Purification and characterization of a fungal glucosidase from Aspergillus niger

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PURIFICATION AND CHARACTERIZATION OF A FUNGAL GLUCOSIDASE FROM ASPERGILLUS NIGER

by

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et al. at school. In Eastern Asia, particularly Japan and
the use of exogenous activating agents for the production of
perhaps the largest potential use for fungal enzymes
simultaneously by the same microorganisms. We
have emphasized the multiplicity of natural enzymes produced
and have been drawn to the fact that, a number of reports
of enzymes are microorganisms within nature, direct, indirect,
little-known, but until recently, little-used sources
are dependent upon these naturally occurring enzyme systems.
In many industries, enzyme processes are expensive, and more readily available sources of en-
tirely new to be directly toward the search for better,
are preparations were found to yield remarkable results;
the textile, leather and brewing industries, as these vary-
partly from certain enzyme and plant tissues were used in
nature and function. At the beginning, crude enzyme prepa-
repeated their experiments on efforts to understand their
utitnted in the home and in industry long before men ever
active in the field. A number of these hydrolytic were
investigated in most biochemical laboratories are presently
seen in industry and are still staggeringly unknown although
the enzyme system which catalyzes the metabolic proc-

I. INTRODUCTION
China, it was early recognized that molds can perform the function of the more expensive and less readily available malt in the conversion of starches to fermentable sugars for the production of alcohol. More recently, the reports of many investigators in the United States also have indicated that the use of fungal amylolytic enzymes leads to higher alcohol yields than does the use of malt. This increased efficiency has stirred interest in the various fungal enzymes which can hydrolyze starches. This group of enzymes, now designated as fungal carboxydrases, has many and varied industrial applications in addition to its present and potential use in the grain-alcohol fermentation.

Fungal carboxydrases are now widely used in the food industries, such as in bread making, and in the conversion of acid-modified starches to sweet sirups of the desired sweetness, viscosity, and other physical and chemical properties. The pharmaceutical industries as well as the manufacture of sizing materials, adhesives and dry-cleaning preparations for the textile and paper industries have found extensive uses for these fungal enzymes.

It appears from the literature available that progress in the knowledge of the amylolytic content of fungal enzyme preparations has been rapid in recent years. Lately, the importance of amylglucosidase in the hydrolysis of starch
has been emphasized. This enzyme, formerly well known as maltase, has been demonstrated to be non-specific for the disaccharide maltose as substrate, but is also capable of hydrolyzing starch and other oligosaccharides having 1,4-glucosidic linkages. The presence of this glucosidase in fungal enzyme preparations has offered an alternative explanation for the increased yields of alcohol from grains when molds are used instead of malt for saccharification. A number of workers have indicated the importance of this factor in the production of fermentable sugars from starch by fungal enzyme preparations.

The present investigation was carried out with the hope of finding ways for the purification of the glucosidase of Aspergillus niger NRRL 330. This strain of mold has been reported to produce high yields of alcohol as a saccharifying agent, and enzyme preparations from it are known to be high in amyloglucosidase activity. In this work attempts were directed towards obtaining the glucosidase from the mold filtrate in a highly purified form which is desirable for an increased understanding of the exact nature and function of the enzyme as well as for defining the conditions for additional studies about it. The availability of pure fungal carbohydrases had in the past and will in the future
contribute much to our knowledge of starch structure and to our understanding of the mechanisms of action of these enzymes on their specific substrates.
II. REVIEW OF LITERATURE

A. Maltase and Amyloglucosidase

1. **Carbohydrate linkages split by maltase and amyloglucosidase**

Two major alpha glucosidic linkages are known to occur in the starch components. Amylose contains the alpha-1,4-linkage and this, together with the alpha-1,6-linkage, is found in amylopectin. Whelan, in a recent paper on the enzymic breakdown of starch (166), has divided the enzymes known to attack the alpha-1,4-linkages in the starch molecule or partially degraded starch molecule into two well defined groups. Enzymes of the first group can only hydrolyze a 1,4-linkage situated at the terminal non-reducing end of the molecule. The product of the hydrolytic action is therefore glucose, and the enzyme progresses along the molecule in an end-wise fashion until the linear substrate is completely converted to glucose. Chains of glucose residues united only by alpha 1,4 linkages are attacked. Maltase from bakers' yeast seems to exercise some specificity with respect to the size of the substrate, showing no action on amylose. *Rhizopus delemar* gluc amylase,
Aspergillus niger amylglucosidase, and Clostridium aceto-
butylicum maltase on the other hand, seem to exert their
action independently of the molecular size of the substrate.
The second group of the enzymes hydrolyzing alpha-1,4
linkages, namely the alpha- and beta-amylases, differ from
the first group in their inability to hydrolyze the terminal
linkages at either end of the molecule. The beta-amylase
shows some similarity to the first group of enzymes in that
its action begins at the non-reducing chain end. The final
products of the hydrolytic action of both alpha- and beta-
amylases are maltose and maltotriose. The beta-amylase acts
in an end-wise fashion while the alpha-amylase acts by
random scission.

