CONTROL OF MALTASE SYNTHESIS IN YEAST

by

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Abstract

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Methods are presented for isolating maltase negative and maltase constitutive mutants from strains carrying MAL 1, 3, and 6. All the negative mutants are noninducible and recessive to wild type. Those allelic to the corresponding MAL gene produced basal levels of maltase indistinguishable from the wild type enzyme. The constitutive mutants were all allelic to the corresponding MAL gene, and were generally recessive to wild type. Maltase was isolated in pure form from strains carrying MAL 1, 2, 3, and 6. No significant differences in the physical properties of the enzyme were found. These results are consistent with a model in which the MAL genes are involved in the control of maltase synthesis, and are therefore not structural genes for maltase.

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INTRODUCTION

The α-glucosidases of yeast have been an active field of research for over a hundred years, beginning with the work on invertase by Dumas and Boullay (1828). Despite the importance of these enzymes in practical industry (Coppock, 1958), very little is known about them in terms acceptable to a modern enzymologist. The enzymological work on the maltases and α-methyl-glucosidases has been particularly confused by the fact that crude extracts or extracts from strains of unknown genotypes have been used although at least seven "structural genes" (the reason for the quotation marks will become clear later) are known in Saccharomyces. This difficulty is compounded by the fact that the yeast glucosidases have widely overlapping specificities; maltase, for example, hydrolyzes sucrose as well as maltose. A major goal of this thesis was to characterize the α-glucosidases purofied from strains of known genotype and to establish the number of distinct enzyme species necessary to account for the ability of crude extracts from yeast to hydrolyze a variety of α-glucosides including maltose, sucrose, α-methylglucoside, melezitose, turanose, and palatinose.

The α-glucosidase system has also played an important role in the early investigations of the phenomena of enzymatic adaptation; the ability of cells to metabolize, after a delay, substances which are not immediately utilizable (Winge and Roberts, 1948). This phenomon became a major experimental tool in molecular biology and the results of these investigations solved the major problem of twentieth century biology:

the nature of the link between the genes and the cell phenotype. The major advances which formed the basis of this knowledge were accomplished, however, with the lactose system of E. Coli; work on the control of enzyme synthesis in yeast and other fungi was essentially abandoned in favor of work on the simpler bacterial systems. During the last ten years (Gross, 1969) there has been a revival of interest in the control of protein synthesis in the fungi.

The study of the regulation of gene expression in the fungi is especially important because of the intermediate position that these organisms occupy between the procaryotes and the higher eucaryotes. The yeast cell possesses the complex organelle structure of the higher eucaryotes (a nucleus, multiple chromosomes, mitochondria, vacuoles, and peroxisomes) and the metabolic compartmentalizations which such an organization implies (Matile, et. al., 1969). Yeast also lack those features, including obligate polyploidy (diploidy), absence of a sexual cycle, and long generation times which greatly complicate genetic investigations in higher eucaryotes such as mammalian cells. Even so standard a technique as gene mapping by recombinational analysis is as yet impossible in studies of mammalian cells.

The maltase system has several major advantages for the study of the control of enzyme synthesis in yeast:

1. Maltase is an inducible enzyme and is produced in large quantities by fully induced cells (0.8-1.1% of the total protein). This quantity of enzyme represents an increase of about one hundred fold over the basal level in uninduced cells. This relatively high induction ratio facilitates the study of the kinetics of enzyme induction.

2. Maltase is easily isolated in pure form, and a convenient chromagenic assay utilizing para-Nitrophenylglucoside is available for the assay of either cell extracts or permeabilized whole cells.

3. Several of the Maltose genes have been mapped and some have closely linked markers.

4. The enzyme is involved in an early step of catabolism so that the possible number of effectors is limited.

The amount of enzyme present in cells can be increased or decreased by the operation of various mechanisms. These include induction, product repression, catabolite repression, specific inactivating proteins, and protein degradation (Schimke and Doyle, 1970). The paradigm for the study of these processes involves the isolation of mutants affecting the amount of enzyme produced in a given environment under conditions of induction or repression. A second major goal of this thesis was to establish methods for the isolation of control mutants for the maltase system in order to elucidate the nature of the controls operating to regulate synthesis in a eucaryotic cell.

The Genetic System

Maltose fermentation depends upon the presence of any one of seven unlinked genes. Winge and Roberts (1950a,b) isolated the genes MAL 1,2, and 3 from Saccharomyces cerevisiae (Yeast Foam). MAL 4 was obtained by x-ray irradiation of a diploid maltose negative strain. The diverse origins of the MAL 4 gene (some spontaneous mutants were also selected) is particularly important to remember since certain claims have been made on the basis of the nonidentity of the enzymes produced by several MAL 4 strains (Kahn and Eaton, 1971). The gene MAL 5 was isolated by Gilliland (1954) from a strain of S. diastacticus. Although

no detailed studies with MAL 5 are available, preliminary evidence indicates that this gene produces a glucoamylase rather than a maltase (Gilliland, 1958) and this locus should be renamed. Two genes have been isolated from S. carlsbergensis, MAL 6 by Winge and Roberts (1957) and MAL 7 by Oeser (1964).

A set of genes having an equivalent function is referred to as a polymeric system. The five invertase genes are another well-known example since a cell possessing any one of five unlinked genes can ferment sucrose. It is important to note that this equivalence is required only at the functional level and that the gene products themselves, as in the case of MAL 5, may be entirely different. It is also important to emphasize that during the original isolation of the MAL genes from "wild" (i.e., nonlaboratory bred) strains usually only one gene was present in any given strain (Winge and Roberts, 1950a,b). This observation, along with the high induction ratio for maltase synthesis, makes unlikely the hypothesis that the multiplicity of MAL genes is a result of a selective advantage that is conferred by the possession of several MAL genes by a single strain during the growth on maltose (a gene dosage effect for an otherwise limiting enzyme).

Strains Used in Maltase Studies and Their Genes

Yeast systematics is a particularly difficult subject due in part to the limited number of morphological differences available for classification and to a penchant for the use of single gene characters in many classification schemes (van Rij, 1970). Recent classifications based on nucleic acid homology have indicated that the species of Saccharomyces are extremely diverse, with differences greater than those

observed between various classes of vertebrates (Bichell and Douglas, 1970).

The following strains have been used in work relevant to the subject of this thesis: S. cerevisiae (100), S. carlsbergensis (96), S. Chevalieri (94), S. italicus (92), S. oviformis (89), S. uvarum (40), and S. globulus (20). The number that appears in parenthesis represents the amount of DNA homology on a percentage basis between the indicated species and S. cerevisiae as determined from DNA-DNA binding stuides (Bichel and Douglas, 1970). The group consisting of S. cerevisiae, S. italicus, S. carlsbergensis, and S. chevalieri are interfertile. In the experiments reported in this thesis, S. carlsbergensis has been crossed with S. cerevisiae strains with little difficulty, and viable spore progeny isolated; and indication that the DNA homology extends to the arrangement of the genes on the chromosome. Although this thesis concerns the regulation of maltase synthesis in S. cerevisiae, we do not believe that any useful distinction between S. cerevisiae and S. carlsbergensis can be made and that the differences are, at most, at the racial level. The recent work of the Utrecht group (ten Berge et. al., 1973) on the regulation of maltase synthesis in S. carlsbergensis is therefore fully applicable to the systems reported upon in this thesis.

The gene called M2 by Winge and Roberts (1955) is now referred to as MAL 3 and vice versa. Oshima's (1967) MALa is allelic to MAL 1. The relationship of Lindegren's MZ gene to the maltase series, MAL 1-7, is not known (but see this thesis). None of the seven MAL genes is linked to a centromere nor are they linked to each other. MAL 1 and 3 possess tightly linked sucrose genes. It is not known whether the other MAL genes are associated with invertase genes or not. For a map dis-

playing the position of the MAL genes in relation to other markers, see Mortimer and Hawthorne (1969).

The Products of the Maltose Genes

There are two competing claims in the literature on the number of distinct maltases present in S. cerevisiae. The prevalent claim is that only a single species of maltase is made in the presence of MAL 1,2,3,4, and 6 (Halvorson, et. al., 1963). This result is usually paraphrased to read "The genes MAL 1,2,3,4, and 6 are structural genes coding for a single species of maltase," a translation which amounts to a serious abuse of language since the experimental results reported by Halvorson, et. al. are consistent with the view that there is only one structural gene for maltase in yeast and that the MAL genes, MAL 1,2,3,4, and 6 play an entirely different, although equivalent role--for example, as control genes. The claim of identity for the products of the maltase genes made in these experiments of Halvorson, et. al. rests on the behavior of partially purified maltase prepared from strains of known genotype. The criteria included heat inactivation, starch gel electrophoresis, CM cellulose and DEAE cellulose chromatography, and response to antiserum. In addition, identical Ki values were found for PNPG, Maltose, Turanose, and Phenyl- glucoside.

The other claim is that of Terui et. al. (1959) that at least two distinct maltases exist. They were able to separate two maltases by starch gel electrophoresis. The enzymes had different Vm's with α-methyl glucoside, phenylα-glucoside, and maltose as substrates; one enzyme hydrolyzed both phenylα-glucoside and α-methylglucoside at a higher rate than the other with respect to maltose hydrolysis. Yau and Lindegren have also observed two distinct maltases after gel

electrophoresis (Yau et. al, 1967). The observations of Terui et. al. however, are impossible to correlate with any of the known MAL genes since genetically undefined strains were used. Therefore, one trivial explanation of these differences is that Terui was observing the proudct of the MAL 5 gene, a gene which is known to code for an enzyme which differs from those of the other MAL loci (Gilliland, 1954, 1958). If this were the case, the substrate specificity of the enzymes separated by starch gel electrophoresis would be widely different; and Lindegren did notice a qualitative difference in the ability of his two maltase fractions in hydrolyzing various substrates. The immunological results of Halvorson et. al (1963) are also ambiguous to a certain degree since partially purified preparations (about 30 fold) were used to elicit antibodies. This use of impure enzyme, as well as the presence of basal levels of enzyme in maltose negative strains, raises serious questions about the specificity of the prepared antibodies.

A further goal of this thesis was to reopen the question of identity of the maltases, obtain pure preparations of maltase in strains of known genotype, and use as many criteria as possible in comparing them. The existence of differences between the maltases would allow us to test whether the maltose alleles were control or structural genes.

Materials and Methods

A. Strains.

The strains of Saccharomyces used in these studies, and their genotypes, are presented in Table 1.

B. Media.

1. Growth media. The standard rich broth medium used for general purposes was YEPD, 1% Yeast Extract (Difco), 2% Peptone (Difco), and 2% Glucose. With the substitution of 2% Maltose for the Glucose, this medium becomes YEPM.

Two less rich media were also used. The minimal medium for experiments with catabolite repression was: 2gm. KH_2PO_4, 6gm. $(NH_4)_2SO_4$, 2.5 gm. Yeast Extract, 0.25 gm. $MgSO_4 \cdot 7H_2O$ in one liter of distilled water (van Wijk, 1968). With the addition of 0.3% Glucose, this is called CAT Glu; with the addition of 2% Galactose, it becomes CAT Gal. The defined minimal medium used was 6.7 gm. Difco Yeast Nitrogen Base w/o Amino Acids in one liter of distilled water. Usually 2% Glucose served as the carbon source and appropriate amounts of Amino Acids were added when auxotropic strains were grown. This medium is called YNB or Minimal.

2. Sporulation media. Sporulation was induced on YNB agar plates containing 0.5% Potassium acetate.

C. Mating of haploid strains.

Mixtures of the two parents were heavily inoculated into YEPD broth and incubated for one to four days on a rotary shaker.

Diploids were isolated either on the appropriate minimal media or by selecting large colonies from YEPD plates and checking for sporulation.

D. Genetic techniques.

After sporulation, the washed asci were digested with the gastric enzymes of Helix pomatia, "Glusulase," obtained from Endo Laboratories. The asci were washed with distilled water, spread on an agar slab and dissected. For a more detailed description of these standard techniques, see the article by Mortimer and Hawthorne (1969). In every dissection all nutritional markers were scored in order to eliminate spurious tetrads.

E. Enzyme assays

1. Extracts. Whole cell extracts were prepared by washing the cells twice in distilled water, resuspending them in 0.05 M. Potassium Phosphate buffer (pH 6.8-7.4, depending on the specific purpose) and crushing them in the press described by Eaton (1962). Cellular debris and whole cells were eliminated by a centrifugation at 25,000g for 20 minutes in a Sorvall RC-2B centrifuge, and the supernatent was retained for assay.

2. Measurement of activity with PNPG (para-Nitroα-phenyl-glucoside). An appropriate amount of enzyme extract was suspended in a total of one ml of 0.1M Potassioum phosphate buffer, pH 7.0, in the presence of either 1mM EDTA or 1mM β-mercaptoethanol. One tenth ml of PNPG (5 mg./ml) was added, and the reaction was stopped with an equal volume of 1M Sodium Carbonate. Reactions were usually run at 30°C. The p-nitrophenol was measured by its absorbance at 400

nanometers in a Spectrophotometer. Enzyme activity was expressed in nMoles/min mg protein. These values are not Vm's since the substrate was not at a saturating concentration. Whole cell preparations were assayed by lypholyzing 0.1ml of washed yeast cells and then adding 1ml of 0.1M Phosphate buffer, pH 7.0. After one hour incubation at 30°C, 0.1 ml of PNPG (5mgs/ml) was added, and the reaction was stopped with an equal volume of 1M Sodium Carbonate. The absorbance was read at 400 and at 550 nmeters. The 550 reading was used as a measure of cell number. The activity was expressed as Abs. 400-(1.1) Abs. 550/Abs. 550 min. The numerator includes an experimentally determined correction for the effect of the scattering of the cells alone on the absorbance at 400 nmeters. This assay was linear with time and with cell density. It was used primarily for preliminary screening of presumptive constitutive mutants and is not recommended for precise determinations since some variation was noted. It is, however, reliable for differentiating constitutive and inducible cells.

3. Maltase and α-methylglucosidase determinations. The release of glucose from maltose amd α-methylglucoside (and from other disaccarides as well) was followed by the use of a coupled glucose oxidase and peroxidase system (Lloyd and Whelan, 1969). The activities were measured, unless otherwise noted, with substrate concentrations for maltose and α-methylgulcoside of 2.5 and 5 mM respectively. Mercaptoethanol interfered with the assay and 1mM EDTA was included instead. Enzyme activities were expressed in nM substrate/min. mg. These values also do not represent maximum velocities. A low concentration of substrate was used because of the high degree of

glucose contamination present in all grades of maltose and α-methyl glucoside which are available. Calculations showed that a correction for glucose inhibition of the enzyme and for the changing substrate concentration of the reaction mixture was unnecessary. The complete procedure was as follows:

A sample of enzyme extract and substrate in a total volume of 0.4 ml in 0.05M Phosphate buffer pH 7.0 was incubated for the appropriate time in a 30°C water bath, removed and the reaction stopped by boiling for two minutes. 0.8ml, of the following mixture was added:

30 mg Glucose oxidase (Sigma type II)
3 mg Horseradish peroxidase
10 mg o-Dianisidine dihydrochloride

in 100 ml Tris-phosphate (0.o5M, pH 7.0) made 40% (v/v) in glycerol. After one hour of incubation at 30°C, the reaction was stopped by the addition of 1.6ml of 5N HCl. The absorbance was read at 525nm, and the amount of glucose released was calculated from a standard curve. The appropriate blanks and a glucose standard were run simultaneously.

F. Fermentation tests.

Fermentation was scored by noting the amount of gas produced in Durham tubes. The medium used was YEP with the addition of 2% of the carbon source. Bromocresol purple was added as an acid indicator.

G. Mutagenesis.

Cells were mutagenized by allowing cultures to grow in YEPD containing 0.1mg/ml NTG (Nitrosoguanidine). Generally a survival level of about 30% was used. The cells were washed three times with

water and allowed to grow several hours to allow for mutation fixation (Mortimer and Hawthorne, 1969).

For mutagenesis on plates, heavy lawns of cells were grown overnight on YEPD agar, a crystal of NTG was placed in the center and the cells were allowed to grow for 18 hours. The plates were then replicated to selective media. This technique is a variant of one recommended by Lowenstein and Fink (1969).

H. SDS gel electrophoresis.

Polyacrylamide gels were prepared by mixing the acrylamide solution with 0.05 of its volume of 1% Ammonium persulfate. The bisacrylamide: acrylamide ratio was 29:1. The gels were 0.1% in SDS, 0.1M in Sodium phosphate buffer pH 7.2 and 0.1% (v/v) in TEMED (N,N,N,N tetramethylethylenediamine).

The sample was dialyzed against 0.01 Sodium phosphate buffer pH 7.2. SDS was added to a final concentration of 1% and after the addition of 1% (v/v) β-mercaptoethanol, the sample was boiled for two minutes. 5% gels were run 2-3 hours, 10% gels for 4-5 hours in 10cm. long tubes. Electrophoresis was carried out with a regulated power supply at 90V and 10ma/tube. The gels were stained either by fixing in 20% sulfanosalycylic acid for 24 hours followed by staining with an aqueous solution of 0.25% Coomassie Blue, or by immersing the gels in 0.03% Coomassie made up in Acetic Acid: Methanol: Water (14:40:180). Destaining was carried out by washing with several changes of 7% Acetic Acid or in the Acetic acid, Methanol and Water mixture described above.

I. Transport of maltose and α-methylglucoside.

The transport of maltose and α-methylglucoside was measured with C^{14}-maltose and $C^{14}\alpha$-methylglucoside (uniformly labelled, New England Nuclear). For consistency in interpretation, the protocols of Zimmerman et. al. (1973) were used. The results were expressed in cpm/mg dry weight. The dry weight was measured by filtering aliquots of the cells into Millipore (0.45u) filters, washing with 15 ml of water and weighing the filter after it had been dried to a constant weight. Determinations of the equilibrium level of internal counts was based upon an internal volume of 1.12ul/mg. dry weight as given by Kroon and Koningsberger (1970).

J. Synthesis of Phenyl- glucoside

Phenylα-glucoside was synthesized by the method of Trevelyan (1966).

K. Protein Determinations

Protein was determined by the microbiuret method according to Zamenhoff (1957).

