 2% maltose (63)	5.542	0.065
HZ1-113D (suc0 MAL3)	YEP 2% sucrose	- b	- b
	CS 2% maltose (90)	0	21.6

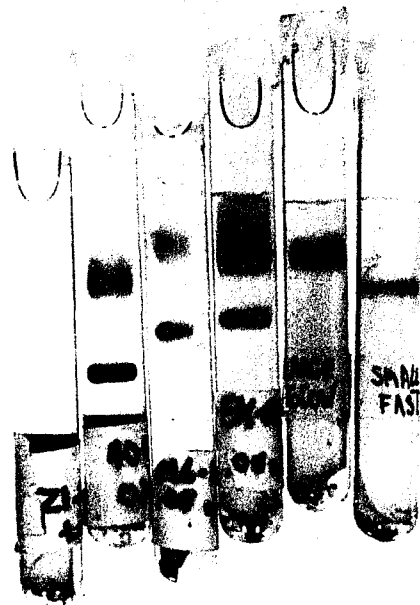
^a Extracts were prepared and enzymes assayed as described in "Materials and Methods". For growth of CS cultures, 0.1 ml of a 24 hour YEPD inoculum was added to 50 ml of CS medium containing the appropriate sugar.

^b Enzyme activities were not determined as the culture did not grow at all.

Fig. 9. Invertase activities of segregational sucrose nonfermenter (Z1-2D), three sucrose fermenters and the two forms of invertase of EK-6B separated on Sephadex C-150 (see Fig. 7), as visualized by SDS-polyacrylamide gel electrophoresis. Preparation of crude extracts, electrophoresis and invertase staining were performed as described in "Materials and Methods". Gel 1 represents Z1-2D (suc0), gel 2 4059-358D (SUC1), gel 3 M6-1D (SUC2), gel 4 EK-6B (SUC3), gel 5 external invertase of EK-6B (fraction 16 of EK-6B, Fig. 7), and gel 6 internal invertase of EK-6B (fraction 23 of EK-6B, Fig. 7).

EXTERNAL INVERTASE

INTERNAL INVERTASE



1 2 3 4 5 6

strains: EK-6B (SUC3), M6-1D (SUC2) and 4059 (SUC1). Sucrose non-fermenting (suc3) mutants lacked both invertase bands and were no different in appearance from Z1-2D (results not presented). The slower moving band represented the external invertase; the faster moving band, the internal invertase. This is presented in Fig. 10. The two invertases from strains bearing the SUC3 gene were separated on gels. The first gel showed that the SUC3 mal10 recombinant from the tetratype tetrad of cross EK-6B x Z1-2D (HZ1-113B) had the two bands of invertase activity. The second gel showed the invertase banding pattern of the SUC3 MAL3 strain HZ1-27D. The third and fourth gels represented the invertases of HZ1-27D partially fractionated on Sephadex G-150 (see Fig. 8, lower panel). The third gel showed that fraction 18 of the gel filtration of crude extract of HZ1-27D, the peak fraction of external invertase activity, gave a single slow-moving, broad band. The fourth gel in contrast showed that the peak fraction for the internal invertase gave a very strong band of fast-moving internal invertase, and a much fainter band of external invertase. It may be concluded that the Sephadex gel filtration of the invertases in crude extract of HZ1-27D did not separate the two forms of invertase completely, since the internal invertase was still mixed with material that migrated as a slow-moving band. It did appear that there was a correspondence between the external invertase eluting first from the Sephadex column and the slow-moving band of invertase activity, and the internal invertase with the fast-moving band. While not presented here, it was found that the SUC3 external invertase, partially purified according to the method of Goldstein and Lampen (1975), when applied to a SDS-polyacrylamide gel and electrophoresed,

Fig. 10. Visualization of fractionated invertase activities on SDS-polyacrylamide gels. Methods were as described in "Materials and Methods". Gel 1 represents the two invertases of HZ1-113B (SUC3 ma10), gel 2 HZ1-27D (SUC3 MAL3), gel 3 external invertase of HZ1-27D fractionation on Sephadex G-120 (Fig. 8), gel 4 the internal invertase from HZ1-27D (Fig. 8).

EXTERNAL INVERTASE

INTERNAL INVERTASE

TRACKING DYE



1

2

3

4

showed only the slow-moving band.

The co-segregation of both invertases already shown in Table 8 and in Fig. 8 was further demonstrated by electrophoresing aliquots of crude extracts of HZ1 ascospore clones 27A through 27D on separate gels and staining them for invertase activity (Fig. 11). Only 27A and 27D showed two bands of invertase activity; 27B and 27C showed neither.

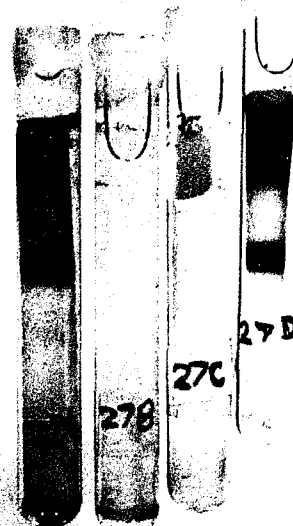
It was concluded that the two invertases separated on these SDS-gels were the same two invertases separable on Sephadex G-150, and that all SUC3 strains examined had both while sucrose non-fermenters had neither.

In summary, a series of sucrose nonfermenting mutants (suc3 mutants) were isolated from the strain EK-6B carrying the SUC3 gene. Genetic data in this Chapter suggest that suc3 mutants are allelic to the SUC3 gene, fail to complement each other, and are recessive in nature. The suc3 mutants fail to produce either external or internal invertase. It is concluded that a single point mutation in the SUC3 gene led to the loss of both invertases.

Fig. 11. Co-segregation of the external and internal invertases in cross EK-6B x Z1-2D, tetrad 27: visualization of invertase activities in SDS-polyacrylamide gels. See Fig. 8. Gel 1 represents ascospore 27A (SUC3); gel 2 ascospore 27B (suc0); gel 3 ascospore 27C (suc0); and gel 4 ascospore 27D (SUC3).

EXTERNAL INVERTASE

INTERNAL INVERTASE



1 2 3 4

CHAPTER IV

ISOLATION AND CHARACTERIZATION OF DE NOVO SUCROSE FERMENTERS FROM MUTATIONAL *suc3* NONFERMENTERS

The *suc3* mutants derived from SUC3 lacked the ability to ferment both sucrose and raffinose as a result of the lack of invertase activity. The nature of these *suc3* mutants and hence of the SUC3 gene, however, remained unresolved. In order to investigate whether the known SUC genes were structural genes for invertase or regulatory genes, a search for revertants was conducted. Revertants of these *suc3* mutants might be expected to possess altered levels of invertase, have thermolabile invertase or some function related to B-fructoside fermentation in a thermolabile state, have invertase with altered enzymatic properties or have altered regulatory properties. "Revertants" will be considered to be any strains derived from a mutational *suc3* nonfermenter with a restored ability to ferment raffinose and sucrose and having invertase activity. This chapter presents genetic and enzymatic data on a number of revertants derived from strains bearing *suc3* mutants; these "revertants" were the result of mutations at genes other than SUC3. These strains are thus called de novo sucrose fermenting strains.

Spontaneous Reversion Frequencies of *suc3* Mutants

To detect possible differences in the lesions responsible for the various *suc3* mutants, spontaneous reversion frequencies were determined. The *suc3* mutants were grown in YEPD broth for 20 hours at 30°C and the

cells were harvested and washed twice with sterile, distilled water. Aliquots of cells containing up to 5×10^7 cells were plated onto YEP 2% raffinose agar plates and incubated for 14 days. The following suc3 mutants have reversion frequencies of 10^{-8} : 2, 3, 20, 34, 220, 40 and 236; 2×10^{-8} , mutant 47; 5×10^{-8} , mutant 94. These frequencies were determined on agar plates containing raffinose as the sugar. When spontaneous revertants were selected on sucrose-containing agar plates, however, the reversion frequency was higher. Since yeast invertase is known to have a lower affinity for raffinose than for sucrose (Gascón, Neumann and Lampen, 1968), this might be part of an explanation for this disparity in the magnitude of spontaneous reversion frequencies. Alternatively, the revertants arising on sucrose plates might be the result of spontaneous mutations at genes other than those of the suc mutants. This second explanation seemed to have some validity in view of the observation that many of these "sucrose revertants" failed to ferment raffinose; some were examined for invertase activity and failed to show any. These might have been mutants able to utilize sucrose because of its hydrolysis by α -glucosidase (cf. Khan, Zimmermann and Eaton, 1973). Revertants were therefore routinely picked on raffinose-containing agar plates. These revertants were found to ferment sucrose as well as raffinose.

Of the few spontaneous revertants obtained from suc3-94, only SR3 (spontaneous revertant) appeared to be of interest. Revertant SR3 fermented sucrose in one to two days and raffinose in 5 to 7 days at 30°C , which was somewhat slower than the parental SUC3 strain EK-6B (see Table 10).

TABLE 10

**Fermentation Properties of Spontaneous and Induced Sucrose Fermenting
Strains Derived from suc3 Mutants**

Strain	Days to Ferment at 30°C		
	Raffinose	Sucrose	Maltose
EK-6B (SUC3)	+(2)	+(1)	+(2)
suc3-94 (suc3)	-	-	+(2)
Mutants induced with MNNG			
" MR2	+(3)	+(1)	+(2)
" MR3	+(2)	+(1)	+(2)
" MR4	+(2)	+(1)	+(2)
Mutants induced with EMS			
" ER501	+(2)	+(1)	+(2)
" ER502	+(2)	+(1)	+(2)
" ER509	+(2)	+(1)	+(2)
" ER512	+(2)	+(1)	+(2)
" ER520	+(2)	+(1)	+(2)
" ER536	+(2)	+(1)	+(2)
Spontaneous sucrose fermenter			
" SR3	+(5-7)	+(2)	+(2)
EMS-induced sucrose fermenter of suc3-40			
suc3-40 ERR1	+(4)	+(2)	+(2)
Spontaneous sucrose fermenter of suc3-20			
suc3-20 SR1	+(3)	+(1)	+(2)

A minus (-) sign indicates the failure to ferment a particular sugar. A plus (+) sign means the ability to ferment that sugar. The number of days needed to ferment a sugar is indicated in parentheses.

A spontaneous revertant of suc3-20, designated suc-20 SR1, was also picked from a raffinose plate. It was able to ferment sucrose in one day and raffinose in three days at 30°C (see Table 10).

Induced Revertants of suc3 Mutants

Attempts were made to obtain mutagen-induced revertants from various suc3 mutants. Mutant suc3-94 was the subject of most of the mutagenesis experiments because it, along with suc3-40, was the mutant best characterized as being allelic to SUC3. One mutant induced with EMS in suc3-40, designated suc3-40 ERR1 (for raffinose revertant) was also obtained. Sucrose fermenter suc3-40 ERR1 fermented raffinose in four days and sucrose in two days at 30°C and did not differ significantly in this regard from the parental SUC3 strain EK-6B (see Table 10).

Induction of SUC revertants with N-Methyl-N'-nitro-N-nitrosoguanidine (MNNG) and Ethyl Methane Sulfonate (EMS) derived from suc3-94

The first MNNG mutagenesis experiments using suc3-94 gave three SUC fermenting strains (designated MR) with restored ability to ferment sucrose and raffinose (Table 10). These induced sucrose fermenters did not differ in their fermentation properties from the SUC3 parent EK-6B. Later mutagenesis experiments with EMS yielded various sucrose fermenters (designated ER) whose fermentation properties are also presented in Table 10.

Genetic Analysis of Sucrose Fermenting Revertants

Dominance relationships of the sucrose fermenting strains, both induced and spontaneous, were investigated by crossing them to strains bearing the SUC3 gene and to segregational sucrose nonfermenters. Diploids were isolated from these crosses and examined for their fermentation properties. These results are presented in Tables 11 and 12. Diploids of the sucrose fermenting strains crossed to SUC3 strains were able to ferment raffinose in three days, sucrose in one day and maltose in three days at 30°C (Table 11).

Diploids of sucrose fermenters crossed to the segregational sucrose nonfermenter Z1-2D (suc0 mal0) were able to ferment raffinose and sucrose (Table 12).

These crosses were sporulated and tetrad analysis was undertaken to determine the genetic relationship of these revertants to suc3-94 (also to suc3-40 and suc3-20), and hence to SUC3. The results are presented in Table 13 for crosses of suc3-94 MR2 to Z1-2D (suc0 mal0) and to R. S. 5-1 (SUC3 mal0). In the cross MR2 x Z1-2D the number of parental ditype, tetratype and nonparental ditype tetrads for sucrose and maltose fermentation were those expected for a two-gene segregation (ratio of 1 PD:4 TT:1 NPD). Ascospore clones were obtained which fermented sucrose and raffinose but not maltose; conversely maltose fermenting ascospore clones able to ferment both raffinose and sucrose were obtained. If MR2 were allelic to SUC3, one expected only parental ditype tetrads. Tetrad data from the cross MR2 x R. S. 5-1 showed the ratio of parental ditype : tetratype : nonparental ditype tetrads close to the 1:4:1 ratio expected for a revertant at a gene unlinked to the

TABLE 11

Fermentation Properties of the SUC Revertants Crossed to SUC Strains

Crosses				Days to Ferment at 30°C		
				Raffinose	Sucrose	Maltose
R. S. 5-1 (SUC3 mal10) x suc3-94 MR2				+(3)	+(1)	+(2)
"	x	"	MR3	+(3)	+(1)	+(2)
"	x	"	MR4	+(3)	+(1)	+(2)
"	x	"	ER520	+(3)	+(1)	+(2)
"	x	"	ER536	+(3)	+(1)	+(3)
HZ1-27B (SUC3 MAL3) x suc3-40 ERR1				+(3)	+(1)	+(3)
R. S. 5-1 (SUC3 mal10) x suc3-94 SR3				+(3)	+(1)	+(3)
R. S. 5-1	"	x	suc3-20 SR1	+(3)	+(1)	+(3)

TABLE 12

Fermentation Properties of SUC Revertants Crossed to the Segregational
Sucrose Nonfermenter Z1-2D

Diploid of SUC Revertant crossed to Z1-2D (suc0 mal0)	Days to Ferment at 30°C		
	Raffinose	Sucrose	Maltose
suc3-94 MR2	+(3)	+(1)	+(3)
" MR3	+(3)	+(1)	+(3)
" MR4	+(3)	+(1)	+(3)
" ER509	+(3)	+(1)	+(3)
" ER512	+(3)	+(1)	+(3)
suc3-40 ERR1	+(3)	+(1)	+(3)
suc3-94 SR3	+(7-10)	+(1)	+(3)
suc3-20 SR1	+(3)	+(1)	+(3)

TABLE 13

Segregation of SUC Fermenter MR2 in Crosses with Z1-2D (suc0 mal0) and
R. S. 5-1 (SUC3 mal0)

Tetrad Types					
	PD	TT	NPD	Chi ²	p
MR2 x Z1-2D (suc0)	SUC MAL	SUC MAL	suc MAL		
	SUC MAL	suc MAL	suc MAL		
	suc mal	SUC mal	SUC mal		
	suc mal	suc mal	SUC mal		
Observed	2	8	2		
Expected ^a	12	0	0	∞	≪.001
Expected ^b	2	8	2	0	1.0
MR2 x R. S. 5-1 (SUC3)	PD	TT	NPD		
	^c MR SUC MAL	MR SUC MAL	suc MAL		
	MR SUC MAL	suc MAL	suc MAL		
	SUC mal	MR SUC mal	MR SUC mal		
	SUC mal	SUC mal	MR SUC mal		
Observed	4	13	4		
Expected ^b	3.5	14	3.5	0.214	0.9-1.0

^a Results expected if MR2 were allelic to SUC3.

^b Results expected if MR2 were single gene unlinked to suc3-94 MAL3.

^c MR represents sucrose fermenter gene causing fermentation of sucrose and raffinose unlinked to suc3-94 MAL3.

Chi² values were used to determine the p (probability) value based on two degrees of freedom.

suc3-94 mutant, hence not allelic to the parental SUC3 and MAL3 gene. Thus MR2 was a de novo sucrose and raffinose fermenter unlinked to suc3-94 MAL3.

No definite conclusions could be reached on the allelism of MR3 and MR4 to SUC3 because no complete and very few three-spored asci were recoverable from crosses of these strains to Z1-2D. For the cross MR3 x Z1-2D it was possible, however, to recover ascospores that were SUC MAL, SUC mal, suc MAL and suc mal; this suggests that the mutation in this strain may not have been allelic to SUC3.

The nature of the genetic lesions in EMS-induced sucrose fermenters of suc3-94 was studied in strains with low invertase activity (ER509, ER512 and ER520) and one with high invertase activity (ER536). Invertase activity is presented in Table 19. The ability of diploids of ER509 and ER512 crossed to Z1-2D to ferment raffinose and sucrose (Table 12) indicated that the genetic factors responsible for fermentation in these strains was dominant. Diploids of ER520 and ER536 crossed to R. S. 5-1 were also able to ferment raffinose and sucrose (Table 11), because of the presence of the parental SUC3 gene in addition to the function contributed by the sucrose fermenters.

These ER strains were allele-tested with respect to SUC3 by sporulating the above diploids and performing tetrad analysis of these crosses. The results for the cross ER512 x Z1-2D are presented in Table 14. If ER 512 were allelic to suc3-94 MAL3 (hence to SUC3) one would expect that all tetrads dissected would have the fermentation of both sucrose and raffinose co-segregating with maltose fermentation since SUC3 and MAL3 are closely linked; this hypothesis is not fulfilled. From the numbers of the three tetrad types present

TABLE 14

Segregation of Sucrose Fermentation in Cross ER 512 x Z1-2D

	Tetrad Types			Chi ²	p
	PD	TT	NPD		
	SUC MAL	SUC MAL	suc MAL		
	SUC MAL	suc MAL	suc MAL		
	suc mal	SUC mal	SUC mal		
	suc mal	suc mal	SUC mal		
Observed	1	6	3		
Expected ^a	10	0	0	∞	<.001
Expected ^b	1.67	6.68	1.67	0.68	0.7-0.8

^a Results expected if ER512 were allelic to SUC3.

^b Results expected if ER512 were result of mutation at a gene
unlinked to SUC3 (unlinked to suc3-94).

(1 parental ditype (PD): 6 tetratype (TT): 3 nonparental ditype (NPD)), it is most probable that the mutation to sucrose and raffinose fermentation in ER512 occurred at a gene unlinked to mutant suc3-94 and MAL3. The Chi-square value of 0.68 with two degrees of freedom gives a probability of 70 to 80 per cent that these results do not significantly deviate from the theoretically expected numbers. It was previously established that mutant suc3-94 was allelic to SUC3 (see Chapter III, Table 5).

The inability to recover any complete tetrads and the low frequency of three-spored asci rendered it impossible to perform tetrad analysis of the cross ER509 x Z1-2D. Considering all isolated ascospore clones in a random fashion, the following numbers of each kind were obtained: SUC MAL, 3; SUC mal, 5; suc MAL, 4; and suc mal, 6. The recombinant ascospore types SUC mal and suc MAL were about as frequent as the parental types SUC MAL and suc mal. It may be tentatively concluded that ER509 was also the result of a mutation at a gene unlinked to SUC3.

Tetrad analysis of the cross ER520 x R. S. 5-1 revealed 7 nonparental ditype tetrads and two tetratype tetrads (Table 15). This suggested that this revertant was the result of mutations at more than one gene unlinked to SUC3.

For the cross ER536 x R. S. 5-1 (Table 15) tetrad data showed a ratio of tetrad types that was very close to that expected for a two-gene segregation for sucrose and maltose fermentation: 1 PD:4 PD:1 NPD. ER536 was most likely the result of a mutation at a gene unlinked to suc3-94 (SUC3).

The EMS-induced sucrose fermenter derived from suc3-40, suc3-40

TABLE 15

Segregation of Sucrose Fermentation in Crosses of ER520 and ER536 to
SUC3 mal0 strain R. S. 5-1

Cross		Tetrad Types							
R. S. 5-1 x		PD		TT		NPD		Chi ²	p
ER Strain		suc MAL (SUC) ^a	suc MAL (SUC)	suc MAL (SUC)	suc MAL (SUC)	suc MAL (SUC)	suc MAL (SUC)		
		suc MAL (SUC)	suc MAL (SUC)	suc MAL (SUC)	suc MAL (SUC)	suc MAL (SUC)	suc MAL (SUC)		
		SUC mal	SUC mal	SUC mal	SUC mal	SUC mal	SUC mal		
		SUC mal	SUC mal	SUC mal	SUC mal	SUC mal	SUC mal		
ER520	Observed	0		2		7			
	Expected ^b	9		0		0		∞	<.001
	Expected ^c	1.5		6		1.5		5.67	.05-.1
ER536	Observed	4		11		2			
	Expected ^b	17		0		0		∞	<.001
	Expected ^c	2.8		11.4		2.8		.757	.5-.7

^a suc MAL (SUC) indicates ascospore clones carrying the genetic factor responsible for sucrose and raffinose fermentation in the ER strains.

^b Results expected if revertant allelic to SUC3.

^c Results expected if revertant were result of mutation at a gene unlinked to SUC3.

ERR1, appeared to be the result of a mutation at a gene unlinked to SUC3 (see Table 16). As was the case with the various de novo sucrose and raffinose fermenters induced in suc3-94, suc3-40 ERR1 crossed to Z1-2D yielded tetrads in which sucrose and raffinose fermentation (SUC) segregated independently of maltose fermentation (MAL).

Genetic analysis of the spontaneous revertants suc3-94 SR3 and suc3-20 SR1 revealed them to be most probably the result of mutations at genes unlinked to SUC3 (Table 17). The tetrad analysis data agreed more closely with the hypothesis that each of these strains was the result of a mutation at a gene unlinked to the suc3 mutant.

The genetic analysis of these induced and spontaneous sucrose and raffinose fermenting strains derived from suc3 mutants may be summarized by stating that each appeared to be the result of a mutation at a gene unlinked to SUC3. This other gene(s) may be a nonfunctional sucrose gene (suc0) at least superficially analogous to that present in segregational sucrose nonfermenters. Allele testing of these strains to other known SUC genes (SUC2, etc.) was not attempted, however.

Enzymatic and Physiological Studies of Sucrose Fermentors Derived from suc3 Mutants

Even though none of the sucrose fermenters derived from strains containing suc3 mutants was shown to be allelic to SUC3, these fermenters were used to characterize invertase in crude extracts. Invertase specific activity was determined in crude extracts of derepressed cells of MNNG-induced sucrose fermenters (Table 18), and

TABLE 16

Segregation of the Genetic Factor Responsible for Sucrose Fermentation
in the Cross *suc3-40* ERR1 x Z1-2D

	Tetrad Types			Chi ²	p
	Parental Ditype (PD)	Tetra- type (TT)	Nonparental Ditype (NPD)		
	SUC MAL SUC MAL suc mal suc mal	SUC MAL suc MAL SUC mal suc mal	suc MAL suc MAL SUC mal SUC mal		
Observed	4	6	2		
Expected ^a	12	0	0	∞	≪.001
Expected ^b	2	8	2	2.5	0.2-0.3

^a Results expected if *suc3-40* ERR1 were allelic to *suc3-40* (hence to SUC3).

^b Results expected if *suc3-40* ERR1 were result of gene unlinked to *suc3-40* (hence not allelic to SUC3).

TABLE 17

Segregation of Sucrose Fermentation in Crosses suc3-94 SR3 x Z1-2D and
suc3-20 SR1 x R. S. 5-1

Cross	Tetrad Types			Chi ²	p
suc3-94 SR3 x	PD	TT			
Z1-2D	suc MAL (SUC) ^a suc MAL (SUC) suc mal suc mal	suc MAL (SUC) suc MAL suc mal (SUC) suc mal	suc MAL suc MAL suc mal (SUC) suc mal (SUC)		
Observed	8	12	5		
Expected ^b	25	0	0	∞	<.001
Expected ^c	4.2	16.6	4.2	4.82	.05-.1
suc3-20 SR1 x	PD	TT			
R. S. 5-1	suc MAL (SUC) ^a suc MAL (SUC) SUC mal SUC mal	suc MAL (SUC) suc MAL SUC mal (SUC) SUC mal	suc MAL suc MAL SUC mal (SUC) SUC mal (SUC)		
Observed	1	9	3		
Expected ^b	13	0	0	∞	<.001
Expected ^c	2.2	8.8	2.2	.95	.3-.5

^a (SUC) represents gene responsible for sucrose and raffinose fermentation in the revertants; suc MAL represents the suc3 mutant. SUC represents the ability to ferment both sucrose and raffinose determined by the SUC3 gene.

^b Number of tetrads expected if revertant were allelic to SUC3.

^c Number of tetrads expected if revertant were result of mutation at a gene unlinked to SUC3.

TABLE 18

Invertase and α -Glucosidase Activities in EK-6B, suc3-94 and MNNG-induced Revertants from suc3-94

Strain	A590 nm	Specific Activity	
		Invertase	α -Glucosidase
EK-6B	1.303	1.49	0.219
suc3-94	1.228	0	0.303
" MR2	1.23	1.6	0.233
" MR3	1.256	2.2	0.283
" MR4	1.050	0.67	0.295

Crude extracts were prepared from harvested and washed 50 ml cultures grown on YEP + 0.5% glucose for 18 hours at 30°C, as described in "Materials and Methods".

compared with that of EK-6B. While there was some variation, none of these de novo fermenters had either significantly higher or lower invertase activity to warrant anything other than a superficial investigation of the properties of the invertases.

To determine if these de novo sucrose fermenters possessed both forms of invertase shown to be present in various SUC3 strains (see Chapter III), polyacrylamide gels were run with aliquots of crude extracts made from derepressed cultures of EK-6B, suc3-94 and the three MR strains (Fig. 12). All three de novo MR strains had two bands of invertase activity which had relative mobilities in the gels similar to their counterparts in EK-6B, although this was not proof that they were identical to (had the same amino acid sequence, etc.) the external (slow-moving) and internal (fast-moving) bands in the parental strain SUC3 strain EK-6B. The suc3-94 mutant lacked both bands of invertase activity.

Invertase levels were also examined in a number of de novo sucrose fermenters of suc3-94 induced with EMS (Table 19). While these fermenting strains showed no variation in the time required to ferment sucrose and raffinose (see Table 10), they showed considerable variation in invertase levels. Fermenter ER501 had about the same invertase specific activity as EK-6B, but another group of fermenters had less invertase activity than EK-6B (ex. ER512, specific activity 0.172). De novo fermenter ER536 was representative of a group having about two-fold more invertase activity than EK-6B. α -Glucosidase levels showed less than a two-fold variation from highest to lowest (as was the case with the MR strains in Table 18). Further enzymatic investigations were not conducted with the invertases of these strains as none had a "reversion" that was allelic to SUC3.

Fig. 12. SDS-Polyacrylamide gel electrophoresis of invertase activities in EK-6B, suc3-94 and MNNG-induced sucrose fermenters derived from suc3-94. Crude extracts were prepared from cells of derepressed cultures as described in "Materials and Methods". Aliquots of 0.05 ml of crude extract were applied to gels. Gel 1 is EK-6B; gel 2 is suc3-94; gel 3 MR2; gel 4 MR3; gel 5 MR4. Invertase activity was stained for as described in "Materials and Methods".

EXTERNAL INVERTASE

INTERNAL INVERTASE



1 2 3 4 5

TABLE 19

Invertase and α -Glucosidase Levels of EMS-induced Sucrose Fermenting
Strains Derived from suc3-94

Strain	A590 nm	Specific Activity	
		Invertase	α -Glucosidase
EK-6B (SUC3)	1.244	0.690	0.091
ER 501	1.137	0.535	0.111
ER 502	1.095	1.265	0.086
ER 509	1.122	0.364	0.105
ER 512	1.171	0.172	0.135
ER 520	1.291	0.239	0.131
ER 536	1.213	1.967	0.119

Enzyme specific activities were determined in crude extracts of cells grown on 10 ml of YEP + 0.5% glucose for 18 hours at 30°C, except for ER 520 which was grown for 20 hours. Crude extracts were prepared using the Braun homogenizer as described in "Materials and Methods".

suc3-94 SR3: A spontaneous de novo sucrose fermenter derived from
suc3-94 with altered sucrose fermentation and invertase

Of the few spontaneous revertants obtained from suc3-94, only SR3 appeared to be of interest since SR3 fermented sucrose in one to two days and raffinose in five to seven days at 30°C. This was somewhat lower than the wild type SUC3 strain EK-6B.