2. Sources and some preparations of maltase and
amylglucosidase

Maltase is found widely distributed in nature in most
plant and animal tissues. Some yeasts, molds and bacteria
are good sources of the enzyme. For a true maltase, the
specific substrates are maltose and alpha-glucosides. Cer-
tain enzymes having maltase activity or causing hydrolysis
of maltose, however, have been found to hydrolyze higher
carbohydrates such as starch and dextrins into lower
molecular weight dextrine and glucose. These enzymes are therefore now preferably designated as amylglucosidases (157). The names applied to the enzymes or enzyme preparations by the original workers, however, have been retained in this review.

a. Fungal amylglucosidase. The presence in Aspergillus niger of an enzyme converting maltose into glucose was demonstrated in 1883 by Bourquelot (11). The same enzyme was found in koji (Aspergillus oryzae) in 1890 by Kellner and co-workers (68). In 1933, Weidenhagen (161) reported a maltase preparation he obtained by dialyzing commercial taka-diastase or an extract from pure cultures of Aspergillus oryzae against water and by concentrating the residue under vacuum.

Feigenbaum (29) obtained an enzyme preparation from taka-diastase which contained only maltase activity. Commercial taka-diastase dissolved in water was dialyzed against water, and in order to destroy the saccharase present, was filtered and treated with sodium hydrosulfite. After 24 hours at room temperature, the reducing agent was removed by dialysis, and the enzyme solution was concentrated by dialysis against 95 per cent ethyl alcohol.

The stability of taka-maltase toward acid pH, high temperature and aqueous alcohol (85) were used by Schwimmer
(131) in producing from taka-diatase a maltase preparation free of alpha-amylase. The use of acid and precipitation by alcohol was found particularly effective. A maltase preparation was made from taka-diastase which was suspended in water and fractionated with alcohol. The resulting precipitate with 70 per cent alcohol was suspended in water and adjusted to pH 3.0. The suspension was kept at 30° C. for 1 hour, then dialyzed, filtered and dried. Another maltase preparation was obtained from commercial Aspergillus oryzae mold bran. The enzyme was extracted from the bran with water at room temperature. The extract was squeezed through cheese cloth, centrifuged, and ammonium sulfate was added to complete saturation. The precipitate was filtered on purified diatomaceous earth, dissolved in water and brought to pH 3.0 with 1 normal hydrochloric acid. After incubation at the acid pH at 30° for 30 minutes, the preparation was cooled to 0° and precipitated with cold 95 per cent ethyl alcohol at a final concentration of 70 per cent. The resulting precipitate was dialyzed against water. The insoluble matter remaining after dialysis was removed by filtration and the resulting filtrate was dried.

Limit dextrinase, alpha-amylase and maltase were obtained from submerged cultures of Aspergillus niger by Lipps and co-workers (89). In their separation and purification of
maltase, the complete removal of limit dextrinase presented
the most difficulty. Their procedure eliminated all alpha-
amylase activity but caused destruction of much of the
original maltase and appreciable limit dextrinase activity
was still retained in their purest preparation. Their best
separation of maltase was obtained after adsorption on
Fuller's earth. The mold filtrate from submerged cultures
of Aspergillus niger after adjusting to pH 7.0 was treated
with 30 per cent lead acetate followed by 10 per cent
disodium phosphate. The mixture after stirring for 20
minutes was centrifuged, and the pH of the supernatant was
adjusted to 4.1 to 4.5 before addition of 2 per cent Fuller's
earth. Maltase was eluted from the adsorbent by a 0.2 molar
phosphate buffer, pH 7.1. The activity of the enzyme
preparation obtained was 15.8 per cent hydrolysis for
maltase and 2.7 per cent hydrolysis for limit dextrinase.
It was found that additional treatments with Fuller's earth
did not afford any further separation of the two enzymes.

Corman and Langlykke (23), in their study on the action
of mold enzymes in starch saccharification demonstrated
that the evaluation of fungal preparations should include
measurement of glucogenic activity as well as alpha-amylase
activity since the rate and completeness of starch hydroly-
sis depend to a large extent on the activity of the
carbohydrase enzyme system that can be measured by its action on maltose. For this purpose, they investigated a number of organisms of the *Aspergillus* and *Rhizopus* genera 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 were found interesting in that they produced only traces of alpha-amylase, but were still fairly efficient in the production of alcohol from starch. The culture filtrate from *Rhizopus* sp. "Boulard" NRRL 1891 was reported to contain intermediate glucogenic activity with almost no alpha-amylase activity. Glucose was liberated fairly rapidly but without any detectable traces of maltose.