Results

Purification of Maltase and α-Methylglucosidase

Maltase was first obtained in a demonstrably pure form by Kahn (1967) although Halvorson and Ellias (1958) reported a purification of the enzyme earlier. The purification method used by Kahn (1967) had three steps: Ammonium sulfate precipitation, Sephadex G-100 gel filtration, and ion-exchange chromatography on DEAE-Sephadex (A50). For the purposes of this thesis, a method was needed which would effect a better separation of α-methylglucosidase and maltase and which would eliminate the critical A-50 equilibration. During a visit to our laboratory in December 1971, A.M.A. ten Berge and the author worked out the following procedure for the isolation of enzyme from strains carrying MAL 6. The procedure is satisfactory for the purification of the maltases in general, and an example using enzyme from strain 76-8D (MAL 1) is presented in Table 2.

Growth and Preparation of Extracts

A small inoculum of the desired strain was grown overnight at 30°C in ml of YEPD. One-half ml of this was inoculated into six 2.5l Fernbach flasks, each of which contained 1 liter of YEPM. The flasks were incubated in a New Brunswick rotary shaker at 30°C. After 16-18 hours of growth, the cells were harvested, washed twice with distilled water, and crushed (Eaton, 1962).

Seventy to one hundred ml of 0.05M Potassium phosphate buffer

pH 7.4 (referred to subsequently as "isolation buffer") was added to the crushed cells. This crude extract was centrifuged at 25,000g for 20 min. The pellet, which contained whole cells, cell wall fragments, and mitochondria was discarded. Twenty ml of the supernatent,containing between 40 and 60 mgs of protein per ml., was applied to a 70x2.5 cm. column of Sephadex G-100 equilibrated with isolation buffer. Elution of the column took place with the same buffer, and fractions of 5-7mls were collected. Under these conditions the PNPGase activity appears as a single peak emerging just after the bulk of the proteins (see Fig. 1), cofractionating with a yellow pigment. If the column load was decreased, the peak was bifrucated, due possibly to interaction with the glucose (1-6) glucose links in the Sephadex gel. Contral fractions of the peak were combined and loaded on a 30x2.5 cm. column of Hydroxylappitite which was equilibrated with isolation buffer. After loading (total loads of 10-100mgs, were satisfactory), the column was washed with one volume of isolation buffer. Fractionation was accomplished with a linear gradient of 0.05M to 0.5M Potassium phosphate buffer pH7.4 containing 1mM β-mercaptoethanol. The total volume of the buffer used in elution was 1 liter; 500 ml of the starting buffer was used in the mixing chamber of a two chamber liner gradient apparatus with an equal volume of 0.5M buffer in the other chamber. Stirring with a magnetic bar ensured adequate mixing.

Three to four ml volumes were collected, and three peaks of PNPGase activity were detectable (Fig. 2). The first two peaks lacked maltase activity and correspond to the activity as assayed with α-methylglucoside as the substrate. The third peak, cleanly separated

from the first two, corresponded to maltase activity. The sharp separation of α-methylglucosidase and maltase ensures that the substrate specificity studies reported here are not complicated or compromised by cross contamination of the enzymes involved. This is of particular importance since all strains, even those negative for the corresponding fermentations, exhibit significant basal levels of the enzymes. The isolated enzymes showed a single band after electorphoeresis in either the standard Davis system or SDS gels, even when loads of over 150 microgms were applied.

Comparison of the Ki's of the Maltases produced by Strains carrying MAL 1,2,3, and 6.

The Ki values of various substrates or complexants were measured by measuring the inhibition of PNPG hydrolysis observed in their presence. Table 3 shows the Ki's for purified preparations of maltase. No significant differences were found.

Heat inactivation of the Maltases produced by Strains carrying MAL 1,2,3, and 6.

Figure 3 shows the heat inactivation curves at 51°C in in Potassium phosphate buffer pH 7.4 with BSA (Bovine serum albumin, 0.1%) and with BSA and Ammonium sulfate (5%). At least five different points were taken in each experiment, and all can be fitted to a single straight line for the buffer containing BSA alone. The addition of Ammonium sulfate gives a biphasic curve; but the behavior of each enzyme is the same.

Substrate specificity of the Maltases produced by Strains carrying MAL 1,2,3, and 6.

Table 4 shows the specific activity of the various maltases compared to their ability to hydrolyze PNPG. The data are presented in this form to correct for slight variations in the specific activity toward PNPG found in the preparations. PNPGase activity was in the 160-110 nM/min mg range. Once again no significant differences were found.

Molecular weights of the Maltases produced by strains carrying MAL 1,2,3, and 6.

Three independent molecular weight estimates of the maltases from yeast have been made. Kahn and Eaton (1972) used gel filtration on Sephadex G-100 and obtained a value of 68,500 for the enzyme from a strain carrying MAL 3. Halvorson and Ellias (1958), using enzyme from a MAL 1 strain, found a value of 85,000 from the sedimentation coefficient. McDermott (1968) reported a value of 44,000 as measured by G-200 gel filtration and a molecular weight of 44,000 by equilibrium centrifugation as well. In the latter study, genetically undefined strains were used; one of S. oviformis and one of "brewers yeast." Figure 4 shows the values obtained from several strains by the use of SDS polyacrylamide gels. Once again no significant differences were found and the molecular weight agrees best with the value of 68,500 proposed by Kahn and Eaton (1972). The value is about 63,000 daltons.

Several attempts were made to dissociate the enzyme into smaller subunits. These included succinylation, boiling in 4% SDS with 2% (v/v) mercaptoethanol for ten minutes, and treatment with

8M urea with subsequent dialysis against 0.1% SDS. None of these treatments succeeded in altering the molecular weight. It is therefore reasonable to conclude that maltase has a single polypetide chain of about 63,000 daltons molecular weight.

Each of the enzymes, regardless of the source, gave the same molecular weight.

This chapter has shown that the maltases present in strains which are genotypically MAL 1 mal2mal3mal4mal5mal6, mal1 MAL 2 mal3mal4mal5mal6, etc., are, if not identical, then very similar in many physical properties. In succeeding chapters we shall show that mutations at MAL 1 and at MAL 3 affect the rate of synthesis of the enzymes but not the enzymatic activity. There are several possibilities for the location of the structural gene. These possibilities need not be mutually exclusive but for convenience we shall assume that each MAL gene plays the same role in ensuring the production of maltase in response to inducer.

1. Each MAL gene represents a control gene but has a closely linked structural gene for maltase.

2. There is but one structural gene and it responds to the dominant form of <u>any</u> of the control genes (the MAL genes).

Assuming the first hypothesis, it is reasonable to expect, given the virtual identity of the maltases as described in this chapter, that the gene complex(es) arose as duplications of an original complex. This in turn implies that the control genes are identical in their fundamental character (i.e., repressors or activators or mixed); the second hypothesis is a stronger statement of this conclusion.

This requirement for functional identity should be kept in mind during the two succeeding chapters which describe mutations affecting MAL 1 and MAL 3. It leads to certain difficulties in explaining the dominance properties of the constitutive mutants since most of these interact only with their allelic recessive forms. Some divergence in function of the control genes may therefore have taken place. This will be discussed more fully later.

Maltase Negative Mutants

Isolation methods. The existence of various sugar fermentation indicator media for bacteria (McConkey, Tetrazolium, Eosin-Methylene Blue) has made the isolation of negative mutants routine. In yeast, however, no fully satisfactory medium is available. The usual Bromothymol Blue indicator plates have very poor resolution due to the diffusion of the acid produced by isolated colonies. Screening of masters is possible only if the plates are checked frequently. Attempts were made preliminary to this study to develop a suitable medium. Plates containing Tetrazolium were found to induce (or select) petites while various pH indicator media had the same deficiencies as Bromothymol Blue plates. The final medium chosen was an EMB medium based on the formulation of Spiegleman, et. al. (1950), modified by adjusting the pH to 5.3 with HCl. In later experiments the unadjusted medium was used with the addition of the necessary amino acids for auxotropic strains as suggested by ten Berge (personal communication). The major difficulty encountered with this medium is that many yeast strains produce excessive amounts of acid even when grown on nonfermentable substrates. To overcome this problem, all strains are given a preliminary screening on EMB-mal and EMB-glycerol plates, and only those strains which give a clear positive reaction on the former and a clear negative reaction of the later are selected for mutagenesis.

The EMB medium used in the selection of MAL negatives

contains glycerol and maltose as carbon sources. The acid produced by Maltose positive strains lowers the pH in an area below and adjacent to the colony, thereby precipitating a black Eosin-methylene blue complex.

Analysis. Two crosses are necessary for preliminary analysis of the maltose negative strains. The first, mal x by MAL x (MAL x denotes a Maltose positive strain carrying a gene MAL x allelic to the MAL gene of the wild type strain from which the negative, mal x was derived) determines whether or not a single gene is involved; this is indicated by a 2:2 segregation of the ability to ferment maltose. The second cross, mal x by mal- (seg), indicates whether a gene inhibiting maltose fermentation has been induced in the mutated strain (mal [seg] denotes a segregational negative). Occasionally this cross is also useful to insure that back mutation is not a relevant factor in crosses where unusual segregational patterns are observed. All of this presumes, of course, that the chosen mal (seg) contributes no genes that interact to restore the parental phenotype (i.e., by complementation, supression, etc.), an assumption which in this case is partially checked by observing the fermentation behavior of the diploid.

The interpretation of the crosses is made difficult because of the possibility that under the strong selection pressure for maltose fermentation experienced by negatives kept in Durham tubes, revertants and not complementation may be observed. Although the negatives were originally selected for stability, revertants do occur. As an additional check, the data were always interpreted with

this possibility in mind (i.e., on the assumption that the reversion frequency was independent of genetic background). None of the unusual segregation patterns observed could be accounted for on the basis of reversion. As an additional control in the fermentation tests, tubes were checked for signs of fermentation periodically; and, depending on the cross, an arbitrary maximum period was determined as the cutoff point for determining whether a segregant is fermenting or not.

To simplify the discussion of the genetic data, let us consider several theoretical possibilities where complementation is observed between a mutant and a negative strain carrying a single maltose gene. We assume that no genes are centromere linked.

Case I The maltose negative phenotype of the mutant is due to a mutation which leaves the control/structural gene, MAL 1,2,etc. (designated by MAL y below), intact.

Observations:

1. If the wild-type allele of mutant gene (X) is present in the segregational negative to which the mutant is crossed:

a. If X is not linked to the control/structural gene, MAL y, we recover 2:2, 1:3, and 0:4 tetrads in the ratio of 1:4:1. The tetrad ratios are always expressed as Maltose positive: Maltose negative. Linkage of X and Mal y increases the number of 0:4 tetrads and decreases the others.

b. If crossed to a strain carrying the MAL y ellele, which ferments maltose, all tetrads are 2:2.

The important point is that the 2:2 segregation observed in

case b. does not prove that the Mal y gene has been mutated.

Case II The maltose negative phenotype is due to a mutation in the MAL y gene (i.e., mal y) and this gene complements a gene X in the negative strain.

a. If X is not linked to mal y, we find 2:2, 1:3, and 0:4 tetrads in the ratio of 1:4:1.

b. If crossed to an allelic MAL y strain which also carries a gene X unlinked to MAL y but which, unlike the situation in case a., is unnecessary for maltose fermentation when the dominant allele MAL y is present. In this case we find 4:0, 3:1, and 2:2 tetrads in the ratio of 1:4:1.

c. If the gene X, described in 2b., is linked to mal y, we find fewer positive spores, since it is in repulsion to the gene which it is complementing. In the case of infinitely tight linkage, we recover only 2:2 tetrads.

d. If crossed to a nonallelic maltose positive strain MAL z which carries a gene X which complements mal y, we find 4:0, 3:1 and 2:2 tetrads in the ratio of 1:16;19, assuming no linkage between any of the genes. In the limit of infinitely tight linkage between MAL z and X, we recover only 2:2 tetrads. If X is tightly (infinitely) linked to MAL y, the same situation arises since X and MAL y are present in repulsion.

Strains exhibiting the pattern shown in Case Ia. were isolated originally by A.M.A. ten Berge and G. Zoutewelle (personal communication) from a strain constitutive for MAL 4 (1403-7A). Similar mutants were later investigated by Zimmerman, et. al. (1973)

and were shown to inhibit the utilization of maltose and α-methyl-glucoside. They were designated DSF (Dissacharide fermenting). They fall into at least six complementation groups, but their true nature is obscure. It is important to note that they are present in every maltose negative strain in our collection; if they were not, maltose fermentation would not segregate 2:2 in crosses between these negative strains and strains carrying a single MAL gene.

Negatives derived from strain 76-8D (MAL 1)

Maltose negative mutants were selected on EMB-Maltose plates after NTG mutagenesis as described in Materials and Methods. After purification the mutants were screened in YEPM fermentation tubes and only those which exhibited no fermentation every 7 days were retained for study. The mutants studied therefore were relatively stable in respect to reversion, and nonleaky in their phenotype. Twenty-four mutants were found after this procedure and these were scored further for growth on YEPD and YEPGal. Strains exhibiting growth rates significantly different from those of the wild type were eliminated from further consideration. Similarly, strains showing additional nutritional requirements, and strains which were petite were also discarded.

Complementation

Table 5 presents the results of the complementation tests with maltose positive and maltose negative strains. All the mutants complement 1315-2C which carries MAL 1: all mutants are therefore recessive. In contrast, two distinct types of complementation behavior

are observed in the crosses with the maltose negative strains. One class (Class A) shows complementation for maltose fermentation with every segregational negative; the other (Class B) shows no complementation. Strain E-4B complements all known dsf genes so it is DSF. When two additional maltose negative strains containing an amber and an ochre suppressor (SUP 7-2 and SUP 7-1) were crossed to the mutants, the complementation patterns were unaltered; the mutants are not suppressible by these suppressors.

Allelism

The results of the complementation tests made it likely that Class B represented mutations in the MAL 1 gene. Class B mutants were crossed to 1315-2C (MAL 1) and the diploids were sporulated and dissected. Table 6 shows the results of this test. All Class B mutants showed a single gene segregation for Maltose fermentation. It must again be emphasized that this result by itself is not a proof of mutation in the MAL 1 gene (see the previous theoretical discussion, Cases I and II). The complementation data, however, does strengthen this conclusion. As an additional test, a cross was made between 1315-2C and a maltose nonfermentor 99C. The tetrads were dissected and the maltose nonfermentors crossed back to the Class B mutants. Once again no complementation was observed. We can conclude therefore that the Class B mutants are either mutant in the MAL 1 gene or in a gene closely linked to MAL 1 which complements it. The latter assumption would necessitate that all of our stock strains which are maltose negative would be lacking this hypothetical, closely linked complementing gene, an assumption which is not only ad hoc but completely unnecessary to

to explain the above results. This possibility is, of course, always a possibility in tests for allelism; there is no reason, however, for raising this theoretical spectre. As a further test, the negative segregants of the 1315-2C x Class B mutants, of the opposite mating type to 76-8D, were crossed back to each of the Class B mutants and the diploids scored for fermentation. Once again, none of the diploids fermented. Either they are mutants of a single allele, MAL 1, or mutants of a single gene (Oshima, 1967) of a gene complex.

Both Oshima (1967) and Naumov (1969) have found complementation between certain wild type (i.e., nonmutagenized maltose negative strains), and Oshima believes that the MAL 1 locus is a complex of two genes M and N which exhibit interallelic complementation. We have not recovered any mutants which show an interallelic complementation of the type described by Oshima; the complementing mutants we have discussed, 76-6, 76-7, etc., complement genes which are present in MAL 1 strains. Presumably, these maltose positive strains contain intact MN cistrons. Since we have shown that maltase is a single polypeptide chain, this eliminates the hypothesis that the M and N cistrons code for nonidentical polypeptides of maltase. The remaining possibility is that the complementation between permease, structural, or control genes. Alternatively, since the maltose negatives he described originated in stock strains, it is impossible to exclude the possibility that only two nonallelic mutations in a single cistron are being compared and that the maltose positive phenotype is the result of mitotic gene conversion. This is a possible interpretation because the procedure he uses in scoring complementation did not make use of isolated diploids, but uses a mass

mating mixture obtained by growing yeasts of opposite mating type in YEPD broth. An alliquot was then inoculated into Maltose fermentation tubes. The complementation test results obtained in Table 5 are not totally unambiguous either, since these results are obtained only if the fermentation is scored within five days. After two weeks, 3:1 and 4:0 segregations appear in crosses with 76-5 and 76-3, two strains which also show a higher spontaneous reversion rate. The wild type ferments within three days, however, and the stable mutant 76-8 shows a strictly 2:2 pattern so that the proof of allelism for mutants of Class B is firmly based. It is also possible that a larger collection of mutants at the MAL 1 locus will reveal complementation.

For simplicity and since we have no evidence to indicate the contrary, we consider the Class B mutants to be mutant at the MAL 1 locus.

Enzymatic analysis of the mutants allelic to MAL 1 (Class B)

The mutants were grown in various media and extracts prepared (Materials and Methods). Table 7 presents the levels of PNPGase and Maltase found in the mutant strains after growth on Cat-Gal or Cat-Mal+Glu (2% Maltose, 0.3% Glucose). In subsequent experiments it was found that Cat-Glu and Cat-Gal gave equivalent enzyme levels for all the strains tested. All of the mutants contain significant levels of PNPGase and Maltase. These basal levels are equivalent to those found in the wild type after glucose repression on YEPD, and are about 10% of the level found in wild type cells after growth under nonrepressing conditions on Cat-Gal. The striking feature of these results is that the enzyme is not inducible by maltose. Since it was possible that this lack of inducibility and the low basal level were both due to the

presence of another enzyme having a marginal maltase activity, and not to maltase itself, the enzyme was further characterized. Because of the low levels present, this work was carried out in extracts which had been partially purified by hydroxylappitite fractionalation to eliminate the other major PNPGase enzyme, α-methylglucosidase. Table 8 presents the results obtained for two maltose negative mutants. In all respects the properties of the enzyme present in each of the strains are indistinguishable from purified maltase. Since these levels are not increased upon the addition of maltose, we may conclude that the mutants are impaired in the induction of the enzyme and not in a structural eomponent of maltase.