The slower growth of strain SR3 on raffinose compared with the wild type strain prompted further investigation of this phenomenon by comparing growth of SR3 and EK-6B on YEP-based media containing various sugars as carbon sources (Fig. 13). On media containing 0.5% glucose the growth curves for these two strains were virtually superimposable; furthermore, EK-6B did not grow significantly faster on sucrose than SR3. SR3 grew more slowly on raffinose than did EK-6B. While the data presented here are absorbance readings at 590 nm, viable counts for these strains at the same time points also revealed that SR3 was growing more slowly than EK-6B (data not presented).

When invertase levels were compared in derepressed cultures of EK-6B and SR3, the parental SUC3 strain had about five-fold higher activity than the spontaneous fermenter (Table 20). α -Glucosidase activity was about one-half that seen in the de novo fermenter SR3.

In order to determine if the SR3 invertase were structurally different from that of EK-6B in such a way as to explain the former's slower growth on raffinose, the K_m for sucrose and thermal lability were compared in both SR3 and EK-6B. There was a significant difference in the K_m for sucrose; SR3 invertase had a K_m for sucrose about five-fold greater than that of EK-6B (Fig. 14: 0.167 M sucrose for SR3 versus 0.028 M sucrose for EK-6B). While this difference was not large enough to cause any significant diminution of the de novo

Fig. 13. Comparison of growth of EK-6B and suc3-94 on YEP-based media with various sugars at 30°C. The upper panel shows growth of EK-6B and SR3 on YEP 0.5% glucose and on YEP 2% sucrose. To one liter volume of media in 2.2 liter Fernbach flask was added 10 ml of 24 hour old YEPD broth inoculating cultures.

0.5% glucose: Δ EK-6B □ SR3

2% sucrose : ▲ EK-6B ■ SR3

The lower panel shows growth on YEP2% raffinose.

○ EK-6B ● SR3

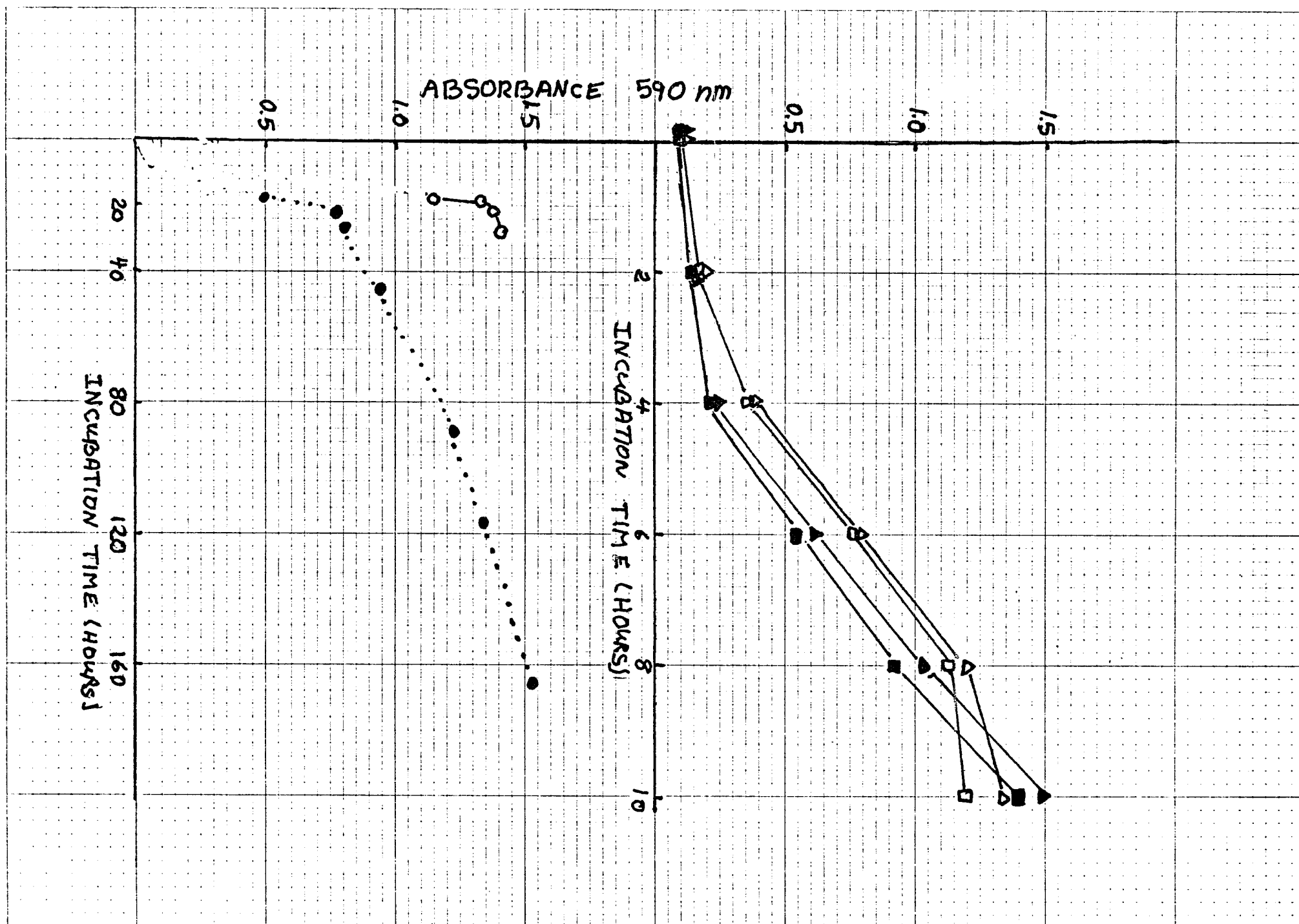


TABLE 20

Invertase and α -Glucosidase Activity in EK-6B and Sucrose Fermenting
Strains Derived from *suc3* Mutants

Strain	A590 nm	Specific Activity	
		Invertase	α -Glucosidase
EK-6B (SUC3) ^a	1.418	1.1	0.158
<i>suc3</i> -94 SR3 ^a	1.916	0.203	0.259
<i>suc3</i> -40 ERR1			
YEP 0.3% glucose	1.2 (18 hr)	2.18	0.336
YEP 2% sucrose	1.4 (18)	0.81	0.693
<i>suc3</i> -20 SR1			
YEP 0.5% glucose	1.2 (19½)	0.33	0.260

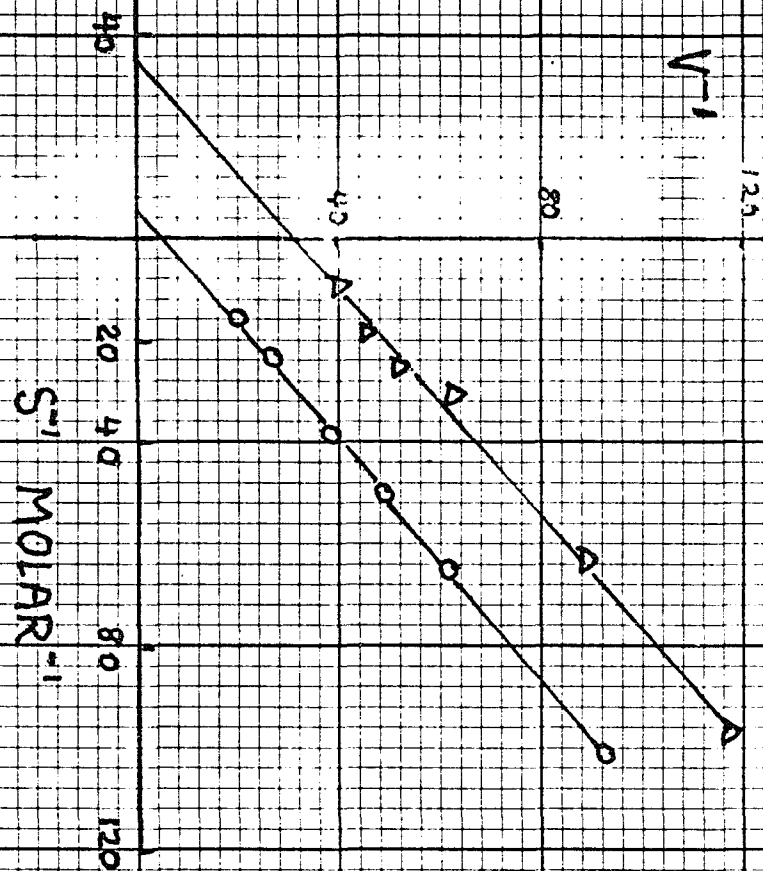
^a Enzyme activities were determined in crude extracts prepared from cells grown on YEP 0.5% glucose for 24 hours at 30°C.

Fig. 14. Lineweaver-Burk Plot of invertase activity of suc3-94 SR3 and EK-6B. Crude extracts were prepared from cells grown on YEP 0.5% glucose for 24 hours at 30°C, as described in "Materials and Methods". The K_m for sucrose at pH 4.5 (0.1 M sodium acetate buffer, 30°C) was obtained directly from the Lineweaver-Burk plot.

Legend

Δ EK-6B

○ suc3-94 SR3



fermenter's growth on sucrose compared with EK-6B, growth on raffinose would be much slower if the K_m for raffinose were increased proportionately. (from 0.150 M raffinose to 0.750 M, although this was not measured). Yeast invertase from a strain bearing the SUC2 gene was reported to have a K_m for raffinose six-fold larger than that for sucrose (Gascón, Neumann and Lampen, 1968).

Thermal lability was also investigated in strain SR3 and the SUC3 strain HZ1-27D (see Chapter III). Both invertases appeared to be inactivated to the same overall extent (Fig. 15); 30% of strain SR3 invertase remained after 30 minutes versus 26% for the SUC3 strain. An interesting difference appeared to be the nearly logarithmic decay of SUC3 invertase activity with time, whereas the de novo fermenter's invertase showed what may be a biphasic curve of inactivation. This difference may have been the result of differing amounts of the two forms of invertase (external and internal) present in the crude extracts of SR3 and the SUC3 strain. Crude extracts of strain SR3 have, however, both forms of invertase. If the difference in the shape of the inactivation curves is not a result of experimental error, a difference in amino acid sequence of these invertases from SUC3 and SR3 affecting thermal lability of these invertases may be another possible explanation.

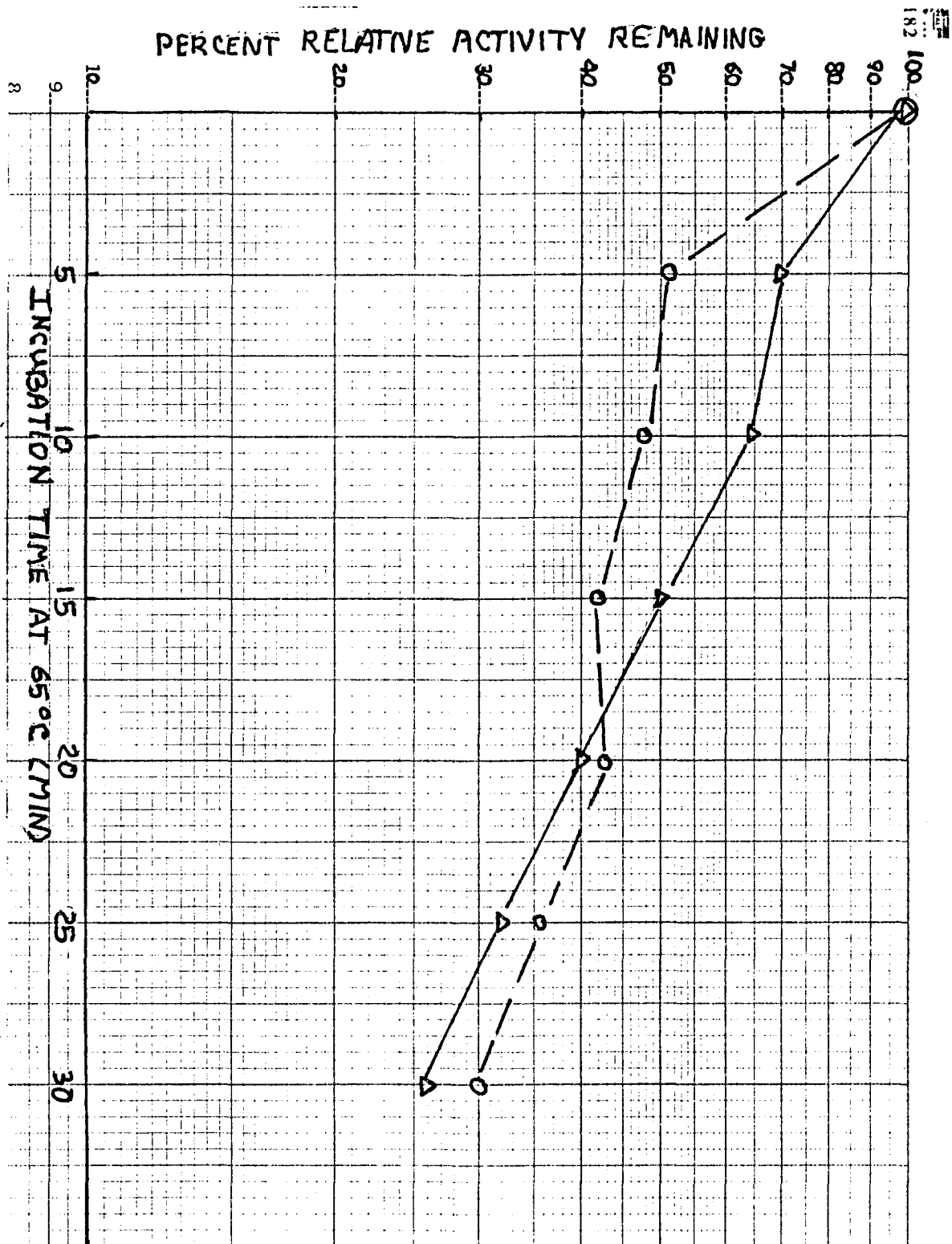
Whether de novo fermenter SR3 possessed the two forms of invertase that SUC3 strains (as well as other SUC genes) have was examined by running aliquots of crude extract of this revertant on polyacrylamide gels and staining for invertase activity as described in "Materials and Methods". Crude extracts of cells grown under derepressed conditions were used. As already described in Chapter III SUC3 strains such as EK-6B and HZ1-27D had two bands of invertase

Fig. 15. Thermal lability of invertase of strains suc3-94 SR3 and the SUC3 strain HZ1-2D at 65°C at pH 5.0. Crude extracts were prepared from cells grown on YEP 2% sucrose as described in "Materials and Methods". Crude extracts contained 8.9 and 9.5 mg of protein per ml for suc3-94 SR3 and HZ1-27D respectively. To follow heat inactivation of invertase at 65°C at pH 5.0, aliquots of 0.4 ml of the above crude extracts, warmed to room temperature, were pipetted into 1.6 ml of 0.025 M sodium acetate buffer pH 5.0 preheated to 65°C. At appropriate time intervals aliquots of 0.25 ml were removed and rapidly pipetted into 0.75 ml of ice-cold sodium acetate buffer. Invertase activity was then determined as described in "Materials and Methods".

Legend

Δ EK-6B

○ suc3-94 SR3



activity: a slow-moving band with a Rf (relative mobility) of 0.2 to 0.3 corresponding to the external invertase, and a fast-moving band with a Rf of 0.6 to 0.7 corresponding to the small internal invertase. Gels with extract from SR3 also had two band of activity that corresponded with those of the SUC3 strains.

In summary, strain SR3 derived from suc3-94, while not allelic to SUC3, had several properties which made it interesting: (1) this de novo sucrose fermenter fermented and grew more slowly on raffinose than the parental SUC3 strain; (2) this fermenter had about one-fifth the invertase specific activity of EK-6B; (3) the Km for sucrose of strain SR3 was about five-fold larger than that of EK-6B. It may be tentatively concluded from these results that SR3 has invertase(s) which are different in amino acid sequence which consequently affected substrate affinity and enzyme stability. The Km for sucrose was several-fold that reported for any of the invertases determined by known SUC genes (Ottolenghi, 1971b). SR3 may contain an allele of one of the known SUC genes other than SUC3, or of a new structural gene for invertase.

suc3-40 ERR1: An EMS-induced sucrose fermenter in mutant suc3-40

Enzymatic studies of this induced de novo sucrose fermenter derived from suc3-40 indicated that it had higher specific activity of invertase in extracts of cells grown on glucose and lower activity in extract from sucrose-grown cells (Table 20; see also Table 18). These results were interesting since for the parental SUC3 strain EK-6B invertase specific activity was somewhat higher in sucrose-grown than in cells grown in 0.5% glucose (see Chapter III).

The possibility that the invertase present in sucrose fermenter suc3-40 ERR1 was different from that of EK-6B was investigated by

determining the K_m for sucrose. This de novo fermenter had a K_m for sucrose of 0.039 M sucrose in crude extract of derepressed cells; this was about 30 per cent higher than that for EK-6B invertase (0.028 M sucrose; see Fig. 14).

suc3-20 SR1: A spontaneous sucrose fermenter

Strain SR1 derived from suc3-20 was found to have less invertase activity than the parental SUC3 strain (Table 20). The possibility that this sucrose fermenter had an invertase different from that of EK-6B was examined by determining the K_m for sucrose. For strain SR1 the K_m was 0.03 M sucrose which was not significantly different from that for invertase from EK-6B, nor from that of invertases determined by other known SUC genes (Ottolenghi, 1971b).

The results presented in this chapter may be summarized as follows:

- (1) "Revertants" of various suc3 mutants, obtained spontaneously or induced with MNNG or EMS, were able to ferment sucrose and raffinose.
- (2) These revertants were the result of dominant mutations at genes unlinked to SUC3; these sucrose fermenters were thus not true revertants but were de novo fermenters arising from nonfunctional genes other than suc3 mutants.
- (3) All de novo fermenters had invertase activity; the level of invertase activity varied somewhat among the various fermenters compared with the parental SUC3 strain EK-6B.
- (4) One sucrose fermenter, suc3-94 SR3, had a lower affinity for sucrose (and presumably for raffinose also) compared with invertase from SUC3 or other SUC genes.

While enzymatic studies of these de novo sucrose fermenters suggest that at least one of them may have an invertase with properties

different from that of the SUC3 strain, the major problem faced in this study was that none of the sucrose fermenters were true revertants of the suc3 mutants, hence none was allelic to SUC3. This might have been the result of any of the following alternatives: (1) the suc3 mutants allelic to SUC3 were deletions which could not be reverted; (2) there were various nonfunctional sucrose genes present in EK-6B and the suc3 mutants derived from it; reversion to sucrose and raffinose fermentation was perhaps just as likely to occur at any of these nonfunctional sucrose genes as at the induced suc3 nonfermenters. That there may be such nonfunctional sucrose genes present in the strains bearing the suc3 mutants is supported by the observation that sucrose and raffinose fermenters could be induced in segregational sucrose nonfermenters such as AZ-5A; these de novo fermenters were able to ferment both sugars since they had invertase activity.

The possibility that suc3 mutants derived from EK-6B have genes which can mutate into functional SUC genes other than SUC3 greatly complicates any genetic investigation of the role of a particular SUC gene, in the present case SUC3.

CHAPTER V

GENETIC AND BIOCHEMICAL STUDIES OF SUCROSE NONFERMENTING MUTANTS INDUCED IN SUC1 STRAIN 4059-358D AND REVERTANTS DERIVED FROM THEM

Isolation and Characterization of Mutants Defective in Sucrose and Raffinose Formation

Two mutants defective in their ability to ferment sucrose and raffinose were isolated from the SUC1 strain 4059-358D, which carried the MAL1 gene closely linked to SUC1; this strain was also able to ferment α -methylglucoside. The suc mutants were induced using EMS. The fermentation properties of the SUC1 parent, hereafter designated strain 4059, and the two suc mutants are as follows:

	Days to ferment at 30°C			
	Raf	Suc	Mal	MG
4059 (SUC1)	+(4)	+(1)	+(2)	+(5)
suc D10	-(14)	+(7)	+(3)	+(5)
suc CII7	-(14)	-(14)	+(2)	+(5)

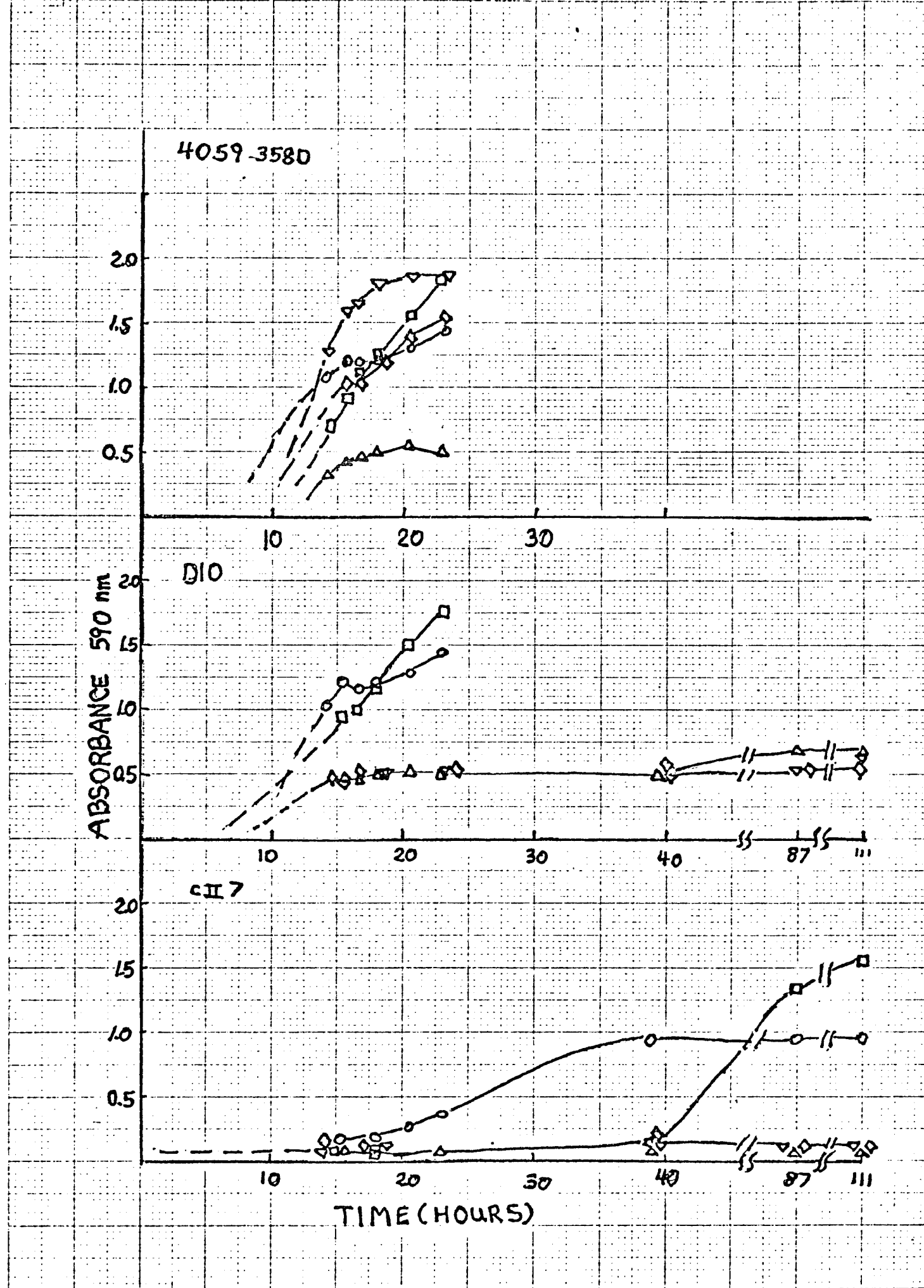
Growth Characteristics of SUC1 Strain 4059 and the suc Mutants Derived From It

Growth of SUC1 strain 4059 and the suc mutants D10 and CII7 on various sugars was compared (Fig. 16). Strain 4059 and sucD10 were respiratory competent and grew equally well on YEP alone, or on YEP supplemented with glucose or maltose. Growth of 4059 on sucrose was initially more rapid than on maltose. Growth of strain 4059 on raffinose

Fig. 16. Growth of strain 4059-358D (SUC1 MAL1 MGL) and two suc mutants derived from it (suc MAL1 MGL) on various YEP-based media. Flasks containing 50 ml of YEP-based media supplemented with different sugars were inoculated with 0.05 ml of a cell suspension or culture to give a final cell concentration of 2×10^5 per ml. Cultures were incubated at 30°C and growth was followed by reading absorbance at 590 nm, at appropriate times, using a Beckman DU spectrophotometer.

Legend

- Δ YEP alone
- YEP 0.5% glucose
- YEP 2% maltose
- ▽ YEP 2% sucrose
- ◇ YEP 2% raffinose



was about equal in magnitude to that on 0.5% glucose, probably due to the fact that only one-third of raffinose (the fructose moiety which was one-third of 2% raffinose present) was actually used (see Table 1 in Chapter II, "Materials and Methods"). Growth of the parental SUC1 strain on YEP alone was considerably less than on any of the added sugars.

Mutant sucD10 grew on YEP or YEP plus glucose or maltose about as well as the SUC1 parent. Growth on sucrose and raffinose, however, was virtually nil since there was no difference in the amount of growth on these sugars when compared with the YEP control. Thus sucD10 did not utilize these B-fructosides.

Mutant CII7 was respiratory deficient as it was unable to grow on glycerol-containing media. This defect, however, was unlinked to the suc mutation (results not presented). The respiratory defect resulted in less growth on the various media including YEP alone. Growth on glucose was slow and on maltose even slower than in the other strains, but was still more than on YEP alone. Sucrose and raffinose, however, were not utilized.

These results indicated that the suc mutants were defective in their ability to utilize sucrose and raffinose.

Genetic Analysis of suc1 Mutants Induced in Strain 4059

To determine the genetic nature of suc mutants derived from strain 4059, the parental strain and suc mutants D10 and CII7 were crossed to the segregational sucrose nonfermenter EZ-5B. Diploids were isolated from these crosses and examined for their fermentation properties

(Table 21). The diploids of all of these strains crossed to EZ-5B fermented maltose in one day at 30°C; the diploid 4059 x EZ-5B, however, was the only one to ferment sucrose in one day and raffinose in three. The crosses involving the two suc mutants failed to ferment raffinose in fourteen days, and took seven days to ferment sucrose at 30°C. The basis of this slow sucrose fermentation is unknown since these diploids do not have detectable levels of invertase (see Table 26). The two suc mutants were thus considered to be recessive, and were not complemented by the segregational sucrose nonfermenter.

To answer the question of whether or not the suc mutants were actually allelic to the SUC1 gene, the diploids of the above crosses were sporulated and tetrad analysis was performed (Table 22). As expected, the cross 4059 x EZ-5B gave only tetrads with two SUC MAL ascospore clones and two suc MAL ascospore clones; thus strain 4059 contained a single SUC gene closely linked to the MAL1 gene. Data from incomplete tetrads (not presented here) supported this conclusion. Dissection of asci from the crosses of suc mutants to EZ-5B yielded only 2 suc MAL: 2 suc mal tetrads; incomplete asci yielded data (results not presented) which also suggested that the suc mutants were due to a single gene difference which was probably at or near the SUC1 gene. Analysis of the random ascospore clones yielded nearly equal numbers of suc MAL and suc mal ascospores, which further supported this conclusion.

The suc mutants were crossed to a SUC1 MAL1 segregant (4059-3A) derived from the cross 4059 x EZ-5B, and diploid clones were isolated (Table 23). Examination of the fermentation properties of these

TABLE 21

Fermentation Properties of Diploids of 4059 and *suc1* Mutants Crossed
to Segregational Sucrose Nonfermenter EZ-5B

Cross	Days to Ferment at 30°C				
	RAF	SUC	MAL	MG	GLUC
4059 x EZ-5B	+(6)	+(1)	+(5)	+(5)	+(1)
D10 x EZ-5B	-(16)	+(6)	+(5)	+(5)	+(1)
CII7 x EZ-5B	-(16)	+(4)	+(2)	+(6)	+(1)

The numbers in parentheses represent the number of days necessary
to ferment a particular sugar.

TABLE 22

Crosses of Strain 4059 (SUC1) and Two Induced suc1 Mutants to the
Segregational Sucrose Nonfermenter EZ-5B

Cross	Tetrad Types		
	SUC MAL	suc MAL	suc MAL
	SUC MAL	suc MAL	suc MAL
	suc mal	suc mal	suc MAL
	suc mal	suc mal	suc MAL
4059 x EZ-5B	15	0	0
suc1-D10 x EZ-5B	0	7	0
suc1-CII7 x EZ-5B	0	5	0
suc1-D10-2C x suc1-CII7	0	0	10

Ascospore clone suc1-D10-2C was an a suc1 MAL1 trp1 segregant
derived from the cross suc1-D10 x EZ-5B.