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 the strain of mold used (30, 116). Workers at the Northern Regional Research Laboratory at Peoria, Illinois, have investigated the factors influencing the production of alpha-amylase and maltase by certain *Aspergilli* (152). The use of different media affected the yields of alpha-amylase and maltase, but as different media produced varying amounts of acid when metabolized, it was difficult to separate the effects of the medium itself from the attendant effects of the acid
produced. Thus, it was found that an increase in the carbon source lowered the pH while an increase in the nitrogen source raised the pH. An attempt to take care of any variation from this source was made by adjusting the media to various pH 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 essentially the same between pH 4.25 and 7.25. Increased use of thin stillage solids, a good nitrogen source, raised the terminal pH with an attendant increase in alpha-amylase production by the mold. Increased use of corn meal, a good carbon source, lowered the pH, and thus the yield of alpha-amylase. However, the increased use of corn meal increased the yield of maltase. The use of calcium carbonate, previously incorporated into the media to control the pH level (87, 28) was found to be detrimental to the highest yields of maltase. By adjusting the concentrations of distillers' thin stillage and corn meal in the medium, it was reported possible to control, to some degree, the yields of both alpha-amylase and maltase. Variations of the concentrations of the ingredients of the media affected the production of alpha-amylase and maltase in cultures of *Aspergillus niger* NRRL 330, and *Aspergillus oryzae* NRRL 458 but less markedly in cultures of *Aspergillus niger* NRRL 337. The maximum
The relative amounts of ethane- and methane and methanol were determined by the carbon dioxide and protein content contoured by the thin and blackwood axes by varying the termitene

interpretations of the common nitrogen and sodium nitrate and supplemented with pyridine 0.001 M of nitrogen. However, there is a higher yield of methane and ethylene were obtained with pyridine as the nitrogen source. However, higher yields of the enzyme were obtained with pyridine added to the enzyme, where the amount of nitrogenase was least dependent on the substrate. Variation of the enzyme, with glucose, and the production of lumin dex

rather, good yields of methane were obtained with glucose, and the production of dimethyl ether were carbon source present, methane and dimethyl ether were obtained with the culture filtrates varied widely, depending on the enzyme activity by the enzyme. The enzyme activity on the carbon and nitrogen sources on the production of ethylene tenable investigation on the effect of the variation of the

soil and per cent ground corn. The oxygen need was obtained from a medium containing 5 per cent thiamine Yield of methane reported was 21 units per milliliter

12
as well as proteolytic activity, with a corresponding
trate was obtained which was free of any measurable
addition of cold absolute alcohol. A dry matrass was
After evaporation with phosphate buffer, the matrass was preprepared by
adsorbed for a second time on butter earth. After evaporation
phosphate buffer was used to etrate the enzyme which was
then preprepared at pepsin as susceptible to absorb the matrass.
extracted with phosphate buffer at a pH of 7.4. The matrass was
extracted with phosphate buffer at a pH of 7.4. The matrass was
Direct yeast extract, calcium carbonate, and water, solid
extract, and a medium containing yellow corn,
In 1971, Roy and Underkoffer (126) reported a procedure
as ammonium acetate, served as a suitable nitrogen source.
Any nitrogen source which was a potent material donor,
for the production of high yields of the enzyme. Amount
reported that preparations containing some of these were not necessary
were kept for 6 to 8 days. In a later paper (127), it was
containing 2 per cent stachy and 6 per cent casein hydrolysate
content was obtained by culturing the matrass on a medium
or low enzyme-activity, but high matrass and initial determarch
maximun yields of the amylolytic enzyme. A culture of stachy
to achieve the maximum enzyme yield as well as to increase the
either sepharose or protein increased the time required
of the matrass. It was found that an increased amount of
unsuccessful methods tried to separate the two activities were reported.

found to be associated with matease activity. The eucarbohydrase activity was always
complete destruction of all other enzymes except the se- 
complete at 300 °C. For 1 or 2 days. Okazaki et al. reported the

Superrenatant was treated with 1 to 2 per cent mercuric 
Superrenatant completely with ammonium sulfite and the deadned

ovarian from take-dextrose. Take-dextrose solution was 
of a partially purified eucarbohydrase enzyme of Aspergillus

Very recently, Okazaki (112) reported the preparation

and 1.1 per cent dextrose activities.

there was a tight correlation, however, between the matease
and 1.1 per cent dextrose activity of matease activity were found.

A 300 Yelled the highest matease and 1.1 per cent dextrose
system of 12 different modes, found Aspergillus niger

MITER (100) in the studies on the carboxydrase

preparative was less than 10 per cent.

that the total recovery of oritlated matease in the final
procedures were reported to be extensive. It was estimated

