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INDUCTION, PURIFICATION AND CHARACTERIZATION OF MULTIPLE FORMS OF ISOMALTASE FROM *SACCHAROMYCES CEREVISIAE*

by

Gary L. Youngleib

A dissertation submitted to the Graduate Faculty in Biochemistry in partial fulfillment of the requirements for the degree of Doctor of Philosophy, The City University of New York

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Abstract

INDUCTION, PURIFICATION AND CHARACTERIZATION OF MULTIPLE FORMS OF ISOMALTASE FROM SACCHAROMYCES CEREVISIAE

by

Gary L. Youngleib

Adviser: Professor Morton D. Glantz

Four forms of isomaltase, (E.C.3.2.1.10), an enzyme possessing substantial hydrolytic activity at pH 7.0 towards isomaltose, α-methyl-D-glucoside, palatinose, sucrose and p-nitrophenyl-α-D-glucopyranoside (PNPG) have been isolated from a strain of Saccharomyces cerevisiae. Yeast cells grown on media containing maltose as the only added sugar produce predominantly the more acidic form (M), pI 4.86, while growth on α-methyl-D-glucoside induces predominantly the more basic one (α), pI 5.00. Growth on media containing both α-methyl-D-glucoside and maltose induces substantial amounts of both forms of the enzyme. The third form (B) is present at very low concentration with a pI intermediate between the other two (4.93), and can be concentrated into a protein peak upon hydroxylapatite chromatography. The fourth form (A) is present at low concentration with a more acidic pI than the M form of isomaltase. Each of the two major forms when added to a crude extract of the other shows no interconversion. Extraction in the presence of phenylmethanesulfonylfluoride, a serine protease inhibitor, produces

the same distribution pattern as in the absence of inhibitor.

A near homogeneous preparation of the α and M form was achieved after 91 fold purification (31% yield). Homogeneity of the M form was established by isoelectric focusing in a pH 4-6 gradient, polyacrylamide disc gel electrophoresis and sodium dodecyl sulfate polyacrylamide disc electrophoresis. Homogeneity of the α form was established by isoelectric focusing in a pH 4-6 gradient. All four forms were subject to amino acid analysis and showed significant differences in amino acid composition with the M form containing half the glycine and three times the proline of the α form. A comparison of Km values at pH 7.0 and 25°C with PNPG for the maltose induced form (M) and the α-methyl-D-glucoside induced form (α) gave values of $8.54X10^{-4}M$ and $3.2X10^{-4}M$ respectively and Vmax values $3.18X10^{4}nmolmin^{-1}mg^{-1}$ and $3.03X10^{4}nmolmin^{-1}mg^{-1}$ respectively. Km values of the M form at pH 7.0 and 25°C for α-methyl-D-glucoside , isomaltose, palatinose, and sucrose were $2.02X10^{-2}M$, $2.22X10^{-2}M$, $2.25X10^{-2}M$, $5.68X10^{-2}M$ respectively, and Vmax values of 8.42 $\mu molmin^{-1}mg^{-1}$, 12.9 $\mu molmin^{-1}mg^{-1}$, 9.16 $\mu molmin^{-1}mg^{-1}$ and 2.30 $\mu molmin^{-1}mg^{-1}$ respectively.

Thermolability studies performed on the M and α form of isomaltase gave first order decay constants $k=-0.090\pm0.02 1min^{-1}$ and $k=-0.100\pm0.19min^{-1}$ respectively. Both the M and α form of isomaltase exhibited a pH optimum of 7.0-7.2, the α form demonstrating detectable activity from pH 4.6-7.7 and the M form from pH4.6-8.5.

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RESULTS AND DISCUSSION

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1)	PNPG	p-nitrophenyl-α-D-glucopyranoside
2)	α-MG	α-methyl-D-glucopyranoside
3)	PMSF	phenylmethanesulfonylfluoride
4)	IEF	isoelectric focusing
5)	PCMB	p-chloromercuribenzoate
6)	DFP	diisopropylfluorophosphate
7)	G-6-PD	glucose -6-phosphate dehydrogenase
8)	SDS	sodium dodecyl sulfate
9)	EDTA	ethylenediaminetetraacetate
10)	HAc	acetic acid
11)	TEMED	tetraethylmethylethylenediamine
12)	O.D.	outside diameter
13)	I.D.	inside diameter
14)	HPLC	high pressure liquid chromatography

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CHAPTER I

INTRODUCTION

HISTORICAL BACKGROUND

Isomaltase (E.C.3.2.1.10) from Saccharomyces cerevisiae catalyzes the hydrolysis of isomaltose, α-methyl-D-glucoside, palatinose, sucrose and p-nitrophenyl-α-D-glucopyranoside (1). Yeast cells fermenting either α-methyl-D-glucopyranoside or maltose, produce increased levels of the enzyme, α-methyl-D-glucopyranoside being the preferred inducer for isomaltase (2,3). Either of the aforementioned glucosides will induce maltase (E.C.3.2.1.10), an enzyme catalyzing the hydrolysis of maltose, sucrose, turanose and p-nitrophenyl-α-D-glucopyranoside, maltose being the preferred inducer (4,5,6). Maltase possesses minimal hydrolytic activity towards α-methyl-D-glucopyranoside.

The first chromatographic separation of the two activities was achieved by Khan and Eaton (1) and by Axelrod et al. (2), facilitating the establishment of a set of criteria to distinguish maltase from isomaltase, both of which had been reported as PNPG hydrolyzing activity, α-glucosidase, by Halvorson and Elias (3). A comparative study of the thermolability, pH stability and sensitivity to chymotrypsin activity (2) showed differences, with isomaltase exhibiting a greater thermolability, a different pH optimum, 7-8 as compared to 6.4 - 6.8, and an insensitivity to chymotryptic activity. Khan and Eaton reported a pH optimum of 7.2 for both enzymes, a difference in thermolability, with isomaltase exhibiting a greater thermolability, and a molecular weight of 68,500 ± 1200 for maltase in contrast to 64,700 ± 2870 for isomaltase as determined by Sephadex gel

filtration.

Also in 1968, Yau and Lindegren (6) reported the chromatographic separation of isomaltase from two discrete maltases, each enzyme eluting in different fractions upon DEAE chromatography.

A comparative study of the thermolability, pH stability and sensitivity to chymotrypsin of maltase induced with maltose versus the α-methyl-D-glucoside induced maltase showed no differences (2). Despite extensive purification, controversy remained as to the actual number of α-glucosidases present in yeast.

In 1976, Eaton and Zimmerman (7) reported preliminary findings suggesting the existence of three maltases in a variety of strains of Saccharomyces cerevisiae. Evidence rested on observations of the behavior of crude extracts when subjected to heat denaturation at 48°C. A plot of logarithm of percent remaining activity as a function of time of exposure to the temperature showed a deviation from the linear relationship expected if inactivation were of a single maltase species. When extracts were subjected to gel filtration chromatography and samples removed from either side of the resultant protein peak no differences in thermolability could be detected, suggesting no differences in molecular weight.

The most recent purification of maltase from yeast resulted in a preparation judged homogenous by the criteria of isoelectric focusing and sodium dodecyl sulfate electrophoresis (4). Multiple forms of maltase were not observed and thermal inactivation experiments performed on the purified enzyme showed monophasic heat inactivation profiles indicative of a single enzyme species.

This thesis reports the induction of four forms of isomaltase and

the purification and characterization of the two major forms. In the process of isolating isozymes of isomaltase, two forms of maltase were separated by column chromatography and their identity confirmed by isoelectric focusing and activity staining.

SURVEY OF ASSAY TECHNIQUES

The methods utilized for the determination of the catalytic activity of isomaltase involve the detection of the products of hydrolytic cleavage of the disaccharide linkage.

PNPG (p-nitrophenyl-α-D-glucopyranoside) assay - This method involves measuring the release of p-nitrophenol upon hydrolysis of p-nitrophenyl-α-D-glucopyranoside. The equilibrium between p-nitrophenol and the phenoxide ion can be shifted towards the latter by raising the pH, the phenoxide ion having a characteristic absorption maximum at 400 nanometers. The assay is sensitive in the nanomole range and can be performed by following the continuous release of p-nitrophenol or in a discontinuous fashion by terminating the reaction with sodium carbonate (4). It suffers from a lack of selectivity in that both maltase and isomaltase hydrolyse the substrate, but is useful in detecting minute quantities of enzyme and as a colorimetric qualitative tool for distinguishing active enzyme from nonenzymatic protein.

Glucostat assay - Release of free glucose from glucose containing disaccharides can be monitored by a coupled glucose oxidase-peroxidase assay (8):

$$\beta\text{-D-glucose} + H_2O + O_2 \xrightarrow{\text{glucose oxidase}} H_2O_2 + \text{gluconic acid}$$

$$H_2O_2 + \text{O-dianisidine} \xrightarrow{\text{peroxidase}} H_2O + \text{oxidized dianisidine}$$

Oxidized dianisidine can be detected by a characteristic absorption

maximum at 400 nanometers or the reaction can be terminated with strong acid shifting the absorption maximum of the dye to 525 nanometers. Preparation of the reagent in a tris-phosphate buffer is necessary to suppress maltase activity endogenous to the glucose oxidase preparation and the incorporation of glycerol further stabilizes the reagent. Glucose oxidase is specific for β-D-glucose and it is necessary to allow mutarotation to proceed to equilibrium prior to assay. The enzyme is relatively specific for β-D-glucose with relative rates of oxidation of D-glucose, D-mannose and 2-deoxy-D-glucose of 100:20:20. It exhibits negligible activity on other hexoses (10).

Hexokinase - glucose-6-phosphate dehydrogenase (G-6-PD) assay system.

The enzymatic reaction sequence utilized in the assay of glucose is as follows; (11,12):

(1) Glucose + ATP $\xrightarrow{\text{hexokinase}}$ glucose-6-phosphate + ADP

(2) Glucose-6-phosphate + NAD^+ $\xrightarrow{\text{G-6-PD}}$ 6-phosphogluconate + NADH + H^+.

NADH can be detected by its characteristic absorption maxima at 340 nanometers. Contraindications to the use of this assay are the presence of metal chelators and phosphate, hexokinase requiring Mg^{+2} for activity and glucose-6-phosphate dehydrogenase being activated by phosphate, sulfate and bicarbonate (13).

Non-enzymatic assay systems for the quantitation of glucose abound but the specificity of chemical methods is of a questionable nature and enzymatic assay is the preferred method.

GENERAL PROPERTIES OF YEAST α-GLUCOSIDASES

α-glucosidase activity has been reported (3) to be completely

inhibited by Cu^{++}, Hg^{++}, Ag^{+}, Pb^{+2}, and Zn^{+2} and moderately inhibited by Fe^{+2} at 3.3×10^{-4}M. The rate of p-nitrophenyl-α-D-glucopyranoside (PNPG) hydrolysis was unaffected by the addition to K^{+}, Na^{+}, Li^{+} or NH_4^{+} as chlorides, phosphates and malonates up to concentrations of 0.1M.

Incubation with 5×10^{-5}M p-chloro-mercuribenzoate (PCMB) leads to a complete loss of activity as does incubation with iodoacetate, the inhibition being competitive with cysteine and maltose, implying a reactive sulfhydryl group (3).

Hydrolysis of PNPG has been reported to be reversibly inhibited by histidine, trishydroxymethylaminomethane (tris), quinine, benzylamine, napthylamine and aniline (3). The inhibition is pH dependent and observable only at pH values above the pK of the amine suggesting that the non-protonated form of the amine is the inhibitory species. Ouwehand and Wijk (5) have utilized the inhibitory properties of tris as a alternative to heat denaturation for terminating enzymatic reactions prior to assay.

Kinetic studies show typical Michaelis-Menten kinetics with PNPG as substrate and plots of pKm versus pH show a break in slope at pH 6.8 implicating an essential histidine residue (3). Needleman et al. (4) report a Km for PNPG hydrolysis by maltase of 3.1×10^{-4}M, Vmax=134×10^{-6} molmin^{-1}mg^{-1} and Km amd Vmax values for maltose of 1.7×10^{-2}M, and 45×10^{-6} mol^{-1}mg^{-1} and respectively for sucrose 1.5×10^{-2}M, and 53 umol min^{-1}mg^{-1}. Km(PNPG) is reported to be 0.7×10^{-3}M for isomaltase (14).

FORMAL REACTION MECHANISM

Maltase and isomaltase from Saccharomyces cerevisiae are specific for the hydrolysis of α-D-glucosides, the hydrolysis of p-nitrophenyl

α-D-glucopyranoside proceeding with the production of glucose with $[\alpha]_D^{20} \gtrsim +100^\circ$ at initial time. Mutarotation yields a final equilibrium value of $[\alpha]_D^{20} = +58^\circ$ (15). Extrapolation of the equilibrium reaction to zero time gives a $[\alpha]_D^{20} = +120^\circ$, approximating the value for α-D-glucose. Retention of configuration about the anomeric carbon can proceed by one of two mechanisms as reviewed by Koshland (16). In hydrolytic reactions in which an OR is replaced by an OH group, bond cleavage may actually occur between the O and the R rather than between the O and the C. In this case, no displacement occurs at the assymmetric carbon atom, and no inversion would be expected. An alternate explanation is a double displacement mechanism with the formation of an enzyme-substrate complex with inversion of configuration at the C-1 position of the anomeric carbon of glucose followed by a second displacement to yield the free sugar in its original configuration (2).

Lai and Axelrod (17) have identified a stable enzyme-glucose intermediate obtained from the reaction between isomaltase and α-CH_3-D-^{14}C-glucopyranoside. A covalent linkage between enzyme and glucose was confirmed by the observation that radioactivity was retained in a tryptic peptide isolated from the mixture by gel filtration, and acid hydrolysis of the peptide released over 80% of the radioactivity as a compound with the same chromatographic mobility as glucose. The presence of the inhibitor glucosylamine, prevented the formation of the intermediate.

The isomaltase subunit of the sucrase-isomaltase complex from rabbit small intestine possesses similar activities to yeast isomaltase, demonstrating hydrolytic activity towards isomaltose,

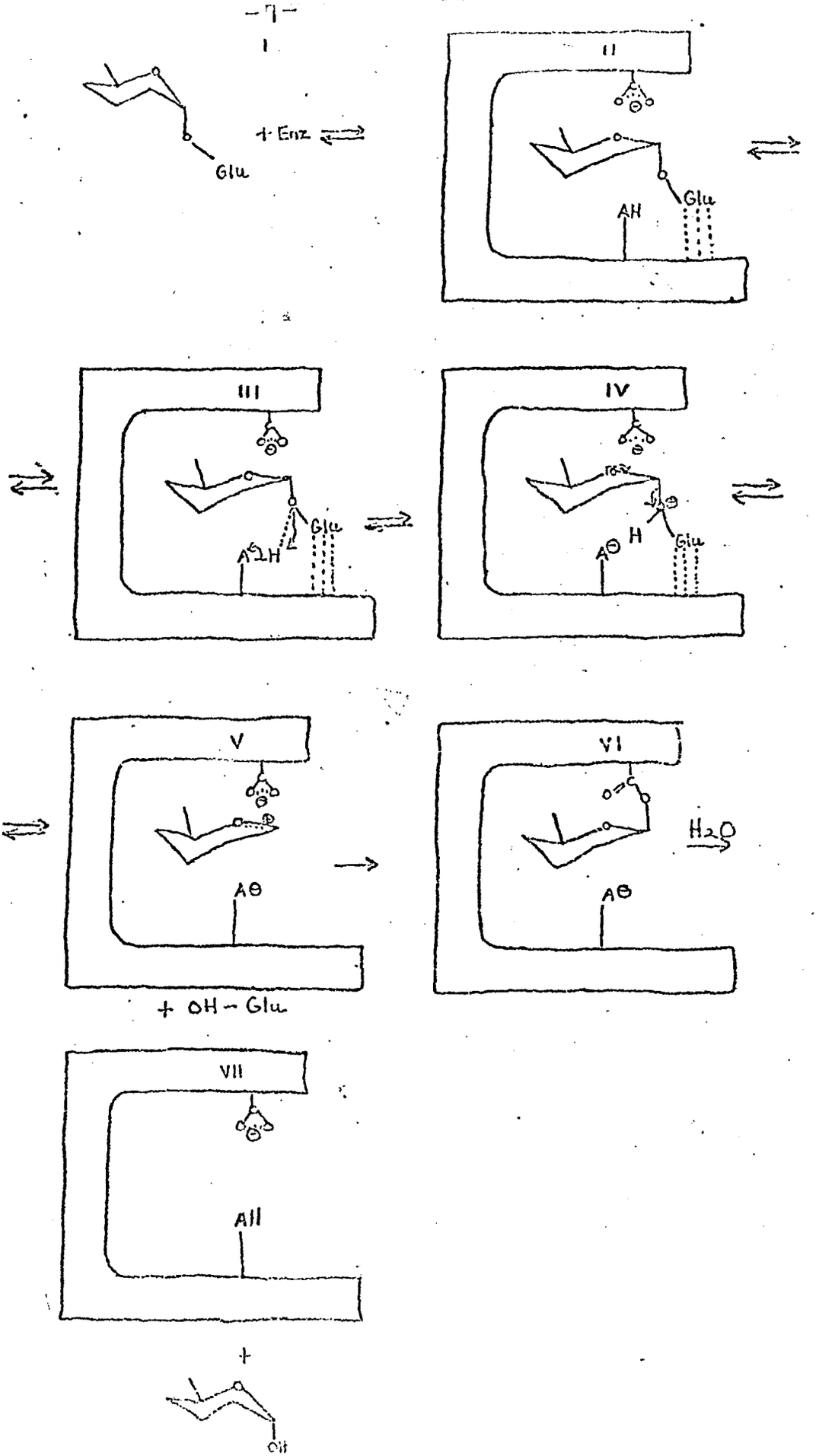


Fig. 1 suggested common hydrolytic mechanism for sucrase and maltase

maltose, sucrose and palatinose (18). [^{3}H]-Conduritol-β-epoxide is reported to be an active site directed inhibitor for both subunits of the complex, 1 mole of inhibitor being bound covalently per mole of subunit. The inhibition is irreversible and prevented by the presence of competitive inhibitors and substrates. After labelling and peptic digestion of the radiolabelled peptide, the label was in each case bound to the carboxyl group of an aspartic acid residue (19).

A formal mechanism for small intestinal sucrase and isomaltase has been proposed by Cogoli and Semenza (20) based on studies with p-Cl-phenyl-α-D-glucopyranoside. They demonstrated that substitution of ^{1}H with ^{2}H at C_1 of the glucosyl moiety leads to a decrease in k_{cat} of both sucrase and isomaltase, the k_1H/k_2H ranging between 1.14 and 1.20. The magnitude of the secondary deuterium effect is strongly indicative that the rate limiting step goes through the formation of an oxocarbonium ion. The data suggested a reaction mechanism for the two glucosidases; protonation of the glycosidic oxygen is followed by the liberation of the aglycone with formation of an oxocarbonium ion, which is temporarily stabilized by a carboxylate group (20) (Fig. 1).

BIOLOGICAL SIGNIFICANCE OF ISOMALTASE

Because of the multiple hydrolytic activities of isomaltase the in vivo function of the enzyme in yeast is still unproven. In yeast and in some mammals (21,22) storage polysaccharides are degraded by the concerted action of amylo-1,4-glucosidase and amylo-1,6-glucosidase yielding the disaccharides maltose and isomaltose. Neither of these two activities possesses hydrolytic activity towards isomaltose or maltose. Presumably, isomaltase and maltase function to catalyze the hydrolysis of isomaltose and maltose, respectively, yielding free α-D-

glucose.

A sucrase-isomaltase complex has been identified in homogenates of human small intestine mucosa (23) and dissociated into subunits by treatment with mercaptoethanol. The isomaltase subunit demonstrated hydrolytic activity towards α-(1 $\rightarrow$6) limit dextrins and little hydrolytic activity towards isomaltose. The sucrase subunit possessed hydrolytic activity towards sucrose, maltose, maltotriose and towards α-(1 $\rightarrow$ 4) limit dextrins (24). The authors suggest a more appropriate name for the complex might be sucrase-α-dextrinase. Gray et al. (24) suggests that the function of the complex in vivo is to degrade α-dextrins by removal of individual glucose residues from the nonreducing end of an oligosaccharide by complementary action of both sucrase and isomaltase.

A sucrase-isomaltase complex has been isolated from rabbit small intestine, the sucrase subunit possessing hydrolytic activity towards sucrose and maltose and the isomaltase subunit hydrolyzing isomaltose, palatinose, and maltose (25). No attempt was made to ascertain the physiological significance of the complex.

Current available literature on α-glucosidases avoids comparison of the enzymes structure and function from different systems. Indeed function is still speculative within each given system.

PROTEOLYTIC ARTIFACTS IN ENZYMES FROM YEASTS

A wide variety of enzymes purified from Saccharomyces cerevisiae exist in multiple forms. In many of these cases it has subsequently become apparent that many of these isozymes were produced by a partial proteolysis of the native form of the enzyme (26). In some cases the

modified forms have altered affinities for their substrates but in many cases degradation can occur with essentially full retention of activity.

Schulze and Colowick (27) demonstrated that crytalline hexokinase preparations consisted largely of an enzyme with altered chromatographic properties from those demonstrated with the native form. When precautions were taken to inhibit the activity of a serine protease possessing hydrolytic specificity for casein and benzoyl-L-arginine at neutral pH, the relative distribution of the different forms was altered, as determined by the chromatographic profiles. Inclusion of phenylmethanesulfonylfluoride, (PMSF; a serine protease inhibitor) in all buffers, enabled these authors to isolate two forms of hexokinase believed to be genuine in vivo isoenzymes.

Clark and Jacoby (28) demonstrated that amino terminal end group analysis of homogeneous aldehyde dehydrogenase purified from autolyzed yeast, revealed gross microheterogeneity which they ascribed to proteolysis. By substitution of a Manton-Gaulin homogenizer for the autolysis step and by purification of the enzyme in the presence of the esterase inhibitors phenylmethanesulfonylfluoride (PMSF) and diisopropyl fluorophosphate (DFP), the conversion of the native dehydrogenase, designated A, to the degradation products B and C was prevented. DFP proved more effective than PMSF in preventing the conversion. Amino terminal analysis of dehydrogenase A showed serine to be the amino terminal end group.

The yeast endopeptidase, protease B, (29) hydrolyses casein rapidly between pH 5.7 and 10. It possesses considerable activity towards azocoll (30,31) and weak activity for α-N-benzoyl-L-arginine

ethyl ester and α-N-acetyl-L-tyrosine ethyl ester. It is completely inhibited by PMSF, DFP or p-chloromercuribenzoate (PCMB) and is most likely responsible for the production of multiple active species of many enzymes in the neutral pH range (26).

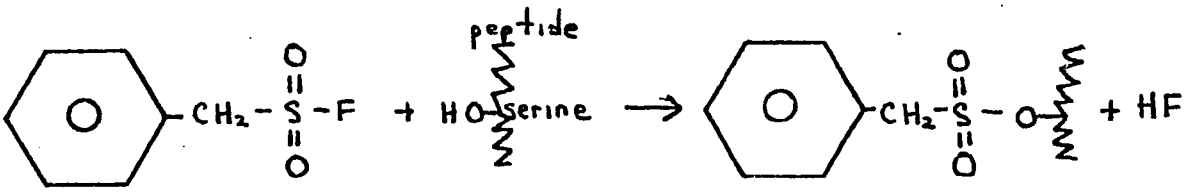
A carboxypeptidase from yeast, protease C, can remove carboxy terminal amide and ester groups as well as carboxy terminal amino acids from a wide variety of peptide and protein substrates in the pH range 4-9.5 (32). It too is completely inhibited by PMSF, DFP and PCMB.

Subcellular fractionation of yeast cells demonstrated the localization of these proteases in the vacuole (33,34) and the presence of polypeptide inhibitors in the extravacuolar cytoplasm (35). Upon cellular disruption, protease-inhibitor complexes are formed in the crude extract, the stability of each type of complex being influenced by the pH of the extract and the relative level and activity of various proteases. The pathway whereby inactive protease complexes are activated involves a metabolic cascade whereby protease A inactivates or cleaves a protease B-inhibitor complex, the active B protease in turn cleaves protease A and protease C inhibitor complexes producing higher levels of free protease A and C (26,30,31). The complexes are stable at pH 7.0 but will dissociate in the acid pH range (16) possibly because of the existence of a pH optima of 2-4 for protease A. This fact might explain the immediate loss of α-glucosidase activity in certain strains of yeast upon addition of ammonium sulfate to crude extracts (36).

Proteolytic enzymes have been found to contaminate highly purified preparations of other proteins (26). Their existence becomes evident upon SDS gel electrophoresis as a series of low molecular weight

peptides, the number and intensity increasing with increasing time of incubation in SDS and mercaptoethanol. Proteolysis during denaturation was prevented by adding PMSF a few minutes before SDS with an immediate treatment at 100°C for 4 minutes (37). An explanation for the appearance of proteases in purified protein preparations is offered by Pringle (26). The yeast cell contains at least five different proteases. These, and their naturally occurring inactive complexes, can distribute themselves in different fractions during purification procedures, increasing the probability that at least one protease will copurify with any particular protein (26).

PMSF is the reagent generally employed for inhibiting serine type proteases. It is hydrolyzed with a half-life of about 100 minutes at pH 7 (38). Its mechanism of action is as follows:



Both PMSF and DFP form covalent derivatives with any accessible serine hydroxyl groups. The use of PMSF-^{14}C is a useful control in checking for incorporation of the reagent in the protein under study in addition to a serine protease (39). Impurities in commercial PMSF have been reported to produce artifacts (40,41). As an alternative to the use of PMSF it has been reported that glycerol, sodium phosphate, or $(NH_4)_2SO_4$ are effective in reducing proteolytic activity in some strains of yeast (30,42).

It is suggested by Pringle (26) that the vast number of studies on proteins of yeast that have subsequently proven to be done on partially degraded proteins is a problem prevalent in the isolation of proteins from many different organisms (26).

OCCURRENCE OF MULTIPLE GLYCOSIDASES

The glycosidase enzymes exist in multiple forms in a wide variety of organisms. No attempt will be made to speculate on a common function for this phenomena of multiplicity, but a brief review is in

order.

Dimond and Loomis (43) describe two β-glucosidase isozymes in developing cells of Dictyostelium discoideum. Type 1 is present in vegetative cells and increases in specific activity during early development while type 2 is not present until late in development. The isozymes differ in their Km for p-nitrophenol substrates and in their thermolabilities. Type 1 is excreted into the medium, and the authors suggest the enzyme acts as a block to selfing in macrocyst formation since strains lacking the enzymes do not require the presence of the opposite mating type to form macrocyst-like structures. Mutant strains were derived from parental strains producing forms of the isozymes differing in Km and thermolability. The isozymes were not characterized with regard to the specificity of the susceptible glycosidic linkage (β 1→2 β1→4 etc.). The presence of an unknown factor in the cell homogenate caused the loss of 15% of activity per day and only a 20 fold purification of type 2 was achieved. Antisera prepared against type 1 did not react with type 2. Neither enzyme was purified to homogeneity and the structural basis for the difference remains unknown.

It has been reported by Weeks (44) that cells of Dictyostelium discoideum are markedly agglutinated by low concentrations of concanavilin A and that cells from the vegetative phase of growth are far more susceptible to agglutination than differentiating cells. The possibility exists that agglutination is being mediated by degradation of cell surface sugars by β-glucosidases.

It has been observed by Jolly et al. (45) that in the lysosomal storage disease α-mannosidosis there is an altered α-mannosidase

possessing a decreased affinity for substrate and an altered stability. The authors suggest a mutation in the structural gene coding for α-mannosidase as the basis for the disease. Hirani and Winchester (48) have identified four forms of α-D-mannosidase in normal plasma in addition to the acidic α-D-mannosidase. These forms of the enzyme differ in molecular weight and in affinity for a concanavilin A Sepharose column and are present in the plasma of patients with the lysosomal storage disease, mannosidosis, while the acidic form is absent (48).

Mueller and Rosenberg (46) report that the membrane bound β-glucosylceramide: β-glucosidase of normal cultured human skin behaves as an acidic protein isoelectric at pH 4.80 with minor β-glucosidase fractions isoelectric at pH 4.55 and pH 4.67. The residual β-glucosidase in cells from enzyme-deficient donors with types I and II glucosylceramidosis (Gaucher's disease) displays precisely the same isoelectric point, pH 4.55, as the most acidic, trace, β-glucosidase component in normal cells, and is present in roughly the same amount as in normal cells, suggesting that the enzyme deficient cells produce only a minor structural variant.

Jolly (47) observed that upon isoelectric focusing in a sucrose density gradient column the α-glucosidase activity from Gaucher's fibroblasts existed in as many as 10 or more discrete forms as measured by assay of the eluted fractions. No attempt was made to characterize further these fractions and the specificity of the α-glucoside linkage hydrolysed was undetermined.

Nakagawa (49) et al. report the presence of two pH optima for β-glucosidase in fibroblast cultures from controls and patients with

adult Gaucher's disease. With intact cell suspensions from controls and Gaucher's, the pH optima for β-glucosidase are 3.8-4.0 and 4.2-4.4 respectively. Lysates prepared by freeze-thawing and sonication show a shift in pH optima to 4.2 and 4.8 for control and Gaucher's cells respectively. Maler et al. (50) report the following: when cultured lymphoblasts derived from patients with cystic fibrosis and normal individuals were compared in terms of the level of activity of several lysosomal glycosidases, differences were observed primarily in α-L-fucosidase. The specific activity of the enzyme in homogenates of cystic fibrosis cells was consistently lower than in normal cells. The Km and SDS molecular weight of the enzyme from cystic fibrosis cells were identical to that of normal cells, being 40-60uM and 35,500 daltons respectively. The only indication of structural change in the enzyme of cells from patients with the disease was a reduced susceptibility to precipitation by concanavilin A. These findings provide evidence for a reduction in α-L-fucosidase level (probably with altered mannose containing oligosaccharides) in cystic fibrosis which may correlate with reports of elevated amounts of L-fucose in some glycoproteins of patients with the disease (50). Maler, upon performing isoelectric focusing and activity staining, observed six or seven multiple forms of the enzyme in both normal and cystic fibrosis cells on a preparation judged homogenous in SDS gel electrophoresis.

Bishop and Desnick (51) purified human α-galactosidase A from spleen, and placenta to homogeneity as judged by SDS gel electrophoresis. Isoelectric focusing and activity staining on the purified enzymes showed multiple banding patterns that were modified when treated with neuraminidase. The authors conclude that the

multiple forms observed in the isoelectric focusing patterns almost certainly represent varying degrees of sialylation. The precise carbohydrate content and differences among the various forms of α-galactosidase A have not been determined.

This thesis reports the induction of genes regulating multiple forms of isomaltase and the isolation and characterization of the two major forms of the enzyme (61). The purpose of this study was to characterize the two differentially inducible forms of the enzyme, and to determine whether the structural differences would support the notion of a distinct gene for each enzyme. An alternate explanation would be that of a single gene coding for one enzyme, followed by a postransational modification resulting in multiple forms. Differences in amino acid composition between the two differentially inducible forms would support the two gene hypothesis.

CHAPTER II

MATERIALS AND METHODS

MATERIALS

A haploid strain of yeast with the designation number 1412-4D was obtained from Norman Eaton and sustained on glucose 2%, peptone 2%, yeast extract 1%, agar 2%. From Sigma (Saint Louis, Missouri) and Calbiochem (La Jolla, California) was purchased α-methyl-D-glucoside which was recrystallized from ethanol when necessary. Ethanol recrystallization was unnecessary with the Calbiochem product. Maltose was from Difco (Detroit, Michigan), Sigma and Mann (Orangeburg, New York). Palatinose and isomaltose were from Sigma. Standard growth media components (peptone and yeast extract) were purchased from Difco. Sephadex and the Sepharose ion-exchanger were from Pharmacia (Piscataway, New Jersey) and hydroxylapatite from Bio-Rad (Hicksville, Long Island) as were acrylamide, bis-acrylamide and Coomasie R-250 electrophoresis reagents. Coomasie G-250, glucose oxidase, O-dianisidine hydrochloride, horseradish peroxidase type II, and 2,3,5 triphenyl tetrazolium chloride were purchased from Sigma. Ampholines were from LKB (Hicksville, Long Island) and Bio-Rad. PNPG was from Calbiochem. All other chemicals were of reagent grade and obtained from Fisher and Baker.

EQUIPMENT

An LKB Ultrorac fraction collector, model number 7000 was used. Electrophoresis cells were from Bio-Rad and Canalco (Division of Miles; Elkhart, Indiana) and were used with a 0-2000 volt constant power supply from LKB, model number 2103. All chromatography columns were from Pharmacia. The enzyme assays and protein determinations were

performed with a Gilford 240 uv, visible spectrophotometer. The Sorvall (Division of Dupont; Newton, Connecticut) model RC-2B refrigerated centrifuge was used in conjunction with a continuous flow apparatus for recovery of yeast from large culture volumes. A Beckman (Irvine, California) 116 amino acid analyzer was used to determine amino acid compositions. A Rainin (Woborn, Massachusetts) repipet system was used for all routine pipetting operations.

ANALYTICAL PROCEDURES

Protein estimation - Protein concentrations were determined by the microbiuret method of Zamenhoff, using bovine serum albumin as a standard (52). Most protein determinations were performed at two different concentrations particularly in the presence of ammonium sulfate and the results averaged, the deviation between the two values was typically 2%. Since glycerol interfered with the protein assay, it was dialyzed out before the determination. The volume pickup upon dialysis was corrected for by direct measurement of volume before and after dialysis and confirmed by calculation of before and after PNPG hydrolysis rates. Standard curves were determined in triplicate.

PNPG assay

The enzyme was assayed during the course of purification procedures by incubating 1.7 ml of enzyme solution with 0.2 ml. of 0.5M phosphate buffer, pH 7.0, and 100μl of PNPG (5 mg/ml). The reaction was followed spectrophotometrically by monitoring the production of phenoxide ion at 400nm.. Absorbance values were read every 30 seconds and rates were proportional to time over the first 2 minutes. Enzyme dilutions were performed occasionally to check proportionality between velocity and enzyme concentration. All solutions were preincubated at

30°C and the assay was performed in a Gilford spectrophotometer containing a water jacketed cell compartment. The reaction rate was expressed as moles $min^{-1}ml^{-1}$ for enzyme kinetics by terminating the reaction with 1.0M Na_2CO_3 and measuring the released p-nitrophenol read against a standard curve.

Glucostat assay

The enzyme was assayed during the course of purification procedures by incubating 0.8ml of a suitable enzyme-solution with 0.1ml of 0.5M phosphate buffer, pH 7.0, and 100µl of 0.05M substrate (palatinose, α-methyl-D-glucoside, isomaltose, sucrose or maltose). Aliquots of 0.2ml were withdrawn at various times and the reaction was terminated by heat denaturation at 98°C. Each aliquot was treated with 0.2ml of glucostat reagent (8), incubated for 30 minutes at 30°C and the reaction terminated upon addition of 0.8ml 6N HCl. Resultant colors were read at 525nm and total glucose produced extrapolated from a standard curve. All colors were read vs. substrate blanks made up in an identical manner as the reaction mixture, protein (heat denatured) included when precise measurements were necessary (kinetics). Rates were expressed as µg glucose formed min^{-1} ml^{-1}. Enzyme dilutions were performed occasionally to check proportionality between velocity and enzyme concentration. A special adaptation of the assay was necessary to perform enzyme kinetics. The reaction mixture containing a total volume of 0.5ml, with variable composition and quantity of substrate, was terminated by the precise addition of 100µl of the glucostat buffer without glycerol and at 3 times the ordinary concentration(5). A 0.2ml aliquot was withdrawn and the enzyme denatured by heating at 98°C for 3 minutes. The balance of the procedure was performed as described

above.

Isoelectric focusing

A procedure for isoelectric focusing was developed from a composite view of current literature much of which is reviewed by Righetti and Drysdale (53) and will be briefly summarized. The technique rests on the generation of a pH gradient formed by the application of an electrical potential across a mixture of polyamino-polycarboxylic acids, the anode being immersed in acid and the cathode in base. Ionization of the amphotere is dependent upon proximity to an electrode, and a pH gradient is formed when all polyamino-polycarboxylic acids assume a position such that the number of protonated amine groups is balanced by the number of ionized carboxyl groups, at which point the amphotere has a net charge of zero. The acid electrolyte at the anode serves to protonate ampholines at the positive end. The resultant positive charge proximal to the positive electrode prevents migration of the amphotere out of the gel. The details of the technique are as follows:

Ten centimeter length glass tubes, 7mm O.D., are cut and fire polished, soaked in cleaning solution and washed thoroughly so as to eliminate any ionic residue. Each tube is marked for the desired length of the gel, 8-9cm.