Nature of the Defect in the Class B Mutants

The failure of maltose to induce the enzyme in the mutant strains could be due to a variety of reasons including:

1. Lack of maltose uptake
2. Inability to form the true inducer
3. Inability to retain the true inducer in sufficient concentrations to allow induction
4. Mutation in a regulatory product which interacts with the inducer to turn on maltase synthesis
5. Mutation in a region which is a binding site for a protein involved in making the structural gene for maltase available for transcription.

These possibilities need not, of course, be mutually exclusive. The lac operon of *E. coli* provides some examples of the above possibilities.

Noninducible phenotypes can occur by mutation in the structural gene (y) for the permease protein which binds lactose; by mutation in the structural gene (z) for β-galactosidase thereby preventing the synthesis of the true inducer (presumably allolactose) by transglucosidation; and by uncoupling the carrier protein from the energy coupling device with resultant leakage of allolactose (Wong et. al, 1970). In this system, then, certain z- mutants, producing an inactive β-galactosidase, would appear to be regulatory mutants.

Revertant Analysis

The analysis of suppressed mutants can help distinguish among these possibilities. The isolation of temperature sensitive revertants is of particular value. Many are second site mutations in the same locus which restore protein function at one temperature but not at another, generally higher temperature. The existence of a temperature sensitive phenotype is generally considered to be proof that the function affected involves either a protein or a t-RNA. Their value in dissecting the nature of the gene product lies in the ability to alter activity in vivo and in vitro. If the MAL 1 gene is a control gene, the identification of a temperature sensitive mutant at the MAL 1 locus which affects the inducibility of maltase without affecting the thermostability of maltase would be expected.

Revertants of Class B mutants derived from 76-8D (MAL 1)

Revertants were obtained by NTG mutagenesis. The appearance of the revertants varied greatly, with many colonies exhibiting a wrinkled morphology unlike the wild type strain. Both temperature

sensitive (growing at 25 C° on YEPM but not at 35 C°) and temperature independent strains were isolated; they were assayed for enzyme activity after growth in Cat-Glu (0.3% Glucose) or Cat-Mal+Glu (2% Maltose and 0.3% Glucose).

The enzyme levels found in some of the twenty-one revertants analyzed are presented in Table 9. None of the revertants was constitutive. This is in marked contrast to revertants obtained from mal 6 mutants where almost all are constitutive (ten Berge, et. al., 1974). One temperature sensitive revertant, 76-8-1A, was selected for further study.

Analysis of a revertant derived from 76-8, 76-8-1A

The fermentation of maltose at the permissive temperature was considerably slower than that of the original wild type strain (76-8D), and required about one week for full expression. Overnight growth in Cat-Mal+Glu was insufficient for induction (Table 9). No significant differences in enzyme levels were found after growth in nonrepressing media at either temperature. The revertant was crossed to 1315-2C (MAL 1) and diploid sporulated and dissected. Maltose fermentation was scored at both 25 and at 35 C° (Table 10). Viability was poor and only seven complete tetrads were isolated. The segregation pattern of both maltose fermentation and the temperature sensitivity can be accounted for by assuming that a gene X complements 76-8 (mal 1-8), and that the combination mal 1-8 X leads to temperature sensitivity of maltose fermentation. If this gene is unnecessary in strains carrying MAL 1, we would expect the following pattern, assuming nonlinkage of X and MAL 1:

Parental Ditype 16.6% 4:0 (Mal:mal), two temperature sensitives
Tetratype 66.6% 3:1 (Mal:mal), one temperature sensitive
Nonparental Ditype 16.6% 2:2 (Mal:mal), no temperature sensitives

In seven tetrads the theoretical number of PD, T, and NPD types would be 1.1, 4.6, and 1.1 respectively; the observed number was 1, 4, and 2. Because of the spore viability, we can only state that the hypothesis is completely consistent with the data; additional tetrad data are probably necessary to confirm it. Nevertheless, the interesting point is that a mal 1 mutant can be complemented by a gene which is probably unlinked to the MAL 1 locus. This, then, represents a case of interallelic complementation in the MAL system.

Transport of Maltose and α-methylglucoside in 76-8-1A

Transport was measured at 25 C° after growth in Cat-Glu at 25 C° and at 35 C°. The transport of maltose and α-methylglucoside was unaffected by the temperature of growth when the wild type 76-8D or the mal 1-8 mutant, 76-8 was tested. In contrast the transport in the revertant 76-8-1A was markedly affected by the growth temperature (Fig. 5). The transport of both substrates was extremely rapid at 25 C°; the uptake at 35 C°, however, was indistinguishable from 76-8. The MAL 1 constitutive strain, 27-11C is included in Figure 5 for comparison. The gene X therefore allows cells to ferment maltose by providing an alternate mechanism for maltose (and concomitantly α-methylglucoside) uptake but the absence of the MAL 1 protein prevents rapid fermentation.

The defective MAL 1 allele mal 1-8 (76-8) complements the unlinked gene X for maltose fermentation at the permissive temperature; neither gene alone can ferment maltose. Gene X is involved with the transport of maltose. The mal 1-8 mutation, then, somehow affects

maltose utilization. There are at least two possibilities:

1. The MAL 1 allele is a complex locus and the mal 1-8 mutation affects one gene concerned with permeation; another gene functions normally in the presence of inducer.

2. The MAL 1 locus codes for a regulatory protein with two functions: the induction of maltase and the induction of a separately controlled transport system.

Under the second hypothesis, one must assume that the mal 1-8 mutation affects the induction of the permease more strongly than the induction of maltase. In the presence of inducer supplied by gene X, this functionally leaky (but phenotypically tight) mutant is sufficiently active to allow maltose fermentation. The hypothesis that the basal levels of maltase present in strain mal 1-8 are sufficient for maltose fermentation can be eliminated since the possession of gene X, by itself, does not allow the cells to ferment. This hypothesis is also consistent with the temperature sensitivity of the fermentation of α-methylglucoside in the temperature sensitive constitutive mutant 27-11C (next chapter). In this mutant the uptake of maltose and α-methylglucoside is temperature sensitive but the constitutivity is not.

The first hypothesis implies that complementation should occur for mutants at the MAL 1 locus; none of the nine mal 1 mutants isolated complements any of the others; but this number may be too small for any definite conclusions.

If cells of strain 76-8 (mal 1-8) are treated with Amphotericin B at concentrations of 0.3 microgms/ml in YNB-Mal fermentation tubes, fermentation occurs after seven days. This polyene antibiotic increases

the permeability of yeast cells to normally impermeable substances (Keith, et. al., 1973), and mimics the effect of gene X.

While we can not exclude the possibility that the MAL 1 locus is a complex of a regulatory gene and permease gene, the MAL 1 locus cannot be simply a permease gene. The evidence upon which this conclusion is based will be presented in the following chapter on constitutive mutants.

Maltose negative mutants derived from MAL 3

Maltose negative mutants were obtained from strain 48-9A (MAL 3) by the procedure used for obtaining MAL 1 negatives. Although the mutageneic treatment was identical, the mutant yield was much higher and over fifty negative mutants were obtained.

Characterization of the parent strain 48-9A

The strain 48-9A was crossed to a MAL 3, sucrose negative strain, 1359-11B; the diploid was sporulated and dissected. In eleven tetrads, maltose fermentation segregated 4:0, indicating that the strains had allelic MAL 3 genes. Sucrose fermentation showed 2 (4:0), 7 (3:1), and 2 (2:2) ratios; this compares to the theoretical 1.8 (4:0), 7.3 (3:1), and 1.8 (2:2) ratios expected for a two gene segregation. When 48-9A was crossed to a sucrose negative, maltose negative strain 1300-14B, all maltose fermentors also fermented sucrose (Fifteen complete tetrads). Strain 48-9A therefore has two sucrose genes and one of these is linked to MAL 3. This strain also complements the MGL 1 tester 122-1C for α-methylglucoside fermentation. Results of other crosses in this laboratory show that, as in all MAL 3 strains which we have looked at, there

is a complementing gene, MGL 2, linked to the MAL 3 locus (Hawthorne 1958; unpublished data). Strain 48-9A therefore has the gene complex MAL 3 MGL 2 SUC 3 (the order of the genes is unknown.) It also has a gene gal 3 which in the petite condition interferes with Matlose fermentation. Because of this complication, all maltose negative mutants which were petite were eliminated from further consideration. The presence of the gal 3 gene can be easily recognized by a requirement for tryptophan in this strain; the parent 48-9A has the trp 1 gene which is tightly linked to gal 3.

Characterization of the mutant 48-8

Several mutants were studied in detail, but for convenience the results will be described in detail only with mutant 48-8 since identical features were found in the other cases (48-7, 11, 14). Strain 48-8 did not complement the DSF strain 1300-14B; its defect was therefore not of the dsf type. When the diploid 1300-14B x 48-8 was sporulated and dissected, all six tetrads showed a 0:4 segregation for maltose fermentation. Strain 48-8 therefore does not contain a nonlinked suppressor of maltose fermentation. All six tetrads remained 0:4 for several months, demonstrating the stability of the negative mutation. This stability was confirmed in extensive reversion trials with this strain; no revertants were obtained even after severe UV (less than 1% survival) or NTG (less than 10% survival) mutagenesis.

In order to see whether the mutation in 48-8 was allelic to MAL 3, cross C11 (48-8 x 1359-11D) was examined for maltose fermentation. Table 11 presents the results of these tests. Maltose segregation was irregular; ratios determined at early intervals deviated considerably

from the expected 2:2 segregation pattern. Since the negative character was not leaky, one possible explanation is that the strain 1359-11C contains a gene(s) which can complement 48-8 for maltose fermentation. This gene(s) must be nonessential for maltose fermentation in 1359-11D. The segregation ratios were 1 (4:0), 7 (3:1), and 11 (2:2); a result consistent with a nonlinked complementing gene in strain 1359-11C (see the theoretical discussion on page 21). The theoretical ratios would be 6.3 (4:0), 12.5 (3:1), and 6.3 (2:2). The observed ratios are consistent with MAL 3, mal 3-8, and two complementing unlinked genes (theoretical: 0.5 (4:0), 8.0 (3:1), 915 (2:2).

If a single gene complementing 48-8 was present in 1359-11D, it should be possible to separate it from the MAL 3 gene present in this strain and obtain unambiguous evidence for complementation. Toward this goal, strain 1359-11D was crossed to 1071-11B (mal) and random spores isolated. If interallelic complementation is involved the prevalence of the irregular segregations (3:1, 4:0) suggests that the involved gene(s) are only loosely linked (or not linked) to MAL 3. Twelve maltose negative spores from this cross (C68) were selected and crossed back to 48-8. The diploids were purified and tested for maltose fermentation. None of the diploids fermented maltose.

Three maltose negative spores from tetrads of C11 were back-crossed to 1359-11D. All showed the irregular gene segregation patterns for maltose fermentation. The results of one of these crosses are presented in Table 12. The rationale behind this experiment was to see whether the allele trans to MAL 3 played a role in complementation. If it did, then the probability of MAL 3 being mutated in strain 48-8 would

be increased. An inspection of the ratios obtained in this cross shows 5 (4:0), 3 (3:1), and 1 (2:2). This is not consistent with the results obtained with cross C11, and the samples must represent two distinct populations. The segregations at early time intervals were, however, exclusively 2:2 and only showed late deviations from this pattern. As a control the parent 48-9A was crossed to strain 1359-11D (C43) and eleven tetrads dissected. All spores filled the YEPM Durham tubes completely with gas within two days.

These tests indicate that the maltose negative mutation occurred at the MAL 3 locus in strain 48-8. The irregular segregation patterns observed after long intervals could be due either to weakly complementing genes present in 1359-11D, or to altered cellular permeability of the ageing cell suspensions in the Durham tubes. While no complementing genes could be isolated from strain 1359-11D, the late fermentors could have a genetic basis. In order to test this hypothesis further, the maltose negative strains (48-7, 8, 11, 14) were crossed to another MAL 3 strain, 1412-4D. In a total of 27 tetrads isolated from these crosses, maltose fermentation segregated 2:2, and the ratios remained 2:2 for periods up to three weeks.

Since these strains did not complement 1300-14B (DSF), we can conclude that these four mutants are mutant in the MAL 3 gene.

Additional Defects Present in the Mal 3 Mutants

The mutants were crossed to the MGL 1 tester 122-1C and the diploids tested for α-methylglucoside fermentation. A positive result implies that the mutants retain an unaltered MGL 2 gene. Table 13 shows that many mutants are unable to complement MGL 1 for α-methylglucoside

fermentation. Four mutants, 48-7, 48-8, 48-11, and 48-14 were chosen and tested for the presence of an unaltered SUC 3 gene. These strains were crossed to 1359-11D (MAL 3 suc 3), and the diploids were sporulated and dissected. Rapid sucrose fermentation segregated 2:2, indicating that only one sucrose gene was present in the mutants. After one week of testing, additional sucrose fermenting spores appeared. If the sucrose gene SUC 3 is present in the mutants (i.e., if they carry a mutation in their unlinked SUC gene), one would expect that sucrose fermentation would segregate in repulsion to MAL 3. Since this is not observed, we can conclude that the SUC 3 gene is defective in these strains. Since three of the above mutants have a wild type MGL 2 gene (Table 13), the pleiotropic negative phenotype (mal-suc-) does not necessarily affect the closely linked MGL 2.

If the maltose negative phenotype of strain 48-8 is due to a mutation of MAL 3, then the concomitant defect in the closely linked SUC 3 gene may be due to either a double mutation in this region or to a single mutation in a common unit of expression. The possibility that MAL 3 and SUC 3 are identical can be excluded since Hawthorne (1969) has been able to separate the loci. The likelihood of a double mutation in this region is made more probable by the fact that NTG mutagenesis was used; this mutagen is known to cause a high frequency of double mutants in a limited region since it preferentially mutagenizes the DNA replicating point (Cereda-Olmendo, 1968).

Dominance Relations of the mal 3 Mutations

The mal 3 mutations had properties identical to the mal 1 (described previously in this thesis) and the mal 6 (ten Berge, 1974)

loci. They had basal levels of maltase, but were not induced by maltose. When crossed to MAL 3 strains, the diploids were inducible by maltose and rapidly fermented this sugar.

Discussion

The properties of the mal 1 and mal 3 mutations described in this chapter are consistent with a purely positive control system. In this model, the MAL 1 and MAL 3 alleles produce an inactive product, which, in the presence of maltose, converts to an activator for the maltose structural gene(s). In negative control system the noninducible phenotype occurs by mutations which affect the inactivation of the repressor by the inducer without changing the affinity of the repressor for the operator. It is doubtful that the mal 1 or mal 3 loci code for such a repressor (i.e., a "super repressor") since there is no difference in maltase levels in mal 1 x MAL 1 and MAL 1 x MAL 1 diploids after growth on CAT-Glu (data not shown).

A purely positive type of control system can, however, be eliminated as a hypothesis because of the nature of the constitutive mutants which occur at the MAL 1 and MAL 3 loci. These are described in detail in the next chapter.

Methods for the Isolation of Constitutive Mutants

A major method for isolating constitutive mutants for catabolic inducible enzymes in bacteria makes use of the relative advantage induced cells have over noninduced cells during the early stages of their growth on the inducing substrate. Mutagenized cells are exposed to the inducing (adapting) substrate for a limited time and then placed in noninducing media for a time sufficient to allow deadaptation. The cycle is then repeated. Alternatively, the cells may be exposed in a chemostat to a limiting concentration of the inducing substrate. These protocols are applicable where the growth substrate (or its immediate product) is also the inducing substrate. In cases where noninducing substrates are available, the procedure is less complex since unlimited exposure to the substrate is then permissible.

The first step in developing a method for constitutive mutant isolation in the maltase system was, therefore, to characterize the substrate specificity and the induction response of maltase. Pure preparations of maltase were obtained by the methods described in this thesis and then characterized. The enzyme hydrolyzed phenyl α-glucoside, turanose, sucrose, PNPG, maltose, α-methylglucoside, and melezitose. This list of substrates differs slightly from the results of Halvorson (1963) and Kahn (1964). Halvorson found that melezitose was not a substrate although Philips (1959) in an earlier paper did report a weak melezitose activity. Kahn could not detect any α-methylglucoside hydrolysis in preparations of pure MAL 3 enzyme although the ability of maltase to hydrolyze α-methylglucoside weakly has been confirmed by

others (van Wijk [1968]; ten Berge, [1973]). All pure enzymes tested had identical substrate specificities (this thesis) and all (MAL 1, 2, 3, and 6) hydrolyzed α-methylglucoside and melezitose weakly. Since melezitose was a poor substrate, the ability of cells to ferment this sugar was tested in fermentation tubes containing 2% melezitose. Strains carrying MAL 1, 2, 3, 4, 5, or 6 as well as MGL 1 MGL 2, MGL 3 MGL 3 strains were tested. The only strain to ferment melezitose (and turanose) within a period of four days was strain 1403-7A which has a constitutive MAL 4 gene. The MAL 4 inducible strain tested, 1394-9B was completely negative. Strain 119-16 showed a slow fermentation of melezitose and turanose.

Lindegren has described a locus, MZ, which confers upon the cells the ability to ferment melezitose, maltose, sucrose, α-methyl-glucoside, and turanose. Originally it was assumed that a single enzyme "melezitase", was coded for by the MZ locus; subsequently, three distinct enzymes, two maltases (one having melezitase activity), and α-methylglucosidase were found in strains carrying a single MZ gene (Yau 1967). Mutants mapping at the MZ locus and having altered induction specificities. Some which fail to adapt to the fermentation of sucrose, α-methylglucoside, or melezitose; others to α-methylglucoside and melezitose have been recovered. In each case, however, all three enzymes are present and it is therefore likely that the MZ locus controls either premeability or the induction response itself rather than (or in addition to) coding for a particular enzymatic activity.

Regardless of the mechanism of melezitose fermentation, both the ability of the constitutive strain described above to ferment this substrate and the lack of fermentation in the corresponding inducible

strain suggested that constitutives could be obtained by selecting for melezitose fermentation in maltose inducible strains. Such mutants would be expected to have increased transport of melezitose, increased enzyme levels, or altered enzyme in which the Vm for melezitose would be increased.