activity. Losses of oritlated during the concentration

true of 1.1 per cent dextrose activity but with a high matease
The enzyme was extracted from extracts of fruit and juice concentrate of the enzynzyme from Phizoporus and co-workers (38) have reported the purity and concentration of 35 to 37% and 40% w/v, respectively. The procedure used for the purification and concentration of the enzyme was described in detail in the original paper. The enzyme was then purified and concentrated to a final concentration of 70% w/v. The enzyme was then dialyzed against a 0.01 M phosphate buffer, pH 7.0, and then lyophilized. The enzyme was then reconstituted by dissolving it in a 0.01 M phosphate buffer, pH 7.0, and then stored at -20°C. The enzyme was then assayed for activity using a standard assay procedure.
by 50 to 70 per cent saturation with ammonium sulfate, dialyzed, and then treated with lead acetate. The resulting precipitate was discarded, and the amylase in the supernatant was precipitated with 55 to 65 per cent saturation with ammonium sulfate, dialyzed, treated with rivanol and filtered. The filtrate was fractionated with acetone, and a fraction precipitated with 45 to 55 per cent acetone was dissolved in a small amount of water and precipitated with 42 per cent acetone, yielding hexagonal plates. The enzyme had both starch saccharifying and liquefying activities. Another crystalline amylase having the same properties as the gluc amylase of Phillips and Caldwell was reported by the same workers. The crystals were obtained when the crystalline amylase prepared as described above was left at pH 2.6 in the cold for 10 days, dialyzed and crystallized with 42 per cent acetone. The crystals so obtained were of long prismatic shape.

b. Yeast maltase. In 1894, Emil Fischer (31) discovered that an extract from dried brewers' yeast was active not only toward maltose but also toward methyl alpha-D-glucoside. Fischer established that this yeast enzyme is not identical with yeast invertase and termed it "yeast maltase".
In a later paper (66)', the same workers and Efferoén, spraying sodium thioacetate solution on corn kernels, showed some inhibitory effect upon hydrolase metathese, and suggested some hypothetical enzyme not beta-amylase but could be methyme. Wittenberg (76)' prepared a yeast extract from yeast extract together with sodium hydroxide for the extraction of methyme. Sodium dihydrogen phosphate and dihydrogen phosphate potassium, seen and Lorentz (67)', need sodium hydrogen carbonate, extractable methyme from yeast witek dihydrogen phosphate. Wittenberg, Wittenberg (76)', as a good adsorbent for yeast methyme, Wittenberg and Jamsma (77)', as a good adsorbent for the yeast methyme and Wittenberg and Jamsma (77)' and later, Wittenberg and Steedman (78)', and later, Wittenberg and Steedman (78)', and later, Wittenberg and Steedman (78)', and later, Wittenberg and Steedman (78)', and later, Wittenberg and Steedman (78)', and later, Wittenberg and Steedman (78)', and later, Wittenberg and Steedman (78)', and later, Wittenberg and Steedman (78)', and later, Wittenberg and Steedman (78)', and later, Wittenberg and Steedman (78)', and later, Wittenberg and Steedman (78)', and later, Wittenberg and Steedman (78)', and later, Wittenberg and Steedman (78)', and later, Wittenberg and Steedman (78)', and later, Wittenberg and Steedman (78)', and later, Wittenberg and Steedman (78)', and later, Wittenberg and Steedman (78)', and later, Wittenberg and Steedman (78)', and later, Wittenberg and Steedman (78)', and later, Wittenberg and Steedman (78)', and later, Wittenberg and Steedman (78)', and later, Wittenberg and Steedman (78)', and later, Wittenberg and Steedman (78)', and later, Wittenberg and Steedman (78)', and later, Wittenberg and Steedman (78)', and later, Wittenberg and Steedman (78)', and later, Wittenberg and Steedman (78)', and later, Wittenberg and Steedman (78)', and later, Wittenberg and Steedman (78)', and later, Wittenberg and Steedman (78)', and later, Wittenberg and Steedman (78)', and later, Wittenberg and Steedman (78)', and later, Wittenberg and Steedman (78)', and later, Wittenberg and Steedman (78)', and later, Wittenberg and Steedman (78)', and later, Wittenberg and Steedman (78)', and later, Wittenberg and Steedman (78)', and later, Wittenberg and Steedman (78)', and later, Wittenberg and Steedman (78)', and later, Wittenberg and Steedman (78)', and later, Wittenberg and Steedman (78)', and later, Wittenberg and Steedman (78)', and later, Wittenberg and Steedman (78)', and later, Wittenberg and Steedman (78)', and later, Wittenberg and Steedman (78)', and later, Wittenberg and Steedman (78)', and later, Wittenberg and Steedman (78)', and later, Wittenberg and Steedman (78)', and later, Wittenberg and Steedman (78)', and later, Wittenberg and Steedman (78)', and later, Wittenberg and Steedman (78)', and later, Wittenberg and Steedman (78)', and later, Wittenberg and Steedman (78)', and later, Wittenberg and Steedman (78)', and later, Wittenberg and Steedman (78)', and later, Wittenberg and Steedman (78)', and later, Wittenberg and Steedman (78)'
attempted to show that there is another enzyme, amyloglucosidase, present in yeast juice.

The production of yeast maltase has been demonstrated to be enhanced by the addition of an inducer for the enzyme synthesis. Spiegelman and co-workers (143), have shown that maltose can induce the production of maltase. Methyl alpha-glucoside has also been shown to be an inducer of maltase at concentration levels at which utilization could not be detected (144).

c. Bacterial amyloglucosidase. Maltase activity has been quite frequently reported in conjunction with bacterial amylases. Rose (124) in his studies on the amylase of Bacillus polymyxa reported the presence of maltase in the enzyme preparation together with the amylase. The two enzymes were separated by filtering through Hyflo supercel several times which seemed to selectively remove the maltase almost entirely.

