1954

Purification and characterization of fungal carbohydrases

Edgar Erwin Stinson
Iowa State College

NOTE TO USERS

PURIFICATION AND CHARACTERIZATION OF FUNGAL CARBOHYDRASES

by

Edgar Erwin Stinson

A Dissertation Submitted to the
Graduate Faculty in Partial Fulfillment of
The Requirements for the Degree of
DOCTOR OF PHILOSOPHY

Major Subject: Biochemistry

Approved:

Signature was redacted for privacy.

In Charge of Major Work

Signature was redacted for privacy.

Head of Major Department

Signature was redacted for privacy.

Dean of Graduate College

Iowa State College
1954

ACKNOWLEDGEMENT

The author wishes to express his deepest appreciation to Dr. L. A. Underkofler for his suggestion of the research subject and for his guidance throughout the course of this work.

The author also desires to thank Miss Ampero Vicenti, who did much of the analytical work.

TABLE OF CONTENTS

TABLE OF CONTENTS (Continued)

TABLE OF CONTENTS (Continued)

TABLE OF CONTENTS (Continued)

TABLE OF CONTENTS (Continued)

I. INTRODUCTION

Ethyl alcohol, or ethanol, is one of the basic chemicals of modern commerce. It is produced in tremendous quantities for such purposes as the preparation of other organic compounds, the manufacture of smokeless powder, and for use as an organic solvent. The importance of this material stretches back even to antiquity, when it was valued as a beverage.

The two methods of preparing ethyl alcohol on a commercial scale are hydration of ethylene and fermentation of carbohydrate materials. The process involving the hydration of ethylene is becoming more widely used as the source of ethanol for industrial purposes, but the process involving fermentation is still used for producing alcohol for industrial and beverage purposes.

At least several ages of antiquity knew of the use of carbohydrate-containing materials for preparing alcoholic beverages. The ancient peoples were undoubtedly familiar with the alcoholic fermentation which occurred spontaneously with several fruits (particularly the grape) if the fruit or its juice were stored with limited access to air. The use of starchy materials as carbohydrate substrates for the fermentation, however, required a more complex technology. The yeast organism responsible for the alcohol fermentation assimilates sugars and is unable to utilize any type of starch. However, early Egyptian tomb drawings indicate that the Egyptians achieved the saccharification necessary by the crude method of allowing a partially baked loaf of ground wheat and barley to ferment. The barley, under these conditions, liberated enzyme mixtures capable of forming fermentable sugars from the starch.

In Europe and the United States malt has traditionally been used for saccharification of starches for grain fermentation. Malt, made by kilning partially germinated barley, contains enzymes which act upon starch to yield sugars such as maltose as well as the nonfermentable dextrins. These nonfermentable dextrins are materials which are resistant to further hydrolysis and are fermented with difficulty, if at all.

In Eastern Asia, particularly Japan and China, an alternative method of saccharifying starches has been developed. This method involves the use of amylolytic molds which have the ability to convert starches into fermentable sugars. This method was introduced into the Western world largely through the pioneering effort and skill of Mr. J. Takamine, the founder of the company that bears his name.

Recent developments in the alcohol fermentation industry have been to utilize the amylolytic mold preparations for preparation of neutral spirits, while retaining the malting process for the products in which the public has learned to expect the incidental flavors and aromas imparted by the malt. The mold preparations have several advantages when compared with malt.

Grain treated with mold preparations produces a higher yield of alcohol upon fermentation than does grain treated with malt.

The mold may be grown on very cheap, often waste, materials. Bran from the milling of grains is an excellent substrate for mold growth and the dry material after mold growth is known as mold bran. Stillage, a waste material from alcohol distillation, has been used for cultivating molds, as have other materials. Malt is made from barley, the supply of which is limited for any given year and which is difficult to expand when

necessary upon short notice. Also, barley is quite expensive when compared to the waste products mentioned before which have little or no economic value.

Other factors enter in, such as equipment investment, speed of action, ease of handling, the conservative nature of industry, and off-flavors and odors, but the general consensus of opinion is that the use of mold preparations for producing neutral spirits is the preferable method.

The production of ethyl alcohol from grain is highly competitive and it is of great interest to the industry to determine the specific factors responsible for variations in alcohol yields. Despite the fact that the yields are generally higher with molds than with malt, there is still considerable variation from batch to batch despite rigid control of physical conditions. Considerable attention has been focused on the possibility that the variation in alcohol yield may be due to the differences in enzyme concentrations in the saccharifying agent, although the results of work based on this hypothesis have failed to demonstrate any definite correlation between alcohol yield and any enzyme concentration.

One phenomenon observed with the use of malt which may explain the incomplete conversion of carbohydrate to alcohol in the fermentation is the accumulation of limit dextrins. These dextrins are short chain molecules that are resistant to further hydrolysis and thus are not fermented. The existence of an enzyme named "limit dextrinase" has been postulated which is able to hydrolyze the alpha-1,6-glucosidic bonds known to exist in starch at the points of branching. After hydrolysis of this branched structure at the end of the limit dextrin molecule, the maltase and alpha-amylase could act on the residue to produce fermentable sugars and thus

increase the final yield of alcohol.

An alternative explanation for the increased yield of alcohol when Aspergillus niger filtrate is used for saccharification instead of malt would be the presence of an alpha-1,4-glucosidase, capable of acting upon short chain dextrins (not necessarily branched) which are attacked only slowly by the enzymes alpha-amylase or maltase.

This present investigation was conducted to carry out work on the purification and characterization of the amylolytic enzyme system of Aspergillus niger NRRL 330, a strain of mold which has been found to result in high yields of alcohol, with particular emphasis upon the enzymes known as maltase and limit dextrinase.

The first work of this investigation was an attempt to purify for further study an appreciable quantity of limit dextrinase by a procedure already developed for the isolation of the limit dextrinase of Aspergillus oryzae. However, our starting material, a filtrate from submerged culture of Aspergillus niger, failed to give comparable results. The results did indicate possible correlation between the presence of limit dextrinase and maltase, lending support to the hypothesis of limit dextrinase being an alpha-1,4-glucosidase capable of attacking short chain molecules. The possibility was not excluded, however, that limit dextrinase was an alpha-1,4-glucosidase which was also capable of splitting the alpha-1,6-glucosidic linkages.

With this in mind, emphasis was placed on the isolation of this glucosidase, following the course of the purification by the maltase analysis rather than the cumbersome limit dextrinase analysis.

II. REVIEW OF LITERATURE

A. Structure and Composition of Starch

Recent literature contains several excellent reviews of the current concepts of starch structure and composition. The substances known as starches are composed of two main components called amylose and amylopectin. Amylose molecules are composed of a large number of repeating glucose units in helical spirals connected by alpha-1,4-glucosidic linkages, while amylopectin is considered to be an irregular network of glucose units connected largely by alpha-1,4-glucosidic linkages but with a large number of branched positions at irregular intervals. The branched positions are points at which a glucose unit in a chain is attached to another chain through an alpha-1,6-glucosidic link.

B. Alpha-amylases

Alpha-amylases are classified as dextrinizing enzymes due to their hydrolytic action in breaking the alpha-1,4-glucosidic linkage in starch to create smaller dextrin units. These links are attacked apparently at random throughout the molecule of amylose or amylopectin, with the possible exception of the two or three alpha-1,4-glucosidic linkages at either end of the amylose chain. The action of this enzyme causes a rapid decrease in the average chain length and complexity of the molecule, and thus causes a decrease in the viscosity of the solution with little increase in detectable reducing sugar. The dextrin fragments formed from amylose are hydrolyzed to still smaller fragments which impart no color to an iodine

solution. A second, much slower, action of alpha-amylase then occurs upon the fragments to produce a mixture of maltose and maltotriose. The maltotriose is eventually hydrolyzed to form glucose and maltose.

Alpha-amylases are of frequent occurrence in nature. They are present in many animal organs and fluids, higher plants, and particularly germinated cereals such as wheat or barley, and many bacteria and molds such as Aspergillus oryzae and Aspergillus niger. A number of crystalline alpha amylases have been prepared recently, among them human and swine pancreatic alpha-amylases (63), Bacillus subtilis amylase (64), malt alpha-amylase (88), and Aspergillus oryzae alpha-amylase (21), (103).

An excellent summary of the literature concerning alpha-amylase is already available (9).

C. Beta-amylases

The beta amylases attack only the second alpha-1,4-glucosidic linkage from the non-reducing end of the carbohydrate molecule, liberating beta-maltose and the residue of the carbohydrate molecule (67), (71), (86). The conversion from the alpha-glucosidic link occurring in starch into the beta configuration of the free hydroxyl group at the reducing end of the beta-maltose molecule apparently occurs simultaneously with the hydrolysis (56). This suggests that the hydrolysis proceeds by means of a Walden inversion (95). Maltose is split from the molecule of amylopectin about 20 times as rapidly as from the molecule of amylose (37), although amylose is hydrolyzed virtually completely to such simple saccharides as maltose, and traces of higher saccharides (68), (69), while a dextrin

unreactive to beta-amylase is an end-product of the hydrolysis of amylopectin (36). Approximately 60 per cent of the amylopectin is ultimately converted to maltose (28).

The beta-amylases that have been described in the literature thus far are principally of plant origin (12) and will not be treated further in this discussion.

D. Alpha-D-glucosidases

The types of enzymes discussed thus far, the alpha- and beta-amylases, have in common the ability to attack the oxygen of the alpha-1,4-glucosidic link of amylose, amylopectin, or other comparatively large molecules. There exist, however, a large group of enzymes which have the ability of hydrolyzing the linkages in molecules of intermediate size which are classified as alpha-D-glucosidases because of their specificity of action upon compounds possessing this particular linkage. The alpha- and beta-amylases, as mentioned before, may in some instances be able to act on these smaller compounds but in general have a much more rapid action upon the larger molecules.

The various alpha-glucosidases are capable of action upon a number of substrates, the specificity of the enzyme depending upon the species of origin and even the environmental conditions of the organism during its growth and development of the enzyme.

Gottschalk (27) has presented an extensive review of these enzymes.

1. Action

The term "alpha-D-glucosidase" is a general term for those enzymes which are capable of hydrolyzing the alpha-D-glucosidic link, both in molecules that consist solely of sugar residues (holosides or oligosaccharides), and in molecules that consist of glucose residues connected by alpha-links to non-sugar residues (alpha-glucosides). The main specificity is directed toward the alpha-D-glucopyranosyl radical, so that activity is generally incompatible with any alteration of the configuration on carbon atoms 1, 2, 3 and 4 of the glucose portion, or with a change from the glucopyranose to glucofuranose structure. In at least several cases substitution at carbon atoms 5 and 6 will not alter the enzyme action, but in other cases it has a marked effect (27).

Although the steric requirements of an alpha-glucosidase for the glycone are, as indicated by the name itself, quite specific, the enzyme will often tolerate alteration in the structure of the aglycon. Hence many enzymes which operate at an optimal rate when the substituent is a glucose unit attached through the fourth carbon (that is, when the molecule is the disaccharide maltose), will still act on the molecule, although perhaps at altered speed, with a different substituent. Melizatose, which is a trisaccharide, is acted upon by the same enzyme which acts upon sucrose, a disaccharide. In many cases it is not even essential that the aglycone be a carbohydrate residue. The crystalline maltase isolated from malt, for example, displays activity toward both the methyl and phenyl alpha-glucosides.

There are numerous references in the literature concerning the diversity of substrates that may be acted upon by enzymes, but unless the enzyme is homogenous the possibility cannot be discounted that the diverse activity is due to several enzymes. An example of a controversy of this nature is the possible identity of fungal sucrase and maltase.

By definition, a sucrase is an enzyme that acts upon sucrose to yield glucose and fructose. The enzyme may act primarily upon either the fructofuranoside moiety or upon the glucopyranoside moiety. Yeast saccharase is an enzyme possessing the former type of action, for it requires an unsubstituted beta-D-fructofuranosyl residue before it will act (57), (10). The enzyme is inactive toward melezitose and is definitely not an alpha-glucosidase (58). However, a glucosaccharase exists in Aspergillus oryzae (55), (102), which evidently combines with the glucose portion of the sucrose molecule and results in the hydrolysis of a molecule of sucrose to yield a molecule of glucose and fructose.

Weidenhagen (106) has hypothesized that all alpha-glucosidic linkages are split by enzymes that are essentially alpha-D-glucosidases. He observed that yeast maltase, purified by the method of Willstatter and Bamann (112) and freed from beta-fructofuranosidase activity, is able to hydrolyze maltose, sucrose, and melezitose. Regardless of whether Willstatter's method for the purification of yeast maltase results in a homogenetically pure enzyme, it certainly cannot be claimed that such a wide adaptability is typical of all alpha-D-glucosidases. For example, maltase preparations from sources such as Escherichia coli (38) are without effect upon sucrose. As the alpha-D-glucosidases from different sources

are distinct chemical entities, as shown by quantitative analysis for the various elements as well as their different isoelectric points, solubilities, temperature optima, it is impossible to make a generalization for the entire class of enzymes. To describe an enzyme adequately, then, it is necessary to specify the source and the method of preparing the enzyme, the criteria of purity that have been observed, and activities that the specific enzyme preparation has been found to possess. However, all maltases, regardless of their origin or preparation (27), probably maintain contact with the glucon moiety of the maltose molecule by forming an enzyme-substrate complex with the oxygen atoms of the hydroxyl groups on carbon atoms two, four, and six, as well as the glucosidic oxygen.

2. Occurrence

The alpha glucosidases are among the most widely occurring enzymes in nature, being very frequently found in both plants and animals. Enzymes designated as maltases, for example, have been demonstrated in Schizomycetes such as Escherichia coli (7) and Clostridium acetobutylicum (34), fungi such as Aspergillus oryzae (14) and Aspergillus niger (44), and the Saccharomyces (60) as well as numerous higher plants.

In animals, alpha-D-glucosidases, and particularly the maltases, occur in the digestive tract in the pancreas and intestine, and in the blood serum. Numerous examples of the occurrence of alpha-D-glucosidases are given by Gottschalk (27) in his review.

3. Specific examples of alpha-D-glucosidases

The term alpha-1,4-glucosidase is actually a very general term which could be used to designate any enzyme capable of hydrolyzing an alpha-1,4-glucosidic link. The size of the substrate molecule could vary from maltose to the original starch molecules. However, we propose to use the term alpha-1,4-glucosidase to designate those enzymes which are primarily active upon the shorter molecules. We shall first consider the groups of enzymes which have historically been designated as maltases.

(a) Maltase: The first mention of the activity of an alpha-D-glucosidase in the literature was that made by Dubrunfaut (19), who observed that a distinct entity was formed as the result of the interaction of starch and kiln dried malt. Dubrunfaut did not characterize his product further and it remained for a later worker (71) to characterize this compound as a disaccharide and give it the name "maltose". Musculus and von Mering (66) found that glucose, maltase, and dextrins are produced by the action upon potato starch of such crude amylolytic preparations as malt diastase, saliva, and pancreatic extract. The first workers to observe the enzymic hydrolysis of maltose itself were Brown and Heron (11) who used preparations of pancreatic extract and intestinal cell wall. The hydrolytic cleavage of maltose by saliva and malt diastase was observed independently by von Mering (62), who called the active ingredient responsible for the cleavage "glucase". The presence of materials in Aspergillus niger filtrate and koji (45) with similar activity upon maltose was observed by later workers.

That the hydrolytic action of Aspergillus niger upon maltase was due to the action of an enzyme and not to the action of the living cells of the mold was proved by stopping the growth, and presumably the metabolic activity, by adding chloroform. The hydrolytic activity was followed by observing the optical rotation (8).

The literature contains a number of methods which have been used at different times to determine the concentration of maltase or alpha-glucosidase activity.

The change in optical rotation resulting from the disappearance of maltose and the appearance of glucose has been used both as a qualitative and quantitative method of analysis for maltase activity. It was the observation of the change in optical activity of a solution of starch and malt extract that led Dubrunfaut to discover the sugar that he called "le glucose de malt", and which we now call maltose.

The specific rotation of maltose is + 137°, while that of glucose is + 52.5°. Thus, the rotatory power of a solution of maltose will progressively diminish if a maltase is present. Quantitative methods for the determination of maltase by polarimetric methods are given by Waksman (105), and Gottschalk (27), as well as in other sources.

Another method commonly used for the quantitative determination of maltase activity is that of measuring the increase in reducing power. When a molecule of maltose is hydrolyzed, two molecules of glucose are produced, and the total reducing value is increased. The reducing value of the resulting solution may be analyzed by using either a buffered cupric salt solution (92), (102), or a phosphomolybdate solution (54).

Other methods which have not been exploited utilize a photobacterium which phosphoresces in the presence of maltose but not starch or dextrins (106) or employ organisms that selectively utilize various sugars (3). A review of the methods for the determination of malt amylases has been published recently (93).

Both filter paper chromatography and the filter paper electrophoresis technique (25), indicate a greater complexity of the enzyme systems of Aspergillus oryzae than has been suspected. A spot of the filtrate of an Aspergillus oryzae culture was placed upon filter paper and subjected to a voltage differential of six volts per centimeter for six hours. At the end of the time the protein spots were stained with bromthymol blue dye. The spots possessing enzyme activity were determined from a companion strip of filter paper. This strip was either cut into smaller strips vertical to the direction of the current flow, and any active enzyme eluted and determined, or the entire strip was sprayed with a chromogenic substrate, and after allowing the enzyme to act, the spots where enzyme activity had occurred were determined by suitable means. The results indicated that crude Aspergillus oryzae filtrate contained at least two components with protease activity, etc. A later paper by the same group (43) disclosed the presence of at least eight components in Aspergillus oryzae filtrate capable of breaking beta-glucosidic linkages in various substrates, which confirmed the general nature of the previous work. The components were not only active in splitting simple glucosides but were also capable of depolymerizing sodium carboxymethylcellulose. The experimental evidence admitted neither the postulate for a single beta-glucosidase nor of a specific enzyme for each substrate. The postulate of an enzyme specific

for polymeric beta-glucosidoses which is inactive upon other beta-glucosidoses was inadmissible from this evidence. Presumably, the enzyme fraction called "maltase" has a similar multiple nature.

An investigation of the relative quantities of enzyme fractions called alpha-amylase, beta-amylase, and maltase in wheat bran cultures of Aspergillus oryzae, Aspergillus awamori, Rhizopus javanicus, and an albino variant of Aspergillus awamori disclosed (50) that the diastase of Aspergillus oryzae was composed chiefly of alpha- and beta-amylase, those of Aspergillus awamori and Rhizopus javanicus chiefly beta-amylase and maltase, and the white variety of Aspergillus awamori contained large quantities of all three enzymes. As most workers in the field feel that molds do not produce beta-amylase (12), it is probable that these workers used a definition of beta-amylase activity other than the commonly accepted one.

It was significant that the alpha-amylase of Aspergillus oryzae, Rhizopus javanicus, and a white Aspergillus could be separated from beta-amylase and maltase, but that beta-amylase and maltase could not be separated because, according to these workers, their physical properties were identical.

Beta-amylase obtained from Aspergillus awamori (50) displayed practically no maltase activity. However, it produced large amounts of glucose in addition to maltose (a 4:1 ratio) by its action upon soluble starch. These workers considered this enzyme to be a distinctly new enzyme which they called gamma-amylase.

The characteristics of the diastase of Aspergillus awamori led the authors to the hypothesis that the enzyme carriers of alpha- and

beta-amylase could be interchanged.

In ordinary mold: alpha-amylase = coenzyme alpha + carrier A.
beta-amylase = coenzyme beta + carrier B.

In Aspergillus awamori: alpha amylase = coenzyme alpha + carrier B or A.
beta-amylase = coenzyme beta + carrier B or A.

An article in the Russian literature (51) concerned a series of tests conducted on the composition and activity of the amylolytic activity of a number of Aspergillus species including Aspergillus niger and Aspergillus oryzae. The results indicated wide variation in the composition and activity of the enzymes. The amylase type was said to characterize Aspergillus oryzae while the phosphorylase type characterizes Aspergillus niger.

The results of a number of workers have indicated that the production of enzymes by molds is definitely influenced by the substrate as well as by the variety of mold used. For example, Goodman (26) reported that amylase production by Aspergillus flavus was dependent upon the substrate used.

Workers at the Northern Regional Research Laboratory, Peoria, Illinois have investigated the factors influencing the production of alpha-amylase and maltase by certain Aspergilli (95). Low yields of alpha-amylase of Aspergillus niger NRRL 337 in media which have a low pH at the termination of the culture appeared to be due to inactivation of alpha-amylase at low pH. This enzyme became increasingly instable at pH values below 4.5, but was stable over the pH range from 4.5 to 7.25. Maltase from the same

source was stable over a range from 4.2 to 7.25, the highest pH tested. The enzymes were held at 30°C. for 18 hours.

The use of different media affected the yields of alpha-amylase and maltase, but as various media produced varying amounts of acid when metabolized, it was difficult to separate the effects of the media itself from the attendant effects of the acid produced. Thus an increase in the carbon source lowered the pH while an increase in the nitrogen source raised the pH. An attempt to determine the compensation necessary from this source was made by adjusting the pH of the media to various levels prior to inoculation. Alpha-amylase production was shown to be quite sensitive to changes in the pH level of the medium, while maltase production was substantially the same between pH 4.25 and 7.25. Increased use of thin stillage solids (a nitrogen source) raised the terminal pH with an attendant increase in alpha-amylase. Increased use of corn meal (a carbon source) lowered the pH, and thus the yield of alpha-amylase. However, the increased use of corn meal increased the yield of maltase, and this effect would seem to be independent of the change in acidity.

The use of calcium carbonate, previously incorporated into the media to control the pH level (59), was found to be detrimental to the highest yields of maltase. As calcium chloride does not have this deleterious effect, Tsuchiya, Corman and Koepsell (99) recommended that calcium chloride be used to replace the calcium carbonate and that the pH level of the media be controlled by variation in the concentration of thin stillage. The influence of the variation in media upon the yield was the most pronounced with Aspergillus niger NRRL 330 and Aspergillus oryzae NRRL 458, but less pronounced with Aspergillus niger NRRL 337. The optimum yield of

maltase reported was 21 units per milliliter which was given by 5 per cent thin stillage solids and 5 per cent ground corn. The organism was Aspergillus niger NRRL 330.

A mold designated as Aspergillus niger PRL 558 was used by Shu and Blackwood (91) to study the effect of carbon and nitrogen sources upon the production of amylolytic enzymes by submerged culture. Variations in the specific type of carbon source affected the yield of alpha-amylase to a marked degree, while the yield of maltase was affected to a smaller extent, and the yield of limit dextrinase was affected least of all. Variations in the source of nitrogen were quite influential in determining the amounts of the three enzymes produced. Highest yields of the enzymes were obtained with hydrolyzed casein as the nitrogen source. However, still higher yields of maltase and amylase were obtained by adding inorganic nitrogen compounds such as ammonium nitrate and sodium nitrate as supplementary nitrogen.

In a later paper (90), it was found that proteins or amino acids were not necessary. Almost any nitrogen source which was a potential alkali donor served as well. Thus ammonium acetate was a suitable nitrogen source.

A procedure for the purification of fungal maltase was developed in 1951 by Roy and Underkofler (85). These workers purified maltase of Aspergillus niger grown on a substrate of yellow corn, Difco yeast extract, $CaCO_3$, and tap water. The procedure involved adsorption upon freshly precipitated calcium oxalate and Fuller's earth. No nitrogen determinations were reported so that no idea of the specific enzyme activity could be obtained. The final yield was estimated as less than 10 per

cent of the original enzyme activity present.

Miller (65) investigated the carbohydrase systems of 12 molds in regard to their production of alpha-amylase, maltase, and limit dextrinase. The alpha-amylase activity among the different molds ranged from less than 0.1 unit per milliliter to 37.7 units per milliliter. The activity of maltase varied from 0.3 to 9.6 units per milliliter, and the limit dextrinase activity varied from 0.8 to 10.8 units per milliliter. The highest maltase and limit dextrinase activities were found in Aspergillus niger NRRL 330. Miller found no correlation between alpha-amylase activity and the maltase or limit dextrinase activity. There was a slight correlation between maltase and limit dextrinase activity.

(b) Amylo-glucosidases: A great deal of attention has been directed recently to a group of enzymes for which the name "amylo-glucosidase" (47) has been suggested. This group of enzymes is postulated to attack molecules of varying sizes up to the starch molecules themselves, liberating glucose directly. In other words, this group of enzymes would be comprised of alpha-D-glucosidases capable of acting on the end alpha-1,4-glucosidic linkages of a molecule regardless of the size of the remainder of the molecule.

It has long been known that the starch-splitting enzyme system of Clostridium acetobutylicum is capable of converting starch almost quantitatively to glucose with neither starch nor limit dextrins occurring in the conversion products to any significant degree. Early workers (34) considered the hydrolysis of starch to be due to two distinct enzymes, an alpha-amylase capable of dextrinizing the starch and breaking it down into

maltase, and a "true" maltase capable of hydrolyzing maltase to form glucose. The maltase was not capable of action upon alpha-D-glucosides such as sucrose.

However, French and Knapp (22) separated the maltase fraction from any detectable trace of alpha-amylase activity by adsorbing the fraction possessing maltase activity upon starch granules from an acetone-water solution and examined its action upon oligosaccharides that would be expected as products of the action of alpha-amylase upon starch. A study of the products formed by its action upon maltase, amyloheptaose, whole starch, and limit dextrins indicated that the maltase of Clostridium acetobutylicum was an alpha-glucosidase capable of the hydrolytic removal of individual glucose units from the non-reducing terminus of a starch chain or starch oligosaccharide. The enzyme solution was also capable of hydrolyzing the link in isomaltose although at a slower rate. The apparent first order rate constant was 1.0×10^{-6} for isomaltose as compared to 6.7×10^{-5} for maltose. It was presumed, although the action of the enzyme upon isomaltose was quite slow, that the total conversion of isomaltose would have reached 100 per cent. No detectable hydrolysis was observed with alpha-methyl glucoside, or with dextran after a very slight initial hydrolysis.

Unfortunately, no criteria were given for the purity of the enzyme preparation obtained from the starch adsorption.

Hydrolysis of starch by either malt diastase or inorganic acids results in residues that are more resistant to further hydrolysis by either malt diastase or inorganic acid than is the original starch. However, fungal enzyme preparations were found capable of acting upon these

dextrins to liberate glucose units in a piecemeal fashion (49). It was stated that the enzyme responsible for the hydrolysis of the dextrins attacked only the terminal glucose units of the dextrins (48).

Bernfeld and Studer-Pecha (2) made an exhaustive study of the action of several crystalline alpha-amylases and found that the final limit of the hydrolysis of amylose by these enzymes was attained in a prolonged second phase of the reaction with complete conversion of amylose to 13 per cent glucose and 87 per cent maltose.

Workers at the Corn Products Refining Company (47) have proposed that all of these starch-splitting, glucose-producing enzymes be given the generic name of amylo-glucosidases, following the suggestion by Cori and Larner (15). These workers (47) reported the presence of an amylo-glucosidase in an enzyme preparation mutant of Aspergillus niger NRRL 330.

The extent of hydrolysis of corn amylose by an enzyme preparation of the Aspergillus niger was determined by the total reducing material formed, the glucose formed, and by the change in the wavelength for maximum light adsorption by the iodine-hydrolysate complex. The two types of action apparent were an endwise attack on some amylose molecules to produce relatively large percentages of glucose early in the reaction, and the random hydrolysis of the others. As the alpha-amylase is deactivated by acid to a greater degree than amylo-glucosidase, alpha-amylase activity was eliminated by lowering the pH level to 2.2. The enzyme amylo-glucosidase produced glucose at the same rate from equimolar solutions of amyloses of widely varying sizes. During the hydrolysis of large polymers such as corn amylose or amylopectin the adsorption spectra of the complex of iodine with the remainder of the molecule did not vary appreciably. Glucose was

the only sugar found in the hydrolysate by paper chromatography. The initial rate of the hydrolysis of amylopectin was greater than the initial rate of the hydrolysis of corn amylose, but after approximately 60 per cent hydrolysis of the amylopectin to glucose, the rate of amylopectin hydrolysis dropped sharply. The hydrolysis of the linear substrate, corn amylose, was found to be first order. The results of the study of the activity of amylo-glucosidase indicate the presence of an enzyme that will hydrolyze starch molecules from the end to liberate individual glucose units.

With linear molecules at least, the enzyme probably acts by a "single-chain" mechanism. That is, the enzyme will pick off successive glucose units on a molecule and complete the hydrolysis of the molecule before proceeding to the next. This would be similar to the action of beta-amylase proposed by Kerr, Cleveland and Katzbeck (47).

Other workers who obtained similar results with Aspergillus niger were Weill, Burch and Van Dyk (107). A relatively high yield of glucose was obtained from the hydrolysis of starch by Aspergillus niger (107). Glucose was the only reducing sugar present even in the early stages of the hydrolysis of starch. By comparison it was demonstrated that during deactivation the maltase and saccharogenic activities were denatured to the same extent. Fractionation of the enzyme preparation caused a slight change in the ratios of amyloclastic to saccharogenic and saccharogenic to maltase activities of the different fractions. However, glucose was still found to be the only reducing sugar formed, and as the changes in the ratios were not large, there was little positive evidence for the presence of more than one enzyme.

Corman and Langlykke (17) during an investigation upon the correlation between the ultimate alcohol yield upon fermentation and the enzyme activities of various molds found that the amylase system contained both an alpha-amylase fraction and a glucogenic fraction.

The fungal alpha-amylase converted starch into dextrins and maltose, whereas the glucogenic enzyme system was capable of converting maltose, dextrins, and even starch into glucose. The efficiency of the saccharification of the starch was more highly correlated with glucogenic activity than with alpha-amylase. High glucogenic activity caused rapid and almost quantitative formation of glucose from starch. The species examined were a number of organisms of the Aspergillus and Rhizopus groups including various strains of Aspergillus niger, Aspergillus oryzae, Rhizopus delemar, and others. Two Rhizopus species, Rhizopus delemar NRRL 1705 and Rhizopus sp. "Boulard" NRRL 1891 (the strain used in the Amylo process for alcohol production) were interesting in that they were still fairly efficient for the production of alcohol, but produced only traces of alpha-amylase. The culture filtrate from Rhizopus sp. "Boulard" NRRL 1891 contained intermediate glucogenic activity but almost no alpha-amylase activity. Glucose was produced fairly rapidly but there were no detectable traces of maltose. The blue starch-iodine color persisted for more than 24 hours, indicating that the dextrin fraction still contained starch that was essentially unaltered. After 24 hours the hydrolyzate contained 70 per cent glucose, 30 per cent dextrin and starch, and no detectable maltose. The filtrates of the Aspergillus cultures gave comparable results, although there were appreciable amounts of maltose found after the hydrolysis. Evidently the glucogenic enzyme system attacked the starch directly to produce glucose.