Random Spore Analysis

	suc MAL	suc mal
suc1-D10 x EZ-5B	59	63
suc1-CII7 x EZ-5B	19	14

TABLE 23

Fermentation Properties of SUC1 x SUC1, SUC1 x suc1 and suc1 x suc1
Diploids

Cross	Days to Ferment at 30°C		
	RAF	SUC	MAL
SUC1 x SUC1			
4059 x 4059-3A	+(3)	+(1)	+(1)
4059-3A x 4039-3C	+(3)	+(1)	+(1)
SUC1 x suc1			
4059 x D10-2C	+(6)	+(1)	+(1)
4059-3A x D10	+(6)	+(1)	+(1)
4059-3A x CII7	+(6)	+(1)	+(3)
suc1 x suc1			
D10-2C x D10	-(14)	+(6)	+(3)
D10-2C x CII7	-(14)	+(6)	+(3)

Strain 4059-3A is an a trp1 SUC1 MAL1 segregant derived from the cross 4059 x EZ-5B. Strain 4059-3C is an Δleu1 SUC1 MAL1 segregant derived from the same cross. D10-2C is an a trp1 suc1 MAL1 segregant derived from the cross D10 x EZ-5B.

diploids revealed that they fermented maltose and sucrose at 30°C in the same number of days as the SUC1/SUC1 homozygous diploid (4059 x 4059-3A); this was one day. They fermented raffinose, however, in six instead of three required by the homozygous SUC1 diploid. This result may be explained by a gene dosage effect operating for fermentation on raffinose; diploids having only one functional SUC1 gene will take twice as long to ferment raffinose as diploids having two functional SUC1 genes. The homoallelic (suc1-D10/suc1-D10) or heteroallelic (suc1-D10/suc1-CII7) crosses of suc1 mutants failed to ferment raffinose within 14 days but fermented sucrose in six (Table 23). This may be the result of the presence of other genetic factors affecting sucrose fermentation in the absence of SUC genes, since these diploids had no detectable invertase activity (see Table 27).

The diploids of suc mutants crossed to 4059-3A were sporulated and tetrads were dissected (Table 24). Most of the tetrads were 2 SUC MAL: 2 suc MAL, indicating a single gene difference between the suc mutants and the SUC1 segregant. The few tetrads showing 3:1 or 1:3 segregation of sucrose fermentation versus inability to ferment sucrose may have been aberrant, although the other genetic markers segregated in the expected ratios in these tetrads. If these are not false tetrads, however, they may represent gene conversion events which are very frequent. S. Fogel (personal communication) reported finding a high incidence of gene conversion of SUC3 MAL3; perhaps the above findings have a similar basis.

The genetic data presented above suggested that the suc mutants were allelic to SUC1 or closely linked to it. They will hereafter be designated suc1.

TABLE 24

Crosses of suc1 Mutants to a SUC1 Strain (4059-3A)

Cross	Tetrad Types		
	SUC MAL	SUC MAL	suc MAL
	SUC MAL	SUC MAL	suc MAL
	suc MAL	SUC MAL	suc MAL
	suc MAL	suc MAL	SUC MAL
suc1-D10 x 4059-3A	13	1	1
suc1-C117 x 4059-3A	17	3	0

Ascospore clone 4059-3A was a SUC1 MAL1 segregant (a trp1)
 derived from the cross 4059 x EZ-5B.

Invertase and α -Glucosidase Levels in Strain 4059 (SUC1) as Functions of Culture Age

In order to know when invertase and α -glucosidase activities were maximal in 4059 and strains derived from it, strain 4059 was grown on YEP-based 0.5% glucose and 2% sucrose and invertase and α -glucosidase levels were determined in crude extracts made from cells harvested at various culture ages. Growth was most conveniently monitored as absorbance at 590 nm because strain 4059, both suc1 mutants and the revertants derived from them formed small balls of cells which made it difficult to accurately determine cell numbers per ml (although this clumping will have some effect on the absorbance).

In strain 4059 grown on 0.5% glucose (see Fig. 17), both invertase and α -glucosidase specific activities appeared to increase as the culture approached late exponential growth phase. Both invertase and α -glucosidase had maximal specific activities between 16 and 18 hours when the culture was in late exponential or early stationary growth phase. After 20 hours invertase activity decreased more rapidly than α -glucosidase. Therefore, cultures were routinely harvested about 18 hours to obtain maximal levels of these enzymes.

Enzyme levels as a function of culture age were also determined in 4059 grown on YEP 2% sucrose (Fig. 18). Invertase and α -glucosidase activities increased from low levels in middle exponential growth phase to maximal levels during early stationary growth phase. Shortly thereafter activities of both enzymes declined. Therefore cultures grown on sucrose were usually harvested between 18 and 20 hours in order to obtain maximal levels of invertase and α -glucosidase.

Fig. 17. Invertase and α -glucosidase specific activities as functions of the culture age in strain 4059-358D (SUC1 MAL1 MGL) grown on YEP 0.5% glucose. 0.2 ml of a 24 hour culture of strain 4059 grown on YEPD broth was added to 200 ml of YEP 0.5% glucose. This culture was incubated at 30°C. At appropriate times aliquots of 30 ml were removed and treated as described in "Materials and Methods". Enzyme assays were performed in crude extracts as described in "Materials and Methods".

Legend

- ▲ Absorbance at 590 nm (read in Beckman DU spectrophotometer)
- Invertase specific activity
- α -Glucosidase specific activity

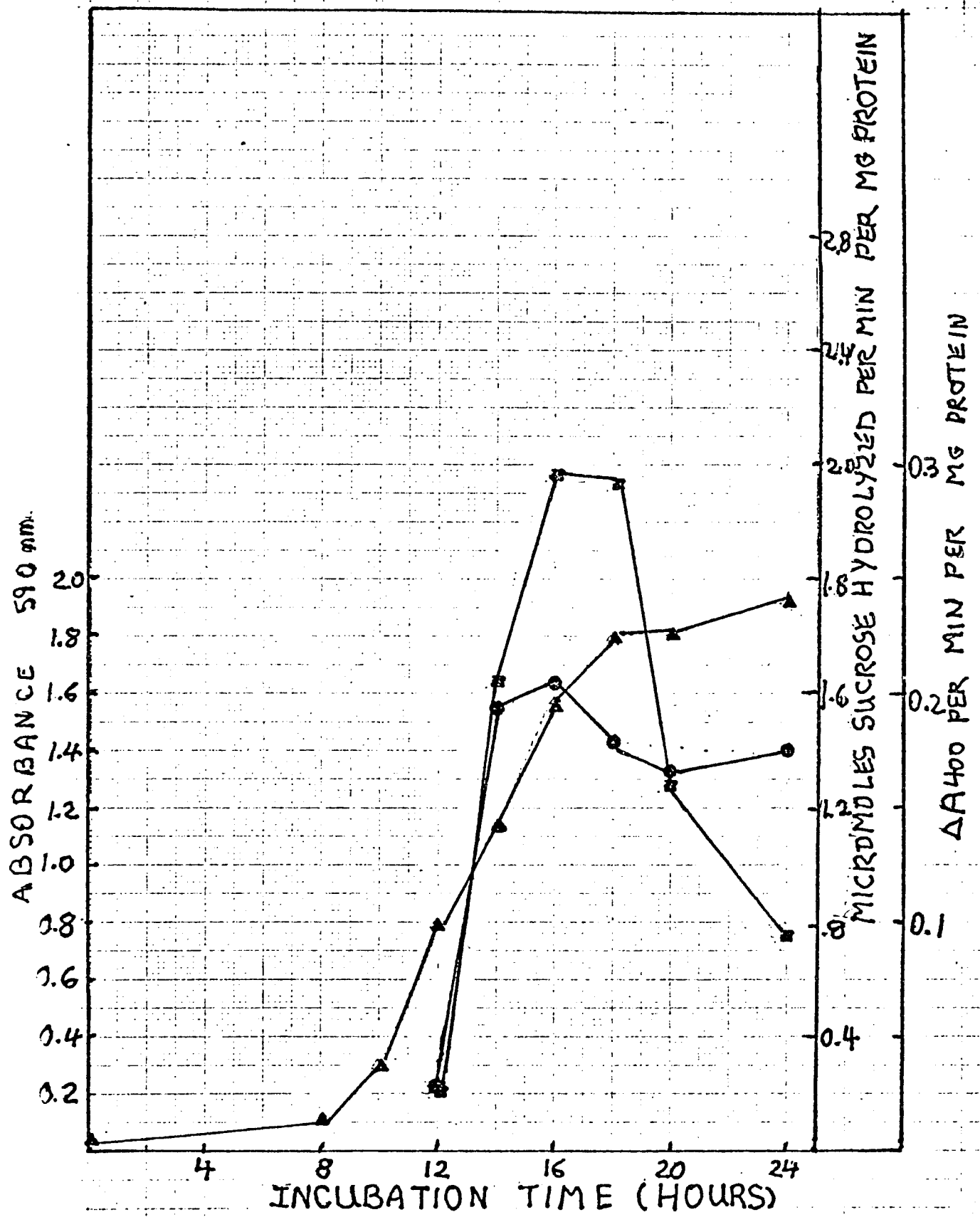
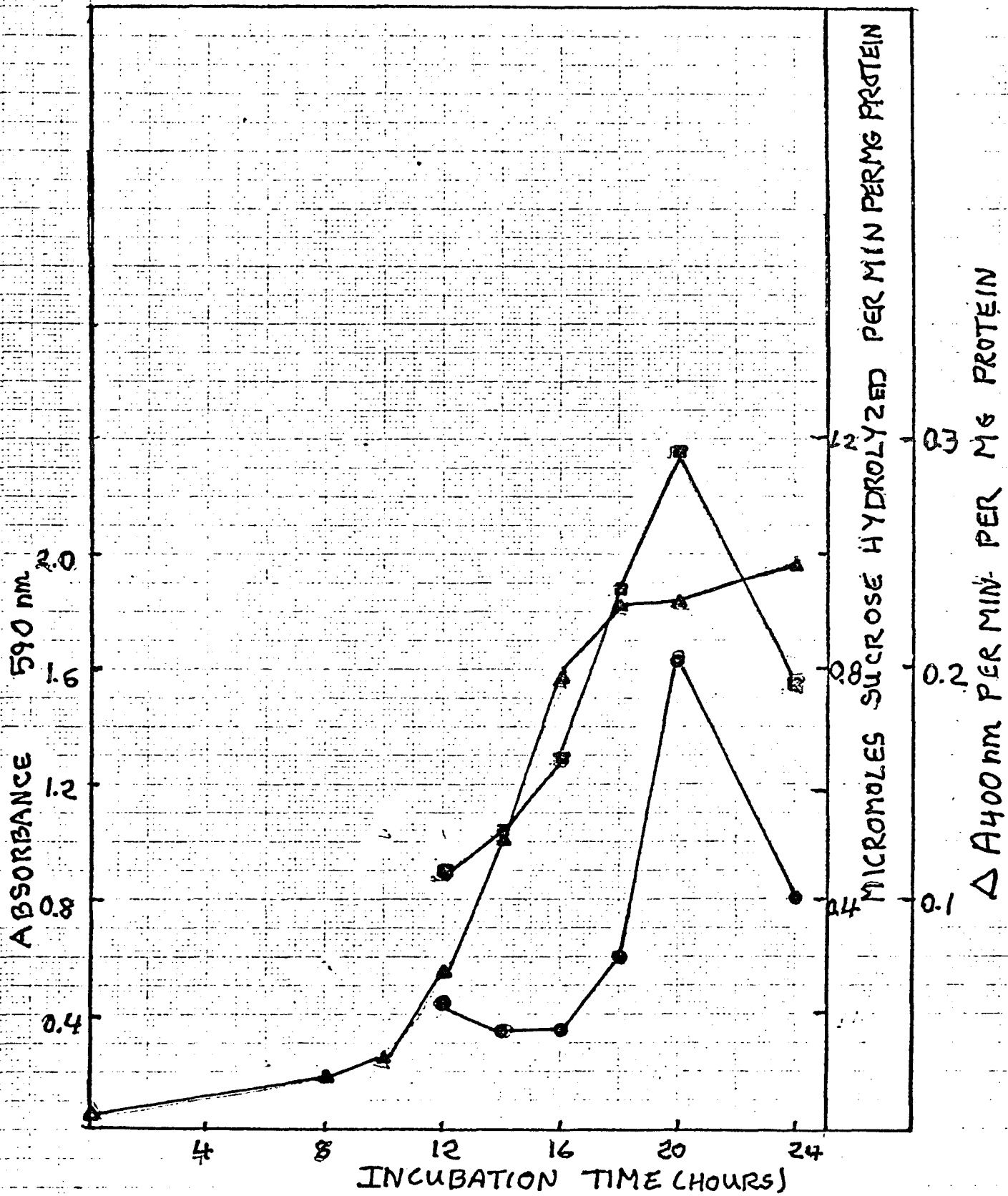


Fig. 18. Invertase and α -glucosidase specific activities as functions of the culture age of strain 4059-358D (SUC1 MAL1 MGL) grown on YEP 2% sucrose. Experimental conditions were as described in Fig. 17.

Legend

- ▲ Absorbance at 590 nm
- Invertase specific activity
- α -Glucosidase specific activity



Enzymatic Studies

The SUC1 parental strain 4059 and the two suc1 mutants were examined for their invertase activity. All three strains were grown to late exponential or early stationary growth phase on YEP-based 0.5% glucose and 2% sucrose; invertase and α -glucosidase levels were determined in crude extracts made from the harvested cultures (Table 25). In YEP 0.5% glucose invertase activity was substantial in the parental strain 4059, but was virtually nil in the two suc1 mutants (definitely less than one per cent of the wild type value). That the cultures were roughly similar physiologically was suggested by the comparable specific activities for α -glucosidase in all three strains. Mutant CII7 grows more slowly and more poorly on fermentable substrates as it has a respiratory defect that is unlinked to the suc1 MAl1 combination (results not presented).

Invertase levels could not be measured in crude extracts of suc1 mutant D10 on sucrose because of its poor growth on that sugar compared with strain 4059. Mutant CII7 had essentially no invertase activity in crude extracts of cells grown on YEP 2% sucrose; comparisons may be difficult in view of the poor growth of CII7 on this sugar. It was concluded that the suc1 mutants D10 and CII7 have essentially no invertase activity compared with the parental strain.

Invertase levels were determined in diploids isolated from crosses of 4059, D10 and CII7 to the segregational sucrose nonfermenter EZ-5B (Table 26). Since only the diploid 4059 x EZ-5B was able to ferment sucrose and raffinose (Table 21), it was expected that only this diploid should have invertase activity. The data indicate that this was the case irrespective of the medium in which the cells were grown.

TABLE 25

Comparison of Invertase and α -Glucosidase Levels in SUC1 Strain 4059
and suc1 Mutants D10 and CII7 Grown on YEP 0.5% Glucose and YEP 2%

Sucrose

Sugar	Strain	A590 nm (Age - hrs)	Specific Activity	
			Invertase	α -Glucosidase
0.5% glucose	4059 .	1.463 (18½)	1.74	0.327
	D10	1.334 (18½)	0	0.299
	CII7	0.973 (40½)	0	0.277
2% sucrose	4059	1.999 (18½)	0.754	0.154
	D10	1.034 (66½)	- ^a	- ^a
	CII7	1.379 (159)	- ^a	- ^a

^a Due to the poor growth of these strains on sucrose, invertase and α -glucosidase levels were not determined.

TABLE 26

Comparison of Invertase and α -Glucosidase Levels in SUC1 x suc0 and
suc1 x suc0 Diploids Grown on Various Sugars

Cross	Sugar	A590 nm (hrs)	Specific Activity	
			Invertase	α -Glucosidase
4059 x	0.5% glucose	1.598 (17)	1.69	0.145
EZ-5B	2% sucrose	1.908 (17)	2.61	0.268
	2% maltose	1.955 (20)	3.38	31.84
	2% raffinose	2.035 (22½)	1.64	1.94
D10 x	0.5% glucose	1.72 (15½)	0	0.136
EZ-5B	2% sucrose	1.38 (22½)	0	0.207
	2% maltose	1.956 (20)	0	26.12
	2% raffinose	1.786 (21)	0	0.152
CII7 x	0.5% glucose	1.587 (17)	0	0.136
EZ-5B	2% sucrose	1.504 (21)	0	0.207
	2% maltose	1.878 (21)	0	23.38
	2% raffinose	1.698 (21)	0	0.132

Invertase and α -glucosidase activities were determined in crude
extracts of cells prepared as described in "Materials and Methods".

Crude extracts made from cells grown on maltose appeared to have the highest invertase specific activity; sucrose also gave substantial activity while glucose and raffinose gave somewhat less. The diploids D10 x EZ-5B and CII7 x EZ-5B had no detectable invertase on any of these sugars.

While 4059 and EZ-5B grew somewhat better on raffinose than either of the *suc1* x EZ-5B diploids, the latter still showed considerable growth which could not be readily explained since these diploids failed to ferment sucrose and raffinose and crude extracts of raffinose-grown cells of these diploids had no detectable invertase activity.

Determination of α -glucosidase levels in these diploids revealed similar values for cells grown on glucose and sucrose for all three diploids. α -Glucosidase levels were highest in crude extracts of all three diploids grown on maltose although the *suc1* x EZ-5B diploids had less activity than 4059 x EZ-5B. The observation that α -glucosidase activity in the *suc1* x EZ-5B diploids grown on raffinose was about one-tenth that of 4059 x EZ-5B grown on the same sugar may have been related to the different amounts of growth shown by these diploids on raffinose medium.

Invertase levels were compared in *SUC1/SUC1* and *suc1/SUC1* as well as *suc1/suc1* diploids grown on YEP 0.5% glucose (Table 27). While all three diploids were able to ferment sucrose and raffinose at 30°C, the *SUC1/suc1* diploids did so more slowly than the *SUC1/SUC1* diploid (see Table 23). This was reflected in the invertase activity measured in crude extracts of derepressed cells (Table 27). Both 4059-3A x D10 and 4059-3A x CII7 (*SUC1/suc1* diploids) had about one-half of the invertase specific activity of the 4059-3A x 4059 (*SUC1/SUC1*) diploid.

TABLE 27

Invertase and α -Glucosidase Levels in SUC1 x SUC1, SUC1 x suc1 and
suc1 x suc1 Diploids

Cross	A590 nm	Specific Activity	
		Invertase	α -Glucosidase
4059-3A x 4059	1.815	2.233	0.134
4059-3A x D10	1.969	1.018	0.113
4059-3A x CII7	1.764	0.998	0.167
D10-2C x CII7	1.947	0	0.082

Invertase and α -glucosidase levels were determined in crude
extracts of cells grown on YEP 0.5% glucose for 18 hours at 30°C.

α -Glucosidase levels showed less of a difference; 4059-3A x D10 had about 15 per cent less activity, and 4059-3A x CII7 about 25 per cent more activity than the 4059-3A x 4059 diploid.

Invertase activity was also measured in crude extract of the diploid D10-2C x CII7 (suc1/suc1; see Table 23). The data presented in Table 27 show that this diploid lacked invertase activity (too insignificant to measure reliably) and this may explain its inability to ferment sucrose and raffinose rapidly.

The above studies lead to the following observations: (1) the suc1 mutants had virtually no invertase activity although α -glucosidase levels were comparable to those of the parental SUC1 strain; (2) suc1 x suc0 diploids had no invertase activity while SUC1 x suc0 diploid did; (3) SUC1 x suc1 diploids ferment sucrose and raffinose but have one-half the invertase activity of the SUC1 x SUC1 diploid; the diploid of D10 x CII7 failed to ferment sucrose and raffinose and had virtually no detectable invertase. The two more likely hypotheses to explain the behavior of these suc1 mutants are as follows: the suc1 mutants are in the structural gene for invertase; or the suc1 mutants are in a regulatory gene affecting expression of invertase structural genes. The suc1 mutants are thus similar to the suc3 mutants in their total lack of invertase.

Reversion Studies of suc1 Mutants

To elucidate the nature of the genetic defects in the suc1 mutants, studies of spontaneous and induced revertants were performed.

Spontaneous reversion frequencies were determined for the suc1

mutants D10 and CII7 by plating cells of each mutant on YEP 2% raffinose agar plates and incubating them at 30°C. The spontaneous reversion frequencies for both mutants were on the order of 10^{-8} . Since these spontaneous reversion frequencies were so low, it was considered more efficacious to induce revertants in the *suc1* mutants.

Reversion mutagenesis experiments were performed using EMS or nitrous acid (1 mg sodium nitrite per ml of 0.1 M sodium acetate buffer, pH 4.5 at 30°C for 30 minutes). No revertants were obtained for *suc1*-CII7 with any of these mutagens. With mutant *suc1*-D10 EMS and nitrous acid yielded one revertant for each mutagen. The EMS-induced revertant was designated D10-ER1 (or ER1), and the one induced by nitrous acid, D10-NAR1 (or NAR1).

The revertants had the following fermentation properties:

Revertant	Days to ferment at 30°C			
	Raf	Suc	Mal	MG
ER1	+(4)	+(1)	+(2)	+(3)
NAR1	+(4)	+(1)	+(2)	+(3)

Thus these revertants were not different from the parental *SUC1* strain 4059 in the amount of time needed to ferment sucrose and raffinose.

Genetic Studies of Induced Revertants of *suc1* Mutant D10

Revertant D10-ER1 was crossed to the segregational sucrose nonfermenter EZ-5B and diploids were isolated. The diploid *suc1*-D10-ER1 x EZ-5B was able to ferment sucrose in one day and raffinose in three days at 30°C. This diploid was sporulated and asci were dissected; the tetrad data are presented in Table 28. All ten tetrads showed two SUC MAL ascospore clones and two *suc mal* ascospore clones, indicating that the revertant ER1 was segregating with MAL1.

TABLE 28

Segregation of Sucrose Fermentation Ability in Revertants of suc1-D10
Crossed to EZ-5B and 4059-3A

Cross	Tetrad Types			
	SUC MAL	SUC MAL	SUC MAL	SUC mal
	SUC MAL	SUC MAL	SUC mal	SUC mal
	SUC MAL	suc mal	suc MAL	suc MAL
	SUC MAL	suc mal	suc mal	suc MAL
suc1-D10-ER1 x EZ-5B	0	10	0	0
suc1-D10-ER1 x 4059-3A	6	0	0	0
	SUC MAL	SUC MAL	SUC MAL	
	SUC MAL	SUC MAL	SUC MAL	
	SUC MAL	SUC MAL	suc MAL	
	SUC MAL	suc MAL	suc MAL	
suc1-D10-NAR1 x 4059-3A	1	6	1	

While not presented, the two three-spored asci contained only SUC MAL or suc mal ascospore clones.

Revertant D10-ER1 was also crossed to 4059-3A (a SUC1 segregant also containing MAL1) and diploids were isolated. This diploid fermented sucrose and raffinose as expected. It was sporulated and asci were dissected (Table 28). The six complete tetrads examined all showed 4 SUC MAL:0 suc MAL ascospore clones; furthermore, four 3-spored asci showed only SUC MAL ascospores (data not presented).

The above two crosses involving D10-ER1 suggest that this revertant is most probably the result of a mutation at or near the suc1 mutant D10 which was previously shown to be linked to MAL1 and thus was allelic to SUC1 (see Tables 22 and 24). Thus it is most likely that ER1 is allelic to SUC1 or at least closely linked to it.

Revertant D10-NAR1 was crossed to strains EZ-5B and 4059-3A and diploids were isolated. The diploid D10-NAR1 x EZ-5B failed to ferment sucrose and raffinose even after two weeks at 30°C. The diploid D10-NAR1 x 4059-3A fermented these sugars since it contained a functional SUC1 gene contributed by the 4059-3A parent. This diploid was sporulated and tetrads were dissected (Table 28); the diploid D1-NAR1 x EZ-5B was not dissected. For D10-NAR1 x 4059-3A the numbers of PD : TT : NPD tetrads were 1 : 6 : 1, which suggest that this revertant was the result of a mutation at a gene distinct from SUC1. This conclusion was supported by the failure of the diploid D10-NAR1 x EZ-5B to ferment raffinose and sucrose.

Enzymatic Studies of D10 Revertants

Invertase levels in these revertants were examined in crude extracts made from cells of these strains grown on YEP 0.5% glucose

(Table 29). It is evident that both revertants have invertase activity, although both have less activity than the parental SUC1 strain 4059. α -Glucosidase activity was less in the revertants than in 4059 as well.

Invertase levels were examined in diploids of these D10 revertants crossed to EZ-5B and 4059-3A (Table 30). Comparison of the diploids of 4059 and the D10 revertants crossed to the segregational sucrose nonfermenter EZ-5B revealed a difference between the revertants. The diploid D10-ER1 x EZ-5B had about one-half of the specific activity of 4059 x EZ-5B. The diploid D10-NAR1 x EZ-5B, however, had no detectable invertase activity. Whether the lower α -glucosidase activities of the revertants crossed to EZ-5B compared with 4059 x EZ-5B was related to their lower invertase activities is unknown.

Comparison of invertase levels in diploids of SUC1 and revertants crossed to the SUC1 strain 4059-3A showed some difference in invertase activity; the diploids involving the revertants had less invertase than the SUC1 x SUC1 diploid. α -Glucosidase levels were roughly the same in all three diploids (see Table 30).

It is evident from the above data that the D10 revertants ER1 and NAR1 differ in their expression when crossed to EZ-5B. Revertant ER1 is dominant while NAR1 is recessive. These data give further support to the previously described genetic differences between these revertants (see Table 28).

Whether the revertants had both forms of invertase possessed by the parental SUC1 strain was examined by running SDS-polyacrylamide gels containing aliquots of extracts of derepressed cultures of 4059,

TABLE 29

Invertase and α -Glucosidase Levels in the SUC1 strain 4059 and Two
Induced SUC Revertants Derived from suc1 Mutant D10

Strain	A590 nm (hrs)	Specific Activity	
		Invertase	α -Glucosidase
4059 (SUC1)	1.463 (18)	1.7	0.327
D10-ER1 (SUC)	1.458 (20½)	1.4	0.241
D10-NAR1 (SUC)	1.531 (20½)	0.8	0.204

Enzyme activities were determined in crude extracts prepared from cells grown on YEP 0.5% glucose for the times indicated in parentheses next to the value for absorbance at 590 nm.

TABLE 30

Invertase and α -Glucosidase Levels in Diploids of SUC1 and
D10 Revertants Crossed to Segregational Sucrose Nonfermenting
and SUC1 Strains

Diploid	A590 nm (hrs)	Specific Activity	
		Invertase	α -Glucosidase
EZ-5B x 4059	1.598 (17)	1.7	0.145
" x D10-ER1	1.638 (16)	0.7	0.097
" x D10-NAR1	1.657 (16)	0	0.066
4059-3A x 4059	1.815 (18)	2.2	0.134
" x D10-ER1	1.692 (16)	1.7	0.217
" x D10-NAR1	1.669 (16)	1.5	0.101

Enzyme Activities were determined in crude extracts prepared from
cells grown on YEP 0.5% glucose at 30°C for the times indicated in
parentheses next to the value to A590 nm.

the *suc1* mutants D10 and CII7, and the D10 revertants ER1 and NAR1 (also a spontaneous revertant SR1; Fig. 19). It is evident that the *suc1* mutants lack both invertase activities possessed by SUC1 strain 4059. 4059 had a relatively thick slow-moving band of invertase activity corresponding to the heavy external invertase, and a sharper fast-moving band corresponding to the small internal invertase. The thickness and intensity of color of the two bands of invertase activity is probably a function of the amount of crude extract added to each gel, the relative amounts of each invertase activity present and the duration of the invertase assay which is part of the procedure for staining for invertase. Examination of the gels of D10 revertants (spontaneous SR1 and induced ER1 and NAR1) revealed only the slow external invertase band of varying thickness and intensity (see gels 5 to 7 in Fig. 19). The faster internal invertase band was absent either because there was no internal invertase in crude extracts, the fast invertase of these revertants was more unstable than that of the parental SUC1 strain or the amount of internal invertase applied to each gel was too small to detect. Gels 8 and 9 show the invertase banding pattern of ER1 x EZ-5B and NAR1 x EZ-5B grown under derepressed conditions (YEP 0.5% glucose for 16 hours, see Table 30). Gel 8 had a single slow band of invertase activity characteristic of the haploid ER1, whereas gel 9 showed neither band of invertase activity (compare with data in Table 30).