Hockenhull and Herbert (56) found that the organism Clostridium acetobutylicum produced a maltase when grown in media containing maltose as the sole carbohydrate. The maltase thus formed showed no starch-splitting activity. When the organism was grown on starch media, the culture filtrates contained both amylase and maltase. The maltase
was reported to be specific for maltose and not an alpha-glucosidase.

Whelan and Naer (167, 168) stated that despite the seeming similarity between the amylases of *Clostridium acetobutylicum* and *Clostridium butyricum*, the enzyme production by the two organisms reveals an interesting difference. When grown on starch, the former secretes both amylase and maltase, but when grown on maltose, only maltase is secreted. The latter organism when grown on starch secretes only amylase but no maltase, but secretes maltase when the medium contains both starch and maltose.

Roy and Roy (125) have prepared cell-free amylase filtrates of butyl organism R-38 (Wisconsin) and 2 pure strains of *Clostridium acetobutylicum*. The filtrates were shown by established methods to contain alpha-amylase, cyanide-labile saccharifying amylase (beta-amylase type), limit dextrinase, and maltase.

French and Knapp (34) separated the maltase fraction from any detectable trace of alpha-amylase activity in an enzyme preparation from *Clostridium acetobutylicum* by adsorbing the fraction carrying the amylase activity upon starch granules from an acetone-water solution.

A pseudo-maltase activity was reported by Monod and Torriani (101). The enzyme preparation was obtained from
cultures of *Escherichia coli*, and the enzyme seemed to be able to produce a starch-type polysaccharide along with glucose. The workers proposed the name amylomaltase for this enzyme which is present in extracts of the bacteria only when cultured on maltose. In a later paper, (102) the same workers described their procedure for the preparation and partial purification of amylomaltase which yielded an enzyme solution which could decompose 5000 micromoles of maltose per hour per milliliter.

The occurrence of maltase has been pointed out in still other organisms such as *Lactobacillus delbruckii*, *Corynebacterium diphtheriae*, *Neisseria meningitidis* and others (44).

d. Other plant and animal maltase and amyloglucosidase.

Numerous other examples of the occurrence of maltase are given by Gottschalk (44) in his review of alpha-D-glucosidases. Germinated seeds of maize, barley, oat, sugar cane, soy bean, potato and peas are among those mentioned as maltase-containing species. In animals, the presence of maltase has been demonstrated mainly in the digestive tract and in blood serum. The enzyme is also known to occur in extracts from liver of some animals and in the skeletal muscle of others, as well as in the kidney of some.
3. Nature of action on different substrates and properties

a. Maltase. The enzymatic hydrolysis of maltose into glucose was first described by Brown and Heron (14) who used pancreas extract and dried, shredded intestinal wall as sources of the enzyme. A similar hydrolytic cleavage of maltose by saliva and by malt diastase was observed independently by von Mering (158). A first attempt to purify the maltose-splitting enzyme from the grains of many cereals by repeated alcohol precipitation was made by Geduld (41). He called the enzyme "glucaze", but it was not until Fischer (32) found the enzyme in yeast with a similar activity towards maltose, that the enzyme got the name "maltase" as previously suggested by Bourquelot (11). Since then, the name maltase has been used to refer to that enzyme which has for its biological substrate, maltose (4-alpha-D-glucopyranosyl-D-glucose), the disaccharide end product of amylase action on amylase, amylopectin, and glycogen. However, as has been previously stated, maltase from a number of different sources has been found to hydrolyze not only maltose but also starch and dextrins as well. Hence, a number of different names for the enzyme have appeared in the literature.
The specificity of maltase is discussed rather thoroughly by Gottschalk (44) in his review of alpha-D-glucosidases. Besides maltose, a number of maltose derivatives and some alkyl and aryl alpha-D-glucopyranosides are acted upon by the enzyme.

Leibowitz (85) has differentiated two types of maltases. One type, designated as "glucosidomaltase", is represented by yeast maltase and is characterized by a high degree of tolerance toward structural changes in the aglucon part of its substrate. This type can act upon alkyl and aryl alpha-D-glucosides and many maltose derivatives. The second type from barley malt and molds can hydrolyze only maltose, and therefore does not tolerate any structural change in the aglucon moiety of maltose.

Later investigations, however, by other workers have disagreed with Leibowitz's classification of maltases. Weidenhagen (161) claimed that in sufficient enzyme concentrations even malt and taka-diastases hydrolyze methyl alpha-glucoside. He considered all maltases to be glucosidomaltases.