For a typical run of 12 gels the following solutions are mixed in individual test tubes:

0.41ml acrylamide (30g acrylamide-1gbis/100ml)

0.41ml TEMED (0.2ml TEMED/100ml)

40-50μl ampholines pH 4-6

0.688ml protein in 20% glycerol; the mixture of TEMED,

acrylamide and ampholines is cooled to 0-4°C prior to addition of protein.

0.1ml filtered riboflavin (15mg/100ml)

Riboflavin solutions are prepared fresh when required. The protein is exhaustively dialyzed into 20% glycerol-10^{-3}M EDTA prior to use to eliminate all buffer salts. It is advisable to add the riboflavin last to a precooled solution and to minimize exposure to laboratory fluorescent lighting prior to addition to the 10cm glass tubes.

Glass tubes are filled to the desired mark and if necessary additional mixture is added to set gel heights to exactly the desired marks for direct comparison of focusing profiles.

Each gel is carefully overlayed with water and the mixture is set directly before a (Canalco) fluorescent light for a full 2 hours. It is advisable to deaerate all non-protein reagents prior to use to prevent extensive cracking in the gels during the polymerization process. The gel mixture is allowed to stand undisturbed for 15 minutes prior to the photopolymerization at 0-4°C. As polymerization proceeds the developing gel will turn opaque within 15-20 minutes.

Gels are freed from surface water, and are set in the electrofocusing bath at the same level.

Catholyte is ethanolamine 0.2% $^{w}/_{v}$. Anolyte is sulfuric acid 0.2% $^{w}/_{v}$. In a pH 4-6 gradient, the cathode is the bottommost electrode. The catholyte gel surfaces are wetted with ethanolamine and the apparatus is assembled according to manufacturers instructions. The minimum volume of electrolyte was used so as to just cover the bottom of the gel tubes and the electrode (7). Focusing is initiated by applying a voltage such that the total power applied to the system is

0.3 watts/gel at constant power. The current on the constant power supply is set to maximum such that the current cut-off is inoperative. The voltage limit cut-off is set to either 400 or 500V. Voltage maximum is held for 5 hours and gel tubes are removed individually at intervals thereafter to check for completion of focusing. Occasionally, large protein loads will require additional time and runs up to 12 hours are not uncommon. To increase the resolution of the system 20cm gel length focusing was performed using a final ampholine concentration of 1.7% $^{w}/_{v}$ as compared to 0.9-1.2% $^{w}/_{v}$ for 8cm focusing. Gels were prepared with a final concentration of 15.6% reagent grade glycerol and the anolyte and catholyte were 0.01M glutamic acid (pH 3.3) and 0.01M histidine (pH 7.4), respectively. Optimal focusing time for 20cm gels was at least 36 hours with a cathodic drift setting in immediately prior to completion of focusing. The maximum voltage was set to 750V and the cell was cooled with recirculating water at 0-4°C. All focusing operations were performed in the cold room to minimize temperature rise in the gel during electrofocusing.

Protein staining

The G-250 instant staining technique was used as described in the LKB staining procedure (54). The reagent was prepared by dissolving 0.7g of G-250 in 59ml conc. perchloric acid and diluted to 1 liter, stirring one hour and filtering. Gels were incubated in the staining solution until bands of sufficient intensity were visible and the gel transferred to 7.5% HAc - 5% methanol. Occasionally, gels required 1 hour staining time and excess background was eliminated by briefly soaking in 3% perchloric acid. The gels were stored in 7.5 HAc-5% methanol-10% glycerol.

Activity staining

An aliquot of enzyme was applied to the gel that had been previously determined to hydrolyse substrate at the rate such that 100-150µg of glucose was generated from 0.05M α-methyl-D-glucoside in 5 minutes. The gels were immersed in a solution containing 0.05M α-methyl-D-glucoside and 0.1% 2,3,5 triphenyltetrazolium chloride in 0.1M phosphate buffer pH 7.0;10^{-3}M EDTA for a period of time such that 100-150µg of glucose was generated. Optimal time for activity staining was 5 minutes and protein concentration was adjusted accordingly. Reactions were terminated by addition of 0.1 volume 10N NaOH. The tubes were transferred to an 80°C water bath and colors visualized upon conversion of tetrazolium to insoluble formazan. In the reaction, the tetrazolium salt acts as a hydrogen acceptor, glucose serving as the reducing compound. Alternatively, sucrose could be used to perform activity staining of both α1 → 6 and α1 → 4 glucosidases. Staining was terminated with 7.5%HAc- 5% methanol.

Protease B assay

A reaction mixture containing 10mg azocoll dye, 0.5ml 0.1M phosphate buffer pH 7.7 and 0.1ml 1% triton X-100 was treated with 10µl or more of enzyme extract and constantly agitated at room temperature for varying time periods, 30 min, 1 hr, 2 hrs being representative of times of reaction. The reaction was terminated by the addition of 3.5ml of 0-4°C H_2O and the undissolved solid azocoll was filtered out. The absorbance at 520nm was compared to control tubes that contained the reaction mixture made up with azocoll and without protein. Absorbances were proportional to the time of incubation.

SDS gel electrophoresis

SDS gel electrophoresis was performed according to the method of Weber and Osborn (55) with controls incorporated from Weber et al. (56). All samples were treated with 1% SDS and 1% β mercaptoethanol in 0.01M sodium phosphate buffer separately at room temperature and at 100°C. The chymotrypsinogen A standard was treated with PMSF in addition to the SDS treatment. The enzyme (1mg) was incubated in 2ml of 1% SDS and 1% β mercaptoethanol for varying periods of time - 5 minutes, 30 minutes, 6 hours and 24 hours and with or without 4% PMSF. The PMSF control and the varying times of incubation were deemed necessary so as to ascertain the effects of a possible proteolytic contaminant upon the observed molecular weight of the enzyme. The balance of the procedure was according to Weber and Osborn (55).

Heat denaturation

Heat denaturation studies were performed by the method of Eaton and Zimmerman (57) utilizing PNPG as substrate. An amount of enzyme

which liberated 40-60 nanomoles of p-nitrophenol min^{-1} (in all cases less than 0.5ml) was added to buffer (10^{-3} M EDTA in 0.1M phosphate, pH 7.0 equilibrated at 45°C) to give a total volume of 2.5ml. The mixture was equilibrated at 45°C for 2 minutes. At 2 minute intervals over a period of 22 minutes 0.2ml samples were removed to chilled tubes for subsequent PNPG assays. Points were fitted to a straight line according to the least squares method. The correlation coefficients (r) were 0.98-0.99.

Amino acid analysis

Amino acid analysis was performed according to the procedure of Moore and Stein (58). A volume of 70ml of pure enzyme (containing 12mg of protein) in 0.1M phosphate buffer-10^{-3}M EDTA; 20% glycerol (standard storage buffer) was dialyzed exhaustively against H_2O at 0-4°C and lyophilized to dryness. The dry sample was taken up in 8ml of constant boiling HCl, made up to 0.15% in βmercaptoethanol, and the suspension distributed equally to 4 Carius combustion tubes. Each tube was subject to 4 cycles of freeze-evacuate-thaw and the tubes then sealed with a methane-oxygen torch. Evacuation was performed with an oil vacuum pump and samples were frozen in liquid nitrogen. The tubes were placed in an oven set at 110°C±1°C, and incubated for 18,24,48 and 72 hours, respectively, after which the top portion of each tube was scored, broken off and the HCl was removed under nitrogen and at a temperature of 40°C. To the dry residue of amino acids was added 4ml of pH 2.2 citrate buffer, the sample buffer for the Beckman 116 amino acid analyzer. The samples were then stored at 0-4°C prior to the analysis. This procedure was carried out for isomaltase from maltose induced cells. The same procedure was followed with 280µg of protein

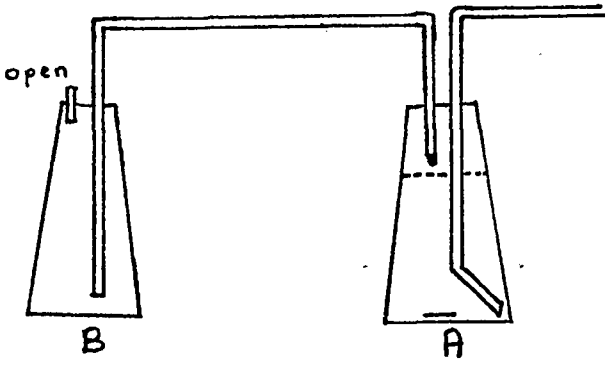
for the α-methyl-D-glucoside induced isomaltase. Both forms of the enzyme were subject to amino acid analysis using standard column techniques and the fluorescamine reagent in the detection system. Peaks were integrated with a Hewlett Packard 3390 A integrator. In addition to analysis by the above methods both forms of isomaltase were subject to isoelectric focusing, stained with G-250 and the excised bands subject to amino acid analysis with the fluorescamine reagent. This amino acid analysis was kindly performed by S. Stein of the Roche Institute of Molecular Biology using a laboratory built instrument (66).

Operation of the Beckman 116 amino acid analyser

The Beckman 116 amino acid analyzer was operated according to the procedures of Beckman Instrument Co. Ninhydrin was prepared in methyl cellosolve solvent and stored under nitrogen and all buffers were purchased from Beckman Instrument Co. An Infotronics integrator specially designed for amino acid analysis was connected to channel 3 of the analyser, baseline tracking being under correction by the integrator. A sample injector with a 0.5ml capacity was used. All standards were run in triplicate with 2 standards being analyzed prior to actual sample analysis and one at the completion. The product of the proline and ninhydrin reaction was measured by integration of the absorption peak at 440nm, the machine being set to switchover to 440nm from 570nm at the preset time of the proline peak. Standard reproducibility was within 2% on all standard amino acids. A 1ml ampule containing 1.25 μmole cystine and 2.50 μmoles of each of the other amino acids was quantitatively diluted 1:10 with sample dilution buffer and a 0.5ml aliquot was used for the standard analysis.

A concentration of sample hydrolysate was analyzed such that the concentration of amino acid in greatest abundance was nearly equal to 0.125 μmoles (the concentration of the amino acids in the standard). The number of μmoles of amino acids in an unknown hydrolysate of 0.5ml was quantitated by dividing the integrator readout for the unknown by the readout for the actual standard and multiplying by 0.125, the number of μmoles of each amino acid (excepting cystine) in the standard. In all cases the same sample was used for both long column (acidic and neutral amino acids) and short column (basic amino acid analysis).

Preparation of gradient maker



Flask A was capped with an air tight 2 hole rubber stopper into which 2 glass tubes were inserted, one leading through tygon tubing to the column and the other to a high molarity feeder flask. Upon elution the partial vacuum created in the space above the liquid in A drew solution from B through the glass tube and into A which was being stirred magnetically. Typically, glass tubing with a 7mm O.D. was used. However, when solutions containing 20% glycerol were used it was imperative to use glass tubing with an inner diameter of 4-5mm in order to insure uniform draw. The gradients produced by this type of gradient maker are hyperbolic, and quite reproducible.

Purification of Maltose

Glucose, which contaminated the maltose preparation to be used as a substrate for maltase, was removed as follows:
A 1.5x100cm column with packing reservoir was packed with G-15 swelled in H_2O. Maltose was dissolved in H_2O such that the resulting viscosity was no more than 2x that of pure H_2O as measured by an Ostwald viscometer. The column was run in the manner recommended by Pharmacia with H_2O as eluent. Reducing sugars in the effluent flow were identified by reduction of a tetrazolium dye. Glucose was located by the glucostat assay and maltose by hydrolytic cleavage with maltase. A representative elution profile is illustrated in Fig.2. Purified maltose was lyophilized to dryness and stored at -10°C.

Flat bed isoelectric focusing

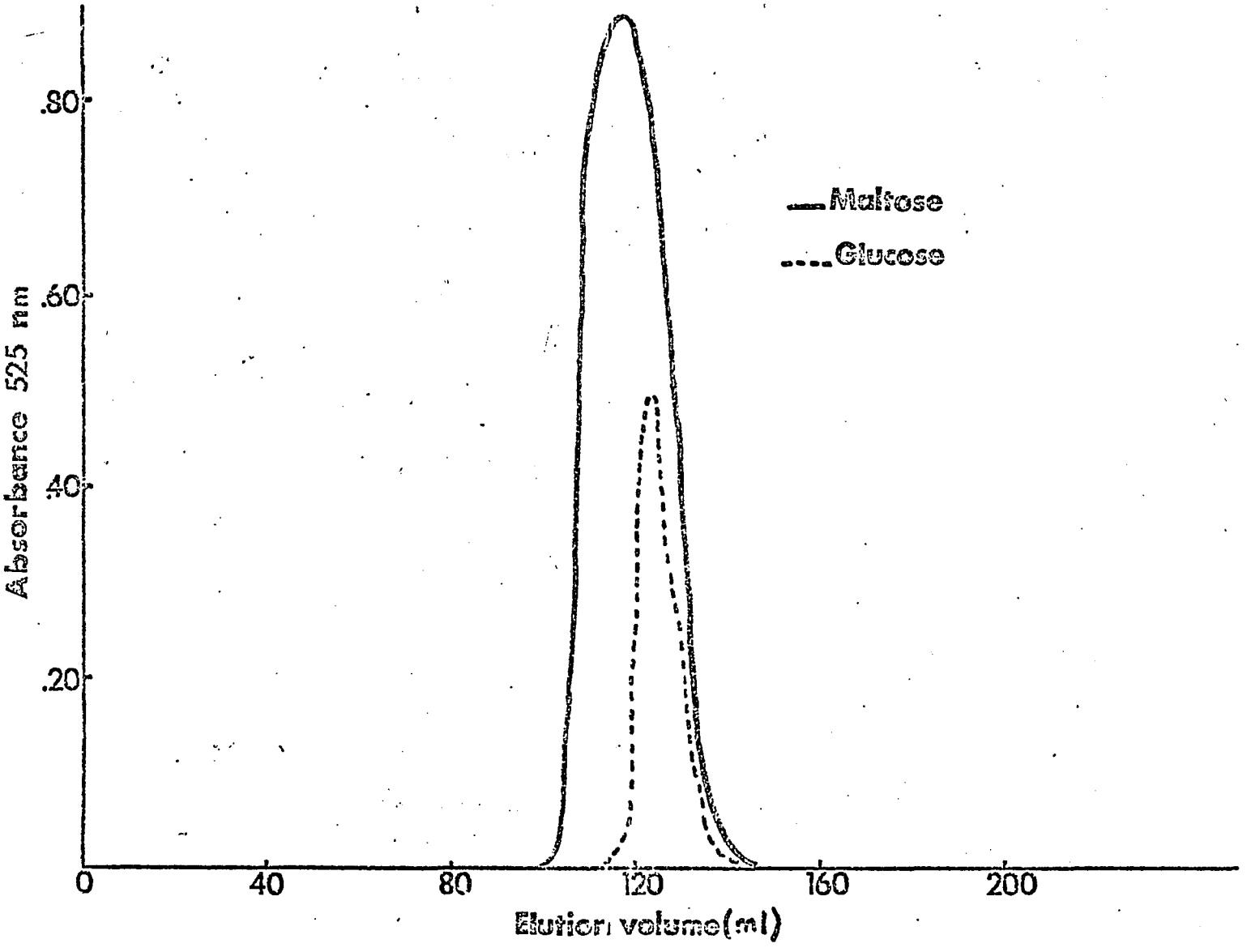
Preparative flat bed electrofocusing in granulated gel with the LKB multiphor was performed according to LKB application note 198. The gel was ultrogel as supplied by LKB and the salt free sample was included in the original gel slurry from which the gel bed was prepared. Excess water was evaporated by allowing the tray to remain open to the air for several days. After 16 hours a fractionating grid was used to section the gel and samples were recovered from each section by elution. The pH gradient was linear over the region of interest. Analytical polyacrylamide isoelectric focusing on the fraction purified in this way is shown in Figure 3.

Fractionation of narrow range ampholines

Ampholines of pH 4-6 were fractionated in Sephadex G-15 according to the method of O'Brien (60). A 2.5x40cm Pharmacia column (bed height

Fig. 2
Sephadex G-15 chromatography of maltose contaminated with glucose. Glucose was measured by the glucostat assay and maltose by hydrolytic cleavage with maltase. The chromatography was performed in the manner described in the text.

FIG.2 SEPARATION OF GLUCOSE FROM MALTOSE



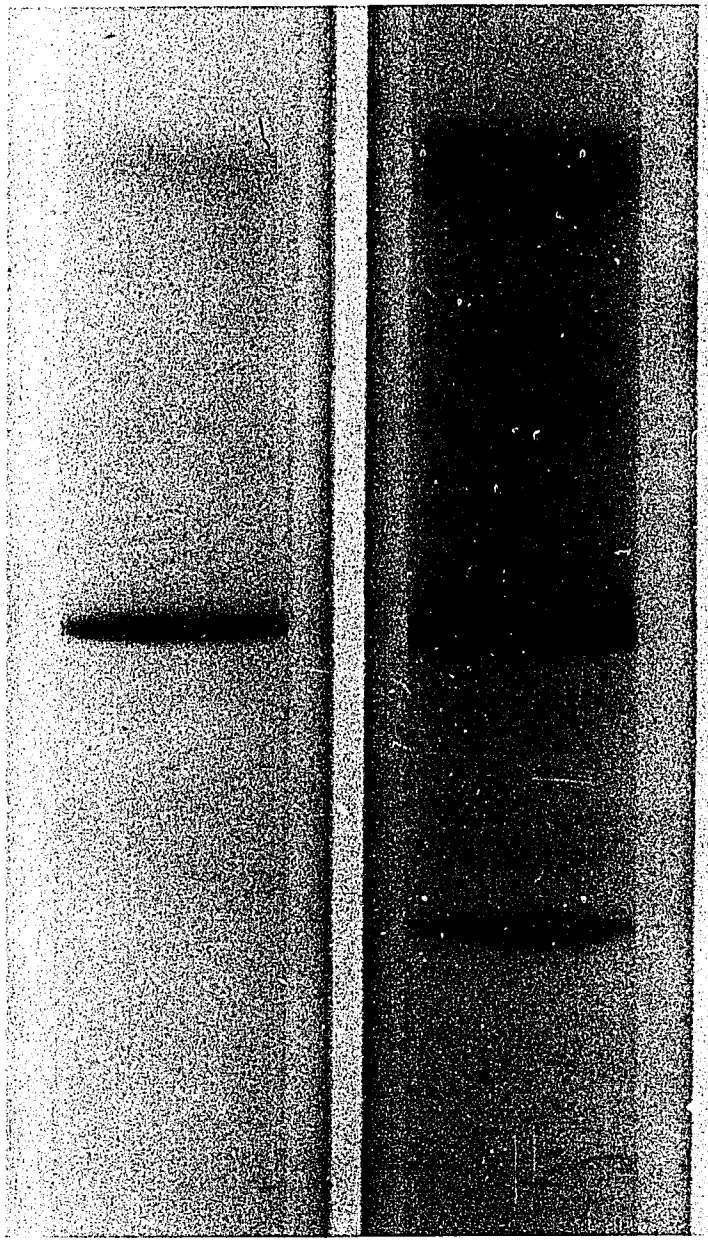


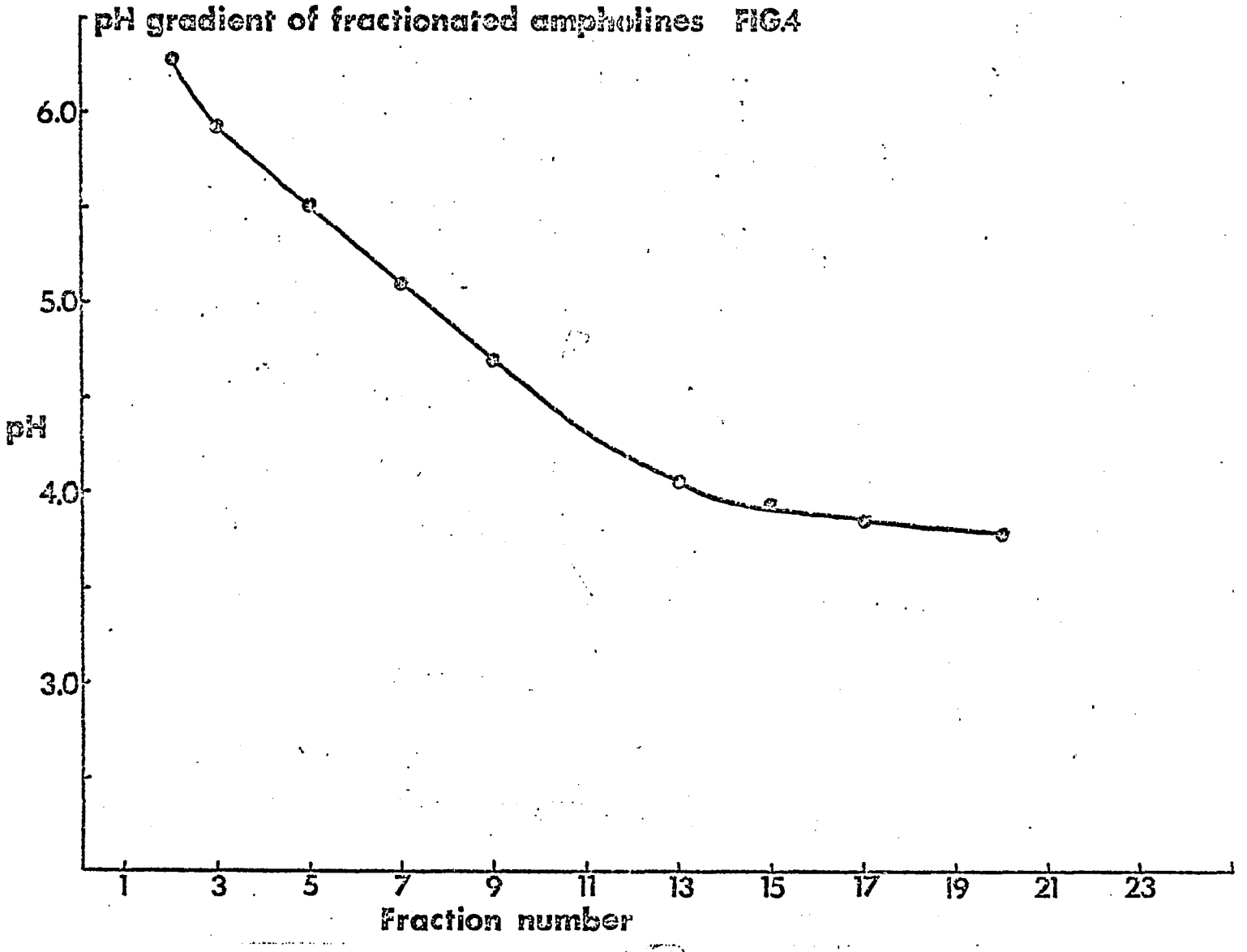
Figure 3
Analytical polyacrylamide isoelectric focusing pH 4-6 on M form of isomaltase purified by preparative isoelectric focusing in granulated gel on the LKB Multiphor. Gels were stained with Coomasie G-250
Lane 1 25 ug protein
Lane 2 100 ug protein

as to remove all charged substances. The column was then equilibrated with two bed volumes of a solution containing 1.5% carrier ampholytes in 10% glycerol. The column inlet flow adaptor was removed and a nylon net support was lowered on the surface of the bed by means of 3 lengths of monofilament fishing line tied to the net. Ten ml of an acrylamide polymerization solution (60) was layered over the bed and net, and the solution allowed to gel. The monofilament facilitated the removal of the polyacrylamide plug after termination of IEF. The column was inverted and a second plug was cast. The balance of the procedure was according to O'Brien (60). A representative pH gradient of eluted fractions is shown in Figure 4.

Growth of yeast

Nine liters of media in a Virtis Model fermenting vat and 300 ml of culture media in a Erlenmeyer flask were prepared containing 1% yeast extract, 2% peptone and 2% maltose. One ml 30% silicone antifoam solution was added to the fermentation vat. The fermentation vat was autoclaved for 50 minutes at 121°C and 115lbs/sq. in., whereas the flask was autoclaved for 15 minutes at the same temperature and pressure. Alternatively, when it was desired to induce both major forms of the enzyme simultaneously, the culture media consisted of 1% yeast extract, 2% peptone, 1% maltose and 1% α-methyl-D-glucoside. A volume of 300ml of culture media were inoculated with yeast strain 1412-4D and incubated in a Brunswick incubator at 30°C with shaking at 200 r.p.m. for 24 hours. The slant containing the stock culture was inoculated several days in advance and used immediately without refrigeration to inoculate 300 ml of starter culture. Starter culture was counted in a hemocytometer and the 9 liter Virtis fermenting vat

Fig.4
pH gradient of eluted ampholines fractionated by isoelectric focusing in Sephadex G-15 as described in the text. Fraction volumes of 15ml/ tube were collected.


pH gradient of fractionated ampholines FIG.4
6.0
5.0
4.0
3.0
pH
1
3
5
7
9
11
13
15
17
19
21
23
Fraction number

was counted in a hemocytometer and the 9 liter Virtis fermenting vat was inoculated with 300 ml of starter culture containing 2.72 X 10^8 cells/ml (A_{600}=2.429). The cells were resuspended in cold (0^oC) water and centrifuged at 10,000 r.p.m. in the Sorvall RC-2B using a GSA rotor. Washing was repeated once. The wet weight of the harvested cells was 102 grams and the cells were frozen immediately at -15^oC.

Disruption of yeast and preparation of crude extract

One hundred and two grams of maltose-induced cells were allowed to thaw and disrupted in the Eaton press (63) at 20,000 lbs/sq.inch. To the disrupted cells, 816 ml extraction buffer containing 0.1M phosphate buffer pH 7.0-0.2M $(NH_4)_2SO_4$-10^{-3}M EDTA was added and the mixture was stirred at $0-4^oC$ for 2 1/2 hours. Unless otherwise indicated, all subsequent operations were performed at $0-4^oC$ in the cold room. After stirring, the suspension containing cell debris was centrifuged at 10,000 r.p.m. for 30 minutes in the GSA rotor and the yellow supernatant retained for purification procedures.

First ammonium sulfate fractionation

The supernatant was brought to 35% saturation with solid $(NH_4)_2SO_4$ at pH 7.0 and 4^oC. The precipitate was removed by centrifugation, the supernatant containing all the enzyme activity. The yellow supernatant was brought to 95% saturation by the slow addition of solid $(NH_4)_2SO_4$. The pH was maintained at 7.0 by the slow dropwise addition of 1N NaOH into the vortex created by the stirring bar. The suspension containing precipitate obtained at 95% $(NH_4)_2SO_4$ saturation was centrifuged at 10,000 r.p.m. in a GSA rotor for 30 minutes and contained essentially all the enzyme activity. The supernatant, containing considerable protein, was found to be essentially devoid of activity. At this

point, the precipitate was separated from the supernatant and suspended in a solution 95% saturated in $(NH_4)_2SO_4$, adjusted to pH 7.0. The enzyme preparation was stable and could be stored at 0-4°C for several months without any loss in activity.

Second extraction of cell debris and non-disrupted cells

The cellular debris from the first extraction was immediately frozen for storage and thawed when ready to be used. It was extracted in a Braun homogenizer with 0.45mm glass beads in the same buffer as used in the first extraction. Eight hundred ml of extraction buffer was added to the disrupted cells and stirred for 2 1/2 hours. The procedure including the $(NH_4)_2SO_4$ cut was identical to that reported for the first extraction. The second extract was found to contain 12% of the total activity. The 95% $(NH_4)_2SO_4$ precipitate from the 2nd extract was pooled with the first and the balance of the purification procedure performed on a single pool of enzyme.

G-100 Sephadex Chromatography

The 95% $(NH_4)_2SO_4$ precipitate was redissolved in a minimum amount of 0.1M phosphate buffer-10^{-3}M EDTA containing $(NH_4)_2SO_4$ at 20% saturation. The concentrated enzyme solution was dialyzed against two 4 liter changes of 0.1M phosphate buffer-10^{-3}M EDTA-20% $(NH_4)_2SO_4$-pH 7.0. (As reviewed by Pringle (26), $(NH_4)_2SO_4$ is a potent inhibitor of protease B). Dialysis against buffer in which 20% $(NH_4)_2SO_4$ was not included resulted in a loss of 50% of enzyme activity occurring within 8 hours of dialysis and the production of a large amount of insoluble material. Upon termination of dialysis the volume of the enzyme preparation was 200ml containing material imparting an opaque appearance to the preparation. A 5 X 100cm column fitted with flow adaptors was packed with Sephadex G-100 swollen in 0.1M phosphate buffer-10^{-3}M EDTA-20% $(NH_4)_2SO_4$-pH 7.0. The bed height after packing was 92cm at a head of hydrostatic pressure of 80cm and a flow rate of 70ml/hour. The flow rate was increased to 90ml/hour during sample

application through the flow adaptors. Two hundred ml of the extract were applied to the column, representing approximately 11% of the total bed volume. During sample application, the head of hydrostatic pressure was 100cm. The flow rate was 70ml/hour at 80cm hydrostatic pressure and the column was developed with 0.1M phosphate buffer -10^{-3}M EDTA-20% $(NH_4)_2SO_4$-pH 7.0. As protein eluted from the column outlet there was a considerable drop in flow rate. Fractions of approximately 10ml/tube were collected. The major peak of enzyme activity eluted just beyond a peak of white turbid material with some overlap of the two materials occurring. The enzyme activity was pooled, and subsequent assay showed essentially full recovery of enzymatic activity. Purification and recovery are shown in Table 1. Occasionally, the major pool of activity was rechromatographed and essentially all the turbidity producing material was removed. The enzyme solution was characterized at this point by a crystal clear yellow appearance. A representative elution profile is illustrated in Fig.5. No separation of the multiple isomaltases occurred on this column, and 80% of the total non enzymatic protein was eliminated from the preparation.

Stability studies

Dialysis of the pooled active fractions from the G-100 column against 0.1M phosphate buffer -10^{-3}M EDTA resulted in a precipitous loss of enzyme activity accompanied by the appearance of a large amount of insoluble protein in the dialysis bag. The loss was not apparent when the preparation was dialyzed against the same buffer with the inclusion of 20% $(NH_4)_2SO_4$. Enzyme preparations without $(NH_4)_2SO_4$ added contain a substance possessing hydrolytic activity towards

Table 1

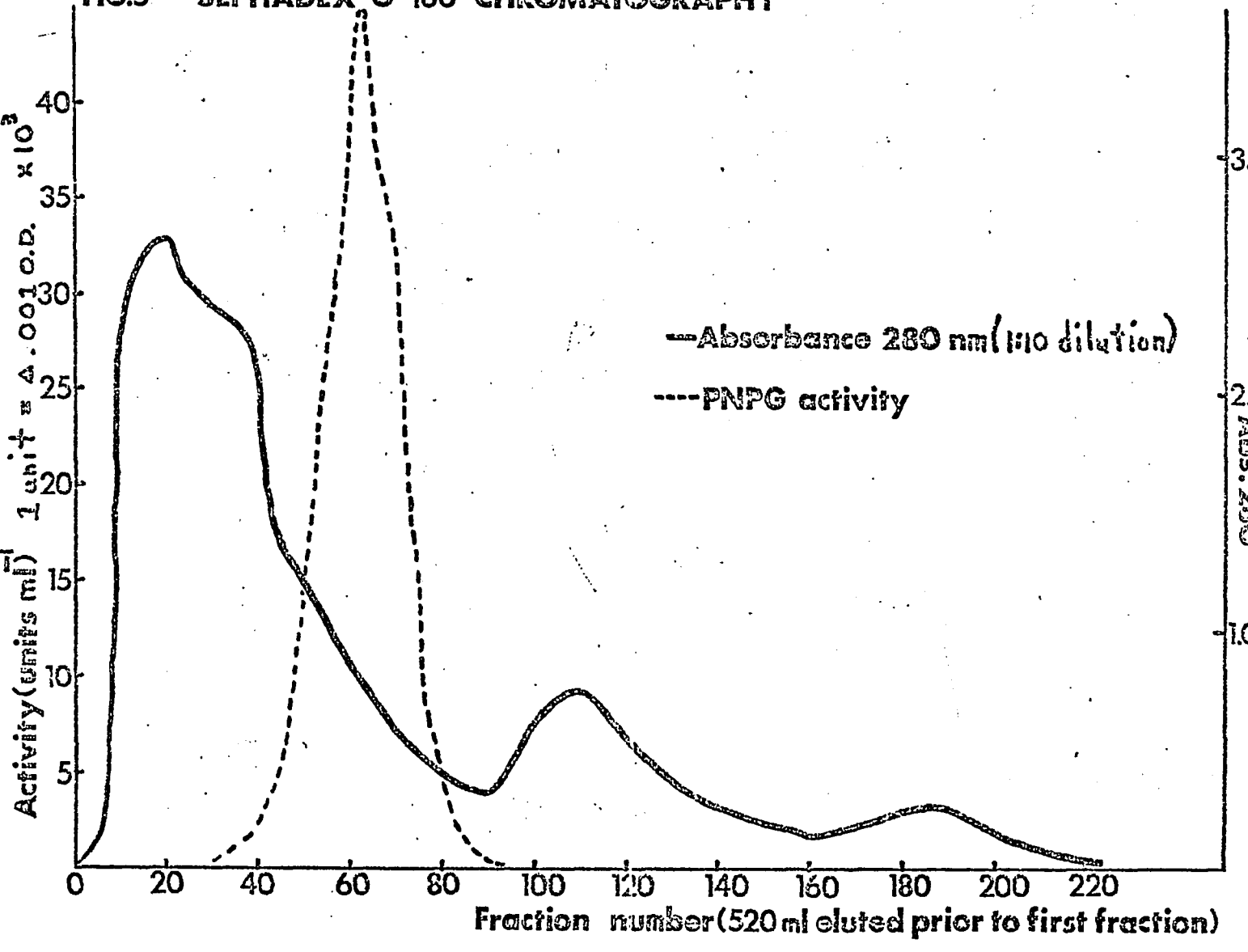
Purification of the maltose induced isomaltase(M) from a maltose induced extract

	Volume (ml)	Palatinose Activity $\mu g min^{-1} ml^{-1}$	Protein mg ml^{-1}	Specific Activity	Total protein grams	Total Activity	Yield	Fold Purification
1) Crude extract - Eaton press	885	336	10.4	32.3	9.2	297,360	100	1
Second extract - Braun homogeniser	850	40.3	2.8	14.4	2.4	34,255		
				28.7(combined)				
2) First and second extracts combined and enzyme recovered from a 35-95% $(NH_4)_2SO_4$ precipitate. The precipitate was dissolved in 0.1M phosphate buffer pH 7.0-10^{-3}M EDTA-20% $(NH_4)_2SO_4$ and dialysed against two 4 liter changes of the same buffer	200	1158	22.9	50.6	4.6	231,600	70	1.8
3) G-100 column Fractions 55-97 pooled	350	704	4.8	147	1.7	246,400	74	5.1
4) DEAE-Sepharose in the presence of 20% glycerol. All fractions containing only the maltose induced form (130-150) pooled	370	359	0.456	787	0.169	132,830	40	27
5) Second DEAE Sepharose. Fractions 175-200 pooled and assayed	240	432	0.165	2618	0.040	103,680	31	91

Homogeneous upon isoelectric focusing pH 4-6

Fig. 5
Sephadex G-100 chromatography on a crude extract of maltose induced cells as described in the text.
——— , Absorbance 280nm; - - -, PNPG activity.

FIG.5 SEPHADEX G-100 CHROMATOGRAPHY



azocoll dye in the neutral pH range.

Stability studies performed on the enzyme preparation equilibrated with concentrations of glycerol ranging from 15-50% are illustrated in Fig. 6. Glycerol was added directly to the preparation to achieve the desired concentrations and the preparation was dialyzed against 2 changes of buffer containing glycerol.