Corman and Langlykke suggested that the term "glucogenic activity" would probably be more accurate than "maltase activity", due to the fact that the glucogenic enzyme systems examined hydrolyzed higher glucose polymers as well as maltose.

These results were confirmed, at least in the case of Rhizopus delemar, by the subsequent investigations of Phillips and Caldwell (81), (82). These workers isolated a glucose-producing amylase which they called "gluc amylase".

Phillips and Caldwell (81) purified their glucose-forming amylase, "gluc amylase", from the mold Rhizopus delemar, which was free of all detectable traces of alpha-amylase activity. The purified gluc amylase possessed maltase as well as amylase activity. All attempts to fractionate the preparation into portions containing varying proportions of amylase activity and maltase activity were unsuccessful, and any treatment that caused a destruction or loss of maltase activity caused a proportional loss in amylase activity.

At relatively low temperatures of 5° to 10°, the gluc amylase was less sensitive than the alpha-amylase to exposure to relatively acid conditions. The traces of alpha-amylase were removed in this manner. Glucose was responsible for practically all of the reducing value of the hydrolysates formed from starch by gluc amylase until approximately 90 per cent of the possible glucose had been formed.

Gluc amylase apparently was capable of splitting glucose from the non-reducing end of the glucosidic chain (81) somewhat after the manner of the formation of maltose from its substrates through the action of beta-amylase. However, with gluc amylase there was no evidence of the formation of limit

dextrins from its action upon either the linear and branched fractions of corn starch, defatted waxy maize starch, glycogen, or residual beta dextrins. Neither the highly purified gluc amylase nor the accompanying alpha-amylase had any noticeable action upon the alpha or the beta Schardinger dextrins, dextran, or upon isomaltose. However, the gluc amylase must have either hydrolyzed the alpha-1,6-glucosidic linkages of the substrate or by-passed them in some manner, as shown by the extensive hydrolysis of substrates known to be branched. The failure to hydrolyze branched substrates completely (actually 92 per cent for glycogen, 89 per cent from residual beta dextrins, etc.), together with the inactivity shown toward dextran and isomaltose suggested that gluc amylase was inactive toward the alpha-1,6-D-glucosidic linkages and that the branching points were by-passed. Thus gluc amylase must be capable of attacking non-terminal alpha-1,4-glucosidic links.

The Michaelis constants of gluc amylase were found to be 6.6×10^{-3} M for maltose, 4.4×10^{-5} M for the linear substrate, and 4.1×10^{-7} M for the branched substrate. The values of the affinities (the reciprocal of the Michaelis constant, or 1/Ks) of gluc amylase for the three substrates were 150 for maltose, 22,000 for the linear substrate, and 2,400,000 for the branched substrate. The lower Michaelis constant for the branched substituent indicated that the attack upon the non-terminal linkages proceeded much less rapidly than the attack upon the terminal linkages.

The work of Kitahara and Kurushima (50) has been mentioned before in the "Maltase" section. These workers found evidence of an amylo-glucosidase in a filtrate of Aspergillus awamori which they referred to as gamma-amylase. This enzyme produced principally glucose (ratio of four

molecules of glucose to one of maltose) by its action upon soluble starch and thus should be classified as an amylo-glucosidase.

(c) Amylo-1,6-glucosidases: In this section, the term "amylo-1,6-glucosidase" is used to indicate an enzyme capable of attacking the alpha-1,6-glucosidic linkage between two glucose units either in starch or in a product obtained from the hydrolysis of starch. The size of the substrate molecules may vary from the disaccharide isomaltose to starch itself.

Kerr, Meisel, and Schink (48), (49) reported the presence of an alpha-glucosidase in certain fungal enzyme preparations in addition to the enzymes usually considered to be present. This enzyme was capable of hydrolyzing a fairly high proportion of the more resistant linkages of the starch or the limit dextrins while the other factors of the fungal enzyme preparation were engaged in hydrolyzing the less resistant linkages. The more resistant linkages are, of course, the alpha-1,6-glucosidic linkages.

Kneen (52) also found evidence of glucose-producing enzymes in cereal malt and fungal and bacterial preparations that are active upon certain limit dextrins. Back, Stark, and Scalf (1) devised an analytical procedure for this enzymic agent, for which they provided the name "limit dextrinase". The activity was quantitatively expressed in terms of milligrams of fermentable sugar produced from a standard limit dextrin solution by one gram of enzymic preparation in one hour at 30°C.

Lipps and co-workers (61) developed a method based upon ammonium sulfate fractionation and bentonite adsorption for the separation and purification of the alpha-amylase, limit dextrinase, and maltase produced

by submerged cultures of Aspergillus niger NRRL 337. The activity of the purified alpha-amylase was 40 times greater and the purified limit dextrinase was 5 times greater than that of the original filtrate. The limit dextrinase was more stable than the alpha-amylase, but both enzymes lost activity upon storage.

Limit dextrinase of Aspergillus oryzae was crystallized by Underkofler and Roy (103) by a procedure involving ammonium sulfate precipitation, bentonite adsorption, denaturation and precipitation of impurities by mercuric chloride, and ammonium sulfate-sodium chloride precipitation. The final yield of limit dextrinase was not given but was stated to be quite low.

The investigations of Shu and Blackwood (90), (91) concerning the effect of the carbon and nitrogen sources upon the production of limit dextrinase, maltase and alpha-amylase has been discussed earlier under the "Maltase" section.

A number of enzymes from different sources are known to be effective in forming or hydrolyzing the alpha-1,6-glucosidic bond.

Pigman (83) found that certain enzyme preparations, including those of Aspergillus oryzae and Aspergillus niger, were capable of reacting upon maltose to form unfermentable products. This synthesizing action was advanced as an explanation for the incomplete conversion of starches to fermentable materials. Pan, Andreasen, and Kolachov (74) used an enzyme preparation of Aspergillus niger NRRL 337 and suggested that the unfermentable carbohydrate might consist partly or entirely of isomaltose, 6-[alpha-D-glucopyranosyl]-D-glucose.

Pazur and French (78), (79) described the synthesis of isomaltose, pannose, 6-alpha-isomaltosyl-D-glucose (dextrantriose), and 4-alpha-dextrantriosyl-D-glucose from maltose by an enzyme in a filtrate obtained from Aspergillus oryzae. They considered it probable that only one enzyme was involved. This enzyme was capable of transferring the terminal glucose residue of maltose to the 6 position of the co-substrate molecule. The use of maltose and radioactive glucose confirmed that this enzyme was a trans-isomerase and not a hydrolase. The inactivity of the enzyme towards phosphorylated sugars and the absence of any phosphorylated intermediates seemed to eliminate a phosphorolytic route for the action of transglucosidase.

Tsuchiya, Borud and Corman (98) found that an enzymic preparation from Aspergillus niger NRRL 330 produced only glucose and a trisaccharide from starch, but no maltose or other oligosaccharide. The presence of a trisaccharide could be due either to incomplete degradation or synthesis from glucose or maltose. The latter possibility was favored by the observation that the products of joint action by beta-amylase and Aspergillus niger preparation showed less reducing power than the products obtained by the reducing action of Aspergillus niger preparation alone. This could be explained by the synthesis of higher saccharides from either maltose formed by the action of beta-amylase, or from glucose formed by the action of maltase on maltose. However, glucose alone did not give rise to higher saccharides under the actual conditions used. The enzymic preparation was also capable of synthesizing such products from isomaltose and cellobiose.

Pan, Nicholson and Kolachov (76) reported on the crystallization of a trisaccharide from the unfermentable carbohydrate produced enzymically from maltose by Aspergillus niger NRRL 337.

Preparations of Aspergillus oryzae and Aspergillus niger NRRL 330 having alpha-amylase and limit dextrinase activity were demonstrated (80) to synthesize unfermentable carbohydrates, mainly isomaltose, from glucose.

Tsuchiya, Montgomery and Corman (101) observed the reverse of this synthetic reaction by the filtrate of Aspergillus niger NRRL 330. This preparation was able to hydrolyze the alpha-1,6-glucosidic linkage of isomaltose to form glucose. Although the undiluted enzyme preparation was capable of causing hydrolysis of 90.2 per cent of the isomaltose in five hours, this was much slower than the hydrolysis of maltose by the same solution. The hydrolysis of the same concentration of maltose proceeded to almost the same degree in one hour.

(d) Dextranases: The term "dextran" is used to designate certain glucans produced by bacteria growing on a sucrose substrate. General reviews on dextran have been published by Evans and Hibbert (20), Jeanes (41) and Wistler and Smart (113). The dextrans are long chain polysaccharides made of glucose units linked principally by alpha-1,6-glucosidic bonds. The organisms generally involved in their synthesis are varieties of Leuconostoc mesenteroides, Leuconostoc dextranicum, etc., although other organisms such as Acetobacter capsulatum (31) have been shown to be capable of synthesizing dextrans. Cell-free enzyme solutions of Leuconostoc are capable of producing dextrans that cannot be distinguished from the dextrans produced by living organisms (30).

Cell-free enzyme systems capable of dextran synthesis, named dextransucrase, were first isolated by Hehre (32). The enzyme responsible for the formation of dextran was postulated to be a transferase capable of transfering one half of a unit of a disaccharide (sucrose) into a larger molecular unit (dextran) while liberating the other half as a hexose (fructose).

Hestrin (33) in a provocative review discussed the conventional belief that all disaccharide fermentation involves first a hydrolysis and second a condensation of one of the hexose species with attendant removal of water, and arrived at the belief that dextran production proceeded on sucrose directly. This would be analogous to the growth of a polymer chain of starch by interaction with glucose-1-phosphate.

Hehre and Hamilton (32) obtained an enzyme or enzyme system which they called "dextran-dextrinase" from a culture of Acetobacter capsulatum. This enzyme was capable of transferring an alpha-D-glucopyranosyl radical from a terminal position in a suitable dextrin molecule to a terminal position in a growing dextran molecule, thus causing the simultaneous degradation of a dextrin molecule and growth of a dextran molecule. The net effect was the conversion of dextrin into dextran. The enzyme was unable to act on the unaltered naturally occurring starches or on glucose or maltose.

Ingelman (40) observed that enzymic extracts from the bacterium Cellvibrio fulva were capable of breaking dextran down into comparatively large fragments, with little if any production of glucose or disaccharides from the ends of the molecules as shown by viscometric measurements.

Nordstrom and Hultin (70), (39) found that intracellular dextranases would be produced by Penicillium lilacinum Thom, Penicillium funiculosum Thom, and Verticillium coccorum (Petch) Westerdijk if these molds were grown on media containing dextran. The dextranases appeared to attack the molecule at random positions and not necessarily from the ends. Both low molecular weight fermentable saccharides and higher molecular weight fragments were produced.

An enzymic solution was prepared (109) from an unidentified Aspergillus variety which was highly active in splitting dextran into comparatively large fragments. The enzyme seemed to have an active preference for glucosidic linkages remote from end-groups.

Extracellular dextranases were indicated (100) in twenty strains of Penicillium lilacinum, Penicillium fruniculosum, Penicillium verruculosum and Spicaria violacea. An amylase concentrate of Aspergillus niger NRRL 330 was also capable of degrading dextrans.

The dextranases of the various organisms showed variation in their ability to attack dextrans of different sources and of different sizes. For example, the amylase preparation from Aspergillus niger NRRL 330 hydrolyzed isomaltose readily but dextran (as measured by reducing sugar) only very slowly. Filtrates from Penicillium funiculosum NRRL 1768 on the other hand acted fairly readily upon dextran to produce principally isomaltose, but had very limited activity upon isomaltose itself.

Enzymes have often been observed in various animal tissues and higher plants which are capable of hydrolyzing the alpha-1,6-glucosidic linkage, but as these are apart from our subject matter they will not be discussed here.

III. MATERIALS AND METHODS

A. Production of Mold Enzyme Preparations

The enzyme preparations used in this study were prepared from Aspergillus niger NRRL 330, slant agar cultures of which were obtained from Dr. L. A. Underkofler. These slants were used to inoculate flasks containing bran medium.

1. Preparation of mold bran cultures

The bran substrate was prepared using the following formula:

100 grams wheat bran
10 grams ground corn meal
60 milliliters acid solution containing
0.2 normal HCl
0.62 parts per million $ZnSO_4.7H_2O$
0.62 parts per million $FeSO_4.7H_2O$
0.08 parts per million $CuSO_4.5H_2O$.

Two separate stock solutions were made, one of 0.2 normal hydrochloric acid, and the other containing 0.2 normal hydrochloric acid, and zinc, iron and copper sulfates in such concentration that 5 milliliters of this concentrated minerals solution when diluted to 60 milliliters with the stock acid solution would give the desired concentration of the salts in the final medium.

Concentrated minerals solution:

8.5 milliliters concentrated HCl

0.0074 grams $ZnSO_4 \cdot 7H_2O$

0.0074 grams $FeSO_4 \cdot 7H_2O$

0.0009 grams $CuSO_4 \cdot 5H_2O$

tap water to 1.0 liter.

Stock acid solution (0.2 normal HCl):

8.5 milliliters concentrated HCl

tap water to 1.0 liter.

Five milliliters of the concentrated minerals solution were diluted to 60.0 milliliters with the stock acid solution. The wheat bran, corn meal, and acidic minerals solution were mixed, the preparation divided into ten equal portions and placed in as many 250 milliliter wide-mouthed Erlenmeyer flasks. The flasks were plugged with cotton and sterilized in a steam autoclave at 15 pounds pressure (approximately 250° Centigrade) for 15 minutes. They were then inoculated from agar slant cultures of Aspergillus niger NRRL 330, and placed on their sides in a 30° Centigrade incubator. After about nine days the flasks of mold bran were dry and were turned upright. The mold growth had sporulated and in this dry condition the spores were viable for six to nine months. The mold bran flasks then were stored in the incubator and served as a convenient source of inoculum whenever needed.

2. Production of vegetative inocula

For production of the enzyme preparations on the scale of carboy quantities and larger it was found desirable to use a vegetative growth to improve the inoculation. For this purpose the modification of the basic Shu and Blackwood (91) formula described in the next section was used as substrate. Flasks of mold bran were used to inoculate one liter portions of the media sterilized in wide-mouthed 2.5 liter Fernbach flasks having a base diameter of 8.2 inches and an overall height of 8.5 inches. The flasks were incubated in a reciprocal shaker having a horizontal motion of approximately one inch, 60 to 70 strokes per minute. The length of stroke was adjusted to prevent excessive splashing of the media upon the sides of the flask and the bottom of the cotton plug.

The rapidly growing vegetative inoculum was necessary to avoid the retrogradive degeneration of the starch which would otherwise occur in production cultures and which would make the starch unavailable to the organism.

3. Submerged cultures

Two different substrates were used for cultivating the organism on a large scale. One was the formula developed by Enzymes Incorporated of Eagle Grove, Iowa, for the preparation of an enzyme solution from Aspergillus oryzae, and the other a modification of the formula developed by Shu and Blackwood (91) for the preparation of an enzyme solution from Aspergillus niger PRL 558. The use of either blackstrap molasses, hydrolyzed

casein, or some other natural material was essential for sufficient growth to occur in the case of the Shu and Blackwood formula.

Eagle Grove formula	
material	per cent
starch	3.00
KNO_3	1.00
$CaCO_3$	0.50
KCl	0.02
HCl	0.16 (to pH 6.0)
NaH_2PO_4	0.04
$MgSO_4$	0.02
soybean oil	0.02
Bentonite	0.24
$ZnSO_4$	13.2 ppm.*
$FeSO_4$	13.2 ppm.*
$CuSO_4$	6.6 ppm.*

Shu and Blackwood formula	
material	per cent
soluble starch	2.00
NH_4CL	0.78
$CaCO_3$	0.50
KH_2PO_4	0.10
$MgSO_4.7H_2O$	0.05
soybean oil	0.30
natural material	0.10
$ZnSO_4$	13.2 ppm.*
$FeSO_4$	13.2 ppm.*
$CuSO_4$	6.6 ppm.*

The inorganic salts, with the exception of the $CaCO_3$, were added to tap water and the solution stirred until the solids had dissolved. The solution was then heated to boiling and the starch added with constant stirring. It was necessary to add the soybean oil with the starch when large quantities of material were being sterilized in the large cooker.

---

*These materials were added in a single concentrated stock solution. Corn starch was used in the case of the 50 gallon fermentation mentioned later where the Shu and Blackwood formula was used.

The remainder of the soybean oil and the $CaCO_3$ were added and the solutions sterilized.

The Eagle Grove formula was used for the preliminary work upon the purification of limit dextrinase, whereas the modified Shu and Blackwood formula was used for the large-scale work done later for both the limit dextrinase purification and the maltase (alpha-glucosidase) purification.

4. Production of mold filtrate

The flasks which were used for the small scale preliminary investigation in the limit dextrinase procedure were inoculated directly with mold bran and were incubated in the shaker at 30° Centigrade for 5 to 7 days.

The fermentations in the large fermentation tank were inoculated with vegetative mold growth and allowed to proceed from 5 to 7 days at 30° to 32° Centigrade with the temperature being checked at hourly intervals. Very little attention was needed for the temperature control itself. The incoming air was heated by a steam jacket encircling the air filter, and this counteracted the cooling effect of evaporation. Sterilized soybean oil was added in 300 milliliter portions during the course of the fermentation when there was danger of the foam of the medium rising to the air exhaust and thus being blown out of the tank.

The tank employed for the large scale fermentations had a capacity of 75 gallons. It was equipped with a 1/3 horsepower motor-driven propellor, a steam jacket, a perforated coiled tube of copper near the bottom for aeration, and portholes for observation and inoculation of the medium. The incoming air was filtered by passage through activated carbon which

was itself sterilized prior to use by both a steam jacket and the passage of live steam through the carbon.

The media were tested for contamination both by visual microscopic examination and by collecting samples into sterile tubes, and making agar plates of portions of the samples. The plates were examined after 24 hours. Neither of these methods disclosed the presence of any distinguishable contaminants in any of the fermentations conducted.

B. Methods of Enzyme Analysis

1. Limit dextrinase

The method used for determination of limit dextrinase activity was that developed by Back, Stark and Scalf (1). The unit of activity was the number of milligrams of fermentable sugar produced from a standard limit dextrin solution by one gram (or one milliliter) of the enzymic material in one hour at 30° Centigrade.

(a) Reagents:

(1) Limit dextrin: The limit dextrin employed was a commercial product of the Wahl-Henius Institute, Chicago, Illinois, produced by the method described by Kneen, Beckord, Spoerl, and Foster (53).

(2) Buffer solution pH 4.8: This was made by dissolving 35.32 grams of $Na_2HPO_4.12H_2O$ and 9.73 grams of citric acid in distilled water and diluting to one liter. The pH of the buffer was adjusted to 4.8 by adding more of the salt or acid, the buffer placed in a series of 100 milliliter

volumetric flasks (approximately 75 to 80 milliliters per flask), and the flasks sterilized after plugging with cotton.

(3) Buffered limit dextrin solution: A solution of 0.90 gram of limit dextrin was mixed with 20.0 milliliters of buffer solution in a volumetric flask and diluted to 100.0 milliliters. The solution was always made on the day of use.

(4) Washed yeast: Either Fleischmann or Red Star cake yeast was used for the determination. Active dried yeast contains an alpha-1,6-glucosidase (69). In our experience it was capable of acting upon limit dextrin, thus causing erratic results. The cakes of yeast used were crumbled and divided into approximately equal quantities which were placed in two 250-milliliter glass centrifuge bottles. The bottles were almost filled with distilled water and the mixture stirred until the yeast was completely suspended. The suspensions were centrifuged at 1800 revolutions per minute for 10 minutes. At the end of this time the supernatant solution was discarded and a fresh portion of distilled water added. The yeast was stirred until the solution was a uniform suspension, and then the solution was centrifuged again. This process was repeated five times to remove all traces of reducing sugars and extracellular enzymes of the yeast. The yeast was then removed and excess moisture removed by blotting with absorbent paper.

(5) Sulfuric acid: 1.5 normal.

(6) Sodium hydroxide: 1.5 normal.

(7) Methyl red and phenolphthalein indicators: 0.5 per cent.

(8) Reagents for reducing sugar analysis: The method developed by Underkofler, Guymon, Rayman and Fulmer (112) was used.

(9) Unknown enzyme solution: The sample to be analyzed was centrifuged and a sample withdrawn. Whenever possible, the concentration was estimated before the determination and the sample diluted to an appropriate concentration of limit dextrinase so that the activity fell within the range of linearity.

(b) Procedure: The procedure consisted of three main steps. These were (1), the action of the enzymic preparation upon limit dextrin, (2), the action of washed yeast cells to remove the fermentable sugars produced by the limit dextrinase and (3), the acid hydrolysis of the remaining limit dextrin and analysis of the reducing sugars formed. A blank determination (4) was conducted simultaneously.

(1) Enzymic action upon limit dextrin: Exactly 20.0 milliliters of the limit dextrin solution were transferred by pipette to a 50.0 milliliter volumetric flask. The flask and contents were placed in a water bath maintained at 30° Centigrade for 5 minutes to allow the temperature to become equilibrated. Five milliliters of the enzyme extract or the appropriate dilution were added to the volumetric flask and the contents mixed thoroughly. After 60 minutes the enzymic activity was destroyed by adding 5.0 milliliters of 1.5 normal sodium hydroxide. After 30 minutes the solution was neutralized with 1.5 normal sulfuric acid using methyl red indicator. For reproducible action of the yeast cells it was essential that the final titration with sulfuric acid not be carried past pH 4.8.

The solution was then diluted to 50.0 milliliters and mixed thoroughly. At this point 20.0 milliliters were withdrawn from each flask and added to a 40-milliliter plastic centrifuge tube* in the constant temperature water bath. In addition, from the flask used as a blank (see section (4) below), 10.0 milliliters of solution were withdrawn and added to 10.0 milliliters of 1.4 normal hydrochloric acid in 8-inch test tubes. This solution was treated by the procedure in section (3).

(2) Elimination of the fermentable sugar: Three grams (moist weight) of freshly washed yeast were added to each of the 20.0 milliliter portions of solution in the centrifuge tubes. The contents were stirred thoroughly and kept in the 30° bath for 150 minutes, with occasional stirring or swirling of the contents to insure complete removal of the fermentable sugars.

(3) Acid hydrolysis and analysis for reducing sugars: After 150 minutes the tubes were centrifuged at 1800 revolutions per minute for 10 minutes and 10.0 milliliters were pipetted into an 8-inch test tube containing 10.0 milliliters of 1.4 normal hydrochloric acid.

All samples which had been added to the hydrochloric acid were placed in a bath of boiling water for 150 minutes and then cooled and neutralized to a phenolphthalein end-point with 1.5 normal sodium hydroxide. The contents of each tube were transferred with several rinses to a 100.0 milliliter volumetric flask and diluted to the indicated volume. The

---

*The plastic tubes referred to in this work are made of Lusteroid, a trade name for a pyroxylin plastic material.

contents were well shaken and three 5.0 milliliter portions were withdrawn from each flask for the determination of reducing sugar content.

(4) Blank determination: A blank determination was conducted by varying the order of addition of reagents.

Five milliliters of the solution containing the enzymes were added by volumetric pipette to a 50.0 milliliter volumetric flask containing 5 milliliters of 1.5 normal sodium hydroxide. After 30 minutes to allow for the denaturation of the enzyme, 20.0 milliliters of the standard limit dextrin solution were added and the solution neutralized with 1.5 normal sulfuric acid using methyl red indicator to obtain a final pH in the neighborhood of 4.8. The solution was diluted to 50.0 milliliters. The solution was then carried through the procedure in the latter part of section (1) and all of sections (2) and (3).

(c) Calculation of limit dextrinase activity: The unit of limit dextrinase activity given by the procedure of Back, Stark, and Scalf (who worked with extracts of solid preparations) was in terms of milligrams of fermentable sugar produced from limit dextrin by one gram of enzyme preparation. As our work involved solutions of the enzyme, the effort involved in the calculation was materially reduced, and the unit of enzyme activity used in the present work was that amount of enzyme in one milliliter of solution that was capable of producing one milligram of fermentable sugar in 60 minutes at 30° Centigrade.

The per cent conversion of limit dextrin into fermentable sugars and the units of limit dextrinase activity were calculated by using the following expressions where "B_1" was the milligrams of glucose in a 5

milliliter sample of the blank before yeast sorption, "B_2" was the milligrams of glucose in a 5.0 milliliter sample after the yeast fermentation, "G_2" was the milligrams of glucose in a 5.0 milliliter sample of the blank after yeast sorption, and "E" was the milliliters of enzyme extract or dilution added. The quantity "E" always had the value of 5 in our calculations.

$$\frac{B_2 - G_2}{B_2} \times 100 = \text{per cent conversion.}$$

$$\frac{(B_2 - G_2)\ 100}{E\ \frac{(B_2)}{B_1}} = \text{limit dextranase units.}$$

The factor $(\frac{B_2}{B_1})$ was introduced to correct for the dilution due to the addition of the yeast.

2. Maltase activity

The method of determining maltase activity used was developed by Tsuchiya, Corman, and Koepsell (99). The unit of maltase activity was defined as the quantity of enzyme capable of hydrolyzing one milligram of maltose monohydrate in 60 minutes at 30° Centigrade.

(a) Reagents:

(1) Stock acetate buffer, 6 molar, pH 4.4: Anhydrous sodium acetate (183 grams) was dissolved in distilled water and to this was added 217 milliliters of glacial acetic acid. The solution was diluted to one liter, and if necessary additional salt or acid solution was added to

adjust the final pH to 4.4.

(2) Maltose substrate: The buffered maltose solution that was used as the substrate for maltase determination was prepared by dissolving 2.35 grams of maltose monohydrate (commercial C. P. grade, specific rotation + 131°, produced by Pfanstiehl Chemical Co., Waukegan, Illinois) in 50 to 60 milliliters of distilled water and 5 milliliters of the stock acetate buffer, and then diluting to 100.0 milliliters in a volumetric flask.

(3) Sulfuric acid: 1 normal.

(4) Sodium hydroxide: 1 normal.

(5) Phenolphthalein indicator: 0.1 per cent.

(6) Reagents for reducing sugar analysis: The method developed by Somogyi (92) was used.

(b) Procedure: The general method of procedure involved allowing the enzyme solution to act on the buffered maltose solution for a definite interval and then denaturing the enzyme by sulfuric acid. The sulfuric acid was neutralized with sodium hydroxide to a phenolphthalein end-point. The solution was then diluted to a definite volume and analyzed for reducing sugar content.

Five milliliters of enzyme solution and 10.0 milliliters of buffered maltose solution were attempered to 30° Centigrade. The solutions were then combined and held in a constant temperature water bath at 30° Centigrade. After 15 minutes 3.0 milliliters were withdrawn by a volumetric

pipette and added to 3 milliliters of one normal sulfuric acid in a 100.0 milliliter volumetric flask. Again, 120 minutes after the start of the reaction another 3.0 milliliters were added by pipette to 3 milliliters of one normal sulfuric acid in a 100.0 milliliter volumetric flask.

After the acid deactivation of the enzyme had proceded for 10 minutes, the acidified reaction mixture was neutralized to the phenolphthalein end-point with one normal sodium hydroxide and diluted to 100.0 milliliters with distilled water. Five milliliter aliquots were analyzed for total reducing sugar by the method of Somogyi (92) using the 20-minute heating period.

(c) Calculation of maltase activity: The units of maltase activity of a solution were expressed in terms of milligrams of maltose hydrolyzed per milliliter of enzyme preparation per hour. The following expression gave the units of maltase activity; "a" was the reducing value (in terms of milliliters of thiosulfate solution) of the 15 minute reaction mixture and "b" was the corresponding value for the 120 minute reaction mixture.

$$\frac{(b-a)}{0.78} \times (\text{glucose equivalent of } Na_2S_2O_3 \text{ solution} \times 1.78) \times 20 \times \frac{60}{105}$$

= milligrams maltose hydrolyzed per milliliter of enzyme preparation per hour

= units of maltase activity.

3. Alpha-amylase activity

There are two general methods of analyzing alpha-amylase activity in common use, one dependent upon the viscometric influence of alpha-amylase upon starch solutions, and the other dependent upon the color produced by the addition of iodine to the solution. The method dependent upon the iodine complex with starch or starch fragments is the more common one used in this country. The method used in this investigation was the Wohlgemuth (114) procedure as modified by Sandstedt, Kneen, and Blish (87), together with the inorganic color standard recommended by Olson, Evans, and Dickson (72). The unit of alpha-amylase activity was defined as the amount of enzyme required to dextrinize one gram of soluble starch in one hour at 30° Centigrade (4) in the presence of an excess of beta-amylase.

(a) Reagents:

(1) Buffer solution: One hundred and sixty-four grams of anhydrous sodium acetate were dissolved in distilled water. Then 120 milliliters of glacial acetic acid were added, and the solution diluted to 1000 milliliters.

(2) Beta-amylase: A commercial enzyme preparation produced especially for the alpha-amylase determination was purchased from Wallerstein Laboratories, New York, New York. This material has also been used for the determination of fungal alpha-amylase by other workers (17) and (95).

(3) Stock iodine solution: Eleven grams of resublimed iodine crystals and 22.0 grams of potassium iodide were dissolved in a volumetric flask and diluted to 500.0 milliliters with distilled water.

(4) Dilute iodine solution: Two milliliters of the stock iodine solution and 20.0 grams of potassium iodide were diluted to 500.0 milliliters with distilled water.

(5) Color standard (Olson, Evans and Dickson): Twenty-five grams of $CoCl_2.6H_2O$ and 3.89 grams of $K_2Cr_2O_7$ were dissolved in 100.0 milliliters of 0.01 normal hydrochloric acid.

(6) Soluble starch: The reagent-grade soluble starch preparation labeled "suitable for iodometry" was purchased from Merck and Co., Inc., Rahway, New Jersey.