Tauber and Kleiner (151) reported some enzymes from Solanum indicum, a solanaceous fruit from Siam. Some carbohydrases were observed including a maltase. It was found that not all the alpha-glucosides were hydrolyzed to
the same extent by the fruit maltase studied. Some were not hydrolyzed at all, such as methyl alpha-glucoside even after 90 hours, and others were hydrolyzed only very slowly. Phenyl alpha-glucoside was hydrolyzed only up to 4 per cent in 74 hours. Tauber and Kleiner concluded that there appear to exist 2 groups of maltases: one which splits methyl alpha-glucoside and other alpha-glucosides as well as maltose rapidly, to which they propose the name "true alpha-glucosidases"; the second group of maltases which hydrolyzes maltose rapidly, but which acts very slowly on some alpha-glucosides and does not act on others at all. It was suggested that the latter type be called "pseudo alpha-glucosidases".

While Leibowitz maintained his view of the 2 types of maltases which he named gluco-maltase and glucosido-maltase, and Weidenhagen claimed that all maltases are glucosido-maltases, Leibowitz and Hestrin (35) investigated further the specificity of the maltose-splitting enzyme. In the course of a series of tests on the heat stability of different lots of taka-diastase, Leibowitz and Hestrin, however, found some taka-maltase preparations which were peculiarly resistant to inactivation by heat. When a solution of the enzyme was heated to the boiling point, the resistant preparations lost their ability to split sucrose and also
what slight activity they formerly possessed to split methyl alpha-glucoside. They retained, however, or regenerated after heating most of their ability to split maltose. It was concluded, therefore, that the inversion of sucrose by taka-diastase is not even partially due to taka-maltase but to a distinct taka-sucrase. A further proof that yeast maltase and taka-maltase are different was furnished by a study of the effect of taka-diastase on yeast maltase solution. Yeast maltase solution incubated for a definite period of time with a sufficient concentration of taka-diastase lost completely its ability to hydrolyze either methyl alpha-glucoside or maltose. The ability of the taka-maltase to split maltose was not removed under those conditions.

To refute further Weidenhagen's theory that the maltose-splitting factor of taka-diastase is an alpha-glucosidase identical with the sucrose-splitting factor, glucosucrase, Leibowitz and Hestrin (86) reported that brief incubation at pH 3.5 completely destroyed taka-sucrase, while the heat stable taka-maltase retained full activity. This provided further proof that the two enzymes are distinct from each other.

A rather extensive study on the specificity of mold maltase was later undertaken by Hestrin (53). The hydrolytic
activity of different taka-diastase preparations on a number of natural and synthetic saccharides was investigated. After incubation for a long period, a very sluggish and slight hydrolysis of methyl alpha-glucoside was observed. When, however, suitable taka-diastase preparations were exposed for a brief period of time to the temperature of boiling water, their specificity range was radically changed. Taka-maltase was distinguished from other taka-oligases by its relative heat stability. Heat treated taka-diastase preparations could split maltose and maltobionic acid, but were inactive towards the other substrates studied. Taka-maltase was also found acid resistant and was separated from sucrase by acid treatment. It was found unable to effect any condensation of glucose with methanol under the same conditions in which an equal concentration of yeast maltase showed a very marked synthesis of methyl alpha-glucoside. Hestrin concluded that mold maltase specificity is not determined alone by either the glycosidic or non-glycosidic residue. It was suggested that it may be well to assume tentatively that the specificity is determined both qualitatively and quantitatively by the whole maltose molecule. The results obtained, however, were shown to be consistent with the two-enzyme theory of specificity by Leibowitz. It was demonstrated that yeast alpha-glucosidase
or yeast maltase is not the same as mold maltase, and it was suggested that mold maltase must be classed as a glucomaltase. The enzyme was presumed to be specific for the maltose molecule, and as such splits no alpha-glucoside and no C-4 substituted derivative of glucose other than maltose.

While Leibowitz attributed the maltose-splitting and the sucrose-splitting activities of taka-diastase to two distinct and specifically different enzymes, Goldhaber (43) investigated the inactivation of the enzymes by irradiation. Taka-maltase and taka-sucrase showed identical inactivation curves when irradiated by ultraviolet light. The inactivation curve of yeast sucrase, on the other hand, followed a different course. The worker stated that it appears that it is the nature of the accompanying substances (the colloid carriers) rather than the enzyme as such that is of decisive importance for the behavior of the enzyme under irradiation.

Results of numerous other investigations seem to indicate that the degree of maltase specificity toward the aglucon may vary in an unpredictable manner not only from organism to organism, but also even in the same organism depending on the conditions of growth. Three different species of Schizosaccharomyces were found to have a maltase acting upon maltose and methyl alpha-D-glucoside
(59). Sulfatase bacteria, when cultivated on maltose-agar, were noted to contain a maltase which could hydrolyze maltose, and phenyl and methyl alpha-D-glucosides. When the same organism was grown on a meat extract-peptone medium, the bacterial maltase was inactive toward the alkyl glucoside (58). Gotschalk (44) has stated that differences in the specificity of maltase of different origin, referring to the aglucon only, may be regarded as of secondary nature and minor importance. They may be due to slight variations in the chemical structure of that part of the enzyme protein to which the aglucon is adsorbed. He concluded that all maltases, irrespective of their source, must establish close contact with the glucon moiety of their specific substrates. There must be a variation in the mode of contact between the enzyme protein and the aglucon depending on the chemical structure of the latter.