Selection of Maltase Constitutive Mutants Using Melezitose

Several attempts were made to obtain melezitose fermentors from several maltose positive inducible strains. Heavily mutagenized cells were inoculated in melezitose fermentation tubes or spread at various dilutions on YEPMelz agar. No fermenting colonies were obtained. One possibility for this failure was that the heavy inoculum used in the fermentation tubes was depleting the medium of essential nutrients before growth of the few melezitose positive cells could be observed. To avoid this, a different method was devised. Cells were mutagenized and then passed alternatively through melezitose and YEPD tubes. This method was successful when applied to strain 48-9A (MAL 3) and TB 1 (MAL 6). Two constitutives were selected, MZ48.1 and MAL6C19.

Melezitose Fermentation

Since the melezitose selection method had been used to select MAL 6 C19 and MZ 48.1, it was possible that a separate study of this system would yield some insight into the mechanism of this selection. Lindegren and coworkers (see Yau, et. al. 1967) have proposed the existence of a single locus, MZ which either codes for, or controls, the synthesis of an α-glucosidase which is able to hydrolyze melezitose, sucrose, turanose, and α-methylglucoside. It was therefore of interest

to determine whether the MZ locus was alleleic to any known maltose gene and whether it was a constitutuve MAL gene rather than a distinct enzyme system.

A culture of strain 11339, kindly supplied by Ms. Gertrude Lindegren, was purified and tested for sugar fermentation. It was able to ferment melezitose, maltose, sucrose, and α-methylglucoside. Turanose fermentation was not tested. It was sporulated and a haploid strain MZ 3 with the same fermentation characteristics was crossed to strain 1300-14B, which was unable to ferment any of these sugars. Table 15 presents the results of this cross (C60). Six tetrads were analyzed for sugar fermentation. Maltose fermentation segregated 1 (4:0), 3 (3:1), and 2 (2:2), indicating that two MAL genes were present. Sucrose fermentation segregated 4 (3:1), 2 (2:2) in a typical two gene pattern. Alpho-methylglucoside fermentation was more complex with 3 (2:2), 1 (1:3), and 1 (0:4) after five days. After two weeks this became 1 (3:1) and 5 (2:2). Melezitose fermentation segregated as 1 (2:2) and 5 (1:3). Every melezitose fermentor fermented maltose and sucrose but not necessarily α-methylglucoside. The one ascus which gave four maltose positive spores and two melezitose fermenters was chosen for further study since in this case the gene presumably responsible for maltose and melezitose fermentation had been separated from another maltose gene. The melezitose system on the basis of this preliminary evidence seemed to be composed of a complementing gene system with a single maltose gene playing a major role.

Since constitutive mutants had been selected on the basis of increased growth on melezitose, the clone 60-6A was tested for PNPGase after growth on Cat-Glu. The clone was not constitutive and had extremely

low PNPGase activity. The value was 0.5nM/min/mg, a level lower than any previously observed, even in maltose negative mutants. (Some Mal 6-6 malx strains have values in the same range).

To identify the MAL gene involved in 60-6A, the strain was crossed to tester strains carrying known MAL genes. The strains used were 1315-2C (MAL 1), D6-5A (MAL 2), 48-9A (MAL 3), 1394-9B (MAL 4), and BC 35 (MAL 6). Spore 60-6B was crossed to 645-1A (MAL 5) and 1300-14B (mal). Only a limited number of spores was scored since the identification of a single spore which does not ferment maltose suffices to prove nonalleleism of the genes involved in the cross. At least five negative spores were obtained from each of the crosses listed above. The MZ gene is therefore not allelic to MAL 1, 2, 3, 4, 5, or 6.

The results of the cross between the maltose negative strain 1300-14B and 60-6B are presented in Table 16. Only a single maltose gene is present and it is linked with the fermentation of sucrose. Melezitose fermentation does not segregate as a single gene difference; but, once again, every melezitose fermentor ferments maltose and sucrose.

The experiments described here demonstrate, in contrast to the claims of Yau and Lindegren (1967) that the fermentation of Melezitose is controlled by a single gene, that this fermentation is the property of a polymeric gene system. One gene of this system is either identical to, or, closely linked to a MAL gene. This MAL gene is not allelic to MAL 1, 2, 3, 4, 5, or 6.

The two constitutives selected by the Melezitose transfer technique, MZ 48.1 and MAL 6 C19, differ from other constitutives; MZ 48.1 is the only <u>derived</u> constitutive (the stock strain MAL 4^c,

1403-7A is the only other) in which the constitutive character segregates in a pattern more complex than that of a single gene difference, and MAL 6 C19, unlike other constitutives at the MAL 6 locus, was dominant and not suppressed by mal x. (ten Berge et. al., 1974) Constitutives derived by other methods were therefore tested in Melezitose fermentation tubes. Fermentation was considerably faster in 2% melezitose than in 1%, an indication that transport may be the rate limiting factor in the fermentation of this sugar. All constitutives tested, which included MAL 6 C2, MAL 6 C17, and 76-C1 (see next chapter) were able to ferment melezitose within one week. Melezitose fermentation in MZ 48.1 and MAL 6 C19 started much sooner and was completed in a shorter period of time (two days). These strains show a much higher level of α-methylglucosidase transport than other constitutive strains (ten Berge, Zoutewelle, and Needleman, 1974). The Melezitose selection method may therefore select mutants with an increased rate of α-methyl-glucoside and melezitose transport. The transport of α-methylglucoside in strain 60-6A after growth on Cat-Glu is very high, with the uptake essentially identical with the uptake kinetics of MZ 48.1 (Figure 6). This strengthens the contention that the fermentation of melezitose depends on the presence of a transport system that can cotransport α-methylglucoside.

Although the melezitose selection method was successful, alternative methods were sought since there was the possibility that the constitutives selected by this method would be a subset of the most general class of maltase constitutives. This in fact proved to be the case.

Selection of Maltose Constitutives using α-methylglucoside

It has been repeatedly observed that the induction of the maltase system increases the rate of uptake of α-methylglucoside and α-ethylthiogluside (Halvorson, et. al., 1964; Avigad, 1959; Kroon and Koningsber, 1970). Maltose also induces the synthesis of α-methylglucosidase in strains carrying the dominant form of the MAL genes (Table 14; Owehand and van Wijk, 1972). Basal levels of α-methylglucosidase are present in all strains which we have examined; even in those which contain the recessive forms of the alleles for α-methylglucoside fermentation mgl 1 mgl 2 mgl 3. The α-methylglucosidase activity found in crude extracts of these strains is due to α-methylglucosidase and not to maltase, since the activity is separable on hydroxylappitite. A.M.A. ten Berge has also reported the presence of α-methylglucosidase in strains of genotype, mgl a mgl b mgl c (ten Berge, 1973).

Alphamethylglocuside fermentation requires one of the following gene sets: MAL 1 MGL 4, MGL 1 MGL 2, MGL 2 MGL 3, MAL 4 MGL 1, or MGL a MGL b MGL c (Hawthorne, 1958; Kahn 1967; ten Berge, 1971). With the exception of MGL 2 which is required for the facilitated transport of α-thioethylglucoside, the function of the genes is unknown although the available evidence suggests that MGL 1 and MGL 3 are involved in the control of enzyme snythesis and active transport (Halvorson et. al. 1964). Alpha-methylglucoside is not a natural carbon source for yeast and therefore the existence of a specific transport system seems unlikely. The tight linkage of MGL 2 and MAL 3 is suggestive in this regard, and MGL 2 may normally function in maltose metabolism. The active transport systems for maltose and α-thioethylglucoside are coordinately induced in cells containing MGL 2 and MGL 1; the two sugars also compete with each

other for entry (Halvorson, et. al., 1964).

The role of the maltose genes in this system is obscure, but a possible clue is provided by the fact that of the six maltose genes available to us (MAL 1-6), only MAL 4 complements MGL 1. The MAL 4 allele produces enzyme and, presumably, "permease" at a rate which is significantly higher than that of the other maltose genes. Since it can substitute in function for MGL 2, a reasonable hypothesis is that the partially "induced" state of this allele relative to the other maltose genes leads to an increase in a maltose permease which can cotransport α-methylglucoside. The ubiquitous presence of α-methylglucosidase and the cotransport of α-methylglucoside by maltose induced cells suggested that constitutive mutants for the maltase system should exhibit an increased rate of α-methylglucoside utilization and that this increased rate might be sufficient to allow fermentation. Because of these observations, the gene MGL 1 was included in strains used for constitutive selection by this method. The method is inapplicable to strains having MAL 3 or MAL 4. All of our MAL 3 strains carry MGL 2 and therefore complement MGL 1. Although we have no proof of the necessity of including MGL 1 in this procedure, in the only case in which it was omitted (119-16) we did not obtain maltase constitutives, but strains which were constitutive for -methylglucosidase. The selection of constitutives in strain C219 represents a typical protocol for this method.

A MAL 1 MGL 1 diploid (C219) was grown overnight from a small inoculum on YEPD broth. One tenth ml. was spread on a YEPD plate, incubated overnight at 30 C°, and replicated to a series of YEPD plates. Some crystals of NTG were placed in the center of each plate and after overnight growth the plates were replicated to YEP- MG plates which

contained bromothymol blue as an acid indicator. (Fink and Lowenstein, 1969). Fermenting clones were picked into Cat-Gal tubes and after 16 hours of growth they were lypholized and assayed for PNPGase. Constitutives constituted about 20% of the recovered colonies. The constitutive diploids were sporulated, dissected, and the resulting haploids slanted on YEPD.

Use of Other Substrates in the Selection of Maltase Constitutives

The rate of substrate utilization is, of course, an aggregate measure of both transport and hydrolysis. A convenient way of measuring this sum is to use p-nitrophenyl glucoside as a substrate in whole cell suspensions since the nitrophenyl split after substrate transport and hydrolysis appears in the external medium and is easily measured colorimetrically. When these experiments were carried out in both induced and noninduced MAL 6 cells, it was apparent that the induced cells exhibit a much higher rate of nitrophenylglucoside hydrolysis (data not shown). A selection for constitutive mutants based on this observation was therefore possible. Instead of using nitrophenylglucoside, phenylglucoside was used since its synthesis is considerable easier due to the difficulty of deacylating the acetylated nitrophenylglucoside without removing the aglycone. Phenylglucoside was synthesized by a modification of the method described by Trevelyan (Trevelyan, 1966). Since the released phenol was expected to be toxic, the cells were grown in YEPD broth between selections. Phenylglucoside, while a substrate of both enzymes, cannot be fermented by maltose positive cells. A typical protocol is presented below:

Cells of strain 48-9A were mutagenized with NTG, washed with

water, and inoculated heavily into YNP+0.5% Phenyl-α-glucoside. After two days a small inoculum was transfered into a fresh tube of the same media and left until growth was evident. After an intermediate growth period in YEPD broth, the cells were transferred back into YNB+0.5% Phenylglucoside. This procedure was then repeated. Generally, after three repetitions, lypholyzed samples showed elevated levels of PNPGase. The cultures were then streaked out onto YEPD and the clones tested for PNPGase.

Selection of MAL 6 Constitutives by Reversion

Constitutive mutants may be obtained from mal 6 mutants by selecting maltose positive revertants (ten Berge and Needleman, submitted for publication). It is unlikely that these revertants represent new amino acid substitutions at the same site as the original mutation since they are recovered from every mal 6 mutant tested. In a pure positive model the noninducible mutations are due to a lack of the activator function even in the presence of inducer; it is impossible to expect that at every site which gives an inactive activator all amino acid substitutions yield an activator which functions in the absence of inducer; true back mutations at a particular site should give at least some wild type (inducible) revertants. These observations would be compatible with a positive model if the noninducible phenotype were due to a mutation in a site which, while not forming part of the activator, nevertheless controls its synthesis. The problem with this interpretation is that if we assume that noninducible mutations occur in some controlling site (e.g., a promotor) it is impossible to account for the suppressing action of mal x (ten Berge, Zoutewelle, Needleman, 1974), since it is

usually assumed that such sites are not translated. The only model that accounts for the prevalence of constitutive mutants in revertants of the noninducible mutants is to assume that noninducibility is due to a super-repression of the type observed for the i^s mutants of the lactose system in E. Coli. (Beckwith, 1970). A genetic map of the MAL 6 region would be of great use in deciding this question.

Starvation Technique for Constitutive Selection

Certain MAL 6 strains grow very slowly on YNP + 2% Maltose. The growth is completely prevented if the cells are starved in distilled water at 4 C° for one week before plating. One such strain, S.C. B 26, was grown in YEPD, mutagenized with NTG to 10% survival, and allowed to grow for three days in YEPD. The cells were washed twice with distilled water and suspended in water at a density of about 10^8 cells/ml. After storage for one week at 4 C°, the cells were plated on YNB+2% Maltose. Colonies were purified and tested for PNPGase. The constitutives, MAL 6-C15, -C16, -C17, and C18, were selected by this method. Strain MAL 6-C14 was a spontaneous mutant selected from a plate used as a control for the mutagenesis. Table 17 shows the levels of PNPGase in these mutants. For wild type values, see Table 14. These mutants are discussed in detail in a recent paper (ten Berge, Zoutewelle, and Needlemen 1974).

Selection of Maltase Constitutives by Serial Transfer

Heavily mutagenized cells of straom 76-8D (MAL 1) were grown overnight in YEPD to fix their mutations and then washed and grown in YEPM for 8-10 hours. They were washed, inoculated into YEPD, and grown

overnight. The process was then repeated. At weekly intervals, samples of CAT-Glu grown cultures (an aliquot was taken from the YEPD culture and grown in CAT-Glu overnight) were lypholized and assayed for PNPGase. The increase in enzyme activity was rather slow, and the selection was terminated after 79 transfers. At this point the PNPGase level was 579; a single haploid 76-C1, purified from this culture, had a level of 1136.

Discussion

Steckowski has described the isolation of mutants constitutive for MAL 1 and MAL 2 by a technique involving paraflurophenylalanine resistence (Steckowski, 1967). We were unable to obtain any mutants by this technique and the method seems to lack a clear rationale. A.M.A. ten Berge has obtained constitutive mutants for MAL 6 by a method devised by Y. S. Oshima (Ten Berge, 1972). This method involves the passage of a noninducible, maltose negative strain alternatively through rich maltose medium and starvation medium. Originally it was thought that this method was successful because the selection works on a relatively limited number of maltose positive back mutations and that a small percentage of these are constitutive. In more recent experiments, however, it was found that revertants from mal 6 strains, whether spontaneous or induced by NIL (1-nitroso-imidazolidone-2), were almost all constitutive for PNPGase. As described in this thesis, no constitutive mutants were obtained as revertants of several mal 1 mutants. When the Oshima method was used, however, several constitutives were recovered; but during the extensive transfers required (over 75), major

genetic alterations were apparent with many nutritional markers lost. Furthermore, at least two of the mutants had acquired additional maltase genes. The alternative methods described in this section avoid these difficulties and are easily adapted to the isolation of specific classes of control mutants (e.g., temperature sensitives).

Characteristics of the Constitutive Mutants

Application of the α-methylglucoside Method to Strains Carrying MAL 1

A selection for α-methylglucoside temperature sensitive mutants was carried out with strain C 219 (MAL 1/ mal 1 MGL 1/mgl 1). One spontaneous (69) and five NTG induced mutants (6,10,50,60,77) were obtained after NTG mutagenesis. All ferment α-methylglucoside at 25 C° but not at 35 C°. Fermentation begins on day 5 and was complete by day 7 at the permissive temperature. Mutant 6 was very tight, but some others (e.g., 17) were leaky and exhibited a fermentation at the restrictive temperature after 13 days. As we shall see, these mutants were constitutive for maltase.

Characterization of the Parent Strain C 219

Strain C 219 is an α-methylglucoside negative diploid derived from a cross between 1323-1B, a flocculant strain carrying MGL 1, and 210-6C, a strain which derives its MAL 1 ultimately from 7972-6C. An added complication is that 7972-6C exhibits a very slow and very weak sucrose fermentation. In order to investigate the relationship between maltase constitutivity and sucrose fermentation, it was necessary to determine if this weak sucrose activity was due to maltase and not to invertase. Toward this end, C 219 was dissected. Maltose fermentation was rapid and segregated 2:2, indicating that single maltase gene was involved. The flocculation character segregated 2:2 and seemed linked to the mal 1 locus. This probably indicates that this character

has a different basis from the flocculation observed in 1323-1B. Sucrose fermentation was vigorous and complete after, at most, 48 hours. This initial fermentation segregated 2:2 in all 10 tetrads. After about two weeks a weak secondary fermentation appeared.

Characteristics of the Mutants Derived from C 219

Mutants 6,50, and 17 were sporulated and dissected. Only strain 50 gave four spored tetrads. Both strain 6 and 17 gave exclusively two viable spores per tetrad.

Eleven complete tetrads were obtained from mutant 50. Maltose fermentation segregated 2:2 in 10 tetrads, with one tetrad showing a 3:1 segretation. In this aberrant tetrad all four nutritional markers and the mating type segregated normally. Sucrose fermentation was complete after three days in two spores of each tetrad. Upon subsequent incubation, however, a vigorous sucrose fermentation evantually occurred. After two weeks the final segregation ratios were: 2 (4:0), 6 (3:1), and 2 (2:2). Since this is essentially a 1:4:1 ratio, it indicates a two gene segregation for sucrose fermentation. Separating the slow sucrose fermentation from the rapid one and ascribing to the latter a classical unlinked sucrose gene (the segregation ratios indicate no linkage to the MAL 1 gene), we can determine if any linkage exists between the MAL 1 locus and the slow sucrose fermentation. None of the sucrose negative spores in the 3:1 and 2:2 tetrads (Suc:suc) was maltose positive.

Let us assume, then, that the slow sucrose fermentation is linked to the MAL 1 locus. If this is the case, the following tetrads are expected, with Su^{fast}, or SUC^{f}, denoting the classical

unlinked surcrase (invertase), and SUC^{slow}, or SUC^{s} the slow sucrose gene. We then have:

PDT	MAL SUC^{s}	SUC^{f}	MAL SUC^{f} : MAL suc	2:0
	MAL SUC^{s}	SUC^{f}		
	--------	----		
	--------	----		
NPD	MAL SUC^{s}	----	MAL SUC^{f}: Mal suc	2:0
	MAL SUC^{s}	----		
	--------	SUC^{f}		
	--------	SUC^{f}		
T	MAL SUC^{s}	SUC^{f}	MAL SUC^{f} : MAL suc	1:1
	MAL SUC^{s}	----		
	--------	----		
	--------	SUC^{f}		

If fermentation occurring within two days is considered to be due to the presence of the fast surcrase SUC^{f}, then the above ratios are (for mutant 50) PD:NPD:T (2:2:6). In the above analysis we assume complete linkage between MAL 1 and the slow sucrose fermentation. Furthermore, we should expect that the sucrose negative spores in the PD types would remain so, that the PD types would exhibit no slow sucrose fermentation in the original sucrose negative spores; that the NPD types would become 4:0 for sucrose fermentation; and that the T types would become 3:1 for sucrose fermentation. This is precisely what is observed.