(b) Procedure: The general plan of procedure was to prepare a solution of soluble starch and add the preparation containing a large excess of beta-amylase and no alpha-amylase. The beta-amylase acted on the non-reducing ends of the starch molecules, producing maltose, until points of branching were reached. A sufficient period of time for the complete action of the enzyme was allowed. Thus any dextrinizing tendency upon the starch displayed by the beta-amylase was complete.

The enzyme solution was then added, at intervals samples were withdrawn and added to an iodine solution. The colors of these solutions were compared with that of the standard. The time that was necessary for the color of the starch-dextrin complex to match the standard was recorded and the concentration of the enzyme calculated.

(1) Preparation of alpha-amylodextrin solution: A slurry of 5.0 grams of soluble starch in 20.0 milliliters of water was added to approximately 120.0 milliliters of vigorously boiling water with rinsing to insure complete transferral. The solution was cooled in running tap water until it could be handled with comfort and 12.5 milliliters of buffer solution were added. The solution was transferred with rinsing into a 250.0-milliliter volumetric flask. The solution was cooled to room temperature and 125.0 milligrams of beta-amylase preparation slurried in a small volume of distilled water was then added with rinsing. The contents of the volumetric flask were diluted to the mark with distilled water and a small amount of toluene was added to prevent bacterial contamination. The solution was mixed thoroughly and used in not less than 24 hours nor more than 48 hours.

(2) Dextrinizing activity: Twenty milliliters of the buffered alpha-amylodextrin solution and 5.0 milliliters of water were transferred by pipette to a 50.0-milliliter Erlenmeyer flask and the flask placed in the 30° Centigrade water bath. After the flask temperature had attained equilibrium, 5.0 milliliters of alpha-amylase solution were added by pipette. The volumes of water and alpha-amylase solution were altered to compensate for low or high activities, but the total volume of the two solutions had to be 10.0 milliliters. After 10 minutes, one milliliter portions were withdrawn by pipette at appropriate intervals and added to a series of plain 12.0-milliliter Pyrex centrifuge tubes, each of which contained 5.0 milliliters of the dilute iodine solution. The tubes were inverted at least twice to insure thorough mixing.

The tube was then placed in the color comparitor between two similar centrifuge tubes, each containing 6.0 milliliters of the cobaltous chloride color standard, and the solutions were compared. If the color of the sample from the enzyme solution was darker than the standards, one milliliter aliquots of the sample were taken at appropriate intervals until the color approached that of the standard. At that point aliquots were taken every half minute until the colors matched and the time required for the matching recorded.

If the color of the solution from the sample was too light after 10 minutes the concentration of the alpha-amylase was too great and another determination was made, using either a smaller volume of enzyme solution (and more distilled water) or a dilution of the enzyme solution.

(c) Calculation of alpha-amylase activity: The unit of alpha-amylase activity was the quantity of enzyme required to dextrinize one gram of "beta-amylase treated" starch in one hour at 30° Centigrade to the point where the dextrin-iodine color matched that of the cobaltous chloride color standard.

The concentration of the enzyme solution was calculated by the following formula:

$$\text{Units of alpha-amylase per milliliter} = \frac{0.4 \times 60}{V \times T}$$

where V represented the original volume of mold filtrate in milliliters or fraction thereof, and T was the dextrinization time in minutes.

C. Determination of Reducing Sugars

Two methods were used for the determination of the concentrations of reducing sugars. The method of Underkofler, Guymon, Rayman and Fulmer (102) was used in the reducing sugar analyses involved in the determination of limit dextrinase activity. This reagent has a high capacity (up to 10.0 milligrams of glucose or glucose equivalent per 5.0 milliliters) and thus the solutions to be analyzed for limit dextrinase activity required no prior dilution. The method of Somogyi (92) was used for the work involving dextran, panose, isomaltose, and the purification of maltase. This was a versatile reagent having a capacity of 0.01 to 3.0 milligrams of glucose or glucose equivalent per 5.0 milliliters of solution. The accuracy of this determination was $\pm$ 0.01 milligrams of glucose. Both of these methods were based upon the reduction of cupric ion in buffered alkaline copper solutions.

1. Method of Underkofler, Guymon, Rayman and Fulmer

(a) Reagents:

(1) Potassium iodide-potassium oxalate: A solution containing 12.5 per cent KI and 25.0 per cent $K_2C_2O_4 \cdot H_2O$.

(2) Dilute sulfuric acid: A 7.5 normal solution of H_2SO_4. To prevent the acid from extracting oxidizable compounds from rubber, the solution was not allowed to come into contact with rubber connections.

(3) Standard thiosulfate: A 0.05 normal $Na_2S_2O_3$ solution was made slightly alkaline by adding traces of sodium carbonate.

(4) Starch indicator solution: One per cent by weight of soluble starch was dissolved in a saturated sodium chloride solution. The purpose of the salt was to discourage bacterial growth.

(5) Sugar reagent "G": One liter of this solution contained the following ingredients:

$CuSO_4.5H_2O$	37.5	grams
$NaKC_4H_4O_6.4H_2O$ (Rochelle salt)	125.0	"
Na_2CO_3 (anhyd.)	53.0	"
KI	1.0	"
Na_2SO_4 (anhyd.)	50.0	"
KIO_3	3.5665	" (exactly)

NaOH . . . approximately saturated solution added to adjust to pH of 9.48.

These solutions were made up in large quantities. The potassium iodide-potassium oxalate solution, and reagent "G" were delivered by inverted pipettes with two-way stopcocks at the bottom of the pipettes and drainage outlets at the top of the pipette. The volumes thus delivered were reproducible. The thiosulfate solution was delivered from a microburette and was stabilized by the addition of 0.5 milliliters of 0.1 normal sodium hydroxide per liter of solution to discourage the growth of the thiosulfate discomposing bacteria (107). The exact method of preparing reagent "G" was important and the details are given in the next paragraph.

Three hundred milliliters of distilled water were placed in a large beaker and the sodium carbonate and Rochelle salt were added and dissolved. The copper sulfate was dissolved in 500.0 milliliters of distilled water and added slowly to the former solution with continuous stirring to minimize evolution of carbon dioxide. The potassium iodide and sodium sulfate were added and the solution stirred until these salts were dissolved. The solution was then diluted to approximately 960.0 milliliters with distilled water and the pH adjusted to 9.48. The resulting solution was heated to boiling and boiled gently for 10 minutes in a covered container, and then cooled to 20° Centigrade. A small portion of the solution was used to dissolve the precise amount of potassium iodate. The potassium iodate solution was returned, with rinsing, to the main solution and the volume made to exactly one liter in a volumetric flask by dilution with boiled distilled water.

The solution was stored in a pyrex container for a week to allow suspended material (mainly cuprous oxide) to settle out. The solution was then carefully decanted from the precipitate and stored in a Pyrex bottle.

(b) Procedure: Five milliliters of the sugar reagent "C" were transferred to a 25 x 145 millimeter Pyrex test tube by an automatic level refilling inverted pipette as previously described. Five milliliters of the solution containing the reducing sugar were added by volumetric pipette and the two solutions intimately mixed by shaking. A short piece of capillary tubing was inserted in a one-hole rubber stopper, and the stopper used to close the test tube. The tube was then immersed at least two-thirds

of its length in a boiling water bath for 30 minutes. The tube was withdrawn and immediately immersed in a cold water bath. Two milliliters of the potassium iodide-oxalate solution were added, the solutions mixed by swirling the test tube several times, and the tube inclined on its side at about a 30 degree angle. One milliliter of 7.5 normal sulfuric acid was carefully introduced by allowing the acid to flow down the side of the tube. Carbon dioxide was released by the reaction of the sodium carbonate and sulfuric acid. The froth was not allowed to reach the lip of the test tube. After the addition of the sulfuric acid, sufficient time was allowed to permit all of the cuprous oxide to dissolve and the solution to become clear.

The excess iodine was back-titrated with the thiosulfate solution. The starch indicator solution was added when the solution became a pale yellow color.

A blank determination was conducted simultaneously in the same manner using 5.0 milliliters of distilled water instead of the sugar solution.

(c) Calculation of reducing sugar concentration: The solutions were calibrated by determing the volume of the thiosulfate solution needed to titrate a series of determinations conducted upon known sugar solutions containing from one to 10.0 milligrams of glucose per 5.0 milliliters.

The volume required for each tube was subtracted from the volume of thiosulfate solution required for the blank, and these resulting volumes were plotted against the sugar concentration.

To determine the concentration of glucose or glucose equivalent in an unknown solution, the volume of thiosulfate required for the solution was

subtracted from the volume required for the blank, and the concentration of reducing sugar per 5.0 milliliters read directly from the graph.

The sugar reagents were calibrated once every month or six weeks.

2. Method of Somogyi

(a) Reagents:

(1) Potassium iodide solution: A solution containing 2.5 per cent potassium iodide and 3.3 per cent potassium oxalate monohydrate was made alkaline with sodium carbonate.

(2) Dilute sulfuric acid: A 2.0 normal solution of sulfuric acid.

(3) Standard thiosulfate: A sodium thiosulfate solution was made alkaline by adding 2.0 milliliters of 10.0 per cent sodium hydroxide solution per liter.

(4) Starch indicator solution: One per cent by weight of soluble starch was dissolved in a saturated sodium chloride solution.

(5) "New" copper reagent: One liter of this reagent contained the following ingredients:

Na_2HPO_4 (anhyd.)	28 grams
1 N sodium hydroxide	100 milliliters
$NaKC_4H_4O_6 \cdot 4H_2O$ (Rochelle salt)	40 grams
$CuSO_4$	8 "
Na_2SO_4 (anhyd.)	80 "

The potassium iodide-potassium oxalate reagent and the "new" sugar reagent were delivered by inverted pipettes. The method of preparing the sugar reagent was that given in the following paragraph.

The anhydrous secondary sodium phosphate and the Rochelle salt were dissolved in 700.0 milliliters of distilled water and the one normal sodium hydroxide solution was added. The cupric sulfate was dissolved in 80.0 milliliters of distilled water and the solutions were combined with stirring. The anhydrous sodium sulfate was added slowly with constant stirring until dissolved. The appropriate volume of one normal potassium iodate solution was added for the expected maximum of glucose in 5.0 milliliters of solution as follows:

25 milliliters = 3.0 milligrams glucose
10 milliliters = 1.0 milligrams glucose
5 milliliters = 0.5 milligrams glucose.

The solution was diluted to one liter in a volumetric flask and allowed to stand 24 to 48 hours. The clear upper portion of solution was decanted and the remainder filtered through a good grade of filter paper.

(b) Procedure: Five milliliters of the copper reagent and 5.0 milliliters of the sugar solution were thoroughly mixed in a 25 x 145 millimeter Pyrex test tube and covered with a one-hole rubber stopper with capillary glass tubing inserted. The tubes were immersed in vigorously boiling water for 20 minutes and then withdrawn and cooled by immersing in cold water. The appropriate amount of potassium iodide solution was added without stirring or agitation. The solution was preferably added by

allowing it to flow down the sides of the inclined test tube. The following quantities of potassium iodide solution were used:

0.5 milliliters of 2.5 per cent potassium iodide solution for 5.0 milliliters of normal iodate per liter.

1.0 milliliters of 2.5 per cent potassium iodide solution for 10.0 milliliters normal iodate per liter.

2.0 milliliters of 2.5 per cent potassium iodide solution for larger quantities of normal iodate per liter.

The solution was acidified with approximately 2.0 milliliters of the two normal sulfuric acid solution. The acid solution was added rapidly by drops with simultaneous agitation of the solution so that the entire contents of the tube were mixed and acidified at once. In practice, it was found to be best to add about half of the acid and agitate the solution until it was a clear color, and then add the remainder of the acid. The solution was then titrated with the 0.005 normal sodium thiosulfate solution. Two drops of the starch indicator solution were added toward the end of the titration.

We found it advisable to add the potassium oxalate to the potassium iodide solution to prevent the following reaction during the course of the titration:

$$2Cu^{+2} + 2I^{-} \rightleftharpoons Cu_2^{+2} + I_2$$

Somogyi minimized this reaction through the use of the minimum amount of potassium iodide, but the undesirable drift in the end-point during the titration was still observable. The oxalate ions eliminated the drift by

forming a stable complex with the cupric ions, thus effectively removing them from the solution and the equilibrium reaction.

(c) Calculation of reducing sugar concentration: The method of calculating the reducing sugar concentration used in the procedure given before was used here also. The reagent solutions were calibrated by using a series of solutions containing known quantities of reducing sugar. A graph was prepared by plotting the volume of thiosulfate solution required to reduce the blank minus the volume required to reduce the unknown versus the reducing sugar concentration. The volume of thiosulfate solution required for the unknown sugar solution was subtracted from the volume required for the blank, and the results were read directly from the graph.

3. Method of Tauber and Kleiner

The alkaline copper reagent of Tauber and Kleiner (96) was used to detect reducing carbyhydrates upon filter paper. It was prepared by dissolving 7.5 grams of copper sulfate in 100.0 milliliters of distilled water. This solution was then added, with stirring, to a solution containing 25.0 grams of Rochelle salt and 40.0 grams of anhydrous sodium carbonate in 300.0 milliliters of water. Five hundred milliliters of methyl alcohol were added and the resulting solution diluted to one liter with distilled water. This solution was sprayed on the chromatogram using a DeVilbiss "31" atomizer and a continuous air stream. The chromatogram was heated in an oven at 110° for 5 minutes. It was then removed and sprayed with a phosphomolybdic acid reagent.

The phosphomolybdic acid solution was prepared by mixing 150.0 grams of molybdic acid anhydride (Baker MoO_3; 99.5 per cent) with 75.0 grams of

anhydrous sodium carbonate. The mixture was dissolved in 500.0 milliliters of distilled water and boiled until all of the molybdic acid had been dissolved. The solution was cooled and filtered and 300.0 milliliters of 85 per cent orthophosphoric acid was added. The solution was cooled to room temperature and diluted to one liter with distilled water.

D. Electrophoresis

Proteins, due to their amphoteric nature, are capable of assuming a net negative or positive charge on the basic or acidic sides of their isoelectric points, and thus are capable of movement in an electrical field. Due to their differing composition and molecular sizes the different proteins and enzymes will move at different rates and thus a mixture of a number of components will give a corresponding number of peaks. The two procedures used in the course of this investigation utilized both solution electrophoresis and filter paper electrophoresis.

1. Solution electrophoresis

The apparatus used in this investigation was the Klett Electrophoresis Apparatus, manufactured by the Klett Manufacturing Co., New York. The standard cell in this instrument contained approximately 11 milliliters and had a cross sectional area of 0.725 square centimeters, while the optical system had a magnification of 0.341.

The solutions that were to be analyzed were dialyzed against distilled water for at least a day and then against a veronal buffer. This buffer was 0.024 molar sodium diethyl barbiturate (veronal), 0.004 molar hydrochloric acid, and 0.080 molar sodium chloride. The solutions were diluted

to approximately 0.5 per cent protein content before dialysis.

2. Filter paper electrophoresis

The apparatus used was essentially that of Cremer and Tiselius (18), but several modifications were found to be desirable.

(a) Apparatus:

(1) Electrodes: The "labyrinth" system of glass tubing surrounding the graphite electrodes was adopted without change from the design of Cremer and Tiselius. The purpose of this labyrinth was to prevent the changes in composition of the buffer that occur in the course of the lengthy procedure from affecting the pH of the buffer solution in direct contact with the filter paper.

(2) Filter paper: The Munktell "Filtrerpapper Nr. 20/150" manufactured especially for electrophoresis was cut into strips 4 x 50 centimeters which during the electrophoresis are enclosed between 5 x 33 centimeter strips of heavy plate glass.

(3) Cooling liquid: The procedure was altered from that of Cremer and Tiselius. The chlorobenzene was dispensable for our purposes, for better results were obtained when the chlorobenzene was actually omitted and the filter paper strips and their plate glass covers were left open to the air. The elimination of the chlorobenzene solution made it necessary to conduct the ends of the filter-paper bridges into a small beaker containing the buffer solution. The ends of the filter paper strips were dipped into the solution to establish contact.

(4) Current source: The direct current source was an electronic tube rectifying system delivering 148 volts. This was fed from the 110 volt alternating current line.

(5) Coloration: The protein on the strips of filter paper after the electrophoresis was dyed by immersing the paper for 10 minutes in one per cent bromphenol blue in 96 per cent alcohol saturated with mercuric chloride. The excess stain was washed off by treating the strips successively with methanol containing one per cent mercuric chloride, ethanol containing one per cent mercuric chloride, and finally ether. The original procedure of Cremer and Tiselius called for a methanol rinse between the rinses with ethanol-mercuric chloride and the ether. We found that such a rinse removed too much of the color and hence eliminated this step. Perhaps if a more anhydrous methanol were used, the solvent property would be destroyed.

(b) Procedure: The filter paper strip was moistened with the buffer solution and the excess blotted off with filter paper. The strip was placed on one of the glass plates at a point previously marked with a lead pencil. At a point 5.0 centimeters from the cathode end of the plate was placed 0.03 to 0.04 milliliters of the enzyme solution. The second glass plate was placed on the strip and clamps applied to minimize the amount of air between the two glass plates. The ends of the paper strip were dipped into beakers of the buffer solution in contact with the filter paper bridges and the current source connected.

After the desired time had elapsed, the current was disconnected and the plates with the filter paper strip removed. The paper strip was

carefully removed and dried at 100° Centigrade in the drying oven for 10 minutes. The strip was placed in the dye solution for 5 minutes, then in each of the dilute mercuric chloride-alcoholic solutions for 15 to 30 minutes. A dip in the methanol (if used) and a dip in the ether removed traces of mercuric chloride and the alcohol.

The strips were examined superficially by eye or were cut into small vertical strips 5.0 millimeters wide. The dye was quantitatively extracted with a methanol soda solution, and the intensity of the blue color determined in a Beckman spectrophotometer at 595 .

E. Filter Paper Chromatography

Protein molecules often show a reversible tendency to be adsorbed upon the surface of various types of substances. This phenomenon is utilized in paper chromatography of protein solution where there is an advancing front of liquid. The protein molecule tends to be sparingly soluble in a solution (on account of its very size among other factors) and thus may be removed from solution by the relatively weak attractive forces between the protein molecule and the cellulose surface. However, this adsorption is reversible, and as the solution front advances, the protein molecules are carried along at a rate inversely proportional to the attractive forces. The protein fractions move at various rates and thus will be found at various distances behind the advancing solution front.

The materials and procedures used for the filter paper chromatography are described below.

1. Filter paper

The paper used was Whatman No. 1, which was purchased in large sheets 46.5 centimeters by 57 centimeters. Smaller sheets 22.5 x 23.25 centimeters were cut from the larger sheets in such a manner that two 23.25 centimeter edges were cut from the 46.5 centimeter edge of the large sheet. It was quite critical that the paper be cut in such a manner that the developing solution flowed in the direction of the grain of the paper for otherwise excessive streaking was encountered.

2. Procedure

A line was drawn 2.5 centimeters from the 23.25 centimeter edge of the filter paper, and 3 to 4 microliters of the solution to be chromatographed were placed on the line on spots about 3 centimeters apart by means of a wire loop. In order to obtain enough material, three or four successive loopfuls of material were applied to the same spot with intervening drying. The 22.5 centimeter edges of the paper were stapled together loosely so that the edges did not touch.

Approximately 75 to 80 milliliters of the appropriate solvent were placed in the jar and the paper cylinder placed in the solution, taking care that the cylinder rested firmly on the bottom of the jar and was perpendicular to the surface of the solution.

After the solution had ascended to the top of the paper cylinder, which required from 90 minutes to 6 hours, depending upon the solvent, the cylinder was withdrawn and dried in the air.

Three methods were used to ascertain the location of the protein material on the chromatograms after development.

The first method was a modification of that of Papastamatis (77) which was based upon bromthymol blue. The dried chromatogram was sprayed first with a 0.2 normal solution of sodium hydroxide and 0.1 per cent solution of the sodium salt of bromthymol blue (13). The paper was dried in the air using an electric fan and the strip was passed quickly through 0.2 normal acetic acid. The areas occupied by protein stood out as blue-violet to green spots against a general background of pale yellow. The areas had a tendency to fade rapidly within 5 to 10 seconds and it was necessary to encircle immediately the colored areas observed.

The second method was a modification of that of Geshwind (24), which was based upon bromphenol blue. The indicator solution was prepared by adding 0.5 grams of bromphenol blue to a solution of 95 per cent ethanol saturated with mercuric chloride. The strip to be developed was dipped into the solution and the excess dye removed by rinsing the strip with a gentle stream of running water without any prior fixation being necessary. The protein areas stood out as blue spots against a white background. Although the spots were fairly stable, excessive washing must be avoided. It might be desirable to use an even stronger solution of dye (i.e., one per cent) in order to obtain an even stronger color.

The third method involved the use of ninhydrin. The cylinder was removed from the bottle and air-dried, after which it was sprayed with a 0.5 per cent solution of ninhydrin in butanol. The colored complex was developed by placing the chromatograms in an oven at 110° Centigrade for 25 to 30 minutes. The areas of protein were pink against a white background.

For the preliminary work indicated below the protein solution used was a 1.0 per cent solution of takadiastase.

The various developing solutions were buffered to a pH of 5.0 by adding 5.0 milliliters of McIlvain's standard buffer per 100.0 milliliters of solution.

Chromatograms developed by buffered ammonium sulfate solutions of various saturations (0.0, 20.0, 40.0, 60.0, 80.0 and 100.0 per cent) produced no resolution of the protein material of the takadiastase. The use of bromphenol blue to develop the chromatogram obtained from distilled water indicated an oblong streak of protein material having no noticeable resolution with an R_f value from 0.5 to 0.8. With increasing concentration of ammonium sulfate the mobility was decreased, but there was still no noticeable resolution. It was impossible to develop these solutions with ninhydrin due to the coloration produced by the interaction of the ammonium radical with the ninhydrin.

Better results were obtained with aqueous acetone solutions. One hundred milliliters of the buffered acetone solutions were placed in each of the jars. The acetone concentrations were increased by increments of 10 per cent from 0.0 to 100.0 per cent. After the solutions had risen to the top of the papers, the cylinders were removed and dried in air by an electric fan. The chromatograms were sprayed with a butanol solution containing 0.5 grams ninhydrin per 100.0 milliliters and developed in a 110° oven for 25 minutes.

With concentrations of 0.0 to 30.0 per cent acetone by volume, the protein material had migrated to the top of the filter paper. With 40.0 per cent acetone there was a single elongated spot between the R_f values

0.744 and 0.893. With 50.0 per cent acetone there were two elongated spots, one between the R_f values 0.097 and 0.215 and the other between the R_f values 0.657 and 0.810. This latter spot showed some tendency to be divided into two spots. With 60.0 per cent acetone there were three distinct spots. The first was on the spot where the enzyme preparation had first been applied. The second had an R_f value between 0.05 and 0.013, and the third had an R_f value between 0.503 and 0.663. With 70.0 per cent acetone the separation was much less distinct, and the movement was much less, but apparently there were still the same spots bearing essentially the same relation to each other as before. Above 80.0 per cent acetone there was slight streaking, but the majority of protein material remained at the spot where it was applied.

The determinations between 30.0 per cent and 70.0 per cent acetone were repeated, using increments of 5.0 per cent acetone. The best separations occurred at 55.0 per cent and 60.0 per cent, with the latter seeming to give the better separation. At 65.0 per cent and 70.0 per cent acetone the separations were not as clear.

F. Preparation of Maltitol

Maltitol was prepared by the reduction of maltose using Raney nickel catalyst. An alternative method, involving reduction of maltose with sodium borohydride, acetylation with acetic acid to destroy the boron complex with the two cis hydroxyl groups, deacetylation, and purification by adsorption on a carbon column, was developed by Suhadolnik (94). A small amount of maltitol prepared by this method was made available for the preliminary investigations by Dr. French of these laboratories.

1. Materials

(a) Hydrogenation apparatus: The hydrogenation apparatus was the low pressure shaker type manufactured by the Parr Hydrogenation Co., Moline, Illinois. The power supply was a quarter horse power motor which was utilized to agitate the shaker 200 times per minute. Any high pressure bottle could be used for the reduction.

(b) Raney nickel: The nickel alloy was purchased from the Raney Catalyst Co., Chattanooga 2, Tennessee. The procedure of Vogel (100) was used to prepare the Raney nickel catalyst.

Procedure: A solution of 190.0 grams of sodium hydroxide in 750.0 milliliters of water was placed in a 2-liter beaker equipped with an efficient air powered glass stirrer (to minimize the danger of igniting the hydrogen). The solution was cooled in an ice bath to 10°, and 150.0 grams of the nickel-aluminum alloy were added in small portions over a period of about 2 hours with constant stirring. The temperature was not allowed to rise above 25°. The stirrer was stopped and the beaker removed from the ice bath and allowed to come to room temperature. The reaction mixture was slowly heated on the steam bath after the evolution of hydrogen had slowed, for about 10 hours—until the evolution of hydrogen again slowed. Distilled water was added to restore the volume, the mixture was stirred, allowed to settle, and the supernatant liquid decanted. The nickel was transferred using distilled water rinses to a stoppered graduated cylinder and the water decanted. A solution of 25.0 grams of sodium hydroxide in 250.0 milliliters of distilled water was added and the mixture thoroughly shaken to suspend the catalyst. The solution was allowed to

settle and the alcohol decanted. The nickel was washed by suspension in distilled water and decantation until the washings were neutral to litmus. The nickel was then washed three times with 95 per cent ethanol and three times with absolute alcohol. The catalyst was stored under absolute ethanol in tightly stoppered bottles completely filled with absolute ethanol.

The material when used was never weighed, but a level teaspoonful was estimated to be about three grams of nickel.

(c) Maltose: The maltose was the C. P. grade purchased from the Pfanstiehl Chemical Co., Chicago, Illinois.

2. Procedure

Fifteen grams of C. P. maltose were placed in a high pressure bottle containing 100.0 milliliters of distilled water and 3.0 grams of Raney nickel. The bottle was then placed in the Parr hydrogenation apparatus and the system flushed several times with hydrogen to remove the air. The pressure was adjusted to 55 pounds and the shaker started.

The hydrogenation was allowed to proceed overnight, after which the Raney nickel was removed by centrifuging and the solution analyzed for reducing sugar. This hydrogenation procedure was repeated several times until the maltitol solution showed virtually no reducing sugar left. The filtrate was evaporated under vacuum to a thick syrup and stored in this form in the refrigerator. It was diluted to approximately a one per cent solution for use as a chromogenic substrate for the paper chromatography. Analysis by paper chromatography showed only a trace of maltose to be present.

G. Determination of Protein Nitrogen

The samples were analyzed for protein after extensive dialysis by the micro-Kjeldahl technique using a solid copper sulfate-potassium sulfate catalyst.

One milliliter of the solution containing an unknown amount of nitrogen was refluxed with one milliliter of nitrogen-free sulfuric acid for one-half hour or until the brown color disappeared. A small amount of catalyst was used to speed the decomposition of organic matter, and for extremely resistant materials, a few drops of hydrogen peroxide were added.

The Kjeldahl tube was placed in an all-glass micro-Kjeldahl distilling apparatus with steam being actively generated. The heat source was momentarily removed from the steam generator unit, and 7.0 milliliters of two normal sodium hydroxide were added. The heat was restored to the steam generator, and an acidic solution buffered by borate brought up around the outlet so that the end of the glass tube was below the surface of the liquid. The steam distillation was continued three minutes after the indicator had changed color. The solution was then titrated with 0.02 normal hydrochloric acid.

IV. EXPERIMENTAL RESULTS

A. Attempted Isolation of Limit Dextrinase

Due to the vagueness of the current ideas concerning limit dextrinase it was considered desirable at the inception of this problem to concentrate upon the purification of limit dextrinase in order to obtain an appreciable quantity of the pure enzyme for further study.

Previous workers (103) had published a method for the purification of the limit dextrinase of Aspergillus oryzae. The application of this procedure had led to a product high in limit dextrinase activity but with only traces of maltase and alpha-amylase activity remaining in the preparation. A later worker (65) made several attempts to apply this procedure to a commercially available product of Aspergillus oryzae but these attempts were unsuccessful.

The filtrate of Aspergillus niger NRRL 330 had been shown by this same worker (65) to have a higher limit dextrinase content than the filtrate of any variety of Aspergillus oryzae and thus the former mold would produce the more desirable starting material.

In the present work several attempts were made to adapt this procedure for isolation of limit dextrinase to the filtrate of Aspergillus niger NRRL 330. The first attempt was made with 1,450 milliliters of filtrate and the second attempt was made with 42.0 liters. The basic procedure used was that of Underkofler and Roy (103) which is given below in diagrammatic form.

1. Procedure for the purification of fungal limit dextrinase (103)

Solid ammonium sulfate added to 50.0 liters of chilled filtrate to three-fourths saturation. Kept overnight in refrigerator and decanted.

A. Precipitate

Supernatant (Discarded)

Dissolved in 5.0 liters distilled water. The pH adjusted to 4.2 using 2 normal acetic acid. Fifty grams Bentonite added, shaken one hour, and centrifuged.

B. Supernatant (5.0 liters)

Precipitate (Discarded)

Solid ammonium sulfate added to three-fourths saturation. Chilled overnight and centrifuged.