One of the early studies on the action of maltase on starch was made by Schwimmer (131), in 1945, when he investigated the role of maltase in the enzymolysis of raw starch. It had previously been observed that starch could be completely converted to fermentable sugars by a mixture of enzymes extracted from hog pancreas and Aspergillus oryzae grown on bran. The difference between the hydrolysis of raw and cooked starch is just one of rate; the conversion
of boiled starch to sugars by the mixture of enzymes used was more rapid and more remarkably complete than that of the raw starch. Pancreas and Aspergillus have been noted to contain much alpha-amylase, but the action of alpha-amylase alone on uncooked starch reaches completion very slowly. Beta-amylase has been shown by other workers to have no effect on raw starch. Schwimmer, therefore, thought it obvious that some factor in addition to the recognized amylolytic ferments is involved in the rapid digestion of raw starch. Previous recorded observations have suggested that that factor may be an alpha-glucosidase such as maltase. Experiments using a preparation from pancreas containing alpha-amylase but practically no maltase, and a preparation from Aspergillus containing much maltase but no alpha-amylase suggested a role of maltase in the hydrolysis of raw starch. The complementary action of mold bran and pancreas in producing relatively complete and rapid digestion of raw starch was attributed to the presence in the mold bran of a non-amylolytic factor. That factor was thought to be probably identical with the alpha-glucosidase or maltase of mold bran.

Hookenhull and Herbert (56) in studying the amylase and maltase of Clostridium acetobutylicum also noted the effect of maltase on starch hydrolysis. It was observed
that when the organism was grown in a medium containing maltose as the only carbohydrate, the culture filtrates contained a maltase with no starch-splitting ability. When it was grown in corn-meal media, the culture filtrates contained a maltase associated with an amylase. When grown in yeast-autolysate medium with glucose or sucrose as carbohydrate source, neither the maltase nor the amylase was produced. The maltase produced in the different media was studied and showed the same properties. The enzyme appeared to be a typical maltase, differing from the other previously known maltases from yeast and pancreas only in its considerably more acid pH optimum of 4.5. The workers felt that the enzyme appeared to be a specific maltase and not an alpha-glucosidase, since it did not hydrolyze other alpha-glucosides such as sucrose. The cell-free filtrates containing both the maltase and amylase were observed to bring about complete conversion of starch to glucose. It was suggested that the action of a completely maltase-free amylase would cease altogether after reducing groups equivalent to 100 per cent maltose had been liberated, and that further conversion to glucose was entirely due to the maltase present, and not to the amylase itself.

A number of observations on maltase from different sources was made by Knapp (78). Yeast maltase was found
not specific for maltose. It could be considered to be an alpha-glucosidase since it attacked methyl alpha-glucoside at approximately the same rate as it did maltose. It failed to attack higher carbohydrates. A pseudo-maltase activity was also reported for the macerans amylase. It was designated as such since the glucose was formed not by hydrolytic scission but by an exchange reaction of the transglucosidase type. The action of saliva on 0.5 per cent maltose also indicated the production of a monose. The apparent maltase activity of saliva, however, did not parallel the amylase activity. The specificity range and mode of action of the maltase of Clostridium acetobutylicum were reported by French and Knapp (34). It was found that the maltase is a glucosidase having as its principal catalytic activity the hydrolytic removal of individual glucose units from the non-reducing terminus of a starch chain or starch oligosaccharide. On encountering a branch point in the starch structure, the enzyme is capable of hydrolyzing at least the majority of the branch linkages, resulting in extensive hydrolysis of starch or open chain dextrins to glucose. While the enzyme showed hydrolytic activity on maltose, amyloheptaose, whole starch and limit dextrins and isomaltose, it failed to hydrolyze methyl alpha-glucoside, glucose-1-phosphate, or the Schardinger
dextrins. Failure to act with any appreciable speed on the latter substrates suggests that the maltase operates best on a substrate in which glucose is joined through an alpha-glucosidic linkage to another carbohydrate unit rather than to an aglucon such as methanol or phosphoric acid. The lack of action of the enzyme on Schardinger dextrin is comparable to that observed with beta-amylase which cannot hydrolyze the cyclic dextrins.

That the enzymatic hydrolysis of maltose by yeast maltase is a reversible reaction was shown by Hill (54), who believed that maltase would not only hydrolyze maltose to glucose but that it would also synthesize maltose from glucose. With an extended reaction time, he observed that the equilibrium point reached was the same whether maltose was being hydrolyzed to glucose or glucose synthesized to maltose provided the total sugar concentration was the same. The position of the equilibrium point was found to be a function of the concentration, an increase in the concentration moving it toward more maltose and less glucose.

Emmerling (27), Armstrong and others contested Hill's view, but Pringsheim and Leibowitz (120) later demonstrated that purified yeast maltase acting at pH 6.4 and 37°C upon a concentrated solution of D-glucose for several weeks could
catalyze the formation of maltose, which he isolated in the crystalline state.