The apparent lack of the fast moving (internal) invertase activity in gels of the revertant D10-ER1 as compared with the parental SUC1 strain 4059 suggested that the invertase made by this revertant might be different from that of the SUC1 strain. This

Fig. 19. SDS-Polyacrylamide gel electrophoresis of invertase activities in SUC1 MAL1 strain 4059, and suc1 mutants and SUC revertants derived from them.

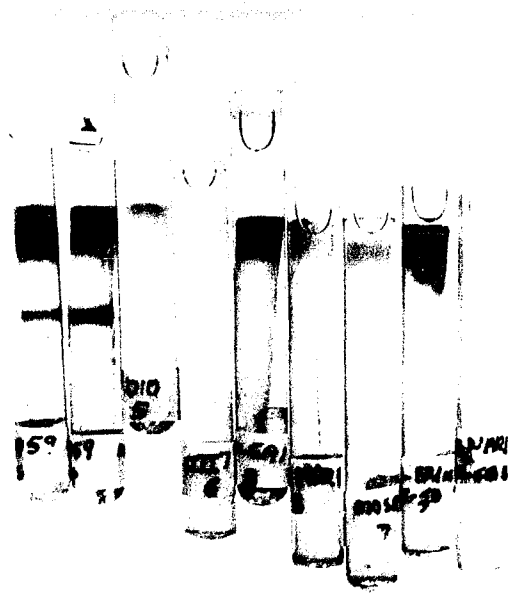
Preparation of crude extracts, gels and invertase staining procedure were as described in "Materials and Methods".

Gel	Strain	Culture Conditions	Volume of extract on gel (ml)
1.	4059	YEP 0.5% glucose	0.05
2.	4059	YEP 2% sucrose	0.05
3.	suc1-D10	YEP 2% maltose	0.10
4.	suc1-CII7	YEP 2% maltose	0.10
5.	D10-ER1	YEP 0.5% glucose	0.05
6.	D10-NAR1	"	0.05
7.	D10-SR1	"	0.05
8.	D10-ER1 x EZ-5B	"	0.10
9.	D10-NAR1 x EZ-5B	"	0.10

All gels were incubated in the invertase activity stain for ten minutes at 37°C.

EXTERNAL INVERTASE

INTERNAL INVERTASE



1 2 3 4 5 6 7 8 9

problem was investigated in some detail.

The possibility that invertases produced by strain 4059 and D10-ER1 were different kinetically was investigated by determining the K_m for sucrose at pH 4.5 for each invertase using crude extracts of derepressed cells (grown for 18 hours at 30°C on YEP 0.5% glucose). For the SUC1 parental strain the K_m was 0.031 M sucrose; for D10-ER1, 0.036M. Thus there was only a slight difference in the K_m for the invertases of these strains. This did not conclusively show that the revertant enzyme was altered, although this possibility could not be ruled out.

To confirm the apparent lack of the small (internal) invertase in revertant D10-ER1, crude extracts of derepressed cells of this revertant and SUC1 4059 were applied separately to a Sephadex G-200 column, and the fractionation of invertase was compared (Fig. 20 and 21). As expected, both strains had the major (external) invertase peak eluting slightly after the protein peak; the invertase from the parental SUC1 strain showing little overlap, the D10-ER1 invertase showing somewhat more overlap with the protein peak. The second, much smaller peak which was presumably the internal invertase, was present in the parental SUC1 strain, but appeared to be absent in the revertant. The minor invertase peak was found just at the front edge of the α -glucosidase peak (fractions 40 to 50 in the SUC1 strain, Fig. 20, but not in the corresponding fractions 36 to 46 in the revertant, Fig. 21).

In order to demonstrate that the major peak of invertase eluting first from the Sephadex column corresponded to the slow-moving invertase of the gels, and the minor invertase peak contained the

Fig. 20. Fractionation of invertase activity of SUC1 strain 4059 on Sephadex G-200. Strain 4059 was grown on one liter of YEP 0.5% glucose (one ml inoculum added) for 18 hours at 30°C. The culture was harvested and washed, and the crude extract was prepared as described in "Materials and Methods". The only difference was that the cells to be broken were suspended in 5 ml of 10 mM potassium phosphate plus 2 mM PMSF (α -toluenesulfonyl fluoride, Eastman; Goldstein and Lampen, 1975) buffer, pH 6.5. Two ml of crude extract containing 118 mg of protein were applied to a Sephadex G-200 column (17 x 2.5 cm; 100 ml total volume). The elution buffer was 25 mM potassium phosphate plus 0.25 mM disodium EDTA, pH 7.0. Gel filtration was performed at 4°C. Fractions of 60 drops were collected. Protein measurement and enzyme assays were performed as described in "Materials and Methods".

Legend

- A280 nm (Protein)
- △ Invertase activity (nmoles of sucrose hydrolyzed per min. per 0.1 ml fraction)
- ▲ α -Glucosidase activity (Δ A400 nm per min. per 0.1 ml fraction)

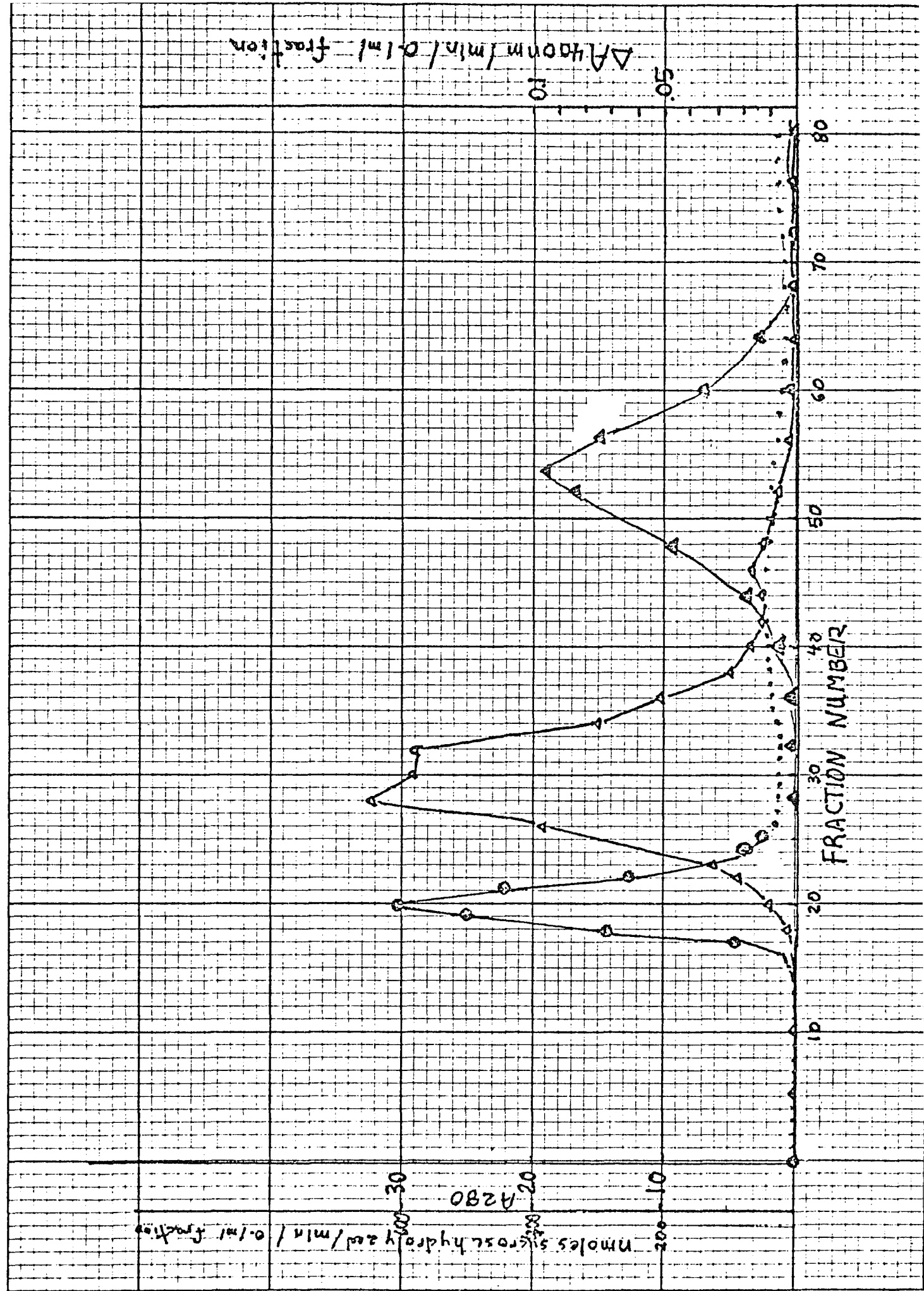
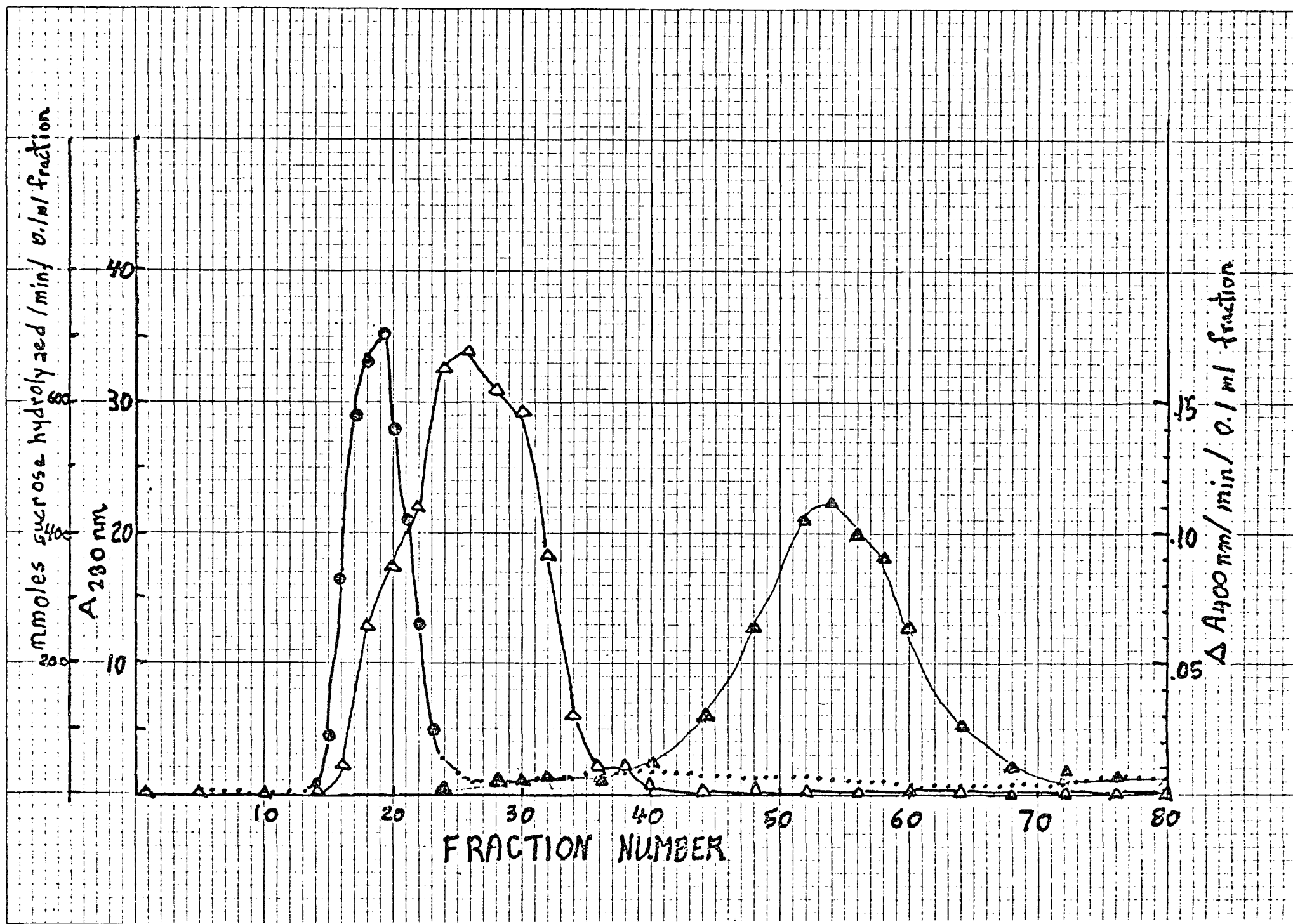


Fig. 21. Fractionation of invertase activity of SUC revertant D10-ER1 on Sephadex G-200. Culture conditions, harvesting of cells, washing of cells and preparation of crude extract were as described in Fig. 20. Two ml of crude extract containing 132 mg of protein were applied to the same Sephadex G-200 column and fractions of 60 drops were collected.

Legend

- A280 nm (Protein)
- Δ Invertase activity
- ▲ α-Glucosidase activity



fast-moving invertase activity, aliquots of fractions from the chromatogram of the parental SUC1 strain major and minor invertase peaks were run on separate SDS-polyacrylamide gels. The results are presented in Fig. 22. The amount of invertase activity added to each gel is given in the description of Fig. 22 for 4059 invertase and Fig. 23 for D10-ER1.

In Fig. 22 gels 1 and 2 represent the invertase banding pattern of the SUC1 crude extract whose chromatogram was presented in Fig. 20. Gels 3 through 7 represent electrophoresis patterns of invertase from fractions of the chromatogram seen in Fig. 20. Gels 3 and 4 represent material from fractions 28 and 32 respectively (peak of external invertase) which migrated as a broad (single ?) slow-moving band of invertase. Fraction 38 (gel 5) contained a mixture of fast and slow invertase bands. Fraction 46 (gels 6 and 7) had different amounts of material from the small invertase peak, and contained mostly the fast-moving small internal invertase and a little slow-moving (external) invertase; a faint band of slow invertase activity was apparent only in gel 7. Thus the slow invertase band corresponded to the major (external) invertase which eluted first from the Sephadex column. The fast invertase band corresponded to material eluting somewhat later (internal invertase). The polyacrylamide gels apparently gave better resolution of the two invertase activities than did the Sephadex G-200 column employed. A longer Sephadex column was probably required to separate the invertase activities completely. While Meyer and Matile (1974) used these gels to quantitatively compare the amounts of the two invertases present, in the present study these gels were used in a semi-quantitative manner. In the description to Fig. 22, decreasing

Fig. 22. SDS-Polyacrylamide gel electrophoresis of invertase activities in SUC1 strain 4059 crude extract and fractions from the gel filtration on Sephadex G-200 (Fig. 20). Gels were run for 5 hours at 2 milliamperes per gel. Invertase activity was stained for as described in "Materials and Methods". Time (min) indicates the duration of the invertase assay. The amount of invertase activity (nmoles sucrose hydrolyzed per min) applied to each gel is obtained by multiplying the relative specific activity (nmoles sucrose per min. per 0.1 ml fraction) by the volume added to the gel.

Gel.	Material		Volume on gel (ml)	Time (min)	Rel. sp. act.	Act. added to gel
1.	Crude extract		.05	4	0.59 mg protein	
2.	" "		.10	4	1.18 mg protein	
3.	Sephadex fraction 28		.05	10	640	320
4.	" "	32	.05	10	580	290
5.	" "	38	.05	10	100	50
6.	" "	46	.05	15	60	30
7.	" "	46	.10	15	60	60
8.	Revertant D10-ER1 crude extract		.02	4	1.32 mg protein	

Tops of gels are at the top of picture.

Slow-moving broad band of invertase activity represents the major invertase which is the external invertase.

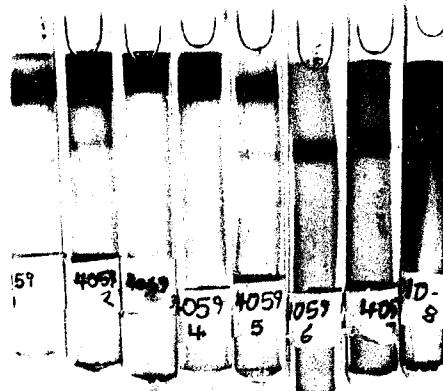
Fast-moving band of invertase activity represents the minor invertase which is the internal invertase.

The "band" visible below the fast invertase in gels 1 and 2 is the bromphenol blue tracking dy.

EXTERNAL INVERTASE

INTERNAL INVERTASE

TRACKING DYE



1 2 3 4 5 6 7 8

amounts of invertase activity added to gels resulted in decreased intensity of staining of the gels. For example, gels 5 and 7 showed external and internal invertases respectively; about equal amounts of activity were applied to each gel (50 and 60 nanomoles of sucrose hydrolyzed per minute respectively) and the gels have bands of about equal intensity, but were in different positions.

When fractions containing invertase activity of the chromatographed D10-ER1 crude extracts were examined for invertase activities by SDS-polyacrylamide gel electrophoresis, the results presented in Fig. 23 were obtained. Crude extract (gel 1) showed one slow band of invertase with little if any detectable fast invertase. Gels 2 through 8 had aliquots of various fractions from the major invertase peak showing only the slow-moving band of invertase activity or nothing. Gels 2 and 3 have material from fractions 26 and 30 respectively. Fractions 34, 36, 38 and 40 ought to have had increasingly intense bands of fast (internal) invertase as was seen in the comparable gels of Fig. 22, but only the slow band was present; as the amount of activity applied to the gels decreased, they became progressively less intense. Gel 8 represented material from the peak fraction of α -glucosidase activity which has no invertase activity. Thus α -glucosidase was not responsible for the fast band.

These data indicated that the small (internal) invertase activity present in the parental SUC1 strain was absent or greatly reduced in amount or activity in the revertant D10-ER1, which appeared to be allelic to SUC1. This difference was shown qualitatively in the polyacrylamide gels run with crude extract or fractionated invertase, and in the gel filtration experiments on Sephadex G-200.

Fig. 23. SDS-Polyacrylamide gel electrophoresis of invertase activity in SUC revertant D10-ER1 crude extract and fractions from a gel filtration on Sephadex G-200 (see Fig. 21 for the corresponding chromatogram of D10-ER1 crude extract). Gels were run for 6 hours at 3 milliamperes. See Fig. 22 for details of electrophoresis.

Gel	Material		Volume on gel (ml)	Time (min)	Rel. sp. act.	Act. added to gel
1.	Crude extract		.01	4	0.66 mg protein	
2.	Fraction 26		.05	15	680	340
3.	"	30	.05	15	580	290
4.	"	34	.05	15	120	60
5.	"	36	.05	15	40	20
6.	"	38	.05	15	40	20
7.	"	40	.05	15	20	10
8.	"	55	.05	15	0	0

EXTERNAL INVERTASE

INTERNAL INVERTASE

TRACKING DYE



1 2 3 4 5 6 7 8

The data presented in Figs. 22 and 23 may be used semi-quantitatively to further demonstrate that revertant D10-ER1 lacked the internal invertase. Activity of as little as 10 nanomoles sucrose hydrolyzed per minute was detectable as a band of invertase activity (see Fig. 23). While gels of both strains showing external invertase had about equal amounts of invertase activity applied, gels of D10-ER1 which would have been expected to have considerable internal invertase, as was the case with 4059, not only seemed to lack internal invertase but also had a lower activity, for example, gel 7 in Fig. 22 had mostly internal invertase and just a trace of external, but gel 7 in Fig. 23 had only external invertase detectable; the first gel had invertase of 60 nanomoles sucrose hydrolyzed per minute but the second had only 10). It appears that revertant is poorer in internal invertase than 4059, either because less of it is actually made or it is present as an unstable species; internal invertase may be present in D10-ER1 but in amounts too low to detect.

The studies presented in this chapter can be summarized:

- (1) The SUC1 gene which determines the presence of the internal and external invertases can be mutated to give mutants lacking invertase and being consequently unable to ferment sucrose and raffinose.
- (2) The suc mutants are allelic to SUC1 or closely linked to it.
- (3) Diploids of suc1 mutants crossed to SUC1 have about one-half the invertase specific activity of SUC1/SUC1 diploids.
- (4) Induced revertants of suc1 mutants were obtained; one is closely linked to or allelic to SUC1 (D10-ER1) and one is unlinked (D10-NAR1). The revertant D10-ER1 lacks the internal invertase or has very little of it.

Several explanations for these results are possible: (1) SUC1 is a regulatory gene inactivated in the suc1 mutants and partially restored in the revertants to allow only the expression of the structural gene for external invertase but not for internal invertase. This makes the assumption that the two invertases are coded for by two different structural genes. (2) SUC1 contains the structural genes for both internal and external invertases in tandem. A polar mutation initially knocks out both to give suc1 mutants, but revertant D10-ER1 has a second mutation which restores the external but not the internal invertase activity. These mutations may have occurred in the structural genes or in regulatory DNA adjacent to them. (3) SUC1 contains a structural gene coding for the external invertase. The internal invertase is a degradation product of the external invertase and not a precursor, since revertant D10-ER1 has the external but apparently not the internal invertase, and is allelic to SUC1. The problem of the biosynthetic relationship of the internal and external invertases was mentioned in the "Introduction". The apparent lack of internal invertase in a SUC revertant allelic to SUC1 suggests that, at least for SUC1, the internal invertase may not be a precursor, but may possibly be a degradation product of external invertase or biosynthetically unrelated. It is not possible to determine which of these possibilities is the correct one, but the evidence suggests that SUC1 may contain a structural gene or genes for the invertases.

CHAPTER VI
SUCROSE NONFERMENTING MUTANTS DERIVED FROM A STRAIN
BEARING THE SUC2 GENE

Isolation and Characterization of suc Mutants Derived from a SUC2 Strain

In order to investigate the relationship between the SUC2 gene and invertase formation, yeast strain M6-1D 1A (hereafter called M6-1D) bearing the SUC2 gene served as the parental strain from which four suc mutants were derived. These mutants were induced using EMS.

The suc mutants were initially identified by their poor growth on sucrose-BTB agar plates (see "Materials and Methods"). Comparison of the suc mutants with the SUC2 parent M6-1D revealed that they were defective in their ability to ferment raffinose and sucrose, while the parent fermented sucrose in two days and raffinose in five days at 30°C. Fermentation properties of these strains are as follows:

Strain	Days to Ferment at 30°C	
	Sucrose	Raffinose
M6-1D (SUC2)	+(1-2)	-(6)
E-1-21 (suc)	-(14)	-(14)
H-1-27 (suc)	+(5)	-(14)
I-1-2 (suc)	-(14)	-(14)
J-1-1 (suc)	-(14)	-(14)

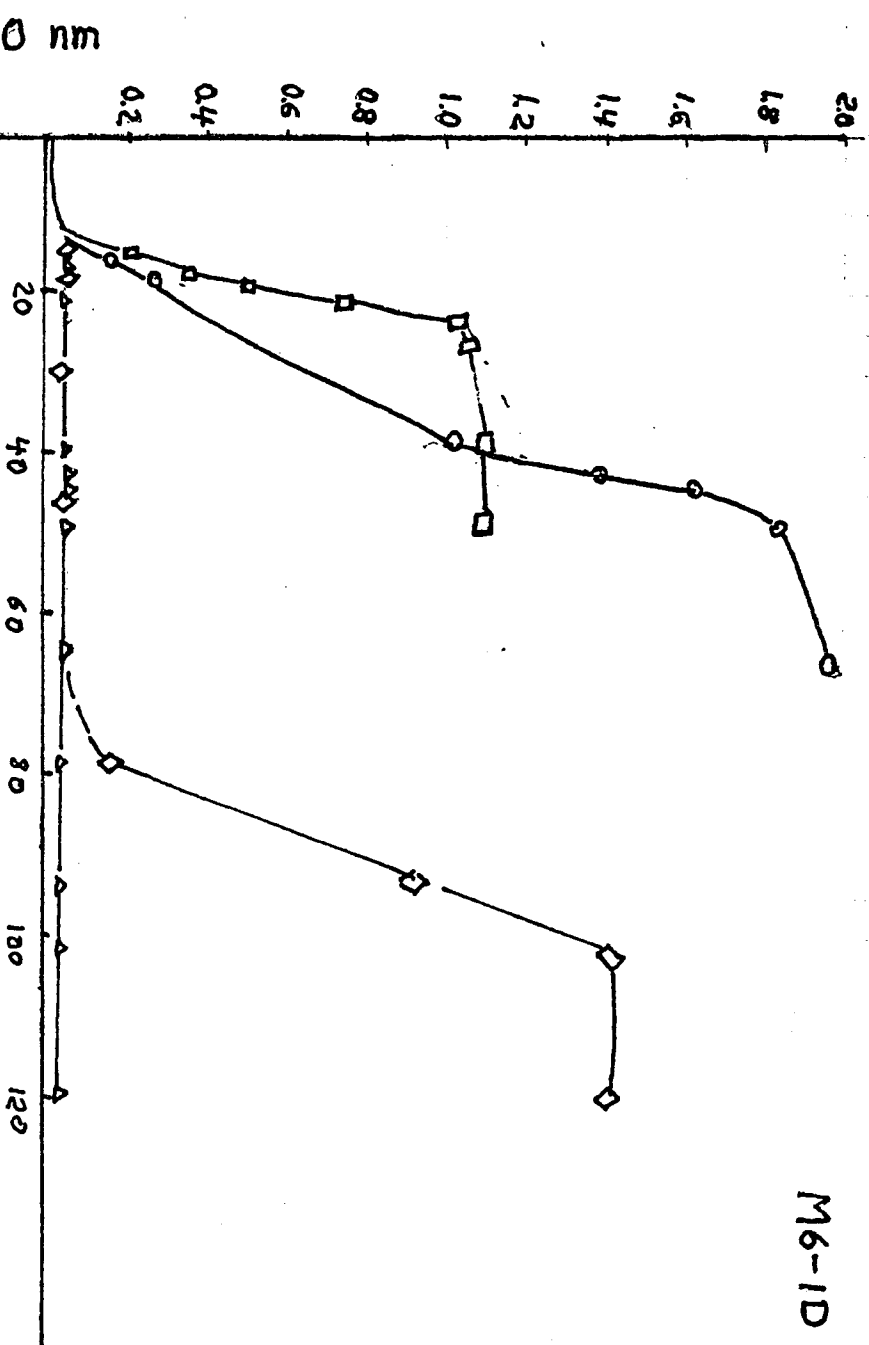
These suc mutants were compared with M6-1D in their ability to utilize various carbon sources in a rich YEP-based medium (Fig. 24). All strains were alike in their ability to grow on glucose (except for mutant J-1-1 which grew to a somewhat lesser extent) and their

Fig. 24. Growth characteristics of SUC2 strain M6-1D and suc2 mutants derived from it. 0.1 ml of inoculum was added to 10 ml of various YEP-based culture media containing different sugars to give a final concentration of 1.5×10^5 cells per ml. The cultures were incubated in a roller drum at 30°C. Growth was monitored by reading absorbance at 590 nm at appropriate times using a Beckman DU spectrophotometer.