C. Precipitate

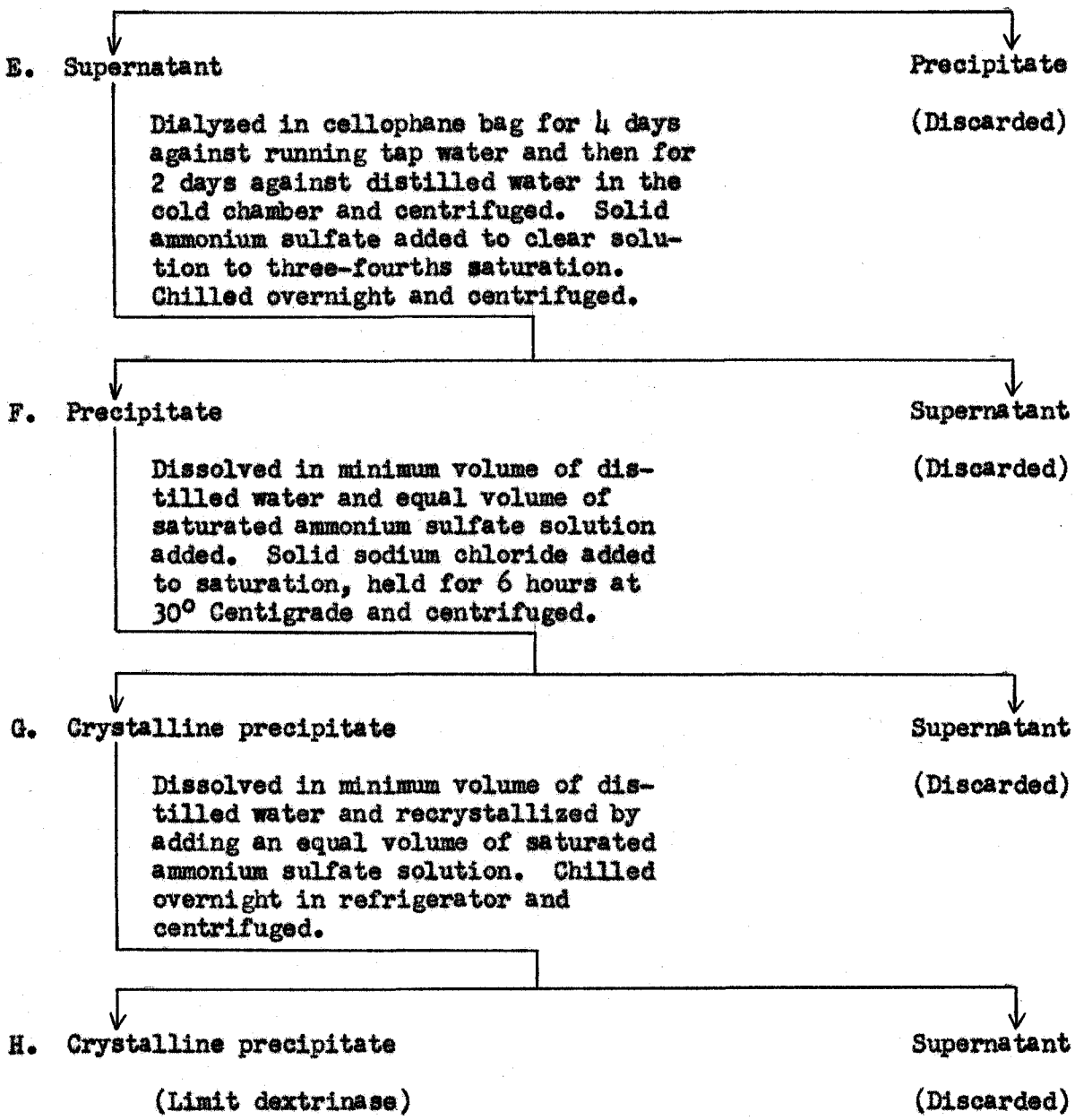
Supernatant (Discarded)

Dissolved in 500.0 milliliters distilled water. Solid ammonium sulfate added to three-fourths saturation, the solution chilled overnight and centrifuged.

D. Precipitate

Supernatant (Discarded)

Dissolved in 50.0 milliliters distilled water, dialyzed in cellophane bag for 3 days against running tap water and then for 2 days against distilled water in the cold room at 10° Centigrade. Centrifuged and 80.0 milliliters clear solution decanted. Eighty milligrams of solid mercuric chloride added to the solution, held at 30° Centigrade for 4 hours, and centrifuged.



The Bentonite adsorption in the article cited was given in a flow chart as occurring after the third ammonium sulfate precipitation, but one of the authors in a private communication indicated that it was actually after the first ammonium sulfate precipitation, as mentioned in the body of the article.

2. First attempt

The following section is an account of the first attempt on a small scale to adapt the procedure of Roy and Underkofler (103) to a filtrate of Aspergillus niger NRRL 330.

Nine 1-liter wide-mouth Erlenmeyer flasks, each containing 200.0 milliliters of the sterilized medium, were inoculated with sporulated mold bran culture of Aspergillus niger NRRL 330 by the following method. One hundred milliliters of sterile water were added to one of the sporulated mold bran cultures and the mixture was vigorously swirled until the mold bran was well suspended throughout the solution. Portions of several milliliters were withdrawn into sterilized glass tubes and transferred to the sterile fermentation flasks. The flasks were placed in the reciprocal shaker in the 30° Centigrade incubator cabinet and the mold allowed to grow for 9 days. At the end of this time, the flasks were removed from the incubator and the contents filtered through cheese cloth and then through filter paper to remove the mycelia. The filtrate, which had a volume of approximately 1,450 milliliters and a limit dextrinase activity of 8.0 units per milliliter (22.5 per cent conversion) was chilled in the refrigerator and for the first precipitation 793 grams of solid ammonium sulfate were added slowly with constant stirring until the salt was dissolved.

The solubility of ammonium sulfate at 0° Centigrade is 70.6 grams per 100.0 milliliters of water and at 100° Centigrade is 103.8 grams per 100.0 milliliters (35). The temperature of the refrigerator was measured to be 10° Centigrade. By interpolation the solubility of ammonium sulfate was calculated to be 73.0 grams per 100.0 milliliters at 10° Centigrade which

was the value used throughout the course of this work.

The supernatant solution was decanted and the excess liquid removed by centrifuging and decanting the expressed solution. One hundred and forty-five milliliters of distilled water were added to the precipitate and the solution was stirred until a uniform suspension was obtained.

Five milliliters were withdrawn for limit dextrinase analysis and diluted to 50.0 milliliters in a volumetric flask. Portions of 2.0, 4.0, 6.0 and 8.0 milliliters of the enzyme solution were added to 10.0 milliliter volumetric flasks and then diluted to volume with distilled water. The resulting solutions had concentrations with respect to the original solution of one to ten (i.e., the original 5.0 milliliters diluted to 50.0 milliliters), two to twenty-five, three to fifty, one to twenty-five and one to fifty. The results of the analysis are given in Table 1. The limit dextrinase concentration was accepted as 55 units per milliliter.

Table 1

Results of Limit Dextrinase Analysis upon Solution Containing the First Precipitate in the First Purification Attempt

	Dilution factor	Per cent conversion	Units per milliliter in solution analyzed	Units per milliliter in original solution
1.	1:50	2.9	1.0	50
2.	2:50	6.2	2.2	55
3.	3:50	10.1	3.6	70
4.	4:50	13.5	4.8	60
5.	5:50	13.5	4.8	48

For the second precipitation 77.0 grams of ammonium sulfate were added to the 140.0 milliliter solution containing the precipitate from the first precipitation and the solution was chilled in the refrigerator overnight.

The solids did not settle to the bottom of the flask spontaneously but did settle upon centrifugation of the solution. The supernatant liquid was decanted and 14.5 milliliters of distilled water added to the precipitate. The precipitate was not entirely soluble, for a slimy black material was not dissolved. This material was removed by centrifugation and saved for possible future analysis. Six-tenths of a milliliter of this solution containing the second precipitate was diluted to 50.0 milliliters in a volumetric flask and analyzed. The per cent hydrolysis was 8.5; the limit dextrinase concentration in the solution actually used for the analysis was 4.8 units per milliliter, which indicated a concentration of 400.0 limit dextrinase units per milliliter in the solution containing the second precipitate, or a total of 5,800 units. The protein material in the remaining solution was precipitated for the third time by adding 7.3 grams of ammonium sulfate and chilling in the refrigerator overnight. When the solution was centrifuged, a gelatinous black mass settled out quite readily, but there were several very fine crystals which could not be separated by centrifuging. The fine crystals appeared to be an insoluble inorganic salt.

The third precipitate was dissolved in 14.0 milliliters of distilled water and the pH adjusted to 4.5 with 2.0 normal acetic acid.

During this adjustment a precipitate was formed, which was insoluble even when the solution was diluted to 26.0 milliliters. After 1.3 grams of Bentonite were added, the container was tightly stoppered and placed in a

reciprocal shaker in the 30° incubator for 2 hours. The suspension was then centrifuged and the clear, colorless liquid decanted. Three milliliters of the solution were withdrawn for experimentation, 10.4 grams of ammonium sulfate were added to the 23.0 milliliter solution remaining after the Bentonite adsorption and filtration, and the solution chilled overnight in the refrigerator. When the solution was centrifuged a very small amount of apparently crystalline material was collected at the bottom of the tube which did not dissolve even when the solution was diluted to 60.0 milliliters with distilled water. A 5.0 milliliter portion of this 60.0 milliliter solution was withdrawn for analysis and diluted to 25.0 milliliters in a volumetric flask. One milliliter of this diluted solution contained 2.8 limit dextrinase units and caused 8.5 per cent hydrolysis. Each milliliter of the 60.0 milliliters therefore contained 14 units of limit dextrinase activity, or the entire solution contained 840 units of limit dextrinase activity.

Work on this solution was abandoned because of the excessive loss of activity that had occurred. It was decided to repeat the work on a larger scale due to the possibility that on the small scale of this work small incidental losses occurring during transfer of precipitates, etc., may have assumed a disproportionately great influence on the per cent of enzyme recovery.

The samples from the second and third ammonium sulfate precipitations were not analyzed.

3. Second attempt

After the previous small-scale purification attempt had failed, attempts were made to grow larger quantities of the culture in 3- and 5-gallon carboys with copper tubing aerators by using the medium of Shu and Blackwood (91) with ammonium chloride and urea as the nitrogen sources. A flask of the dry mold-bran material previously mentioned was used to inoculate each carboy. As the contents of the carboys showed no evidence of mold growth after 72 hours, another attempt was made using the Eagle Grove formula. In this case also, no appreciable growth occurred.

After unsuccessful attempts to grow the mold in carboys, 25 gallons of medium using the Eagle Grove formula were made and sterilized in a 50-gallon stainless steel evaporator which had been equipped with an aerating unit. Due to the lack of reinforcement, it was not considered safe to use the usual pressure of 15 pounds of steam pressure for sterilization, and so the tank and contents were sterilized at a steam pressure of 5 pounds for 2 hours. The solution was inoculated with three 10-gram portions of sporulated mold-bran culture of Aspergillus niger NRRL 330. After 6 days the fermentation was halted and a sample examined visually by microscopic observation and by plating a sample upon sterile agar. Microscopic examination revealed heavy mycelial growth with no visible evidence of contamination and after 48 hours of incubation at 30° the agar plate displayed numerous colonies of Aspergillus niger but no foreign colonies.

The solution was filtered through cheese cloth to remove the mycelial growth and returned to the vacuum evaporator to concentrate the solution. However, the vacuum pump failed before any appreciable concentration took

place and so this step was eliminated. The dextrinase activity of the solution was determined at this stage, two determinations being made.

One was upon the original undiluted solution and the analysis indicated 22 per cent hydrolysis which would correspond to 6.75 limit dextrinase units per milliliter. The other determination was upon a 50 per cent dilution of the mother liquor. The per cent hydrolysis of this diluted solution was 14.5 which would correspond to 4.33 units per milliliter. This indicated a limit dextrinase content of 8.66 units per milliliter in the original solution. As the per cent of conversion in the first determination was out of the range of accuracy, the second determination of 8.7 units per milliliter was accepted as the limit dextrinase content of the solution.

For the first ammonium sulfate precipitation about 7.4 kilograms of the solid salt were added slowly to the contents of three carboys with continual stirring until the salt was dissolved. Each carboy contained 14 liters of solution. The solution was allowed to stand overnight in a refrigerated room at 10° Centigrade and the supernatant liquid was siphoned off. The semi-solid remainder was centrifuged to expel the remaining liquid. This first precipitate was dissolved in 4.2 liters of distilled water and samples withdrawn for analysis, the results of which are given in Table 2.

Table 2

Results of Limit Dextrinase Analysis upon Solution Containing the Second Precipitate in the Second Purification Attempt

Solution	Per cent conversion	Apparent limit dextrinase concentration	Actual limit dextrinase concentration in original solution
1. Undiluted	91.6	30.4	30.4
2. 1 to 10	7.65	2.52	25.2

The limit dextrinase concentration was taken as 27 units per milliliter.

When ammonium sulfate was added to the solution of the first precipitate to three-fourths saturation the precipitate was so flocculent that centrifugation was not possible. In order to remove the inactive sludge the solution was diluted to 3.0 liters and centrifuged. The semi-solid sludge was then extracted twice with successive washes of 500.0 milliliters of distilled water, which were added to the 3-liter solution to make a total volume of 4.0 liters.

Ammonium sulfate, (1.59 kilograms), was added to the 4-liter solution containing the first precipitate to three-fourths saturation. The solution was chilled overnight and centrifuged twice, the precipitates being combined. Four hundred and twenty milliliters of distilled water were added, and the water and precipitate thoroughly stirred to make a uniform suspension of the second precipitate. A 10-milliliter sample of the suspension containing the second precipitate was removed for possible

analysis, and the solution was centrifuged. The semi-solid sludge was discarded, a 10-milliliter sample of the clear supernatant solution was withdrawn and a portion used for the analysis reported under "A" in Table 3. These two samples were later combined, centrifuged, and the resulting solution used for the experiments described later upon dextran, isomaltose, and panose.

Two hundred and twelve grams of ammonium sulfate were added to the 400.0 milliliter solution containing the second precipitate and the solution chilled overnight. The solution was centrifuged, the solid was suspended in 50.0 milliliters of distilled water and a sample of 10.0 milliliters withdrawn for possible analysis. The pH of the remaining 47.0 milliliters of solution containing the third precipitate was adjusted to 4.15 by adding 2.0 normal acetic acid. Then 2.5 grams of Bentonite were added and the solution was agitated in the reciprocal shaker for 2 hours and centrifuged. The supernatant solution was decanted and the precipitate discarded. A 3-milliliter sample withdrawn for analysis gave the results given under "B" in Table 3.

Solid ammonium sulfate (24.4 grams) was added to the solution resulting from the Bentonite adsorption and filtration and the mixture chilled overnight. The solution was centrifuged and the precipitate suspended in 50.0 milliliters of distilled water. The result was a thick grey suspension from which the solid material could not be removed by the high-speed Servall centrifuge. The material was transferred to the plastic centrifuge tubes with rinsing, and so the volume was 53.0 milliliters. A 3.0 milliliter portion withdrawn for analysis gave the results indicated under "C" in Table 3.

Table 3

Analytical Results of Limit Dextrinase Determinations upon Various Solutions in Procedures for Limit Dextrinase Purification

	Ml. of orig. soln. actually used in L. D. analysis	Dilution factor	Per cent conversion	Apparent limit dextrinase concentration, units	Limit dextrinase concentration in original solution, units
A. 1.	2.0	2:5 (10:25)	64.80	21.10	52.7
2.	1.0	1:5 (5:25)	46.60	15.20	76.0
3.	.8	4:25	38.40	12.50	78.2
4.	.6	3:25	34.60	11.30	94.4
5.	.4	2:25	23.90	7.82	97.7
6.	.2	1:25	19.50	6.37	159.0
7.	.1	1:50	10.70	3.49	174.0
8.	.05	1:100	6.30	2.05	205.0
9.	Five ml. of supernatant soln.	- -	61.50	21.50	21.5
B.	.25	1:20	17.40	5.76	155.2
C.	Before dialysis	1:200	5.50	1.89	378.0
D.	After dialysis, before $HgCl_2$	1:200	3.06	1.03	206.0
E.	After $HgCl_2$ and dialysis	1:200	0.62	0.20	40.0
F.	After $(NH_4)_2SO_4$ pptn.	1:200	3.03	1.00	200.0

The remaining 50.0 milliliters of solution were dialyzed* against running tap water for 3 days and then against distilled water for 2 days. A leak developed in the dialysis bag and thus the protein solution was inadvertently diluted to one liter. A 10 milliliter sample was withdrawn for possible analysis and 529.0 grams of ammonium sulfate were added and dissolved. The solution was chilled overnight in the icebox and centrifuged. Fifty milliliters of distilled water were added to the precipitate and the mixture was dialyzed against running tap water at room temperature for 4 days and against distilled water in the cold room. After one day in the cold room a leak was again found in the dialysis bag. As none of the many dialyses made subsequently with the same cellophane tubing and under approximately the same conditions showed any tendency to break, this was interpreted as being a strong indication of the presence of an active cellulase in the preparation being dialyzed. The volume of the solution remaining was 35.0 milliliters, of which 5.0 milliliters were withdrawn for analysis, leaving 30.0 milliliters. The results of this analysis are given under "D" in Table 3.

Thirty milligrams of mercuric chloride were added. The solution was centrifuged after 4.0 hours and the supernatant liquid dialyzed against running tap water at room temperature for 4 days and against distilled water in the ice box for 2 days, with the water being changed periodically. The final volume was 27.0 milliliters, of which 2.0 milliliters were withdrawn for analysis. The results are given under "E" in Table 3.

---

*Visking Cellulose Sausage Casings, 6733 West 65th Street, Chicago 38, Illinois.

The protein material was precipitated by adding 13.3 grams of ammonium sulfate and chilling overnight. The solution was centrifuged and the precipitate dissolved in 30.0 milliliters. A 3.0 milliliter sample withdrawn for analysis gave the results listed under "F" in Table 3.

To the remaining solution, 27.0 milliliters of saturated ammonium sulfate solution were added as well as 17.8 grams of sodium chloride. The solution was placed in the 30° water bath for 6 hours. The only crystalline material observed at this stage were a few undissolved crystals of sodium chloride although there was a considerable quantity of amorphous material.

The solution was centrifuged and the precipitate dissolved in 28.0 milliliters of distilled water. Three milliliters of solution were withdrawn for possible analysis, 25.0 milliliters of saturated ammonium sulfate solution added and the mixture chilled overnight.

After about 12 hours, fine particles of a grey precipitate were observed floating in the solution. The solution was centrifuged to separate the material. The supernatant solution was decanted, and a portion of the material observed under the microscope. There were observed large, massive crystals (evidently sodium chloride), smaller, almost spherical crystals (evidently ammonium sulfate), and small, isolated feathery clusters of apparently crystalline material which resembled the photographs of Aspergillus oryzae limit dextrinase published by Underkofler and Roy.

One milliliter of distilled water was added to this precipitate and one-quarter of this taken for analysis. The quantity of limit dextrinase found was negligible within the limits of experimental error.

Maltase determinations were also conducted upon several of the solutions. Table 4 gives the quantities of maltase activities in the solutions analyzed.

Table 4

Maltase Concentrations at Various Steps of the Limit Dextrinase Purification

	Solution analyzed and dilution factor	Maltase activity per milliliter in solution analyzed	Maltase activity in original solution	Total maltase activity
A.	Original solution after filtration 1:1	6.3	6.3	264,000 units per 42.0 liters
B.	First precipitate dissolved in 4.2 liters 1:1	11.6	11.6	48,700 units per 4.2 liters
C.	Second precipitate dissolved in 420.0 milliliters 1:1	16.2	16.2	6,800 units per 420.0 milliliters
D.	Third precipitate after Bentonite adsorption but before dialysis, dissolved in 55.0 milliliters 1:10	18.2	182.0	10,000 units per 55.0 milliliters
E.	Solution after dialysis but before $HgCl_2$ treatment, dissolved in 32.0 milliliters 1:10	4.3	43.0	1,370 units per 32.0 milliliters

There was a linear relationship between the extent of hydrolysis and the limit dextrinase content as demonstrated in Table 5, when the two values for all of the determinations were compiled.

Back, Stark, and Scalf (1) found the material from Aspergillus oryzae to be very high in limit dextrinase activity at the step corresponding to our last step. Due to the virtually complete loss of enzyme activity with the filtrate from Aspergillus niger, the latter steps of the procedure at least would need to be eliminated and be replaced with another procedure. The three preliminary ammonium sulfate precipitations could be retained as a concentration procedure suitable for much of the inactive material, but even these precipitations resulted in excessive loss of limit dextrinase activity.

Prior to the development of another purification procedure, however, a shorter analytical method would be desirable to avoid the excessively lengthy analysis for limit dextrinase activity. With this end in mind, the action of a concentrated limit dextrinase preparation upon bacterial dextran, panose, and isomaltose was studied. If the enzyme fraction responsible for the limit dextrinase activity could hydrolyze the alpha-1,6-glucosidic linkage, a measurement of the increase of reducing sugar content would afford a convenient index to the limit dextrinase activity of the solution. This was the purpose of the work described in the immediately following sections.

Table 5

Comparison between Limit Dextrinase Concentration and Per Cent Limit Dextrin Conversion to Fermentable Carbohydrates

Per cent conversion	Apparent limit dextrinase concentration	Per cent conversion	Apparent limit dextrinase concentration
91.6	30.4	19.5	6.37
64.8	21.1	17.4	5.76
61.5	21.5	14.5	4.33
46.6	15.2	10.7	3.49
38.4	12.5	8.5	2.80
34.6	11.3	7.6	2.52
31.2	10.3	6.3	2.05
23.9	7.82	5.5	1.89
22.5	8.0	3.06	1.03
22.0	6.75	3.03	1.00

B. Action of Concentrated Enzyme Preparation upon Bacterial Dextran

Four runs were conducted under varying conditions to ascertain the effect of a concentrated solution of limit dextrinase upon dextran. The procedure was largely patterned after the procedures of Tsuchiya, Montgomery and Corman (101), and Back, Stark and Scalf (1).

1. First run on clinical dextran

(a) Materials:

Buffer solution: A 0.3 molar acetate buffer solution, pH 4.7, was prepared by diluting a solution containing 24.6 grams of sodium acetate and 17.1 milliliters of glacial acetic acid to one liter in a volumetric flask using distilled water.

Dextran: The dextran used was the type produced by Streptobacterium dextranicum NRRL B-1254 which contained alpha-1,6- and alpha-1,4-glucosidic linkages in a ratio of about 12 to 1. The material used was obtained from the Northern Regional Research Laboratory, at Peoria, Illinois, and was labeled "Clinical Dextran Solid, Preparation NRRL -35".

Buffered dextran solution: Six-tenths of a gram of dextran was dissolved in 50.0 milliliters of the buffer solution in a volumetric flask.

Enzyme: The enzyme preparation used was from the second attempt to purify limit dextrinase. Twenty milliliters were withdrawn from the 420.0 milliliter solution containing the second ammonium sulfate precipitate. After centrifuging, this solution was used for the work as well as the

hydrolysis of panose and isomaltose. The solution contained approximately 190 units of limit dextrinase activity and 16.2 units of maltase activity per milliliter.

(b) Procedure: Two 20.0 milliliter portions of buffered dextran solution were transferred by volumetric pipette to each of two 40-milliliter plastic centrifuge tubes. The tubes were placed in water baths maintained at 30° and 35°.

Seven milliliters of distilled water and 3.0 milliliters of the enzyme solution were added by pipette to each of the tubes and well shaken. The time of addition was recorded.

The tubes were maintained in the constant temperature water baths and 5.0 milliliter aliquots withdrawn at definite intervals by pipette for analysis.

(c) Analytical method: The 5.0 milliliter aliquots were added to 5.0 milliliters of 1.5 normal sodium hydroxide in 100.0 milliliter volumetric flasks and allowed to stand at room temperature for 30 minutes.

The solutions were neutralized to a methyl red end-point with 1.5 normal hydrochloric acid.

The flasks were diluted to volume with distilled water and analyzed for reducing sugars by Somogyi's method.

(d) Calculation of the per cent dextran hydrolysis: The information accompanying the dextran indicated that the average chain length (as estimated by Somogyi's sugar determination) was approximately 295 glucose

units. The equation for hydrolysis was assumed to be

$$(C_6H_{10}O_5)_n + nH_2O \qquad n\ C_6H_{12}O_6.$$

Each 5.0 milliliter portion withdrawn for analysis contained originally 2.0 milligrams of dextran, which would be equivalent to 2.22 milligrams of glucose if it were fully hydrolyzed. The per cent of hydrolysis was given by the following expression. In this expression the intermediate hydrolysis products were assumed to have reducing power equivalent to glucose and the reducing value of the dextran itself to be negligible.

$$\text{Per cent hydrolysis} = \frac{\text{milligrams glucose equivalent}}{2.22} \times 100.$$

(e) Experimental results: The results obtained at 30° and 35° Centigrade are given in Table 6 under 1A and 1B respectively.

Table 6

Analytical Results of Hydrolysis of Dextran
with Concentrated Enzyme Solution*

Reference to Text		Per Cent Hydrolysis (Time in Minutes)							
		0	30	60	120	180	240	300	1320
1	A	0.0	--	1.35	1.80	1.80	--	--	5.87
	B	0.0	--	0.90	1.44	1.80	--	--	7.67
2		0.0	0.1	1.57	3.51	3.15	--	4.87	--
3	A	0.0	--	--	2.20	--	--	3.60	--
	B	0.0	--	--	1.70	--	--	3.60	--
	C	0.0	--	--	1.90	--	--	2.20	--

2. Second run on clinical dextran

(a) Materials:

Buffer solution: Same as in first run.

Dextran: Same as in first run.

*With the exception of 3C, which used the original filtrate from the second attempt to purify limit dextrinase, the enzyme solution used in all of these experiments was the 20.0 milliliter sample withdrawn from the 420.0 milliliter solution containing the second ammonium sulfate precipitate of the second attempt at purification. The solution was centrifuged prior to use.

Buffered dextran solution: Six-tenths of a gram of dextran was placed in a 50.0 milliliter volumetric flask. Ten milliliters of the 0.3 molar acetate buffer (pH 4.7) were added and the solution diluted to 50.0 milliliters.

Enzyme: Same as in first run.

(b) Procedure: Twenty milliliters of the buffered dextran solution were added by pipette to a 40.0 milliliter plastic centrifuge tube. Seven milliliters of distilled water were added by pipette and the tube and its contents were equilibrated in a constant temperature water bath maintained at 49°. Three milliliters of the sample withdrawn from the 420.0 milliliter solution were added and mixed, the time noted, and aliquots withdrawn periodically for reducing sugar analysis with the Somogyi reagents.

(c) Analysis and calculations: Same as in first run.

(d) Experimental results: The results obtained in this run are given in Table 6, under 2.

3. Third run on clinical dextran, varying pH

The third experiment conducted upon clinical dextran used the temperature of 49° to speed the reaction.

(a) Materials:

Buffer solutions: Two buffer solutions were used. One was the 0.3 molar acetate buffer (pH 4.7) prepared by diluting 24.6 grams of

sodium acetate and 17.1 milliliters of glacial acetic acid to one liter. The other was a phosphate-citrate buffer (pH 4.9) prepared by dissolving and diluting to one liter 35.32 grams (or 0.1 mole) of dibasic sodium phosphate ($Na_2HPO_4.12H_2O$) and 9.73 grams (or 0.05 mole) of citric acid.

Dextran: Same material was used as before.

Buffered dextran solutions: Two solutions were prepared, one by diluting 1.20 grams of dextran and 20.0 milliliters of acetate buffer to 100.0 milliliters, and the other by diluting 1.20 grams of the dextran and 20.0 milliliters of the phosphate-citrate buffer to 100.0 milliliters.

Temperature bath: A water bath insulated with asbestos padding and heated by a thermocouple and a thermo-regulator* was adjusted to 49° Centigrade.

Enzyme solution: Two enzyme preparations were used. One was the solution used before, the sample withdrawn from the 420.0 milliliter solution. The other was the original filtrate prior to any concentration or purification.

(b) Procedure: Three different determinations were conducted.

A. Twenty milliliters of acetate buffered dextran solution and 7.0 milliliters of distilled water were equilibrated in the constant temperature bath. Three milliliters of the sample withdrawn from the 420.0 milliliter solution were added, the solution well mixed, and 5.0 milliliter aliquots withdrawn for analysis after 0, 2 and 5 hours.

---

*H - B Instrument Company, 2633 Trenton Avenue, Philadelphia 25, Pennsylvania.

B. Twenty milliliters of phosphate buffered dextran solution and 7.0 milliliters of distilled water were added to 40.0 milliliter plastic centrifuge tubes and equilibrated in the 49° bath. Three milliliters of the sample from the 420.0 milliliter solution were added, the solutions mixed thoroughly, and 5.0 milliliters withdrawn for analysis after 0, 2 and 5 hours.

C. Twenty milliliters of phosphate buffered dextran solution and 10.0 milliliters of the sample of the original filtrate were equilibrated in the 49° bath in separate tubes. The filtrate solution was added to the dextran solution and the solutions were mixed by pouring from one tube back and forth several times. The solution was placed in the plastic centrifuge tube, and the tube replaced in the water bath. Five milliliter samples were withdrawn at 0, 2 and 5 hours.

(c) Analysis and calculations: Same as in first run.

(d) Experimental results: The results obtained with the solutions A, B and C which were described under "Procedure", are given in Table 6 under the headings 3A, 3B and 3C respectively.

The results obtained from the preceding work upon dextran indicated that the extent of hydrolysis was so slight that measurement of the reducing power of the resulting solution could not be used as a reliable index of enzyme concentration. The extent to which the viscosities of these dextran solutions were affected was not determined.

Studies were then made of the effect of the enzymic concentrate upon the smaller molecules of panose and isomaltose, since previous workers (101)

had reported the hydrolysis of isomaltose by filtrates of cultures of Aspergillus niger NRRL 330.

C. Action of Concentrated Enzyme Solutions upon Panose

1. Materials

Panose: The necessary amount of anhydrous chromatographically pure panose prepared by Dr. Pan was supplied by Dr. Dexter French.

Buffer solution: The 0.3 molar acetate buffer at pH 4.7 described under the procedure for the hydrolysis of dextran using fungal enzyme concentrate was used for this experiment.

Buffered panose solution: Twenty milliliters of the acetate buffer solution and 0.1168 grams of panose were diluted to 100.0 milliliters in a volumetric flask with distilled water.

Constant temperature bath: The constant temperature bath described under the section on the hydrolysis of dextran was used for this determination. The temperature was adjusted to 39.5°.

Reducing sugar determination: The alkaline copper reagent of Somogyi (92) having a maximum capacity of 0.5 milligrams of glucose or glucose equivalent per 5.0 milliliters was used for this work.

A series of determinations made to determine the optimum reduction time for solutions containing panose indicated that the solutions should be immersed in the boiling water bath for 25 to 30 minutes.