First DEAE Sepharose Chromatography

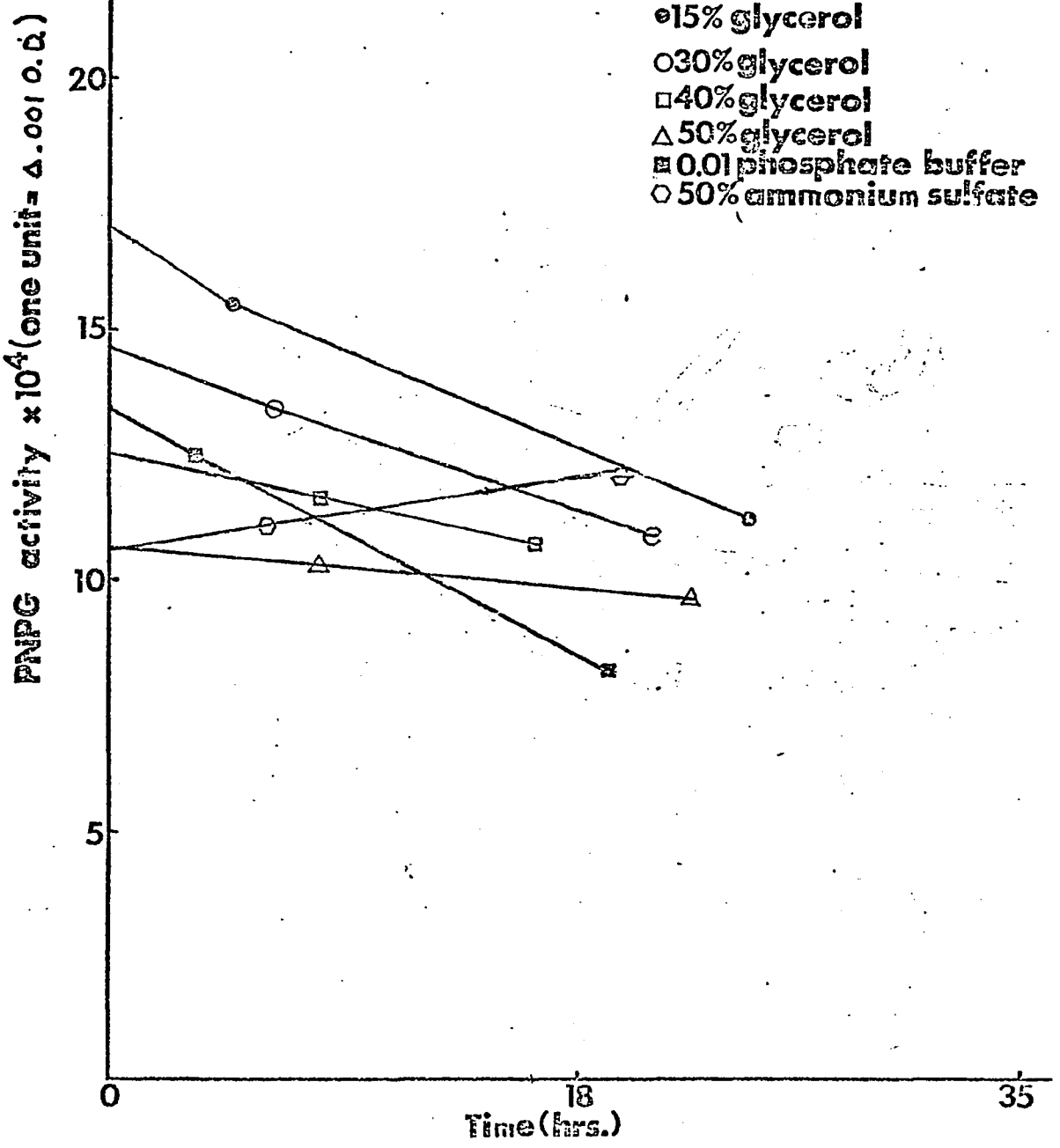
A 5X60cm column (bed height 46cm) with flow adaptors was packed with DEAE Sepharose and equilibrated with 2 liters of 0.05M phosphate buffer -10^{-3}M EDTA- pH 7.45-20% glycerol followed by 4 liters of 0.01M phosphate buffer -10^{-3}M EDTA pH 7.45-20% glycerol. Flow was maintained by a peristaltic pump.

Glycerol (final concentration 20%) was added to the enzyme preparation obtained from G-100 chromatography and the enzyme was dialyzed against six 4 liter changes of equilibration buffer or until the dialysate showed the absence of SO_4^{-2}.

The dialysed enzyme solution, clear yellow in appearance, was applied to the column at a flow rate of 240ml/hour, a bright yellow band of bound protein appearing in the top 1-2cm of the gel. The column was washed with 2 volumes of equilibration buffer followed by a hyperbolic salt gradient of 0-0.3M NaCl. A flow rate of 70ml/hour was maintained by a peristaltic pump, and fractions of 15ml were collected. The gradient maker was prepared as described with the low molarity chamber containing 4 liters of 0.01M phosphate-20% glycerol-10^{-3}M EDTA and the high molarity flask containing 8 liters of 0.01M phosphate-20% glycerol-10^{-3}M EDTA-0.3M NaCl. A representative elution profile of the chromatography is illustrated in Fig.7 with accompanying photographs

Fig. 6
Stability study at 0-4°C of a crude extract containing all forms of isomaltase. Samples were supplemented at the indicated glycerol concentrations and dialyzed against glycerol at the same concentration. Activity was measured by rate of hydrolysis of p-nitrophenyl- α-D-glucopyranoside at 30°C.

FIG.6 STABILITY STUDY ON A CRUDE EXTRACT



of isoelectric focusing and activity staining confirming the chromatographic separation (Fig.7A).

Second DEAE Sepharose Chromatography

The major peak of α-methylglucosidase (isomaltase) was pooled and dialyzed against 0.01M phosphate buffer-10^{-3}M EDTA-20% glycerol-pH 7.45 prior to DEAE chromatography. The enzyme was applied to a 2.6X106cm column at a flow rate of 100ml/hour. The column was washed with one volume of equilibration buffer at a flow rate of 60ml/hour followed by a hyperbolic salt gradient of 0-0.1M NaCl. The flow rate was 40ml/hour and fractions of 15ml were collected. The gradient maker was prepared as described above with the low molarity chamber containing 4 liters of 0.01M phosphate buffer-20% glycerol-10^{-3}M EDTA and the high molarity flask containing 8 liters of 0.01M phosphate-20% glycerol-10^{-3} EDTA-0.1M NaCl. A representative elution profile of the chromatography is illustrated in Fig.8 along with accompanying photographs of isoelectric focusing and activity staining confirming the chromatographic separation (Fig.8A).

Criteria of Purity

The degree of purity was judged by isoelectric focusing profiles, SDS gel electrophoresis, and by polyacrylamide gel electrophoresis, the latter according to the method of Gabriel (65). The running gels were pH 8.9 and the buffer system was Tris-glycine pH 8.9. The gels were stained as described by the method of Weber and Osborn (55). Isoelectric focusing gels stained with the G-250 stain required no destaining, while Coomasie R-250 stained gels were destained electrolytically in a Canalco fast destainer according to Weber and Osborn (55).

Purification of α-glucosidases From 1412-4D Under Conditions of Simultaneous Induction

The purification scheme reported earlier was for the purification of isomaltase from maltose induced yeast cells. When it was desired to induce two major forms of isomaltase in approximately equal amounts, cells were grown on 2% peptone, 1% yeast extract, 1% maltose and 1% α-methyl-D-glucoside. The purification scheme was essentially identical as that reported for the maltose induced enzymes until the first DEAE-Sepharose column and the methods of purification of a simultaneously induced extract begins at that point.

DEAE-Sepharose Chromatography

A 2.6X40cm column (bed height 35cm) was packed with DEAE-Sepharose and equilibrated with 2 liters of 0.05M phosphate buffer pH 7.45-20% glycerol followed by 4 liters of 0.01M phosphate buffer pH 7.45-20% glycerol. Flow was maintained by a peristaltic pump. All solutions contained 10^{-3}M EDTA.

The enzyme preparation was prepared as previously described and 14,620,000 PNPG units (One O.D. unit=Δ0.001/min at 400 nm) were adsorbed onto the column. The column was washed with 2 volumes of equilibration buffer followed by a hyperbolic salt gradient of 0-0.3M NaCl. Flow rate was 18ml/hour and fractions of 5ml were collected. The gradient maker was prepared as described with the low molarity chamber containing 750ml of 0.01M phosphate buffer-20% glycerol and the high molarity flask containing 750ml of 0.01M phosphate-20% glycerol-0.3M NaCl. A representative elution profile of the chromatography is illustrated in Fig. 9 with accompanying photographs of isoelectric focusing and activity staining confirming the chromatographic

separation (Fig. 9A). The α-methyl-D-glucoside induced isomaltase was concentrated into fractions 75-103 and the maltose-induced isomaltase into fractions 103-137 along with one form of maltase (α 1 $\rightarrow$ 4 glucosidase).

Hydroxylapatite Chromatography

Following dialysis against 0.01M phosphate buffer-20% glycerol-10^{-3} EDTA-pH 7.0 fractions 75-103 and 103-137 were applied to separate 1.6X30cm columns (bed height 25cm) packed with hydroxylapatite and equilibrated with 500ml of 0.05M phosphate buffer-20% glycerol-10^{-3}M EDTA pH 7.0 followed by 500ml of 0.01M phosphate buffer-20% glycerol-10^{-3}M EDTA-pH 7.0. Flow was maintained by a peristaltic pump. After application of the enzyme the column was washed with 2 volumes of equilibration buffer followed by a hyperbolic salt gradient of 0.01-0.1M potassium phosphate. After attaining approximately 0.1M potassium phosphate in the effluent flow the column to which fractions 103-137 was applied was subject to a hyperbolic gradient of 0.1-0.3M potassium phosphate in order to elute maltase. Flow rate was 8ml/hour and fractions of 4ml were collected. The gradient maker was prepared as described with the low molarity chamber containing 200ml of 0.01M phosphate buffer-20% glycerol-pH 7.0 and the high molarity flask containing 1,000ml of 0.1M phosphate-20% glycerol-10^{-3}M EDTA-pH 7.0. A representative elution profile of the chromatography is illustrated in Fig. 10 and Fig. 11 along with accompanying photographs of isoelectric focusing and activity staining confirming the chromatographic separation (Fig.10A and Fig. 11A). Aliquots of the eluted peaks were mixed and subject to IEF and activity staining to ascertain the identity of the various forms.

Induction studies

A volume of 300ml of culture media in Erlenmeyer flasks was prepared containing;

Flask 1 1% yeast extract, 2% peptone, 2% maltose

Flask 2 1% yeast extract, 2% peptone, 2% α-MG

Flask 3 1% yeast extract, 2% peptone, 0.8% α-MG + 1.7% maltose

Flask 4 1% yeast extract, 2% peptone, 1% α-MG + 1% maltose

The media were inoculated with yeast strain 1412-4D and incubated at 30°C with swirling for 20 hours. Cells were harvested by centrifugation at 10,000 r.p.m. for 30 minutes in the GSA rotor and disrupted in a Braun homogenizer with glass beads of 0.45mm diameter. Extraction buffer was 0.1M phosphate pH 7.0-5% $(NH_4)_2SO_4$-10^{-3}M EDTA. The suspension was centrifuged at 10,000 r.p.m. for 30 minutes in a GSA rotor and the supernatant was exhaustively dialysed against a solution containing 20% glycerol-10^{-3}M EDTA pH 7.0. Isoelectric focusing and activity staining with α-methyl-D-glucoside as substrate were performed in 20cm gels as described. Results are illustrated in Fig. 12 with lanes 1,2 and 3 corresponding to flasks 1,2 and 3 and Fig.13 corresponding to flask 4 (10cm gel).

Preparative electrofocusing (20cm)

Individually per gel

1.26ml acrylamide (30g acrylamide-1g bis/100ml)

1.26ml TEMED (0.23ml TEMED/100ml)

0.225ml ampholines pH 4-6

0.975ml 75% glycerol

1.12ml enzyme-H_2O

0.315ml fresh filtered riboflavin (15mg/100ml)

Total volume=5.155ml

Catholyte was 0.01M histidine and anolyte 0.01M glutamic acid.

The resolution achieved in 10cm gels in a pH 4-6 gradient was insufficient to recover material from parallel unstained gels. The staining reaction produced a size change in the gel typically of 0.5-1.0cm and the separation of active components was insufficient for accurate alignment and recovery. For preparative focusing, gel tubes of either 7 or 8mm O.D. were used and the focusing was performed as described. Ampholine concentration (1.7% $^{W}/v$) was the minimum consistent with good separation, with higher concentrations of ampholines requiring longer focusing times. Gels were sampled at 36 hours and at intervals thereafter if focusing was incomplete (as evidenced by undefined background staining), until a final stable pattern was attained. The correct protein load was ascertained by staining 10cm IEF gels with Coomasie G-250, slightly more protein being applied to 20cm gels. Loading was a function of band separation and consistent with ampholine buffering capacity. The solution for the entire focusing run was prepared and pipetted into tubes and adjusted to the same height (from 18 - 19.5cm). It was imperative to set gel heights exactly so as to make accurate cuts and it was desirable to make slightly more gel solution than needed. IEF was performed in 17-25cm gels, the gels were stained for activity as described and dehydrated to their former size by incubation in 40% ethanol-20% glycerol. Gels were measured prior to the staining reaction and intermittently during the course of dehydration. Representative gels

were removed from opposite sides of the gel bath as a measure of reproducibility of the banding position. Unstained gels were aligned with 2 stained gels and gel slices were taken corresponding to active bands. The polyacrylamide gel slices were then completely macerated in a 50ml beaker with the pestle of a Potter Elvehem apparatus and taken up in 10ml of 0.1M phosphate buffer-pH 7.0-10% glycerol, allowed to stir overnight and the gel separated from the extracted enzyme by centrifugation. The process was repeated once more and the combined extracts were pooled, assayed and dialyzed against 0.1M phosphate-pH 7.0-10% glycerol to remove ampholines. The solution containing enzyme was dialyzed against 20% glycerol-10^{-3}M EDTA to remove all buffer salts, refocused in a 10cm IEF gel and stained for protein to ascertain the homogeneity of the material. Phosphate buffer was included in the initial dialysis so as to prevent charge interactions between ampholines and protein.

CHAPTER III

RESULTS AND DISCUSSION

Purification of α-glucosidases From 1412-4D Strain of Saccharomyces Cerevisae Under Conditions of Simultaneous Induction

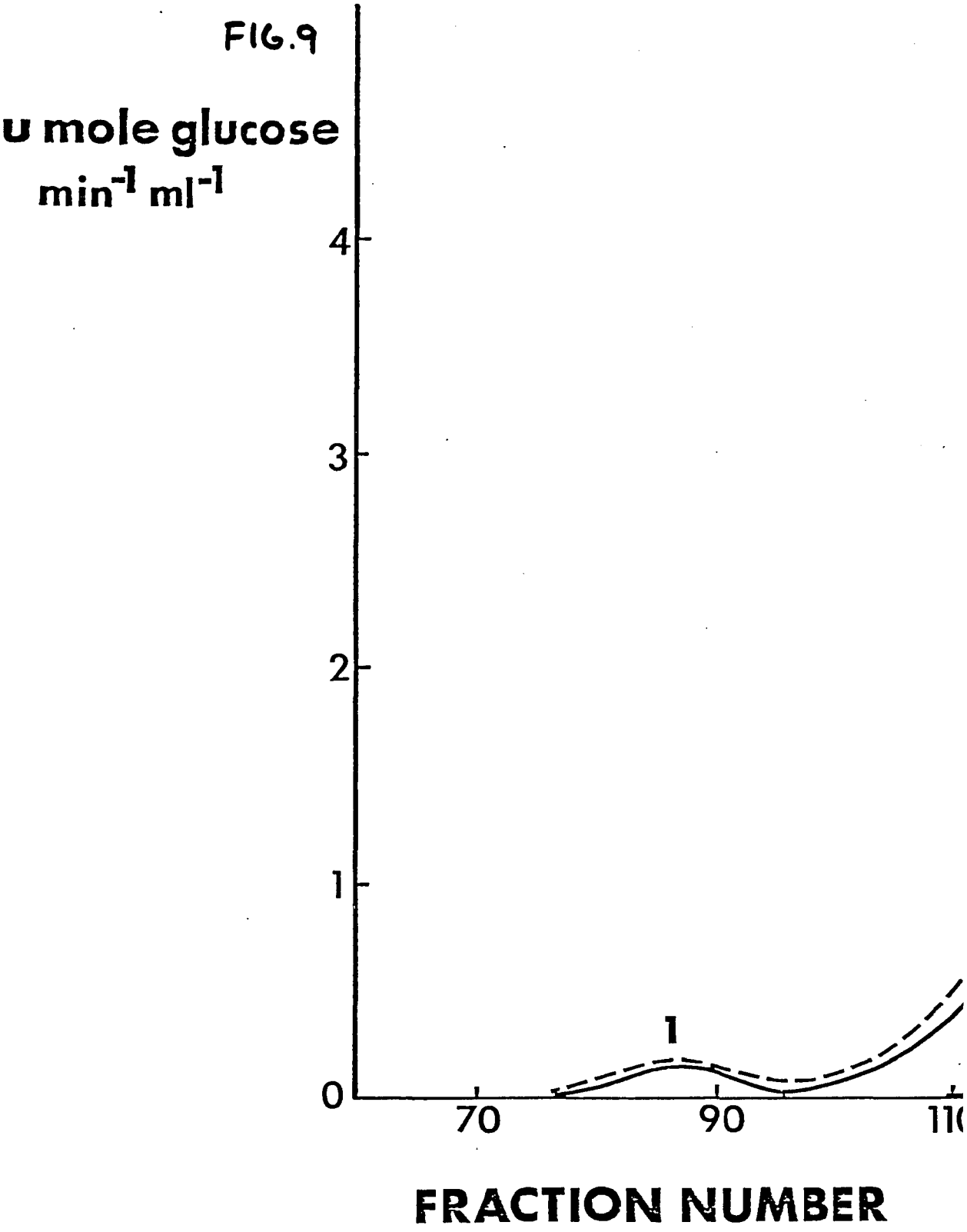
The existence of multiple forms of isomaltase was substantiated by chromatographic separations and IEF and activity staining of the eluted peaks. For purposes of proof of the reality of multiple forms, it is more illustrative to consider separations where all forms of α-glucosidases were present in approximately equal amounts as under simultaneous induction conditions. Despite symmetrical and well resolved protein peaks, complete separation of the three forms of isomaltase was not achieved because of tailing from one peak to another. For the purposes of isolation it is desirable to induce with only one sugar, producing predominantly one form of the enzyme. Chromatographic separation was maximal when samples applied to the column contained a preponderance of one form of the enzyme. The discussion begins with the separation on DEAE-Sepharose chromatography of two forms of isomaltase from simultaneously induced yeast cells.

DEAE Sepharose Chromatography

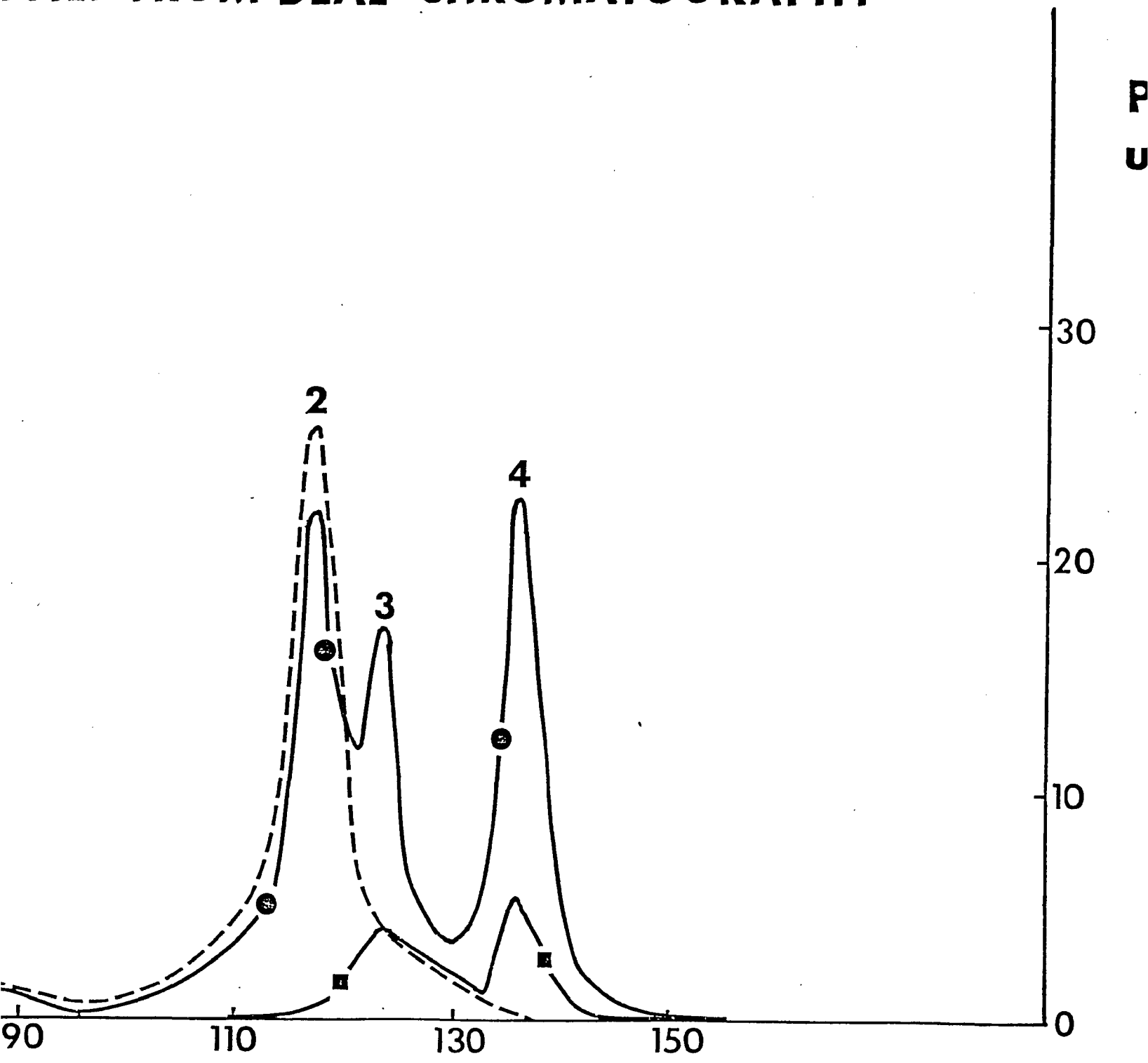
(Fig. 9, 9A). Enzyme activity was determined qualitatively in porcelain spot plates to determine protein peaks possessing hydrolytic activity towards PNPG. The active peaks were then assayed quantitatively with the PNPG assay. The nonspecific PNPG assay showed four peaks of α-glucosidase activity. Two of the peaks (1 and 2) demonstrated substrate specificity for isomaltose, α-methyl-D-glucoside and palatinose and two were active towards maltose (3 and 4). Isoelectric focusing and activity staining were performed on pooled fractions 75-103 and on fractions 103-137.. DEAE-Sepharose

Fig. 9
DEAE Sepharose chromatography of pooled fractions 30-90 from G-100 chromatography. —●— , p-nitrophenyl α-D-glucopyranoside hydrolysis;——, isomaltose hydrolysis; —■— , maltose hydrolysis. Elution peaks are designated 1, 2, 3 and 4.

FIG.9


ELUTION PROFILE FROM
u mole glucose
min-1 ml-1
4
3
2
1
0
1
70
90
FRACTION NUMBER

DFILE FROM DEAE CHROMATOGRAPHY



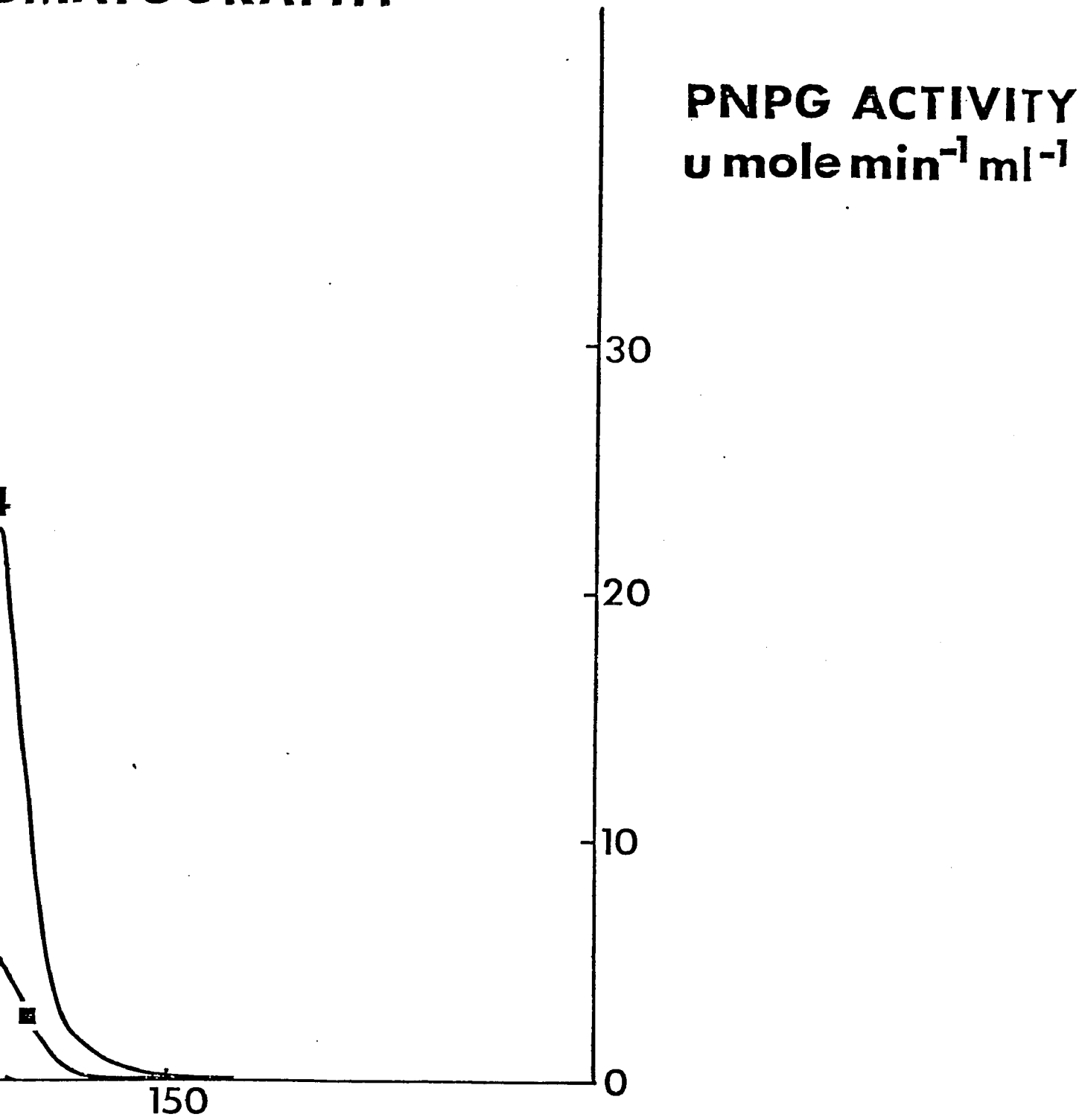
NUMBER

—●— p-nitrophenol α-D-glucopyranoside

----isomaltose(αCH_3-glucoside) hydrolysis

—■— maltose hydrolysis

DMATOGRAPHY



phenol α-D-glucopyranoside hydrolysis

ose(αCH$_3$-glucoside) hydrolysis

hydrolysis

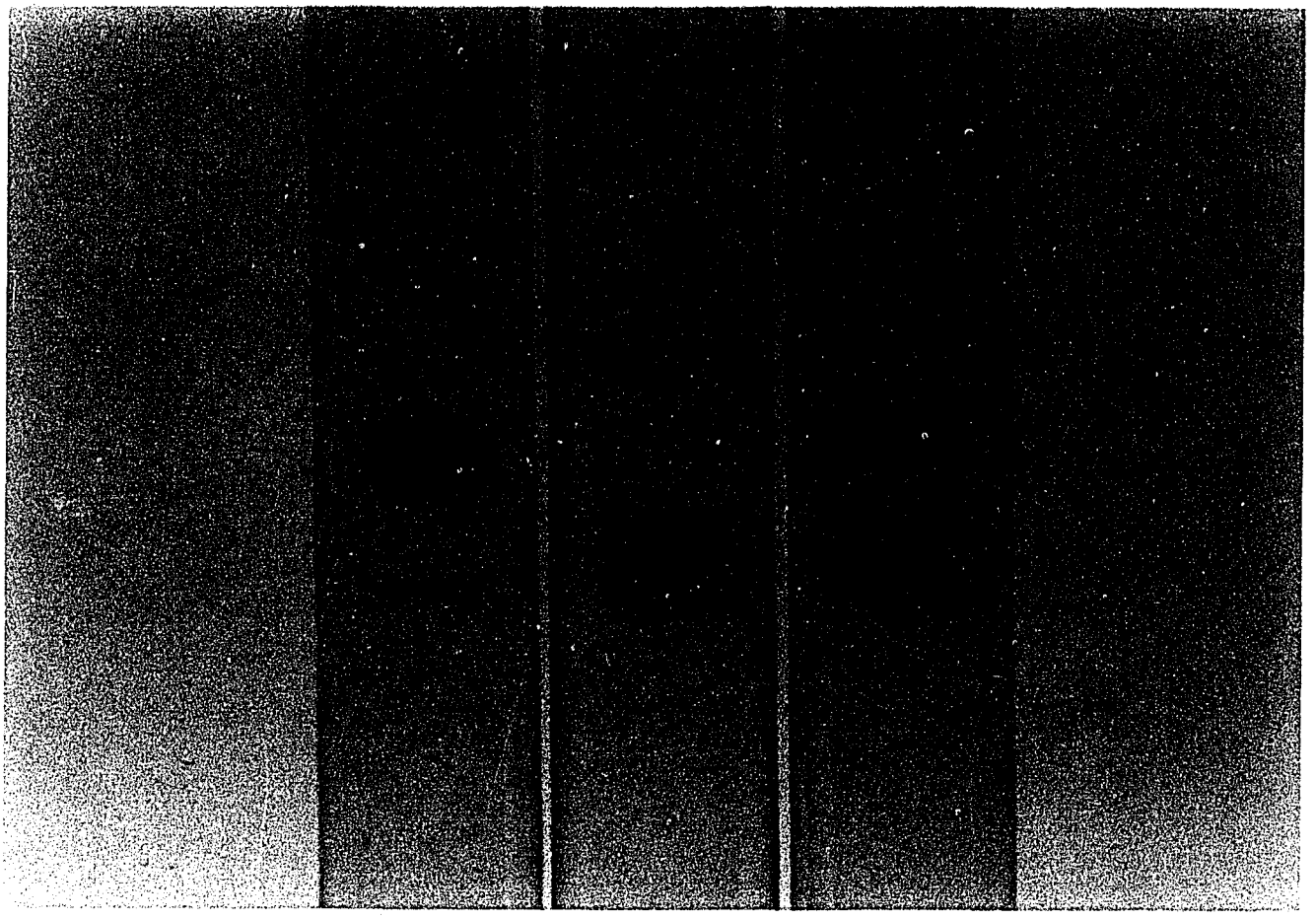


Figure 9A
Analytical polyacrylamide isoelectric focusing pH 4-6 and activity staining with αMG as substrate of fractions separated by DEAE chromatography
Lane 1 Fractions 103-137
Lane 2 Fractions 75-103
Lane 3 Combined aliquots

chromatography resulted in separation of the α-methyl-D-glucoside induced form of the enzyme (Fig. 9A fractions 75-103) from the maltose induced form (fractions 103-137). Since simultaneous induction produced approximately equal concentrations of both forms of the enzymes, the disproportionate size of peak 1 compared to peak 2 can be explained by the fact that the α-methyl-D-glucoside form (peak 1) tailed into peak 2. Fractions 75-103 and fractions 103-137 were concentrated and applied to separate hydroxylapatite columns. The column also produced separation of two forms of maltase, the identities of which were corroborated by isoelectric focusing and activity staining. Either sucrose or α-methyl-D-glucoside could be used as a substrate for maltase but hydrolytic cleavage of sucrose was much faster than cleavage of α-methyl-D-glucoside. The bottommost active band in Fig. 12, in maltose-induced cell extracts is maltase, and the intensity of the band is not proportional to the actual amount of maltase in the cell since the α-methyl-D-glucoside used in the activity stain is a poor substrate for maltase. Isomaltases focus in a cluster in a pH 4-6 gradient while maltases focus at a more basic pI and in a discrete cluster at that pI. Identities of isomaltase and maltase were confirmed by recovering active enzyme from unstained gels and testing for substrate specificities.

Samples dialysed against 20% glycerol prior to DEAE-Sepharose chromatography lose activity slowly due to proteolysis and it is desirable to perform the chromatography as quickly as is consistent with removal of NH_4^+ from the preceeding G-100 column pool. Enzyme activity in the eluted protein peaks is quite stable and samples can be stored for months at 0-4°C with very little loss in enzyme activity. Samples selected for long term storage were supplemented with 20%

$(NH_4)_2SO_4$ in addition to the glycerol and were stable indefinitely at 0-4°C.

Hydroxylapatite Chromatography

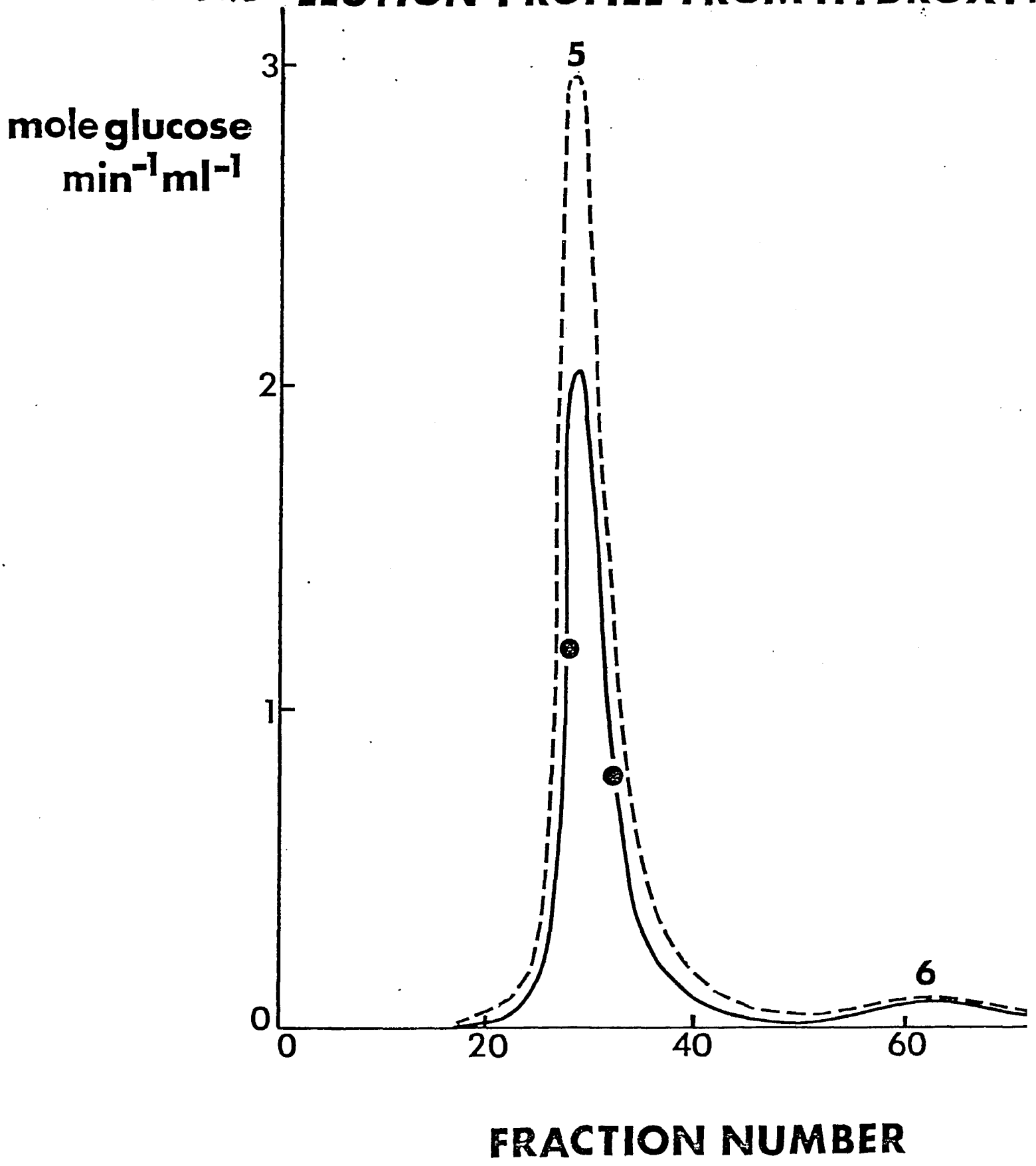
(Fractions 103-137 Fig. 10, 10A). Nonspecific PNPG assay showed three peaks of enzymatic activity. Two of the peaks (5 and 6) showed substrate specificity for isomaltose, α-methyl-D-glucoside and palatinose and one was active towards maltose. Combined aliquots of peak 5 and peak 6 show a high concentration of a middle form of the enzyme that was concentrated into peak 6. This form appears in a α-methyl-D-glucoside induced crude extract (Figure 12 Lane 2) and therefore is not an artifact of separation. The fact that it can be concentrated into a protein peak proves its existence. Peak 7 is a maltase that was included in pool 103-137.