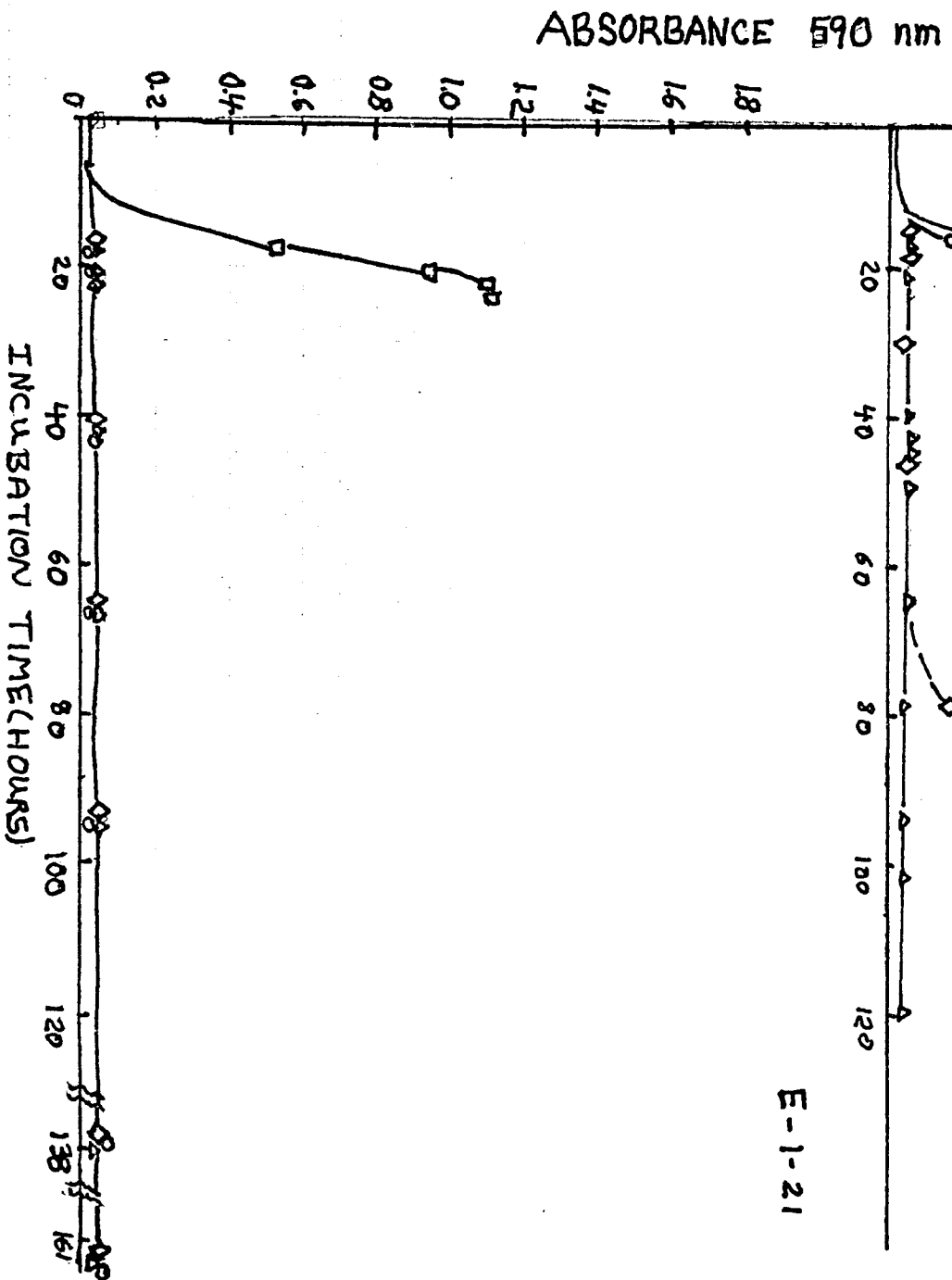
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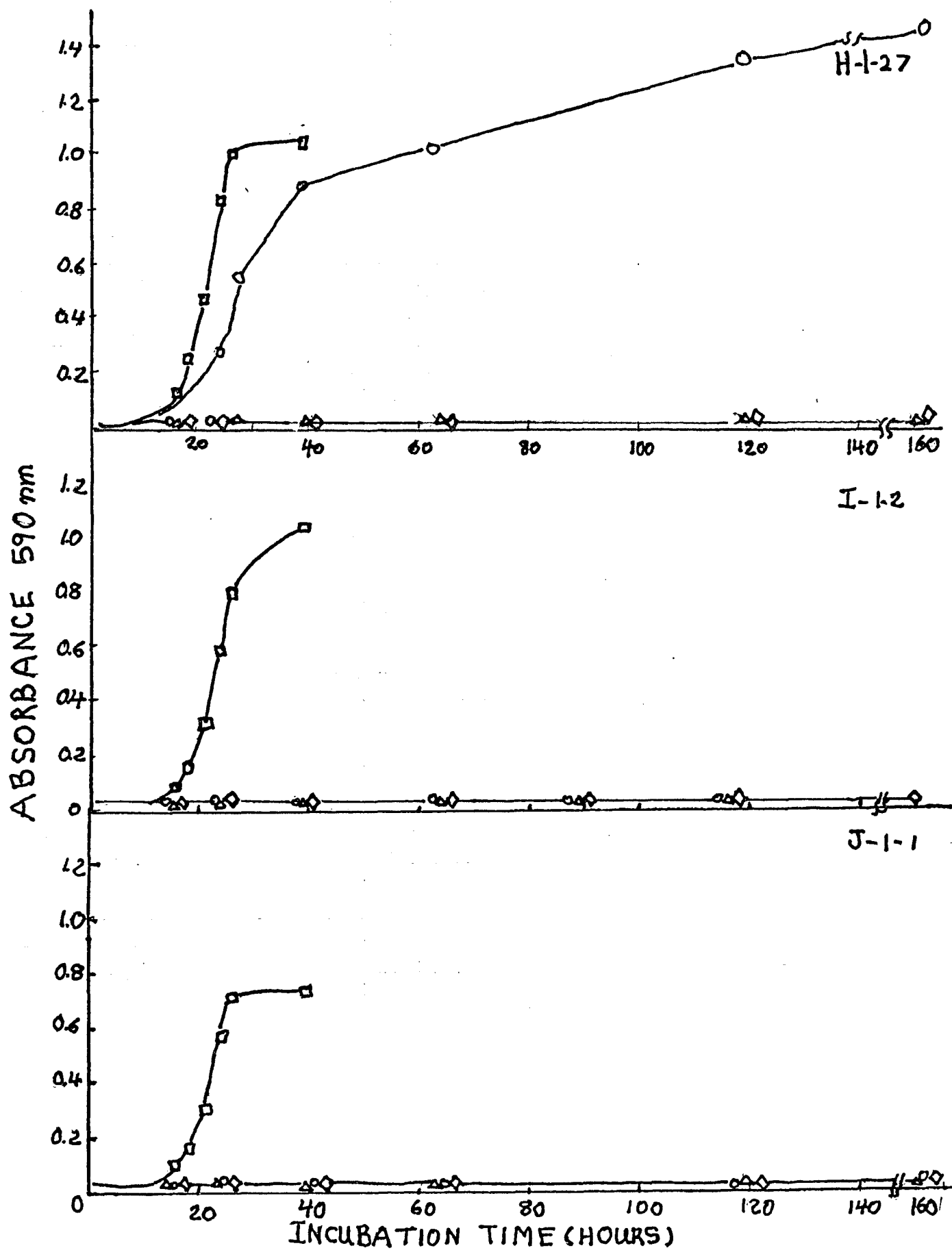
- △ YEP alone
- YEP 0.5% glucose
- YEP 2% sucrose
- ◇ YEP 2% raffinose

M6-1D



E-1-21





inability to grow on glycerol. This inability to utilize glycerol was determined by a single nuclear gene, although the results were not presented. All strains grew extremely poorly on YEP without any added sugar. Strain M6-1D showed a short lag in growth on sucrose as compared to growth on glucose; on raffinose the lag was longer. All four suc mutants (E-1-21, H-1-27, I-1-2 and J-1-1) failed to utilize raffinose. Of the four mutants only H-1-27 appeared to grow on sucrose. The growth curves for these mutants on sucrose (with the exception of H-1-27) and on raffinose were virtually superimposable on that of YEP alone. The poor growth of all of these strains on YEP and YEP plus nonutilizable carbon sources were similar to observations made with suc3-34, a strain bearing a suc3 mutation allelic to SUC3 which also had a (mitochondrially determined) respiratory defect ("petite", see Chapter III). The inability of these "petite" strains to utilize efficiently the components of the YEP medium may have been responsible for the comparative ease in obtaining suc mutants from strain M6-1D.

Genetics of suc Mutants Derived from SUC2

Strain M6-1D and the four suc mutants derived from it were crossed to the segregational sucrose nonfermenter EZ-5B and diploid clones were isolated. These diploids were examined for their fermentation properties. While the diploid M6-1D x EZ-5B fermented sucrose in one day and raffinose in five days at 30°C, none of the diploids of the suc mutants crossed to EZ-5B were able to ferment raffinose (raffinose fermentation tubes were negative after 21 days at

30°C), although diploid H-1-27 x EZ-5B fermented sucrose in one day and diploid I-1-2 x EZ-5B fermented sucrose in five days; diploids E-1-21 x EZ-5B and J-1-1 x EZ-5B failed to ferment sucrose in 21 days.

Genetic analysis of these diploids was performed by sporulating them and dissecting asci. Tetrad analysis was hampered by the difficulty in digesting asci with Glusulase; treatments of up to one hour with Glusulase gave only a few dissectable tetrads (see Table 31). As a result random spore analysis was also performed (Table 31). The cross M6-1D x EZ-5B gave only tetrads containing two sucrose-raffinose fermenters (SUC) and two sucrose-raffinose nonfermenters (suc), indicating that only a single gene for sucrose and raffinose fermentation was present in the parental strain M6-1D.

Dissection and tetrad analysis of asci of diploids of suc mutants x EZ-5B gave only 0 SUC:4 suc tetrads (see Table 31). Incomplete tetrads contained only sucrose-raffinose nonfermenting (suc) ascospore clones. It was concluded from these data that these suc mutants were due to single-gene mutations which were not complemented by the segregational sucrose nonfermenter EZ-5B, and that the suc mutants were probably allelic or closely linked to the SUC2 gene. The random spore analysis data in Table 32 support this conclusion, since among the random ascospores sampled, no sucrose-raffinose fermenters were detected.

Enzymatic Studies

Invertase Activity of SUC2 Strain M6-1D as a Function of Culture Age

Strain M6-1D was grown on YEP 0.5% glucose and aliquots of

TABLE 31

Tetrad Analysis of Crosses of SUC2 Strain M6-1D and suc Mutants
Derived from it to the Segregational Sucrose Nonfermenter EZ-5B

Strain	Tetrad Types		Incomplete Tetrads						
	2 SUC : 2 suc	0 SUC : 4 suc	2:1	1:2	0:3	2:0	1:1	0:2	
M6-1D (SUC2)	15	0	2	5	0	3	5	2	
E-1-21 (suc)	0	7	0	0	3	0	0	8	
E-1-27 (suc)	0	3	0	0	3	0	0	2	
J-1-1 (suc)	0	3	0	0	8	0	0	3	

Tetrads could not be dissected in the cross I-1-2 x EZ-5B because of the resistance of the ascus wall to digestion with Glusulase.

SUC indicates the ability to ferment both sucrose and raffinose, and suc indicates the inability to ferment both of these sugars at 30°C.

TABLE 32

Random Spore Analysis of Crosses of *suc2* Mutants to the
Segregational Sucrose Nonfermenter EZ-5B

Strain	Number of SUC Ascospores	Number of suc Ascospores
E-1-21	0	71
H-1-27	0	66
I-1-2	0	94
J-1-1	0	128

SUC ascospores were unable to ferment sucrose and raffinose; *suc* ascospores were unable to ferment both of these sugars at 30°C. The random spore analyses were performed as described in "Materials and Methods".

culture were harvested at various times during late exponential growth and stationary growth phase. Invertase activity was determined in crude extracts of these cells (see Fig. 25). Invertase activity was found to increase as the culture went from late exponential growth phase (20 hours) to stationary growth phase (about 29 hours). It gradually decreased by 45 hours which was late stationary growth phase. It was concluded that invertase activity could be sampled from 26 to 45 hours and still show relatively little variation.

Variation in invertase activity with culture age when strain M6-1D was grown on YEP 2% sucrose was also investigated (Fig. 26). Invertase levels were somewhat higher than at comparable growth stages in cells grown on 0.5% glucose. Invertase levels reached maximal values as the culture entered stationary growth phase on sucrose (about 45 hours). It was concluded that invertase activity should be examined in sucrose grown cells harvested between 29 and 45 hours.

Invertase Activity in M6-1D and suc Mutants Derived from it

Invertase activity was measured in suc mutants induced in M6-1D and compared with levels in the parental SUC2 strain. The results are presented in Table 33. The suc mutants lacked detectable invertase activity, whereas the SUC2 parent had substantial activity whether grown on 0.5% glucose, sucrose or raffinose.

The presence or absence of the two forms of invertase was studied by SDS-polyacrylamide gel electrophoresis (see "Materials and Methods") as it had been investigated in strains bearing the SUC3 (Chapter III) and the SUC1 (Chapter V) genes. As expected, crude extracts of derepressed cells of strain M6-1D had a slow invertase band (presumably corresponding to the external invertase as shown for SUC3 and SUC1) and

Fig. 25. Invertase specific activity as a function of the culture age in strain M6-1D (SUC2) grown on YEP - 0.5% glucose. 0.2 ml of a 24 hour culture of strain M6-1D grown on YEPD broth was added to 200 ml of YEP 0.5% glucose. This culture was then incubated at 30°C. At appropriate times aliquots of 30 ml were removed and treated as described in "Materials and Methods".

Legend

- ▲ Absorbance at 590 nm (read in a Beckman DU spectrophotometer)**
- Invertase specific activity**

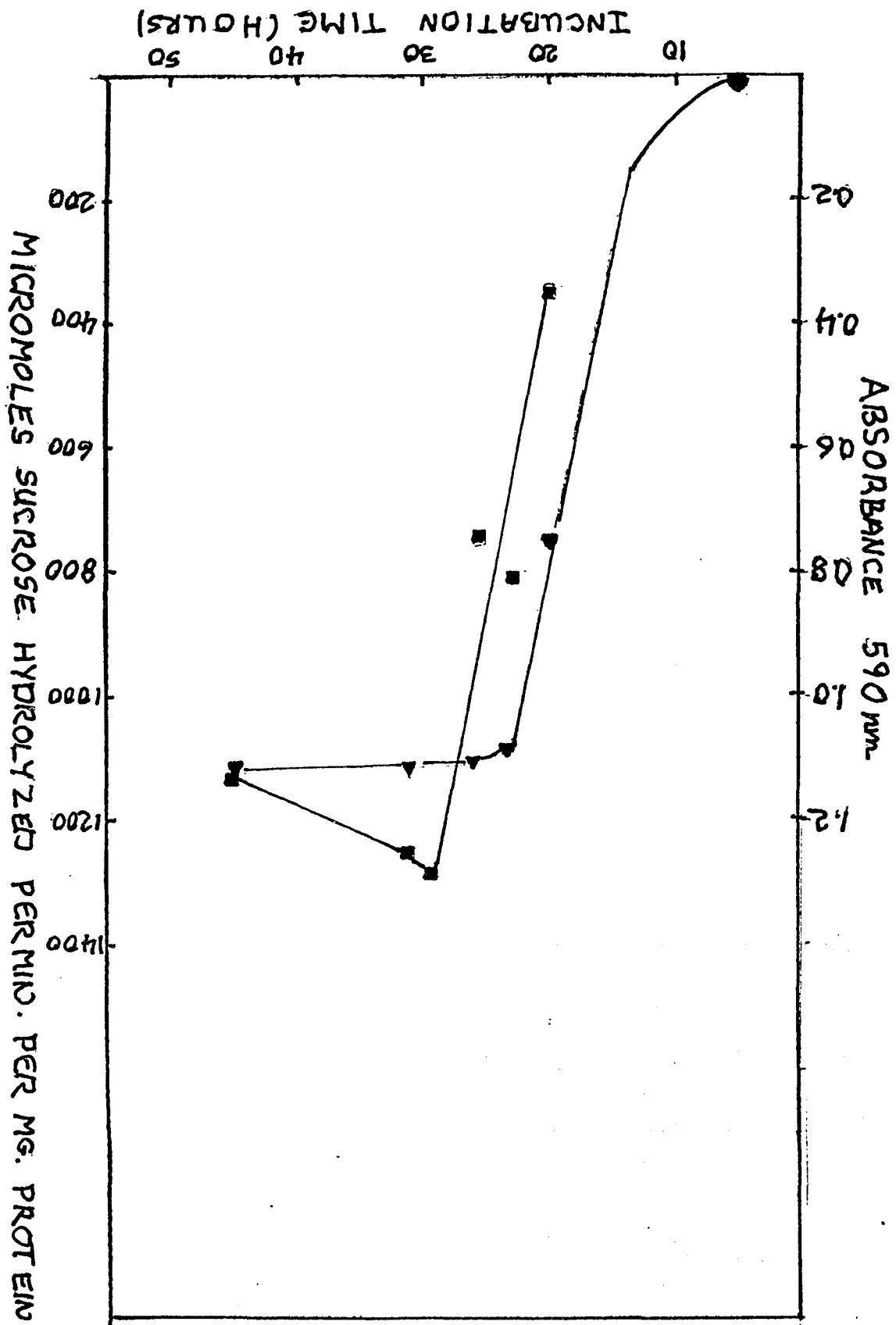


Fig. 26. Invertase specific activity as a function of the culture age in strain M6-1D (SUC2) grown on YEP + 2% sucrose. 0.2 ml of a 24 hour culture of strain M6-1D grown on YEFD broth was added to 200 ml of YEP + 2% sucrose. This culture was then incubated at 30°C. See Fig. 25 for details.

Legend

- ▲ Absorbance at 590 nm
- Invertase specific activity

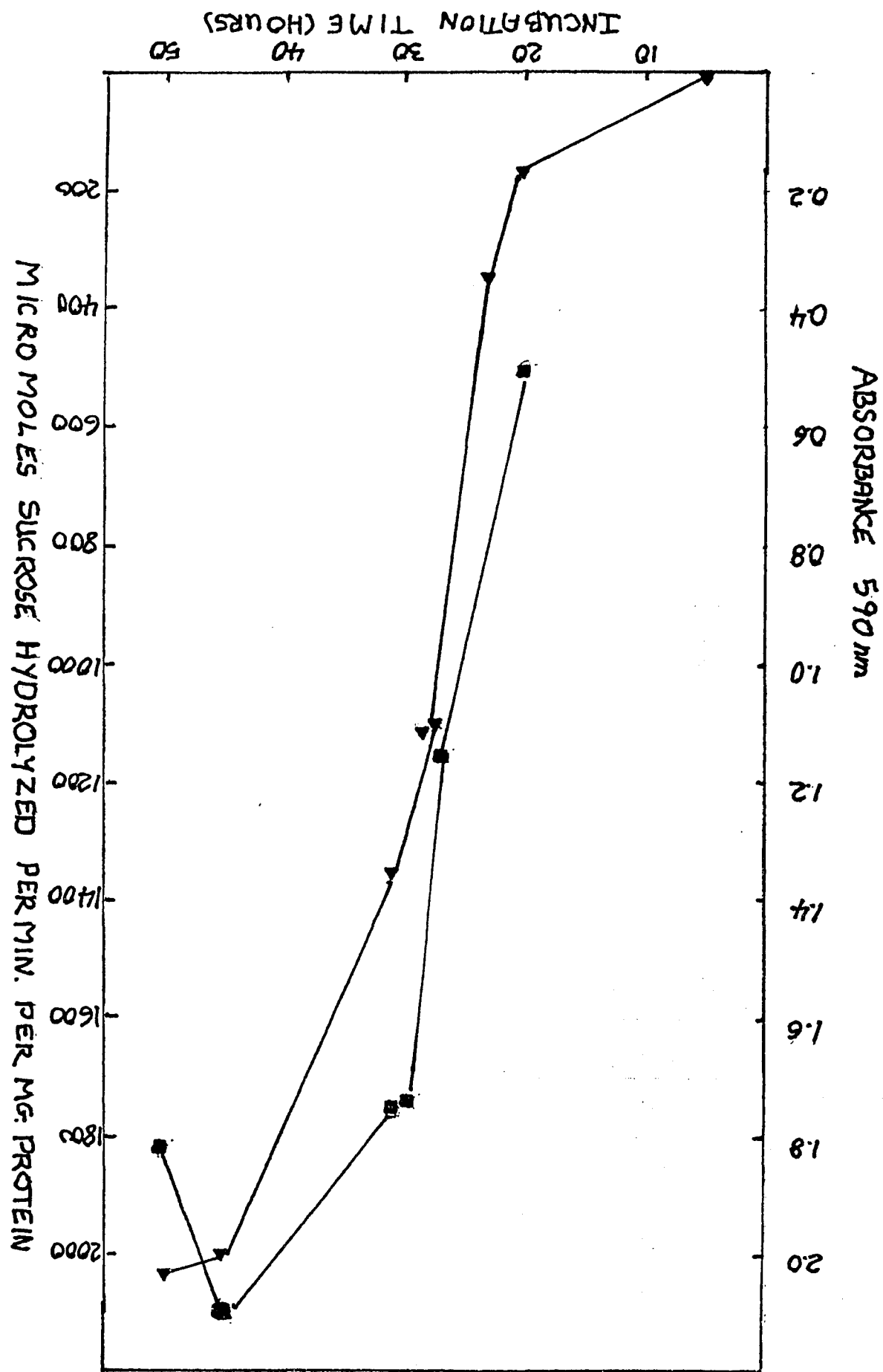


TABLE 33

Invertase Activity of SUC2 Strain M6-1D and suc2 Mutants

Strain	Culture Conditions	A590 nm (hr)	Invertase sp. act. umoles sucrose hydrolyzed per min per mg protein
M6-1D (SUC2)	YEP 0.5% glucose	1.047 (23½)	0.77
	YEP 2% sucrose	1.388 (42½)	1.42
	YEP 2% raffinose	1.096 (42½)	2.03
E-1-21 (suc2)	YEP 0.5% glucose	1.118 (23½)	0
H-1-27 (suc2)	YEP 0.5% glucose	1.041 (39½)	0
I-1-2 (suc2)	YEP 0.5% glucose	1.041 (39½)	0
J-1-1 (suc2)	YEP 0.5% glucose	0.732 (39½)	0

Invertase activity was assayed in crude extracts of cells grown and prepared as described in "Materials and Methods".

a fast invertase band (presumably the internal invertase). None of the suc mutants, however, had detectable bands for either form of invertase (Fig. 27).

Invertase activity was also measured in crude extracts of derepressed cells of diploids of the segregational sucrose nonfermenter EZ-5B crossed to M6-1D and the suc mutants. The results are presented in Table 34. The data indicate that the ability to ferment sucrose and raffinose is related to the ability of these diploids to make invertase or to have a functional invertase. The diploid M6-1D x EZ-5B, as expected, had considerable invertase activity whether grown on glucose or sucrose. None of the suc2 mutant x EZ-5B diploids, however, had significant invertase activity.

The studies described above may be summarized as follows:

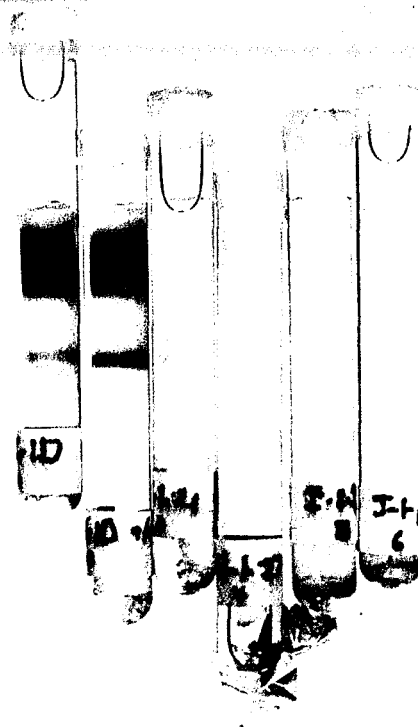
- (1) Mutations can be induced in a yeast strain bearing the SUC2 gene which result in the inability to ferment the sugars sucrose and raffinose.
- (2) Genetic analysis indicated that these suc mutants, designated suc2, were probably the result of single-gene mutations either allelic or closely linked to SUC2.
- (3) The suc2 mutants lacked detectable invertase activity.
- (4) Diploids of suc2 mutants x suc0 (segregational sucrose non-fermenter) could not ferment sucrose and raffinose and lacked detectable invertase activity whereas the SUC2 parent x suc0 diploid fermented both of these sugars and had invertase activity. Since single point mutations at SUC2 result in the loss of the ability to make both invertases, these suc2 mutants appear to be similar to the suc3 and suc1 mutant already described.

Fig. 27. SDS-polyacrylamide gel electrophoresis of invertase in SUC2 strain M6-1D and suc2 mutants derived from it. Crude extracts were prepared from cells grown on YEP 0.5% glucose for 39 hours at 30°C. Gels and crude extracts were prepared as described in "Materials and Methods".

Gel	Strain	Volume of crude extract added (ml)	Invertase incubation (min)
1.	M6-1D (0.5% glucose)	0.05	10
2.	M6-1D (2% sucrose)	0.05	"
3.	E-1-21 (0.5% glucose)	0.10	"
4.	H-1-27 "	"	"
5.	I-1-2 "	"	"
6.	J-1-1 "	"	"

EXTERNAL INVERTASE

INTERNAL INVERTASE



1 2 3 4 5 6

TABLE 34

Invertase Activity in Diploids of M6-1D (SUC2) and suc2 Mutants
Crossed to the Segregational Sucrose Nonfermenter EZ-5B

Sugar	Diploid EZ-5B (suc0) x	A590 nm (hr)	Invertase Sp. Act.
YEP 0.5% glucose	M6-1D	1.44 (15)	0.758
	E-1-21	1.68 (20)	0
	H-1-27	1.42 (17)	0
	I-1-2	1.56 (18)	0
	J-1-1	1.65 (20)	0
YEP 2% sucrose	M6-1D	1.78 (15)	1.35
	E-1-21	0.98 (24)	0
	H-1-27	1.39 (17)	0
	I-1-2	0.908 (17)	0
	J-1-1	1.17 (22)	0

Invertase specific activity was determined in crude extracts as described in "Materials and Methods".

CHAPTER VII

ISOLATION AND CHARACTERIZATION OF MUTANTS CONFERRING INVERTASE

HYPERPRODUCTION IN STRAIN EK-6B CARRYING THE SUC3 GENE

Effect of 2-deoxy-D-glucose on growth of EK-6B on various sugars

It has been reported that the D-glucose analog 2-deoxy-D-glucose (2DG) can inhibit invertase formation in Saccharomyces (Kuo and Lampen, 1972; Kratky, Bialy and Bauer, 1975). The effect of 2DG in inhibiting the growth of EK-6B on various sugars is shown in Table 35. It is apparent that 2DG inhibited raffinose fermentation to a greater extent than any other sugar, although maltose fermentation was greatly retarded. As previously suggested these results indicated that raffinose would be better than sucrose for testing the inhibitory effect of 2DG on invertase synthesis or activity (F. K. Zimmermann, personal communication).

Isolation and Characterization of DGR Mutants from EK-6B

Mutants which grow on raffinose plates in the presence of 2DG (designated DGR) were induced in strain EK-6B using MNNG or EMS (as described in "Materials and Methods"). These DGR mutants were able to ferment raffinose in the presence of 2DG (250 μ g per ml) while the parental strain did not (Table 36).

DGR mutants differed in the levels of invertase and α -glucosidase that cells of these strains had when grown on YEPD broth, as compared with the parental strain (Table 37). Considerable variation in the

TABLE 35

Effect of 2-Deoxy-D-Glucose (2DG) on Fermentation of Various
Sugars by Strain EK-6B

Concentration of 2DG (micrograms per ml)	Days Needed to Ferment at 30°C			
	Raffinose	Sucrose	Maltose	Glucose
0	2	< 1	1	< 1
20	2	1	3	1
40	3	1	4	1
100	7	2	9	1
200	- ^a	3	10	1
300	- ^a	4	26	1

To fermentation tubes containing 5 ml of YEP + 2% sugar,
3.5 x 10⁵ cells of strain EK-6B were added. Varying concentrations
of 2DG were added and the tubes were incubated at 30°C.

^a A minus (-) sign means that raffinose was not fermented even
after 26 days.

TABLE 36

Effect of 2DG on the Ability to Ferment Raffinose by Strain EK-6B and
DGR Mutants Derived from It

Strain	Mutagen	Days needed to ferment raffinose at 30°C + 250 µg 2DG per ml
EK-6B	-	- (26)
DGR2	MNNG	3
DGR3	"	3
DGRA1	EMS	5
DGRB3	"	5
DGRC1	"	3

Strain EK-6B failed to ferment raffinose in the presence of 2DG
(250 µg per ml) even after 26 days.

TABLE 37

Invertase and α -Glucosidase Levels in EK-6B and DGR MutantsGrown on YEPD Broth ^a

Strain	Culture Age (hours)	A590 nm	Specific Activity ^b	
			Invertase	α -Glucosidase
EK-6B (Parental)	16	1.77	0.20	0.20
DGR2	16	1.758	2.6	0.739
DGR3	16	1.85	2.0	0.367
DGRA1	21	1.797	10.6	2.23
DGRB3	16	1.901	4.27	0.628
DGRC1	25	1.144	12.5	2.34

^a Cultures of 50 ml of YEPD broth were started by adding 0.05 ml of 24 hour old starting cultures of the above strains. The cultures were incubated at 30°C. Growth was monitored by reading the absorbance at 590 nm in a Beckman DU spectrophotometer.