2. Experimental procedure

Two 20.0 milliliter portions of the buffered panose solution were transferred to each of two 40.0 milliliter plastic centrifuge tubes immersed in the constant temperature bath. To one tube was added 7.0 milliliters of distilled water (solution A) and to the other was added 10.0 milliliters of distilled water (solution B). The tubes were well mixed and replaced in the constant temperature bath for 10 minutes.

Solution A: In the second attempt to purify limit dextrinase, a 20.0 milliliter sample was withdrawn from the 420.0 milliliter solution containing the second ammonium sulfate precipitate. This solution was centrifuged and the supernatant solution was used for this experiment. Three milliliters of this solution were added to the plastic centrifuge tube containing 20.0 milliliters of the buffered panose solution and 7.0 milliliters of water. The solution was well mixed, replaced in the constant temperature bath, and 3.0 milliliter aliquots withdrawn periodically for analysis.

Solution B: To the plastic centrifuge tube containing 20.0 milliliters of the buffered panose solution and 10.0 milliliters of water was added 0.30 grams of Takamine Clarase*. The solution was well mixed, replaced in the constant temperature bath, and 3.0 milliliter aliquots withdrawn periodically for analysis.

---

*Takamine Laboratory, Inc., Clifton, New Jersey

3. Analytical procedure and results

(a) Analytical procedure: Three milliliter aliquots were withdrawn by pipette from the solution and placed in a 25.0 milliliter volumetric flask containing 5.0 milliliters of 1.5 normal sodium hydroxide, after which the solution was shaken to insure thorough mixing.

The solution was allowed to stand 30 minutes and was then neutralized to a methyl red end-point with 1.5 normal sulfuric acid.

The solution in the flask was diluted to the proper volume, and 5.0 milliliters were withdrawn and analyzed for reducing sugars using Somogyi's solution. The solution was capable of oxidizing an expected maximum of 0.5 milligrams of glucose or its equivalent per 5.0 milliliters of solution. The time of immersion was 25 minutes.

(b) Calculation of results: The quantity of panose used for the analysis was selected so that complete hydrolysis would yield 0.5 milligrams of glucose per 5.0 milliliters of solution analyzed if the hydrolysis were complete.

The calculation of the per cent of hydrolysis for the intermediate stages between no hydrolysis and total hydrolysis involved the same calculations regardless of which of the two following possibilities was the more accurate: first, that one type of linkage, either the alpha-1,4- or alpha-1,6-glucosidic linkage was hydrolyzed preferentially, leaving essentially a solution of the more resistant disaccharide which was then hydrolyzed at a slower rate, or, second, that the two types of linkages were hydrolyzed at the same rate. From our knowledge that the 1,4 linkage is hydrolyzed at a rate four times as great as that for the 1,6 linkages by

acid hydrolysis (111), the first postulate was more probable.

The formula used to calculate the per cent of panose hydrolysis was derived by the following method.

$$\text{Per cent hydrolysis} = \frac{\text{milligrams panose hydrolyzed}}{\text{milligrams panose in original solution}} \times 100$$

$$= \frac{\text{milligrams panose hydrolyzed}}{0.467} \times 100.$$

Let g = milligrams reducing sugar equivalent calculated as glucose in each 5.0 milliliters of solution analyzed.

Let m = milligrams panose hydrolyzed in each 5.0 milliliters of solution analyzed.

$$g = \frac{(0.467 - m) \text{ mg. panose residue}}{5.0 \text{ ml. solution}} \times \frac{180 \text{ mg. glucose equiv.}}{504 \text{ mg. panose residue}}$$

$$+ \frac{m \text{ mg. panose hyd.}}{5.0 \text{ ml. soln.}} \times \frac{3 \times 180 \text{ mg. glucose equiv.}}{504 \text{ mg. panose hyd.}}.$$

$$g = (0.467 - m) \times \frac{180}{504} + m \frac{(3 \times 180)}{504}$$

$$g = \frac{180}{504}(0.467 + 2m),$$

or

$$m = 1.40\,g - 0.234.$$

$$\text{Per cent hydrolysis} = \frac{1.40g - 0.234}{0.467} \times 100$$

$$= (3.00g - 0.502) \times 100.$$

The preceding equation was derived upon the assumption that panose as well as any other disaccharide present (i.e., maltose or isomaltose) would

give the stoichiometrically correct value for glucose equivalent under the conditions of analysis. However, the use of this equation led to the obviously erroneous conclusion that 27 per cent hydrolysis had occurred immediately upon combination of the enzyme and panose solutions. This was the result obtained by substituting 0.258, the number of milligrams of glucose found at 0 time, into the equation.

The explanation lies in the fact that sugar determinations are quite empirical and that different monosaccharides give different reducing values, while di- and tri-saccharides may diverge quite widely. A correction factor was obtained by the following method.

The assumption was made that no hydrolysis had occurred at 0 time, and that m was equal to 0. The basic equation

$$g = (0.467 - m) \times \frac{180}{504} + m \times \frac{3 \times 180}{504}$$

then became, with a correction factor included,

$$g = 0.467 \times \frac{180}{504} \times \text{correction factor.}$$

Substituting the values found,

$$0.258 = 0.167 \times \text{correction factor}$$

$$1.54 = \text{correction factor.}$$

When this factor was inserted, the equation then became

$$g = (0.467 - m\ 0.553) + m \frac{(3 \times 180)}{(504)} .$$

The final equation for per cent of hydrolysis became

$$\text{Per cent hydrolysis} = (4.13g - 1.07) \times 100.$$

This last equation was used to calculate the values reported in Table 7. Due to the absence of any data concerning the accumulation of disaccharide in the reaction, it was not possible to attempt to make any correction for the error introduced from this source. However, the relative extent of copper reagent reduction within a series was a fairly good reflection of the extent of hydrolysis.

(c) Experimental results: Solutions A and B gave the analytical results listed in Table 7 under 4A and 4B respectively.

Although the results were not clear-cut, it was evident that the concentrated and partially purified limit dextrinase solution was capable of hydrolyzing the alpha-1,6-glucosidic bond in small molecules.

The action of the enzyme solution upon isomaltose was next investigated.

D. Action of Concentrated Enzyme Solution upon Isomaltose

1. Materials

Isomaltose: Isomaltose was prepared from dextran by a modification of the procedure of Wolfrom, Georges and Miller (115).

Table 7

Analytical Results of Hydrolysis of Panose and Isomaltose with Concentrated Enzyme Solution*

Reference to Text		Per Cent Hydrolysis (Time in Minutes)							
		0	15	30	60	120	180	240	1320
4	A	0.0	- -	32.6	50.0	68.6	70.7	62.4	48.5
	B	3.3	- -	72.7	72.0	79.0	79.0	96.8	- -
5		0.0	13.0	20.5	38.5	55.0	57.5	68.0	

Seventy-two grams of dextran were added to 2,180 milliliters of 30 per cent hydrochloric acid at room temperature. The initial specific optical rotation was + 180°. The solution was maintained at 30° for 8 hours until the specific optical rotation was + 105°. Lead carbonate was added to neutralize the acid. The lead chloride was removed by filtration, rinsed with 500.0 milliliters of distilled water and the rinses combined with the main solution. Hydrogen sulfide was passed through the solution to remove the excess lead, and the solution was filtered. The solution was concentrated under partial vacuum distillation at 50° Centigrade to 500.0

*The enzyme solution used for the first experiment with panose, 4A, and the experiment with isomaltose, 5, was the same solution as that described in Table 6. That is, during the second attempt to purify limit dextrinase, a 20.0 milliliter sample was withdrawn from the 420.0 milliliter solution containing the second ammonium sulfate precipitate. This 20.0 milliliter sample was centrifuged and used for these experiments. The enzyme solution used for the second experiment with panose, 4B, was Takamine Clarase, a commercial product of the Takamine Laboratory, Inc., Clifton, New Jersey.

milliliters. The solution was filtered and the sugars separated by the carbon-celite chromatographic column separation technique developed by Whistler and Durso (108). The fraction eluted by the 5.0 per cent ethanol solution was concentrated to 50.0 milliliters. Paper chromatography (42) indicated that the main sugar present was isomaltose, but there were also traces of glucose and higher carbohydrates. Further attempts at purification of the isomaltose were not successful and so the impure solution was used for the experiments described below. The yield of crude isomaltose was approximately 1.05 grams. The isomaltose was stored as a syrup and withdrawn by a pipette as used.

Concentrated enzyme solution: The 20.0 milliliter sample withdrawn from the 420.0 milliliter solution was the source of the enzyme.

Buffer solution: A 0.3 normal acetate buffer at pH 4.7 was used.

2. Procedure

The experimental and analytical procedure described in the experiments with panose were used here also.

One milliliter of the impure solution of isomaltose, which contained 1.09 grams of isomaltose per 50.0 milliliters, 5.0 milliliters of the acetate buffer solution, and 1.0 milliliter of the enzyme solution were mixed and diluted to 25.0 milliliters. The maltase activity in the resulting 25.0 milliliter solution which contained the enzyme and isomaltose was 0.65 units per milliliter. The limit dextrinase activity was 7.6 units per milliliter. Three milliliter samples were withdrawn periodically by pipette and placed in 3.0 milliliter portions of 1.4 normal sodium hydroxide for 30

minutes. The solutions were neutralized with 1.9 normal sulfuric acid to methyl red end-points, and the solutions diluted to 25.0 milliliters. Five milliliter samples were withdrawn by pipette for reducing sugar analysis.

3. Analytical results

(a) Calculation of results: The formula used to calculate the per cent of isomaltose hydrolysis was derived by the following method.

$$\text{Per cent hydrolysis} = \frac{\text{milligrams isomaltose hydrolyzed}}{\text{milligrams isomaltose in original solution}} \times 100.$$

$$= \frac{\text{milligrams isomaltose hydrolyzed}}{0.523} \times 100.$$

Let g = milligrams reducing equivalent calculated as glucose in each 5.0 milliliters of solution analyzed.

Let m = milligrams isomaltose hydrolyzed in each 5.0 milliliters of solution analyzed.

$$g = \frac{(0.523 - m) \text{ mg. isomaltose residue}}{5.0 \text{ ml. solution}} \times \frac{180 \text{ mg. glucose equiv.}}{342 \text{ mg. isomaltose residue}}$$

$$+ \frac{m \text{ mg. isomaltose hyd.}}{5.0 \text{ ml. soln.}} \times \frac{2 \times 180 \text{ mg. glucose equiv.}}{342 \text{ mg. isomaltose hyd.}}.$$

$$g = (0.523 - m) \times \frac{180}{342} + \frac{m(2 \times 180)}{342}$$

$$g = \frac{180}{342} (0.523 + m),$$

or

$$m = 1.90g - 0.523.$$

$$\text{Per cent hydrolysis} = \frac{1.90g - 0.523}{0.523} \times 100$$

$$= (3.64g - 1)\ 100.$$

It must be borne in mind that while within the series the relative values for the degree of hydrolysis were reliable, actually there were unknown quantities of glucose and higher carbohydrates in the isomaltose solution, and thus the absolute values gave only an approximation. The actual per cent of hydrolysis was probably higher than indicated by the experimental results, for acid hydrolysis indicated less isomaltose than expected.

An empirical correction factor similar to that used for panose would have been desirable here also, although apparently isomaltose was not subject to serious deviation from the stoichiometric factor. The results were quite satisfactory for qualitative comparisons.

(b) Experimental results: The experimental results given in Table 7 under 5 indicated that appreciable hydrolysis of isomaltose had occurred. This procedure, or some modification of it, could be used as an analytical method for estimation of alpha-1,6-glucosidase activity if isomaltose can be made available.

E. Purification of Maltase

The persistence with which maltase activity accompanied limit dextrinase activity in all of the present purification attempts as well as those of Miller (65) underlined the possibility that limit dextrinase was actually a glucosidase of a type similar to that found by Phillips and

Caldwell (81).

Accordingly it was decided to investigate the properties of the alpha-1,4-glucosidase system of Aspergillus niger NRRL 330. The analytical method to be used to indicate alpha-glucosidase activity was the standard method for maltase determination.

1. First purification procedure

Fifty gallons of the semi-synthetic medium of Shu and Blackwood (91) in the large fermenter tank were inoculated with a vegetative culture of Aspergillus niger NRRL 330.

Preparation of the inoculum. One liter of the medium, the ingredients of which are given below, was added to each of three wide-mouth Fernbach fermentation flasks and the flasks and contents sterilized after plugging with cotton.

Material	Per cent
Corn starch	2.0
$CaCO_3$	0.5
KH_2PO_4	0.1
$MgSO_4 \cdot 7H_2O$	0.05
$(NH_4)_2SO_4$	0.2
Molasses	0.1
Stock minerals solution	0.1

The mineral materials with the exception of the calcium carbonate were dissolved in one liter of tap water. The solution was heated to boiling and the corn starch was added slowly with stirring. The calcium carbonate

was added and the flasks were plugged with cotton and sterilized.

Each of the flasks, after being cooled to room temperature was inoculated with a mold bran flask containing 10.0 grams of the sporulated mold culture. The inoculated flasks were placed on the shaker in the 30° Centigrade incubator for 24 hours. Abundant growth had occurred in all the flasks. The two flasks in which the most abundant growth had occurred were selected to inoculate the 50.0 gallons of medium.

The 75.0 gallon fermenter was cleaned, rinsed and then filled with 50.0 gallons of tap water. The nutrients were added with constant stirring in the same manner as before. When the soluble starch was added, 250.0 milliliters of soybean oil were added to prevent foaming. The tank was sterilized at 15 pounds pressure for 30 minutes, cooled to 86° Fahrenheit (30° Centigrade), and inoculated with the 2.0 liters of vigorous 24-hour vegetative mold culture. A sterilized 250.0 milliliter portion of soybean oil was added to the tank. Vigorous aeration and stirring was started and the temperature was maintained at 86° Fahrenheit for 4 days. The maltase content on the second day was 3.8 units, the third day was 4.7 units, and the fourth day was 4.7 units per milliliter. The aeration and stirring were stopped at this time and approximately 23.0 gallons of the unfiltered culture were placed in the cold room and allowed to stand overnight. The mycelia settled to the bottom, leaving a clear solution on top. The upper solution, which had a pH of 4.9, was siphoned off and saved, while the slurry containing the mycelia was discarded. The volume of the clear solution was about 13.0 gallons. Previous attempts to separate the mycelia from the unsettled filtrate with the Sharples super-centrifuge were unsuccessful, probably due both to vibration of the machine and the hydration

of the suspended material. The pH was 4.8

(a) Preliminary ammonium sulfate precipitation: Before the addition of solid ammonium sulfate to 16.0 liters of enzyme solution, two experimental ammonium sulfate precipitations were conducted in order to determine the optimal degree of saturation for the first concentration of the protein fraction containing the enzymic activity.

First experimental ammonium sulfate precipitation: Eleven 25.0 milliliter portions of the mold filtrate were added by pipette to a series of 50.0 milliliter Erlenmeyer flasks. Sufficient solid ammonium sulfate was added to each flask with stirring to increase the saturation to the desired degree. For example, the first flask contained no ammonium sulfate, while the second flask was 10.0 per cent saturated, the third flask 20.0 per cent saturated, etc.

The solutions were stored in the cold room for 48 hours, and the contents were then transferred to 40.0 milliliter plastic centrifuge tubes and centrifuged at approximately 3,000 revolutions per minute for 20 minutes. The supernatant liquids were decanted and 25.0 milliliters of distilled water were added by pipette to each precipitate. The contents of the plastic centrifuge tubes were mixed thoroughly and 5.0 milliliters were taken for analysis. However, even at complete ammonium sulfate saturation only a small fraction of the maltase activity known to be present was found in the precipitate.

Due to the physical observation that the large basket centrifuge was not capable of exerting enough force to completely settle the material forced out of solution by salting out, the experiment was repeated with a

Servall conical head centrifuge capable of approximately 12,000 revolutions per minute.

Second experimental ammonium sulfate precipitation: A 150.0 milliliter portion of the original mold filtrate was used for the second experiment. The ammonium sulfate saturation was increased in increments of 10 per cent. The solution was centrifuged 20 minutes after each addition of ammonium sulfate. The precipitates were dissolved in 15.0 milliliter portions of distilled water so that the first solution contained the material suspended in the original solution, the second solution contained the precipitate formed between 0 and 10 per cent ammonium sulfate saturation, the third solution contained the precipitate formed between 10 and 20 per cent ammonium sulfate saturation, etc. The process was repeated until the solution was completely saturated with ammonium sulfate.

Five milliliter portions were withdrawn by pipette determination of maltase activity. These results are given in Table 8 and Figure 1.

Preliminary ammonium sulfate precipitation of 16.0 liters: Sixteen liters of the original mold filtrate were used to obtain a more concentrated enzyme solution for further work. Solid ammonium sulfate to 50 per cent saturation was added slowly with constant stirring to the 16.0 liters of original mold filtrate and the solution chilled overnight in the cold room.

The resulting solution was centrifuged using the Sharples supercentrifuge at 45 pounds pressure. The precipitate was discarded and the supernatant liquid, which was still opalescent, was preserved. Solid ammonium sulfate was slowly added up to 90 per cent saturation and the suspension stirred until solution was complete. After the solution was chilled

Table 8

Precipitation of Maltase Activity from Crude Filtrate at Various Ammonium Sulfate Concentrations

Per cent saturation	Units of maltase activity precipitated per milliliter of conc. solution	Units of maltase activity precipitated per milliliter in original soln.	Total units of maltase activity precipitated per milliliter in original soln.	Total per cent of maltase activity precipitated
0 - 10	2.61	0.261	0.261	5.53
10 - 20	0.914	0.091	0.352	7.46
20 - 30	0.487	0.049	0.401	8.50
30 - 40	0.653	0.065	0.466	9.86
40 - 50	0.653	0.065	0.531	11.25
50 - 60	1.83	0.183	0.714	15.1
60 - 70	4.24	0.424	1.138	24.1
70 - 80	8.75	0.875	2.013	42.6
80 - 90	11.0	1.10	3.11	65.9
90 - 100	4.70	0.47	3.58	75.8

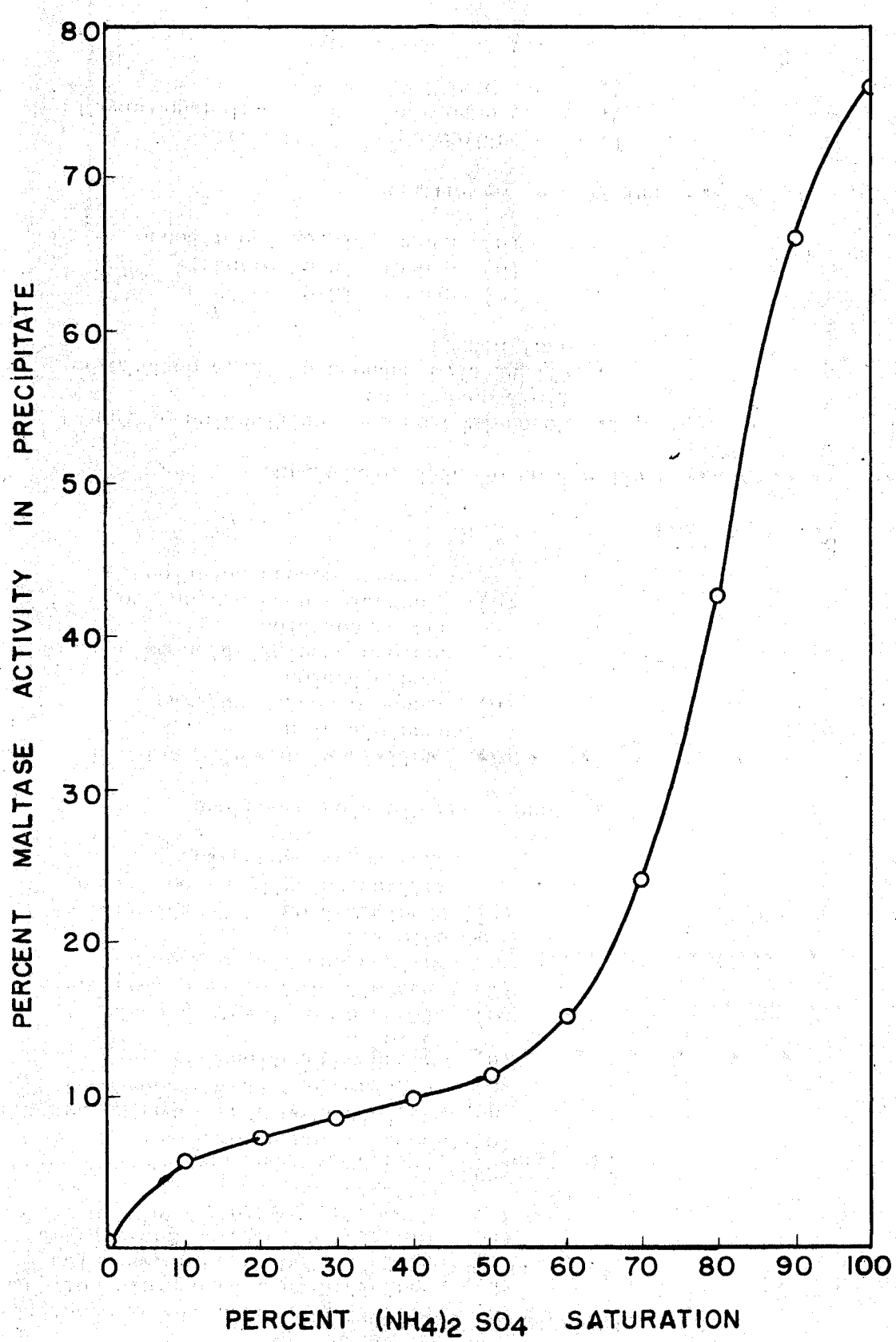


Fig. 1 Precipitation of Maltase Activity from Crude Filtrate at Various Ammonium Sulfate Concentrations

overnight a flocculent precipitate rose to the top. The underlying solution was removed by siphoning and the upper layer of precipitate was centrifuged in small portions in the Servall centrifuge for 15 minutes. The clear supernatant liquids were decanted and the precipitates combined and dissolved in 1.6 liters of distilled water.

The maltase activity after the purification was 12.3 units per milliliter. The pH of the solution was 4.7. This solution was labeled "enzyme concentrate B" for future reference.

(b) Determination of isoelectric point: The solubility of the protein fraction possessing maltase activity was determined at various pH levels and concentrations of ammonium sulfate in order to determine the isoelectric point.

Pipettes were used to withdraw seven 20.0 milliliter portions of enzyme concentrate B. Prior to the addition of ammonium sulfate, the pH levels of the solutions were adjusted using the Beckman pH meter using approximately 0.05 normal sodium hydroxide or sulfuric acid. Each solution was transferred to a 40.0 milliliter plastic centrifuge tube. The desired quantity of solid ammonium sulfate was added with constant stirring until the salt was completely dissolved and the solution was allowed to stand 20 minutes in the cold room. It was then centrifuged at 12,000 revolutions per minute for 20 minutes and the supernatant solution poured into another plastic centrifuge tube. The solution was saved for the determination of the solubility of the enzyme at the same pH but a higher ammonium sulfate concentration. The pH of the solution was readjusted if necessary.

The precipitate was dissolved in 10.0 milliliters of distilled water, the solution thoroughly stirred, and 5.0 milliliters withdrawn for the maltase activity determinations reported in Table 9 and Figures 2 and 3.

Despite the most vigorous attempts, it was impossible to get any more than about three-fourths of the eighth addition of ammonium sulfate to dissolve. Assuming then that the 1.6 liters of solution was already about 13 per cent saturated (from the liquid adhering to the precipitate when centrifuged), this would mean that the first solution was approximately 12 per cent saturated, the second 22 per cent saturated, and so on for the entire series. This would not influence the isoelectric point but would merely mean that the proteins are salted out of solution sooner than otherwise.

From Table 9 it was apparent that the pH optima for precipitation was near 5.0, and that the collection of a fraction between 30 and 70 per cent saturation would contain the bulk of the enzyme activity.

(c) Second ammonium sulfate precipitation: The pH of the enzyme concentrate B remaining after the previous samples had been withdrawn (about 1,380 milliliters) wad adjusted to 5.0. Solid ammonium sulfate was slowly added with constant stirring to 30 per cent saturation. The solutions were centrifuged twice at 12,000 revolutions per minute for a total time of approximately 50 minutes. The precipitate was discarded and to the supernatant liquid was added, with constant stirring until dissolved, solid ammonium sulfate to 70 per cent saturation. The pH was readjusted to approximately 5.0 using pH-Hydrion paper to gauge the pH level and the

Table 9

Precipitation of Maltase Activity from Enzyme Concentrate B at Various pH Levels

Per cent of ammonium sulfate saturation	pH level						
	4.0	4.5	5.0	5.5	6.0	6.5	7.0
	Units of maltase activity per milliliter in solution containing precipitate						
0	1.04	1.57	1.57	2.35	1.31	(1.44)	1.57
0 - 10	0.52	0.78	0.78	0.52	0.52	0.52	0.78
10 - 20	0.78	0.52	0.78	0.78	1.31	2.52	1.05
20 - 30	1.83	2.61	1.83	1.57	1.31	1.05	1.57
30 - 40	5.22	9.92	10.50	8.61	8.20	7.32	10.50
40 - 50	14.40	14.90	15.40	15.20	14.90	13.90	15.20
50 - 60	13.80	9.40	15.47	8.08	12.00	11.50	7.80
60 - 70	2.61	2.60	1.50	9.90	2.60	3.40	1.80
Total	40.20	42.30	47.83	47.01	42.15	40.21	40.27
	4.0	4.5	5.0	5.5	6.0	6.5	7.0
0 - 40	9.39	15.40	15.46	13.83	12.65	12.85	15.47
0 - 50	23.79	30.30	30.86	29.03	27.55	26.75	30.67
0 - 70	40.20	42.30	47.83	47.01	42.15	40.21	40.27

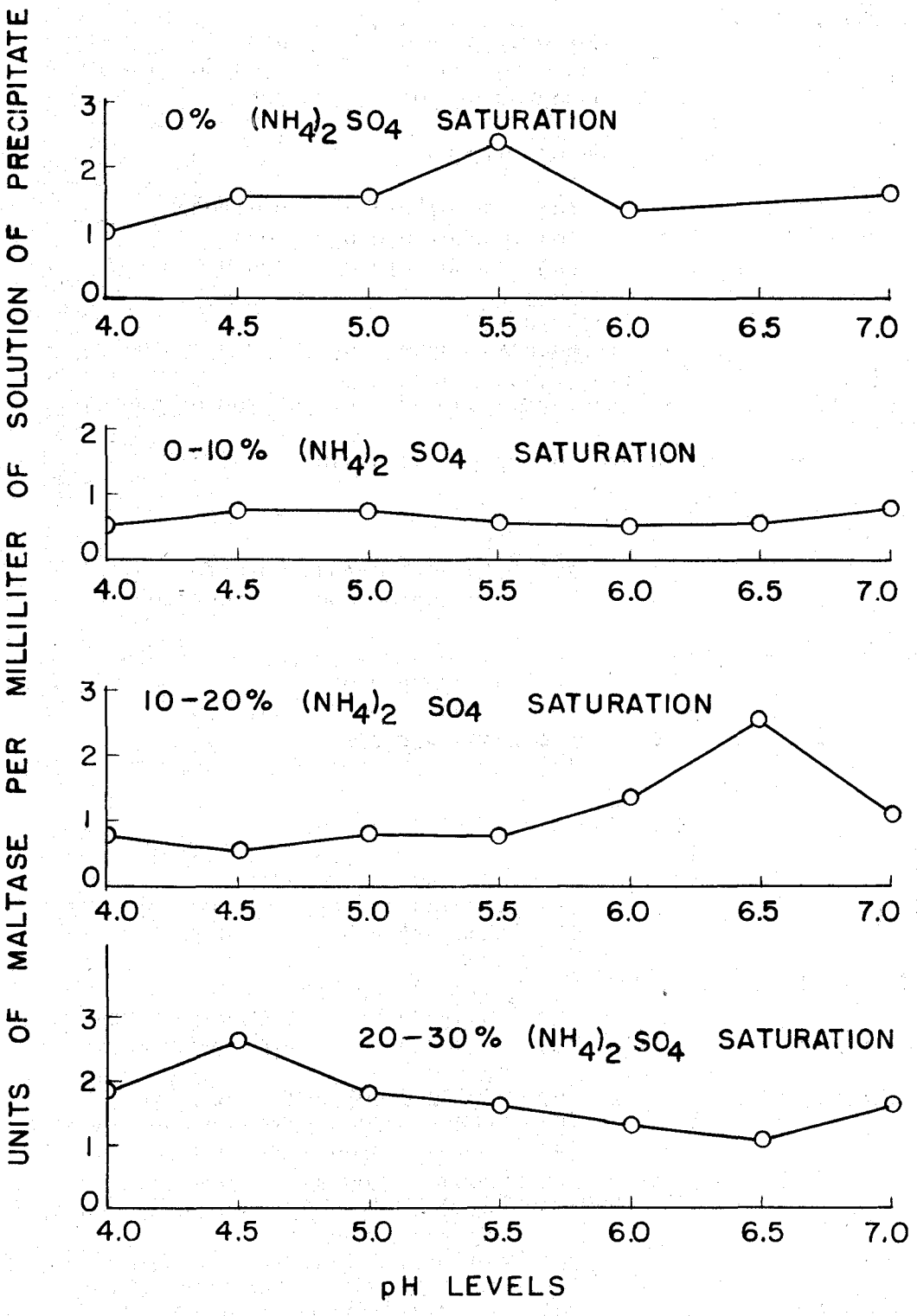


Fig. 2 Precipitation of Maltase Activity from Concentrated Filtrate with Ammonium Sulfate at Various pH Levels