Enzymatic Analysis

The temperature sensitive, α-methylglucoside fermenting

mutant 2-50-4 was grown in Cat-Glu and assayed for maltase and α-methylglucosidase. Table 18 shows that the PNPGase activity was higher in the mutant than in the wild type (C219).

The diploid 2-50-4 was sporulated and dissected. Each spore was grown in 100 ml of Cat-Glu and assayed for PNPGase (Table 19). Maltose fermentation segregated 2:2, indicating that only a single maltose gene was present. Every maltose positive spore was constitutive (For wild type levels see Table 14). The constitutive character is therefore linked to the MAL 1 locus. Every maltose positive spore also ferments sucrose within two weeks; this, however, is also true of the parent C 219. The constitutive strain is still subject to glucose repression (Table 14).

Dominance Relations

The MAL 1 constitutive strain 27-11C (MAL 1C) was crossed to strains 1412-4D (MAL 3), 5-3B (mal 1-8), 1300-14B (mal), and 27-1A (MAL 1C). The diploids were purified, grown in various media, and assayed. Table 20 presents the results of this assay. Although slightly elevated basal levels are observed in each of these crosses (compared to wild type MAL 1), the levels of PNPGase are significantly lower than those of the constitutive haploid 27-11C. Constitutivity is therefore recessive to inducibility.

Alphamethylglucoside Fermentation in Mutants Derived From C219

Alpha-methylglucoside fermentation was scored in the segregants of mutants 6, 17, and 50 at the permissive temperature of 25 C°. Surprisingly, only two spores out of 88 fermented α-methyl-

glucoside. This is certainly lower than expectations based upon a two or three gene complementation, assuming nonlinkage of the factors involved. For example, a three gene model would predict 2:2, 1:3, and 0:4 segregations in the ratio of 1:16:19. There are several possibilities:

1. More than three genes are involved in α-methylglucoside complementation in this strain. While this possibility cannot be logically eliminated, the genetic analysis needed to settle it is prohibitively tedious.

2. Linked genes are involved in complementation. The genes would have to be present in the diploid in repulsion since, otherwise, the number of fermenting spores would be increased over the observed values. The genes MGL a, b, c are not linked to one another (ten Berge, personal communication). Neither are MGL 1, 2, or 3. MGL 1 segregates independently of MAL 1 as well (Eaton, unpublished). Since both α-methylglucoside fermentors are maltose positive and since the α-methylglucoside positive mutants are also constitutive for the MAL 1 maltase, one would expect that this gene plays a role in the observed complementation. Let us assume then, that two of the genes involved in the complementation are the constitutive MAL 1 gene and a gene trans to MAL 1 donated by strain 1323-1B to the diploid C 219. One tetrad of C27 (C 219, mutant 50), tetrad 2, exhibiting an aberrant 3:1 segregation for maltose fermentation, allows us to test this hypothesis. The following is the tetrad in which this unusual segregation is observed:

	ur	lys	ad	leu	Gly	mating type	Mal	MG
2A	+	0	+	0	+	α	0	0
2B	0	+	0	+	+	α	+	0
2C	+	+	+	+	+	a	+	+
2D	0	0	0	0	+	a	+	0

The mating type locus and four independent nutritional markets showed a normal 2:2 segregation, so the tetrad can be considered to have arisen by gene conversion at, or near, the MAL 1 locus. Spore 27-2C was the only spore in this cross that fermented α-methylglucoside (out of 44 spores). This spore fermented maltose and had a constitutive level of PNPGase (1357, after growth on CAT-Glu).

Let us assume, then, that two of the genes involved in the complementation for α-methylglucoside fermentation are the constitutive MAL 1 gene and a gene trans to MAL 1 donated by 1323-1B. If this is the case, and assuming that any other genes participating in the complementation are being contributed by 1323-1B as well, then the α-methylglucoside fermenting spores should show an increased frequency of α-methylglucoside positive spores in a backcross with 1323-1B. Spore 27-2C was crossed to 1323-1B (C 55). Seven tetrads were scored for maltose and α-methylglucoside fermentation. Every segregant which fermented a α-methylglucoside fermented maltose (Table 21). The segregation pattern of α-methylglucoside fermentation was consistent with the pattern expected for two independent complementing genes, with PD : NPD : T ratios of 2:1:4 compared to the theoretically expected 1.2:1.2:4.6. Two gene segregations in this case can be interpreted in the sense of a gene complex at the MAL 1 locus and an independently segregating, complementing gene. In this interpretation the conversional event at the MAL 1 locus coupled two genes involved in -methylglucoside

fermentation which were present in repulsion in the diploid. Alternatively, the apparent two gene ratios, in comparison to the ratios involved in C 27, where very few α-methylglucoside positive spores were recovered, could be due to the homozygosity of unmutated genes contributed by 1323-1B. This later interpretation is not convincing since all maltose positive spores (of the proper mating type) complemented strain 1323-1B for α-methylglucoside fermentation in cross C 55 (data not shown). Furthermore, this 2:2 pattern was not observed in a cross with the other α-methylglucoside spore isolated from a tetrad where MAL segregated 2:2.

Temperature Sensitivity of α-methylglucoside Transport

Alpha-methylglucoside transport was studied in diploid 2-50-4 (selected by the technique described on p.53) and in a haploid 27-11C derived from it by sporulation. Strain 27-11C is a MAL 1 constitutive. The strains were grown overnight in CAT-Glu and the transport of α-methylglucoside measured as described in Materials and Methods. The wild type parent of 2-50-4, C 219 was included as a control. Figure 6 shows the results of the transport assay. The MAL 1^c constitutive (MAL 1^c/mal 1) mutant 2-50-4 showed a temperature sensitive transport of α-methylglucoside, which, while significantly higher than the parent C 219 at both temperatures, is not as rapid as in 27-11C. The transport in the later strain is also temperature sensitive, and much higher at 25 C°. This result would be expected if the maltase of α-methylglucosidase of the mutant strains was temperature sensitive. If this were the case, the decrease in the transport

of α-methylglucoside would be a reflection of a decrease in utilization of this sugar and not a decrease of transport per se. This is an unlikely possibility, however, as far as the maltase is concerned since the mutant ferments maltose quite rapidly at 35 C°. As an additional check, 2-50-4 and C 219 were grown at 25 C° and at 35 C° and assayed for PNPGase, maltase, and α-methylglucosidase. The results of the assay are shown in Table 18. The levels of maltase and α-methyl-glucosidase are higher after growth at 35 C° than they are at 25 C°, in both the control and mutant; a temperature dependence in a direction opposite to that expected from the above hypothesis. The constitutivity is therefore not temperature sensitive in these strains although the ability to grow on and to transport α-methylglucoside is.

Analysis of 76-C1: (Derived from 76-8D, MAL 1)

Strain 76-C1 was crossed to 1315-2C (MAL 1), sporulated and dissected. All the tetrads were incomplete, so the diploid was analyzed in terms of random spores. One of the constitutive spores R6 was chosen; like the parent 76-C1, it fermented α-methylglucoside as well. Strain 76-8D does not ferment this sugar. Strain R6 was backcrossed to the wild type MAL 1 parent, 76-8D (C10). Diploid C10 was sporulated and eight four spore tetrads scored for maltose fermentation. Maltose fermentation segregated 4:0. The spores of one tetrad were grown in CAT-Glu overnight and assayed for PNPGase. The main purpose of the cross was to isolate a constitutive strain which would pass through crosses easily so that the linkage of the constitutive character could be tested. Surprisingly the assay gave PNPGase values of:

10-2A	1230
10-2B	435
10-2C	1007
10-2D	3

Maltose fermentation segregated 4:0 (MAL:mal), but only spore 2C was α-methylglucoside positive. R6 therefore has at least the original MAL 1 gene but may contain additional genes affecting the level of maltase/alpha-methylglucosidase. Since it was possible that R6 had picked up multiple MAL genes during the serial transfer, two additional crosses were made. Spore 10-2C was crossed to the segregational negative 1300-14B (C24) and dissected. All six tetrads were incomplete and all had only three spores. Two of these three spores were always maltose positive. A spore, 24-3C (maltose politive) was crossed to 1315-2C (C46). This spore had two MAL genes with the probability of .33.

Four complete asci were isolated and tested for maltose fermentation. Maltose segregated 1 (4:0) and 3 (3:1). This demonstrates that 24-2C has a single maltose gene which is not allelic to MAL 1. The same must be true for strain 76-C1. Because of this complication, this method for selecting constitutive mutants was not pursued further. At least one other mutant selected by this procedure had also picked up another maltose positive gene (i.e., a gene nonallelic to the parent).

Maltase Constitutive MZ 48.1

Strain MZ 48.1 was selected from 48-9A (MAL 3) by the Melezitose transfer technique. Table 14 presents the results of assays for PNPGase, maltase, and α-methylglucosidase after growth on various media. The mutant is constitutive for both maltase and α-methylglucosidase. It is still repressible by glucose.

The linkage of the constitutive character to MAL 3 was tested by crossing MZ 48.1 to the maltose negative strain 1300-14B (C36). If the constitutive character is linked to MAL 3, then every maltose fermenting spore should be constitutive. Maltose fermentation segregates 2:2 but constitutivity segregates as a complex character with a deficiency of constitutive spores (data not shown). This cross also shows that no new maltose fermenting genes have been selected by the repeated serial transfers in melezitose.

Sucrose displayed a two gene segregation pattern like the parent 48-9A. Unlike the parent strain, strain MZ 48.1 fermented -methylglucoside. Every α-methylglucoside positive spore ferments maltose as well, suggesting that a gene closely linked to MAL 3 complements a gene contributed by either 1300-14B or MZ 48.1. The 2:2 segregation pattern for α-methylglucoside fermentation indicated either that the complementing gene is homozygous in the cross (i.e., if complementation with MGL 2 is involved, the new gene must be present in MZ 48.1 but not in the parent, 48-9A), or that the mutation has occurred at the MAL 3 locus which results in the ability to ferment α-methylglucoside. These interactions raise the possiblity that the mutation causing the constitutivity of maltase and α-methylglucosidase is linked to MAL 3 but that full constitutive expression depends on other genes.

The transport of -methylglucoside in both wild type and mutant strains is shown in Figure 6. Growth of 48-9A overnight on maltose induces an increased transport of α-methylglucoside. The constitutive strain MZ 48.1 is also constitutive for α-methylglucoside

uptake. The peculiar two phase kinetics seen in the uptake curves when the cells are constitutive or induced by maltose is a constant feature in these experiments. In contrast, the noninduced uptake of the substrate possesses only the first phase and reaches saturation at considerably lower levels. The second phase corresponds either to active transport or utilization of α-methylglucoside. In the experiments depicted in Figure 6 the equilibrium concentration, assuming an internal volume of 1.12 ul per mg dry weight (de Kroon, et. al., 1971) corresponds to 1120 cpm/mg.

Alpha-methylglucoside fermentation in the segregants of C36 as well as in MZ 48.1, is not very rapid despite levels of α-methylglucosidase which are about four times the amount normally found in MGL 2 MGL 3 strains fully induced for the enzyme by growth on α-methylglucoside (e.g. strain 1412-4D, 131nM/min/mg). The constitutive strain has a high level of α-methylglucoside transport, α-methylglucosidase, and the presumed 'transporteur' MGL 2, but this is not sufficient for a rapid fermentation of this sugar.

Dominance Relations

The MAL 3 constitutive MZ 48.1 was crossed to strains having MAL 3, mal 3-7, and mal genes. Table 20 presents the results of the assay performed on purified diploid cells. The dominance relations are the same as those observed in the case of the MAL 1 constitutives. That is, the constitutivity is recessive to inducibility, and the diploids have only slightly elevated levels of PNPGase.

These dominance relations are consistent with the hypothesis that the MAL loci, even in their recessive, noninducible form, produce

a product which is active in repressing the constitutive synthesis of maltase in strains carrying the constitutive allelic form of the MAL genes. The interaction observed is compatible either with repressor synthesis by these genes or with an interference of synthesis due to negative complementation.

The observation that the MAL 1 constitutive 27-11C shows a strict linkage of the constitutive character and the MAL 1 locus implies that it does not interact with the nonallelic negative forms of the other mal alleles, namely, mal 2 mal 3 mal 4 mal 5 mal 6 etc. present in the cross. Since all the mal 6 alleles, for example, that have been tested also produce an active product (ten Berge, 1974; this laboratory), the implication is that the MAL 1 and MAL 6 alleles are functionally different. The irregular segregation pattern observed with strain MZ 48.1 and with a constitutive MAL 4 allele (1403-7A, unpublished observations) may be due to interaction of these alleles with the products of other mal genes.

The -methylglucoside Selection Method applied to Strain 119-16

Twelve mutants capable of fermenting α-methylglucoside were derived from the α-methylglucoside negative strain 119-16 (MAL 2). A lypholization assay showed that eight of these mutants had elevated levels of PNPGase. In addition, most were very flocculent, grew poorly on glycerol, and either did not mate readily or failed to sporulate. After extensive trials in which the PNPGase constitutives derived from 119-16 were crossed to stock strains, one strain (119a4) capable of mating and sporulating was found.

Characterization of 119a4

The enzymatic analysis of this mutant shows that the elevated levels of PNPGase are due mainly to high levels of α-methylglucosidase and not to constitutive levels of maltase (Tabel 14). The mutant also exhibits a high degree of resistance to glucose repression. The parent, 119-16, complements strain E58 (mal⁻ MGLa mglb mglc) for α-methylglucoside fermentation rapidly (3 days), but complements strain E59 (mal⁻ mgla mglb mglc) slowly (10 days). It does not complement strains T4 (mal 6-13 mgla MGLb MGLc) or E65 (mal⁻ mgla mglb mglc). It therefore has a gene equivalent in function to MGLb MGLc. When the mutant 119a4 was crossed to strain T4 (see above), the diploid failed to ferment α-methylglucoside. The mutant gene(s) is therefore recessive for α-methylglucoside fermentation. Diploid C 30 (119a4xE59) was assayed after growth on Cat-Glu, and had a PNPGase activity of 47. The diploid T4x 119a4 had a PNPGase level of 12. The constitutive production of α-methylglucosidase was therefore recessive as well.

The phenotype of 119a4 is similar to that of a mutant discovered by A.M.A. ten Berge (personal communication). This mutant called, flk, is flocculent, grows poorly on glycerol, mates poorly, and in the presence of the gene mal 6-13 shows a partially constitutive level of maltase and α-methylglucisidase. The mutant has a pleiotropic effect and renders several enzymes partially insensitive to glucose repression. Despite high levels of maltase, mal 6-13 flk strains are unable to grow on maltose. The mutant is recessive and is not centromere linked. In addition the mutant has a greatly increased rate of α-methylglucoside uptake. Since the flk mutant so closely resembled the 119a4 strain, several tests were carried out to ascertain whether

the mutants were allelic.

A cross was made between 119a4 and 1926-4B (mal 6-13 flk). The diploid was assayed after growth on Cat-Glu. It had PNPGase values of 41, compared with control values of 453 for 119a4 and 650 for 1926-4B. This result indicates that strain 119a4 carries the dominant allele Flk and further illustrates that the α-methylglucosidase constitutivity is recessive. Diploid 119a4xE59 (C30) was sporulated and dissected. The flocculent character noted in 119a4 did not segregate 2:2, but gave a great excess on nonflocculent spores-showing that this character depends on more than one gene. The segregation of the enzyme levels was equally complex with seven complete tetrads yielding only two constitutive spores. When the tetrads were scored for α-methylglucoside fermentation, the ratios were 2:2 after three days but after seven days additional slow fermentors appeared. The lack of a clear cutoff point for scoring fermentation in this cross makes further genetic analysis unproductive.

Although the results obtained with this mutant are only preliminary, they are included here because the existence of specific α-methylglucosidase constitutive mutants is extremely important in dissecting the relationship between the maltose and α-methylglucoside system. The nature of the mutation is still unclear. The tests described above indicate that it is not an flk mutant although its phenotype is similar. The mutant is recessive and may be a mutation in a repressor for α-methylglucosidase synthesis. When temperature sensitive mutants for α-methylglucoside fermentation were isolated from strain 1071-3B (mal$^-$ MGL 2), all 15 were found to ferment at 35 C but not at 25 C. Once again these mutants were difficult to mate, and

where this was possible failed to sporulate. They were also flocculent at the permissive temperature. The direction of the temperature sensitivity also suggests a repressor type control. The poor mating ability exhibited by the strains f1k, 119a4, and 1071 t.s. is not understood, but a similar behavior has been reported by Mountecourt (1968) for a strain FH4C which exhibits an insensitivity to catabolic repression. It is possible that the α-methylglucosidase system plays a role in the synthesis of polysaccharides for the cell wall and does not usually function in a catabolic manner. Since sucrose is the only "natural" sugar substrate for the enzyme, a synthetic function for the enzyme is quite possible. In this case the increase in α-methylglucosidase synthesis observed in these strains may be directly related to an altered wall composition with subsequent self agglutination, as observed in all of the above strains. The relationship may be indirect in the sense that all of the above mutants have a primary defect in the glucose repressibility of a system of enzymes concerned with cell wall synthesis.

Because of the complexities of the α-methylglucosidase system (genes involved include MAL 1, MAL 6, MGL 1, MGL 2, MGL 3, MGLa, MGLb, and MGLc) and the difficulty of crossing the mutant, no further studies were conducted. Since the genes MGLa, b, and c were only recently discovered, no tester strains are available which are completely characterized for all MGL genes. Once this is accomplished, the major question is then more easily posed: Is the mutant allelic to any of the known MGL genes? It is hoped that some of the spores derived from the above cross will exhibit improved mating ability so that this question may be answered.