(Fractions 75-103 Fig. 11, 11A). Peak 8 and 9 although separable chromatographically were indistinguishable by isoelectric focusing patterns. Since volume pickup upon dialysis for peak 9 was identical to peak 6 (middle form previously demonstrated not to be an artifact of separation) and since the salt gradients were identical there is a strong possibility that peak 9 is enriched in the middle form. The degree of binding to the column for the enzyme eluting in peak 9 was identical to that of peak 6. Pure enzyme was recovered from the various fractions by preparative isoelectric focusing, activity staining and recovery from parallel unstained gels.

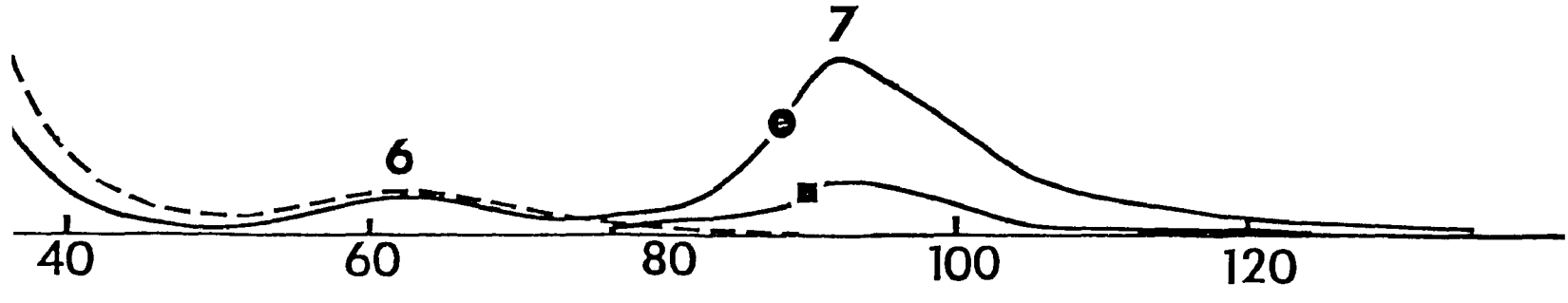
In subsequent purification procedures, it became apparent that separation could be greatly enhanced by performing chromatography in columns 100cm in length and with shallower salt gradients. DEAE Sepharose was chosen since the back pressure of a 100cm column was less than that of hydroxylapatite. In addition to a partial purification of

Fig. 10
Hydroxylapatite chromatography of fractions 103-137 (peak 2) from preceeding DEAE Sepharose chromatography —●— , p-nitrophenol α-D-glucopyranoside hydrolysis; ——, isomaltose hydrolysis; —■— , maltose hydrolysis. Elution peaks are designated 5, 6 and 7.

FIG.10 ELUTION PROFILE FROM HYDROXYL



E FROM HYDROXYLAPATITE CHROMATOGRAPHY



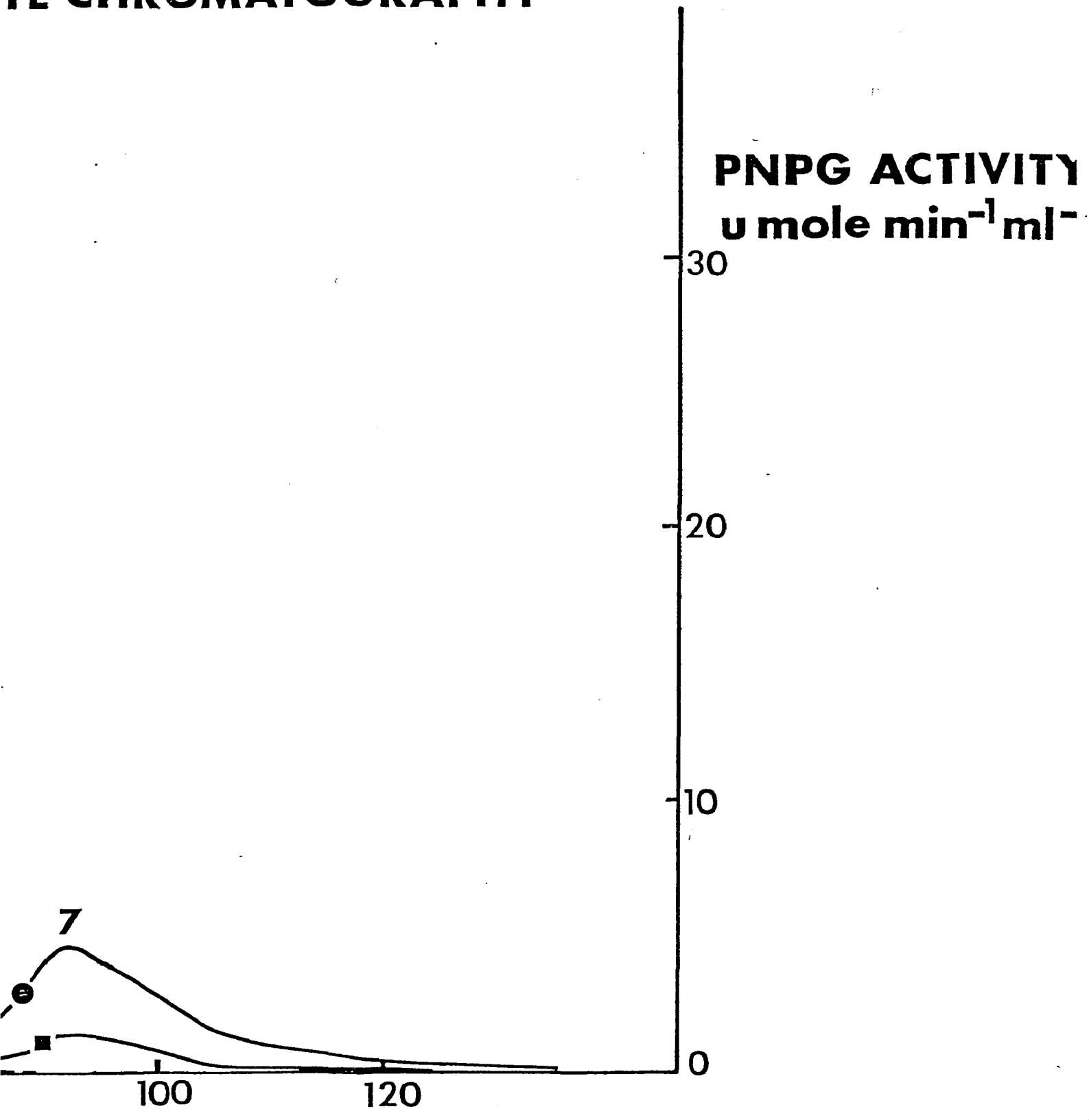
)N NUMBER

—o— p-nitrophenol α-D-glucopyra

----isomaltose(αCH$_3$-glucoside) h

—□— maltose hydrolysis

TE CHROMATOGRAPHY



-nitrophenol α-D-glucopyranoside hydrolysis

omaltose(αCH₃-glucoside) hydrolysis

altose hydrolysis

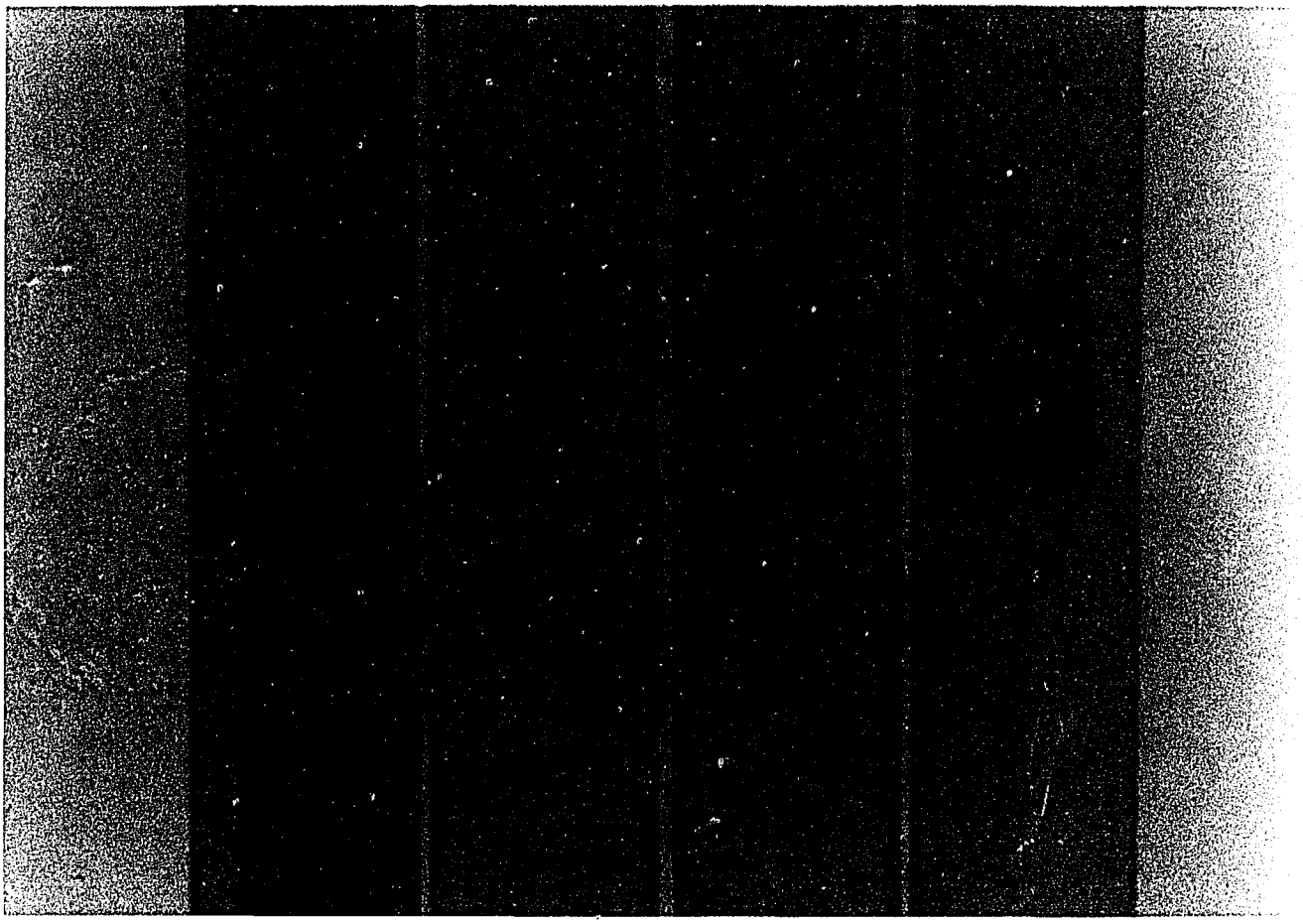
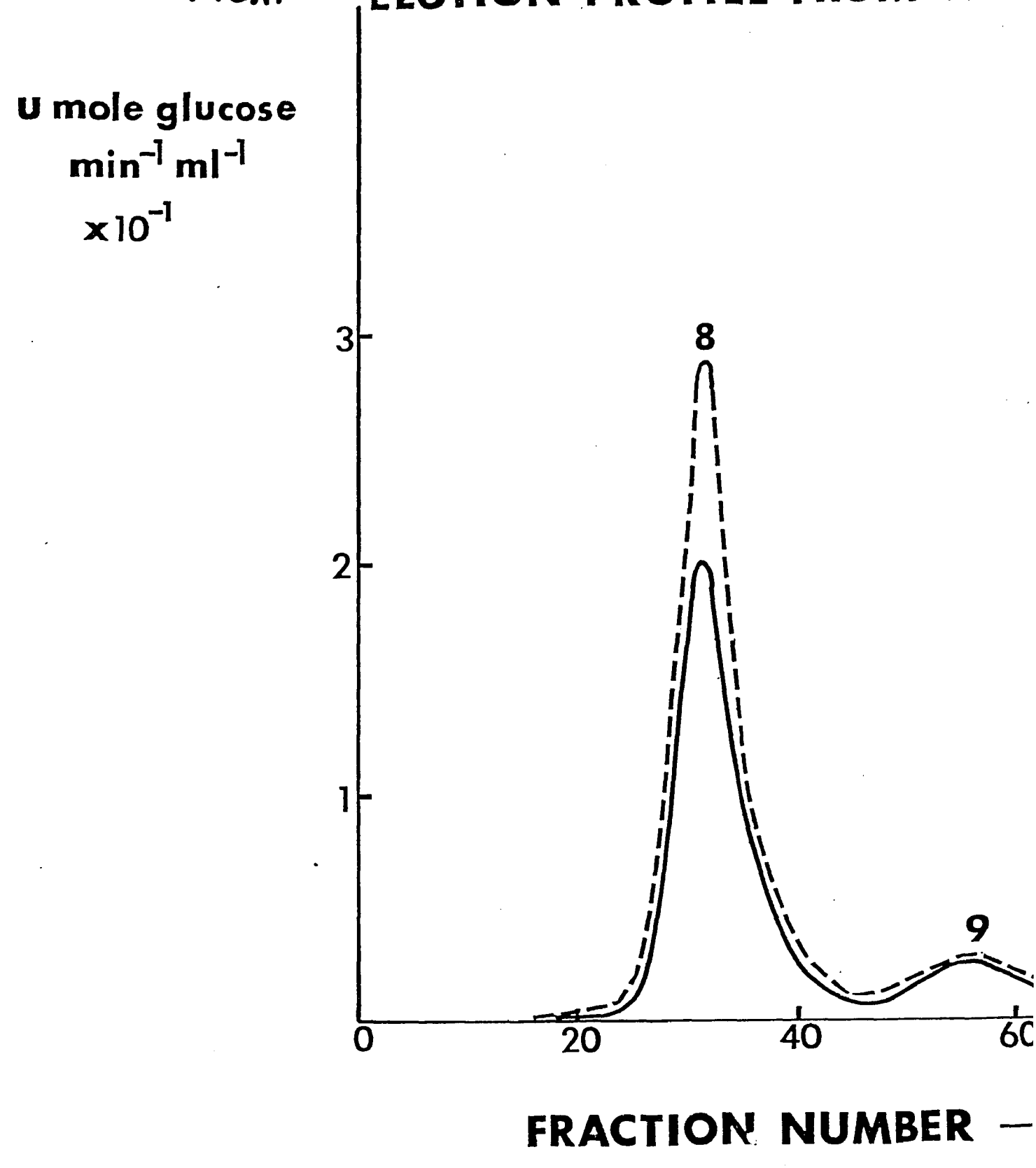


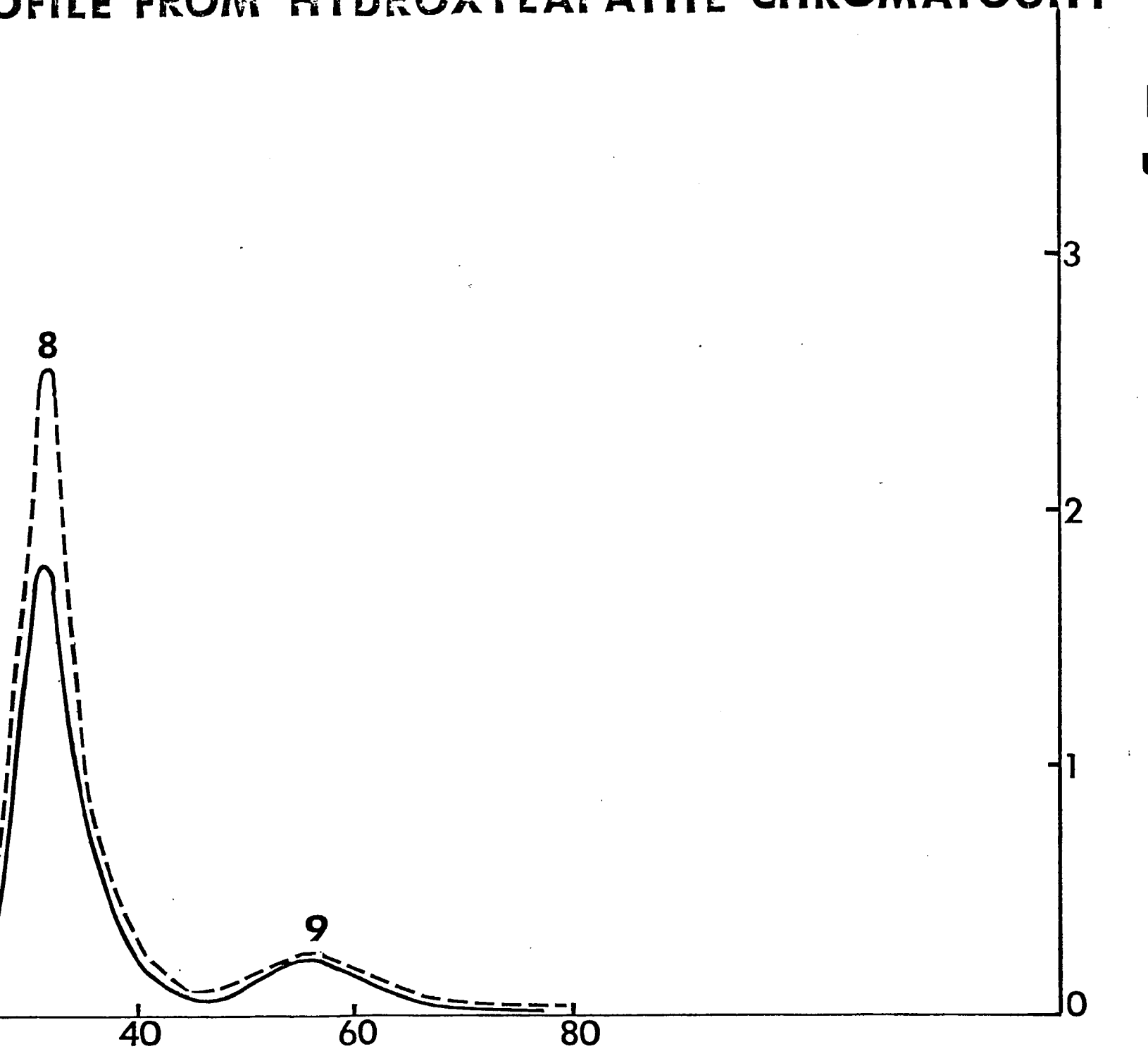
Figure 10A
Analytical polyacrylamide isoelectric focusing pH 4-6 of fractions 103-137 separated by hydroxylapatite chromatography
Lane 1 Protein staining lane 2
Lane 2 Activity staining peak 5
Lane 3 Activity staining peak 5
Lane 4 Combined aliquots

Fig. 11
Hydroxylapatite chromatography of fractions 75-103 (peak 1) from preceeding DEAE Sepharose chromatography ——— , p-nitrophenol α-D-glucopyranoside hydrolysis; ———, isomaltose hydrolysis

FIG.11 ELUTION PROFILE FROM HYD



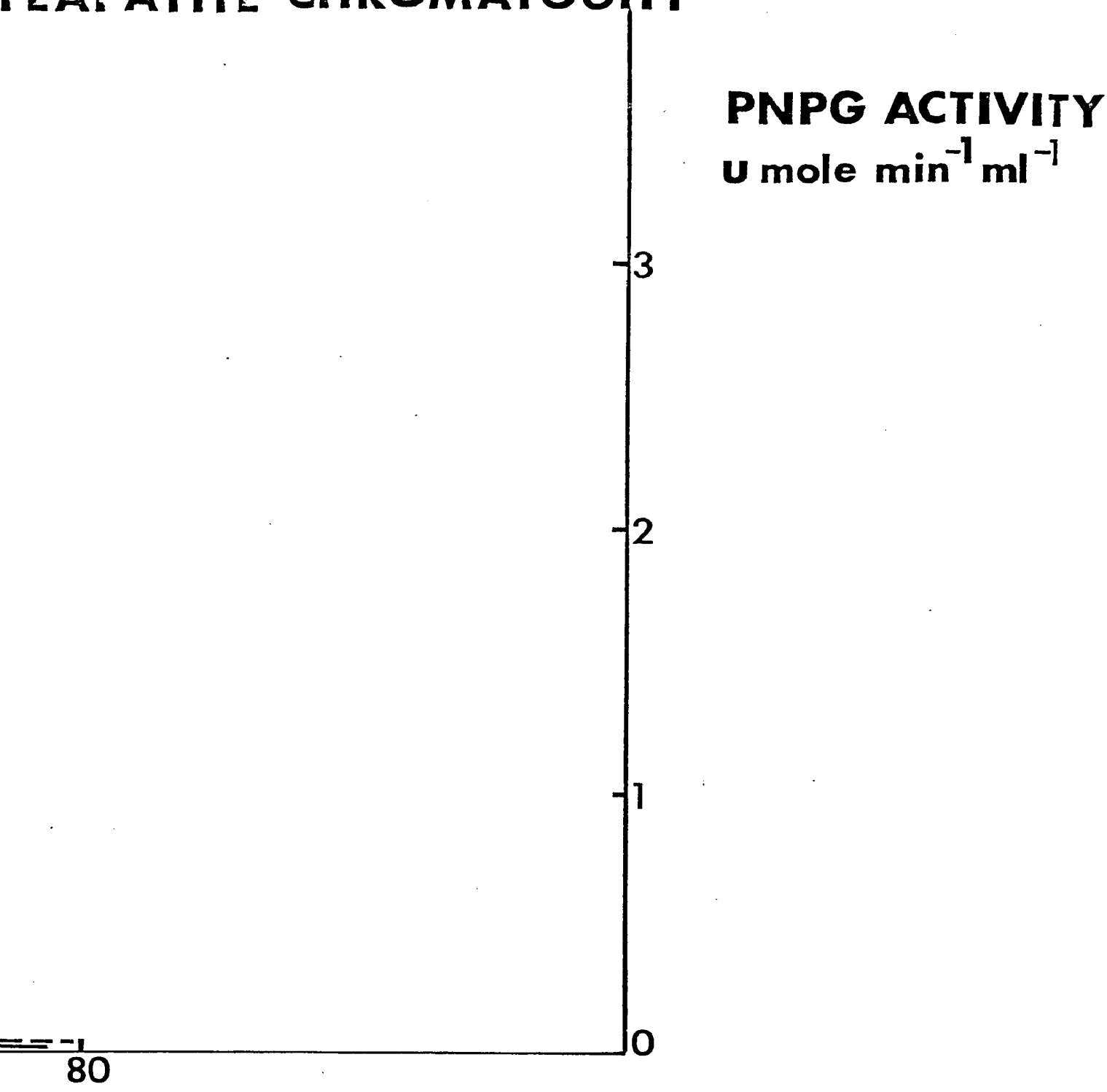
OFILE FROM HYDROXYLAPATITE CHROMATOGHY



TION NUMBER —p-nitrophenol α-D-glucopyranoside

----isomaltose(αCH$_3$-glucoside) hydroly

YLAPATITE CHROMATOGHY



rophenol α-D-glucopyranoside hydrolysis

altose(αCH$_3$-glucoside) hydrolysis

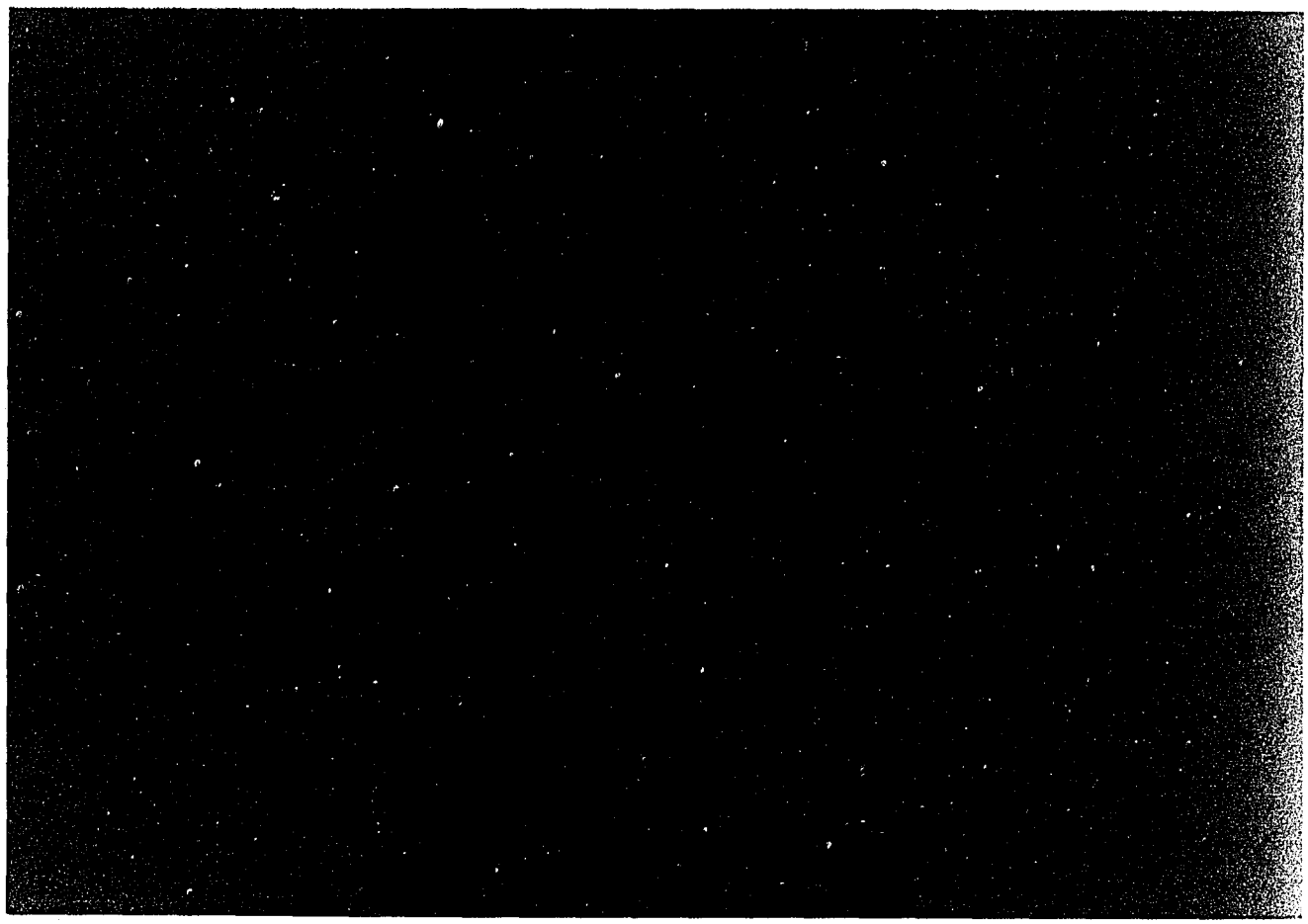


Figure 11A
Analytical polyacrylamide isoelectric focusing pH 4-6 of fractions 75-103 separated by hydroxylapatite chromatography
Lane 1 Protein staining lane 2
Lane 2 Activity staining peak 8

the enzymes, the chromatography corroborated the existence of multiple isomaltases and maltases.

At no time during the purification procedure did the relative levels of the various forms of the enzyme change on storage. At no time during the purification procedure did a previously separated form of isomaltase appear in any fraction. Since the vast majority of isoenzymes from yeast that have subsequently been proven to exist in multiple forms as a result of proteolysis exhibit a change in relative distribution with age of the extract, it is highly improbable that any of these yeast α-glucosidases arise as a result of *in vitro* proteolysis.

Purification of α-glucosidases From 1412-4D Strain of Saccharomyces Cerevisiae Under Conditions of Maltose Induction

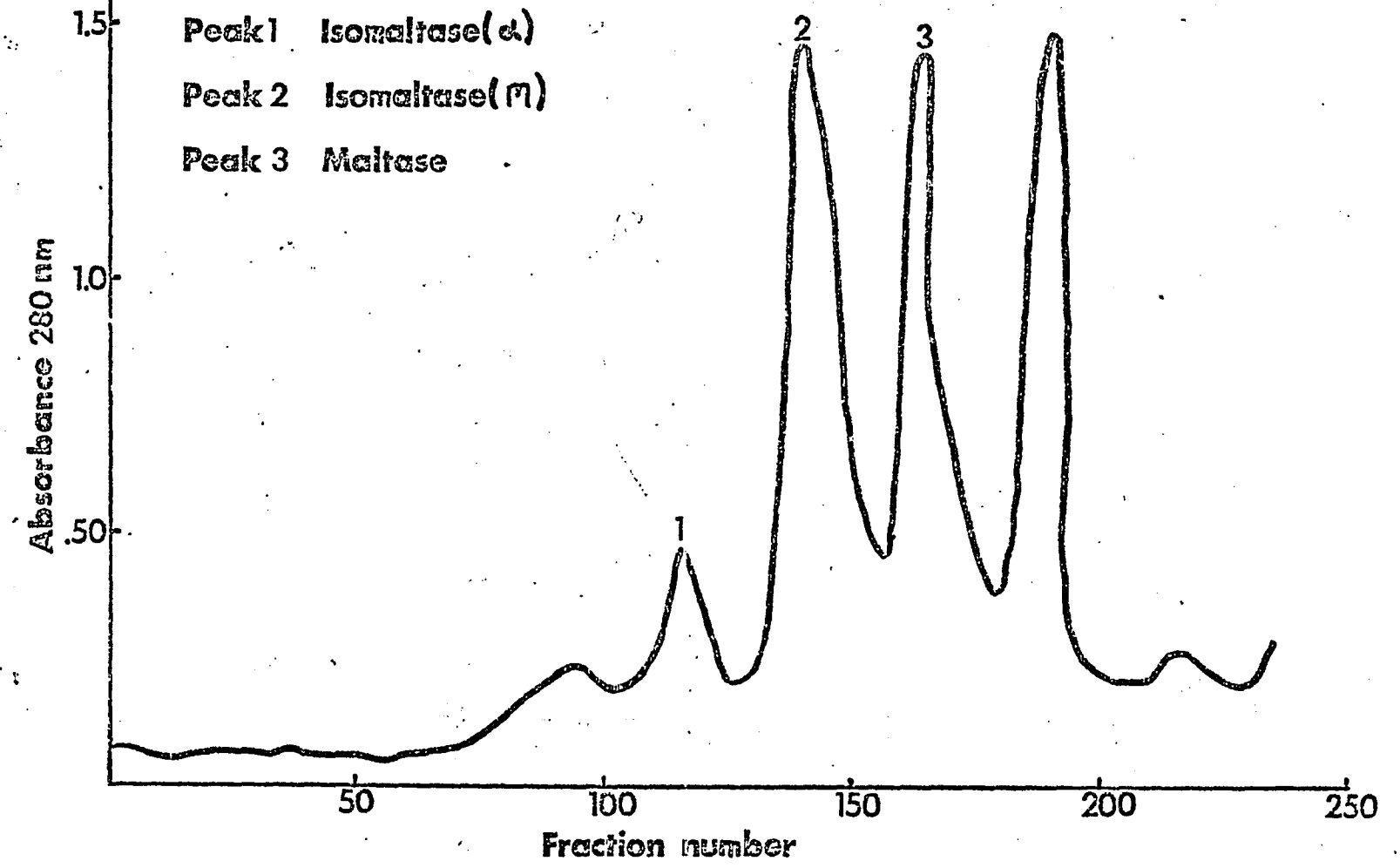
The reason for the existence of a maltose induced isomaltase differing from the α-methyl-D-glucoside induced isomaltase is puzzling, particularly in that maltose is not hydrolyzed by either form of isomaltase. Maltose induced cells were a source of the more acidic form of isomaltase and contained low levels of the α-methyl-D-glucoside induced form. Purification of extracts by G-100 chromatography was identical regardless of inducing sugar and resulted in elimination of 80% of nonenzymatic protein with no separation of multiple forms of α-glucosidases occurring. The discussion begins with the separation achieved upon DEAE Sepharose chromatography.

First DEAE Sepharose Chromatography

(Fig. 7, 7A). Enzyme activity and specificity was determined as previously described. Nonspecific PNPG assay showed 3 peaks of α-glucosidase activity. Two of the peaks (1 and 2) showed substrate specificity for isomaltose, α-methyl-D-glucoside, and palatinose and

Fig. 7
DEAE Sepharose chromatography of pooled fractions 30-90 from G-100 chromatography. Peak 1, α-methyl-D-glucoside induced form of isomaltase; peak 2, maltose induced form of isomaltase; peak 3, maltase.

FIG.7 FIRST DEAE SEPHAROSE CHROMATOGRAPHY



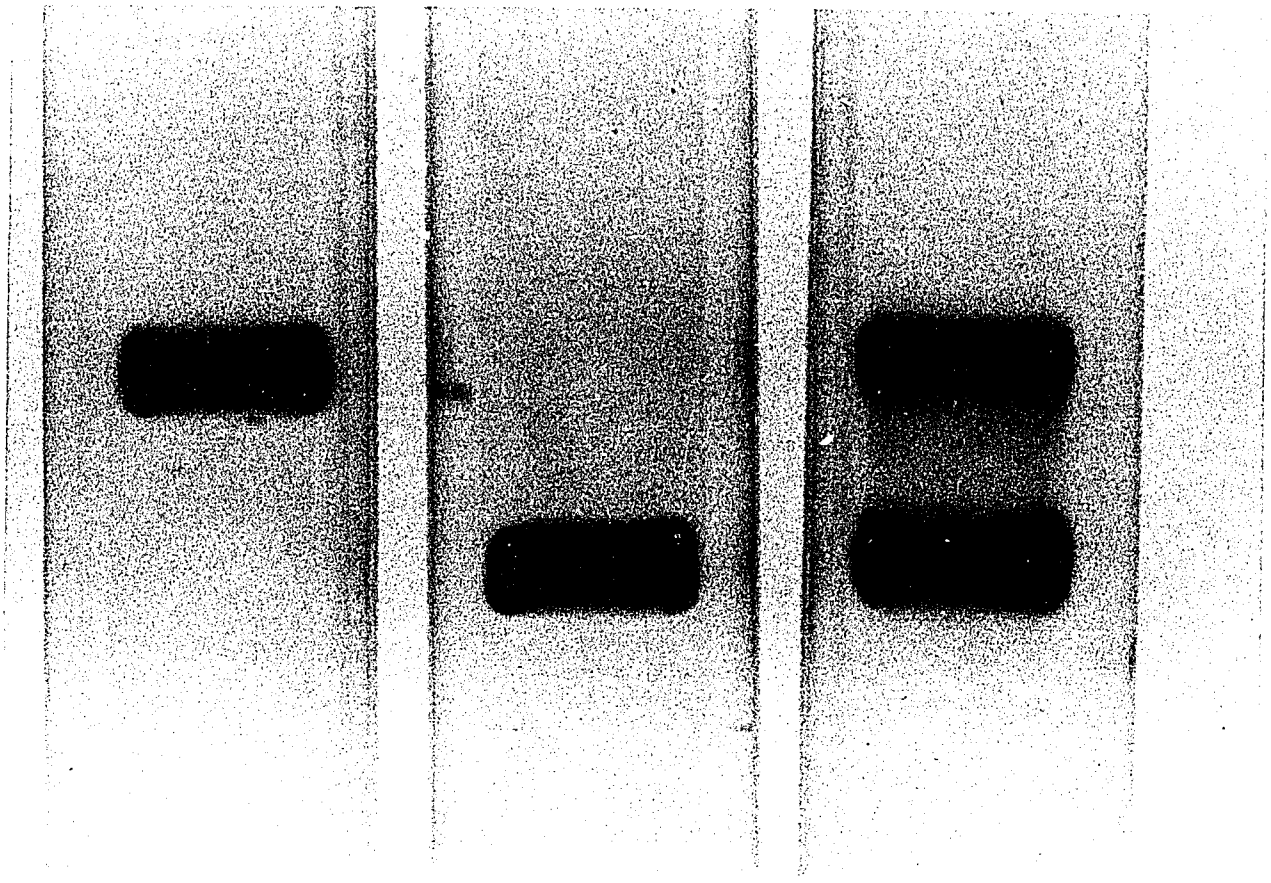


Figure 7A
Analytical polyacrylamide isoelectric focusing pH 4-6 and activity staining with αMG as substrate of fractions separated by DEAE-Sepharose chromatography. Aliquots were selected such that 100-150ug of glucose was liberated from MG in 5 minutes.
Lane 1 M form Fractions 130-150
Lane 2 α form Fractions 110-120
Lane 3 Combined aliquots

one was active towards maltose (3). Isoelectric focusing and activity staining was performed on pooled fractions 110-125 (lane 2) and 130-150 (lane 1). Separation of the two forms of isomaltase was complete primarily because the preponderance of isomaltase activity was the more acidic variant and partly because of the greater column length. Protein loads were adjusted accordingly prior to electrofocusing for equivalent band intensity. Maltase (fractions 155-180) was subject to IEF and activity staining with α-methyl -D-glucoside as substrate (Fig. 14 and 15). Fraction 130-155 was pooled and subjected to a second DEAE Sepharose column.