^b Specific activity was determined using crude extracts of cells as described in "Materials and Methods".

levels of these enzymes was evident, but the values in all cases were higher than those of the parental strain grown under the same conditions, and to the same extent as measured by turbidity (A590 nm). Invertase levels showed a sixty-fold range, α -glucosidase, ten-fold; between the lowest strain values and the DGR mutant showing the highest values in specific activity.

Since it was apparent that the DGR mutants had considerably higher levels of invertase, and in some cases α -glucosidase activity as compared with EK-6B, a comparison of enzyme levels of these strains when grown under different glucose concentrations was made. Enzyme levels of these strains grown on sucrose were also studied. Only mutants DGR2 and DGR3 were compared with EK-6B in this and in subsequent studies. Mutants having high levels of both invertase and α -glucosidase were not studied in detail.

Comparison of Invertase Activity in EK-6B and DGR Mutants as a Function of the Glucose Concentration of the External Medium

The following conclusions can be drawn from the data presented in Table 38. Invertase and α -glucosidase levels varied inversely with the initial glucose concentration of the culture medium for all of these strains. Enzyme levels in EK-6B grown on sucrose were higher than those grown even on the most derepressing (0.5%) glucose concentrations. Invertase levels in sucrose-grown cells of DGR2 and DGR3 were somewhat higher than in EK-6B, although α -glucosidase levels were highest under these conditions, i.e. cells grown on sucrose. Invertase levels were considerably higher in both DGR mutants as compared with EK-6B at all

TABLE 38

Effect of Glucose Concentration on Invertase and α -Glucosidase Levels
in Strain EK-6B and DGR Mutants Derived from It

Initial Glucose Concentration in the medium (%)	Specific Activity of Strain ^a					
	EK-6B		DGR2		DGR3	
	Inv.	α -Glucosidase	Inv.	α -Glucosidase	Inv.	α -Glucosidase
0.5	0.80	0.156	4.7	0.339	3.89	0.246
2.0	0.11	0.21	4.99	0.990	4.13	0.456
5.0	0.121	0.051	2.65	0.288	1.71	0.226
10.0	0.121	0.095	0.563	0.211	0.198	0.137
2% sucrose	1.18	0.382	4.09	0.846	2.73	0.782

Cultures contained 50 ml of the appropriate YEP-based medium inoculated with 0.05 ml of a 24 hour YEPD broth started culture. Cultures were harvested after 20 hours at 30°C.

^a Determination of specific activities of invertase and α -glucosidase in crude extracts was performed as described in "Materials and Methods".

sugar concentrations studied; α -glucosidase levels were not as high but were still significantly greater in the DGR mutants than in EK-6B. Invertase specific activity showed more variation with these initial glucose concentrations than α -glucosidase. Even at an initial glucose concentration which strongly repressed enzyme synthesis in the parental strain (5% glucose), the invertase level was still considerably higher in the DGR mutants than in the parental cells grown under the most derepressing conditions. Mutant DGR2 was slightly less sensitive to catabolite repression by glucose than DGR3; it was evident that the DGR mutants could show some variation in sensitivity to glucose repression (cf. Table 37).

Variation in levels of invertase and α -glucosidase in EK-6B and DGR2 as a function of culture age in YEPD broth was also investigated (Table 39). Invertase levels were considerably higher in DGR2 than in EK-6B, although a progressive decrease in specific activity occurred in both cultures. A parallel trend was seen with α -glucosidase in both strains, although there was only a two-fold difference between DGR2 and EK-6B at any of the times sampled.

Further evidence that invertase activity is much higher in DGR mutants throughout the growth cycle than in EK-6B is presented in Table 40. These YEPD cultures were started with a much heavier inoculum than usual, and were incubated at 36°C rather than at 30°C in order to study invertase levels at a number of different times during the exponential and stationary growth phases. That all three strains (EK-6B, DGR2 and DGR3) show virtually the same growth characteristics is presented in Fig. 28. Little variation in viable counts and Klett unit values is evident among the three strains. The data in Table 40

TABLE 39

Invertase and α -Glucosidase Levels in EK-6B and DGR2 as a Function of Culture Age

Culture Age (hours)	Strain	Specific Activity	
		Invertase	α -Glucosidase
14½	EK-6B	0.062	0.302
	DGR2	2.792	0.655
19	EK-6B	0.049	0.243
	DGR2	1.923	0.412
23½	EK-6B	0.08	0.177
	DGR2	1.127	0.308

Cultures were grown on YEP 2% glucose (YEPD) at 30°C as described in Table 38.

Fig. 28. Growth of strain EK-6B, DGR2 and DGR3 on YEPD broth at 36°C. Culture conditions were as described in Table 40. Upper panel - Klett readings; lower panel - viable counts.

Legend

△ EK-6B

○ DGR2

□ DGR3

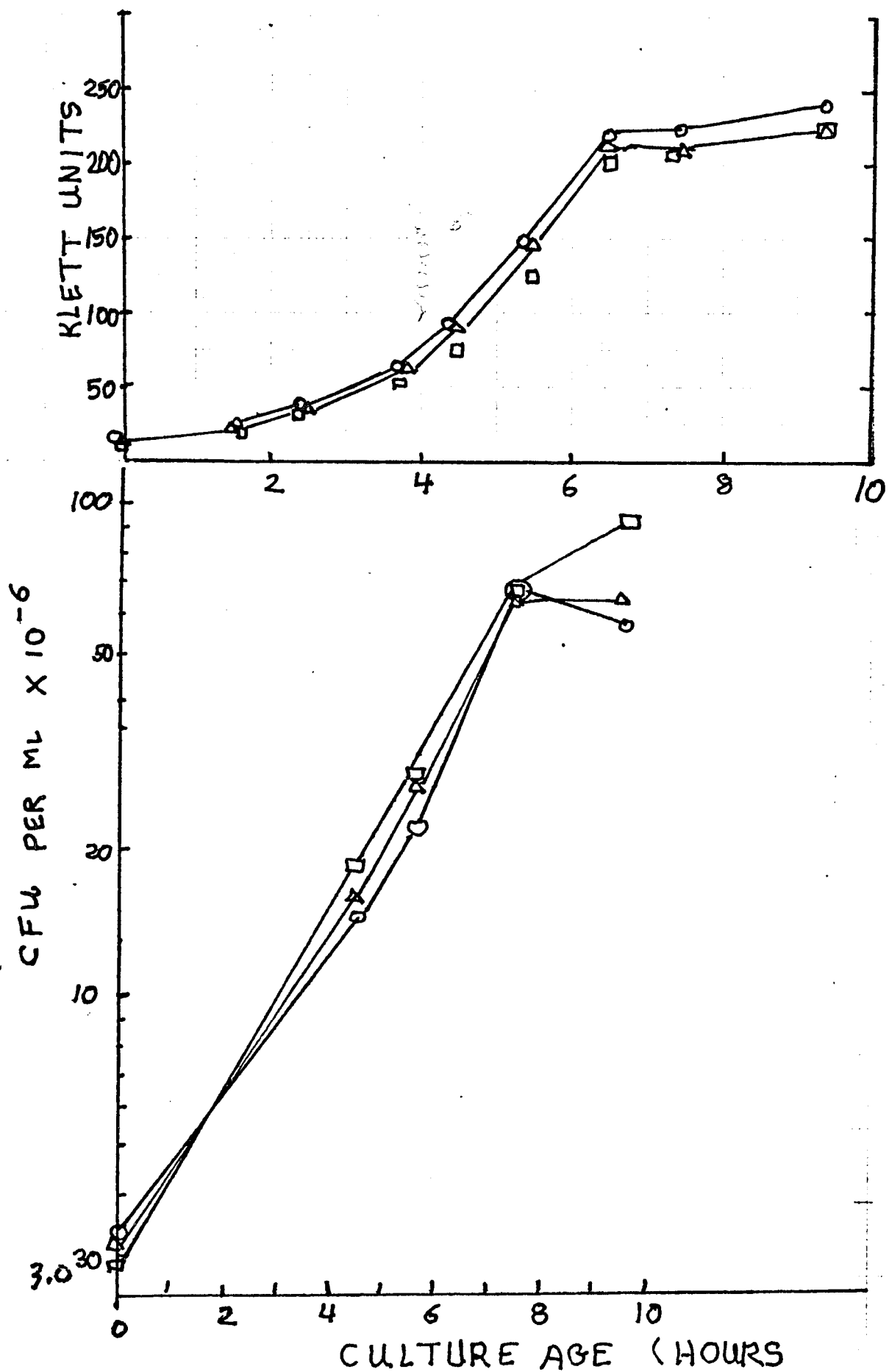


TABLE 40

Comparison of Invertase Levels in Strains EK-6B, DGR2 and DGR3 grown
on YEFD Broth as a Function of Culture Age

Culture Age (hours)	Invertase Specific Activity		
	EK-6B	DGR2	DGR3
4½	0.044	1.720	2.424
5½	0.058	2.184	1.936
7½	0.093	2.356	2.292
9½	0.168	3.368	2.062

One ml of YEFD broth starter cultures of the above strains was added to 30 ml of YEFD, incubated at 36°C. Aliquots of 5 ml were withdrawn at the above culture ages, and the cells were harvested and washed. Crude extracts were prepared using the Braun homogenizer as described in "Materials and Methods".

indicate that DGR2 and DGR3 have much higher invertase activities than EK-6B at culture ages which would normally be expected to have environmental conditions strongly repressing invertase formation. As the two DGR mutants did not grow any faster than EK-6B, it was assumed that environmental conditions were the same at particular culture ages in all three cultures (Fig. 28). Thus, the DGR mutants differed in how much invertase (and presumably α -glucosidase) activity they produced. DGR2 and DGR3 had considerably more invertase activity at 4½ hours (and at all subsequent times sampled) than EK-6B when invertase synthesis of the latter was most derepressed (9½ hours) under the conditions of this experiment.

Comparison of Invertases from EK-6B and DGR2 and DGR3

It was of interest to determine if the greater invertase activity of DGR2 and DGR3 was due to some structural alteration in these invertases conferring greater affinity for substrates such as sucrose. To answer this question the K_m for sucrose at pH 4.5 was determined for invertase in crude extracts of cells of the parent EK-6B and the DGR mutants DGR2 and DGR3 (Fig. 29). The K_m for sucrose for the DGR mutants' invertase was 0.028 Molar which was the same as that of EK-6B. Since the mutations responsible for increased invertase activity also affected α -glucosidase activity (raising it), the DGR mutations were most probably not due to lesions in a structural gene(s) for invertase.

In order to further test for possible structural alterations in the invertases of the DGR mutants, the heat inactivation of invertase in crude extracts of strains EK-6B, DGR2 and DGR3 was compared (Fig. 30).

Fig. 29. Lineweaver-Burk of determination of K_m for sucrose of invertases from strains EK-6B, DGR2 and DGR3. EK-6B was grown on YEP 0.3% glucose for 20 hours at 30°C. Strains DGR2 and DGR3 were grown on YEPD for 18 hours at 30°C. Crude extracts were prepared as described in "Materials and Methods". Invertase activity was determined in 0.1 M sodium acetate buffer, pH 4.5; the sucrose concentration ranged from 0 to 0.05 Molar. Temperature of incubation was 30°C.

Legend

- EK-6B
- DGR2
- Δ DGR3

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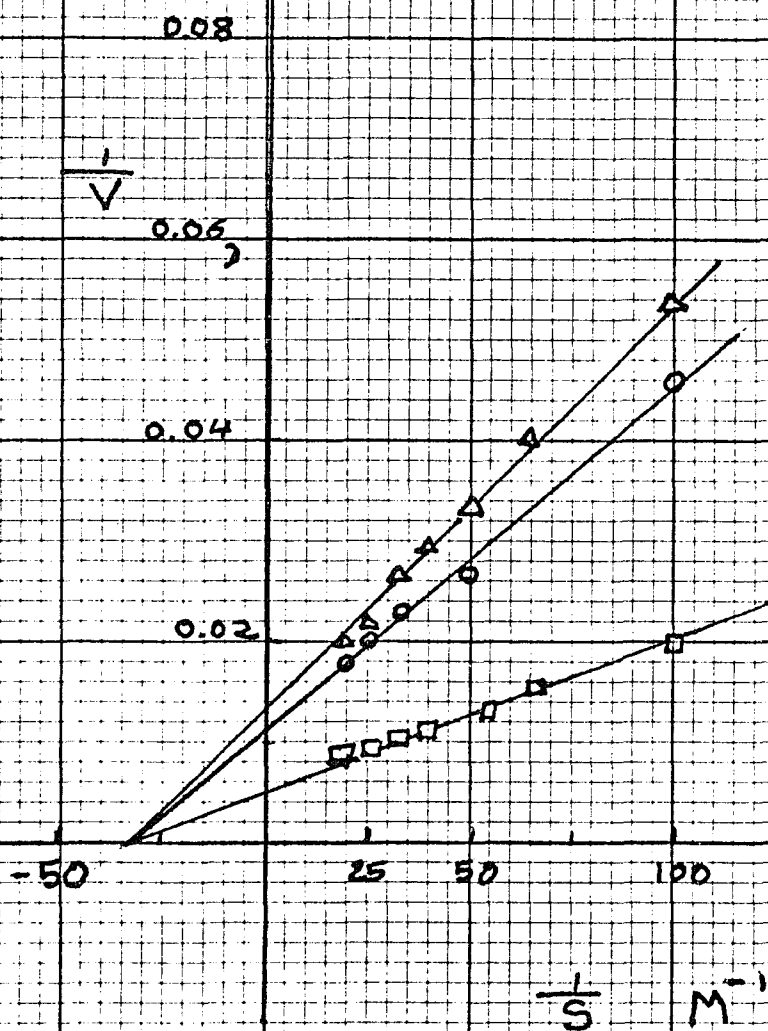


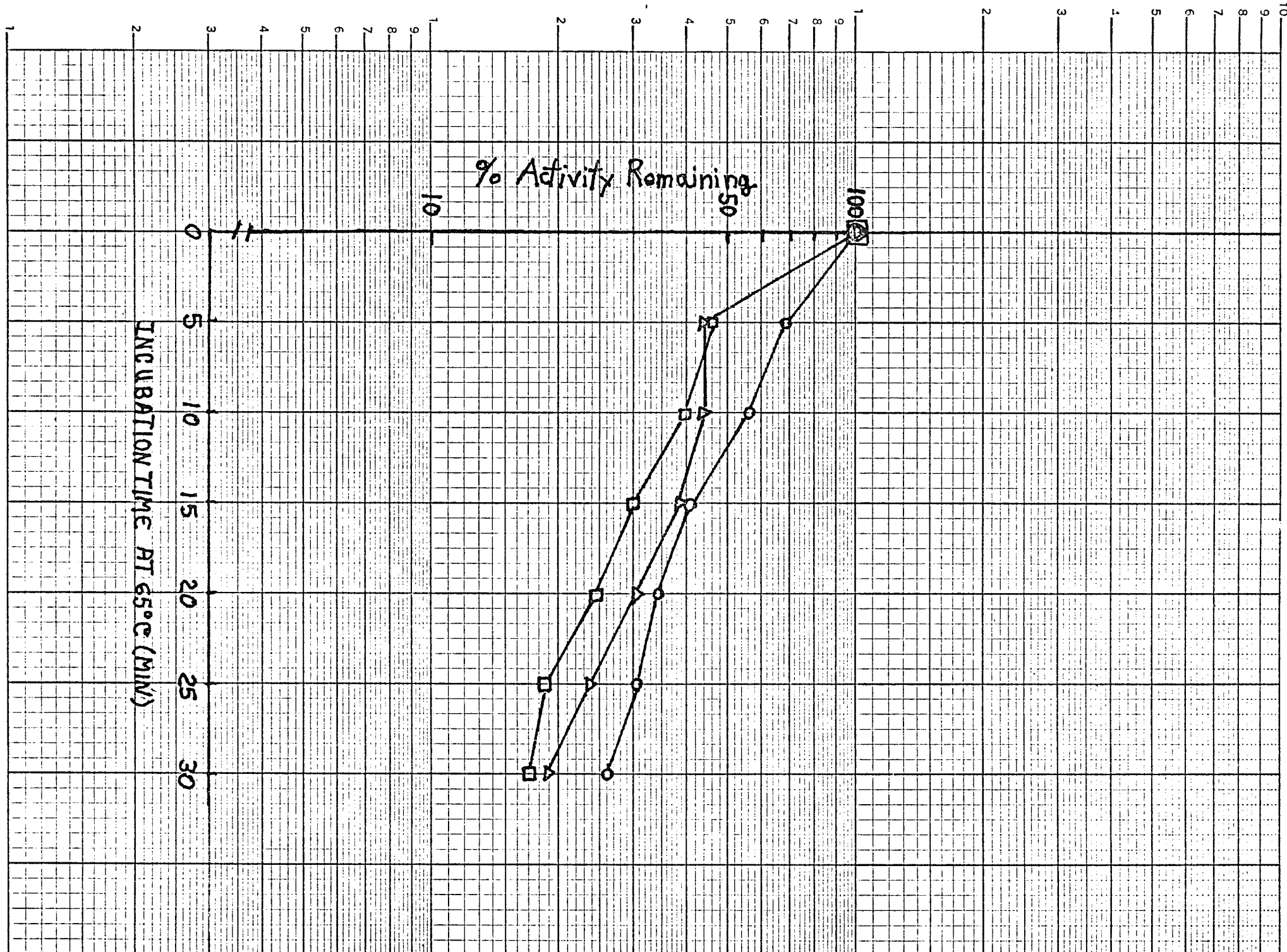
Fig. 30. Heat inactivation of invertases from strains EK-6B, DGR2 and DGR3 at 65°C. The above strains were grown in 50 ml of YEP 0.5% glucose for 16 hours at 30°C. Crude extracts were prepared as described in "Materials and Methods". The crude extracts each contained 2.7 mg protein per ml. Samples were incubated at 65°C in 0.025M sodium acetate buffer, pH 5.0. Invertase assays were performed as described in "Materials and Methods".

Legend

Δ EK-6B

○ DGR2

□ DGR3



There appeared to be little, if any, difference in the heat inactivation profiles at 65°C for invertase activity in these strains. It was concluded that heat inactivation revealed no structural differences in invertase in EK-6B and the DGR mutants that could account for the greater invertase activity of the latter two strains.

The higher invertase activity of the DGR mutants was investigated to determine if both forms of invertase were increased. This was most easily accomplished by SDS-polyacrylamide gel electrophoresis of crude extracts of repressed cells of EK-6B and DGR mutants and their subsequent staining for invertase activity (Fig. 31). Gels 1 through 5 represent parental strain EK-6B and four DGR mutants grown on 5 ml of YEPD; gels 6 and 7 represent EK-6B and DGRB3 grown on 50 ml of YEPD. While EK-6B invertase is virtually undetectable in gel 1, the DGR mutants have heavy bands of slow (external) and fast (internal) invertase activity. DGRB3 has heavier bands of invertase activity in gel 7 than EK-6B in gel 7, although comparable amounts of protein were applied to each gel. It has previously been shown that the slow moving band of invertase activity in these gels corresponded to the external invertase and the fast-moving band to the internal invertase (see Fig. 10 in Chapter III). It has also been shown (Chapter III and V) that the intensity of staining of invertase bands in these gels is directly related to the amount of invertase present and to the duration of the incubation assay. Fig. 31 thus presents evidence that the DGR mutants have increases in both the external and internal invertases.

The above experiments suggested that DGR2 and DGR3 had the same invertase as the parental strain EK-6B. Furthermore, SDS-polyacrylamide gel electrophoresis of invertase from repressed cells of EK-6B and the

Fig. 31. SDS-polyacrylamide gel electrophoresis of invertases from EK-6B and DGR mutants derived from it. Gels 1 through 5 represent invertase patterns of EK-6B and four DGR mutants; crude extracts used were made from 5 ml cultures grown in YEPD broth for 20 hours at 30°C. Gels 6 and 7 represent invertase patterns of EK-6B and DGRB3; crude extracts used were made from 50 ml YEPD cultures grown for 20 hours at 20°C. Aliquots of 0.05 ml crude extracts were applied to each gel.

Preparation of gels, electrophoresis and staining for invertase activity were performed as described in "Materials and Methods".

Gel 1	EK-6B
" 2	DGR2
" 3	DGR3
" 4	DGRA1
" 5	DGRC1
" 6	EK-6B
" 7	DGRB3

EXTERNAL INVERTASE

INTERNAL INVERTASE

TRACKING DYE



1 2 3 4 5 6 7

DGR mutants (Fig. 31) indicated that both the external and internal forms of invertase appeared to be increased in the DGR mutants compared with the parental strain. Therefore, the mutation responsible for the higher invertase activity in the DGR mutants seems to control the level of enzyme rather than the structure.

Genetical Studies on DGR Mutants

In order to investigate the genetic basis for invertase hyperproduction in the DGR mutants, they were crossed to the segregational sucrose nonfermenter Z1-2D, and the SUC3 MAL3 strain HZ1-27D, and diploids were isolated. When invertase and α -glucosidase levels in these diploids grown on YEPD were compared with diploids of EK-6B crossed to the same strains (Table 41) it was evident that there was no significant differences in enzyme levels between EK-6B, DGR2 and DGR3 crossed to either Z1-2D or HZ1-27D. Furthermore, none of these values were significantly above those for EK-6B presented in Table 38. It was concluded that the genetic factor(s) conferring invertase hyperproduction were recessive.

To determine the linkage relationship between the gene conferring the DGR-invertase hyperproduction phenotype and SUC3 MAL3, the diploid DGR2 x Z1-2D was sporulated and a number of asci were dissected (Table 42). As expected, the ability to ferment sucrose and raffinose and the ability to make invertase cosegregated. Occasionally ascospore clones (ex. 5B) showed slow sucrose fermentation but no raffinose fermentation; these had no invertase activity and were considered to lack the SUC3 gene. The presence of high invertase

TABLE 41

Invertase and α -Glucosidase Levels in Diploids of EK-6B, DGR2 and DGR3
Crossed to Z1-2D and HZ1-27D

Diploid	Specific Activity	
	Invertase	α -Glucosidase
Z1-2D x EK-6B (suc0)	0.186	0.095
" x DGR2	0.205	0.088
" x DGR3	0.179	0.058
HZ1-27D x EK-6B (SUC3)	0.322	0.128
" x DGR2	0.298	0.175
" x DGR3	0.262	0.159

Cultures of 50 ml YEPD broth were inoculated with 0.05 ml of starting cultures of the above diploids. The cultures were grown at 30°C for 18 hours. Specific activities for invertase and for α -glucosidase were determined in crude extracts as previously described. Z1-2D was a segregational sucrose and maltose nonfermenter (α suc0 mal10 leu1 lys1). HZ1-27D (α SUC3 MAL3 lys1) was a SUC3 MAL3 segregant derived from the cross EK-6B x Z1-2D (see Chapter III).

TABLE 42

Invertase Levels in Tetrads of Cross DGR2 x Z1-2D grown on YEPD

Ascospore	Invertase sp. act.	Fermentation at 30°C ^a		
		Raffinose	Sucrose	Maltose
4A	0	-	-	-
4B	4.69	+ (2)	+ (1)	+ (1)
4C	1.64	+ (2)	+ (1)	+ (1)
4D	0	-	-	-
5A	0	-	-	-
5B	0	-	+ (7)	-
5C	0.168	+ (2)	+ (1)	+ (1)
5D	5.33	+ (2)	+ (1)	+ (1)
6A	0	-	-	-
6B	0.171	+ (2)	+ (1)	+ (1)
6C	0	-	+ (7)	-
6D	3.78	+ (2)	+ (1)	+ (1)
17A	4.8	+ (2)	+ (1)	+ (1)
17B	0.4	+ (2)	+ (1)	+ (1)
17C	0	-	-	-
17D	0	-	-	-

^a Days needed to ferment these sugars.

Ascospore clones of each tetrad were grown on YEPD for 20 hours at 30°C. Culture conditions, crude extract preparation and invertase assay were as described previously.

activity, however, did not always cosegregate with the SUC3 gene, suggesting that the gene(s) responsible for invertase hyperproduction was probably unlinked to SUC3 which determined the presence of invertase; poor spore viability prevented the recovery of more than a few complete tetrads. Of the four tetrads only tetrad 4 was a parental ditype; tetrads 5, 6 and 17 were tetratype tetrads, containing two sucrose fermenting ascospore clones, one with high and one with low invertase activity. It was apparent that for the DGR mutation to be expressed, a fully functional SUC3 gene was required. These data were in themselves not sufficient, however, to demonstrate that the DGR2 mutation was unlinked to SUC3. The data in Tables 41 and 42 suggested that the DGR mutants were the result of a recessive gene which was probably unlinked to SUC3. To determine the linkage relationship between the DGR mutations and SUC3, the DGR mutants and EK-6B were crossed to R. S. 5-1, the SUC3 mal10 strain used for allele-testing the suc3 mutants in Chapter III. Diploids were isolated, and were grown on YEPD broth to determine invertase and α -glucosidase activities (Table 43). No significant differences in enzyme activities were found between any of the diploids. It was concluded that the DGR mutations were recessive in nature. Further support for this conclusion was the inability of any of these diploids to grow on YEP 2% raffinose plus 250 μ g 2DG per ml.

The diploids of DGR2 and DGR3 crossed to R. S. 5-1 were sporulated and asci were dissected. The results are presented in Tables 44 and 45. All tetrads showed a 4:0 segregation for sucrose and raffinose fermentation, and 2:2 segregation for maltose fermentation; thus linkage of DGR mutations to SUC3 MAL3 could readily be tested. The

TABLE 43

Invertase and α -Glucosidase Levels in Diploids of R. S. 5-1 Crossed
Crossed to EK-6B and to the DGR Mutants

Diploid	Specific Activity	
	Invertase	α -Glucosidase
5-1 x EK-6B	0.078	0.088
" x DGR2	0.088	0.077
" x DGR3	0.095	0.092
" x DGRA1	0.044	0.164
" x DGRB3	0.030	0.133
" x DGRC1	0.185	0.131

The above diploids were grown on YEPD broth (10 ml in test tubes) for 16 hours at 30°C. Cultures were harvested and washed according to "Materials and Methods". Crude extracts were prepared using the Braun homogenizer as described in "Materials and Methods".

TABLE 44

Segregation of DGR Mutation in the Cross DGR3 x R. S. 5-1

Ascospore	SUC	MAL	Invertase sp. act.	RAF + 2DG	Tetrad type
1A	+	-	0.030	-	PD
B	+	-	0.109	-	
C	+	+	1.594	+	
D	+	+	1.06	+	
2A	+	-	0.079	-	TT
B	+	-	0.612	+	
C	+	+	0.812	+	
D	+	+	0.104	-	
3A	+	+	0.048	-	TT
B	+	-	0.598	+	
C	+	+	0.618	+	
D	+	-	0.077	-	
4A	+	+	2.75	+	TT
B	+	-	0.081	-	
C	+	-	0.549	+	
D	+	+	0.073	-	
5A	+	+	0.499	+	TT
B	+	+	0.034	-	
C	+	-	0.055	-	
D	+	-	0.587	+	
6A	+	-	0.451	+	NPD
B	+	+	0.121	-	
C	+	-	0.516	+	
D	+	+	0.036	-	
7A	+	-	0.070	-	TT
B	+	-	0.709	+	
C	+	+	0.716	+	
D	+	+	0.026	-	
8A	+	+	0.123	-	TT
B	+	+	0.352	+	
C	+	-	0.059	-	
D	+	-	0.847	+	

TABLE 44 (Continued)

Ascospore	SUC	MAL	Invertase sp. act.	RAF + 2DG	Tetrad type
9A	+	-	0.447	+	NPD
B	+	+	0.054	-	
C	+	+	0.104	-	
D	+	-	0.371	+	
10A	+	+	0.118	-	TT
B	+	+	1.146	+	
C	+	-	0.107	-	
D	+	-	0.598	+	
11A	+	-	1.074	+	TT
B	+	+	0.411	+	
C	+	+	0.046	-	
D	+	-	0.038	-	
12A	+	-	0.005	-	TT
B	+	+	0.047	-	
C	+	+	0.913	+	
D	+	-	0.670	+	
13A	+	+	0.573	+	PD
B	+	-	0.023	-	
C	+	-	0.034	-	
D	+	+	0.404	+	

Invertase activity was determined in crude extracts prepared from 16 hour old YEPD cultures (10 ml) grown at 30°C. Crude extracts were prepared using a Braun homogenizer as described in "Materials and Methods". The ability to grow on raffinose in the presence of 2DG (250 µg per ml) was determined in plates and in fermentation tubes. For the different types of tetrads, PD designated ditype tetrads; TT, tetratype tetrads; and NPD, nonparental ditype tetrads.

results presented in Table 44 for DGR3 x R. S. 5-1 indicated that a single-gene difference was probably responsible for the ability to grow on raffinose in the presence of 2DG and for high invertase levels on YEPD broth. Each tetrad showed two ascospore clones with high invertase activity and the ability to grow on raffinose plus 2DG (DGR), and two ascospore clones with low invertase levels and the inability to grow on raffinose plus 2DG (dgr).