Summary of Mutants Isolated from Various MAL strains

MAL 1

Twenty-four negative mutants were isolated. Maltase was not inducible in any of these strains. These mutants could be divided further into two categories: those allelic with MAL 1 and those unlinked to this gene. The latter mutants complemented every maltose negative strain. The former mutants did not complement segregational negatives nor did they complement each other.

Several constitutive mutants were isolated by a variety of methods. All were allelic to MAL 1. In all, constitutivity was recessive to inducibility. Temperature sensitive mutants for the utilization of maltose were also isolated. These were allelic to MAL 1. At the restrictive temperature inability to ferment maltose could be suppressed by an unlinked mutation X. These genes, while allowing the utilization of maltose, did not lead to the inducibility of the enzyme. Gene X affected utilization/transport of maltose. These observations suggest that MAL 1 is a regulatory locus which controls the inducibility of maltase and the maltose permease.

MAL 2

Using the αMG selection technique, several PNPGase constitutives were selected. These were not constitutive for maltase but were constitutive for MGase. They were relatively resistant to glucose repression. Inducibility was dominant to constitutivity.

MAL 3

A large number of negative mutants were isolated. Several were allelic to MAL 3. An unusual feature was that several had defects

in two closely linked genes, MGL2 and Suc 3.

Two constitutive mutants were isolated by the MZ and Phenylglucoside technique. Both were allelic to MAL 3. One mutant MZ 48.1 produced unusually high levels of αMGase, as well as maltase. In both mutants the oonstitutivity was recessive to inducibility and to noninducibility.

MAL 6

Using a specialized starvation selection technique, several constitutive mutants were isolated. All were allelic to MAL 6. The single mutant selected by the MZ method was unlike any maltose constitutive previously isolated. In this mutant, constitutivity was dominant to inducibility. Unlike the other constitutive mutants at this locus, this mutant was not suppessed by mal x.

Discussion

Nature of the Control

A model for the control of maltase synthesis must be able to account for the following observations:

1. Noninducible mutants are recovered and these are recessive to the wild type (observed for MAL 1,2,3,4 and 6). These mutants map at the MAL loci.

2. Most constitutives are recessive to the wild type (MAL 1, 3, 4, and 6). The exception is MAL 6 C19.

3. Constitutive mutants are constitutive for maltose and/or α-methylglucoside transport.

4. Diploids constructed from noninducible and the majority of constitutive strains (MAL 6 C19 is the exception once again) while inducible for maltase synthesis (MAL 1, 3, 4 and 6) have elevated basal levels of maltase.

These are the general observations for the genes studied in this thesis. In addition, certain special features of the individual systems have to be explained:

5. Revertants of MAL 6 are generally constitutive but revertants of MAL 1 are inducible.

6. The suppression of most MAL 6 constitutives (save MAL 6 C19) by mal x leads to a noninducible phenotype, but the MAL 1 constitutives are not suppressible by mal x.

The second observation eliminates any model based on the

assumption that the MAL genes code for a protein which has only an activator function. If this were the case, then the constitutives should be dominant since the wild type regulatory gene product has, in the absence of inducer, no function. The possibility of interallelic complementation does not alter this reasoning since at least some activators, fully active in the absence of inducer should be obtained. We are therefore led to consider models in which the regulatory protein has both a repressor and an activator function.

The noninducible phenotype can arise in negative control models by an alteration in the repressor which either increases its affinity for the operator or decreases its affinity for the inducer. (The term "operator" is used in the restricted sense of being the binding site for the repressor protein and does not imply that operons exist in yeast.) If this were the case for the MAL system, one would expect a reduction of basal and induced levels in noninducible/inducible diploids. This does not occur (Table 20). The noninducible mutants are not simply mutants of the i^s type observed in the Lac system (Beckwith, 1970).

The evidence presented in this thesis does not lead to any simple picture of the regulation of maltase synthesis. Models similar to the arabinose model of Engelsberg (Engelsberg, 1971) approximate the observations most closely, but fail to explain the prevalence of constitutive revertants for MAL 6 and the ability of the mal x gene to suppress maltose fermentation. It is more useful to talk about the critical experiments that can be done to decide the nature of the control.

The crucial question is the behavior of the system when the maltose control gene is deleted. This allows an unambiguous determination of its necessity for the induction process. In a positive control model, even where the activator is necessary but not sufficient for induction (i.e., in the arabinose system), a deletion of the control gene must lead to noninducibility of the system. In a negative control system, deletion must lead to constitutivity. Specialized techniques for generating deletions exist in the MAL 6 system; for the other alleles the isolation of nonsense mutations can serve the same purpose.

A genetic map of the constitutive and noninducible mutants for each MAL locus will also be of great value in elucidating the nature of the control. Since both dominant and recessive constitutive mutants map closely, it is necessary to determine if two distinct loci are involved. In addition a genetic map will provide an estimate of the size of each MAL locus. Once again, specialized techniques must be used for the mapping of the constitutive mutants since inducible recombinants must be positively selected from a large population consisting of the parental constitutives. These specialized techniques are available for this system--one of the major goals of this thesis has been the development of efficient selection methods for the isolation of mutants, and two methods have been developed which allow the mapping of the MAL genes. One of these uses the substrate allyl -glucoside (data not presented). Constitutive cells do not grow in the presence of this sugar, but inducible cells do grow on maltose if both are present. Its hydrolysis by maltase leads to the formation of allyl alcohol which is then metabolized to acrolein by alcohol dehydrogenase.

The latter compound is very toxic for yeast.

A more specialized technique is available for the mapping of the constitutives in MAL 6. Strains may be constructed that are heterozygous for two constitutive mutants but homozygous for mal x. These diploids will not grow on maltose. Inducible recombinants will, however, grow since these are not affected by the presence of mal x.

We have presented "circumstantial" evidence in this thesis that the genes MAL 1, 3, and 6 are not structural genes for maltase. It is still possible that they have closely linked structural genes. If they do, then the enzymological data presented suggests that the enzymes are very similar in all of their properties.

The maltase system represents one of the few systems available in eucaryotic organisms where fine structure genetic punctuation may be investigated.

Table 1

Strains and their Genotypes

76-8D	a leu1 thr1 ura ade8 MAL1
1315-2C	α his1 trp met ade2 MAL1
119-16	a his4 MAL2
D8-5A	α MAL2
1412-4D	a ade2 MAL3 MGL2 SUC3 MGL3
48-9A	α gal3 trp1 MAL3 MGL2 SUC3 SUCx
1359-11D	a gal4 his1 leu1 arg4 MAL3
1394-9B	α gal3 gal4 his2 ade8 leu1 lys2 MAL4
1403-7A	a gal3 gal4 trp1 ura3 MAL4
144-22C	a his thr ura leu lys MAL4
645-1A	a MAL5
S.C.-B 26	α ura MAL6
N.Y.C. 74	α MAL6 MGLa MGLb MGLc
E58	α mal MGLa mblb MGLc
T4	α mal6-13 trp5 mgla MGLb MGLc
E56-C2	α ade1 ura MAL6C2 MGLa MGLb MGLc
11339	α MZ
1323-11B	α ura1 lys1 mal MGL1
1071-3B	α gal3 trp1 mal MGL2
122-1C	a leu lys ura ade2 MGL1
1300-14B	a gal4 his4 ura2 mal suc amg
M13-X-C	a mal6-13 malx mgla MGLb MGLc trp5
99C	α mal

Table 2

Purification of Maltase from Strain 76-8D(MAL1)

	Sp. Act.	Total prot.	Total units$\times 10^{-6}$	Purification
Crude	2100	450 mg.	0.94	1
G-100	29,000	9.5 mg.	0.28	14
Hydxlap.	120,000	0.28 mg.	0.034	57

Maltase was isolated as described on p. 14. The hydroxylapatite fraction listed was the peak tube. Total recovery in the maltase peak after this step was over 65% of the units applied. The units are PNPGase: nM/min/mg.

Table 3

Affinity of Maltase for other Sugars
Ki x 10^3

Sugar	MAL1	MAL2	MAL3	MAL6
Maltose	27	15	21	15
AMG	23	15	35	18
Sucrose	19	15	20	20
MZ	35	13	13	17
Turanose	6	3	5	5
Mannose	23	28	34	19
2DG	62	47	51	43
Glucose	3	2	2	2
Fructose	95	82	91	91
Raffinose	21	14	-	-

PNPG hydrolysis was measured with at least four different concentrations of PNPG in the prescence of a constant concentration of sugar(0.1-0.01M). All inhibitors showed competitive kinetics. The Ki values were calculated according to the method of Massart (1950).

Table 4

Activity of Maltase towards other Substrates

Enzyme source	MAL/PNPG	αMG/PNPG	TUR/PNPG	PNPG
MAL1	0.68	0.014	1.1	110
MAL2	0.76	0.015	1.3	150
MAL3	0.77	0.026	1.1	133
MAL6	0.73	0.021	1.2	163

Purified maltase was assayed with four substrates: PNPG, Maltose, α-methylglucoside, and Turanose. The relative rates of hydrolysis and the absolute activity of the particular enzyme preperation are given.

Table 5

Complementation for Maltose Fermentation

Mutant	1315-2C(MAL1)	1071-3B(mal)	1315-2B(mal)	E4B(DSF)
76-8D	+	+	+	+
76-3	+	0	0	0
76-4	+	+	+	+
76-5	+	0	0	0
76-6	+	+	+	+
76-7	+	+	+	+
76-8	+	0	0	0
76-9	+	0	0	0
76-16	+	0	0	0
76-17	+	0	0	0
76-19	+	0	0	0

Table 6

Maltose Fermentation (1315-2C x 76-X)

X	No. of Tetrads	Mal : mal
3	5	2:2
5	19	2:2
8	10	2:2
9	4	2:2
16	5	2:2
17	Poor spore viability	Consistent with 2:2
19	7	2:2

Table 7
Enzyme Levels in Wild Type and mall Mutants

Strain	Medium	PNPGase	Maltase
76-8D	YEPM	1200	52
	Cat-Glu, Cat-Gal	117	10.7
	YEPD	14	1.5
76-3	Cat-Gal	3.2	-
76-4	"	11.0	-
76-5	"	14.0	7.0
76-6	"	11.7	-
76-8	"	3.5	0.7
76-9	"	4.9	0.5
76-16	"	5.1	0.8
76-17	"	6.2	0.8
76-19	"	3.7	0.4
76-3	Mal+Glu	5.3	0.7
76-5	"	18	1.4
76-6	"	7.1	1.2
76-8	"	9.3	1.3
76-9	"	11.7	1.3
76-16	"	9.1	1.4
76-17	"	8.2	0.9
76-19	"	5.3	1.1

Table 8

Comparison of Partially Purified Maltases from Maltase Negative Strains(76-8,76-9) and the Pure Wild Type Enzyme(76-8D)

Strain	Km(PNPG)x10^4	Km(Maltose)x10^3	Half-life 51C°	$\frac{\alpha MG}{PNPG}$
76-8D	2.5	27	2.2 min.	.014
76-8	2.0	29	1.9 min.	.019
76-9	2.1	23	2.3 min.	.011

The Km values were measured as described in the legend to Table 3. Heat inactivation was conducted in 0.05M Potassium phosphate buffer, pH 7.4 containing Bovine serum albumin(0.1%).

Table 9

Enzyme Levels of Revertants

Revertant	Derived from	Media	Growth Temp.	PNPGase
76-8-1A	76-8	Glu	25	18.7
		Glu	35	11.1
		Mal+Glu	25	2.7
		Mal+Glu	35	3.1
76-8-R3	76-8	Glu	25	8.2
		Mal+Glu	26	10.5
76-8-R5	76-8	Glu	25	8.1
76-8-3B	76-8	Glu	25	0.8
76-8-R2	76-8	Glu	25	2.8
76-8-R100	76-8	Glu	25	2.3
76-8-R102	76-8	Glu	25	12.1
76-16-R1	76-16	Glu	25	14
		Glu	35	10
76-19-R3	76-19	Glu	25	1.5

Strains were grown overnight(17 hours) in CAT media with either Glucose(Glu,0,3%) or Maltose and Glucose(2% Maltose and 0.3% Glucose, Mal+Glu) at the indicated temperature. Extracts were prepared and the enzymes assayed as described in Materials and Methods.

Table 10

Maltose Fermentation in Segregants of C72(76-8-1A x 1315-2C)

Maltose Fermentation 25C°/35C°

1A	+/+	5A	+/0
B	0/0	B	+/+
C	0/0	C	+/+
D	+/+	D	0/0
2A	+/+	6A	+/+
B	+/0	B	+/+
C	+/0	C	0/0
D	+/+	D	0/0
3A	+/+	7A	0/0
B	0/0	B	+/+
C	+/0	C	+/+
D	+/+	D	0/0
4A	+/+		
B	+/0		
C	0/0		
D	+/+		

Table 11
Maltose and Sucrose Fermentation in Segregants of C11(48-8x1359-11D)

	Mal	Suc		Mal	Suc		Mal	Suc
1A	+	+	32A	+	+	39A	+	0
B	0	+	B	0	0	B	0	+
C	+	+	C	0	+	C	0	+
D	+	0	D	+	0	D	+	0
2A	+	+	33A	0	0	40A	0	+
B	+	+	B	+	+	B	+	+
C	+	0	C	0	+	C	+	0
D	+	0	D	+	+	D	0	0
3A	+	+	34A	+	+	41A	0	+
B	+	+	B	0	0	B	0	0
C	+	0	C	+	0	C	+	+
D	0	0	D	0	+	D	+	0
4A	0	0	35A	0	0	42A	+	0
B	+	0	B	+	0	B	0	0
C	+	0	C	0	+	C	0	+
D	+	+	D	+	+	D	+	+
5A	+	+	36A	+	0	43A	+	+
B	0	+	B	+	+	B	0	+
C	0	0	C	0	0	C	+	0
D	+	0	D	0	+	D	0	0
30A	+	0	37A	+	0	44A	+	+
B	+	+	B	+	+	B	0	0
C	0	0	C	0	+	C	+	+
D	+	+	D	0	0	D	+	0
31A	0	0	38A	0	0			
B	+	0	B	+	0			
C	0	+	C	+	+			
D	+	+	D	+	+			

Table 12

Maltose Fermentation as a Function of Time(C48,11-40Dx1359-11D)

	Mal(2 days)	Mal(10 days)	Mal(18 days)
1A	0	0	0
B	+	+	+
C	+	+	+
D	0	+	+
2A	0	0	+
B	0	+	+
C	+	+	+
D	+	+	+
3A	0	+	+
B	+	+	+
C	+	+	+
D	0	+	+
4A	0	0	0
B	+	+	+
C	+	+	+
D	0	+	+
5A	+	+	+
B	+	+	+
C	0	+	+
D	0	+	+
6A	+	+	+
B	0	0	+
C	+	+	+
D	0	0	+
7A	+	+	+
B	+	+	+
C	0	+	+
D	0	0	0
8A	+	+	+
B	0	0	0
C	0	0	0
D	+	+	+
9A	0	+	+
B	+	+	+
C	+	+	+
D	0	+	+

Table 13

Complementation between Mutants derived from 48-9A and MGL1

	Fermentation of α-methylglucoside
48-1	+
3	+
4	0
7	+
8	+
10	0
11	+
13	+
15	0
17	0
18	+
23	0
24	+
28	0
29	0
35	+
42	+
44	+

Mutants not listed did not cross with 122-1C(MGL1)

Table 14

Enzyme Levels of Constitutive Mutants

Strain	Genotype	Parent	Selection	Medium	PNPGase	M'ase	MG'ase
45-5C	MAL1	C219 (seg)	-	YM	2600	156	50
				CatGlu	85	2	4
				YD	12	1	1
27-11C	MAL1	C219 (seg)	MG	YM	1942	85	45
				CatGlu	721	28	12
				YD	85	3	3
119-16	MAL2	-	-	YM	807	51	84
				CatGlu	9	-	-
				YD	2	-	-
119a4	MAL2	119-16	MG	YM	1752	82	510
				CatGlu	573	8	329
				YD	222	7	180
48-9A	MAL3	-	-	YM	1626	110	40
				CatGlu	13	1	4
				YD	6	2	-
MZ48.1	MAL3	48-9A	MZ	YM	2223	116	452
				CatGlu	3159	193	400
				YD	15	1	6
TH1	MAL6	-	-	YM	1800	75	41
				CatGlu	85	5	5
				YD	15	2	3
MAL6C19	MAL6	TH1	MZ	YM	6400	169	1130
				CatGlu	4500	168	520
				YD	430	44	-

MG: α-methylglucoside selection; MZ: melezitose selection

Table 15
Fermentation in Segregants of C60(1300x MZ 3)

	MZ	MAL	SUC	αMG
1A	0	+	+	+
B	0	+	+	0
C	0	0	0	+
D	+	+	+	+
2A	0	+	+	0
B	+	+	+	+
C	0	0	+	+
D	0	0	0	0
3A	+	+	+	+
B	0	+	+	0
C	0	+	+	+
D	0	0	0	0
4A	0	0	0	0
B	0	+	+	+
C	0	0	+	+
D	+	+	+	0
5A	0	+	+	+
B	0	+	+	+
C	0	0	0	0
D	+	+	+	+
6A	+	+	+	+
B	+	+	+	0
C	0	+	0	0
D	0	+	+	+

Table 16

Fermentation in Segregants of C76(60-2B x 1300)

	MZ	MAL	SUC
1A	0	0	0
B	+	+	+
C	0	0	0
D	+	+	+
2A	+	+	+
B	0	0	0
C	+	+	+
D	0	0	0
3A	+	+	+
B	0	0	0
C	0	0	0
D	0	+	+
4A	0	0	0
B	0	0	0
C	0	+	+
D	+	+	+
5A	0	0	0
B	+	+	+
C	0	0	0
D	0	+	+
6A	+	+	+
B	+	+	+
C	0	0	0
D	0	0	0

Table 17

MAL6 Constitutives Selected by Starvation:
PNPGase after Growth on Various Media

Mutant	YEPM	CAT-Glu	YEPD
MAL6-C14	1840	1790	20
MAL6-C15	1630	1750	14
MAL6-C16	1305	1470	25
MAL6-C17	1900	1980	15
MAL6-C18	1650	1850	17

Table 18

Enzyme Levels after Growth on CAT-Glu

Strain	Growth temp.	PNPGase	Maltase	αMGase
C219	25	28	10	3
	35	145	30	20
2-50-4	25	96	26	16
	35	455	105	50

The concentration of maltose in this assay was 10mM. The concentration of MG was 50mM. All units are in nM/min/mg of substrate.