Second DEAE Sepharose Chromatography

(Fig. 8 and 8A). Nonspecific PNPG assay showed 2 peaks of enzymatic activity hydrolyzing isomaltose, palatinose, α-methyl-D-glucoside, and sucrose. Fractions 150-170 and 171-200 were pooled and subjected to IEF and activity staining with both α-methyl-D-glucoside and sucrose as substrates. There were no observable differences and insufficient quantity of the minor form to pursue further studies. Protein staining of 10cm IEF gels showed one major band of protein and a minor protein band with a more acidic pI. The minor band is catalytically active and is well separated in 20cm IEF (Fig. 12) in maltose induced cells. It was estimated to represent no more than a few percent of the major band and judged negligible in terms of its effect on subsequent analysis. Polyacrylamide disc gel electrophoresis and SDS gel electrophoresis demonstrated the preparation to be approximately 98% pure and subsequent analysis was performed on this preparation (Fig. 16). The major band of enzyme appeared occasionally as a doublet when stained for protein (Fig. 17). It is not known whether there exist 2 forms of a maltose induced form, the properties

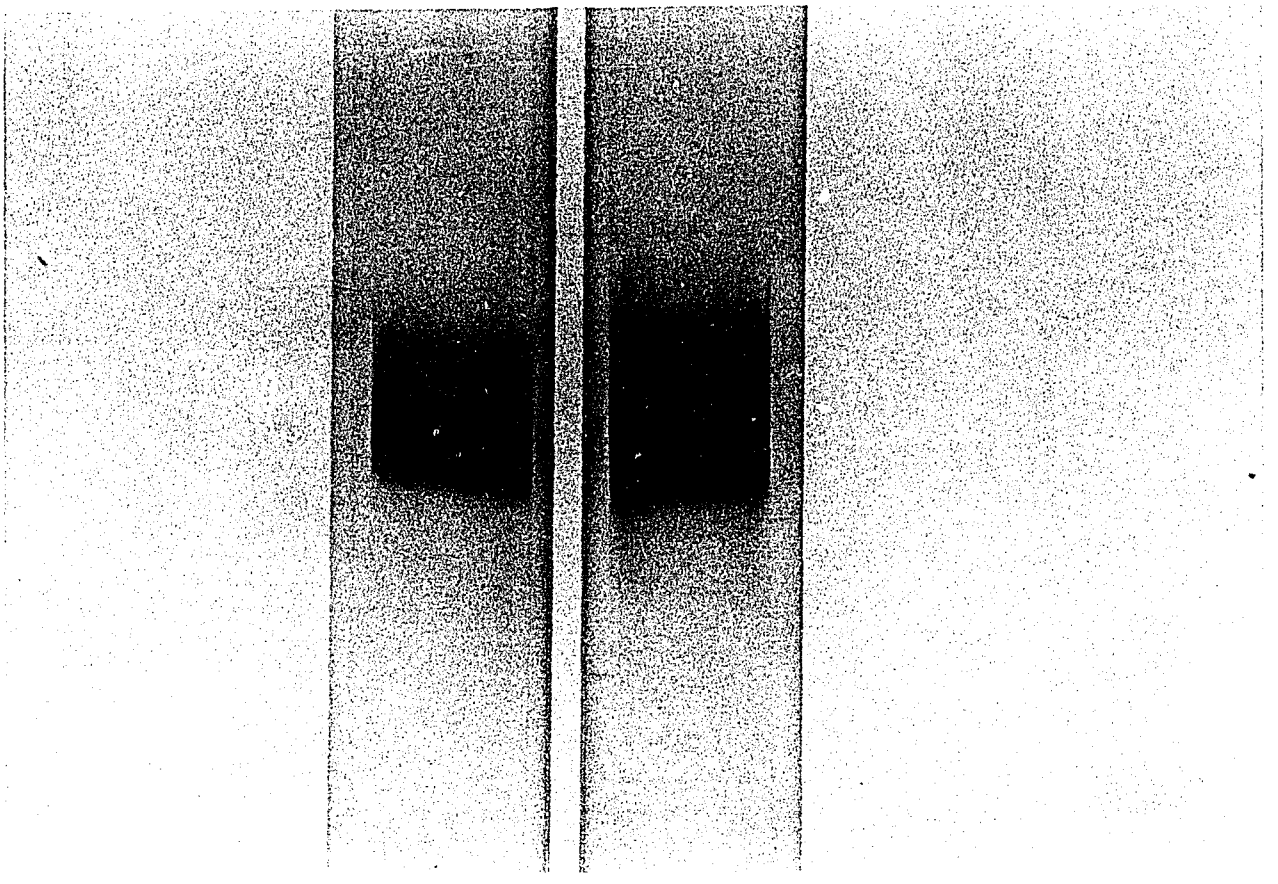


Figure 14
Analytical polyacrylamide isoelectric focusing pH 4-6 and activity staining of maltases in fractions 155-180 of DEAE separation of Figure 7
Lane 1 100-150ug glucose generated from αMG as substrate
Lane 2 300-500ug glucose generated from αMG as substrate

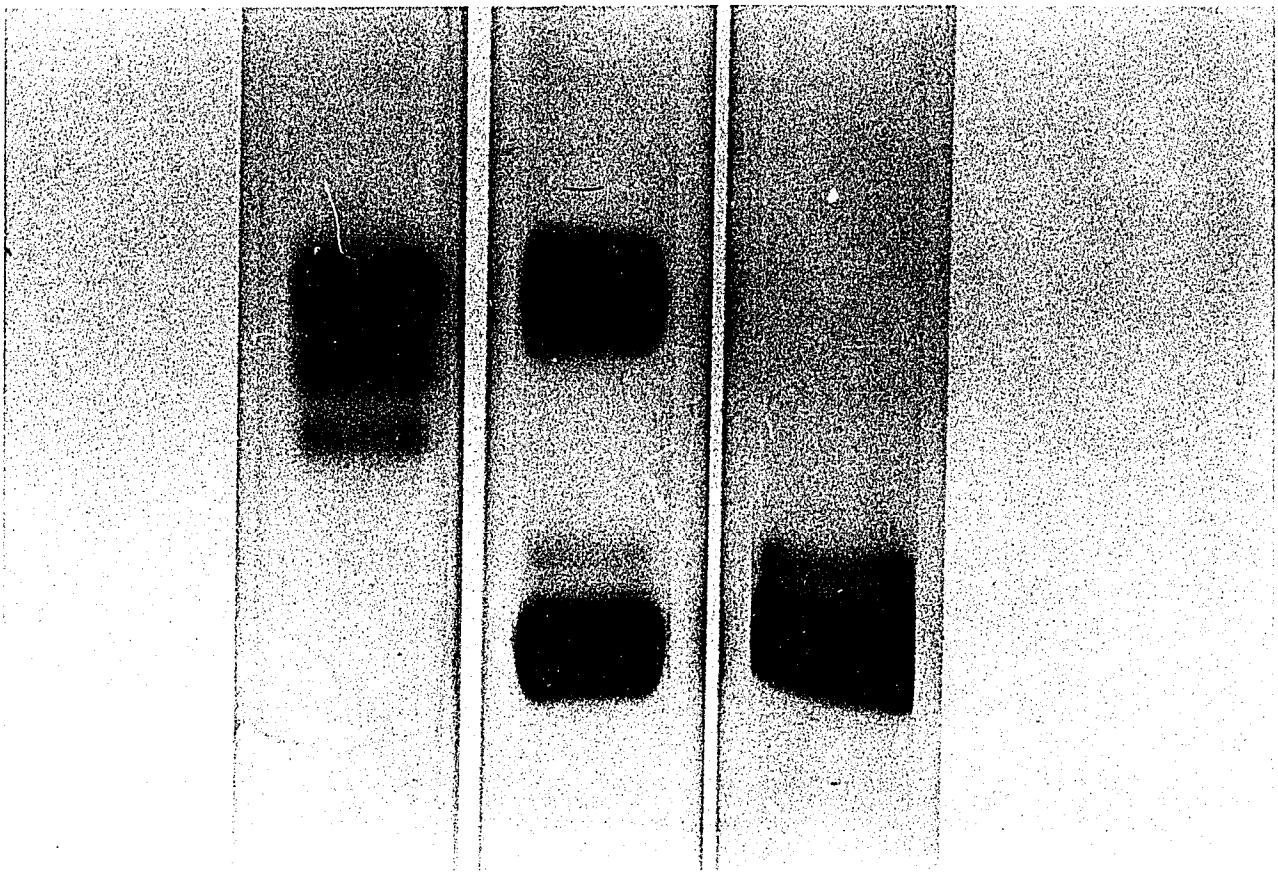
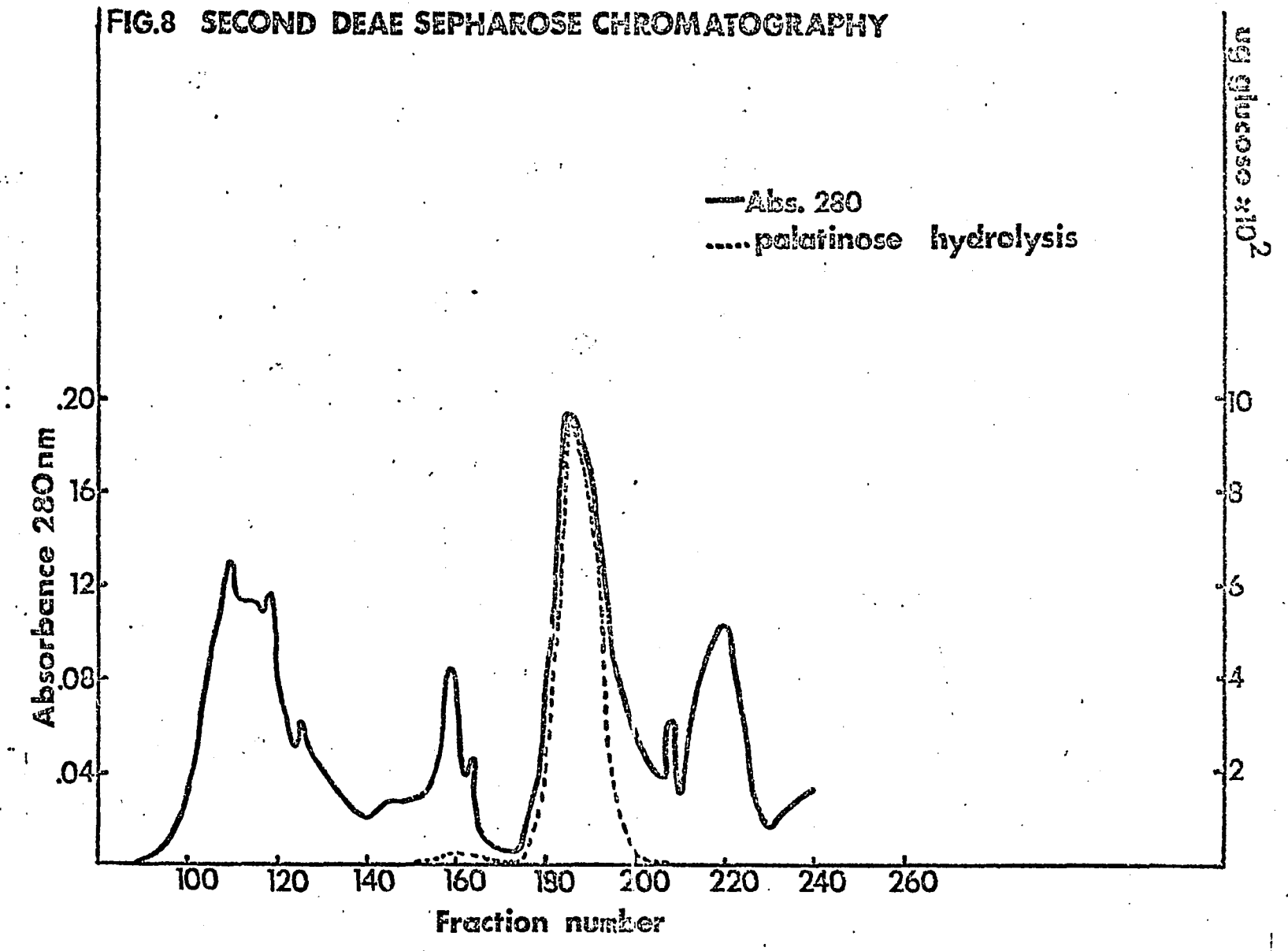


Figure 15
Analytical polyacrylamide isoelectric focusing pH 4-6 and activity staining of fractions from DEAE chromatography illustrated in Figure 7
Lane 1 Marker showing 3 isomaltases
Lane 2 Combination of fractions 130-150 and 155-180
Lane 3 Fractions 155-180

Fig. 8
DEAE Sepharose chromatography of peak 2 from preceeding DEAE Sepharose column.
—— , Absorbance 280nm; —— palatinose hydrolysis

FIG.8 SECOND DEAE SEPHAROSE CHROMATOGRAPHY



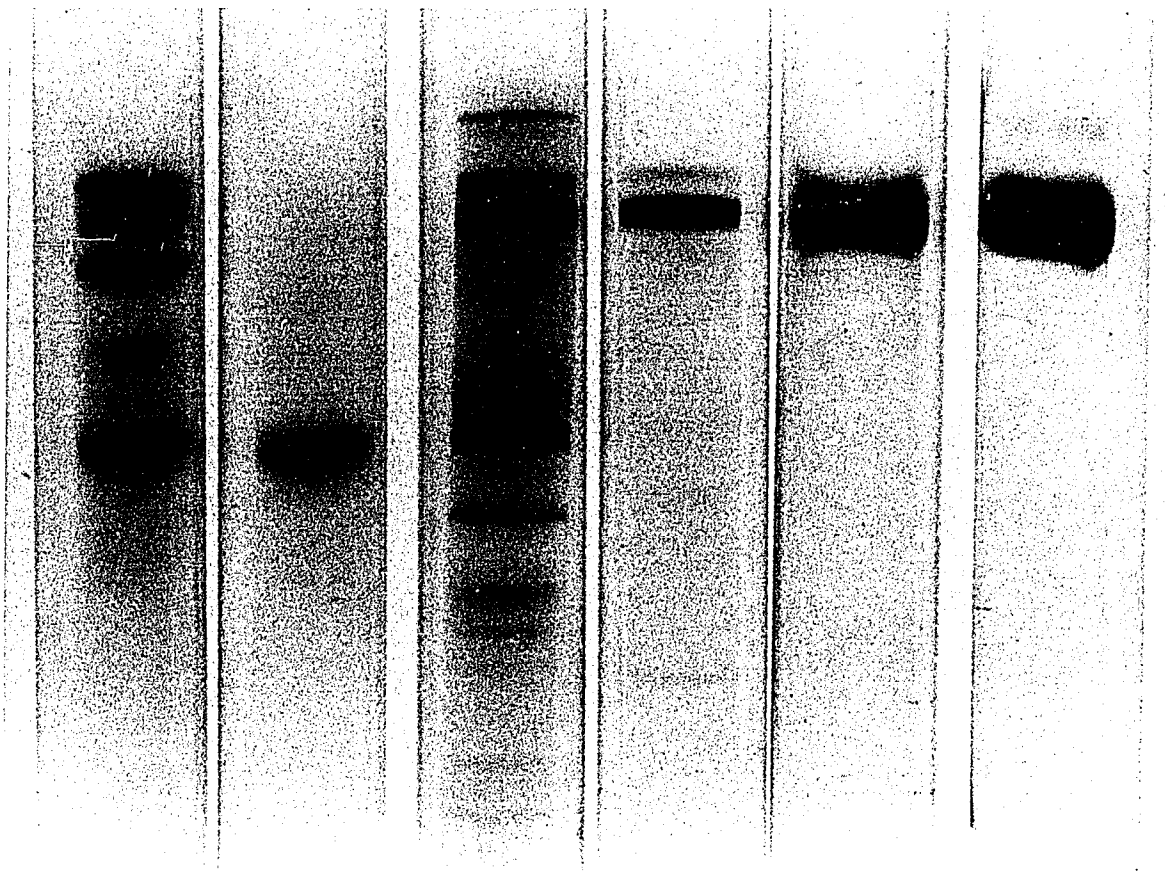


Figure 8A

Analytical polyacrylamide isoelectric focusing pH 4-6 and disc gel electrophoresis confirming separation by second DEAE column

Lane 1 Disc gel electrophoresis and protein staining of protein prior prior to chromatography

Lane 2 Disc gel electrophoresis and protein staining of fractions 175-200 subsequent to chromatography

Lane 3 Analytical polyacrylamide isoelectric focusing and protein staining of protein prior to chromatography

Lane 4 Analytical polyacrylamide isoelectric focusing and protein staining of fractions 175-200 subsequent to chromatography

Lane 5 Activity staining with α MG as substrate of lane 4 gel

Lane 6 Activity staining of IEF at 20cm gel length of material applied to lanes 4 and 5

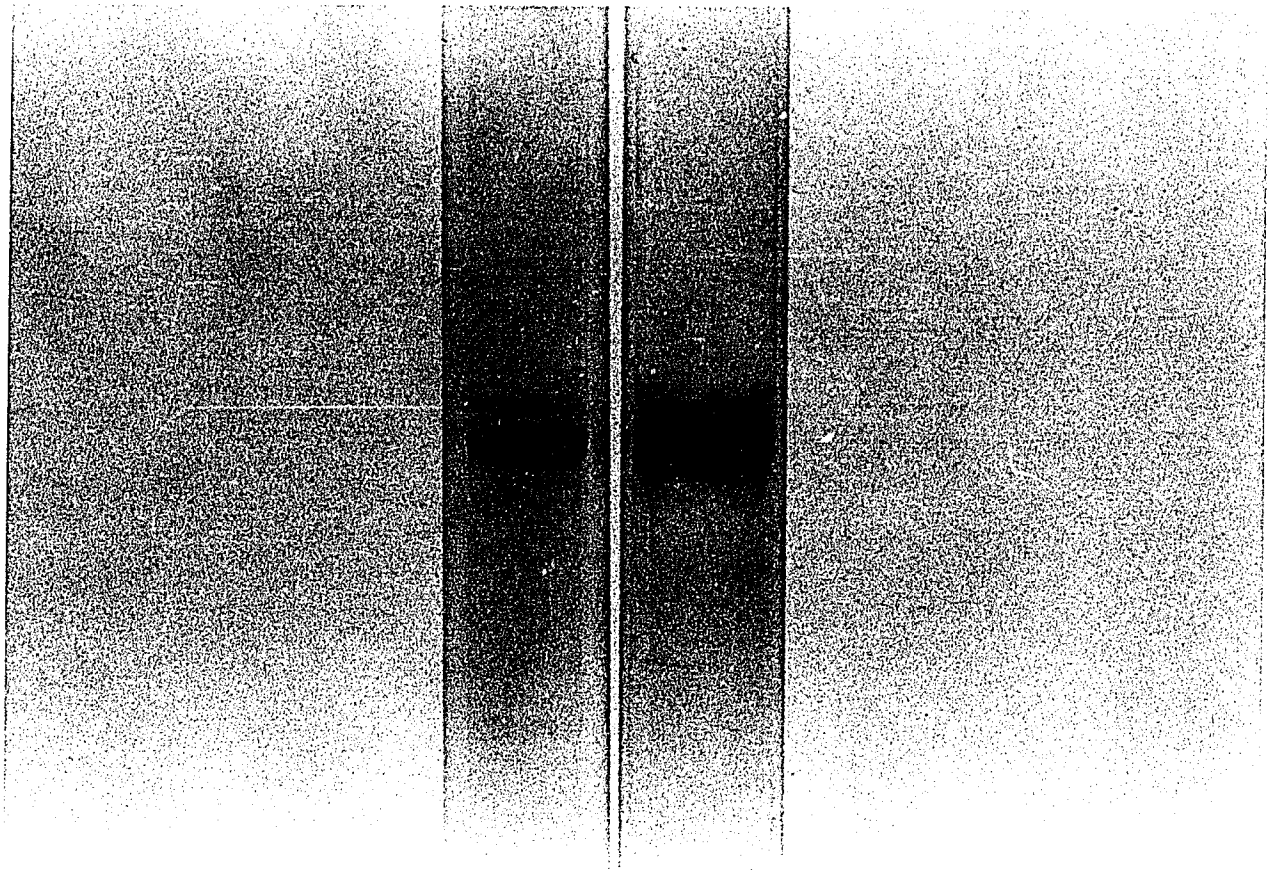


Figure 16
Analytical polyacrylamide isoelectric focusing pH 4-6 of purified M form of isomaltase
Lane 1 M form (100ug) stained with Coomasie G
Lane 2 M form stained for activity with αMG as substrate

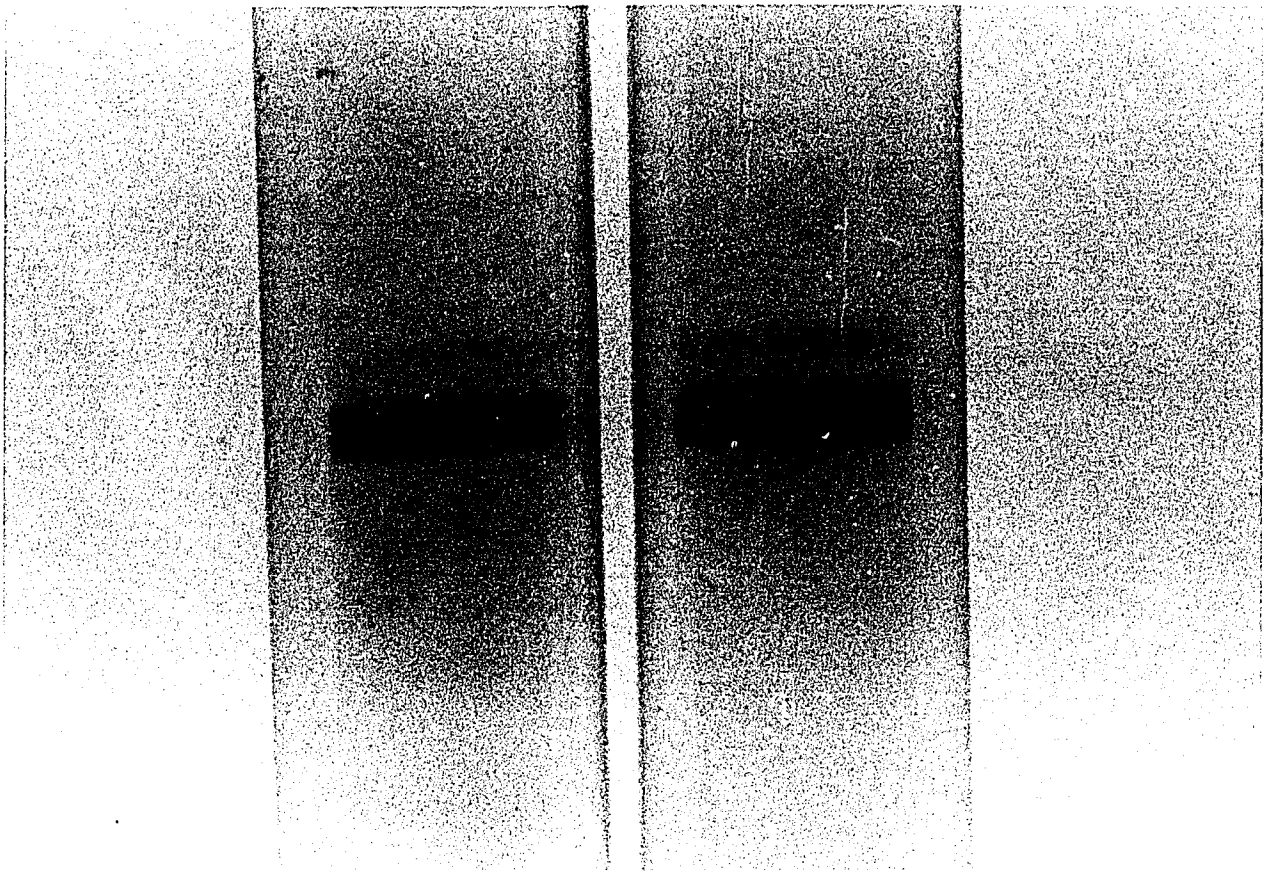


Figure 17
Analytical polyacrylamide isoelectric focusing pH 4-6 of purified M form of isomaltase@
Lane 1 M form (50ug) stained with Coomasie G
Lane 2 M form (100ug) stained with Coomasie G

Fig. 18
Analytical polyacrylamide isoelectric focusing pH 4-6 and activity staining with α-methyl-D-glucoside of a crude extract at 20cm gel length. Uppermost major band is maltose induced form; middle major band is middle form; bottom major band is α-methyl-D-glucoside induced form. Focusing shows the splitting of each major form into multiple bands and is artifactual. See explanation in text.

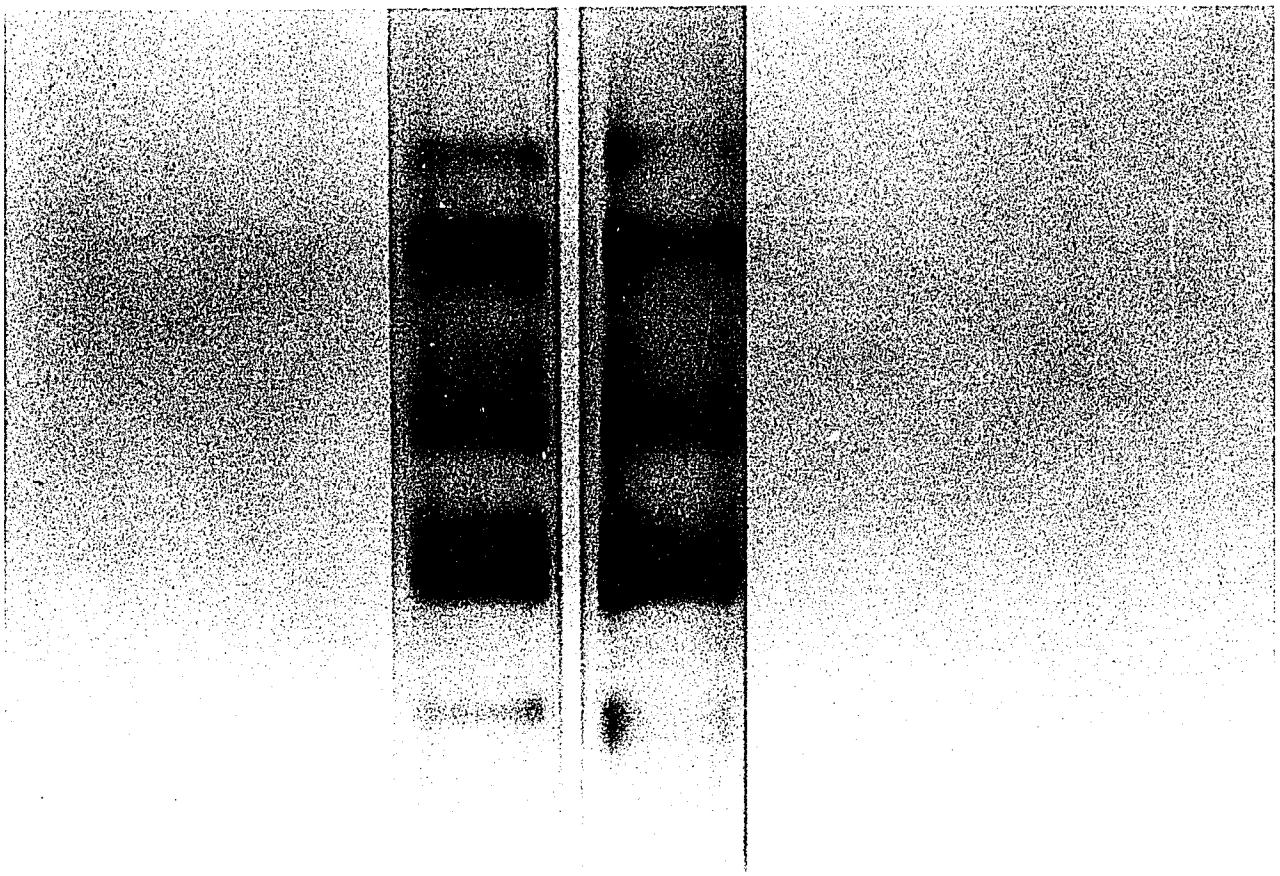


Figure 13
Analytical polyacrylamide isoelectric focusing pH 4-6 and activity staining at 20cm gel length of a crude extract
Lane 1 & 2 Activity staining with αMG as substrate

of which are so similar that they appear fused in single protein band, or whether the infrequent doublets are an artifact of isoelectric focusing. The distribution of the various amphoteres in the pH gradient is not uniform and there exists gaps or regions where there is very little buffering capacity. Should a protein focus proximal to a zone of no ampholine, diffusion away from the isoelectric pI would not result in the protein acquiring a net charge until it impinged upon the next region of high amphotere concentration whereupon it would acquire a net charge and the repulsion of the proximal electrode would then move it back to its pI point. Doublet artifacts apppear with the deposition of unfocused protein between the bands and change with time of focusing and steepness of the gradient. The distance of doublet artifacts is usually one millimeter or less and components focusing as doublets beyond this distance should be considered real. Artifacts can be distinguished by focusing in shallower pH gradients (longer lengths) and by sampling gel tubes at different times.

Proteolysis

Pringle (26) reports that a wide variety of highly purified proteins appearing as single bands in polyacrylamide gel electrophoresis show the presence of an adsorbed proteolytic contaminant upon SDS gel electrophoresis under denaturing conditions. A multiple banding pattern upon SDS gel electrophoresis that changes with time of incubation with SDS and is eliminated by prior treatment with PMSF is indicative of an adsorbed proteolytic contaminant. Purified isomaltases did not exhibit this phenomenon despite the fact that isomaltase is a substrate for protease B. This adsorption can be accounted for by considering general well known principles of enzyme-substrate interactions. Proteins are often purified in the presence of

their substrates so as to stabilize the enzyme by freezing it in its native conformation as when substrate is bound at the active site. Proteases are extremely stable proteins for they are in an environment in an extract where they are in the presence of multiple substrates (other proteins). Should a chromatographic separation be attempted under conditions where proteolysis is actively occurring, the affinity of the protease for its substrate might be more avid than the differential affinity of the protease and substrate for the ion-exchange resin. Commercial enzyme preparations are undoubtedly purified with insufficient attention given to the inhibition of proteolysis prior to column chromatography. In addition, once the metabolic cascade leading to proteolytic activation is initiated and amplified, it is most likely difficult or impossible to interpret or inhibit the events that transpire. The in vivo function of the metabolic cascades that lead to proteolytic amplification has wide ranging implications in terms of intracellular protein degradation and the modulation of cell surface recognition via cleavage of trans membrane controlling mechanisms.

In vitro inhibition of protease B activity and probably all serine type proteases can be achieved by the presence of NH_4^+. Extracts showing enormous proteolytic capacity were completely stabilized when 20% $(NH_4)_2SO_4$ was present at neutral pH, the pH being maintained at 7.0 during the addition of $(NH_4)_2SO_4$. The ammonium ion probably mimics the effects of lysine and histidine residues, the histidine residue being essential for serine type proteolytic activity as reviewed by Lehninger (64).

The ammonium ion quite possibly hinders the general base catalysis effected by the active histidine. Consistent with this,

dialysis of G-100 purified extracts into 0.01M phosphate buffer containing no NH_4^+ demonstrated considerable proteolytic activity and concomitant loss in enzyme activity.

Extracts were supplemented with glycerol (final concentration of 20%) and dialysed until the dialysate showed the absence of NH_4^+. The persistence of NH_4^+ at low levels, despite six 4 liter changes of dialysis buffer, seemed to indicate a binding was occurring between NH_4^+ and yeast protease B.

DEAE Sepharose was chosen because Pringle's review (26) indicated elution properties of protease B were substantially different from isomaltase with this particular resin facilitating the separation of the two activities. DEAE chromatography in the presence of glycerol was successful in eliminating proteolytic activity as the purified enzyme dialyzed against buffer containing neither glycerol nor NH_4^+ was completely stable. Glycerol might function through a viscosity effect, retarding molecular motion and conformational changes essential for catalysis. SDS gel electrophoresis showed the absence of any substantial proteolytic contaminant.

I do not recommend the use of PMSF for a variety of reasons. Protease-polypeptide inhibitor complexes exist such that the polypeptide inhibitor is bound to the active site of the protease. Dissociation of the complex occurs over a period of time (26). It makes no sense to use a reagent such as PMSF with a $t_{1/2}$ =100 minutes at pH 7.0 particularly, when the active site of proteases in protease-polypeptide inhibitor complexes is masked by the presence of the polypeptide inhibitor and is rendered inaccessible to the reagent. In addition, binding of PMSF is specific for serine in any protein and the probability of reagent artifacts is pronounced (26).

I recommend the purification of enzymes in the presence of substantial concentrations of NH_4^+ and glycerol and the selection of resins and conditions where active proteolysis is inhibited. It is hoped that the insights achieved will lead to the production of commercially purified preparations free of proteolytic contaminants.

Induction studies

Yeast cells grown on media containing maltose produce predominantly the more acidic form of isomaltase Fig.12(M) and a low level of the more basic form (α). The form of the enzyme with a slightly more acidic pI (A) than the major maltose induced form was proved not to be an artifact as demonstrated by its persistent appearance during chromatography and its unchanged appearance during the cathodic drift phenomena. The bottommost active band closest to the basic cathode (D) is maltase, exhibiting a minimal hydrolytic activity towards α-MG and demonstrating induction by maltose. Cells induced with α-MG produce predominantly the more basic isomaltase (α) along with low levels of the maltose induced form (M). The form of isomaltase present at low concentrations with a pI intermediate between the 2 major forms(B) was concentrated into a protein peak upon hydroxylapatite chromatography. Simultaneous induction with maltose and α-MG produced substantial amounts of both major forms of isomaltase, the molarity of the inducing sugars being the same in Fig. 12. When simultaneous induction was performed with the same percentage by weight of each sugar (Fig. 13), the 10 cm IEF gels showed approximately equal amounts of the 2 major forms of the enzyme.