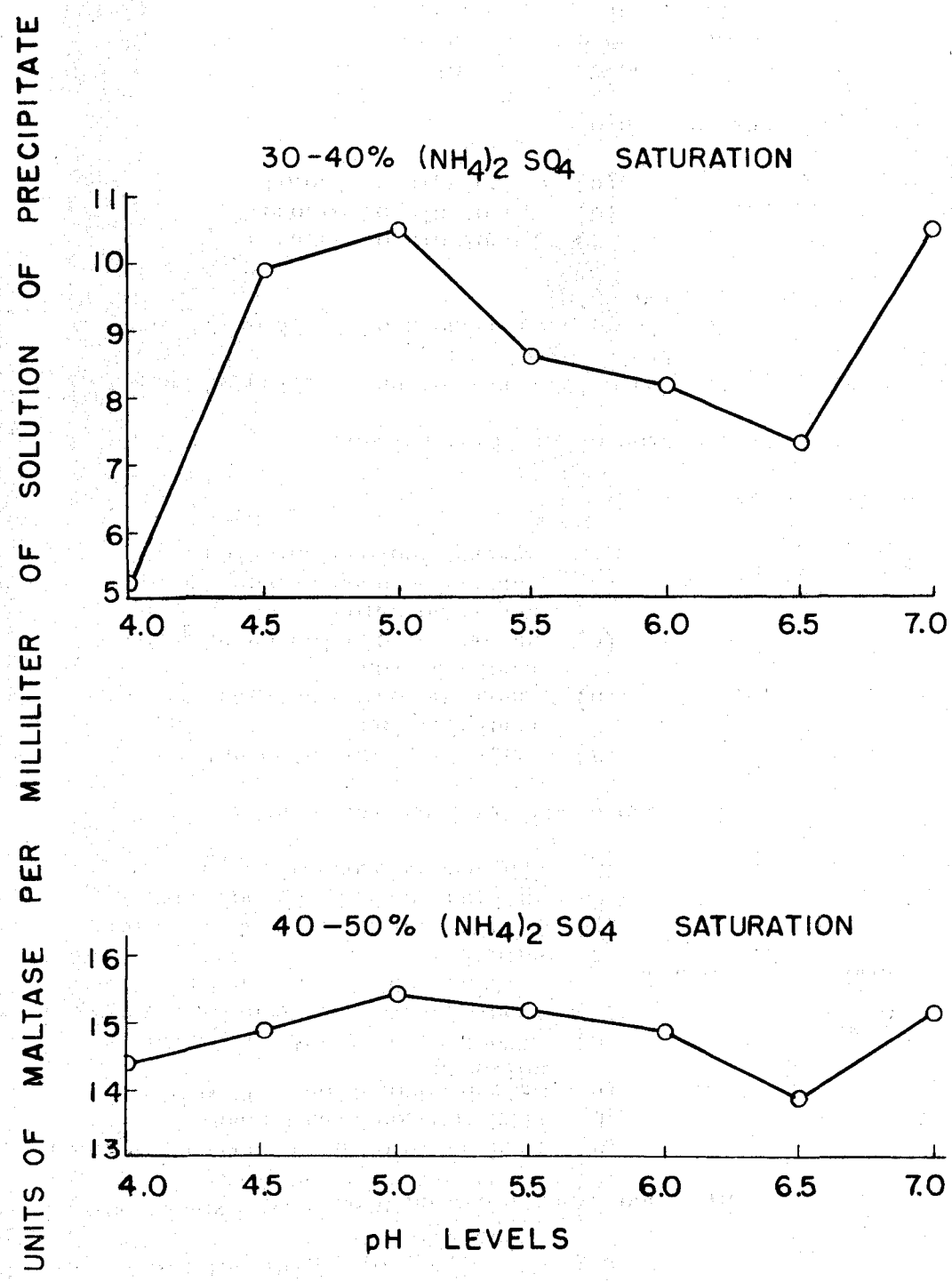


Fig. 3 Precipitation of Maltase Activity from Concentrated Filtrate with Ammonium Sulfate at Various pH Levels

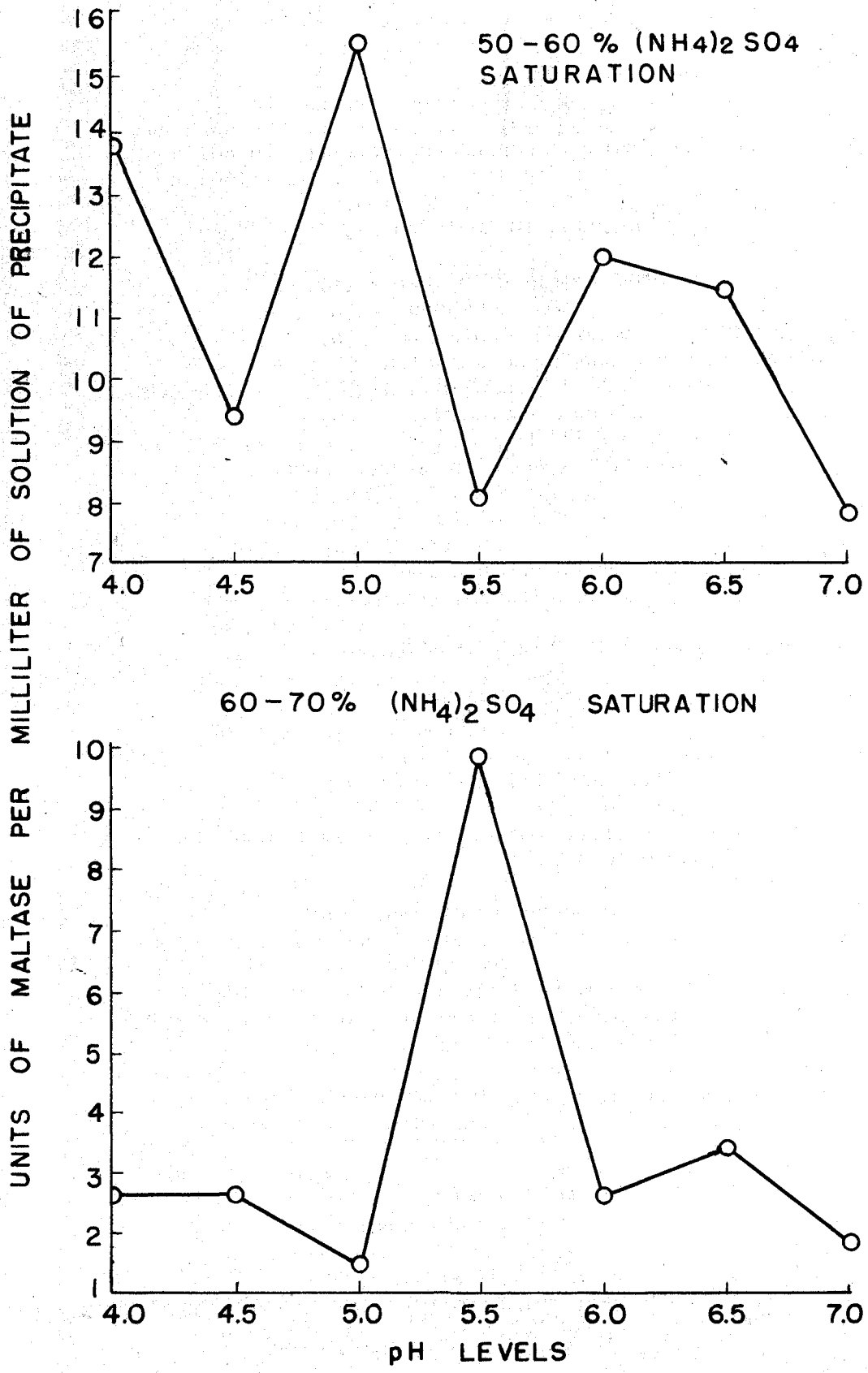


Fig. 4 Precipitation of Maltase Activity from Concentrated Filtrate with Ammonium Sulfate at Various pH Levels

solution was allowed to stand overnight in the cold room. The solution was then centrifuged at 12,000 revolutions per minute. The supernatant solution was decanted and the precipitate was dissolved in 300.0 milliliters of distilled water. This solution was labeled "enzyme concentrate C" for future reference.

(d) Precipitation of fractions having maltase activity by organic solvents:

Procedure: Precipitation of protein material by organic solvents has been a standard method of protein, and enzyme, purification. Accordingly, an investigation was made in this work of the precipitation of protein fractions having maltase activity by the organic solvents acetone, ethanol and dioxane. For these precipitations it was found necessary to use the Servall "316" stainless steel 50.0 milliliter centrifuge tubes and covers to avoid the solvent action of the organic liquids upon the plastic centrifuge tubes.

Ten milliliters of enzyme concentrate C were added to each of four stainless steel centrifuge tubes and the pH adjusted to 5.0. The tubes were centrifuged at 12,000 revolutions per minute for 15 minutes and the precipitate dissolved in 10.0 milliliters of distilled water. The appropriate amount of organic solvent necessary to properly increase the percentage composition of the solvent was added to the solution, by a fine capillary tube below the surface, slowly and with stirring to avoid local concentrations of the organic solvent.

The tubes were centrifuged for 25 minutes at 12,000 revolutions per minute. The supernatant liquids were transferred to other centrifuge

tubes and the precipitates dissolved in 10.0 milliliters of distilled water. The next increment of solvent was added and the process repeated.

The amounts of organic solvents added to 10.0 milliliter portions of enzyme solution were those given below.

0	per	cent	No organic solvent added;								
10	"	"	1.1 milliliters of organic solvent added;								
20	"	"	1.4 milliliters of organic solvent added to preceding solution;								
30	"	"	1.8	"	"	"	"	"	"	"	;
40	"	"	2.4	"	"	"	"	"	"	"	;
50	"	"	3.3	"	"	"	"	"	"	"	;
60	"	"	5.0	"	"	"	"	"	"	"	;
70	"	"	8.3	"	"	"	"	"	"	"	.

Higher concentrations of organic solvents were not used due to fear of inactivation of enzymic activity.

Analytical results: The results are tabulated in Table 10 and Figure 4.

Another 10.0 milliliter portion was withdrawn for possible future analysis, and the remaining solution of 235.0 milliliters was adjusted to a pH of 4.9.

A thin capillary tube below the surface of the solution was used to introduce 192.3 milliliters of acetone (or 45 per cent by volume) while a stirring motor was constantly agitating the solution. The acetone was added over a ten minute period, and as soon as it was added the solution was transferred to the stainless steel centrifuge tubes centrifuged at 13,000 revolutions per minute for 20 minutes. The supernatant liquids were

Table 10

Precipitation of Maltase Activity from Partially Purified Filtrate at Various Concentrations of Acetone, Dioxane and Ethanol*

Volume per cent of organic solvent	Acetone		Dioxane		Ethanol	
	In increment	In total of increments	In increment	In total of increments	In increment	In total of increments
0	2.82	2.82	2.35	2.35	2.88	2.88
0 - 10	1.83	4.65	2.87	5.22	2.61	5.49
10 - 20	2.35	7.00	2.61	7.83	2.61	8.10
20 - 30	(3.22)	10.22	2.09	9.92	2.88	10.98
30 - 40	7.05	17.27	6.25	16.17	2.61	13.59
40 - 50	11.0	28.3	15.4	31.57	14.2	27.8
50 - 60	16.7	45.0	11.3	42.9	12.8	40.6
60 - 70	10.4	55.4	7.84	50.7	4.18	44.8

*The values given are units of maltase activity per milliliter of solution containing the precipitate.

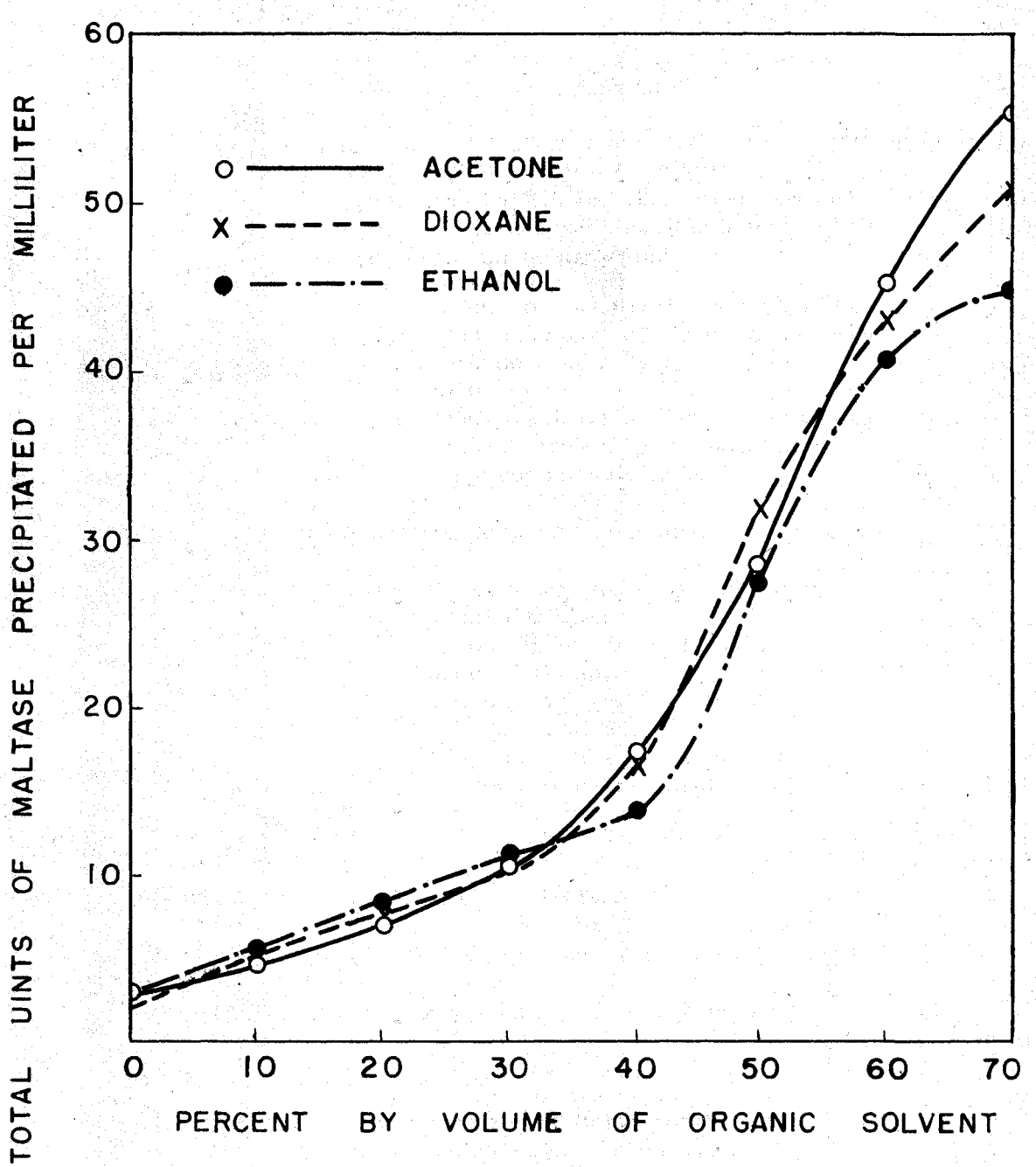


Fig. 5 Precipitation of Maltase Activity from Concentrated Filtrate with Acetone, Dioxane and Ethanol

combined and an additional 244.4 milliliters of acetone were added to increase the concentration to 65 per cent.

The precipitate of the fraction between 45 per cent and 65 per cent was dissolved in 100.0 milliliters of water and the solution labeled "enzyme concentrate D" for future reference.

Enzyme concentrate D was dialyzed against occasional changes of distilled water in the cold room for 48 hours, until the test with barium chloride was negative. This solution was analyzed for alpha-amylase and total nitrogen. The alpha-amylase content was 34.3 units per milliliter, compared to the original concentration of approximately 10 to 12 units per milliliter. The Kjeldahl nitrogen determination was conducted after centrifuging the opalescent solution. The resulting clear solution had a nitrogen content of 1.16 milligrams of nitrogen per milliliter.

A paper chromatogram developed with a 60 per cent acetone solution indicated a spot of protein material remaining at the point where the enzyme was applied with a slight streak extending upward for about 5.0 millimeters. Strips cut from a portion that had not been developed with the dyestuff were sprayed with a 1.0 per cent solution of maltitol and incubated at 30° for 30 minutes in a moist atmosphere. When these strips were then dried and sprayed with the Tauber and Kleiner reagent for reducing sugars, heavy spots of blue appeared at the origin, indicating the presence of a substance capable of hydrolyzing maltitol (i.e., a maltase) at that spot.

The solution electrophoresis conducted upon 10.0 milliliters of the solution withdrawn at this stage indicated the presence of at least three distinct peaks corresponding to protein fractions.

(e) Effect of pH level upon stability: A series of buffer solutions were made by the procedure of McIlvain (35) which involved combining certain volumes of 0.1 molar citric acid solution and 0.2 molar disodium phosphate solution.

The final pH of each buffer solution was adjusted by adding the required citric acid or phosphate solution until the pH value as indicated by the Beckman pH meter was correct.

The solutions of the precipitates from the solvent precipitations were combined and the resulting solution, with an activity of approximately 5.5 to 6.0 units per milliliter and a total volume of 50.0 milliliters, was used for an experiment to determine the effect of pH upon stability.

Five milliliters of the enzyme solution and 10.0 milliliters of the appropriate buffer were withdrawn by pipette and separately equilibrated to 30° Centigrade in the constant temperature water bath. The solutions were mixed and returned to the constant temperature bath. Three milliliters were withdrawn periodically from the solution for analysis. The results are given in Table 11.

Table 11

Stability of Maltase Activity at Various pH Levels

pH	Maltase units per milliliter	
	24 hour exposure	96 hour exposure
4.0	5.61	5.87
4.5	5.48	5.48
5.0	6.55	6.13
5.5	5.87	5.48
6.0	5.48	5.73
6.5	5.74	5.70
7.0	6.00	5.20 (obvious contamination)

(f) Precipitation of fractions having maltase activity by sodium chloride: An attempt to precipitate the protein fraction possessing the maltase activity by means of sodium chloride was made. Three milliliters of enzyme concentrate D were withdrawn by pipette for this experiment. Solid sodium chloride was added slowly with constant stirring, and the solution was centrifuged. Portions of sodium chloride sufficient to increase the saturation by 20 per cent were added each time, but with the exception of inactive sludge obtained by centrifuging the original solution, no precipitate was obtained.

(g) Precipitation of fractions having maltase activity by potassium dihydrogen phosphate: Solid potassium dihydrogen phosphate was added to

the saturated sodium chloride solution without producing any precipitate, either of ensymically active material or any other material.

An additional 3.0 milliliters of enzyme concentrate D were withdrawn by pipette. Potassium dihydrogen phosphate was added to the solution to the saturation point, but with the exception of an inactive sludge present in the original solution, there were no detectable quantities of material precipitated. The results of this and the preceding section indicated that neither sodium chloride nor potassium dihydrogen phosphate was a suitable material for precipitating this protein fraction.

2. Second purification procedure

Although the preceding method resulted in a solution that had high maltase activity, there remained considerable quantities of alpha-amylase activity. Also the electrophoretic pattern displayed, in addition to a large peak, several small humps. For these reasons, it was decided to make a more detailed study of the properties and behavior of the proteins having maltase and alpha-amylase activity.

The supernatant solution from the 50.0 gallon fermentation remaining after the first attempt to precipitate the maltase had been stored for 3 months under toluene in the cold room. When the solution was analyzed for maltase activity it was found to contain 4.52 units per milliliter. This filtrate was therefore concluded to be satisfactory for the ensuing study.

(a) Preliminary ammonium sulfate precipitation: It was desirable first to make a more precise determination of the behavior of maltase activity during ammonium sulfate precipitation. This was carried out in the following manner.

Experimental ammonium sulfate precipitation: Thirty milliliters of the crude enzyme filtrate were placed in each of eleven 40.0 milliliter plastic centrifuge tubes. Solid ammonium sulfate was slowly added to each tube with constant stirring until dissolved. The resulting solutions were saturated with ammonium sulfate to concentrations between 45.0 and 95.0 per cent in 5.0 per cent increments.

The solutions were chilled for several hours, and then centrifuged at the full speed of the Servall centrifuge (about 12,000 revolutions per minute for 25 minutes).

The supernatant solutions were decanted and the precipitates dissolved in 10.0 milliliters of distilled water and dialyzed in the cold room against distilled water. The distilled water was changed every 8 hours. The dialysis was continued until a drop withdrawn from the solution containing the precipitate produced no precipitate when added to a dilute solution of barium chloride. The dialyzed solutions were then transferred, using several rinses of distilled water, to 25.0 milliliter volumetric flasks and the solutions diluted to volume with distilled water.

These solutions were analyzed for maltase, alpha-amylase, and nitrogen content, and the results are given in Table 8 and Figures 5, 6 and 7.

Table 12

Maltase Activity, Alpha-Amylase Activity and Nitrogen-Containing Material Precipitated from the Crude Solution by Ammonium Sulfate

Per cent of ammonium sulfate saturation	Alpha-amylase, units per milliliter	Maltase, units per milliliter	Milligrams nitrogen per milliliter
45	0.040	0.26	0.070
50	0.073	0.21	0.085
55	0.18	0.11	0.114
60	0.40	0.53	0.105
65	1.00	0.79	0.205
70	1.00, 1.33	1.16	0.154
75	1.18, 1.11	3.17	0.183
80	1.48, 1.48	2.29	0.230
85	1.33, 1.38	3.90	0.185
90	1.33, 1.18	3.48	0.260
95	1.60, 1.54	4.32	0.185
orig.	1.60	4.55	0.258

Ammonium sulfate precipitation of 20.0 liters of mold filtrate: The results of the experimental ammonium sulfate precipitations showed that most of the maltase was precipitated between 65 and 95 per cent saturation. To obtain larger quantities of enzyme concentrate for further work, solid ammonium sulfate was added slowly with constant stirring to ten 2.0 liter

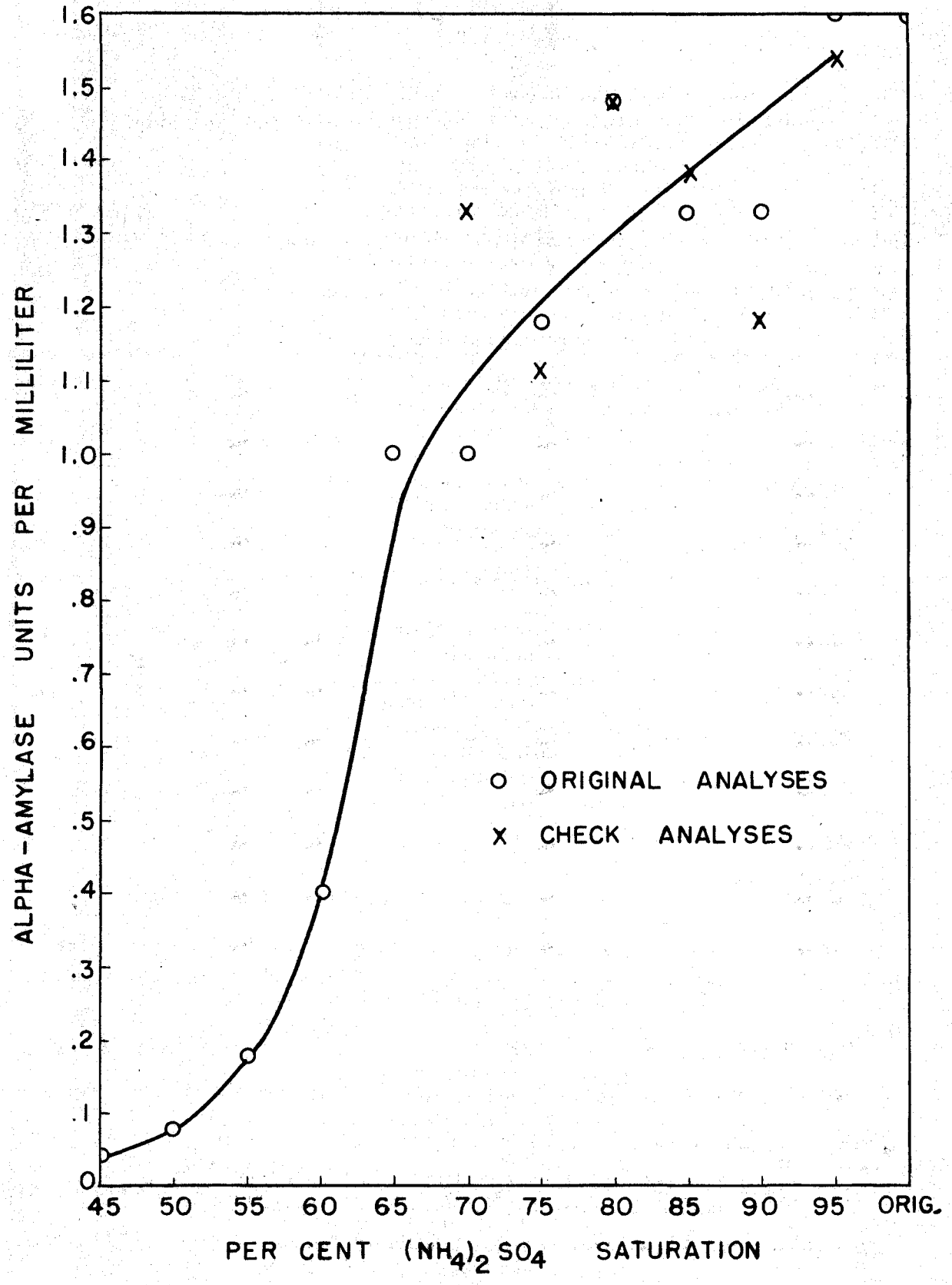


Fig. 6 Precipitation of Alpha-Amylase Activity from Crude Filtrate by Ammonium Sulfate

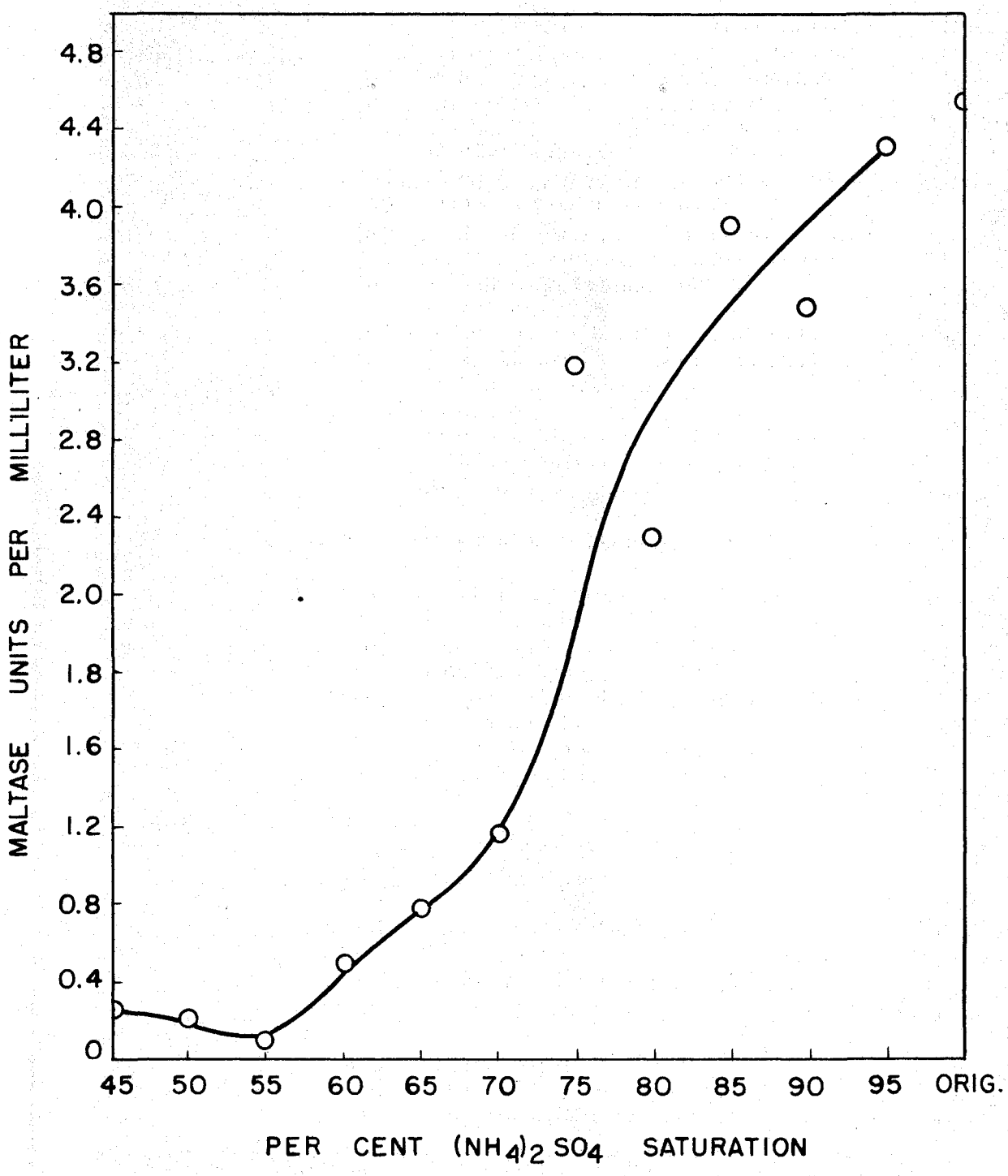


Fig. 7 Precipitation of Maltase Activity from Crude Filtrate by Ammonium Sulfate

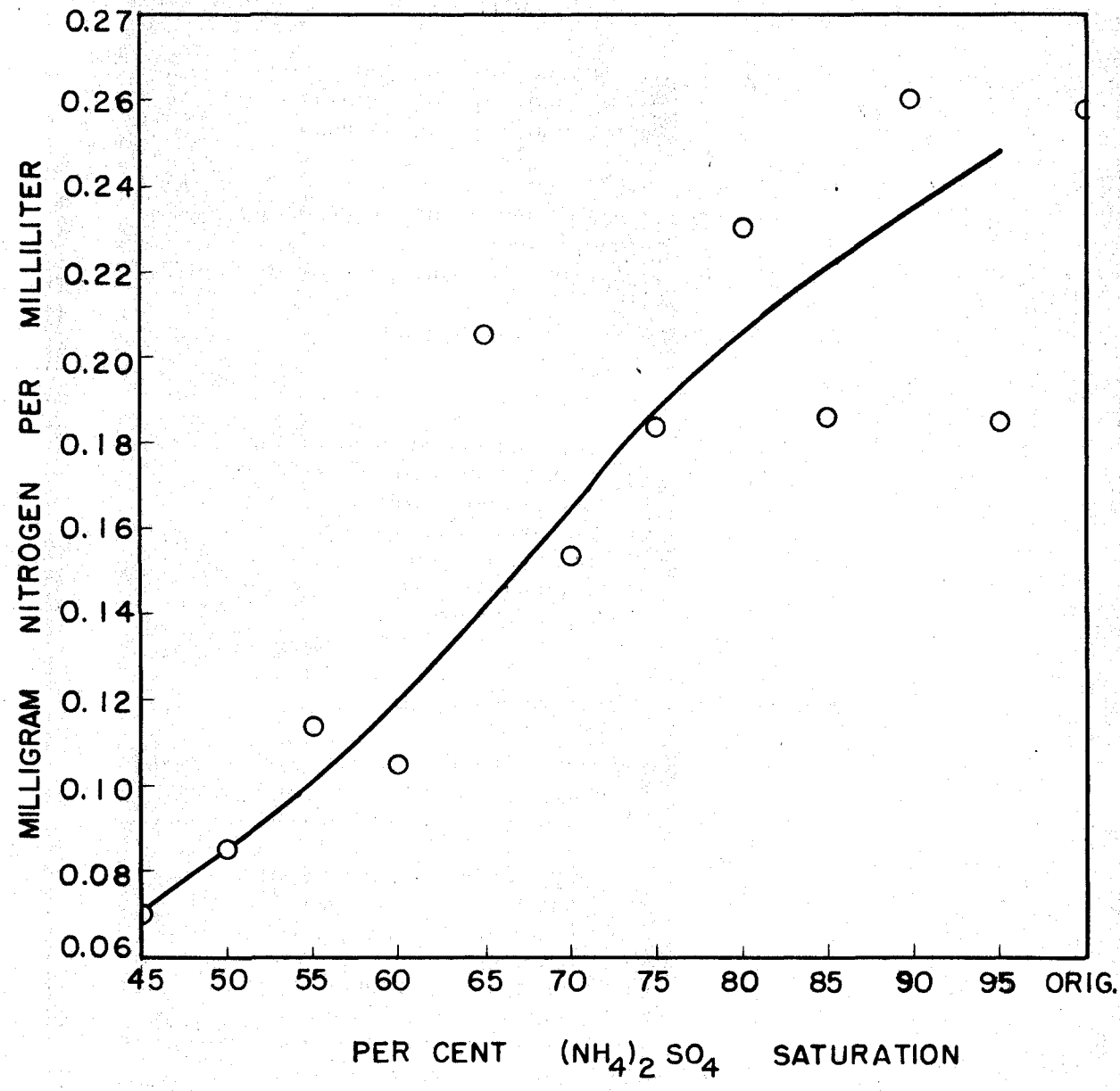


Fig. 8 Precipitation of Nitrogen-Containing Material from Crude Filtrate by Ammonium Sulfate

portions of original mold filtrate until the solutions were 65 per cent saturated. The solutions were combined and chilled overnight in the cold room. The solutions beneath the floating masses of precipitate were removed by siphoning. The semi-solid slurries were combined and the volume measured before they were discarded. The combined slurries had a volume of 350.0 milliliters, which meant that the corresponding volume of approximately 254.0 milliliters of original solution was discarded.