Table 19

PNPGase Levels in Segregants of C27 after Growth on CAT-Glu

	Mal ferm.	PNPGase		Mal ferm.	PNPGase
1A	0	42	8A	+	1210
B	+	1430	B	+	790
C	+	910	C	0	37
D	0	30	D	0	32
2A	0	36	9A	0	43
B	+	750	B	0	20
C	+	650	C	+	810
D	+	932	D	+	971
3A	+	790	10A	+	1210
B	+	1560	B	+	1571
C	0	55	C	0	25
D	0	41	D	0	41
4A	+	890	11A	+	1010
B	+	950	B	0	49
C	0	30	C	+	760
D	0	43	D	0	36
5A	0	31			
B	0	56			
C	+	1150			
D	+	1400			
6A	0	29			
B	0	47			
C	+	810			
D	+	975			

Table 20

Dominance Tests with the Constitutive Mutants

Strain	Genotype	Growth Medium	PNPGase	Maltase
27-11C	MAL1C	YM	1760	75
		CAT-Glu	890	34
27-11C	MAL1C	YM	1751	74
x	x	CAT-Glu	210	8
1412-4D	MAL3			
27-11C	MAL1C	YM	1853	81
x	x	CAT-Glu	173	7
C5-3B	mal1-8			
27-11C	MAL1C	YM	1753	73
x	x	CAT-Glu	253	10
1300	mal			
27-11C	MAL1C	YM	2110	55
x	x	CAT-Glu	1210	55
27-1A	MAL1C			
1412-4D	MAL3	YM	1891	61
		CAT-Glu	51	1
MZ48.1	MAL3C	YM	3570	156
		CAT-Glu	4590	205
MZ48.1	MAL3C	YM	2215	-
x	x	CAT-Glu	1973	-
1412-4D	MAL3			
MZ48.1	MAL3C	YM	1962	-
x	x	CAT-Glu	310	-
7-1A	mal3-7			
MZ48.1	MAL3C	YM	2154	-
x	x	CAT-Glu	273	-
1300	mal			

Table 21

Fermentation of Maltose and α-Methylglucoside in Segregants of Cross C55

C55-27-2C x 1323-1B
(Maltose ferm./α Methylglucoside ferm.), 25C°

1A	+/0	5A	+/+
B	0/0	B	0/0
C	0/0	C	0/0
D	+/0	D	+/0
2A	+/0	6A	0/0
B	0/0	B	+/+
C	0/0	C	0/0
D	+/+	D	+/+
3A	0/0	7A	+/+
B	0/0	B	0/0
C	+/+	C	+/+
D	+/0	D	0/0
4A	+/0		
B	+/+		
C	0/0		
D	+/0		

Figure 1
Gel Filtration of Crude Extract on G-100

Crude extract from strain NYC 74 was prepared as described in the text and applied to a 70 x 2.5 cm. column of Sephadex G-100. Five ml volumes were collected. The single peak represents PNPGase activity, and the specific activity of these fractions is given in nM/min/mg.

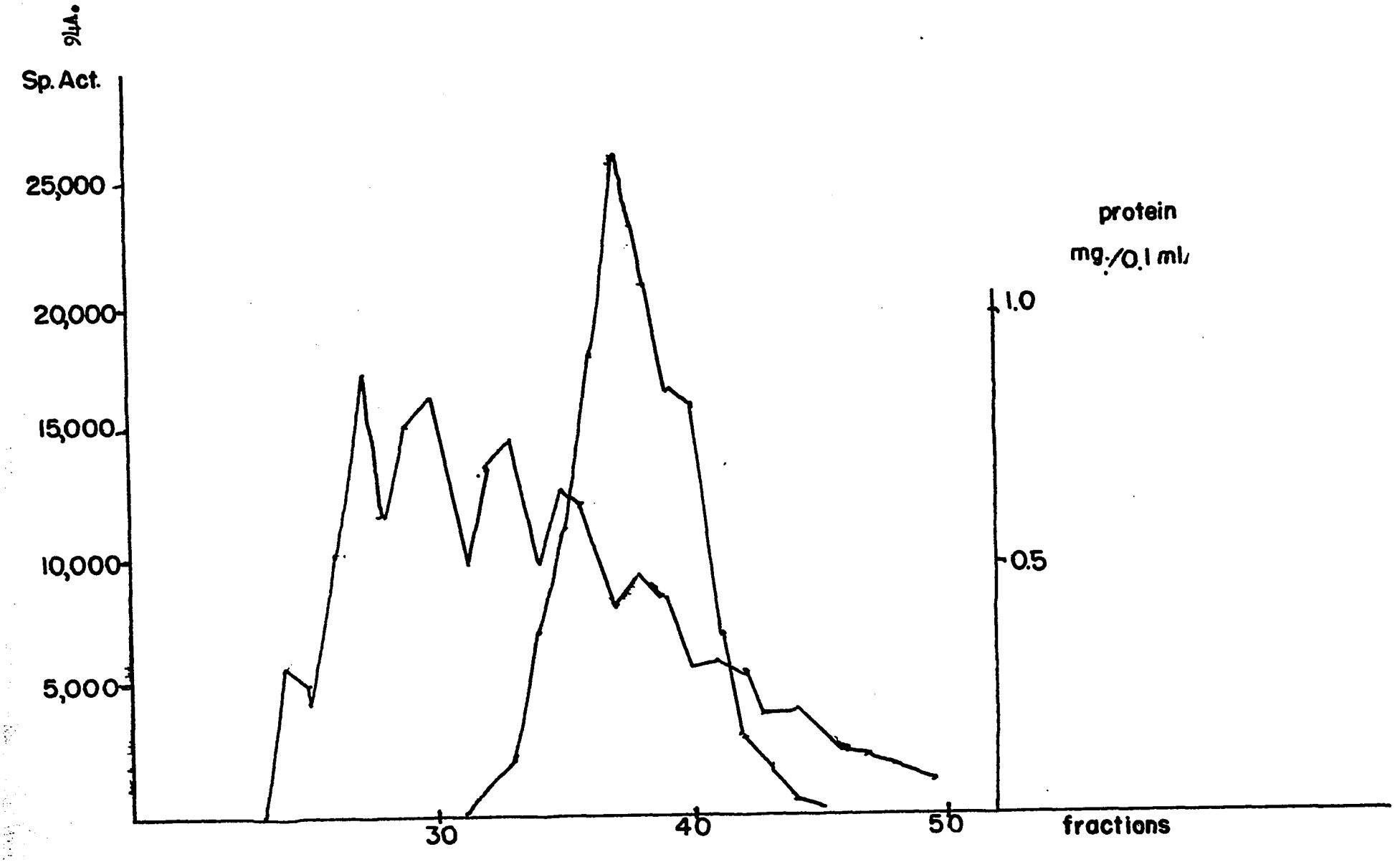

9μA.
Sp. Act.
25,000
20,000
15,000
10,000
5,000
protein
mg./0.1 ml.
1.0
0.5
30
40
50
fractions

Figure 2

Fractionation of G-100 peak on Hydroxylappatite

The peak tubes were combinded and applied to a hydroxylappatite column. Elution was accomplished with a 0.05M-0.5M phosphate gradient. Volumes of 3 mls were collected. For additional detqils, see the text.

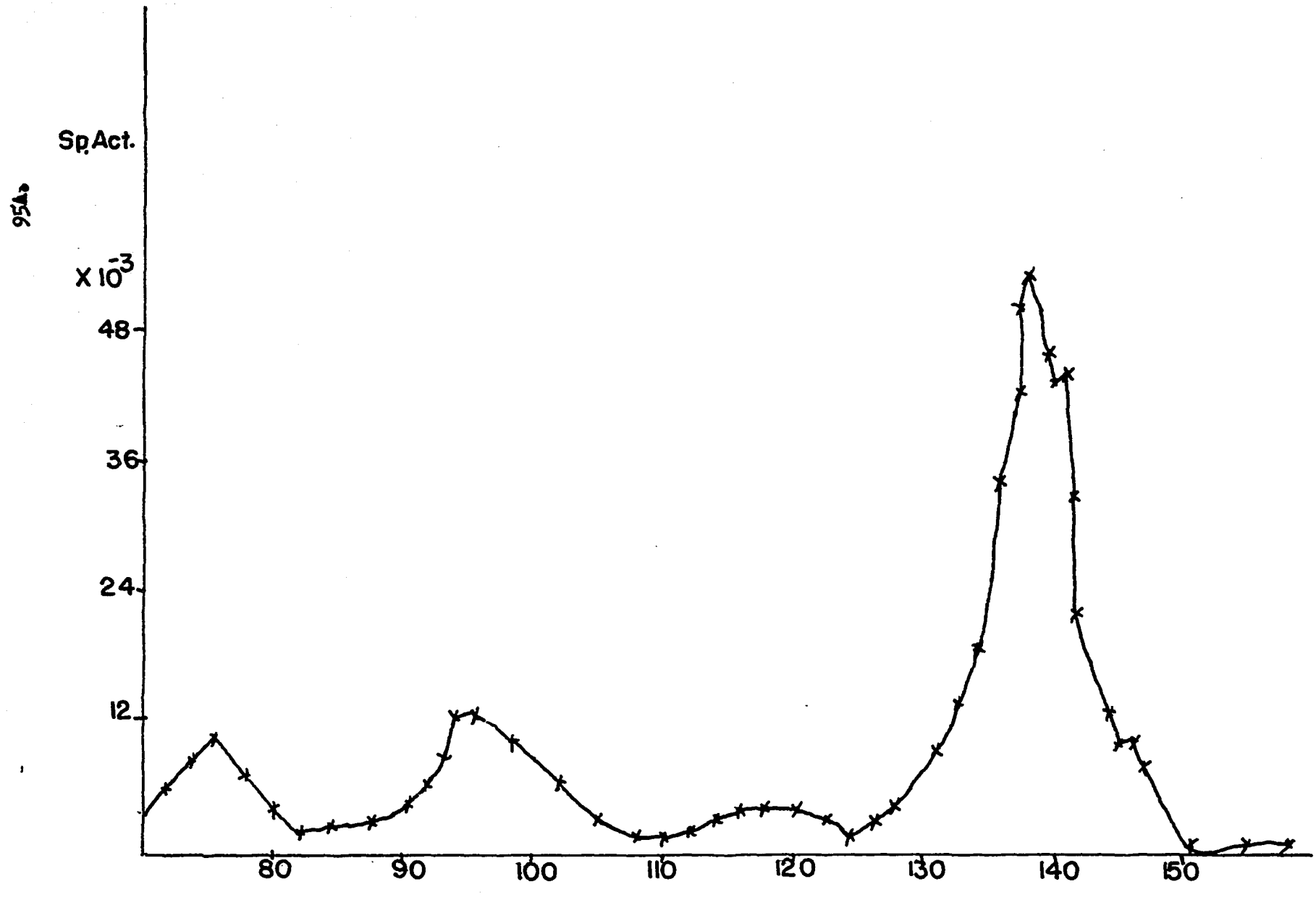

Sp.Act.
95Ab
X 10⁻³
48
36
24
12
80
90
100
110
120
130
140
150

Figure 3

Heat Inactivation of Maltase at 51C°.

Maltase was innoculated into tubes equilibrated at 51C°, containing Bovine serum albumin(0.1%) or Bovine serumn albumin(0.1%) and Ammonium sulfate(5%). The samples were buffered with 0.05M Potassium phosphate buffer pH 7.4 . At intervals, samples were removed into tubes containing only buffer(the same as above) which had been cooled in ice. The samples were then assayed as described in Materials and Methods.

O = MAL1
Δ = MAL2
□ = MAL3
⊗ = MAL6

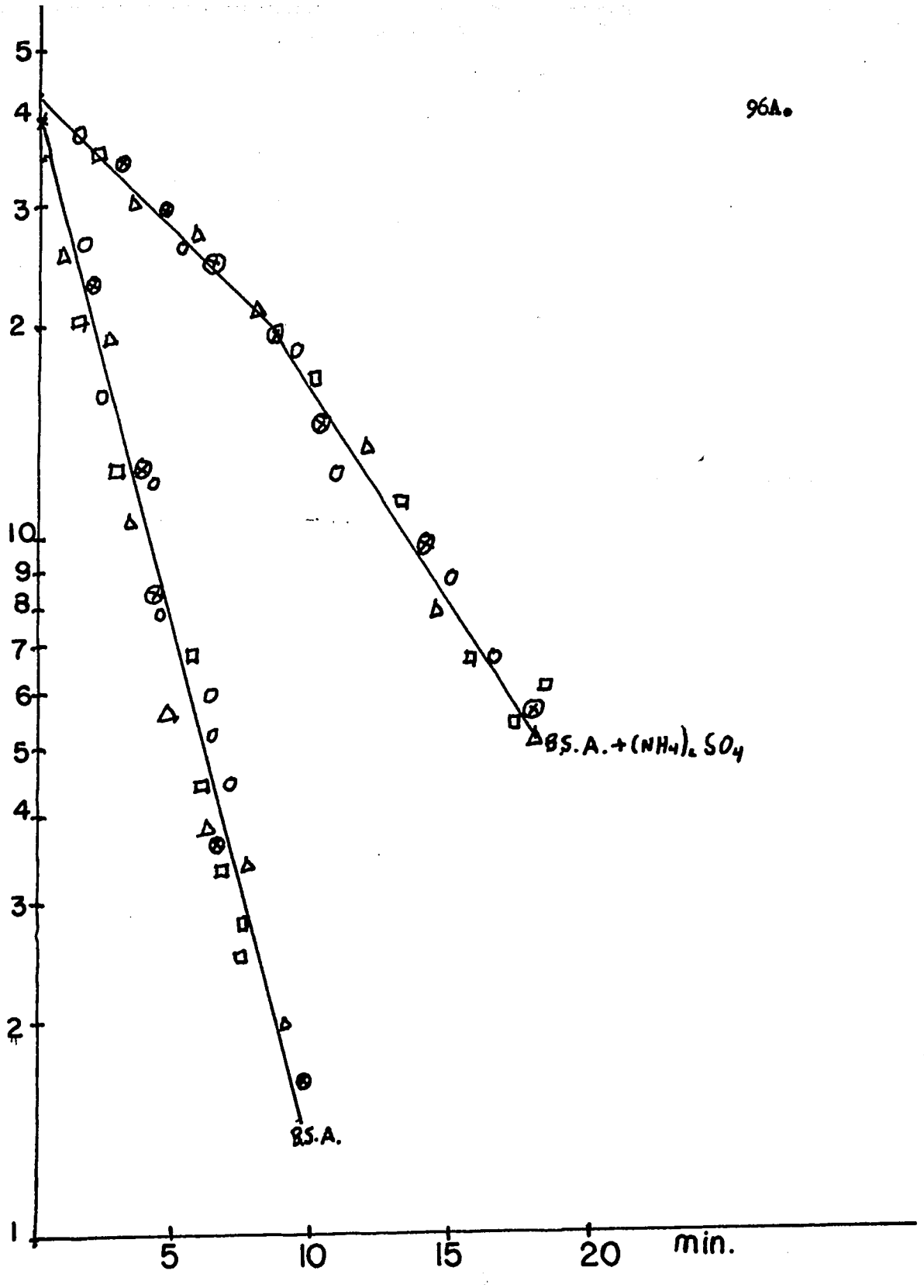

5
4
3
2
10
9
8
7
6
5
4
3
2
1
5
10
15
20
min.
BS.A.+(NH4)2SO4
BS.A.

Figure 4
SDS Gel Electrophoresis of Maltase

Maltase purified from strains having MAL1,2,3, or 6 alleles was electrophoresed on 10% SDS gels as described in Materials and Methods. The R_f values represent the ratio of the distance of migration of the sample to the migration of a Chymotrpsinogen standard which was electrophoresed at the same time. Samples of Bovine serum albumin(Ab), Aldolase(Ad), and Ribonuclease(R) were included as additional Stnadards.

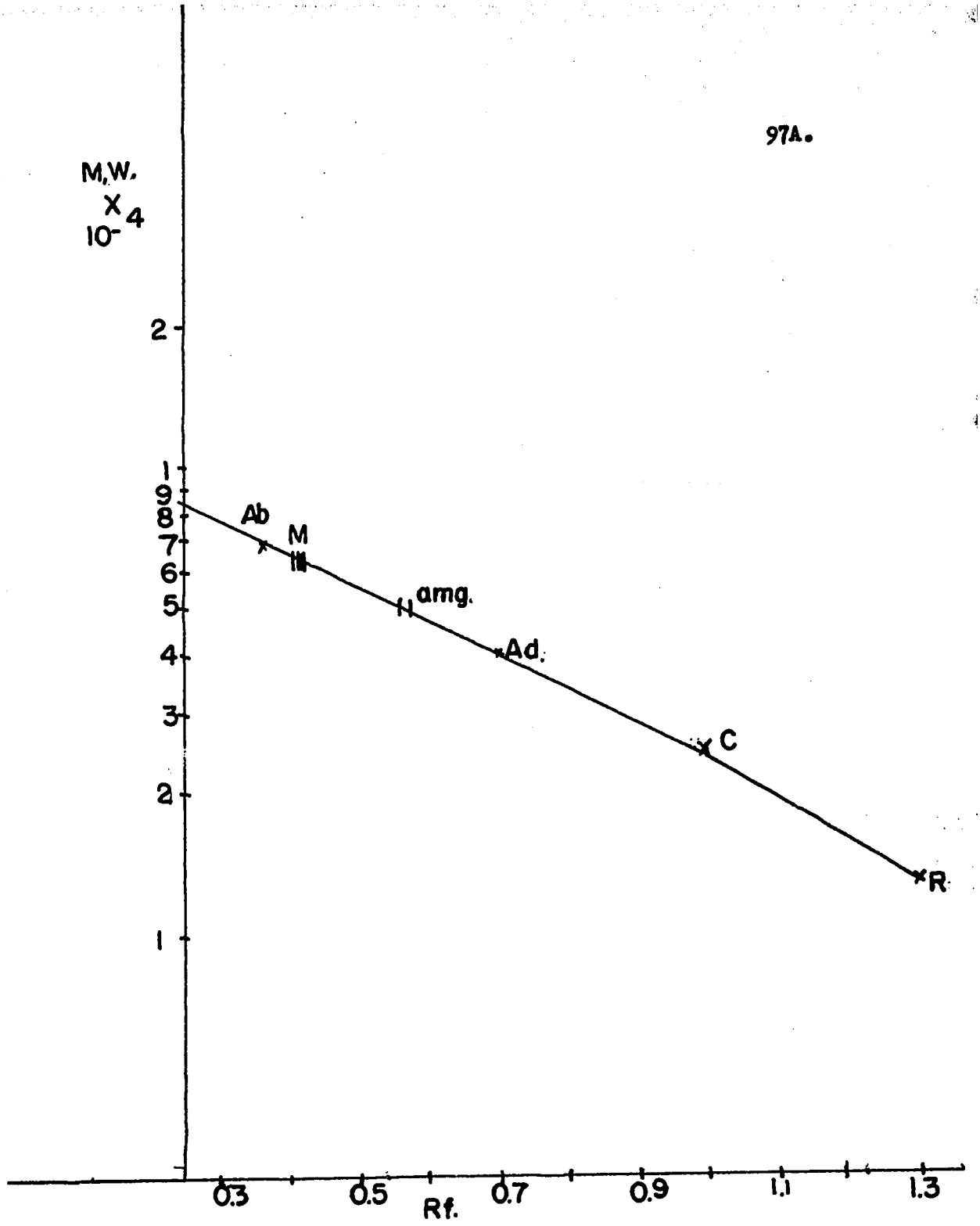

M.W.
X
10⁻⁴
2
1
9
8
7
6
5
4
3
2
1
Ab
M
amg.
Ad.
C
R
0.3
0.5
Rf.
0.7
0.9
1.1
1.3

Figure 5

Maltose Uptake of Strain 76-8-1A After Growth at 25C° and at 35C°

Cells were grown at 25C° and at 35C° in CAT-Glu media, harvested and transport measured at 25C° as described in Materials and Methods. Transport in strain 76-8 was measured simultaneously as a control. Transport in strain 27-11C(a MAL1 constitutive) was measured in a seperate experiment but is included for comparison.