Each of the two major forms when added to a crude extract of the other shows no interconversion over time. Extraction in the presence of PMSF produces the same distribution pattern as in the absence of the

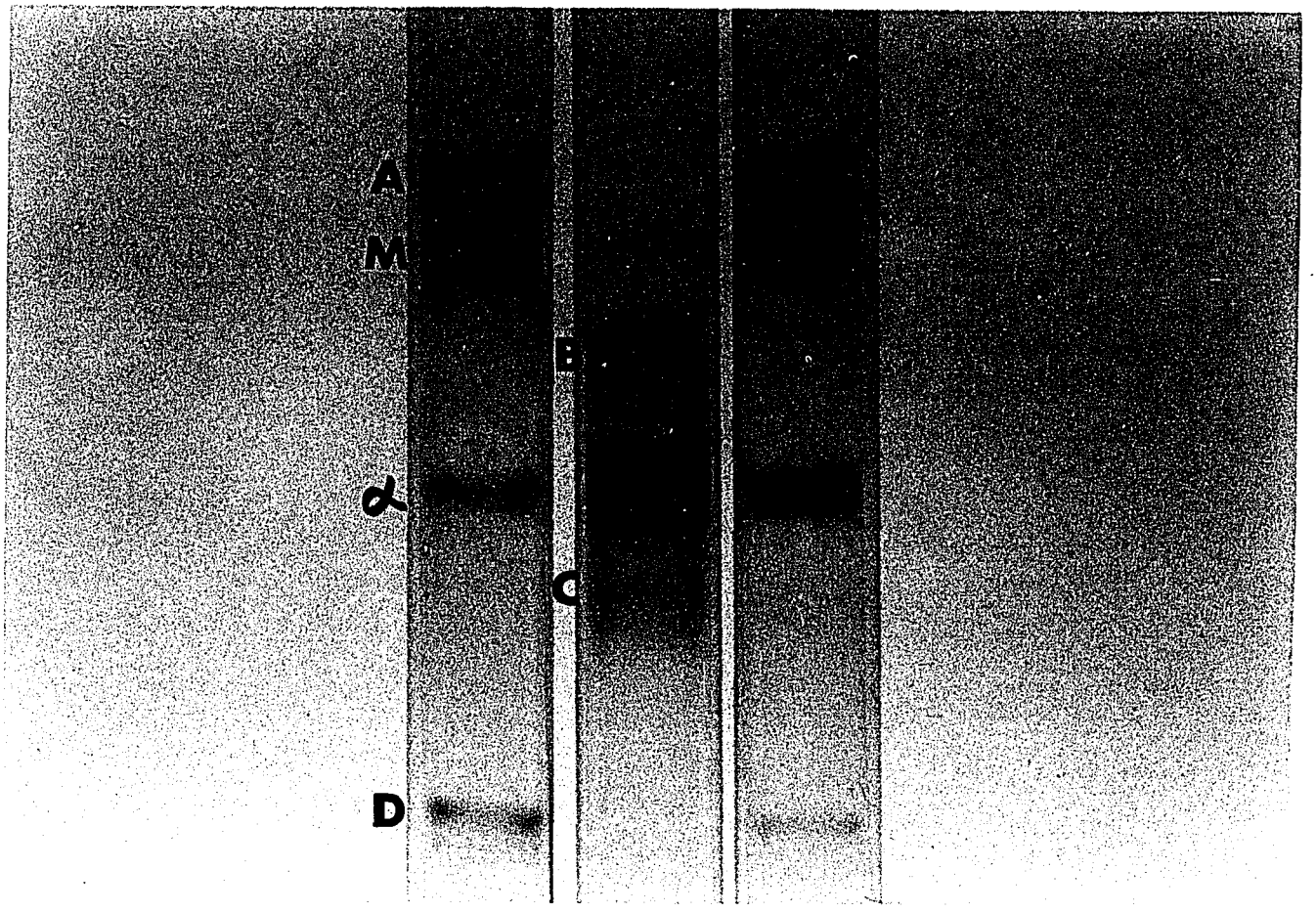


Figure 12

Analytical polyacrylamide isoelectric focusing pH 4-6 and activity staining with α-MG as substrate at 20cm gel length of extracts of Saccharomyces cerevisiae induced with various sugars

Lane 1 Maltose induction corresponding to flask 1 (Materials and Methods)

Lane 2 α-methyl-D-glucoside induction corresponding to flask 2 (Materials and Methods)

Lane 3 Simultaneous induction with 0.8% α-MG and 1.7% maltose corresponding to flask 3 (Materials and Methods)

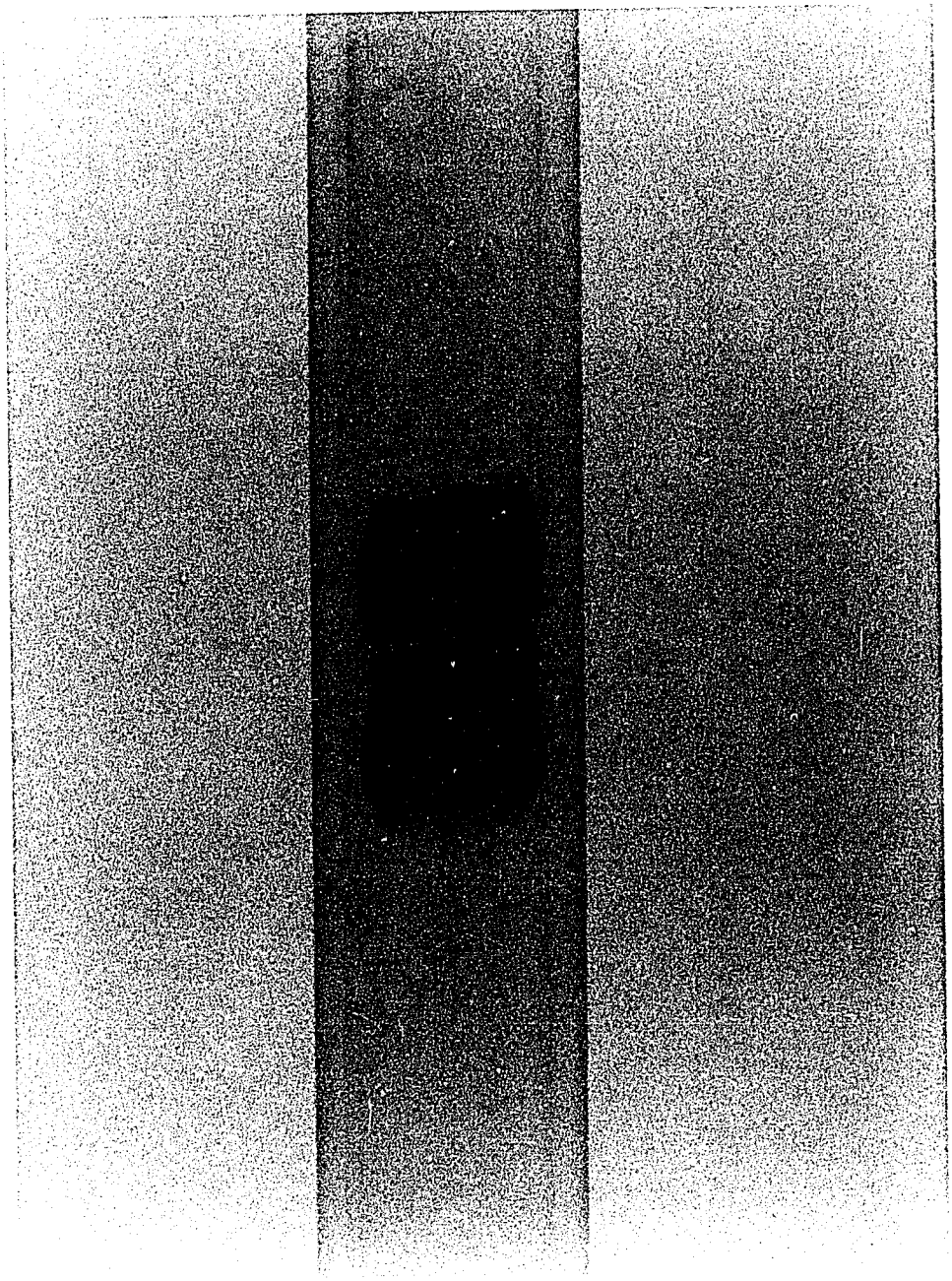


Figure 13
Analytical polyacrylamide isoelectric focusing pH 4-6 and activity staining with α-MG as substrate at 10cm gel length of a simultaneously induced extract of Saccaromyces cerevisiae
with 1% α-MG and 1% maltose

inhibitor. Neither extract was capable of converting one form to the other. Therefore, it is highly improbable that any of these yeast α-glucosidases arise as a result of an in vitro process occurring in the extracts.

Isoelectric focusing

Figure 19 shows IEF and activity stains of crude extracts in the pH 4-6 range and at a gel length of 20cm. Lanes 1,2 and 3 correspond to IEF and activity staining in the absence of glycerol at 24, 36 and 48 hours respectively. Lanes 4, 5 and 6 correspond to IEF and activity staining with the same protein load and in the same bath in the presence of 15.6% glycerol at 24, 36 and 48 hours respectively. The material applied to the 20cm gels had been focused at 10cm gel length and the relative levels of the different forms and final banding pattern was known. Gels focused in the absence of glycerol showed a distribution of multiple isomaltases different from that achieved in the presence of glycerol. The cathodic drift of the bands was pronounced with complete deterioration of the resolution at 48 hours. Gels focused in the presence of glycerol showed the proper banding profile as corroborated by 10cm IEF. A pronounced cathodic shift set in after 36 hours but the drift was controlled and migration of bands towards the cathode actually enhanced the separation with pI band separation greater at 48 hours than at 24 hours. It can be seen that the middle form appears as a doublet at 36 hours and band broadening increases through 48 hours. The determination of this artifact can conveniently be distinguished by sampling gels at various times, an essential prerequisite for IEF. It can be seen that the isomaltase focusing at a slightly more acidic pI than the maltose induced form (A) did not change in appearance during the cathodic drift and for this

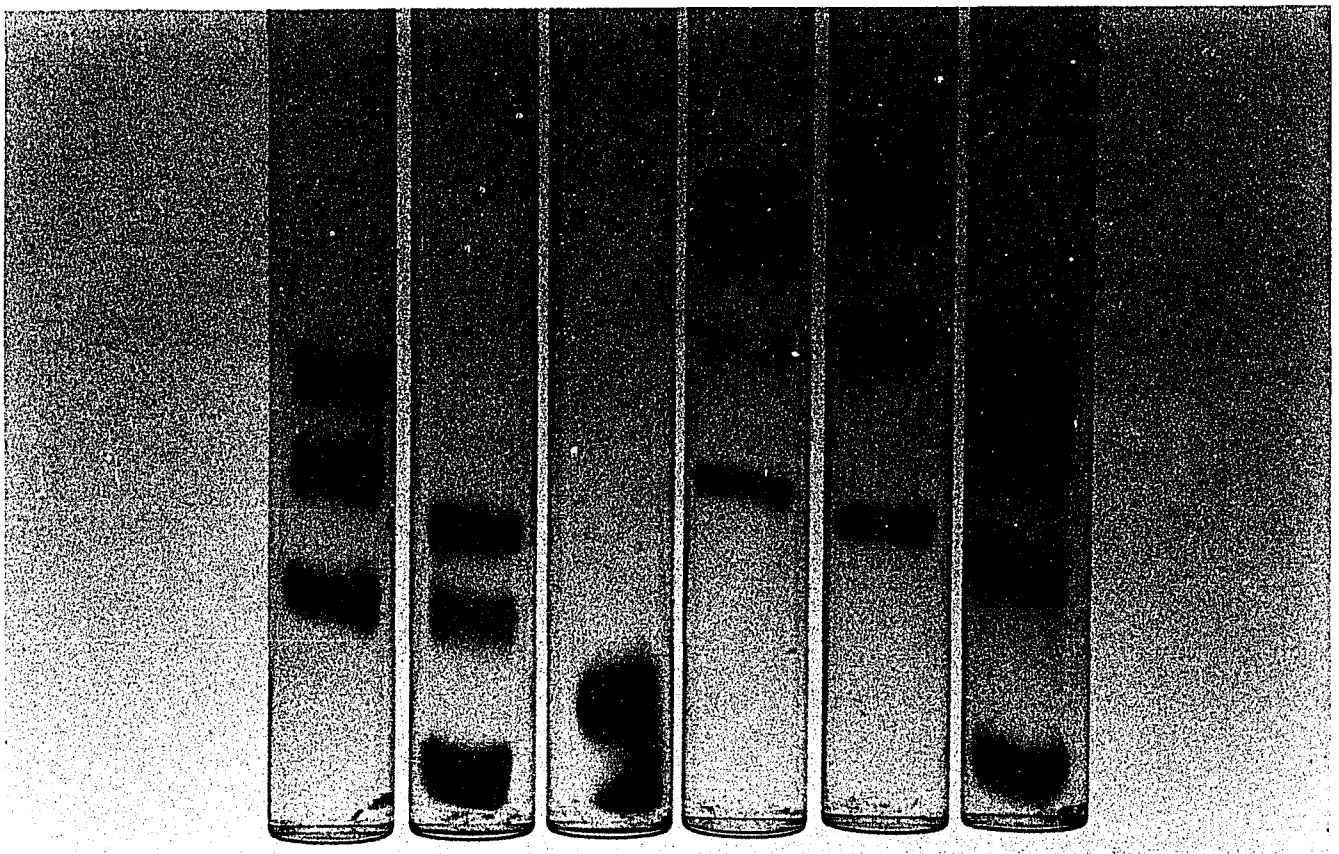


Figure 19
Analytical polyacrylamide isoelectric focusing pH 4-6 (20cm gel length) and activity staining with and without glycerol at vary times of focusing
Lane 1 24 hrs no glycerol
Lane 2 36 hrs no glycerol
Lane 3 48 hrs no glycerol
Lane 4 24 hrs 10% glycerol
Lane 5 36 hrs 10% glycerol
Lane 6 48 hrs 10% glycerol

reason is judged to be genuine.

Even in 10cm gels the presence of glycerol results in stabilization of the pH gradient. It is essential to have the same final concentration of glycerol (10-15%) in all gels so as to make side by side comparisons. It has been reported (61) that the decay of pH gradients with time in IEF is greatly reduced when gels are made viscous by the addition of 12.5% sucrose.

The nature of the instability of the pH gradient in IEF has received wide attention but no consensus of opinion has been formed. Both anodic and cathodic drifts have been observed as evidenced by the fact that protein bands are displaced towards the proximal electrode. Figure 20 is a 10cm IEF gel pH 5-7 demonstrating both anodic and cathodic drift. Bands focusing in the upper region of the gel close to the anode have an inverted appearance. All electrophoresis systems characteristically show discs of protein bands, the edges of which are slightly retarded due to contact with the glass tube. Presumably, the inverted appearance of bands of focusing near the anode likewise demonstrate a movement or anodic shift towards the anode. The bottommost band in Fig. 20 demonstrates a cathodic shift. This inversion is not as pronounced when protein is stained for activity in IEF due to the fact that glucose diffuses away from the site of enzymatic activity prior to staining. Possible explanations of the pH gradient instability are reviewed by Chrambach (59). It has been reported by Nguyen (61) that an anodic drift of the pH gradient occurs in a 6-8 gradient when glycine is the anolyte (pH=6) and arginine is the catholyte (pH=10.3). The direction of the drift is reversed to cathodic when (a) the catholyte is positioned below the gel (in the lower buffer reservoir), (b) tubes are coated by linear polyacrylamide,

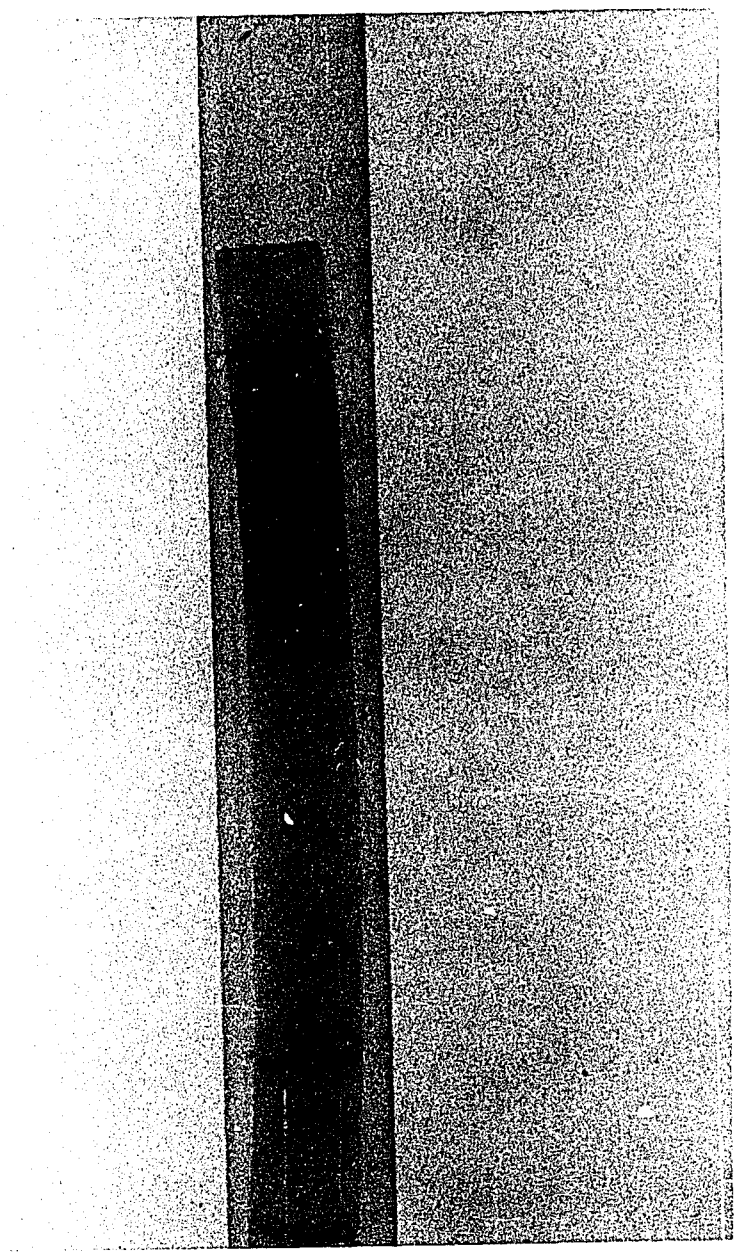


Figure 20
Analytical polyacrylamide isoelectric focusing pH 5-7 stained with Coomasie G of a partially purified extract demonstrating anodic and cathodic shifts

(c) arginine is replaced by lysine (pH=9.5).

The technique of IEF produces an entropically unfavorable situation. The protein bands concentrate next to regions of no protein and there exists a pH gradient with a finite conductivity proximal to electrolyte solutions of different conductivities. The fact that when Nguyen (61) changed the conductivity of an electrolyte (pH 9.5 in place of 10.3) the drift reversed itself is supportive of this view. The drift is a function of the second law of thermodynamics - the entropy of a system tends to assume a maximum and pH gradients tend to migrate into the proximal electrolyte so as to equalize concentrations of all species. Protein bands cannot diffuse away from their pI points as they would acquire a net charge and be repelled by the proximal electrode. The pressure of the band and its tendency to randomize causes it and the entire segment of the pH gradient containing it to migrate into the nearest electrolyte compartment. The reversal of the drift with electrolyte composition is consistent with this view. Consequently, it is desirable to use acids and bases in the electrolyte compartments such that the pH is close to the pH in the extremities of the gel. Strong bases tended to destabilize the gradient at the cathode end more so than strong acids at the anode end. It has been demonstrated by companies marketing carrier ampholines that the number and types of amphoteres in the neutral region of the gel is less and hence the conductivity is less in the neutral region than in the basic or acidic regions. Basic and acidic range ampholines are marketed at 20% $^{w}/v$ while the middle ranges are at 40% $^{w}/v$. For this reason, the cathodic region was problematical in a pH 4-6 gradient. When cathode was uppermost there was rapid migration of base into the gel with concomitant anodic shift of the pH gradient into the acidic

electrolyte. Background ampholine staining was completely absent in the cathode region of the gel and the pH of the gel was identical to that of the catholyte. For this reason, the cathode was made the bottom electrode which necessitated the electrolyte moving upward against the force of gravity upon entering the gel. This proved successful and gradients were linear with only 0.5cm at both cathode and anode end being of a pH identical with the electrolyte. It is imperative to use the minimum volume of electrolyte so as to just cover the bottom of the gel tubes and the electrode and create the minimum hydrostatic pressure on the bottom gel surface. When electrolyte in the bottom compartment was added such that a majority of the gel tube was immersed in solution, the pH gradients were linear over a much shorter distance with significant migration of the electrolyte into the gel from the bottommost compartment. When conducting IEF it is advised to make the low conductivity region of the gradient the bottommost electrode and to use an electrolyte of pH approximately equal to the pH at the extremity of the gradient. Phosphate buffers adjusted to the proper pH did not prove suitable for unknown reasons. There was no increase in resistivity over time, no migration of amphoteres, and no pH gradient. Amino acids are suitable electrolytes particularly when preparative recovery is desired but required longer focusing times than did weak acids and bases. When focusing in pH 3-10 gradients, NaOH was suitable as a catholyte but unsuitable in pH 4-6 gradients and monoethanolamine was preferred. An excellent review article on IEF was written by Righetti and Drysdale (53) the methods proving quite suitable when incorporated with the suggestions mentioned in

this thesis.

The Bio Rad model 155 proved unsuitable for electrofocusing procedures in which the bottom of the gel tube was below the bottom electrode. Twenty centimeter IEF required a voltage maxima of 750V, two hundred volts above the maximum rated voltage of the cell. This and the fact that the inner surface of the power core at the bottom electrode showed the presence of exposed wire produced a pronounced cathodic drift centered on the position where the bottom electrode surfaced from the core (Fig. 21). Proximity to an electrode has been reported (53) to accelerate cathodic drift. Neither cementing over the exposed wire nor baffling off the entire bottom of the core with parafilm alleviated the drift. SDS gel electrophoresis in the cell showed differences in migration rate of the tracking dye dependent on gel position in the bath. The cell was suitable for IEF in gels in which the lower gel surface was considerably above the bottom electrode (10cm) but reproducibility was not quite as good as when IEF was performed in the Bio Rad model 150A. This bath proved ideal for IEF but gel length is limited to approximately 17.5cm and gel number of 12. The Canalco model 1200 might be suitable for IEF provided electrolyte volume in the bottom compartment is kept to a minimum. The Canalco Model 12, kindly supplied by Norman R. Eaton, is ideally suited for IEF as the top electrode is baffled to prevent proximity effects and the bottom electrode is a flat plate. The feature providing for variable length gels is quite convenient and IEF over 25cm was performed with this bath and demonstrated heterogeneity (a multiple banding pattern) within the α form of isomaltase and within the middle form (B) (Fig. 22). Focusing time was 60 hours at 0.3 watt/gel with voltage maximum of 1600V. The heterogeneity was probably artifactual and due to the

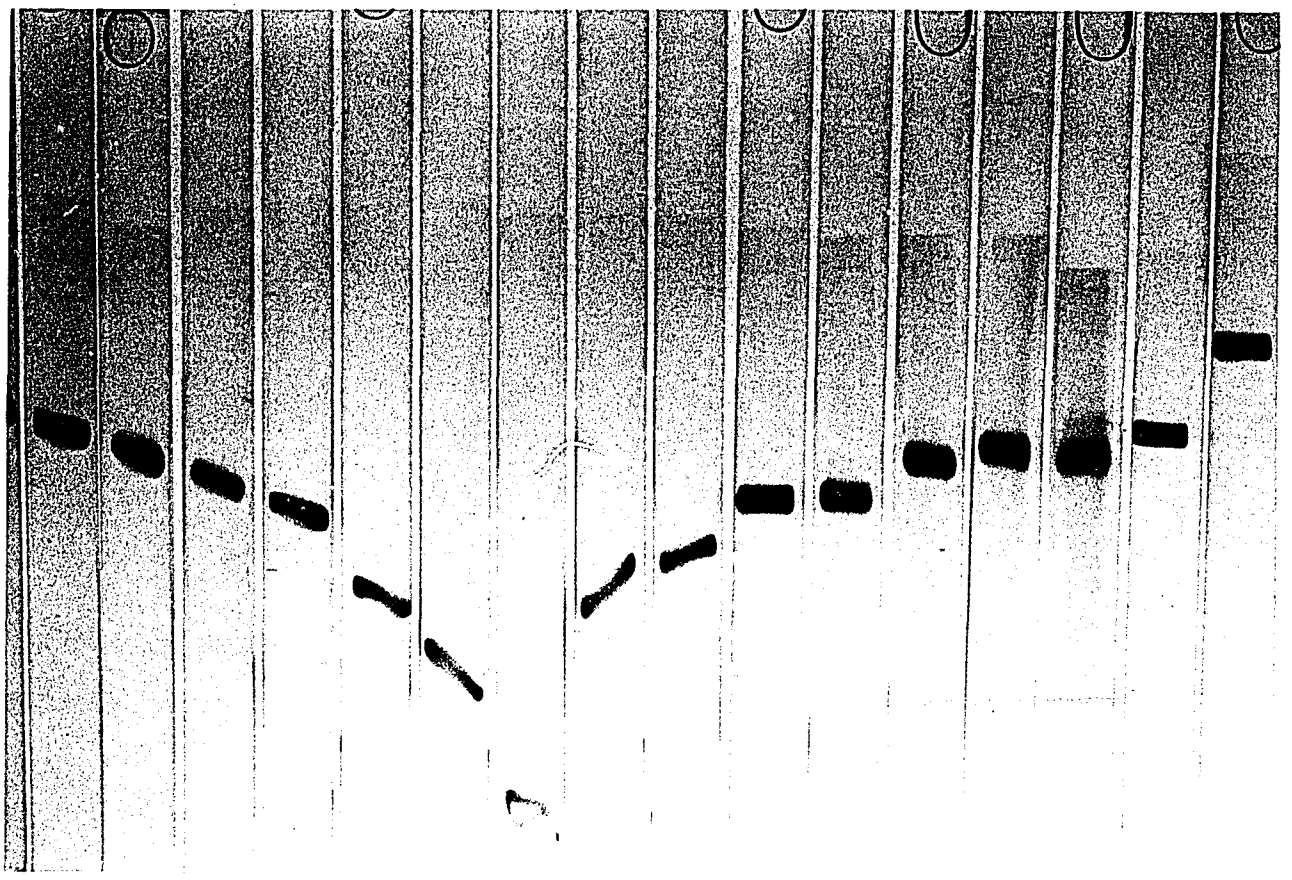


Figure 21
Analytical polyacrylamide isoelectric focusing pH 4-6 (20cm gel length) and activity staining illustrating an Electrode Induced Cathodic Shift in the Bio-Rad Model 155A. Exposed cathode electrode is bottommost
Lane 7 Site of exposed wire
Lane 1-6 Increasing proximity of gel tubes to exposed wire
Lane 8-16 Decreasing proximity of gel tubes to exposed wire

Fig. 22
Analytical polyacrylamide isoelectric focusing pH 4-6 of a fraction containing approximately equal amounts of 3 forms of isomaltase at 10 and 25cm gel length. Lanes 1, 2 and 3 demonstrate splitting of what appear to be single bands in lane 4. An amount of enzyme producing 100-150ug glucose from α-MG in 5 minutes was applied to lane 2, 3, and 4.

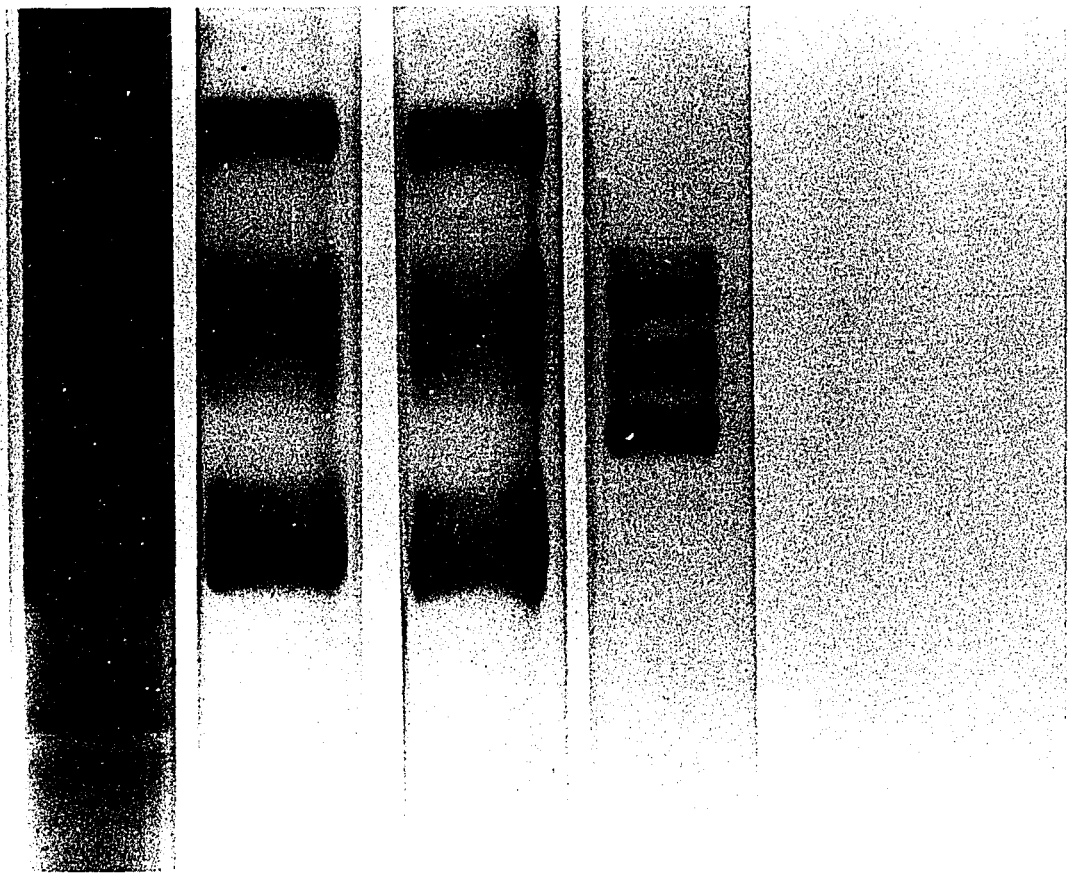


Figure 22
Analytical polyacrylamide isoelectric focusing pH 4-5 at 10 and 25cm gel length illustrating increased resolution at the longer gel length
Lane 1 Protein staining lane 2
Lane 2 & 3 Activity staining at 25cm gel length
Lane 4 Activity staining at 10cm gel length

increased length and increased voltage. Ampholines were distributed over a 25cm length aggravating the problem produced by regions of low conductivity. The heterogeneity observed in 25cm focusing was much more marked than that observed in 20cm, focusing (Fig. 18), moreso than would be expected from the additional 5cm length. The limitations of the technique are a function of nonuniform conductivity of the pH gradient and can possibly be remedied by mixing ampholines of the same range as supplied by different manufacturers. An alternate approach for increasing resolution is to fractionate commercial 2 pH unit ranges as described in methods and perform IEF in 10cm gels. It would be imperative to choose acids and bases as electrolytes such that the pH is very close to the pH in the extremeties of the gel.

A technique known as nonequilibrium focusing has been employed by investigators working with proteins whose pI values lie outside the range of commercial ampholines. It involves termination of IEF prior to completion and attainment of a final stable banding pattern so as to visualize all components. When IEF and activity staining was performed on gels terminated prior to attainment of equilibrium, the number of active bands seemed to be correct but the distribution in level of each form was totally different from that obtained when the technique was performed correctly. Resolution was difficult to obtain, resulting in the deposition of active unfocused isomaltase evident throughout the gel producing a substantial smear of red formazan. I view the validity of nonequilibrium focusing (non-focusing) with great reservation to be used only to ascertain the number of components and then only in conjunction with some other method to verify results.

The use of IEF and activity staining is a powerful tool in the identification of variant enzymes and proteins in crude extracts. It

is best to identify isozymes in crude extracts prior to purification and to use IEF and activity staining to monitor the separations. It is unwise to look for multiple forms of enzymes at the completion of a purification scheme. Proteolysis, differential stabilities, and losses associated with purification might lead to the loss or gain of one or more forms.

Criteria of purity

Upon isoelectric focusing at a 10cm gel length in a pH 4-6 gradient the purified homogeneous maltose-induced form (M) of isomaltase showed the presence of a trace amount of the A form of the enyzme estimated to be less than 5% of the total protein (Fig. 17). Activity staining verified the concomitant migration of protein and activity (Fig. 16). Disc gel electrophoresis demonstrated the presence of one major protein species and failed to resolve the A form of the enzyme. SDS gel electrophoresis demonstrated the presence of one major protein band along with trace impurities.

Upon isoelectric focusing at a 10cm gel length in a pH 4-6 gradient the purified homogeneous α-methyl-D-glucoside induced form of isomaltase demonstrated the presence of a trace amount of the B form of the enzyme estimated to be 5% of the total protein (Fig. 23). Activity staining verified the concomitant migration of protein and activity.

Upon isoelectric focusing at a 20 and 25cm gel length, the α and B form of isomaltase showed heterogeneity existing within what was a single protein on 10cm focusing (Fig. 22). Whether or not the heterogeneity was real or artifactual remains unknown.

All forms of the enzyme demonstrated identical pI values in the purified state and in the crude extracts. By this criteria the purified enzymes retained the structural differences distinguishing

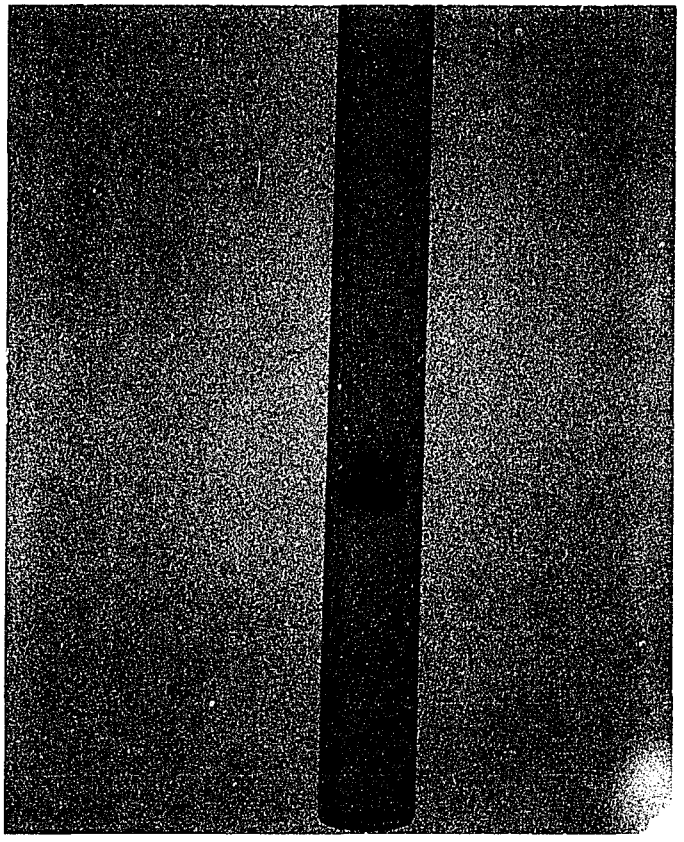


Figure 23
Analytical polyacrylamide isoelectric focusing pH 4-6 (10cm gel length) of homogeneous α form of isomaltase. Protein was stained with Coomasie G.

them from each other despite extensive purification in the presence of considerable levels of proteolytic enzymes. The isoelectric points distinguishing the different forms demonstrated in this study probably reflect in vivo structural differences.

Amino acid analysis

Amino acid analysis performed on a 24,48 and 72 hr. hydrolysate of the maltose induced form on the Beckman 116 is illustrated in Tables 2,3,4. No losses of threonine and serine were observable and no extrapolation to zero time was necessary. The 72 hour hydrolysate showed destruction of tyrosine and concomitant increase in NH_3 content (58). The analysis showed a preponderance of aspartic acid and glutamic acid. No half cystine was detected despite full scale deflection for the aspartic acid and glutamic acid peaks. However, cysteic acid standards were not run. The data in Table 2,3 and 4 illustrate the empirical relationship amongst the constituent amino acids, methionine being the amino acid present at the lowest level and the one which the empirical ratio is based upon.

Table 5 illustrates the reproducibility amongst the standards. Percentage deviation is the range difference between high and low values of the standards divided by the average value times 100. The numbers represent the integration of the area under the peak and correspond to 0.625 umole cystine and 0.125 umole of each of the other amino acids.

Amino acid analysis was performed on all forms of the enzyme by excising protein bands from Coomasie G stained gels of isoelectric focusing at 10cm gel length. The A form was recovered from a 20cm length isoelectric focusing gel. Parallel gels were stained for activity.