There was considerable variation in invertase activity among the ascospore clones showing the DGR phenotype, but the lowest values (e.g. 9D: 0.371 micromoles of sucrose hydrolyzed per minute per mg protein) were still higher than the highest value seen among the dgr ascospore clones (8A: 0.123). These variations could conceivably have been the result of ascospore strain differences in growth indirectly affecting invertase production or genes related to invertase formation. The former alternative is considered more likely as the genetic data suggested only a single-gene difference.

That the DGR mutation was probably at a gene unlinked to SUC3 MAL3 was revealed by the ratio of tetrad types: 2 parental ditype (PD) : 9 tetratype tetrads (TT) : 2 nonparental ditype tetrads (NPD). This was very close to the one PD : four TT : one NPD ratio as expected for the segregation of two unlinked genes (Mortimer and Hawthorne, 1969).

A more limited sample of tetrads obtained from the cross DGR2 x R. s. 5-1 revealed that the DGR2 mutation was also probably unlinked to SUC3 MAL3 (Table 45). The cosegregation of high invertase activity and the ability to grow on raffinose plus 2DG was once again evident.

To determine if the two DGR mutants were allelic to each other, DGR2 and the other DGR mutants were crossed with a DGR3 segregant of

TABLE 45

Segregation of DGR Mutation in the Cross DGR2 x R. S. 5-1

Ascospore	SUC	MAL	Invertase sp. act.	RAF + 2DG	Tetrad type
9A	+	-	0.055	-	PD
B	+	+	0.887	+	
C	+	-	0.124	-	
D	+	+	1.33	+	
10A	+	+	1.87	+	PD
B	+	-	0.160	-	
C	+	-	0.009	-	
D	+	+	1.06	+	
14A	+	+	0.606	+	TT
B	+	-	1.518	+	
C	+	-	0.093	-	
D	+	+	0.247	-	
15A	+	+	1.48	+	TT
B	+	+	0.042	-	
C	+	-	0.072	+	
D	+	-	1.184	-	
16A	+	+	0.086	-	NPD
B	+	-	0.838	+	
C	+	-	0.812	+	
D	+	+	0.056	-	

Cultures of 10 ml of YEPD were grown for 16 hours at 30°C. Crude extracts were prepared in the Braun homogenizer as described in "Materials and Methods".

the cross DGR3 x R. S. 5-1, DGR3-3B (α lys1 SUC3 mal0 DGR3). Diploids were isolated and grown on YEPD broth to determine invertase and α -glucosidase levels (Table 46). Comparison with invertase activity in DGR x dgr diploids (Table 41 and 43) revealed that DGR3 x DGR2, DGR3 x DGR3 and DGR3 x DGRA1 diploids had considerably more invertase activity; α -glucosidase activity was also somewhat elevated. The diploids of DGR3-3B crossed to DGRB3 and DGRC1, however, had no more invertase activity than diploids of the same DGR mutants crossed to R. S. 5-1 (see Table 43). The most likely explanation for this was that DGR2, DGR3 and possibly DGRA1 were allelic to each other, and that mutants DGRB3 and DGRC1 were probably not allelic to DGR3.

To further test the possible allelism of mutants DGR2 and DGR3, the diploid DGR3-3B x DGR2 was sporulated and asci were dissected (Table 47). All but one of the tetrads showed 4:0 segregation for both invertase activity (with considerable variation among the ascospore clones) and the ability to grow on raffinose in the presence of 2DG. The only exception was ascospore clone 2A which turned out to be a sucrose nonfermenter and consequently had no invertase activity; it also failed to grow on raffinose and raffinose plus 2DG. This 3:1 segregation in tetrad 2 for sucrose fermentation was intriguing; the tetrad's other genetic markers segregated in the expected manner. A possible explanation for this observation could be that gene conversion occurred (Fogel and Hurst, 1967; discussed in Mortimer and Hawthorne, 1969). The data suggested that DGR2 and DGR3 were allelic, or at least closely linked.

In summary, a number of induced mutants derived from the

TABLE 46

Invertase and α -Glucosidase Levels in DGR3/DGR Diploids

Grown on YEPD Broth

Diploid	Specific Activity	
	Invertase	α -Glucosidase
DGR3-3B x DGR2	0.910	0.371
" x DGR3	0.713	0.450
" x DGRA1	1.076	0.337
" x DGRB3	0.038	0.199
" x DGRC1	0.049	0.302

Cultures of 10 ml YEPD were grown for 17 hours at 30°C. Crude extracts were prepared in the Braun homogenizer as described in "Materials and Methods". All DGR mutants were crossed to DGR3-3B and diploids were isolated. Ascospore DGR3-3B was an α lys1 SUC3 mal10 DGR3 segregant derived from the cross DGR3 x R. S. 5-1 (see Table 44).

TABLE 47

Segregation of DRG2 and DGR3 Mutations in the Cross DGR3-3B x DGR2

Ascospore	SUC	MAL	Invertase sp. act.	RAF + 2DG
1A	+	+	1.300	+
B	+	-	0.555	+
C	+	+	1.450	+
D	+	-	0.856	+
2A	-	+	0	- ^a
B	+	-	1.054	+
C	+	-	0.500	+
D	+	+	1.550	+
3A	+	+	1.020	+
B	+	+	0.544	+
C	+	-	1.160	+
D	+	-	0.375	+
4A	+	-	0.750	+
B	+	+	0.516	+
C	+	+	1.299	+
D	+	-	1.130	+
5A	+	-	0.563	+
B	+	+	1.202	+
C	+	+	0.327	+
D	+	-	0.876	+
6A	+	+	0.466	+
B	+	+	0.491	+
C	+	-	0.754	+
D	+	-	0.634	+

Invertase activity was determined in crude extracts of YEFD-grown cells as described in Table 44 and in "Materials and Methods".

^a Ascospore 2A was a sucrose and raffinose nonfermenter; it fermented maltose. It had no detectable invertase activity and apparently did not grow on raffinose plus 2DG.

SUC3 MAL3 strain EK-6B were isolated by selecting for their ability to grow on raffinose in the presence of 2-deoxy-D-glucose (2DG). These DGR mutants have the following properties: (1) They produce higher levels of invertase under conditions normally repressing invertase synthesis. (2) The invertases of DGR mutants DGR2 and DGR3 were found to be indistinguishable from the parental invertase with respect to the K_m for sucrose and in thermal stability. (3) The genetic studies revealed that the same gene was responsible for hyperproduction of invertase in both mutants, unlinked to the SUC3 gene and recessive in nature. It is concluded from the data presented in this chapter that DGR mutants DGR2 and DGR3 coming from an altered gene unlinked to SUC3 are responsible for hyperproduction of invertase determined by the SUC3 gene.

CHAPTER VIII

DISCUSSION

This thesis presents results of a genetical and biochemical study of the role of the classical SUC genes of Saccharomyces strains in the fermentation and utilization of the B-fructosides sucrose and raffinose, and in the formation of invertase which is required for their hydrolysis. The two questions asked in the present study were: (1) What are the roles of the SUC genes in invertase formation: are they structural or regulatory genes? (2) What other genetic factors directly or indirectly affect the level of expression of the invertase structural genes? The most recent review on sugar utilization in yeasts (Barnett, 1976) has a comprehensive discussion of the various lines of research into the genetics of maltose utilization. The study of sucrose genetics, on the other hand, has not advanced very far since the classical work of Winge and Roberts in the early 1950's.

In their utilization of sugars such as sucrose and raffinose, maltose and α -methylglucoside, yeasts (Saccharomyces cerevisiae and related species as well as interspecific hybrids) show some superficially comparable features in the genetic systems involved. The SUC and MAL polymeric gene systems have in common the fact that any one of these dominant SUC or MAL genes allows the yeast strain bearing it to ferment the specific sugar. The genetic function of the SUC and MAL genes, however, appears to be different. Furthermore, many more genes have been implicated in maltose utilization than in the utilization of both sucrose and raffinose. These differences may consequently reflect differences in the modes of regulation of sucrose

and maltose utilization.

Induced Sucrose Nonfermenters

The sucrose nonfermenting (suc) mutants derived from SUC1, SUC2 and SUC3 have been shown in this study to share the following properties: (1) All suc mutants failed to ferment raffinose although some could ferment sucrose slowly while others failed to ferment sucrose. (2) The suc mutants were not complemented in crosses to segregational sucrose nonferments (suc0); the diploids failed to ferment raffinose and either fermented sucrose slowly or not at all; suc mutants crossed to one another failed to complement. (3) The suc mutants were recessive in crosses to SUC fermenters, these diploids being able to ferment sucrose and raffinose. (4) Tetrad analysis of the above suc mutant parental SUC fermenter diploids revealed that the suc mutants segregated as single gene mutations which were probably allelic to the SUC gene carried by the parental strain. (5) The suc mutants lacked both internal and external invertases of the parental SUC strains as demonstrated by Sephadex G-150 column chromatography and SDS-polyacrylamide gel electrophoresis (cf. C. III, V and VI). (6) Crude extracts made from derepressed cultures of suc mutants x suc0 diploids had no detectable invertase activity, while suc mutant x SUC diploids had approximately one-half the specific activity of SUC x SUC diploids (cf. Ch. III and V).

One difference among the suc mutants was noted: reversion studies of suc3 mutants failed to yield any revertants allelic to SUC3 but rather de novo sucrose fermenters (Chapter IV), while one revertant of

a suc1 mutant (D10-ER1) was probably allelic to SUC1 (cf. Chapter V).

In the present study the fermentation of both sucrose and raffinose was considered to indicate the presence of a SUC gene and a functional invertase (SUC genes were formerly designated R for raffinose fermentation (Winge and Roberts, 1952)), whereas the inability to ferment both of these sugars meant the lack of a functional invertase resulting from an induced suc mutant or a segregational sucrose nonfermenter (suc0) gene.

The difference in the ability of suc mutants to utilize sucrose may have several possible explanations. It is unlikely that suc mutants fermenting sucrose slowly have an altered invertase since in crude extracts of derepressed cultures of these mutants, even those grown on sucrose (cf. Ch. III suc3-40) there was no detectable invertase activity. Sucrose, however, can be hydrolyzed by the yeast α -glucosidases maltase and α -methyl-glucosidase (isomaltase; Khan and Eaton, 1967) which split the α -glucoside end of the bond between the fructose and glucose moieties. It has been shown that yeast strains with constitutive maltase synthesis are able to hydrolyze and ferment sucrose in the absence of a classical SUC gene (for example the constitutive MAL4 strain 1403-7A of Khan, Zimmermann and Eaton (1973)). These authors reported that sucrose entering cells of this strain is readily hydrolyzed by the α -glucosidase present. Mutant alleles of the SSF (sucrose specific fermentation) were also reported by these same authors to prevent sucrose fermentation by the constitutive α -glucosidase.

The sugar fermentation properties of nearly 500 different yeast strains was recently surveyed (cf. Table XVI of Barnett, 1976). Most

strains able to ferment raffinose were also able to ferment sucrose; the presence of a functional B-fructofuranosidase (invertase) was presumed in these cases. Only two of 497 yeast strains fermented raffinose but not sucrose. In the absence of a functional invertase, raffinose could be hydrolyzed by melibiase (an α -galactosidase) which hydrolyzes the α -galactoside linkage in raffinose (see Fig. 1 of Chapter I). In the present study no ascospore clones were found in any of the crosses dissected able only to ferment raffinose so it was concluded that melibiase fermentation (MEL) genes were absent in the yeast strains employed. A large number of yeast strains able to ferment sucrose but not raffinose were present among the strains surveyed by Barnett; most of these were able to ferment maltose. There is thus considerable evidence for sucrose fermentation in strains fermenting maltose but not raffinose. Barnett reported only four out of 497 strains which fermented sucrose but neither raffinose nor maltose. This may be comparable to the rare ascospore clones apparently only able to ferment sucrose listed in Table 42 of Chapter VII: ascospores 5B and 6C. These disparate results may be explainable by different substrate specificities of α -glucosidases (cf. Table IX of Barnett, 1976) or in differences in permeability barriers to these sugars. Whatever the explanation for the slow sucrose fermentation in the absence of functional classical SUC genes, the above findings complicate the investigation of the relationship between SUC genes and invertase formation.

Comparison of the levels of invertase and α -glucosidases (maltase, etc.) in segregational sucrose (*suc0*) and maltose (*mal0*) nonfermenters revealed the apparent lack of invertase activity while

basal α -glucosidase activity was always present (Khan, Zimmermann and Eaton, 1973; ten Berge, Zoutewelle and van de Poll, 1973). This difference may be related to either or both of the following: (1) Strains of Saccharomyces bear MAL genes which "control" the level of maltase which is an intracellular enzyme, whereas SUC genes determine the presence of invertase which must be outside the plasma membrane of the yeast cell in order for it to hydrolyze sucrose and raffinose (Friis and Ottolenghi, 1959). (2) The MAL genes that have been examined in detail, such as MAL2 (Zimmermann and Eaton, 1974), MAL2 (Khan, Zimmermann and Eaton, 1973) and MAL6 (ten Berge, Zoutewelle and van de Poll, 1973), have been shown most likely to be regulatory genes. The SUC genes have, on the other hand, been claimed on the basis of enzymatic studies to be structural genes (Ottolenghi, 1971b; Plischke et al., 1975).

The mutational studies of the SUC genes apparently yielded, with the possible exception of suc220, mutants which were shown to be allelic to the particular SUC gene in the parental strain. Maltose nonfermenters, on the other hand, can readily be induced at genes other than and in addition to the MAL genes. A strain bearing the MAL6 gene gave rise to mal6 mutants with lower maltase and α -methylglucosidase activities and lower maltose uptake compared with the MAL6 parent (ten Berge, Zoutewelle and van de Poll, 1973). Maltose nonfermenters were induced in the constitutive MAL4 gene of strain 1403-7A resulting in lower maltase activity (Khan, Zimmermann and Eaton, 1973). Mutations resulting in the loss of the ability to ferment maltose have been found at genes other than the MAL genes; the dsf (disaccharide fermentation) genes (Zimmermann, Khan and Eaton,

1973), the UNT mutants temperature-sensitive for maltose fermentation (Khan, 1975), and the gal3 locus which is pleiotropic causing impairment of maltose utilization in addition to that of galactose and the α -galactoside melibiose (Kew and Douglas, 1976).

In the present study the mutant suc220 derived from the SUC3 MAL3 strain EK-6B was unable to ferment maltose in addition to sucrose and raffinose (see Chapter III). This mutant strain probably contained separate unlinked suc and mal mutations since the cross of suc220 to a segregational nonfermenter yielded some ascospores which were unable to ferment sucrose and raffinose but fermented maltose.

What could be concluded about the nature of the SUC genes from the genetic and biochemical studies of the suc mutants? The suc mutants might be any of the following: (1) structural gene mutations affecting invertase; (2) regulatory mutations in "operator-promoter" regions adjacent to invertase structural genes; (3) mutations in regulatory genes unlinked to or at least not adjacent to the structural genes. The third possibility is probably unlikely to be correct because the level of invertase activity in sucx/SUCX diploids is about one-half that of SUCX/SUCX diploids. An unlinked regulatory gene making a gene product operating in the positive mode (normally) would be expected to be compensated for by the product of the normal parental regulatory allele; a regulatory gene product operating in the negative mode normally might block the transcription of invertase structural genes to a greater extent than was observed in sucx/SUCX diploids (cf. Beckwith and Rossow, 1974; Englesberg and Wilcox, 1974). It is more likely that either of the first two possibilities correctly accounts for the various properties of the suc mutants. This will

be discussed further in connection with revertant studies.

Revertants of suc Mutants

The suc mutants lacked any detectable invertase activity and thus did not provide conclusive proof as to whether the SUC genes were structural versus regulatory genes. Therefore, revertants of these suc mutants were sought and examined for possible alterations in properties of invertase or in the regulation of its synthesis.

It should be noted in passing that SUC revertants were selected for on raffinose agar plates and not on sucrose plates because most of the "revertants" appearing on the sucrose plates were found to be unable to ferment raffinose and lacked invertase activity in crude extracts of derepressed cultures. The problem of sucrose fermentation in the absence of raffinose fermentation (indicating the lack of active SUC genes) was discussed in the previous section of this chapter.

Since none of the revertants of suc3 mutants appeared to be allelic to SUC3 but rather were de novo fermenters arising as a result of mutations at other genes, the genotypes of the parental SUC3 strain EK-6B, the suc3 mutants and the de novo sucrose and raffinose fermenters may be designated in the following manner so as to give recognition of the multiplicity of active and silent SUC genes. EK-6B may have the following genotype with respect to sucrose fermentation by SUC genes: SUC3 MAL3 suc₁⁰ suc₂⁰ suc₄⁰, etc. where SUC3 and MAL3 represent the functional sucrose and maltose fermentation genes and suc₁⁰ represents a nonfunctional allele of SUC1 derived segregationally

from some cross; the other suc0 alleles are derived in a similar way. The de novo sucrose fermenters may be the result of a mutation at any of the nonfunctional suc alleles in the mutant suc3 MAL3 suc₁0 suc₂0 suc₄0 suc₅0, etc., from previously unrecognized genes or from genes able to act as suppressors. While not presented in this study, de novo sucrose fermenters making invertase could be induced in segregational sucrose nonfermenters.

Some of the SUC revertants may have been the result of suppression (for a review see Hartman and Roth, 1973). One revertant of suc1-D10, designated D10-NAR1, may have been the result of an unlinked suppressor acting on that suc1 mutant (cf. Chapter V). While little biochemical work on this revertant was undertaken, it appeared that D10-NAR1 had less invertase activity than the parental SUC1 strain and appeared to have only a single faint band of invertase (the slow-moving, external form). This revertant was thus similar in this regard to D10-ER1 which was probably allelic to SUC1, but it was recessive.

Only one suc mutant, suc1-D10, appeared to revert readily giving a sucrose and raffinose fermenting strain that was allelic to SUC1 (cf. Chapter V). The significance of D10 revertants ER1 and NAR1 is that they apparently have only the external invertase and seem to lack or have very little internal. It has been demonstrated that the SUC3 gene determines the presence of both forms of invertase (Chapter III); furthermore, the suc mutants derived from SUC1, SUC2 and SUC3 appeared to lack both invertase activities (Chapters V, VI and III). In addition, the revertants of suc3 mutants described in Chapter IV appeared to have both invertase activities restored. Suc revertant D10-ER1 is thus especially significant because it is probably allelic

to SUC1.

The problem of the biosynthetic relationship between the two forms of yeast invertase was introduced in Chapter I. The biochemical and physiological approaches to solving this problem have generally tended to support the idea that internal invertase does not appear to be the precursor of the external. This view was favored by Lampen and his associates (Lampen, Kuo, Cano and Tkacz, 1972). The alternative, that the internal form is the precursor of the external, is favored by Gascón (Liras and Gascón, 1971). The SUC revertant D10-ER1 provides a possible genetic basis for concluding that the internal invertase is not the precursor of the external, at least for SUC1, and possibly this may be true for the other SUC genes. A recent study of the biosynthetic relationship of invertases in the invertase hyper-producer SUC2 strain 1710 (Gallili, 1975) revealed that the two invertases have different patterns of synthesis during the cell cycle. Furthermore, Gallili reported that the use of the glycoprotein synthesis inhibitor tunicamycin (Kuo and Lampen, 1974) showed that the tunicamycin concentration sufficient to block external invertase synthesis had no perceptible effect on that of the internal form. This information supports the idea that the two invertases are biosynthetically unrelated, although this seems to be in conflict with the many similarities, enzymatic and immunological, reported for these invertases (Gascon, Neumann and Lampen, 1968). Some sort of evolutionary relationship between the two forms of invertase may exist, has has been considered to be the case for the various SUC genes (cf. Ottolenghi, 1971b).

SUC revertant D10-ER1 may also provide evidence not supporting the

existence of a common subunit in both invertases. The possible existence of polypeptide subunits, of different molecular weights, for each of the two forms of invertase was reported (Tkacz, 1971) but this has not been confirmed. The lack of allelic complementation among the suc mutants derived from any one of the SUC genes investigated may also support the absence of polypeptide subunits in the invertases (assuming that the SUC genes are invertase structural genes), although most studies of complementation (see Fincham, 1966) employ many more mutants than were available for the present study.

One suggestion from the genetic and enzymatic studies of SUC revertants (and de novo sucrose fermenters) in the present study is that the SUC genes, at least SUC1, may be complex loci containing structural genes for the two invertases and also regulatory DNA regions adjacent to them, i.e. operator-promoter regions analogous to those of the lac and other operons of bacteria. "Operons" do not appear to have been demonstrated often in yeast: perhaps the HIS4 gene complex (Fink, 1966) and the GAL1, 7 and 10 region (Douglas and Hawthorne, 1964) may qualify as gene clusters which are under some sort of coordinate control. The lack of evidence showing coordinate control of synthesis of the two yeast invertases (Gallili, 1975), however, may be considered as evidence against the SUC genes being "operons". The identification of regulatory and structural gene mutants, closely linked to one another and allelic to particular SUC genes, would aid in the elucidation of this problem.

The Search for suc Mutants and Methods to Obtain Them

Yeast invertase is found mostly in the external form and many studies have pointed out the necessity of having the external invertase outside of the plasma membrane if sucrose and raffinose were to be hydrolyzed (for example, Friis and Ottolenghi, 1959). It is probably realistic to expect to detect only suc mutants with no detectable invertase activity. Also there is evidence that yeast strains have much more invertase activity for sucrose hydrolysis than capacity to ferment the products of sucrose hydrolysis (de la Fuente and Sols, 1962). It is possible that many suc mutants were induced that had significantly less invertase activity than the parental strains, but these were not detected because they did not ferment sucrose and raffinose significantly more slowly than the parental strains.

Maltose nonfermenting mutants can fail to ferment maltose if the maltose is not taken up properly, penetrates but does not produce inducer or act as one, or maltase itself is defective. While none were obtained, it is possible that suc mutants might arise which had invertase activity if the invertase were sequestered away from the substrates. In regard to such suc mutants there is a yeast species, the osmophile Saccharomyces rouxii which makes an invertase which is normally sequestered in a compartment in the yeast cells where it does not have access to and therefore cannot hydrolyze sucrose and raffinose; only upon autolysis of this yeast, occurring naturally or artificially induced, can this invertase be decriptified (Arnold, 1974).

Few sucrose nonfermenting mutants were obtained in this way.

While the selection method employed allowed the detection of the occasional suc mutant, it was desirable to have a method for the enrichment of sucrose nonfermenters, especially of temperature-sensitive mutants. The latter would prove especially useful in distinguishing mutations at structural genes from regulatory genes.

Attempts to achieve enrichment for sucrose nonfermenting mutants were made with the cly1-1 mutant (Hartwell, 1967), the fas1 mutant (Henry, 1973; Henry and Horowitz, 1975), and the double inositol-requiring mutants (Culbertson and Henry, 1975) but none proved successful.

The elucidation of the problem of a structural gene or regulatory gene function for SUC genes was thus pursued by searching for revertants with altered enzymatic properties of activity. Most of the SUC revertants described in this study did not make significantly less invertase than parental strains. Strain ER512, a non-allelic de novo sucrose fermenter derived from mutant suc3-94 (see Chapter IV), had about 25 per cent of the invertase activity of the parental SUC3 strain. The spontaneously derived de novo fermenter suc3-94 SR3 was only identified because it grew slowly on raffinose. Because of its low invertase and larger K_m for sucrose compared with EK-6B (SUC3) or other known SUC genes (Ottolenghi, 1971b), this may have been altered at a structural gene for invertase.

In genetic systems in yeast other than those controlling utilization of di- and trisaccharides, structural genes for external glycoenzymes, for example in the case of acid phosphatase (McLellan and Lampen, 1963; Boer and Steyn-Parvé, 1966) have been sought.

Saccharomyces has a number of regulatory genes controlling the

synthesis of extracellular acid phosphatases (Toh-e, Ueda, Kakimoto and Oshima, 1973). Structural genes for the two different acid phosphatases have recently been identified: PHOE may be the structural gene for the phosphate-repressible acid phosphatase of yeast because revertants allelic to PHOE were shown to have enzymes with altered substrate affinity and heat stability (Toh-e, Kakimoto and Oshima, 1976); the same workers claimed that PHOC was the structural gene for the constitutive acid phosphatase based on the same kind of evidence. The genes PHOE and PHOC, furthermore, were considered to be closely linked. Structural genes for invertases likewise could be identified if the same variety of mutants with altered enzymatic properties were available.

DGR Mutants: Possible Regulatory Mutants of Invertase Formation

DGR mutants which allow growth on raffinose in the presence of 2-deoxy-D-glucose (2DG) may relieve carbon catabolite repression of invertase synthesis in SUC3 yeast strains (Chapter VII). Similar results were also obtained with SUC2 strains, but these results were not presented. DGR mutants also relieve to varying degrees hexose repression of α -glucosidase synthesis. DGR2 and DGR3 are alleles of a gene which is not the structural gene for invertase because these mutants have invertase with the same K_m for sucrose and heat stability as that of the parent EK-6B, and these genetic factors appear to exert an effect on invertase synthesis only in the presence of a functional SUC3 gene. These DGR mutants may be superficially similar to the invertase hyperproducers bearing the SUC80 gene (Montencourt, 1968;

Ghosh, Montencourt and Lampen, 1973); SUC80 is the designation of this mutant in Plischke et al. (1975). The DGR mutants have been subjected to genetic analysis whereas SUC80 has not.

DGR mutants show some variation in the levels of invertase and α -glucosidase produced. Mutants DGR2 and DGR3 had much increased levels of invertase under repressing conditions, whereas α -glucosidase levels were increased to a lesser degree. Mutants DGRA1 and DGRC1, on the other hand, had increased levels of both enzymes.

In comparison with the invertase hyperproducer mutants previously described (Montencourt, 1968) the DGR mutants have a number of advantages. The DGR mutants were relatively easy to obtain and to analyze genetically. DGR mutants were easy to identify because they grew on raffinose in the presence of 2DG whereas the parental strain did not. The previous method for detection of invertase hyperproducers (Montencourt, 1968) involved staining yeast colonies that had been growing on a medium which repressed invertase synthesis for invertase activity; this method was applied in the present study but it was found to be very laborious and not very reliable in distinguishing invertase hyperproducers from the parental strains. The correlation between the ability to grow on raffinose in the presence of 2DG and invertase hyperproduction on YEPD broth seen in tetrad analysis lent further support to the validity of the use of 2DG. A further advantage of the DGR mutants over the previously described mutants of Montencourt is that the DGR mutants have fewer obvious morphological and physiological differences from their parent than did Montencourt's mutants from their parent (see Montencourt, 1968).

DGR mutants were also sought in yeast strains bearing the SUC1 or

SUC2 genes. DGR mutants were readily obtained from various SUC2 strains employed and were found to be invertase hyperproducers when grown on YEPD broth (results not presented). Some DGR mutants derived from SUC2 strains, however, made no more invertase than the parental strain although they appeared to grow better on raffinose plus 2DG than did the parental strains. The SUC1 strain 4059 gave only DGR mutants with "normal" invertase levels. The DGR mutants that were invertase hyperproducers seemed to tolerate higher concentrations of 2DG when growing on raffinose than did these latter DGR mutants. Perhaps these latter DGR mutants involved some glycosylation reaction of glycoprotein biosynthesis which is no longer sensitive to the inhibitory effect of 2DG (for example, see Kuo and Lampen, 1972), but invertase formation is not relieved from hexose repression. The effects of 2DG on yeast metabolism have most recently been reviewed by Barnett (1976). The DGR mutants which were not invertase hyperproducers might be useful in elucidating some of these effects of 2DG.

Various explanations might be advanced for the DGR-invertase hyperproducer mutants described in Chapter VII. 2DG may inhibit enzymatic reactions involved in isomeric interconversion of hexose-phosphates such as glucose-6-phosphate and mannose-6-phosphate. The latter is important in glycosylation of invertase (Kuo and Lampen, 1972). More recently it has been shown that in Saccharomyces, 2DG reacts with dolichol phosphate to form a compound that interferes with the subsequent glycosylation of mannoproteins such as yeast external invertase (Lehle and Schwarz, 1976). Dolichol is a polyisoprenoid clearly shown to be involved in glycoprotein synthesis in yeast and a wide range of other eukaryotes (for reviews: glycoprotein biosynthesis,

Waechter and Lennarz, 1976; glycoprotein structure, Kornfeld and Kornfeld, 1976). Perhaps the DGR mutants have some altered enzyme which allowed more glycosylation of the protein part of external invertase even in the absence of 2DG; as a result more invertase was made (Lampen, Kuo, Cano and Tkacz, 1972).