Solid ammonium sulfate was added until the solution was 95 per cent saturated with ammonium sulfate. Centrifuging the solution at 1,800 revolutions per minute in 250.0 milliliter bottles failed to bring down the precipitate particles. The solution was therefore vacuum filtered with a water aspirator, using Eton and Dikeman 613 filter paper, 18.5 centimeters in diameter. The filtrate came through the filter paper as a clear, yellow solution, whereas the flocculent material was retained by the paper. Due to the tendency of the paper to become clogged, several different sheets of filter paper had to be used. The materials were removed from the filter papers and dissolved in about 500.0 milliliters of distilled water. The filter papers were rinsed with several portions of distilled water and the solutions combined and diluted to 2.0 liters. This solution was labeled "enzyme concentrate E" for future reference. After removal of the precipitates, the filter papers were soaked in two 250.0 milliliter portions of distilled water, but due to disintegration of the cellulose these solutions were discarded.

The solution of enzyme concentrate E had a volume of 2,050 milliliters and a concentration of 15.5 units of maltase activity per milliliter, or a total of 31,800 units. The first 250.0 milliliter portion of distilled

water used for soaking the filter papers had a concentration of 24.5 units per milliliter, or a total of 5,380 units. The second solution of 250.0 milliliters contained 4.42 units per milliliter, or a total of 1,100 units. Thus the total sum recovered by this method was 38,390 units of maltase activity.

The analysis of the mother liquor showed no trace of maltase activity.

(b) Second ammonium sulfate precipitation: The protein material of enzyme concentrate E was again precipitated with ammonium sulfate as before, collecting the fraction between 67.5 per cent and 85 per cent saturation. The material was dissolved in 200.0 milliliters of distilled water, placed in dialysis bags and dialyzed against distilled water in the cold room, changing the charges of distilled water once every 8 hours. The final volume of the solution was 338.0 milliliters. This solution was labeled "enzyme concentrate F" for future reference. The solution contained approximately 106.0 units of maltase per milliliter, 8.56 units of alpha-amylase per milliliter, and 1.50 milligrams of nitrogen per milliliter.

The filter paper electrophoresis conducted upon this last solution for 13 hours indicated a streak of protein material, possibly divided into two portions, extending 2.6 to 6.1 centimeters from the point of origin. The apparent centers of the spots were 3.9 and 5.6 centimeters from the point of origin. A very faint spot was observed at 11.5 to 11.8 centimeters from the origin, but this may have been an artifact. The solution electrophoresis diagram of this solution displayed three peaks corresponding to protein material.

(c) Acetone precipitation of purified enzyme solution: In an effort to further concentrate and purify the enzymes, precipitation by aqueous acetone was employed. Five milliliters of enzyme concentrate F were transferred by pipette to each of seven stainless steel 40.0 milliliter centrifuge tubes. Twenty milliliters of an acetone-water solution were slowly added with stirring to each solution. The relative quantities of acetone and water in the solutions added were varied so that the acetone content of the resulting solutions varied between 40 and 70 per cent in 5.0 per cent increments.

The solutions after addition and mixing were allowed to stand for 15 minutes, and were then centrifuged in the Servall centrifuge for 20 minutes at 13,000 revolutions per minute.

The mother liquors were decanted and saved for analysis, 2.0 milliliters being used for the maltase analysis. The precipitates were each dissolved in 10.0 milliliters of distilled water and analyzed for maltase activity (1.0 milliliter being used for the analysis), and nitrogen. The experimental results are given in Table 13. The analytical values were weighted to refer to quantity of substance per milliliter of original solution.

Table 13

Precipitation of Maltase Activity, and Nitrogen-Containing Material from Enzyme Concentrate F by Acetone

Per cent of acetone by volume	Material in mother liquor*		Material in solution containing precipitate*	
	Maltase activity	Milligrams nitrogen	Maltase activity	Milligrams nitrogen
0	106	1.11		0.38
40	106			
45	104		No activity within limits of experimental error	
50	91			
55	87	0.77		0.58
60	89			
65	73.6	0.53		0.77
70	- -			

(d) Adsorption upon starch from aqueous acetone solution: Several attempts were made to establish the conditions necessary for adsorption of the maltase-active fraction upon starch.

First experimental adsorption: The first experiment was an attempt to adsorb the enzyme upon soluble starch. The soluble starch was washed with acetone and dried. One-tenth of a gram of this material was placed in each

*The units were calculated on the basis of a milliliter of enzyme concentrate F.

of a series of glass centrifuge tubes each containing 12.0 milliliters of an acetone-water solution in such concentration that when the volume was diluted to 15.0 milliliters, the resulting concentration of acetone would be 0, 10, 20, 30, 40, 50 and 60 per cent by volume.

Three milliliters of enzyme concentrate F were added to each tube and the slurry was stirred for 2 minutes (from the time of the addition of the enzyme solution) while immersed in a bath of ice water. The solutions were then centrifuged for 5 minutes at 3,200 revolutions per minute.

The upper solutions were decanted, and 9.0 milliliters of distilled water and 1.0 milliliter of phosphate buffer solution of pH 7.0 were added to the precipitate in each case. The solutions were well stirred and allowed to stand for several hours at room temperature. The mixtures were then centrifuged and the supernatants analyzed.

Within the limits of experimental error, no alpha-amylase or maltase was adsorbed except in the case of 60 per cent acetone, and then only a small amount.

Second experimental adsorption: The second trial was made with a larger concentration of starches (20 grams per 100.0 milliliters of solution) of various kinds for a longer adsorption time.

The starches used for this experiment included C.P. soluble starch,* rich starch,** potato starch,** and corn starch.** These materials were soaked in acetone for 4 hours, filtered and dried.

---

*Merck & Co., Inc., Rahway, New Jersey.

**General Chemical Co., New York, New York.

Two grams of each starch were added to 12.0 milliliters of an equal mixture of acetone and water. Three milliliters of enzyme concentrate F were added by pipette. The suspension was stirred vigorously for 20 minutes and centrifuged. The supernatant solution was decanted and a solution of 14.0 milliliters of distilled water and 1.0 milliliter of Clark and Lubs phosphate buffer* was added to the precipitate. The slurry was well mixed and allowed to stand in the cold room overnight. The suspension was centrifuged and the solutions decanted and analyzed for maltase, alpha-amylase, and nitrogen.

The analytical values reported in Table 14 were weighted so that the units of activity refer to the quantity of substance per milliliter of original solution.

---

*29.63 ml. of 0.1 normal NaOH + 50.0 milliliters of 0.1 normal KH_2PO_4.

Table 14

Adsorption of Maltase Activity, Alpha-amylase Activity, and Nitrogen-Containing Material from the Partially Purified Solution by Various Starch Materials

		Maltase activity		Alpha-amylase activity		Milligrams of nitrogen	
		Units per milliliter	Per cent of possible total*	Units per milliliter	Per cent of possible total**	Per milliliter	Per cent of possible total***
Control	E.#	trace	--	0.18	2.12	--	trace
	M.L.†	98.0	93.1	7.66	90.2	1.49	99.4
Soluble starch	E.	37.9	36.1	6.75	79.5	0.80	53.3
	M.L.	61.7	58.7	0.48	5.6		
Rice starch	E.	48.9	46.6	4.8	56.0	0.63	42.0
	M.L.	25.3	24.1	2.4	28.4		
Potato starch	E.	37.9	36.0	2.54	29.9	0.66	44.0
	M.L.	71.0	67.6	5.41	63.7		
Corn starch	E.	62.3	59.3	8.52	100.0	0.98	65.3
	M.L.	39.5	37.6	0.4	0.47		

*Assuming a maltase content of 105 units per milliliter.

**Assuming an alpha-amylase content of 8.5 units per milliliter.

***Assuming a nitrogen content of 1.50 milligrams per milliliter.

#E = eluate obtained from precipitate.

†M.L. = original mother liquor.

V. DISCUSSION

It was apparent from the Review of Literature that the enzyme fraction known as "limit dextrinase" was in all probability an amylo-1,6-glucosidase, an enzyme capable of hydrolyzing the alpha-1,6-glucosidic linkages found either in starch molecules or fragments occurring from starch hydrolysis. It was by no means certain, however, that the enzyme possessing this action was specifically limited to this type of linkage. In addition, although it was probable that this enzyme was a hydrolase, it was not at all certain that it was not a transferase, capable of transferring the glucose units which were at the ends of the limit dextrin molecule and which were resistant to hydrolysis, to some other linear fraction in which they would be more susceptible to hydrolysis by the other enzymes known to be present.

To study these possibilities it was desirable to purify and study the action of both limit dextrinase and the alpha-1,4-glucosidase enzyme systems. The course of the investigation of the limit dextrinase purification was followed by the analytical procedure of Back, Stark and Scalf (1), although in the latter stages of the work a method was investigated which could probably be developed into a much more convenient procedure. The investigation of the alpha-1,4-glucosidase purification was followed by the standard maltase determination (99), although, of course, this indicated the presence of only alpha-1,4-glucosidase whereas a number of other glucosidic enzymes could be present.

A. Purification of Limit Dextrinase

Underkofler and Roy (103) had published a procedure for purification and concentration of limit dextrinase from the enzyme preparation of Aspergillus oryzae formerly produced by Enzymes Incorporated, of Eagle Grove, Iowa. Unfortunately this plant was no longer in operation and the crude starting material for which this procedure had been developed was no longer available.

Miller (65) used several commercial preparations of Aspergillus oryzae filtrates in attempts to apply Roy's purification scheme. His results indicated that another method would have to be developed for the commercial preparations available.

1. Concentration and purification of fungal limit dextrinase

Attempts were made in the present work to modify the procedure of Roy and Underkofler so that it could be applied to filtrates of cultures of Aspergillus niger NRRL 330, the organism found by Miller to be the best producer of limit dextrinase.

An exploratory attempt was made using a small quantity of filtrate from cultures grown in one-liter wide-mouthed Erlenmeyer flasks. The experimental procedure and results have been described on pages 70 through 73. The first two ammonium sulfate precipitations in the procedure gave yields of 68.8 per cent and 50.3 per cent respectively of the original limit dextrinase present. Due to lack of time the subsequent solutions were not analyzed, but the enzyme activity by the time the step calling for the dialysis had been reached had decreased to approximately 7.9 per

cent of the original content. This yield was too small for the procedure to be of practical use.

Another attempt to apply the procedure was made on a larger scale, as described on pages 74 through 83. Twenty-five gallons of medium were inoculated with sporulated Aspergillus niger NRRL 330 mold bran. The aeration was stopped after 6 days when the limit dextrinase content was 8.7 units per milliliter. Two ammonium sulfate precipitations were made but a modification was necessary after the first precipitation. When the ammonium sulfate was added to the solution of the first precipitate, the mixture was so viscous that all attempts at centrifugation failed. It proved to be necessary to dilute the solution and remove the suspended material by centrifugation before proceeding.

The second precipitate was dissolved and the suspended material removed by centrifuging. The limit dextrinase activity of the solution containing the second precipitate was approximately 190 units per milliliter. This loss was approximately 78 per cent of the original enzyme activity. The third ammonium sulfate precipitation was made with a considerably higher preservation of limit dextrinase activity than the preceding precipitations.

When the directions in the procedure concerning the dialysis were carried out, the nitrocellulose bags had a very decided tendency to rupture, indicating the probable presence of a cellulase.

The treatment with mercuric chloride resulted in a drastic reduction in the limit dextrinase activity of the solution.

The rest of the procedure was conducted as given in the article by Underkofler and Roy (103). Only at the last step was any material obtained

which had crystalline properties, and this material when analyzed had very little, if any, limit dextrinase activity.

The maltase activities of a number of the solutions were also determined and the results are given in Table 15, together with the corresponding limit dextrinase activities.

From these results it was apparent that, although the method of Underkofler and Roy developed for Aspergillus oryzae could be used as the basis for concentration of limit dextrinase of Aspergillus niger NRRL 330, the yields were very low, particularly in the last stages. The early three ammonium sulfate precipitations may be used for the concentration of limit dextrinase and its partial separation from maltase, but the loss of activity was so great in the latter portion of the procedure that an alternative method would be desirable.

As the relative ratios of limit dextrinase and maltase varied during the purification procedure, it was concluded that different protein components were responsible for the limit dextrinase and maltase activity. These values are given in Table 15.

2. Alternative methods of limit dextrinase analysis

The lengthiness of the analytical procedure for limit dextrinase developed by Back, Stark and Scalf (1) severely delimited the possibilities of research and accordingly several possible alternative methods of analysis were investigated. The detailed experimental procedure and results have been described on pages 84 to 100.

If limit dextrinase were an alpha-1,6-glucosidase, it would be expected to attack bacterial dextran, composed predominantly of

Table 15

Comparison of Maltase and Limit Dextrinase Concentrations at Various Steps of the Second Limit Dextrinase Purification

Solution analyzed	Maltase activity per milliliter	Total maltase activity	L.D. activity per milliliter	Total L.D. activity
Original solution (42.0 liters)	6.3 units	264,000 units	8.7 units	3,650,000
First precipitate dissolved in 4.20 liters	11.6 units	48,700 units	25.2	1,060,000
Second precipitate dissolved in 420.0 milliliters	16.2	6,800 units	205	860,000
Third precipitate after Bentonite but before dialysis, in 55.0 ml.	182	10,000 units	1,152	633,000
Solution after dialysis but before $HgCl_2$ treatment	43	1,370 units	206	6,590

alpha-1,6-glucosidic linkages, panose, a trisaccharide possessing an alpha-1,4- and an alpha-1,6-linkage, and particularly isomaltose, a disaccharide with a 1,6-glucosidic bond. Dextran, panose, and an impure preparation of isomaltose were available for experimental purposes, and these materials were used for this investigation.

(a) Hydrolysis of dextran: A detailed account of the experimental procedure and results has been given on pages 84 to 91.

The dextran used was of the type produced by Streptobacterium dextranicum NRRL B-1254, and contained alpha-1,6- and alpha-1,4-glucosidic linkages in a ratio of about 12 to 1. The hydrolysis did not appear to be appreciably affected by the pH at which the experiments were conducted, and was not appreciably affected by the type of buffer used. However, the rate of hydrolysis was influenced by the temperature employed. The greatest degree of hydrolysis, 4.9 per cent of the theoretical maximum, was obtained after 5 hours at 49° Centigrade.

The extent of hydrolysis was so slight that the possibility could not be excluded that the alpha-1,4-glucosidic linkages were the ones being hydrolyzed.

Chromatography indicated the presence of no sugars capable of movement. However, the possibility was not eliminated that the action of the enzyme was to split the dextran molecule into comparatively large fragments having little reducing power.

A possibility for further research suggested by these results was the study of the effect of the enzyme preparation upon the viscosity of dextran solutions. If the method of enzyme action were similar to that of

alpha-amylase, the hydrolysis should produce comparatively large fragments having little reducing power and cause a drastic decrease in the viscosity of the solution. A method of analysis could be developed which would be similar to the method of alpha-amylase determination which involves measurement of the viscosity change in amylose solutions under standardized conditions.

On the other hand, if limit dextrinase does attack the alpha-1,6-glucosidic linkages in the short chain limit dextrins, it does not follow that the enzyme would also act upon such linkages in the high molecular dextrans, at least very rapidly. The "maltase" of Aspergillus oryzae (21) would serve as an analogy. This enzyme does act upon starch slowly, but very much more slowly than upon maltose and other relatively low molecular weight compounds with alpha-1,4-glucosidic linkages.

(b) Hydrolysis of panose: A detailed account of the experimental procedure and results obtained with panose has been given on pages 91 to 96.

Panose is a trisaccharide composed of three glucose residues, joined by one alpha-1,4- and one alpha-1,6-glucosidic linkage. The effects of crude, concentrated Aspergillus oryzae filtrate (Clarase), as well as that of a concentrated, but impure, solution of high limit dextrinase activity, upon a solution containing panose were investigated.

The experimental results were summarized in Table 7 on page 97. The maximum hydrolysis obtained with the first solution was after 180 minutes with 70.7 per cent hydrolysis, after which the apparent extent of hydrolysis began to decrease, reaching a value of 48.5 per cent hydrolysis after

23 hours. The solution containing the crude preparation of Aspergillus oryzae extract reached 96.8 per cent of the possible hydrolysis after 4 hours.

These results could be explained by the presence of an enzyme, present in the Aspergillus niger NRRL 330 preparation but absent in the Aspergillus oryzae preparation, which was capable of converting reducing sugars into non-reducing sugars.

(c) Hydrolysis of isomaltose: The ability of the enzymic preparation to hydrolyze isomaltose, the disaccharide composed of two glucose units connected by an alpha-1,6-glucosidic linkage, was also investigated with the results indicated on pages 96 to 100. Chromatographically pure isomaltose was not available but experiments were conducted on a solution of isomaltose which contained glucose as well as higher carbohydrates.

The experimental results reported in Table 7 under reference 5 indicated that isomaltose was definitely susceptible to hydrolysis by a solution quite low in maltase activity but containing moderate amounts of limit dextrinase activity. After 4 hours, the isomaltose had undergone 68.0 per cent hydrolysis, although the maltase activity of the solution was quite low (0.65 units per milliliter), while the limit dextrinase activity was only moderate (7.6 units per milliliter). The extent of hydrolysis was of the same order of magnitude as that found by Tsuchiya, Montgomery and Corman (101), who worked with the crude filtrate of Aspergillus niger NRRL 330.

The results indicated the presence in the culture filtrate of Aspergillus niger NRRL 330 of an enzyme capable of hydrolyzing the alpha-1,6-glucosidic linkage of isomaltose that was quite distinct from the enzyme

associated with maltase activity. At this stage the work on limit dextrinase was halted, and the behavior of the fraction possessing the maltase activity was studied.

For future work, the author would definitely recommend that the hydrolysis of isomaltose with subsequent determination of reducing sugar be developed as an analytical procedure. Our experiments along this line, limited as they were, were definitely encouraging.

B. Purification of Maltase

1. Production of mold filtrate

Aspergillus niger NRRL 330 was grown in submerged culture upon 50.0 gallons of the medium of Shu and Blackwood (91). Three liters of a 24-hour old vegetative culture were used to inoculate the medium, and the culture was grown for 6 days before the growth was halted. Twenty-three gallons of the unfiltered culture were placed in the cold room and allowed to stand overnight after which the clear solution was removed by siphoning and preserved under toluene. The portion of the solution containing the mycelia was discarded and the remaining portion used for all of the work summarized below and reported in detail on pages 100 through 132.

2. Paper chromatography and electrophoresis

Both buffered aqueous solutions of ammonium sulfate and of acetone were used for the developing solutions in paper chromatography upon Whatman No. 1 filter paper. The buffered aqueous-acetone solution proved to give the better separations. Various techniques for staining the spots containing proteinaceous material were tried.

A modification of the method of Geshwind and Li (24) which utilized bromphenol blue was developed for the paper chromatography of these protein solutions. This method is both sensitive and specific for proteins, according to the reference. Either the bromphenol blue or the ninhydrin method could be used, and either was preferable to the bromthymol blue method. The best separation of protein material occurred at 55 to 60 per cent acetone.

3. First purification procedure

The first purification procedure was discussed in detail on pages 100 to 120. The original mold filtrate contained 4.7 units of maltase activity per milliliter.

(a) Precipitation with ammonium sulfate: An experiment was conducted to determine the desirable concentrations of ammonium sulfate to use for a preliminary precipitation and separation from the remaining mold mycelium and other gummy material. It was found necessary to use the small Servall centrifuge at its full speed (about 12,000 revolutions per minute) in order to effect complete separation of the material possessing the enzyme activity. The results were given in Table 8 and Figure 1.

Sixteen liters of the clear supernatant solution were used for this first precipitation. The fraction precipitated between 50 per cent and 90 per cent ammonium sulfate saturation was dissolved in 1.6 liters of distilled water. The maltase activity of this solution was 12.3 units per milliliter. The recovery of this step was 47.9 per cent of the possible recovery indicated by the preliminary precipitation, or 26.1 per cent of

the enzymic activity in the original mold filtrate. This low yield was probably due in part to the procedure used for handling the large volume. When the concentration of ammonium sulfate had been increased to 90 per cent of saturation the solution was allowed to stand overnight. A flocculant precipitate rose to the top and the under-lying solution was removed by siphoning. Although this procedure served to concentrate most of the precipitate, there were numerous particles observed floating in the solution removed from the precipitate. The suction filtration used in the second purification procedure was preferable.

Another probable cause for low yield was unavoidable and consisted of the pH chosen for the precipitation. The isoelectric point of the protein fraction having the maltase activity was determined upon the 1.6 liter solution containing the first precipitate by determining the quantity of maltase activity in the precipitate at various pH levels and concentrations of ammonium sulfate. It was apparent from the results given in Table 9 that there was one protein fraction possessing maltase activity that had an isoelectric point of 5.0 and another that had an isoelectric point of 7.0 or above. As our precipitation was conducted at a pH of 5.0, the other protein fraction would tend to be eliminated. However, it was possible that there was present in the solution an unknown substance with an isoelectric point of 7.0 or above, which, when precipitated, acted as an adsorbent for the protein fraction with maltase activity. The first possibility, that there were at least two fractions possessing maltase activity, was supported by the discovery by Gillispie, Jermyn and Woods (25) that there were numerous protein fractions in the filtrate of Aspergillus oryzae possessing cellulase and proteinase activity.

A second ammonium sulfate precipitation was conducted upon the remaining solution of 1.38 liters without dialysis. The solution contained an unknown quantity of ammonium sulfate, and thus the absolute ammonium sulfate content was unknown. The fraction precipitated between 30 and 70 per cent apparent ammonium sulfate saturation was dissolved in 300.0 milliliters of distilled water.

(b) Precipitation with organic solvents: A portion of the solution containing the second ammonium sulfate precipitate was used to conduct the preliminary investigation of the precipitation maltase activity using organic solvents as described on pages 113 through 117. The analytical results were reported in Table 10 and Figure 3.

The three organic solvents investigated were acetone, dioxane, and ethanol. The precipitation with the three solvents gave similar results but acetone gave almost quantitative recovery of enzyme. Either incomplete precipitation or partial denaturation occurred with dioxane and ethanol. With all three solvents very little precipitation of enzymic material occurred below 35 per cent solvent by volume, but thereafter the rate of enzyme precipitation rose sharply.

The remainder of the solution containing the second precipitate was then precipitated with acetone, the fraction between 45 and 64 per cent acetone concentration by volume being saved and dissolved in 100.0 milliliters of water. The only area revealed by paper chromatography to contain protein also displayed maltase activity. The maltase activity was detected by spraying strips of the paper chromatogram with a solution of maltitol,

the polyhydroxy alcohol produced by reducing maltose, and incubating the strips in a warm atmosphere. The strips were then dried, and sprayed with the Tauber and Kleiner (96) reagent. Blue spots occurred at the origin, indicating the presence of the enzyme at that location. However, the solution electrophoresis conducted upon this same solution indicated several peaks corresponding to protein components, as would be expected, the electrophoresis analysis being more sensitive than paper chromatography. Analysis of this solution indicated considerable alpha-amylase activity remaining after these precipitations although the paper chromatography had indicated only one component.

A more detailed study of the purification of enzyme by acetone precipitation was made during the second purification procedure.

(c) Precipitation using other salts: The attempts made to utilize sodium chloride or potassium dihydrogen phosphate to precipitate the enzyme possessing maltase activity, either alone, or in combination with acetone were described on pages 119 and 120. The results of these attempts indicated that these salts were ineffective since either ammonium sulfate or acetone alone gave better precipitations.

(d) Stability of maltase activity at various pH levels: An experiment to determine the stability of maltase activity at various pH levels has been described on page 118, and the results given in Table 11 on page 119.

A series of seven buffers were made up varying by increments of 0.5 between the pH levels of 4.0 and 7.0. A solution containing a definite amount of maltase activity was added, the tubes incubated at 30° Centigrade,

and samples were withdrawn periodically for analysis. After 96 hours there was only a slight decrease in enzyme activity in a few of the tubes in the vicinity of the isoelectric point. There was a 6.4 per cent loss of activity at the pH of 5.0, and a 6.7 per cent loss of activity at the pH of 5.5. There was obvious bacterial contamination at pH 7.0, with 7.5 per cent loss of enzymic activity.

4. Second purification procedure

The usual procedure followed in purifying an enzyme is to follow the results of the work by analyzing for the desired enzyme alone for at least the first few steps, trusting that the preliminary purification procedure will eliminate the other enzymes not having a similar nature. However, the final product of the preceding method still contained considerable quantities of alpha-amylase activity. As the electrophoretic pattern still contained several small peaks corresponding to protein material, another, more exact, procedure was developed for purification.

The mold filtrate stored in the cold room under toluene still contained 4.52 units of maltase activity per milliliter after 3 months of storage. This was used for the purification attempt summarized below and described in detail on pages 121 through 132.

(a) Precipitation with ammonium sulfate: A more precise determination of the solubility of the maltase-active fraction in ammonium sulfate solutions was conducted on the original mold filtrate. The ammonium sulfate concentration in 11 portions of the crude mold filtrate was varied in 5 per cent increments from 45.0 to 95.0 per cent saturation. The

precipitates removed from the solutions by centrifuging were redissolved and analyzed for maltase and alpha-amylase activity as well as for nitrogen content. The procedure has been described in detail on pages 121 through 132. The experimental results have been compiled in Table 12 and Figures 4, 5 and 6.

It was found that by collecting the fraction between 67 per cent and 95 per cent ammonium sulfate saturation that 65 per cent of the alpha-amylase activity and 69.9 per cent of the nitrogen content would be eliminated while 66.5 per cent of the maltase activity would be retained.

If the specific activity of an enzyme is defined as units of enzyme activity per one milligram of protein nitrogen, the following terms may be defined (89) as indicated:

Enrichment: the ratio of the specific activity after a step to the specific activity before a step.

Yield: the ratio of the total enzyme activity after a step to the total enzyme activity before the step.

These definitions of the terms were used by the authors of the reference to derive the following equation which may be used to choose the more desirable of several possible procedures for enzyme purification.

$$\log E = (\log e)(\log Y)/(\log y),$$ where

E = overall enrichment

e = enrichment of step

Y = overall yield

y = yield of step.

Tables 16, 17, 18 and 19 were prepared from the results given in Table 12 and Figures 4, 5 and 6 by assuming an overall yield of 10.0 per

Table 16

Enrichment of Maltase Activity, Assuming Ten Per Cent Recovery

Per cent $(NH_4)_2SO_4$ saturation	Specific activity	Enrichment of step (e)	Yield of step (y)	Overall enrichment (E)
80 - 95	37.2	2.14	0.296	4.22
75 - 95	43.7	2.50	0.468	16.2
70 - 95	42.4	2.44	0.680	204
65 - 95	39.1	2.25	0.744	1,480
60 - 95	35.2	2.02	0.818	3,160
55 - 95	31.6	1.82	0.875	30,900

Table 17

Enrichment of Alpha-Amylase Activity, Assuming Ten Per Cent Recovery

Per cent $(NH_4)_2SO_4$ saturation	Specific activity	Enrichment of step (e)	Yield of step (y)	Overall enrichment (E)
80 - 95	6.95	1.13	0.156	1.16
75 - 95	6.60	1.07	0.206	1.10
70 - 95	6.63	1.07	0.281	1.13
65 - 95	7.56	1.23	0.407	1.70
60 - 95	11.1	1.79	0.725	64.6
55 - 95	13.8	1.79	0.862	17,000

Table 18

Enrichment of Maltase Activity, Assuming Ten Per Cent Recovery

Per cent $(NH_4)_2SO_4$ saturation	Specific activity	Enrichment of step (e)	Yield of step (y)	Overall enrichment (E)
72.5 - 77.5	50.6	2.91	.179	3.93
70.0 - 80.0	46.7	2.68	.341	8.28
67.5 - 92.5	42.6	2.44	.452	14.3
65.0 - 85.0	34.8	2.00	.576	17.8

Table 19

Enrichment of Alpha-Amylase Activity
Assuming Ten Per Cent Recovery

Per cent $(NH_4)_2SO_4$ saturation	Specific activity	Enrichment of step (e)	Yield of step (y)	Overall enrichment (E)
72.5 - 77.5	6.25	1.02	0.0625	- -
70.0 - 80.0	6.06	0.982	0.125	- -
67.5 - 82.5	6.18	1.08	0.20	1.12
65.0 - 85.0	6.42	1.04	0.30	1.08

cent. The specific activity of the original solution was 17.0 units of maltase activity per milligram of nitrogen and 6.2 units of alpha-amylase activity per milligram of nitrogen.