Table 2

Amino acid analysis of the maltose induced enzyme on the Beckman 116 24 hr. hydrolysis

Residue	Trial 1[a]	Trial 2[a]	Average	μ moles[b]	Empirical residues[c]	Empirical residues
Asx	727			0.086	10.8	11
Thr	302			0.035	4.38	4
Ser	443			0.051	6.38	6
Glx	570			0.066	8.25	8
Pro	74			0.036	4.5	5
Gly	422			0.050	6.25	6
Ala	417			0.048	6	6
Cystine	N.D.					
Val	294			0.035	4.38	4
Met	72			0.008	1	1
Ile	362			0.039	4.88	5
Leu	446			0.049	6.13	6
Tyr	220			0.026	3.25	3
Phe	292			0.035	4.38	4
Lys	667	643	655	0.048	6	6
His	174	150	162	0.014	1.75	2
NH_3	1202	1174	1188	0.1389	17.4	17
Arg	241	238	240	0.023	2.88	3
Trp	N.D.[d]					

a. Numbers under trial 1, 2 represent area under the peak

b. μ moles = $\frac{\text{readout}}{\text{standard}} \times .125$

c. Empirical value = $\frac{\mu \text{ moles residue}}{\mu \text{ moles methionine}}$

d. N.D. not determined

Table 3
Amino acid analysis of maltose induced enzyme on the Beckman 116 48 hr. hydrolysis

Residue	Trial 1[a]	Trial 2[a]	Average	μ moles[b]	Empirical residues[c]	Empirical residues
Asx	731	729	730	.086	10.8	11
Thr	281	282	282	.033	4.13	4
Ser	386	386	386	.044	5.5	6
Glx	613	611	612	.071	8.88	9
Pro	72	72	72	.035	4.38	4
Gly	424	427	426	.051	6.38	6
Ala	416	417	417	.048	6	6
Cystine	N.D.					
Val	310	313	312	.038	4.75	5
Met	69	69	69	.008	1	1
Ile	371	373	372	.040	5	5
Leu	441	442	442	.049	6.13	6
Tyr	169	170	170	.020	2.5	3
Phe	296	298	297	.035	4.38	4
Lys	639	635	637	.045	5.63	6
His	147	145	146	.013	1.63	2
NH_3	1266	1262	1264	.149	18.6	19
Arg	217	248	233	.023	2.81	3
Trp	N.D.[d]					

a. Numbers under trial 1, 2 represent area under the peak

b. μ moles = $\frac{\text{readout}}{\text{standard}} \times .125$

c. Empirical value = $\frac{\mu \text{ moles residue}}{\mu \text{ moles methionine}}$

d. N.D. not determined

Table 4

Amino acid analysis of the maltose induced enzyme on the Beckman 116 72 hr. hydrolysis

Residue	Trial 1[a]	Trial 2[a]	Average	μ moles[b]	Empirical residues[c]	Empirical residues
Asx	1000	1037	1019	.120	10.9	11
Thr	355	354	355	.041	3.73	4
Ser	463	450	457	.052	4.73	5
Glx	804	792	798	.093	8.45	9
Pro	110	96	103	.050	4.55	5
Gly	576	*	576	.069	6.27	6
Ala	561	626	594	.069	6.27	6
Cystine	N.D.					
Val	393	377	385	.046	4.18	4
Met	105	95	100	.011	1	1
Ile	*	509	514	.055	5	5
Leu	*	590	590	.065	5.91	6
Tyr	116	113	115	.014	1.27	1
Phe	433	430	432	.051	4.64	5
Lys	771	774	773	.074	6.73	7
His	198	183	191	.021	1.91	2
NH_3	2046	1896	1971	.313	28.4	28
Arg	288	243	266	.031	2.81	3
Trp	N.D.[d]					

a. Numbers under trial 1, 2 represent area under the peak

b. μ moles = $\frac{\text{readout}}{\text{standard}} \times .125$

c. Empirical value = $\frac{\mu \text{ moles residue}}{\mu \text{ moles methionine}}$

d. N.D. not determined

* inaccurate baseline tracking

Table 5

Long column standards on the Beckman 116

Residue	Trial 1[a]	Trial 2[a]	Trial 3[a]	Average	% deviation[b]
Asp	1061	1056	1062	1060	.57
Thr	1073	1065	1076	1071	1.03
Ser	1097	1088	1092	1092	.82
Glu	1078	1066	1077	1074	1.11
Pro	257	255	255	256	.78
Gly	1100	1048	1052	1067	4.9
Ala	1132	1075	1080	1096	5.2
Cys	524	498	506	509	5.1
Val	1044	1035	1043	1041	.86
Met	1115	1134	1144	1131	2.57
Ile	1165	1165	1165	1165	0
Leu	1125	1126	1130	1127	.44
Tyr	1061	1052	1055	1056	.85
Phe	1060	1048	1052	1053	1.14

Average % deviation excluding cystine = 1.56%
Range 0 - 5.2%

Short Column Standards					
Lys	1767	1751	1772	1763	1.19
His	1436	1428	1442	1435	.98
NH_3	1042	1047	1069	1053	2.53
Arg	1329	1304	1301	1311	2.14

Average % deviation = 1.71%
Range = .98 - 2.53%

a. Numbers under trial 1, 2 and 3 represent area under the peak and correspond to .125 μ mole of each residue excepting cystine which corresponds to .625 μ mole

b. % deviation = $\frac{\text{range X 100}}{\text{average value}}$

Excised protein bands were hydrolysed for 24 hrs. in 6N HCl at 110°C and amino acid analysis was performed by HPLC with a fluorescamine detection system. Hydrochloric acid blanks were run and showed minimal amino acid contamination. The analyses represents the results obtained from enzyme devoid of any trace impurity (Table 6). Empirical residues were calculated as previously described. The maltose induced form was analyzed in triplicate. Amino acid analysis was performed on the Beckman 116, on the HPLC system with the same hydrolysate, and from an excised protein band. The only pronounced variance was in serine. The Beckman 116 analysis and HPLC analysis on the same hydrolysate gave a value of 6 empirical residues while HPLC analysis of an excised band gave 3 empirical residues. The value of 6 empirical residues based on 4 runs was taken to be correct. As compared to the α-methyl-D-glucoside induced form, the maltose induced form contained one half as much glycine (6 vs. 11) and 3 times as much proline (3 vs. 1) as the α-methyl-D-glucoside induced form. Percentage error on each amino acid on the HPLC system was 10%. The differences between the α form and the M form are well beyond what experimental error would produce and could not have been produced by proteolysis. Both minor forms, A and B, give similar amino acid analysis with the A form showing slightly more aspartic acid, glutamic acid, and glycine than the B form. The major differences exist between the two differentially inducible forms of the enzyme, the maltose induced form (M) and the α-methyl glucoside induced form (α). The A form seems to be induced along with the M form and the B form is induced by both α-methyl-D-glucoside and maltose (Fig. 12).

On the basis of amino acid analysis it is concluded that the yeast cell contains at least two structural genes for isomaltase and possibly

Table 6

Amino acid analysis by HPLC and the fluorescamine reagent

Form	Empirical residues				
Residue	M (48 hr)[c]	M (24 hr)[b]	α(24 hr)[b]	B (24 hr)[b]	A (24 hr)[b]
Asx	10	11	11	12	14
Thr	4	3	4	4	4
Ser	6	3	5	4	6
Glx	10	9	9	10	13
Pro	3	3	1	3	4
Gly	6	6	11	13	15
Ala	6	5	7	6	7
Cystine	N.D.[a]				
Val	5	4	4	4	5
Met	1	1	1	1	1
Ile	5	5	5	5	6
Leu	6	6	6	7	8
Tyr	3	3	3	3	4
Phe	4	5	5	5	7
His	2	2	2	3	3
Lys	7	7	8	8	8
Arg	3	4	3	4	5
NH_3	N.D.				
Trp	N.D.				

a. N.D. not determined

b. ( ) indicate hydrolysis time

c. M (48) run was performed on the same hydrolysate as run on the Beckman 116 and all 24 hr hydrolysates were from excised Coomasie G stained protein bands

d. All analyses are results of duplicate runs

four, each coding for an isoenzyme of isomaltase.

Kinetics

The assay for the liberation of glucose from substrate is performed in two steps. In the first the enzyme in 0.05M phosphate buffer pH 7.01 at 25°C is treated with an aliquot of substrate and the reaction allowed to proceed for one minute after which it is chemically stopped. In the second step the liberated glucose is treated with glucostat reagent (glucose oxidase-peroxidase-O-dianisidine), incubated for 30 minutes at 30°C and the reaction terminated upon addition of 0.8ml 6N HCl. The resultant absorbance of the oxidized dianisidine dye is read at 525nm and total glucose produced extrapolated from a standard curve. Variable concentration of substrate is achieved by varying the aliquot of substrate added, final volume of the incubation mixture is constant in all assays.

A variety of methods were experimented with to terminate the first reaction-the hydrolysis of substrate by isomaltase. Heat denaturation at 98°C proved unsatisfactory for two reasons. Prior to complete denaturation the enzyme most certainly hydrolyzed substrate at an unknown and elevated velocity. In addition, heat denaturation destroyed only 98% of the enyzme activity.

When enzymatic hydrolysis was terminated by the addition of base, shifting the pH to a value greater than 11 the reaction was completely stopped. However, the enzyme was not denatured and readjusting the pH to 7.0 which was necessary in order to perform the glucose oxidase-peroxidase assay resulted in a resumption of hydrolysis. Heating an aliquot of enzyme at pH 11 resulted in complete denaturation of the enzyme, but also resulted in isomerization of D-glucose. Glucose standards heated to 98°C at pH 11 and compared to identical standards

heated to 98°C at pH 7.0 and to unheated standards showed greatly reduced quantities of D-glucose.

Termination of isomaltase activity by lowering the pH with acid was deemed inadvisable because of the lability of the disaccharide linkage to acid necessitating experimentation to determine the lability of each of the five substrates to acid treatment.

The glucose-oxidase buffer (glucostat) is composed of 0.3M tris-0.36M phosphate, tris being included to suppress the activity of maltase endogenous to the glucose oxidase preparation. Tris at pH 7.0 and at concentrations less than 0.05M proved to be a potent inhibitor of isomaltase and did not diminish glucose oxidase and peroxidase activity. Isomaltase hydrolysis was terminated by the precise addition of 100μl of the glucostat buffer without glycerol and at 3 times the tris-phosphate concentration of the glucostat reagent. The pH of the tris-phosphate solution used to terminate enzymatic hydrolysis was 7.0 as was the pH of the glucostat buffer. Identical aliquots of the reaction mixture treated with tris were incubated for varying periods of time. The quantity of measurable glucose in the mixture did not change. Aliquots treated with tris and subject to heat denaturation at 98°C when reacted with glucostat reagent showed the same optical density at 525nm as did identical unheated tris treated aliquots.

Enzyme activity for each Lineweaver-Burk plot was determined at seven different substrate concentrations such that increments on the $^1/S$ axis were identical. Data were fitted to a straight line by the least squares method. Velocities were expressed as molarity changes min^{-1} of D-glucose produced. Vmax is expressed as mM or μM$min^{-1}mg^{-1}$ protein. Velocity versus substrate concentration plots were hyperbolic. Enzyme assays were performed in duplicate for the

measurement of D-glucose and in triplicate when PNPG hydrolysis was measured. Blanks were prepared in duplicate and contained an identical quantity of tris treated, heat denatured enzyme and substrate. Substrates were contaminated with α-D-glucose, as measured by the glucostat assay. It was necessary to work with substrate concentrations such that the background glucose in the highest substrate concentration within the range employed was no greater than 10% of the glucose produced by the enzyme. Sucrose and α-methyl-D-glucoside were purified by recrystallization from ethanol. Prior to glucostat assay tris treated reaction mixtures and glucose standards were allowed to incubate at 30°C for 1 hour to allow mutarotation of α-D-glucose to proceed to equilibrium. Mutarotation was shown to proceed to equilibrium within 30 minutes at 30°C. A standard curve based on varying glucose concentration was prepared, each determination being performed in triplicate. The variance of optical density with glucose concentration was linear for 0-12 μg glucose/0.2ml in accordance with Beer's law. Glucose concentrations beyond 12 μg/0.2ml showed deviation from Beer's law and decrease in optical density with increasing glucose concentration. Visible color changed from pink to purple after the reaction was stopped with acid in the region where Beer's law no longer held true. Maximum absorption of the purple colored oxidized dye was 525nm. In all assays the working range of the Beer's law curve was used.

Error in the determinations was estimated by performing kinetics on the maltose induced form at two independent enzyme and palatinose concentrations giving values for Km of 1.80×10^{-2}M and 2.70×10^{-2}M and for Vmax of 9.61 μmol $min^{-1} mg^{-1}$ and 8.71 μmol $min^{-1} mg^{-1}$. Kinetics with palatinose as substrate and in the presence of 2% glycerol gave a value

Fig. 25
Lineweaver-Burk plot of a M form of isomaltase with palatinose as substrate.

FIG.25 LINEWEAVER-BURK PLOT OF M FORM

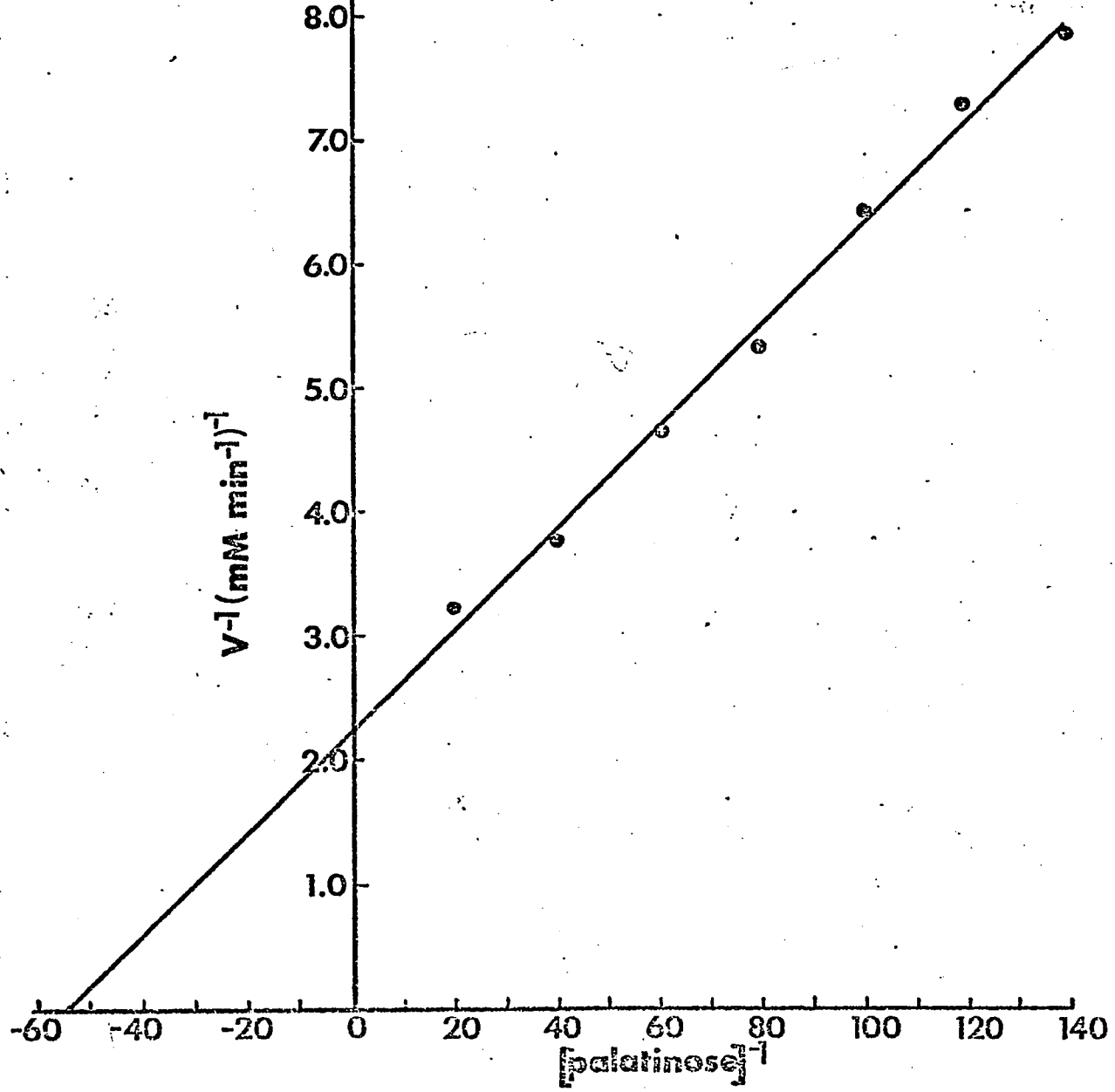


Fig. 26
Lineweaver-Burk plot of M form of isomaltase with palatinose as substrate

FIG.26 LINEWEAVER-BURK PLOT OF M FORM

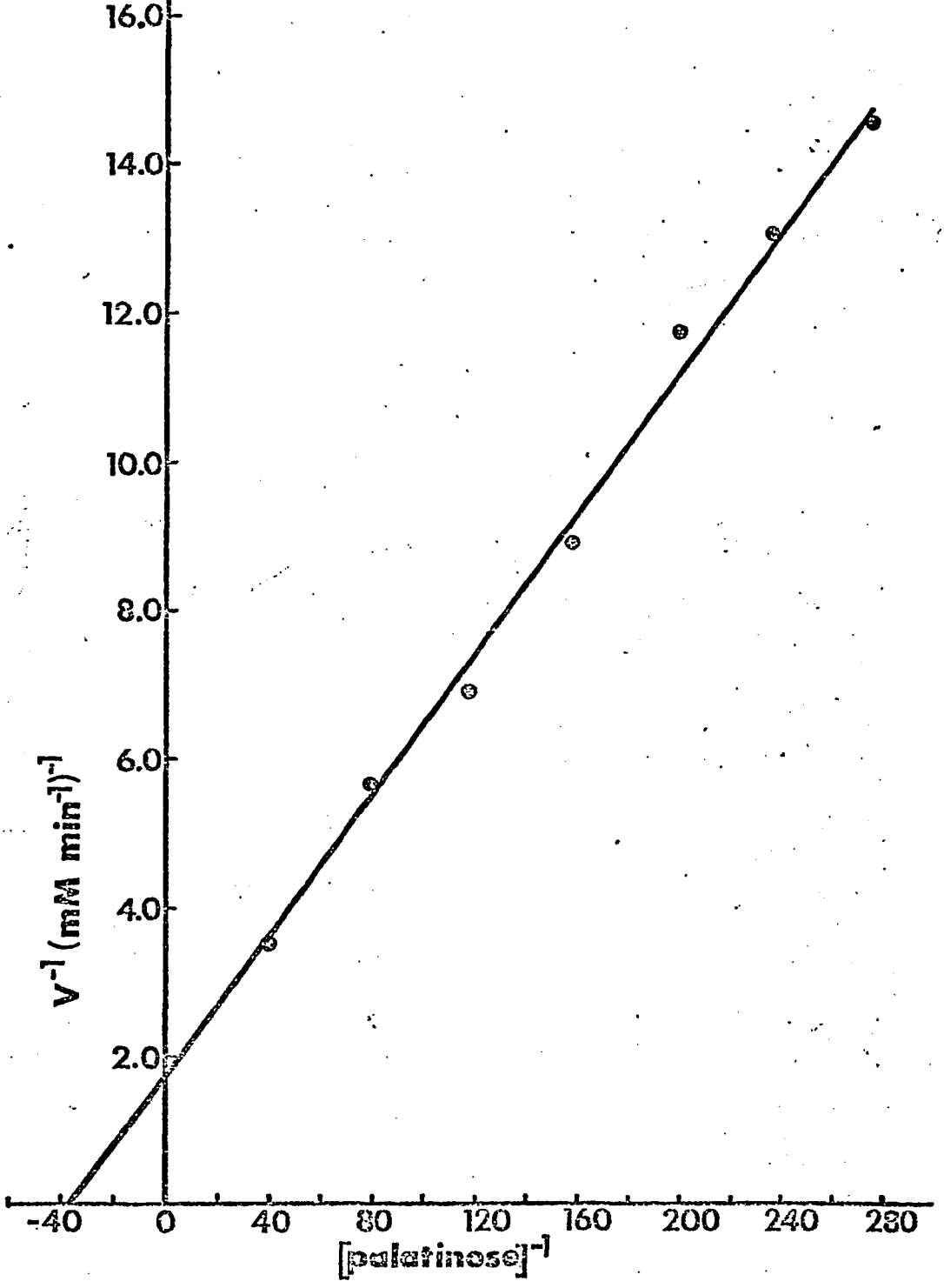


Fig. 27
Lineweaver-Burk plot of M form of isomaltase with palatinose as substrate in the presence of 2% glycerol

FIG.27 LINEWEAVER-BURK PLOT OF M FORM
2% glycerol

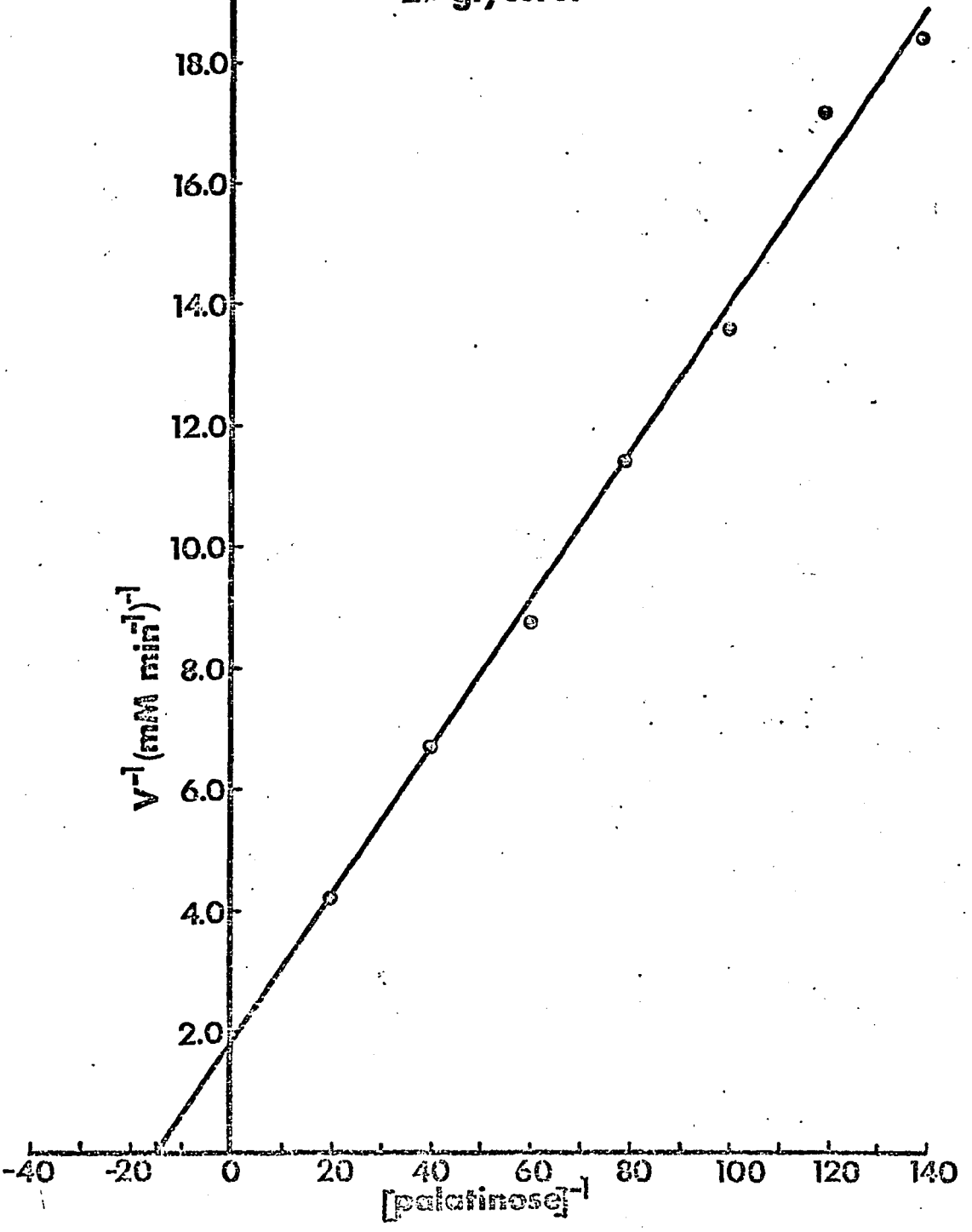


Fig. 28
Lineweaver-Burk plot of M form of isomaltase with isomaltose as substrate

FIG.28 LINEWEAVER-BURK PLOT OF M FORM

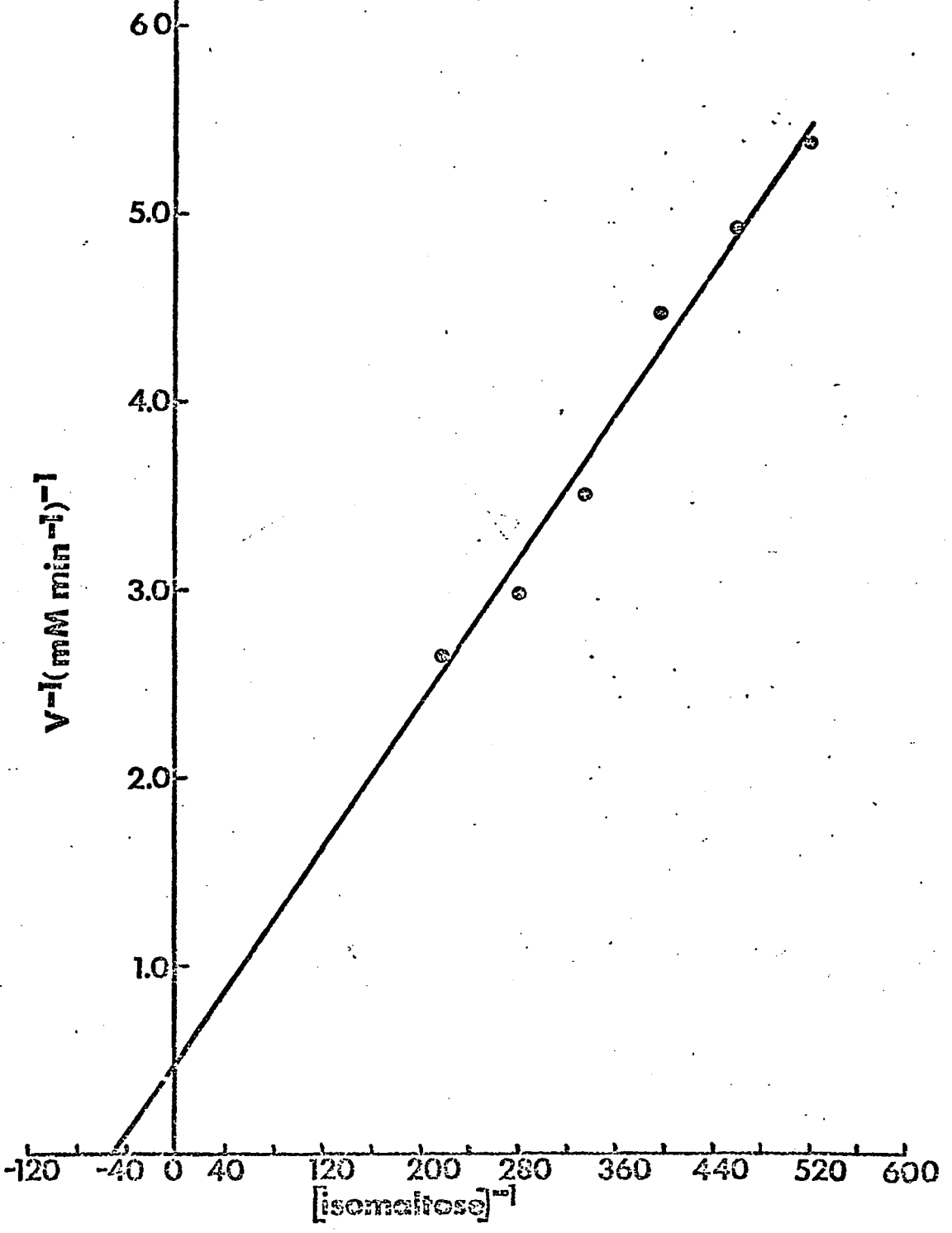


Fig. 29
Lineweaver-Burk plot of M form of isomaltase with α-methyl-D-glucoside as substrate