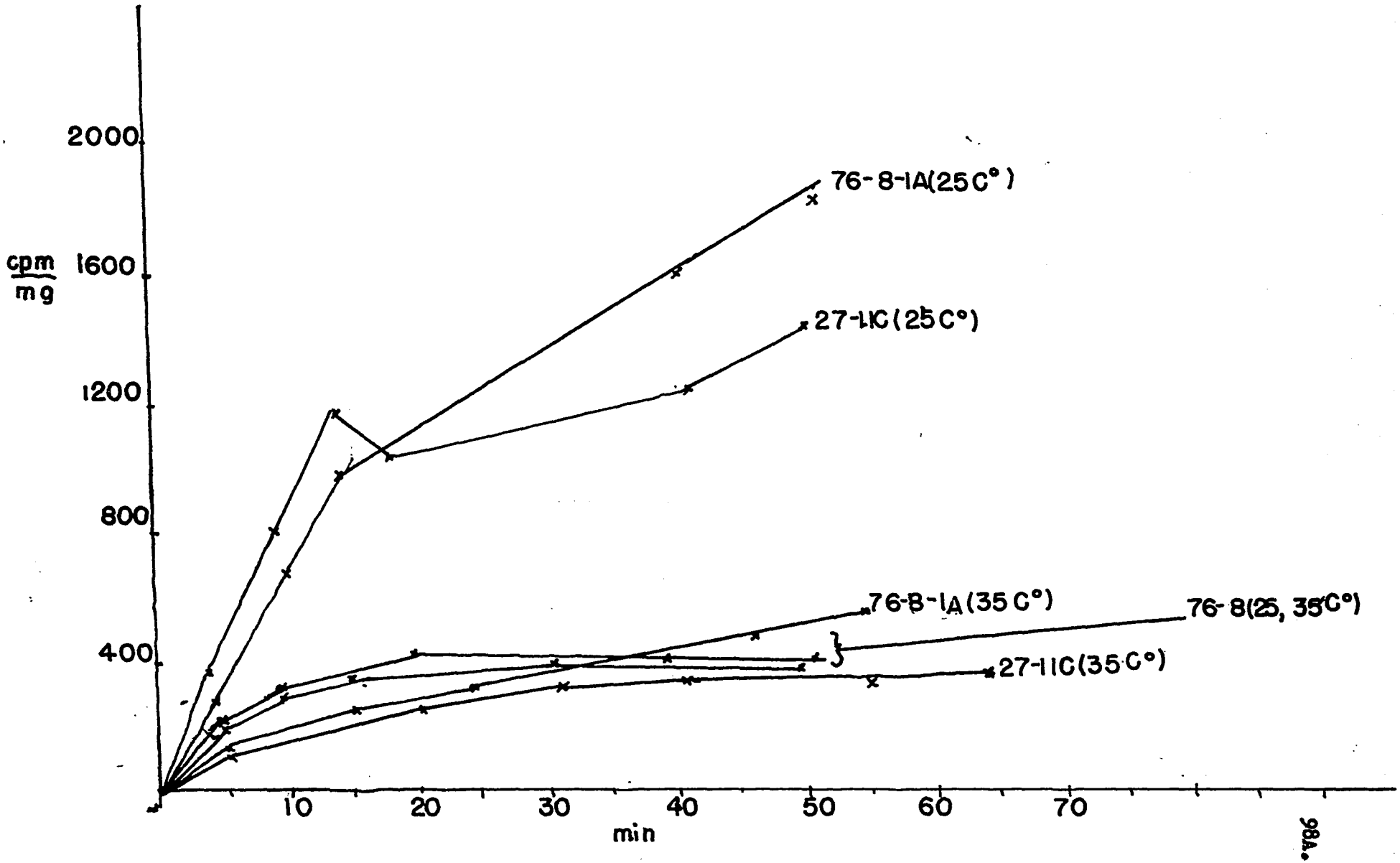

2000
1600
1200
800
400
cpm/mg
76-8-1A(25C°)
27-11C(25C°)
76-8-1A(35C°)
76-8(25, 35C°)
27-11C(35C°)
10
20
30
40
50
60
70
min
98A.

Figure 6

Transport of Methylglucoside

Transport of α-methylglucoside was measured as described in Materials and Methods.

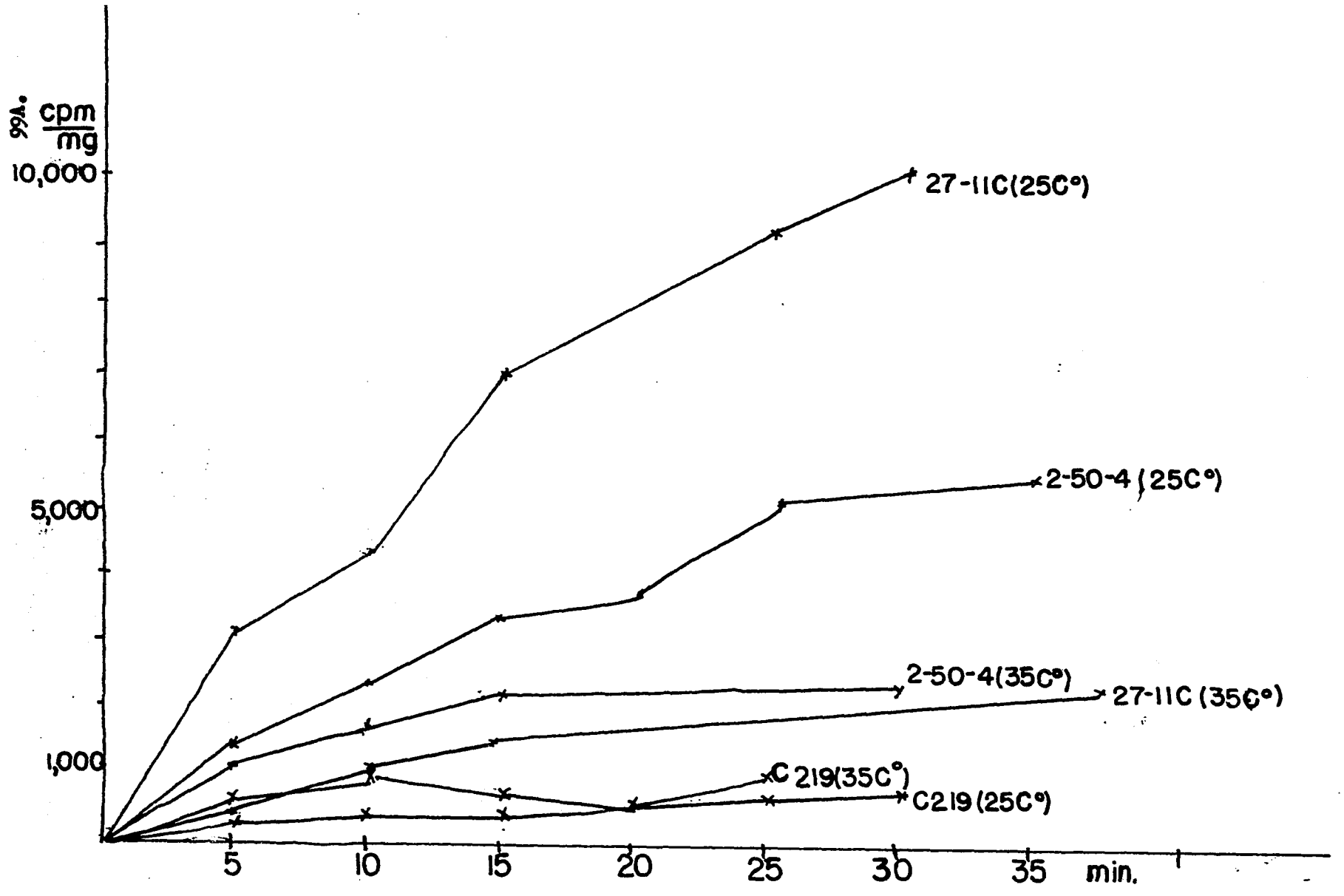

994. cpm/mg
10,000
5,000
1,000
27-11C(25C°)
2-50-4 (25C°)
2-50-4(35C°)
27-11C (35C°)
C219(35C°)
C219 (25C°)
5
10
15
20
25
30
35
min.

Figure 7

Transport of α-Methylglucoside

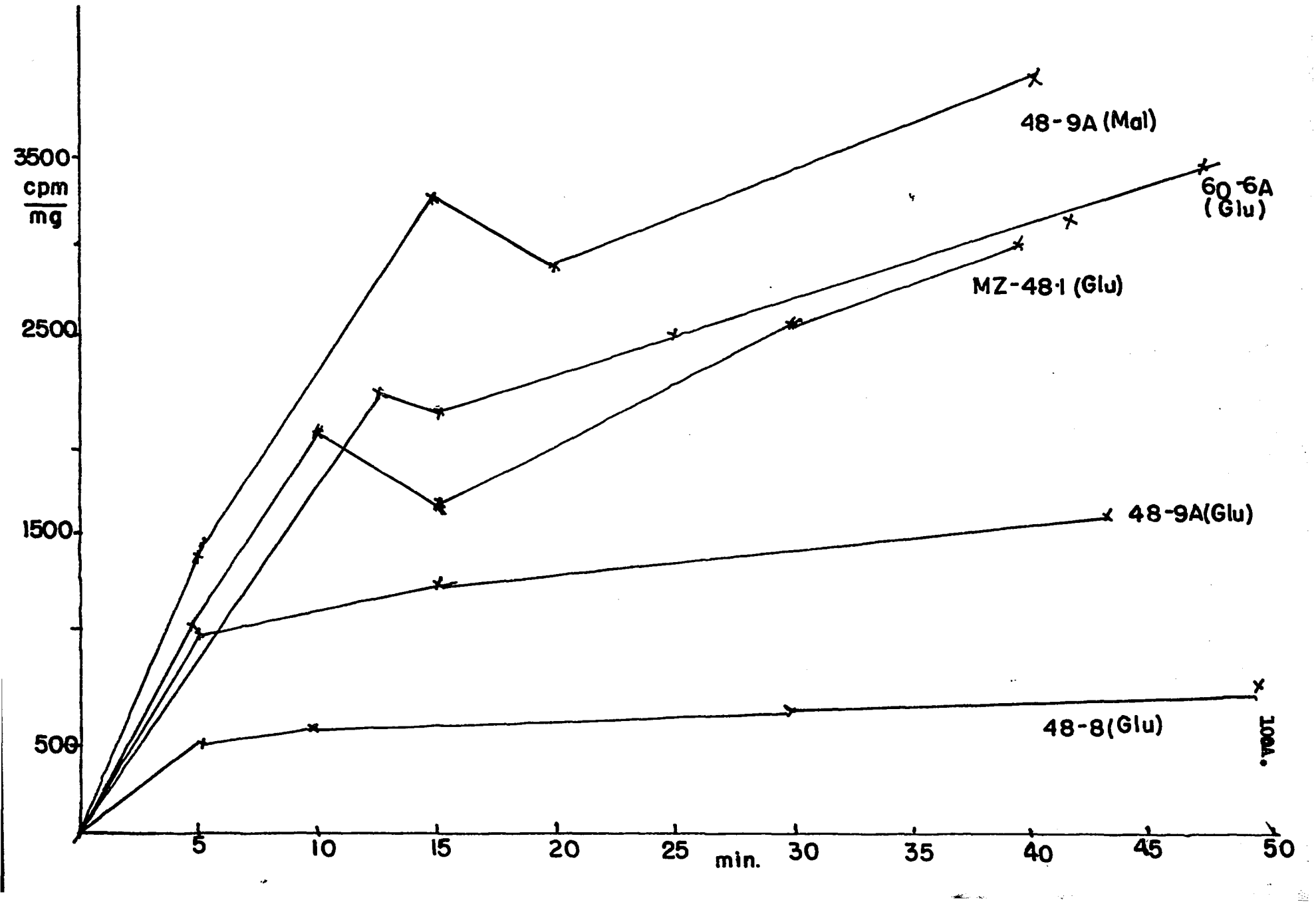
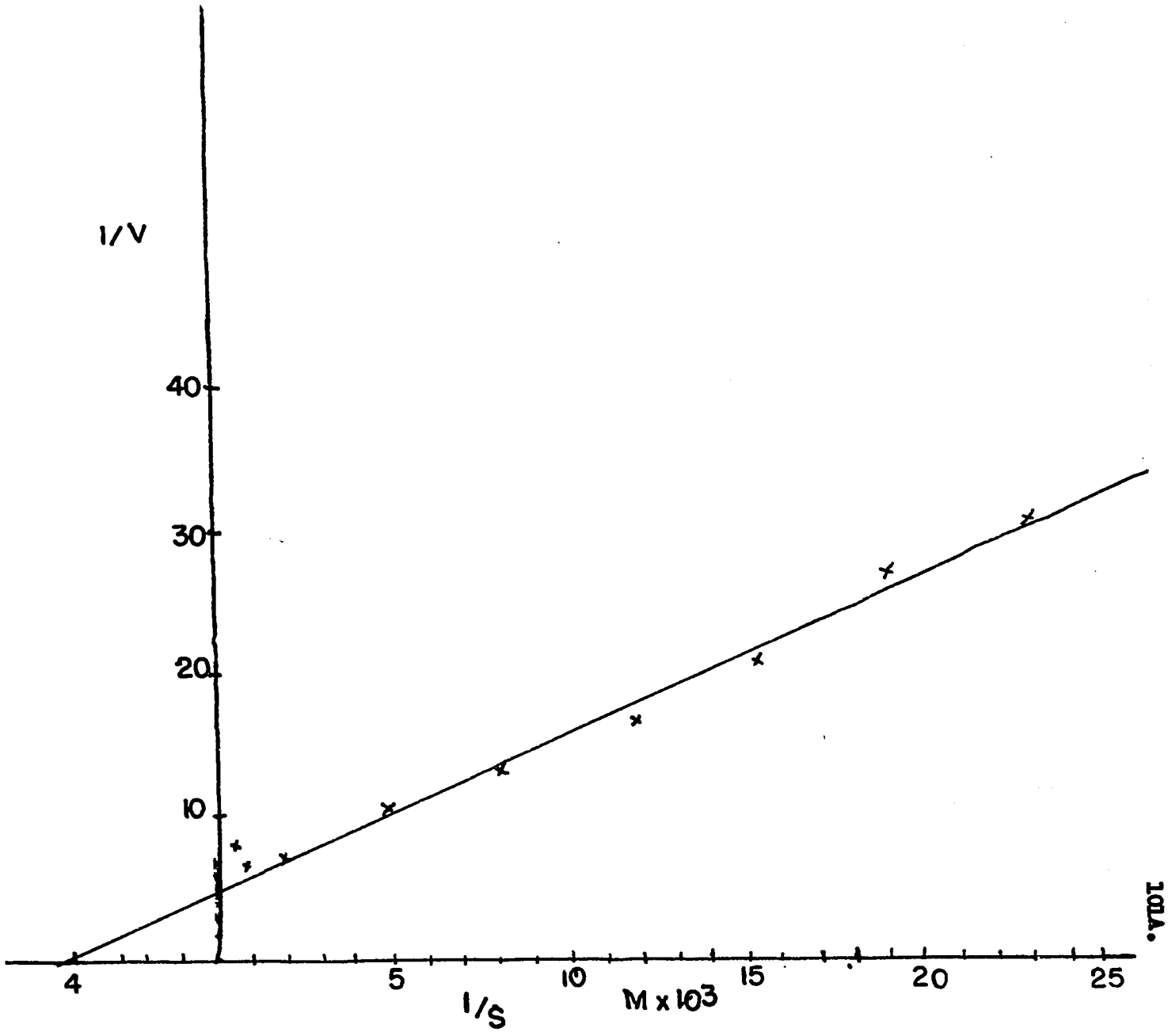

3500
cpm
mg
2500
1500
500
48-9A (Mal)
60-6A
(Glu)
MZ-48-1 (Glu)
48-9A(Glu)
48-8(Glu)
10Å.
5
10
15
20
25
min.
30
35
40
45
50

Figure 8

Michaelis-Menten plot for purified enzyme from 76-8D(MAL1).
PNPG is the substrate.


1/V
40
30
20
10
4
5
10
15
20
25
1/S
M x 10³
10mA.

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