This method of calculating the over-all enrichment may be used to select the method of procedure that will achieve the maximum purification for a given recovery of material. However, the expectations of the extremely high degree of enrichment were not to be taken literally, for as the purification proceeded, the precipitation properties of the enzyme fractions would probably vary, and of course, the extremely high enrichment values could be a physical impossibility due to the very size of the protein molecules possessing the desired enzymic properties.

The ammonium sulfate precipitation described on pages 122 to 127 was conducted upon 20.0 liters of the crude enzyme solution, with the fraction precipitated between 65.0 per cent and 95.0 per cent ammonium sulfate saturation being dissolved in 2.0 liters of distilled water. The total quantity of enzyme recovered in this first precipitation was 65,890 units, of which 59,400 units or 90.2 per cent of the activity was in the main solution of the precipitate in 2,050 milliliters of solution and the remainder in two solutions of distilled water used to rinse the filter paper used to collect the precipitate. The main solution was referred to on page 126 as enzyme concentrate E.

For the first precipitation the expected yield of alpha-amylase was 40.7 per cent, or 14,000 units, the yield of maltase was 73.4 per cent, or 69,300 units, and the yield of nitrogen was 33.1 per cent, or 1.72 grams of nitrogen. The total actual yield of 65,900 units of maltase activity

represented a yield of 95.2 per cent of the expected yield. No analysis was made for alpha-amylase or nitrogen.

After dialysis to remove the entrained ammonium sulfate, the main solution containing the first precipitate was again precipitated with ammonium sulfate, collecting the fraction between 67.5 and 95.0 per cent ammonium sulfate saturation. The second precipitate was dissolved in 200.0 milliliters of distilled water and dialyzed to remove the entrained ammonium sulfate. The volume of the solution containing the ammonium sulfate after dialysis was 338.0 milliliters and was referred to in the experimental section on page 134 as enzyme concentrate F.

It was assumed that the yields of alpha-amylase and nitrogen were in the same percentage of the expected yield (i.e., 95.2 per cent) as the yield of maltase. On this basis, the 2,050 milliliter solution containing the first precipitate would be expected to contain 12,000 units of alpha-amylase (= 14,000 x .952 x .902) and 1.48 grams of nitrogen.

For the precipitation performed upon the solution containing the first precipitate the expected yield of alpha-amylase was 33.2 per cent or a total of 3,980 units, the yield of maltase was 64.4 per cent or a total of 38,200 units, and the yield of nitrogen was 29.2 per cent or 0.432 grams.

The 338.0 milliliter solution containing the second precipitate was actually found to contain 108.9 units of maltase per milliliter, or a total of 36,500 units, 8.56 units of alpha-amylase per milliliter, or a total of 2,890 units, and 1.49 milligrams of nitrogen per milliliter, or a total of 0.504 grams. The specific activities of the solution were 73.1 and 5.74 for maltase and alpha-amylase respectively.

It is a general custom in enzyme purification to employ a given procedure for no more than two steps (89). Accordingly, other methods applicable for this procedure were sought.

(b) Purification with acetone: The precipitation using acetone which has been reported on pages 128 and 129 was not effective in precipitating the enzyme from the partially purified solution, although the concentration of proteinaceous material was actually higher than in the crude and partially purified solution upon which it was effective. This led to the hypothesis that the material with maltase activity in the organic-solvent fractionation of crude solutions was actually adsorbed upon certain impurities which were precipitated from the solution by the acetone.

The maltase fraction was apparently stable at cold room temperatures up to acetone concentrations of 45 per cent by volume, but with higher concentrations the inactivation was quite rapid. As the nitrogen content of the solution decreased at approximately the same rate as the maltase activity, it could be assumed from these results that either the protein fraction possessing the maltase activity was precipitated completely and a portion inactivated in the interval that the precipitate was in contact with the acetone solution (as no detectable traces of maltase activity were found in the solution containing the precipitate), or that the enzyme was unstable in aqueous solutions containing more than 45 per cent acetone. The work of Bovard (9) upon the precipitation of alpha-amylase with acetone at room temperatures suggested that fungal alpha-amylase, at any rate, was unstable in acetone solutions at concentrations above 40 per cent.

Unfortunately, no experiments were conducted to determine the stability of the maltase in acetone solutions.

Due to ineffectiveness of precipitation and the probable inactivation by acetone this procedure was regarded as being unsuitable for a purification step at this point.

The quantities of nitrogen found by the Kjehldahl analysis were in slight disagreement with each other, but this may have been due to incomplete digestion.

(c) Starch adsorption: It was logical to attempt to supply a substitute for the unknown substance that presumably absorbed the enzyme fraction and thus removed it from the solution. After preliminary trials with silica gel had been unsuccessful, starch was used as an adsorbent. The results of these investigations have been described on pages 129 through 132 and summarized in Table 14.

It was apparent from the preliminary attempt at adsorption that, first, no starch was capable of adsorbing the active protein from an aqueous solution, and second, that a comparatively large amount of starch would be desirable when aqueous acetone solutions were used. Accordingly, an experiment was made to determine the adsorption of the enzyme from a solution containing 40.0 per cent acetone by volume and 20.0 grams of solid amylaceous material per 100.0 milliliters. After separation from the supernatant solution by centrifugation the material adsorbed was eluted by a dilute phosphate buffer at a pH of 7.0. Both the mother liquors and eluates were analyzed for maltase activity, alpha-amylase activity, and nitrogen content and the results have been presented in Table 14.

The four materials used as adsorbents, soluble starch, rice starch, potato starch and corn starch, gave variable results. Soluble starch, it would appear, should be excellent to use as an adsorbent to remove extraneous protein and also alpha-amylase activity. It could even be used in a procedure for the isolation of alpha-amylase. Rice starch could be used as an adsorbent for maltase activity, but apparently made no differentiation between maltase and alpha-amylase activity, and the elution under these conditions was not complete. Potato starch was similar, but here, the particles being larger, the tendency for adsorption was less and the elution was complete. Corn starch removed alpha-amylase almost quantitatively from solution, but also eliminated about two-thirds of the maltase activity. Its use would be less desirable than the use of soluble starch.

Adsorption could be used either to remove the impurities, or to adsorb the enzyme selectively and leave the impurities in the solution. Although a thorough investigation of the possible conditions was not made, the results obtained indicated that probably the best use of the adsorption procedure would be to remove the foreign protein material rather than to attempt to adsorb the enzyme itself.

VI. SUMMARY

1. The procedure developed by Underkofler and Roy for the concentration and purification of limit dextrinase from submerged culture filtrate of Aspergillus oryzae was applied to the filtrate of a culture of Aspergillus niger NRRL 330. The first steps involving ammonium sulfate precipitations were suitable for concentration of the enzyme fraction, elimination of extraneous material, and much of the maltase activity. However, even with the ammonium sulfate precipitations it was found to be advisable to investigate the optimum concentrations and temperatures for optimum purification and yields. The subsequent steps involved too much loss of material for practical use.

2. The enzyme fraction possessing the limit dextrinase activity is distinct from that possessing at least the bulk of the maltase activity.

3. Partially purified solutions possessing moderate limit dextrinase activities but rather low maltase activities had only slight hydrolytic activity upon clinical dextran, as indicated by the amount of reducing sugar equivalent produced.

4. These same solutions, however, were quite effective in the hydrolysis of panose and isomaltose. The low maltase activity of the solution and the hydrolysis of isomaltose and panose indicated that the limit dextrinase was actually an alpha-glucosidase with a preference for the alpha-1,6-glucosidic linkage, but capable also of hydrolyzing the alpha-1,4-glucosidic linkage.

5. The rapid and virtually complete hydrolysis of isomaltose suggested the possibility of developing this procedure into a method of analysis

for the enzyme capable of hydrolyzing the alpha-1,6-glucosidic linkages in place of the lengthy procedure now in use for limit dextrinase analysis.

6. The evidence indicated that there were probably at least two protein fractions possessing maltase activity. The isoelectric point of one fraction was near pH 5.0 and the other was near pH 7.0

7. Acetone was capable of precipitating the enzyme from crude or only partially purified filtrates, but not from the solutions from which most of the protein material had been removed. The precipitation of the enzymes in the impure solutions may have been due to their being adsorbed upon some impurity which was insoluble under those conditions.

8. The protein fraction possessing the maltase activity was stable at room temperature between the pH levels of 4.0 and 7.0 for at least 96 hours, with a maximum loss of activity of 7.5 per cent.

9. The solubilities of sodium chloride and potassium dihydrogen phosphate were too slight for these substances to be effective agents for precipitation of materials from these enzyme solutions.

10. The conditions for precipitating maltase and alpha-amylase with ammonium sulfate were determined. Alpha-amylase was precipitated at slightly lower concentrations of ammonium sulfate than maltase. This may have been due to a lower molecular weight. The nitrogen contents of the solutions were also measured, and the specific activities determined.

11. Four materials, corn starch, potato starch, rice starch, and soluble starch, were tested as adsorbents. The conditions included ice-bath temperature, a 40 per cent acetone solution, and 20.0 grams of solid material per 100.0 milliliters of solution. The solutions were agitated

for 20 minutes. Soluble starch and corn starch both removed almost all of the alpha-amylase activity from the solution. One desirable plan of procedure would be first to use soluble starch to remove most of the alpha-amylase activity since it leaves most of the maltase activity in the solution. Then potato starch could be used (with subsequent elution) to remove the maltase activity from the solution and to concentrate the active material.

VII. SELECTED REFERENCES

1. Back, T. M., Stark, W. H. and Scalf, R. E. Measurement of enzymatic activity on limit dextrin. Anal. Chem. 20:56. 1948.

2. Bernfeld, P. and Studer-Pecha, H. Recherches sur l'amidon. 35. La degradation de l'amylose par l'alpha-amylase. Helv. Chim. Acta. 30:1895. 1947.

3. Beyerinck, M. W. Detection and distribution of glucase, the enzyme of maltose. J. Chem. Soc. 72 (II):183. 1897.

4. Bourne, E. J., Haworth, Sir N., Macey, A. and Peat, S. The amylolytic degradation of starch. A revision of the hypothesis of sensitivation. J. Chem. Soc. 1948:924.

5. Bourne, E. J., Sitch, D. A. and Peat, S. The enzymic synthesis and degradation of starch. Part III. The role of carbohydrate activators. J. Chem. Soc. 1949:1448.

6. Bourquelot, E. Recherches sur les propriétes physiologiques du maltose. Compt. rend. 97:1322. 1883.

7. Bourquelot, E. Sur un ferment soluble nouveau dèdoublant le trèhalose en glucose. Compt rend. 116:826. 1893.

8. Bourquelot, E. Über die Hydrolyse der Raffinose (Melitose) durch die löslichen Ferments. Chem. Centr. 1896 I:1169.

9. Bovard, F. C. Purification and characterization of fungal alpha-amylase. Unpublished Ph. D. Thesis. Ames, Iowa, Iowa State College Library. 1952.

10. Bridel, M. and Aagard, T. Sur l'hydrolyse diastasique due turanose. Compt. rend. 184:1667. 1927.

11. Brown, H. T. and Heron, J. Ueber die hydrolytischen Wirkungen des Pankreas und des Dunndarms. Ann. 204:228. 1880.

12. Caldwell, Mary L. and Adams, Mildred. In Anderson, J. A., ed. Enzymes and their role in wheat technology. N. Y., Interscience Publishers. 1946.

13. Clark, W. M. The determination of hydrogen ions. London, Balliere, Tindall and Cox. 1928.

14. Compton, A. Constancy of the optimum temperature of an enzyme under varying concentrations of substrate and of enzyme. Proc. Roy. Soc. London. B88:258. 1914.

15. Cori, Gerty and Larner, J. Amylo-1,6-glucosidase. Federation Proc. 9:163. 1950.

16. Cori, Gerty and Larner, J. Action of amylo-1,6-glucosidase and phosphorylase on glycogen and amylopectin. J. Biol. Chem. 188:17. 1951.

17. Corman, J. and Langlykke, A. F. Action of mold enzymes in starch saccharification. Cereal Chem. 25:190. 1948.

18. Cremer, H. D. and Tiselius, A. Electrophorese von Eiweiss in Filtrierpapier. Biochem. Z. 320:273. 1950.

19. Dubrunfaut, A. P. Note sur le glucose. Ann. chim. et phys. 21: 178. 1847.

20. Evans, T. H., and Hibbert, H. Bacterial polysaccharides. In Pigman, W. W., Wolfrom, M. L. and Peat, S., eds. Advances in Carbohydrate Chemistry. Vol. 2. New York, N. Y. Academic Press, Inc. 1946.

21. Fischer, E. H., and Montmollin, R. de. Purification et cristallisation de l'alpha-amylase d'Aspergillus oryzae. Sur les enzymes amylolytiques. XVIII. Helv. Chim. Acta. 34:1987. 1951.

22. French, D. and Knapp, Doris W. The maltase of Clostridium acetobutylicum. Its specificity range and mode of action. J. Biol. Chem. 187:463. 1950.

23. Geduld, R. Einfluss der Configuration auf die Wirkung der Enzyme. Koch's Jahresberichte Gärungs-Organismen. 1891:220. Quoted by Fischer, E. Ber. 27:2935, 3479. 1894.

24. Geshwind, I. I. and Li, C. H. The reaction of brom phenol blue with amino acids and peptides. J. Am. Chem. Soc. 74:834. 1952.

25. Gillispie, J. M., Jermyn, M. A. and Woods, E. F. Multiple nature of the enzymes of Aspergillus oryzae and of horseradish. Nature. 169:487. 1952.

26. Goodman, J. J. Adaptive production of amylase and lipase by three species of fungi. Science. 112:176. 1950.

27. Gottschalk, A. Alpha-D-glucosidases. In Sumner, J. B. and Myrback, K., eds. The enzymes. Chemistry and mechanism of action. Vol. I, pt. 1. New York, N. Y. 1950.

28. Hanes, C. S. The action of amylases in relation to the structure of starch and its metabolism in the plant. New Phytologist. 36: 101. 1937.

29. Haworth, W. N., Kitchen, H. and Peat, S. The amylolytic degradation of starch. J. Chem. Soc. 1943:619.

30. Hehre, E. J. Production from sucrose of a serologically reactive polysaccharide by a sterile bacterial extract. Science. 93:237. 1941.

31. Hehre, E. J. The biological synthesis of polysaccharides. Trans. N. Y. Acad. Sci. Ser. II. 10:188. 1948.

32. Hehre, E. J. and Hamilton, Doris M. The biological synthesis of dextran from dextrins. J. Biol. Chem. 192:161. 1951.

33. Hestrin, S. Fermentation of disaccharides. II. Sucrose. Wallerstein Labs. Commun. 12, No. 36:45. 1949.

34. Hockenhull, D. J. D. and Herbert, D. The amylase and maltase of Clostridium acetobutylicum. Biochem. J. 39:102. 1945.

35. Hodgman, E. D. Handbook of chemistry and physics. 28th edition. Cleveland, Ohio, Chemical Rubber Publishing Co. 1944.

36. Hopkins, R. H., Jelinek, B. and Harrison, L. E. The action of beta-amylase on potato amylose. Biochem. J. 43:32. 1948.

37. Hopkins, R. H. and Jha, B. K. The selective hydrolysis of amylopectin and amylose by beta-amylase. Biochem. J. 46:319. 1950.

38. Hotchkiss, Margaret. Evidence on the specificity of hexosidases. A comparison of the activity of Excherichia coli and Escherichia communoir. J. Bact. 29:391. 1935.

39. Hultin, E. and Nordstrom, L. Enzymes capable of breaking down dextran and the manufacture of simple sugars from dextran. Swedish Patent 133,157. Oct. 9, 1951. Abstracted in Chem. Abst. 46:3103. 1952.

40. Ingelman, B. Enzymic breakdown of dextran. Acta Chem. Scand. 2:803. 1948.

41. Jeanes, Allene. Dextran--a selected bibliography. U. S. Dept. Agriculture. A. S. C. 288. Peoria, Ill. 1950.

42. Jeanes, Allene, Wise, C. H. and Dimler, R. J. Improved techniques in paper chromatography of carbohydrates. Anal. Chem. 23:415. 1951.

43. Jermyn, M. A., II. The complexity of enzymes from Aspergillus oryzae that split beta-glucosidic linkages, and their partial separation. Australian J. Sci. Research. B 5:409. 1952.

44. Karstrom, H. Zur Spezifität der alpha-Glucosidasen. Biochem. Z. 231:399. 1931.

45. Kellner, O., Mori, Y. and Nagaoka, M. Beitrage für Kenntnis der invertirenden Fermente. Z. physiol. Chem. 14:297. 1890.

46. Kerr, R. W. Action of beta-amylase on amylose. Nature. 164:757. 1949.

47. Kerr, R. W., Cleveland, F. C. and Katzbeck, W. J. The action of amylo-glucosidase on amylose and amylopectin. J. Am. Chem. Soc. 73:3916. 1951.

48. Kerr, R. W., Meisel, H. and Schink, N. F. Corn syrups of high fermentability. Ind. Eng. Chem. 34:1232. 1942.

49. Kerr, R. W. and Schink, N. F. Fermentability of cornstarch products. Relation to starch structure. Ind. Eng. Chem. 33:1418. 1941.

50. Kitahara, T. and Kurushima, M. The diastatic enzyme systems of molds. I. Comparison of diastatic enzyme systems of several important molds. Hakko Kogaku Zasshi [J. Fermentation Tech. (Japan)]. 27:1. 1949. (Original not available. In Chem. Abstracts. 44: 7384. 1950.)

51. Klimovskii, D. N. and Rodzevich, V. J. Amylolytic enzymes and Aspergillus. Mikrobiologiya. 19:60. 1950. (Original not available. In Chem. Abstracts. 44:8423. 1950.)

52. Kneen, E. The amylases. Properties and production. In Kerr, R. W., ed. Chemistry and industry of starch. 2nd ed. New York, N. Y. Academic Press, Inc. 1950.

53. Kneen, E., Beckord, L. D., Spoerl, J. M. and Foster, B. C. Paper presented at the Annual Meeting of the Am. Soc. Cereal Chemists. 1948. Original not available. Cited in Redfern, S. Recent developments in amylase chemistry. Wallerstein Laboratories Communications. 13:41,96. 1950.

54. Krijgsman, B. J. Beiträge zur biochemischen Mikromethodik. VI. Photometrische Mikrobestimmung von Amylase und Maltase. Z. physiol. Chem. 129:57. 1923.

55. Kuhn, R. Vergleich von Hefe- und Takasaccharase. Z. physiol. Chem. 129:57. 1923.

56. Kuhn, R. Uber die Konstitution der Stärke und die verschiedenen Wirkungsweisen der Amylasen. Ber. 57:1965. 1924.

57. Kuhn, R. and Grundherr, C. E. von. Die Konstitution der Melizitose und Turanose. Ber. 59:1655. 1926.

58. Kuhn, R. and Munch, H. Über Gluco- und Fructosaccharase. Z. physiol. Chem. 163:1. 1927.

59. Le Mense, E. H., Corman, J., Van Lanen, J. M. and Langlykke, A. F. Production of mold amylases in submerged culture. J. Bact. 54: 149. 1947.

60. Lindner, P. Gärversuche mit verschiedenen Hefen and Zuckerarten. Wochschr. Brau. 17:713,763. 1900. Original not available. Cited in Chem. Centr. 1901 I:56.

61. Lipps, J. D., Roy, D. K., Andreason, A. A., Vernon, C. C. and Kolachov, P. Properties of limit dextrinase, alpha-amylase and maltase separated from submerged cultures of Aspergillus niger. Oral paper, 114th Mtg. Am. Chem. Soc. Portland, Oregon, Sept., 1948.

62. Mering, J. von. Ueber den Einfluss diastatischer Fermente auf Stärke, Dextrin und Maltose. Z. physiol. Chem. 5:185. 1881.

63. Meyer, K. H., Fischer, E. H., Bernfeld, P. and Duckert, F. Purification and crystallization of human pancreatic amylase. Arch. Biochem. 18:203. 1948.

64. Meyer, K. H., Fuld, M. and Bernfeld, P. Purification et cristallisation de l'alpha-amylase de bactérie. Experientia. 3:411. 1947.

65. Miller, R. E. The carbohydrases of molds with special reference to the content and isolation of limit dextrinase. Unpublished M. S. Thesis. Ames, Iowa, Iowa State College Library. 1951.

66. Musculus, F. and Mering, J. von. Cited in Cohnheim, O. Enzymes. p. 76. N. Y. John Wiley and Sons. 1912.

67. Myrback, K. Zur Kenntnis der Starkespaltung durch pflanzliche Amylasen. I. Biochem. Z. 285:290. 1936.

68. Myrback, K. Der enzymatische Abbau der Stärke und der Bau der Stärkemakromolekule. J. prakt. Chem. 162:29. 1943.

69. Myrback, K. and Leissner, Elsa. Über die Hydrolyse von Maltohexaose und die dabei entstehenden Produkte, vornehmlich die Maltotriose. Arkiv. Kemi, Mineral Geol. 17A:No. 18. 1944.

70. Nordstrom, L. and Hultin, E. Dextranase, a new enzyme from mould. Svenska Kem. Tid. 60:283. 1948.

71. Ohlsson, E. Über die beiden Komponenten der Malzdiastase, besonders mit Rücksicht auf die Mutarotation der bei der Hydrolyse der Starke gebildeten Produkte. J. physiol. Chem. 189:17. 1930.

72. Olson, W. J., Evans, R. and Dickson, A. D. A modification of the Kneen and Sandstedt methods for the determination of alpha- and beta-amylases in barley malt. Cereal Chem. 21:533. 1944.

73. O'Sullivan, C. On the transformation-products of starch. J. Chem. Soc. 25:579. 1872.

74. Pan, S. C., Andreasen, A. A. and Kolachov, P. Enzymic conversion of maltose into unfermentable carbohydrate. Science. 112:115. 1950.

75. Pan, S. C., Nicholson, L. W. and Kolachov, P. Isolation of a crystalline trisaccharide from the unfermentable carbohydrate produced enzymically from maltose. Abstr. 118th Mtg. Am. Chem. Soc. 1950:13A.

76. Pan, S. C., Nicholson, L. W. and Kolachov, P. Isolation of a crystalline trisaccharide from the unfermentable carbohydrate produced enzymically from maltose. J. Am. Chem. Soc. 73:2547. 1951.

77. Papastamatis, S. C. and Wilkinson, J. F. Paper chromatography of proteins. Nature. 167:724. 1951.

78. Pazur, J. H. and French, D. The transglucosidase of Aspergillus oryzae. Communication to the editor. J. Am. Chem. Soc. 73: 3556. 1951.

79. Pazur, J. H. and French, D. The action of transglucosidase of Aspergillus oryzae on maltose. J. Biol. Chem. 196:265. 1952.

80. Pfanmuller, J. and Noe, A. Enzymatic synthesis of higher carbohydrates from dextrose. Science. 115:240. 1952.

81. Phillips, Louise L. and Caldwell, M. L. A study of the purification and properties of a glucose-forming amylase from Rhizopus delemar, gluc amylase. J. Am. Chem. Soc. 73:3559. 1951.

82. Phillips, Louise L. and Caldwell, M. L. A study of the action of gluc amylase, a glucose-producing amylase, formed by the mold, Rhizopus delemar. J. Am. Chem. Soc. 73:3563. 1951.

83. Pigman, W. W. Extent of hydrolysis of starches by amylases in the presence and absence of yeasts. J. Research Natl. Bur. Standards. 33:105. 1944.

84. Rau, R. S. J. and Sreenivasan, A. Influence of pH on the diastatic activity of fungal and malt amylases. J. Am. Pharm. Assn. 37: 513. 1948.

85. Roy, D. K. and Underkofler, L. A. Purification of fungal maltase. Cereal Chem. 28:72. 1951.

86. Samec, M. and Blinc, M. Die neueren Ergebnisse der Stärkeforschung II. Kolloid Beihefte. 49:75. 1939.

87. Sandstedt, R. M., Kneen, E. and Blish, M. J. A standardized Wohlgemuth procedure for alpha-amylase activity. Cereal Chem. 16:712. 1939.

88. Schwimmer, S. and Balls, E. K. Isolation and properties of crystalline alpha-amylase from germinated barley. J. Biol. Chem. 179: 1063. 1949.

89. Schwimmer, S. and Pardee, A. B. Principals and procedure in the isolation of enzymes. In Nord, F. F., ed. Advances in enzymology and related subjects of biochemistry. Vol. 14. New York, N. Y. Academic Press, Inc. c. 1953.

90. Shu, P. Further studies on the nitrogen source for the production of amylolytic enzymes by submerged cultures of Aspergillus niger. Can. J. Bot. 20:331. 1952.

91. Shu, P. and Blackwood, A. C. Studies on carbon and nitrogen sources for the production of amylolytic enzymes by submerged cultures of Aspergillus niger. Can. J. Bot. 29:113. 1951.

92. Somogyi, M. A new reagent for the determination of sugars. J. Biol. Chem. 160:61. 1945.

93. Specht, H. Die Malzamylasen und ihre Bestimmung. Brauerei, wiss. Beilage. 5:13. Feb. 1952.

94. Suhadolnik, R. J. Oxidation of disaccharide alcohols by Acetobacter suboxydans. Unpublished M. S. Thesis. Ames, Iowa, Iowa State College Library. 1953.

95. Sumner, J. B. and Somers, G. F. Chemistry and methods of enzymes. 2nd Ed. New York, N. Y. Academic Press, Inc. c. 1947.

96. Tauber, H. and Kleiner, I. S. A method for the determination of monosaccharides in the presence of disaccharides and its application to blood analysis. J. Biol. Chem. 99:249. 1932.

97. Terui, G., Okada, H. and Fujiwara, H. A method for the separation of taka-amylase. J. Ferment. Technol. (Japan). 28:36. 1950. (Original not available. In Chem. Abstracts. 44:8394. 1950.)

98. Tsuchiya, H. M., Borud, H. J. and Corman, J. The synthetic action of an enzymatic preparation of Aspergillus niger NRRL 330. Abstr. 118th Mtg. Am. Chem. Soc. 1950:14 A.

99. Tsuchiya, H. M., Corman, J. and Koepsell, H. J. Production of mold amylases in submerged culture. II. Factors affecting the production of alpha-amylase and maltase by certain Aspergilli. Cereal Chem. 27:322. 1950.

100. Tsuchiya, H. M., Jeanes, Allene, Bricker, Helen M. and Wilham, C. A. Dextran degrading enzymes from molds. J. Bact. 64:513. 1952.

101. Tsuchiya, H. M., Montgomery, Edna M. and Corman, J. Hydrolysis of the alpha-1,6-glucosidic linkage in isomaltose by culture filtrate of Aspergillus niger NRRL 330. J. Am. Chem. Soc. 71:3265. 1949.

102. Underkofler, L. A., Guymon, J. F., Raymon, M. M. and Fulmer, E. I. A semi-micro method for the determination of reducing sugars in fermentation media. Iowa State Coll.J.Sci. 17:251. 1943.

103. Underkofler, L. A. and Roy, D. K. Crystallization of fungal alpha-amylase and limit dextrinase. Cereal Chem. 28:18. 1951.

104. Vogel, A. Preparation of Raney nickel. Practical organic chemistry. Vol. I. New York, N. Y. Longsman, Green and Company. c. 1951.

105. Waksman, S. A. and Davison, W. C. Enzymes. Properties, distribution, methods and applications. Baltimore, Md. Williams and Wilkins Co. c. 1926.

106. Weidenhagen, R. Zur Spezifität der alpha-Glucosidasen. Erwiderung auf die gleichnamige Arbeit von H. Karstrom. Biochem. Z. 233:318. 1931.

107. Weill, C. E., Burch, R. J. and Van Dyk, J. W. The amylase activity of a preparation of Aspergillus niger. Abstr. 118th Mtg., Am. Chem. Soc. 1950:14 B.

108. Whistler, R. L. and Durso, D. F. Chromatographic separation of sugars on charcoal. J. Am. Chem. Soc. 72:677. 1950.

109. Whiteside-Carlson, V. and Carlson, W. W. Enzymic hydrolysis of dextran. Science. 115:43. 1952.

110. Wijsman, H. P. Diastase considered as a mixture of maltase and dextrinase. J. Chem. Soc. 58(II):998. 1890.

111. Willard, H. H. and Furman, N. H. Elementary quantitative analysis; theory and practice. N. Y. D. Van Nostrand, Inc. 1940.

112. Willstatter, R. and Bamann, E. Trennung von maltase und Saccharase. Siebente Mitteilung uber Maltase. Z. physiol. Chem. 151:242. 1926.

113. Wistler, R. L. and Smart, C. L. Polysaccharide chemistry. N. Y. Academic Press, Inc. 1953.

114. Wohlgemuth, J. Uber eine neue Methode zur quantitativen Bestimmung des diastatischen Fermente. Biochem. Z. 9:1. 1908.

115. Wolfrom, M. L., Georges, L. W. and Miller, I. L. Crystalline derivatives of isomaltose. J. Am. Chem. Soc. 71:125. 1949.

116. Wolfrom, M. L., Tyree, J. T., Galhowski, T. T. and O'Neill, A. N. Acid degradation of amylopectin to isomaltose and maltotriose. J. Am. Chem. Soc. 73:4927. 1951.