FIG.29 LINEWEAVER-BURK PLOT OF M FORM

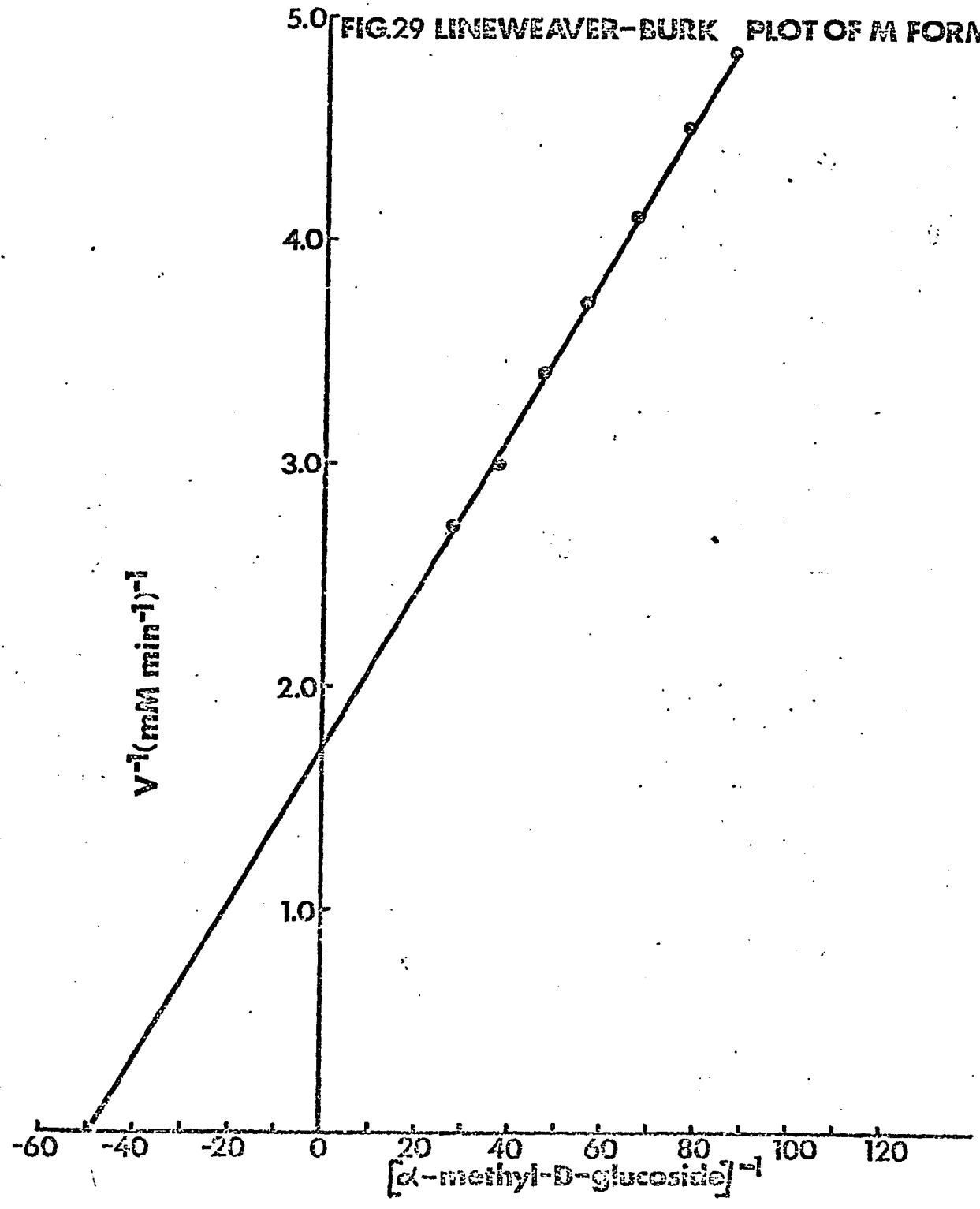


Fig. 30
Lineweaver-Burk plot of M form of isomaltase with sucrose as substrate

FIG.30 LINEWEAVER-BURK PLOT OF M FORM

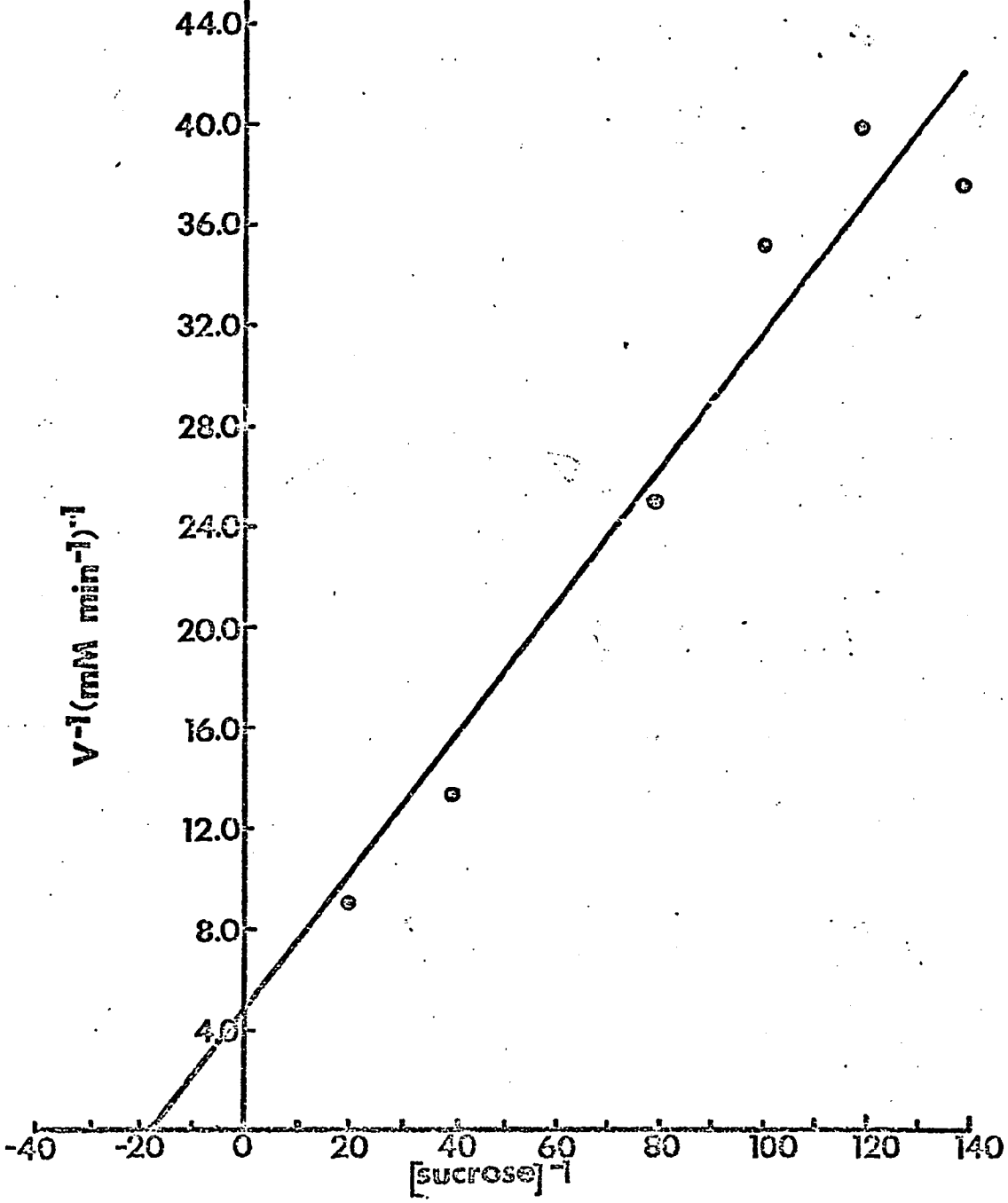


Fig. 31
Lineweaver-Burk plot of M form of isomaltase with PNPG as substrate

FIG.31 LINEWEAVER-BURK PLOT OF M FORM

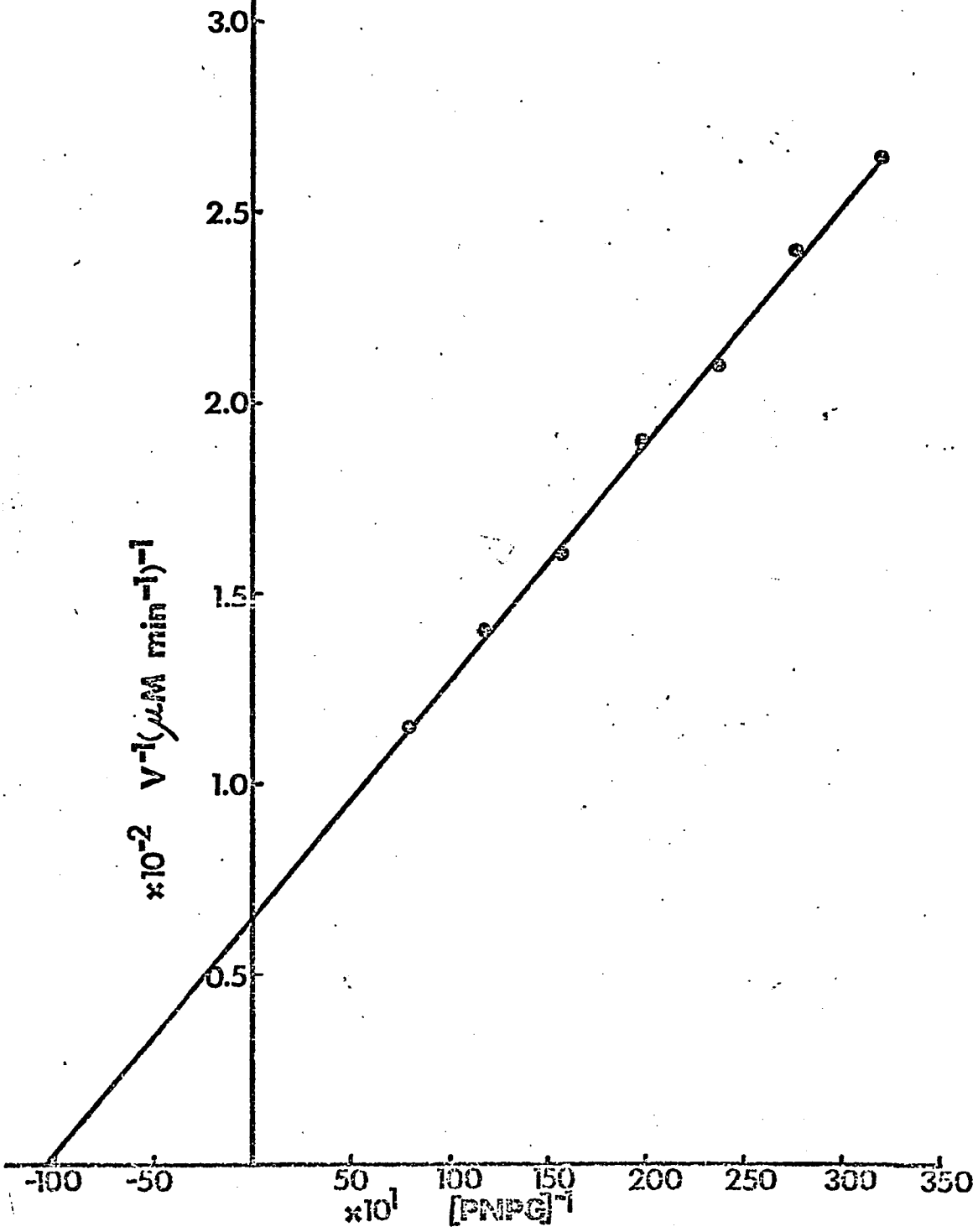
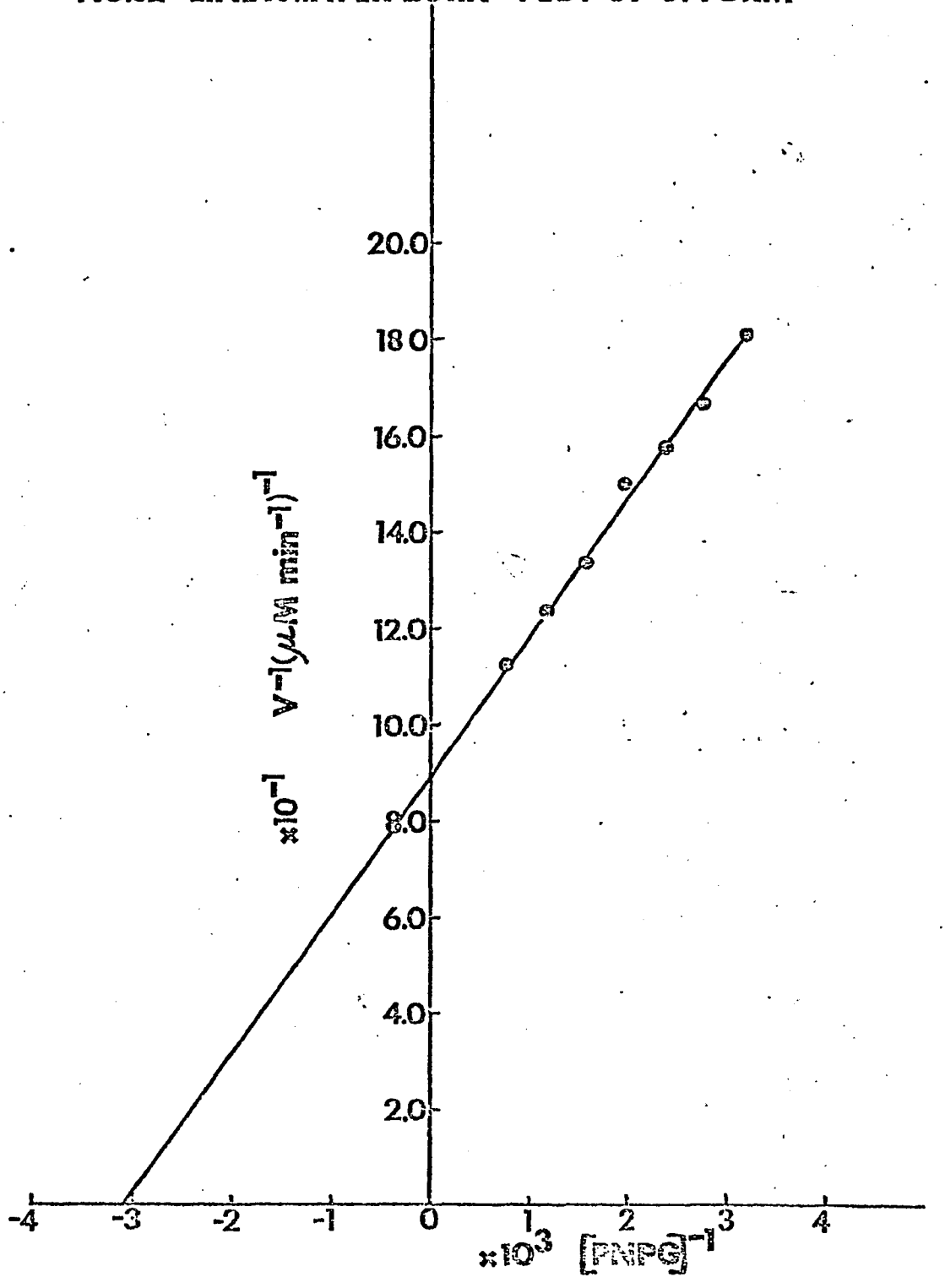


Fig. 32
Lineweaver-Burk plot of α form of isomaltase with PNPG as substrate

FIG.32 LINEWEAVER-BURK PLOT OF α FORM



for Km of 6.90×10^{-2}M. Kinetics for the α-methyl-glucoside induced form was performed with PNPG as substrate. A comparison of Km values for PNPG of the maltose induced form and α-methyl-D-glucoside induced form gave values of 8.54×10^{-4}M and 3.2×10^{-4}M respectively with Vmax values for the M and α form of 3.18×10^{4}nmolmin^{-1}mg^{-1} and 3.03×10^{4}nmolmin^{-1}mg^{-1}. Diffferences in Km values were significant and well beyond the experimental error of $\pm$ 0.9 determined with palatinose as substrate. Lineweaver-Burk plots with PNPG as substrate were considerably more accurate (correlation coefficient r=.998) than kinetics with the coupled glucose oxidase system because of the simplicity of a single assay as compared to a coupled system. The differences in Km values (PNPG) for the two forms was found to be statistically significant.

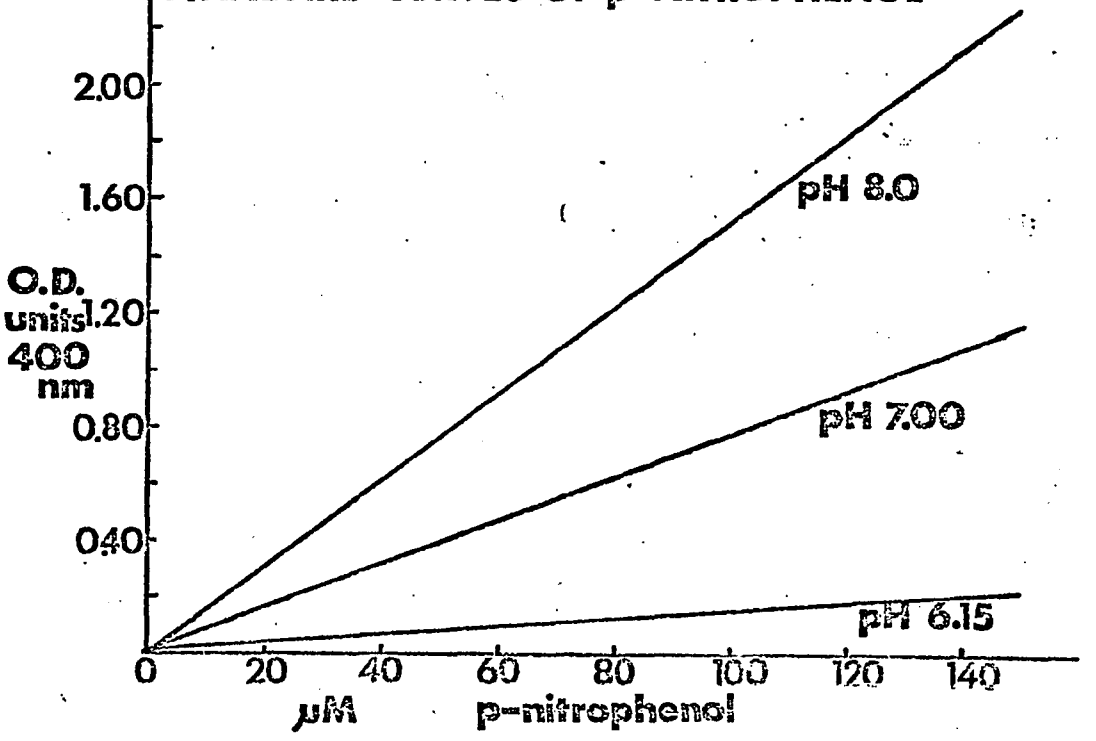
pH optimum studies

Determination of the pH optimum of the enzyme at different pH values by following the continous release of p-nitrophenol from PNPG proved to be problematical. Standard curves of p-nitrophenol concentration versus absorbance at 400nm were pH dependent. At a particular p-nitrophenol concentration absorbance at 400nm increased at higher pH values (Fig. 33). These data can be explained by analysis of the absorption profile of a 50mM p-nitrophenol solution. (Fig. 33). At pH 6.15 there appears an absorption maximum at 315nm corresponding to that reported for free p-nitrophenol and a small absorption peak evident at 400nm. At pH 7.01 there appear two absorption peaks, one at 325nm and one at 400nm corresponding to p-nitrophenol and phenoxide ion respectively. At pH 8.00 there appears only the absorption peak reported for phenoxide ion with an absorption maximum at 400nm.

The equilibrium between p-nitrophenol and phenoxide ion is pH

Fig. 33
Standard curves of p-nitrophenol concentration versus A_{400} at pH 8.00, pH 7.00, and pH 6.15. Bottommost graph shows pH dependence of the absorption profile of a 50mM p-nitrophenol solution at ——, pH 6.15; —— ,pH 7.01; —●— pH 8.00

FIG.33
STANDARD CURVES OF p-NITROPHENOL



ABSORPTION PROFILE OF p-NITROPHENOL

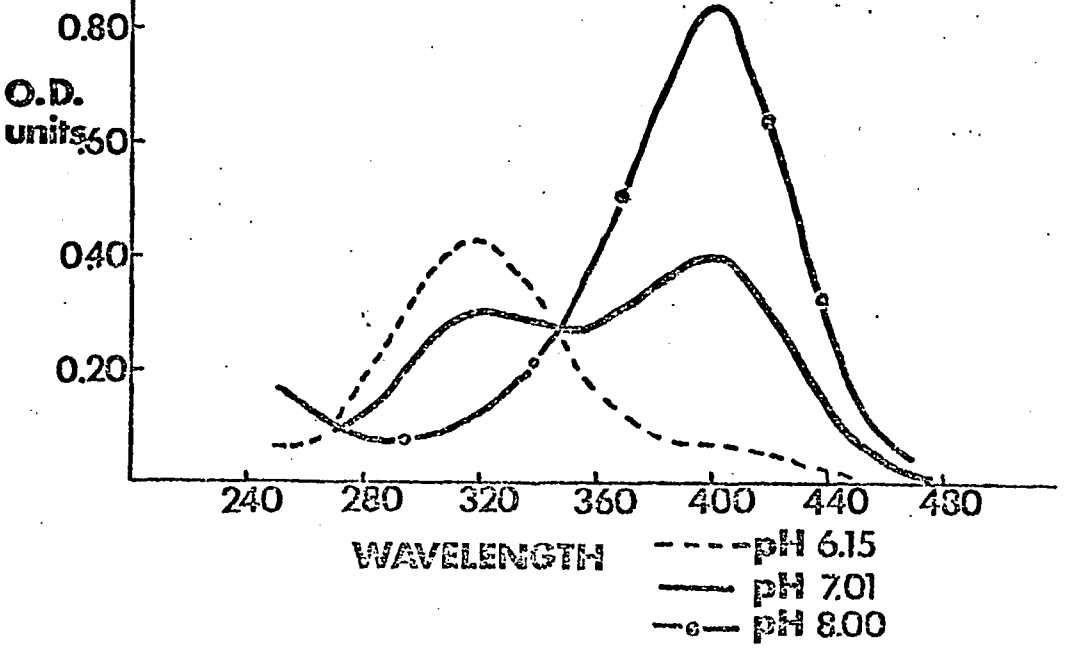
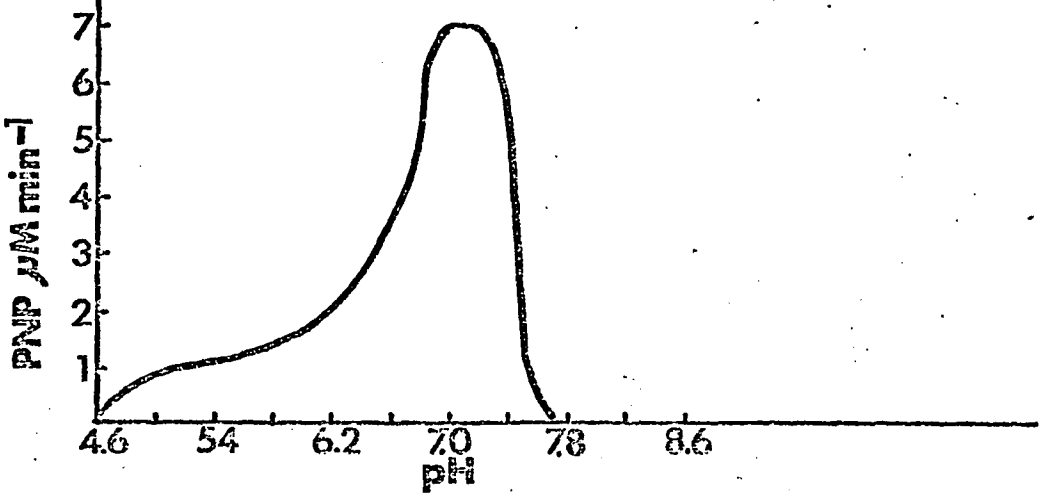


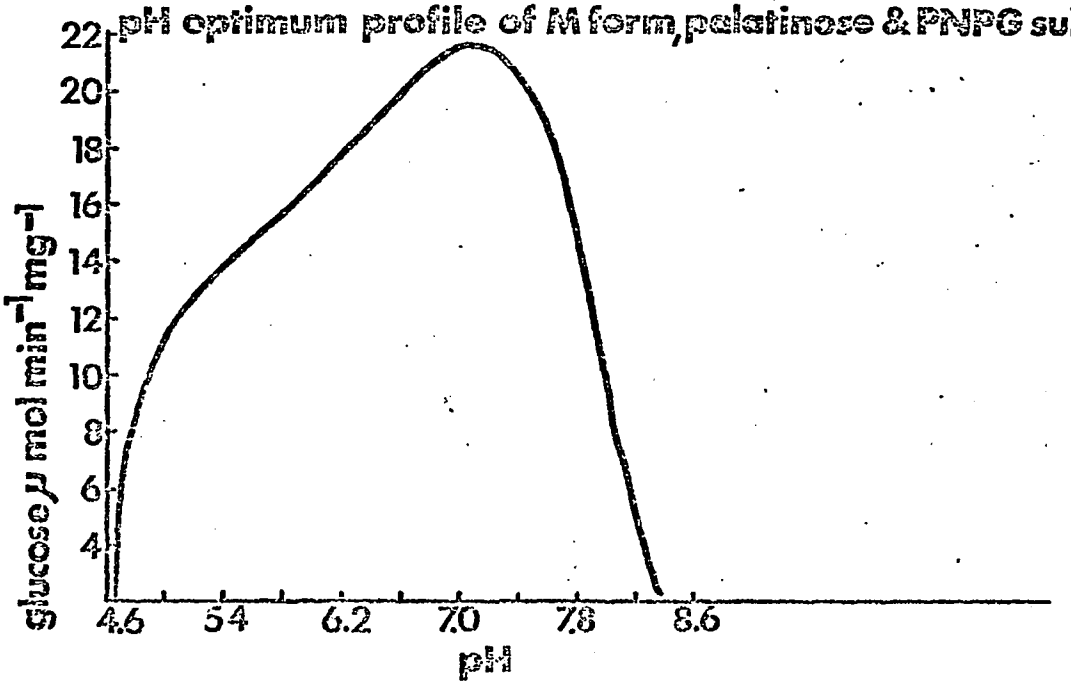
Fig. 34
pH optimum profile of α form of isomaltase with PNPG as substrate and M form of isomaltase with palatinose and PNPG as substrates.

FIG.34

pH optimum profile of α form, PNPG substrate



pH optimum profile of M form, palatinose & PNPG substrates



dependent and shifted towards loss of the phenolic proton at higher pH values.

The pH optimum profile of the enzyme with PNPG was generated by terminating the reaction at each pH with an excess of 1M Na_2CO_3, raising the pH to 10.5 and measuring the absorbance of phenoxide ion at 400nm (Fig. 34). The pH optimum profile of the M form of the enzyme with palatinose as substrate was determined with the glucostat reagent at pH 7.0. Both the α and the M form of the enzyme possess a pH optimum of 7.0-7.2 (Fig. 34). The M form of the enzyme demonstrates a broader pH optimum profile than does the α form. The M form of the enzyme demonstrates detectable activity at pH 7.8 while the α form is inactive at that pH.

SDS gel electrophoresis

The M form of isomaltase migrated nearly coincident with bovine serum albumin upon SDS gel electrophoresis and showed a minor trace impurity. There appeared no evidence of low molecular weight peptides with increasing time of incubation in the presence or absence of PMSF proving the absence of proteolytic contamination. The apparent molecular weight of the M form is approximately 67,000 D (Fig. 35) as determined by a plot of log molecular weight vs. relative mobility (Fig. 37).

Heat denaturation studies

For the homogeneous α and M form of the enzyme a plot of the logarithm of percent remaining activity as a function of time of exposure to the temperature (45°C) showed monophasic heat inactivation profiles indicative of a single enzyme species (Fig. 36). The first order decay constant, k, (slope of the line for 3 determinations) for the M form was $-0.076min^{-1}$, $-0.097min^{-1}$ and $-0.090min^{-1}$ the average

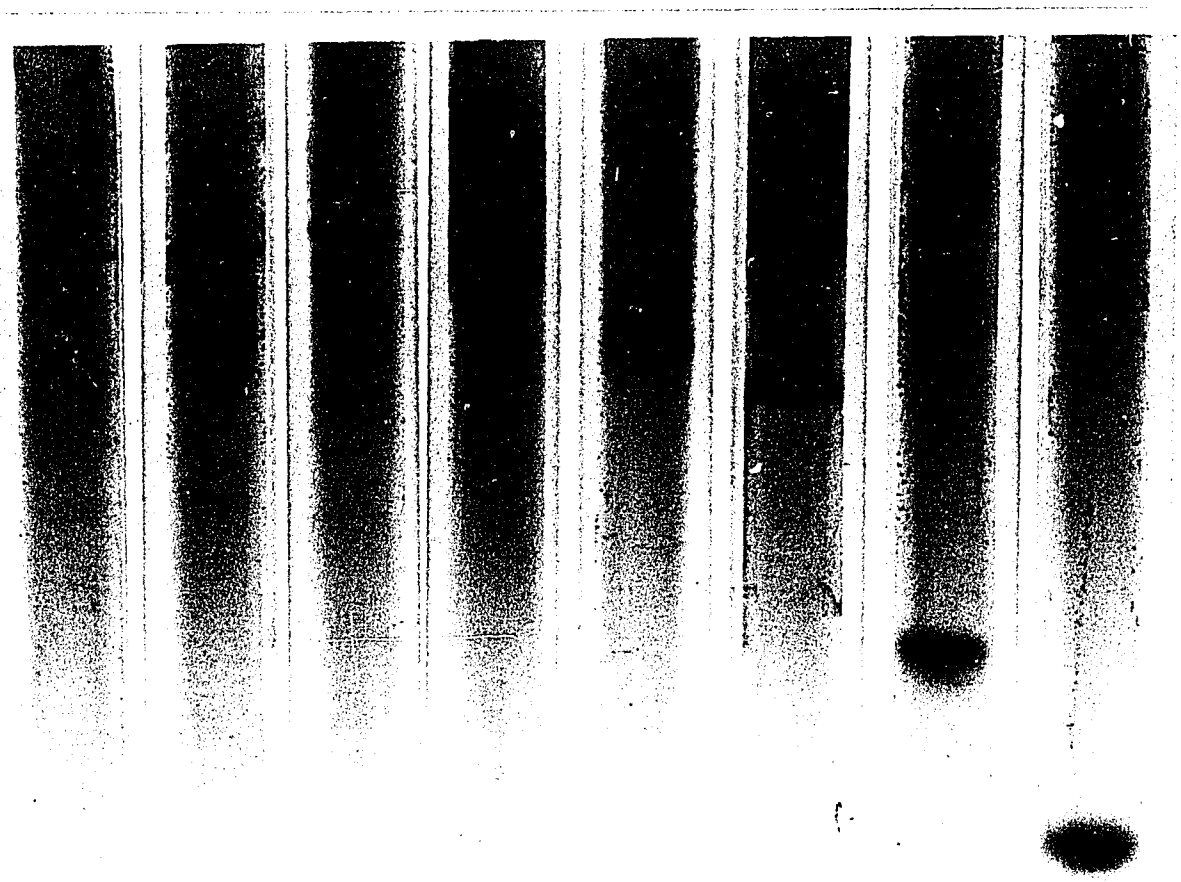


Figure 35
SDS gel electrophoresis of standards and purified M form of isomaltase
Lane 1 & 2 M form of isomaltase
Lane 3 BSA 68,000 D
Lane 4 Catalase 60,000 D
Lane 5 Ovalbumin 43,000 D
Lane 6 Aldolase 40,000 D
Lane 7 Chymotrypsinogen 25,000 D
Lane 8 Ribonuclease 13,700

Fig. 36
Thermal inactivation profile of isomaltase at 45°C. Details in text.

FIG.36
THERMAL INACTIVATION OF ISOMALTASE

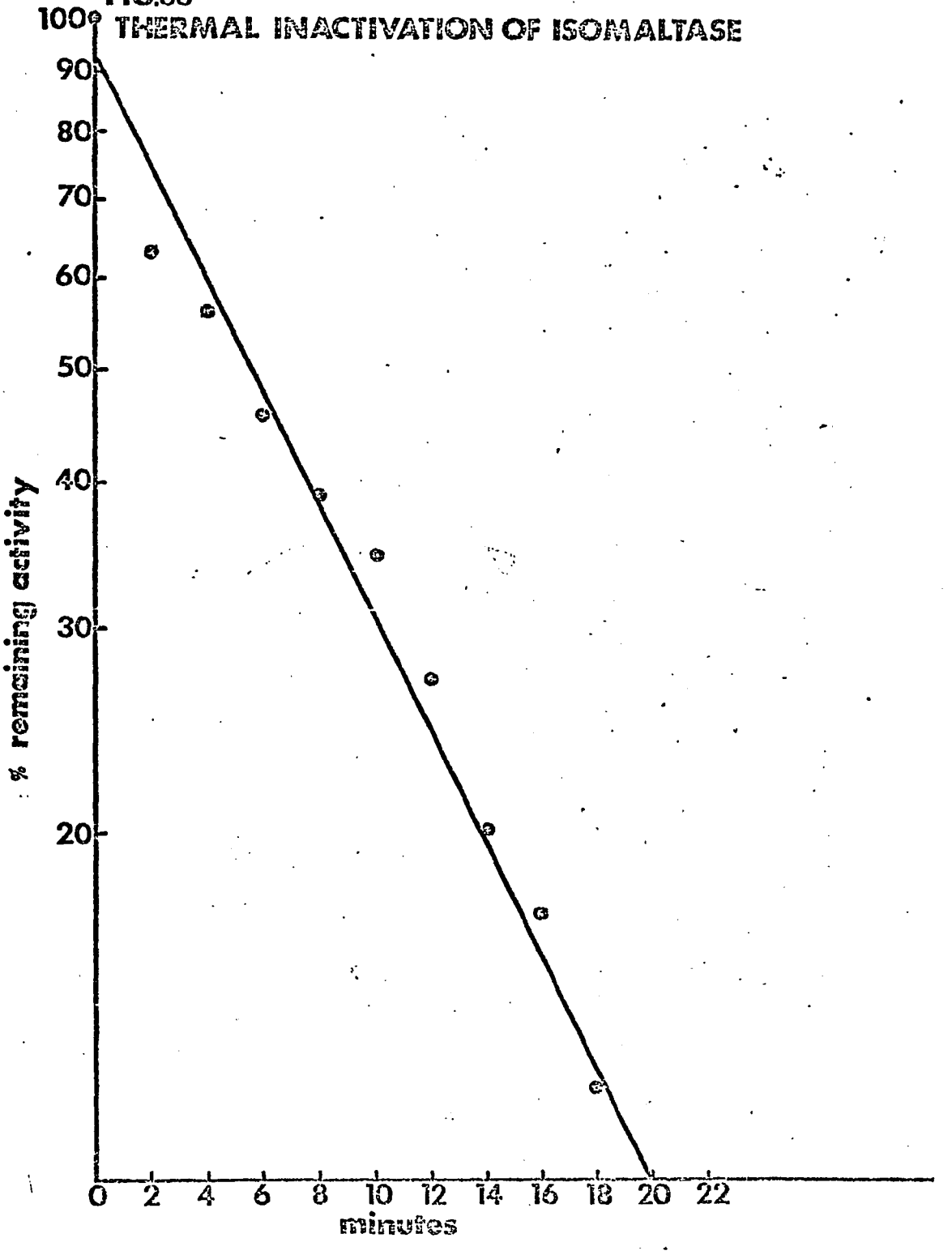
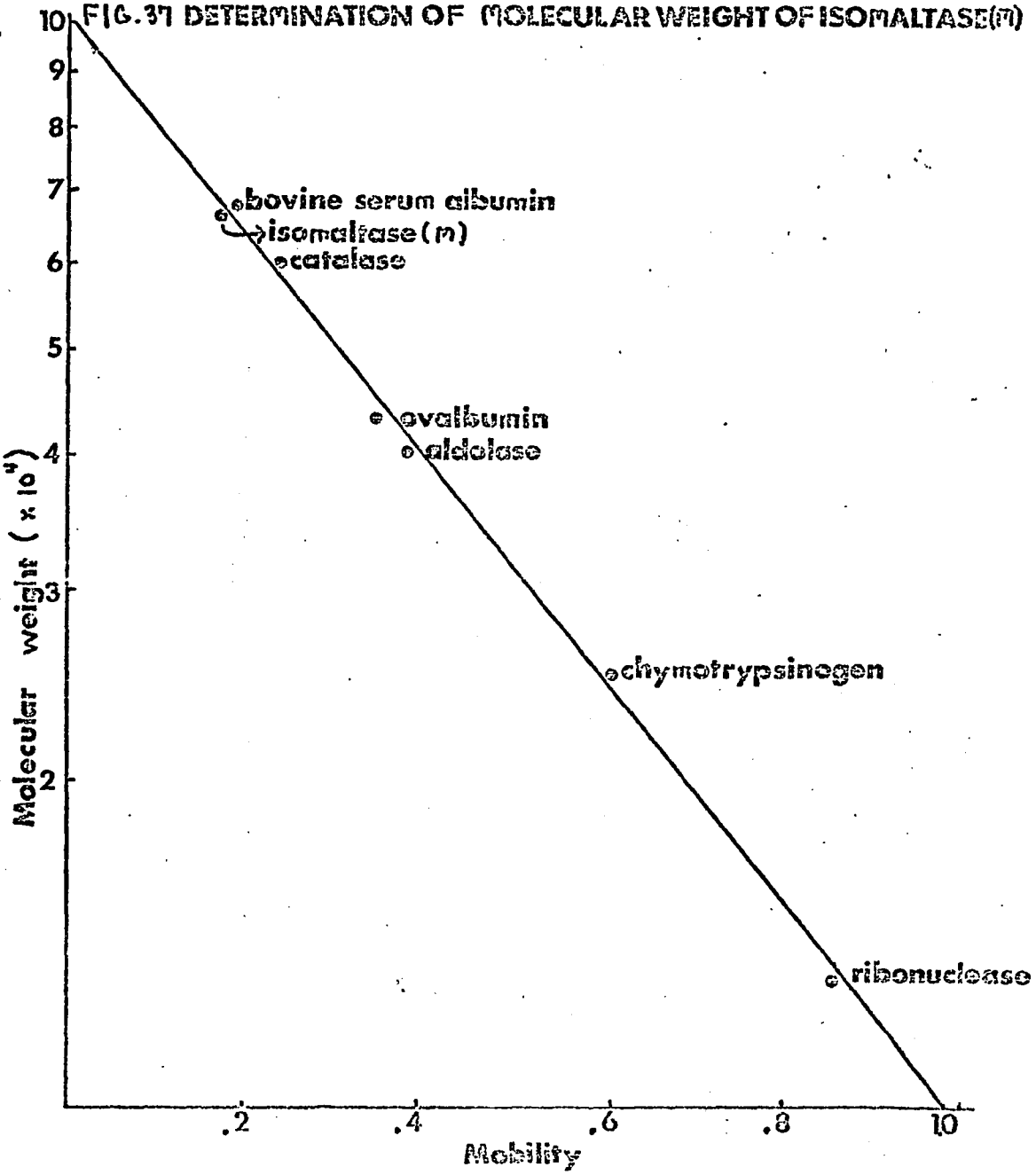


Fig. 37
Determination of apparent molecular weight of M form of isomaltase. Plot of log molecular weight vs. relative mobility for various marker proteins and for M form of isomaltase.

FIG. 37 DETERMINATION OF MOLECULAR WEIGHT OF ISOMALTASE(m)



being. .090min^{-1} ± .021. The first order decay constant, k, for 3 determinations for the α form was -0.092min^{-1}, -0.111min^{-1} and -0.097 min^{-1} the average being -0.100min^{-1} ± .019. Within the experimental error no differences in thermolability between the α and M form could be detected.

Concluding remarks

The properties of the maltose induced form of the enzyme (M) as compared to the α-methyl-D-glucoside induced form of isomaltase are summarized in Table 7. Differences are apparent in amino acid composition, pH optimum profiles, isoelectric points, and kinetic constants with PNPG as substrate. The M form of isomaltase has half as much glycine and 3 times the proline of the α form. Such differences could not be the result of either an in vivo or in vitro proteolysis. This notion is supported by the fact that each of the two major forms of isomaltase when added to a crude extract of the other shows no interconversion over time. This thesis provides evidence that each of the differentially inducible forms of isomaltase (M and α) and probably all four forms are coded for by distinct structural genes. In addition it is highly probable that there exist multiple structural genes coding for isozymes of maltase as postulated by Eaton and Zimmermann (7). Dimond and Loomis (43) describe two β-glucosidase isozymes in developing cells of Dictyostelium discoideum. The isozymes were not characterized with regard to the specificity of the susceptible glucosidic linkage (β 1 → 2/ β 1 → 4 etc.)

Maltase and isomaltase from Saccharomyces cerevisiae are both α-glucosidases but are not considered to be isozymes on the basis of the differences in the specificity of the disaccharide linkage hydrolyzed. On the basis of this work, it is suggested that isozymes of

Table 7
Comparison of properties of M and α form

	M form	α form
pH optimum	7.0 - 7.2	7.0 - 7.2
pH range of activity	4.6 - 8.3	4.6 - 7.7
Kinetic constants (Km)		
PNPG	8.54×10^{-4} M	3.2×10^{-4} M
Isomaltose	2.22×10^{-2} M	N.D.[a]
Palatinose	2.25×10^{-2} M	N.D.
Palatinose (2% glycerol)	6.90×10^{-2} M	N.D.
Sucrose	5.68×10^{-2} M	N.D.
α-methyl-D-glucoside	2.02×10^{-2} M	N.D.
Kinetic constants (Vmax)		
PNPG	3.18×10^{4} nmolmin^{-1}mg^{-1}	3.03×10^{4} nmolmin^{-1}mg^{-1}
Isomaltose	12.9 umolmin^{-1}mg^{-1}	N.D.
Palatinose	9.16umolmin^{-1}mg^{-1}	N.D.
Sucrose	2.30umolmin^{-1}mg^{-1}	N.D.
α-methyl-D-glucoside	8.42umolmin^{-1}mg^{-1}	N.D.
First order decay constant	$-0.090 \pm .021$min^{-1}	$-0.100 \pm .019$min^{-1}
Amino acid analysis	glycine 6 empirical residues proline 3 empirical residues	glycine 11 empirical residues proline 1 empirical residue
Molecular weight	67,000 D	*
Isoelectric pI	4.86	5.00

a. N.D. not determined

* On the basis of identical elution volumes of the α and M form of isomaltase on G-100 chromatography the α form of isomaltase is reported to have a molecular weight of 67,000 D

glucosidases be defined as enzymes that hydrolyze the same specific glycosidic linkage ($\alpha 1 \rightarrow 6$, $\alpha 1 \rightarrow 4$ etc). By this definition the glucosidase isozymes described by Dimond and Loomis do not belong under the classification of isozymes until the specific glycosidic linkage hydrolyzed is ascertained.

The primary practical consequences of my work will be the use of isoelectric focusing and activity staining to identify changes in the isoelectric point of glycosidases in disease processes mentioned in the introduction of this thesis and to serve as a guide to the development of a successful therapy regime.

The physiological significance of the occurence of multiple glucosidases remains unknown. It is possible that random gene duplication and modification has occurred over time to enable Saccharomyces cerevisiae to survive when confronted with variable sugar constituents in its environment. It is equally possible that an important biological function of cells is being mediated by genetic expression and repression of genes coding for glycosidases.

An enormous amount of work is being directed towards identifying elements of the cell division cycle modifying the expression of lectin receptors. In light of findings that differences in lectin mediated agglutinability between tumor cells and normal cells is a result of carbohydrate expression on the cell surface, it is exciting and not entirely speculative to suggest the possibility that the expression of multiple glycosidases might be involved in an enzyme mediated dislocation of trans membrane controlling mechanisms resulting in initiation of cell division!

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