It has been proposed that 2DG may act primarily at the level of synthesis of the mannan moiety of invertase, since Montencourt's invertase hyperproducer FH4C was partially resistant to 2DG (Liras and Gascón, 1971).

2DG may inhibit synthesis of proteins such as invertase and α -glucosidase (Kratky, Biely and Bauer, 1975; Lampen, Kuo, Cano and Tkacz, 1972). These two groups of investigators found increased inhibition of the synthesis of these proteins with increased 2DG concentrations (Kratky et al., 1975). According to Kratky et al., this inhibitory effect was distinct from the inhibition of glycoprotein-mannan biosynthesis in yeast by 2DG. Other workers reported that 2DG blocked the initial glycosylation of mannoproteins such as invertase at the ribosomal level (Ruiz-Herrera and Sentandreu, 1975). Thus it appears that a number of different reactions may be subject to inhibition by 2DG; conceivably a mutation at genes controlling any one of these may be responsible for the DGR-invertase hyperproducer phenotype.

The DGR mutants analyzed in Chapter VII may have arisen in the following way. Yeast invertase from strains bearing SUC genes have a K_m for raffinose about six-fold higher than that for sucrose (Gascón, Neumann and Lampen, 1968). Yeast invertases have been reported to hydrolyze sucrose five-fold faster than raffinose (see Barnett, 1976,

pp. 183-184). Invertase activity is greater in raffinose-grown than in sucrose-grown cells (Montencourt, 1968). Thus 2DG may inhibit either glycosylation of invertase or even the synthesis of the protein itself in cells growing on raffinose as the only sugar. Mutants able to grow on raffinose in the presence of 2DG may "simply" have some relaxation in the control of invertase biosynthesis and perhaps in that of α -glucosidase as well at the level of transcription or translation allowing the production of more than enough invertase to overcome the inhibitory effect of 2DG.

There have been a number of studies on mutants in Saccharomyces strains affecting the level of expression of particular genes. The *flk1* mutant appeared to have synthesis of a number of enzymes insensitive to glucose repression; these were maltase, invertase, galactose-1-phosphouridyl transferase and succinic dehydrogenase (Schamhart, ten Berge and van de Poll, 1973). The *flk1* mutant was unlinked to *MAL6* and was also responsible for the flaky appearance of the mutant. Constitutive alleles of various *MAL* genes have also been observed: *MAL4* (Zimmermann, Khan and Eaton, 1973); *MAL6* (ten Berge, Zoutewelle, van de Poll and Bloemers, 1973) and *MAL2* (Zimmermann, and Eaton, 1974). Zimmermann and Eaton reported that *MAL2* constitutive alleles did not relieve carbon catabolite repression of invertase synthesis when present in a strain together with *SUC3*. More recently regulatory mutants which appeared to exclusively relieve glucose repression of catalase biosynthesis were reported (Rytka, Sledziewsky, Litwinska and Bilinski, 1976). These mutants, designated *cgr1* and *cgr2*, were found to be unlinked to one another; mutant *cgr2* was considered to be epistatic to *cgr1*.

The mechanism by which DGR mutants appear to overcome carbon catabolite repression of invertase synthesis in yeast is unknown. If carbon catabolite repression in yeast is analogous to that in bacteria such as E. coli (see article by Magasanik in Beckwith and Zipser, 1970), then cyclic AMP, or some similar molecule is being produced in response to the level of repressing hexoses, etc., and interacts with regulatory proteins and regulatory DNA sequences to determine whether RNA polymerase can bind to and transcribe genes making products to carbon catabolite repression. Glucose-derepressed yeast cells were reported to have increased levels of cyclic AMP (Wijk and Konijn, 1971; Sy and Richter, 1972). While cyclic AMP levels were higher in derepressed cells of flaky (flkl) or parent grown on galactose than on glucose, the flaky mutant did not have significantly higher levels than the parent strain (Schamhart, ten Berge and van de Poll, 1975). The hexose-repression resistant mutants of Montencourt did not have higher levels of cyclic AMP than did the hexose-repressible parent (Montencourt, Kuo and Lampen, 1973). DGR mutants may conceivably affect adenylate cyclase, causing an increase in cyclic AMP, or perhaps block the degradation of cyclic AMP by phosphodiesterase resulting in the same thing, but the aforementioned studies indicated that hexose repression resistance can occur without an increase in cyclic AMP levels.

Alternatively the DGR mutants may code for an altered regulatory protein involved in hexose repression of invertase biosynthesis in yeast which might bind cyclic AMP more efficiently than the parental strain and thus allow more transcription of hexose-repressible genes. While such proteins have been described in bacteria (for review see Pastan and Perlman, 1970), they have not been studied in yeast although

cyclic AMP-binding protein in yeast has been reported (Sy and Richter, 1972b).

The suc mutants described in the present study may be defective in an operator-promoter-like region adjacent to the structural genes for invertase. They might be expected to be cis-dominant to the structural genes. While such cis-dominant mutants have been known for various bacterial systems (cf. Beckwith and Zipser, 1970), only a few such cis-dominant regulatory mutants have been described in lower eukaryotes. A number have been described in Aspergillus nidulans: adjacent to the acetamidase structural gene amdS (Hynes, 1975), proline catabolic enzymes (Arst and MacDonald, 1975), cis-dominant mutant of uric acid-xanthine permease (Arst and Scazzachio, 1975). In the yeast Saccharomyces cerevisiae a cis-dominant constitutive mutant of ornithine carbamoyl transferase (ArgF) involved in arginine biosynthesis was described (Messenguy, 1976). None of the suc mutants are likely to be regulatory mutants unless they fail to allow transcription to occur; they are then analogous to operator-negative mutants (O^0) which are extreme polar mutants (Comtesse, Crepin and Gros, in Beckwith and Zipser (1970), pp. 129-130).

The previous discussion of regulatory genes involved in hexose repression described regulatory genes which were probably unlinked to the structural genes of enzymes involved in carbon catabolism, there have been reports of cis-dominant regulatory mutants affecting enzymes subject to hexose repression. Such cis-dominant regulatory mutations have been reported to affect glucose-repressible alcohol dehydrogenase (ADHII) of Saccharomyces cerevisiae (Ciriacy, 1976). These were interpreted as being operator-promoter mutants adjacent to the known structural gene

for ADHII (ADR2); they were designated ADR3^C and did not interact properly with the regulatory protein made by the unlinked gene ADR1. These cis-dominant mutants were constitutive. A number of constitutive maltase mutants were previously discussed but since the identity of the maltase structural genes is still unclear, they cannot properly be considered as operator-promoter mutations adjacent to structural genes. It might be possible to look for such cis-dominant mutants in the invertase system if the SUC genes were truly structural genes; the availability of structural gene mutants for invertase would make this a possibility. Only a method to detect such operator-promoter mutants is required to allow for their detection, isolation and study.

The probable presence in yeast strains bearing one SUC gene of one or more inactive suc genes (as was suggested by the reversion studies of suc3 mutants in Chapter IV) may make it difficult to obtain cis-dominant mutants of invertase structural genes, although not impossible. Perhaps SUC revertant D10-ER1, probably allelic to SUC1 (Chapter V), may be considered as an operator-promoter mutant blocking transcription of the structural gene for internal invertase, although this is one of a number of possible explanations for this revertant apparently having only the external invertase.

The identification of structural genes for yeast invertase and genes regulating their expression has just begun. Further studies may help to make the invertase system of Saccharomyces cerevisiae more interesting as a model system in which to study the regulation of a glycoprotein enzyme subject to carbon catabolite repression.

REFERENCES

- Arnold, W. N. 1972. The structure of the yeast cell wall. Solubilization of a marker enzyme, B-fructofuranosidase, by the autolytic enzyme system. *J. Biol.Chem.* 247: 1161 - 1169.
- Arnold, W. N. 1974. Expression of cryptic B-fructofuranosidase in Saccharomyces rouxii. *J. Bacteriol.* 120: 886 - 894.
- Arst, H. N., Jr., and D. MacDonald. 1975. A gene cluster with an internally located cis-acting regulatory region. *Nature* 254: 26 - 31.
- Arst, H. N., Jr., and C. Scazzocchio. 1975. Initiator constitutive mutation with an "up-promoter" effect in Aspergillus nidulans. *Nature* 254: 31 - 34.
- Barnett, J. A. 1976. The utilization of sugars by yeasts. In: *Advances in Carbohydrate Chemistry and Biochemistry*. Tipson, R. S. and D. Horton, Eds. 32: 125 - 134. Academic Press, New York.
- Baseer, A., and S. Shall. 1971a. Properties of the internal invertase of yeast Saccharomyces cerevisiae. *Biochim. Biophys. Acta* 250: 192 - 202.
- Baseer, A., and S. Shall. 1971b. The enzymatic synthesis of fructosides using purified external B-fructofuranosidase from Saccharomyces cerevisiae. *Int. J. Biochem.* 2: 503 - 506.
- Beckwith, J.R., and D. Zipser. Eds. 1970. *The Lactose Operon*. Cold Spring Harbor Laboratory, Cold Spring Harbor.
- Beckwith, J. R., and P. Rossow. 1974. Analysis of genetic regulatory mechanisms. *Ann. Rev. Genetics* 8: 1 - 13.
- Berge, A. M. A. ten, Zoutewelle, G., and K.W. van de Poll. 1973. Regulation of maltose fermentation in Saccharomyces carlsbergensis. I The function of the gene MAL6 as recognized by mal6 mutants. *Molec. gen. Genet.* 123: 233 - 246.
- Berge, A. M. A. ten, Zoutewelle, G., van de Poll, K. W., and H. P. J. Bloemers. 1973. Regulation of maltose fermentation in Saccharomyces carlsbergensis. II Properties of a constitutive MAL6 mutant. *Molec. gen. Genet.* 125: 134 - 156.
- Beteta, P., and S. Gascon. 1971. Localization of invertase in yeast vacuoles. *FEBS Letters* 13: 297 - 299.
- Berge, A. M. A. ten. 1972. Genes for the fermentation of maltose and α -methylglucoside in Saccharomyces carlsbergensis. *Molec. gen. Genet.* 115: 80 - 89.

- Boer, P., and E. P. Steyn-Parvé. 1966. Isolation and purification of an acid phosphatase from baker's yeast (Saccharomyces cerevisiae). Biochim. Biophys. Acta 128: 400 - 402.
- Braun, Hersz. 1976. External yeast B-fructosidase: Affinity labeling of the active site. Biochim. Biophys. Acta 452: 452 - 457.
- Ciriacy, M. 1976. Cis-dominant regulatory mutations affecting the formation of glucose repressible alcohol dehydrogenase (ADHII) in Saccharomyces cerevisiae. Molec. gen. Genet. 145: 327 - 333.
- Comtesse, G., Crepin, M., and F. Gros. 1970. On the mechanism of catabolite repression. In: The Lactose Operon (J.R. Beckwith and D. Zipser, Eds.) 401 - 415. Cold Spring Harbor Laboratory, Cold Spring Harbor.
- Culbertson, M. R. and S. Henry. 1975. Inositol-requiring mutants of Saccharomyces cerevisiae. Genetics 80: 23 - 40.
- Davies, R. 1953. Enzyme formation in Saccharomyces fragilis. I Invertase and raffinose. Biochem. J. 55: 484 - 490.
- de la Fuente, G., and A. Sols. 1962. Transport of sugars into yeasts. II Mechanisms of utilization of disaccharides and related glycosides. Biochim. Biophys. Acta 56: 49 - 62.
- de Ley, J., and M. Vandamme. 1950. The formation of the semiadaptive saccharase in yeast. Enzymologia 14: 329 - 335.
- Dixon, M., and E. C. Webb. 1964. Enzymes. Second Edition. Academic Press, New York.
- Dodyk, F., and A. Rothstein. 1964. Factors influencing the appearance of invertase in Saccharomyces cerevisiae. Arch. Biochem. Biophys. 104: 478.
- Douglas, H. C., and D. C. Hawthorne. 1964. Enzymatic expression and genetic linkage of genes controlling galactose utilization in Saccharomyces. Genetics 49: 837 - 844.
- Douglas, H. C., and D. C. Hawthorne. 1966. Regulation of genes controlling synthesis of galactose pathway enzymes in yeast. Genetics 54: 911 - 916.
- Eaton, N. R. 1962. New press for disruption of microorganisms. J. Bacteriol. 83: 1359 - 1360.
- Englesberg, E., and G. Wilcox. 1974. Regulation : Positive Control. Ann. Rev. Genetics 8: 219 - 242.
- Englesberg, E., Sheppard, D., Squires, C., and F. Meronk. 1969. An analysis of revertants of a deletion mutant in the C gene of the L-arabinose gene complex in Escherichia coli B/r. Isolation of initiator-constitutive mutants. J. Mol. Biol. 43: 53 - 62.

- Fincham, J. R. S. 1966. Genetic Complementation. Benjamin, New York.
- Fink, G. R. 1966. A cluster of genes controlling three enzymes in histidine biosynthesis in Saccharomyces cerevisiae. Genetics 53: 445 - 459.
- Fink, G. R., and R. Lowenstein. 1969. Simplified method for testing mutagens in Saccharomyces. J. Bacteriol. 100: 1126.
- Fogel, S., and D. D. Hurst. 1967. Meiotic gene conversion and the theory of recombination. Genetics 57: 455 - 481.
- Friis, J., and P. Ottolenghi. 1959. Localization of invertase in a strain of yeast. Compt. Rend. Trav. Lab. Carlsberg. 31: 259.
- Gabriel, O. 1971. Locating enzymes in gels. In: Methods in Enzymology (W. B. Jakoby, Ed.) 22: 585 - 604. Academic Press, New York.
- Gabriel, O., and S. F. Wang. 1969. A method for locating enzymes in gels. Anal. Biochem. 27: 545.
- Gallili, G. E. 1975. Synthesis of small and large invertases and the yeast cell cycle. Ph. D. Dissertation. Rutgers Univ.
- Gascón, S., and J. O. Lampen. 1968. Purification of the internal invertase of yeast. J. Biol. Chem. 243: 1567 - 1572.
- Gascón, S., Neumann, N. P., and J. O. Lampen. 1968. Comparative study of the properties of the purified internal and external invertases from yeast. J. Biol. Chem. 243: 1573 - 1577.
- Gascón, S., and P. Ottolenghi. 1972. Influence of glucose concentration of the medium on the invertase content of a strain of Saccharomyces bearing the SUC2 gene. Compt. Rend. Trav. Lab. Carlsberg. 39: 15 - 24.
- Ghosh, B. K., Montencourt, B. S., and J. O. Lampen. 1973. Abnormal cell envelope ultrastructure of a Saccharomyces mutant with invertase formation resistant to hexoses. J. Bacteriol. 116: 1412 - 1420.
- Goldstein, A., and J. O. Lampen. 1975. D-D-fructofuranoside fructohydrolase from yeast. In: Methods in Enzymology. Carbohydrate Metabolism. (W. A. Wood, Ed.) 42: 504 - 511. Academic Press, New York.
- Gross, S. 1969. Genetic regulatory mechanisms in the fungi. Ann. Rev. Genetics 3: 395.
- Halvorson, H., and L. Ellias. 1958. Purification and properties of an α -glucosidase of Saccharomyces italicus Y1225. Biochim. Biophys. Acta 30: 28.

- Hartman, P. E., and J. R. Roth. 1973. Mechanisms of suppression. *Advances in Genetics* 17: 1 - 105.
- Hartwell, L. H. 1969. Macromolecular synthesis in a temperature-sensitive mutant in yeast. *J. Bacteriol.* 93: 1662 - 1670.
- Hartwell, L. H. 1970. Biochemical genetics of yeast. *Ann. Rev. Genetics* 4: 373 - 396.
- Henry, S. 1973. Death resulting from fatty acid starvation in yeast. *J. Bacteriol.* 116: 1293 - 1303.
- Henry, S. A., and S. Horowitz. 1975. A new method for mutant selection in *Saccharomyces cerevisiae*. *Genetics* 79: 175- 186.
- Hawthorne, D. C. 1969. The selection of nonsense suppressors in yeast. *Mutation Research* 7: 187 - 197.
- Hofnung, M. 1974. Divergent operons and the genetic structure of the maltose B region in *Escherichia coli* K12. *Genetics* 76: 169 - 184.
- Hynes, M. J. 1975. A cis-dominant regulatory mutation affecting enzyme induction in the eukaryote *Aspergillus nidulans*. *Nature* 253: 210 - 212.
- Jacob, F., and J. Monod. 1961. Genetic regulatory mechanisms in the synthesis of proteins. *J. Mol. Biol.* 3: 318 - 338.
- Jennings, D. H. 1974. Sugar transport into fungi: An essay. *Trans. Brit. Mycol. Soc.* 62: 1 - 24.
- Johnston, J. R., and R. K. Mortimer. 1958. Use of snail digestive juice in isolation of yeast tetrads. *J. Bacteriol.* 78: 292.
- Kew, O. M., and H. C. Douglas. 1976. Genetic co-regulation of galactose and melibiose utilization in *Saccharomyces*. *J. Bacteriol.* 125: 33 - 41.
- Khan, N. A. 1975. Genetic control of maltase formation in yeast. III Isolation and characterization of temperature-sensitive mutants affecting maltase induction and maltose utilization. *Molec. gen. Genet.* 136: 55 - 61.
- Khan, N. A., and N. R. Eaton. 1967. Purification and characterization of maltase and α -methylglucosidase from yeast. *Biochim. Biophys. Acta* 146: 173 - 180.
- Khan, N. A., and N. R. Eaton. 1968. Relationship between maltase and α -methylglucoside fermentation in yeast. *Genetics* 60: 192.
- Khan, N. A., Zimmermann, F. K., and N. R. Eaton. 1973. Genetic and biochemical evidence of sucrose fermentation by maltase in yeast. *Molec. gen. Genet.* 123: 43 - 50.

- Klar, A. S., and H. Halvorson. 1974. Studies on the positive regulatory gene GAL4 in regulation of galactose catabolic enzymes in Saccharomyces cerevisiae. J. Mol. Biol. 135: 203 - 212.
- Kornfeld, R., and S. Kornfeld. 1976. Comparative aspects of glycoprotein structure. Ann. Rev. Biochem. 45: 217 - 237.
- Kratky, Z., Biely, P., and S. Bauer. 1975. Mechanism of 2-deoxy-D-glucose inhibition of cell-wall polysaccharide and glycoprotein synthesis in Saccharomyces cerevisiae. Eur. J. Biochem. 54: 459 - 467.
- Kuo, S. C., and J. O. Lampen. 1972. Inhibition by 2-deoxy-D-glucose of synthesis of glycoprotein enzymes by protoplasts of Saccharomyces: Relation to inhibition of sugar uptake and metabolism. J. Bacteriol. 111: 419 - 429.
- Lampen, J. O. 1971. Invertases. In: The Enzymes (P. D. Boyer, Ed.) Third Edition 5: 291 - 305. Academic Press, New York.
- Lampen, J. O., Neumann, N. P., Gascón, S., and B. S. Montencourt. 1967. Invertase biosynthesis and the yeast cell membrane. In: Organizational Biosynthesis (H. J. Vogel and J. O. Lampen, Eds.) pp. 363 - 372. Academic Press, New York.
- Lampen, J. O., Kuo, S. C., Cano, F. R., and J. S. Tkacz. 1972. Structural features in synthesis of external enzymes by yeasts. In: Proc. IV Intl. Fermentation Symposium. Fermentation Technology Today. pp. 819 - 824.
- Lehle, L., and R. L. Schwarz. 1976. Formation of dolichol monophosphate 2-deoxy-D-glucose and its interference in the glycosylation of mannoproteins in yeast. Eur. J. Biochem. 67: 239 - 247.
- Leskovac, V., Trivic, S., Pavlov-Pericin, D., and V. Prodanov. 1975. External yeast B-fructosidase. The role of tryptophanyl residues in catalysis. Biochim. Biophys. Acta 405: 482 - 491.
- Liras, P., and S. Gascón. 1971. Biosynthesis and secretion of yeast invertase. Effect of cycloheximide and 2-deoxy-D-glucose. Eur. J. Biochem. 23: 160 - 165.
- Lloyd, J. B., and W. J. Whalen. 1969. An improved method for enzymatic determination of glucose in the presence of maltose. Anal. Biochem. 30: 467 - 472.
- Magasanik, G. 1970. Glucose effects: Inducer exclusion and repression. In: The Lactose Operon (J. R. Beckwith and D. Zipser, Eds.) pp. 189 - 219. Cold Spring Harbor Laboratory, Cold Spring Harbor.
- McLary, D. O., Nutly, W. L., and G. R. Miller. 1959. Effects of potassium versus sodium in the sporulation of Saccharomyces. J. Bacteriol. 78: 362.

- McLellan, W. L., and J. O. Lampen. 1963. The acid phosphatase of yeast: Localization and secretion by protoplasts. *Biochim. Biophys. Acta* 67: 324 - 326.
- Messenguy, F. 1976. Regulation of arginine biosynthesis in Saccharomyces cerevisiae: Isolation of a cis-dominant, constitutive mutant for ornithine carbamoyl transferase synthesis. *J. Bacteriol.* 128: 49 - 55.
- Meyer, J. and P. Matile. 1974. Regulation of isoenzymes and secretion of invertase in bacter' yeast. *Biochem. Physiol. Pflanzen* 166: 377 - 385.
- Montencourt, B. S. 1968. A Saccharomyces mutant with hexose insensitive enzyme formation. Ph. D. Thesis. Rutgers Univ.
- Montencourt, B. S., Kuo, S. C., and J. O. Lampen. 1973. Saccharomyces mutants with invertase formation resistant to repression by hexoses. *J. Bacteriol.* 114: 233 - 238.
- Moreno, F., Ochoa, A. G., Gascón, S., and J. R. Villanueva. 1975. Molecular forms of yeast invertase. *Eur. J. Biochem.* 50: 385 - 460.
- Mortimer, R. K., and D. C. Hawthorne. 1969. Yeast genetics. In: *The Yeasts* (A. H. Rose and J. S. Harrison, Eds.) 1: 385 - 460. Academic Press, New York.
- Myrback, K. 1960. Invertases. In: *The Enzymes* (Boyer, P., Lardy, H., and K. Myrback, Eds.) Second Edition 4: 379 - 396. Academic Press, New York.
- Neumann, N. P., and J. O. Lampen. 1967. Purification and properties of yeast invertase. *Biochemistry* 6: 468 - 475.
- Ottolenghi, P. 1971a. Some properties of five non-allelic B-D-fructofuranosidases (invertase) of Saccharomyces. *Compt. Rend. Trav. Lab. Carlsberg* 38: 213 - 221.
- Ottolenghi, P. 1971b. A comparison of five genetically distinct invertases from Saccharomyces. Some enzymatic characteristics. *Eur. J. Biochem.* 18: 544 - 552.
- Pastan, I., and R. Perlman. 1970. Cyclic adenosine monophosphate in bacteria. *Science* 169: 339 - 344.
- Plischke, M. E., von Borstel, R. C., Mortimer, R. K., and W. E. Cohn. 1975. Genetic markers and associated gene products in Saccharomyces cerevisiae. In: *Handbook of Biochemistry and Molecular Biology* (G. D. Fasman, Ed.) Third Edition. Chemical Rubber Company Press, Cleveland.
- Rickenberg, H. V. 1974. Cyclic AMP in prokaryotes. *Ann. Rev. Microbiol.* 28: 353 - 370.

- 19
- Ruiz-Herrera, J., and R. Sentandreu. 1975. Site of initial glycosylation of mannoproteins from Saccharomyces cerevisiae. J. Bacteriol. 124: 127 - 133.
- Rytka, J., Sledziewski, A., Litwinska, J., and T. Bilinski. 1976. Haemoprotein formation in yeast. II Isolation of catalase regulatory mutants. Molec. gen. Genet. 145: 37 - 42.
- Schamhart, D. H. J., Berge, A. M. A. ten, and K. W. van de Poll. 1975. Isolation of a catabolite repression mutant of a yeast as a revertant of a strain that is maltose negative in a respiratory-deficient state. J. Bacteriol. 121: 747 - 752.
- Schlenderer, G., and H. Dellweg, 1974. Cyclic AMP and catabolite repression in yeasts. Eur. J. Biochem. 49: 305 - 316.
- Sherman, F., Fink, G. R. and C. W. Lawrence. 1971. Methods in yeast genetics. Cold Spring Harbor Laboratory, Cold Spring Harbor.
- Suomalainen, H., and E. Oura. 1971. Yeast nutrition and solute uptake. In: The Yeasts (A. H. Rose and J. R. Harrison, Eds). 2: 1 - 35. Academic Press, New York.
- Sy, J., and D. Richter. 1972a. Content of cyclic-3',5'-adenosine monophosphate and adenyl cyclase in yeast at various growth conditions. Biochemistry 11: 2788 - 2791.
- Sy, J., and D. Richter. 1972b. Separation of a cyclic-3',5'-adenosine monophosphate binding protein from yeast. Biochemistry 11: 2784 - 2787.
- Tkacz, J. S. 1971. Biochemical and cytological examination of the mannan and invertase of Saccharomyces cerevisiae. Ph. D. Thesis. Rutgers Univ.
- Toda, K. 1976a. Invertase biosynthesis by Saccharomyces carlsbergensis in batch and continuous cultures. Biotechnology and Bioengineering 18: 1103 - 1115.
- Toda, K. 1976b. Dual control of invertase biosynthesis in chemostat cultures. Biotechnology and Bioengineering. 18: 1117 - 1124.
- Toh-e, A., Ueda, Y., Kakimoto, S., and Y. Oshima. 1973. Isolation and characterization of acid phosphatase mutants in Saccharomyces cerevisiae. J. Bacteriol. 113: 727 - 738.
- Toh-e, A., Kakimoto, S., and Y. Oshima. 1975. Genes coding for the structural genes of the acid phosphatases in Saccharomyces cerevisiae. Molec. gen. Genet. 143: 65 - 70.
- Waechter, C. J., and W. J. Lennarz. 1976. The role of polyprenol-linked sugars in glycoprotein synthesis. Ann. Rev. Biochem. 45: 95 - 112.

- Waheed, A., and S. Shall. 1971. Chemical modification of the active site of yeast invertase. *Biochim. Biophys. Acta* 242: 172 - 186.
- Wheeler, G.E., Schibeci, A., Epand, R. M., Rattray, J. B. M., and D. K. Kidby. 1974. Subcellular localization and some properties of the adenylate cyclase activity of the yeast Saccharomyces cerevisiae. *Biochim. Biophys. Acta* 372: 15 - 22.
- Wijk, R. van, and T. M. Konijn. 1971. Cyclic-3',5'-AMP in Saccharomyces carlsbergensis under various conditions of catabolite repression. *FEBS Letters* 13: 184 - 186.
- Willstatter, R., Lowry, Jr., C.D., and K. Schneider. 1925. Invertase. IX. Invertase enrichment in yeast. *Z. Physiol. Chem.* 146: 158.
- Winge, O., and C. Roberts, 1952a. The relation between the polymeric genes for maltose, raffinose and sucrose fermentation in yeasts. *Compt. Rend. Trav. Lab. Carlsberg*. 25: 141 - 171.
- Winge, O., and C. Roberts. 1952b. The genes for maltose and raffinose fermentation in Saccharomyces cerevisiae. I. Identification of a raffinose fermentation gene in yeast foam. *Compt. Rend. Trav. Lab. Carlsberg* 25: 241 - 251.
- Zamenhof, S. 1957. In: *Methods of Enzymology* (Colowick, S. P., and N. O. Kaplan, Eds.) 3: 702. Academic Press, New York.
- Zimmermann, F. K., Khan, N. A., and N. R. Eaton. 1973. Identification of new genes involved in disaccharide fermentation in yeast. *Molec. gen. Genet.* 123: 29 - 41.
- Zimmermann, F. K., and N. R. Eaton. 1974. Genetics of induction and catabolite repression of maltase synthesis in Saccharomyces cerevisiae. *Molec. gen. Genet.* 134: 261 - 273.
- Zimmermann, F. K., Schwaier, R., and U. Van Laer. 1966. Nitrous acid and alkylating nitrosamides: Mutation fixation in Saccharomyces cerevisiae. *Molec. gen. Genet.* 98: 152 - 166.