Knapp (78) likewise reported that an equilibrium was apparently established between products and reactants in the action of yeast extract on maltose. There was an observed leveling off of the apparent degree of hydrolysis of maltose with time which was believed to be due not to inactivation of the enzyme since hydrolysis could again be made to proceed at a rapid rate in a digest made by removing an aliquot from the original digest and addition of more fresh maltose.

Inasmuch as yeast maltase can hydrolyze alkyl alpha-D-glucosides, it has been demonstrated by a number of different workers in the field that the enzyme catalyzes also the synthesis of these heterosides. Gottschalk (44) in his review has enumerated numerous instances in support of Hill's original view on the reversion of maltose hydrolysis.

The literature contains a number of methods which have been employed for the determination 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 a quantitative method
of analysis for maltase activity. The specific rotation of maltose is $+137^\circ$, while that of glucose is $+52.5^\circ$. Therefore, the rotatory power of a solution of maltose progressively diminishes in the presence of maltase. From the initial rotation, and the change in rotation that would occur due to the conversion of one molecule of maltose into two molecules of D-glucose, the percentage of hydrolysis can be calculated. Quantitative methods for the determination of maltase by the polarimetric method are given by Waksman (159) and Gottschalk (44). This method, however, presents its problems when other substances that may cause mutarotation or exhibit their own optical rotatory power are present in the hydrolysis mixture.

Another method that has commonly been used for the quantitative determination of maltase activity is that of measuring the increase in reducing power due to the glucose formed. The reducing value of the resulting solution after hydrolysis may be measured by a buffered cupric salt solution followed iodometrically (142, 155), or colorimetrically by a phosphomolybdate solution (150) or an arsenomolybdate solution (108). There are difficulties, however, in the determination of reducing value due to glucose alone in the presence of maltose for an exact measurement of the degree of hydrolysis caused by the maltase action. Several methods
have, therefore, been developed for the determination of glucose in the presence of maltose. Among them is the selective fermentation method of Somogyi (141) who found that alkaline conditions suppressed the fermentation of maltose but not that of glucose. A similar method has been developed by Schultz and Kirby (130) in which they employ certain organisms for the removal of individual sugars from mixtures of several carbohydrates. Several workers have reported the differences that exist in the rate of fermentation of glucose and maltose by a given yeast (9, 51). A procedure that has been used for the determination of maltase activity of mold bran involves the evaluation of the glucose produced by a differential fermentation caused by a yeast having a very low maltose fermentation capacity in the first stages of the fermentation (40). The carbon dioxide production in the first hour following an initial 30 minutes fermentation was measured manometrically and related to glucose content. Easier and faster chemical procedures have been developed to replace the more tedious selective fermentation methods. It is claimed that the chemical methods compare favorably with the accuracy obtained by yeast fermentation. The methods of Zerban and Sattler (174) and of Phillips and Caldwell (119) employ the Soxhlet modification of Fehling's copper solution in
the presence of excess acetate. More recently developed methods involve the use of enzymes specific for glucose. Palmer (114) described an enzymatic procedure for the determination of glucose using the enzyme glucose oxidase or notatin which catalyzes the oxidation of glucose to gluconic acid by means of molecular oxygen and at a much slower rate can also oxidize mannose and galactose but not other sugars (67). The amount of glucose in hydrolysates was calculated from the oxygen uptake of the solutions in the presence of notatin and catalase and compared with the oxygen uptake of standard solutions of glucose measured under similar conditions as the test solutions. A spectrophotometric enzymatic method has been developed and described by Cori and Lerner (22), and Lerner (84). In this test, glucose was converted to glucose-6-phosphate with crystalline yeast hexokinase and adenosine triphosphate. After the reaction had gone to completion, glucose-6-phosphate dehydrogenase and triphosphopyridine nucleotide were added and the reduction of the latter was followed spectrophotometrically. Recovery of glucose from standard solutions containing 10 to 30 micrograms was found satisfactory, and good agreement within ±5 per cent with the copper reduction values for glucose was reported. The procedure is specific for glucose and suitably adapted for the
measurement of micro quantities of the sugar, but the pure enzymes and co-enzymes involved must be available.

Other methods which have not been exploited employ other organisms that selectively utilize various sugars (6). A number of reviews for the determination of the amylolytic activities of various saccharifying agents especially of fungal amylolytic preparations have been published by Drews and Specht (24, 25, 26).

b. Amyloglucosidases. This group of enzymes, like maltase, hydrolyzes maltose to glucose but converts starch and dextrins to glucose as well. Amyloglucosidase seems to be present in most fungal amylolytic preparations, and also the maltase of Clostridium acetobutylicum, as has previously been discussed, exhibits a similar starch-splitting ability in addition to its maltase action.

Members of this class of enzymes which are capable of hydrolyzing higher polysaccharides were recognized in the early work by Kerr and co-workers (73, 74) because of their ability to produce large yields of glucose from starch and starch products. It was observed that alpha-glucosidase-containing fungal preparations could split glucose from configurations in that part of cornstarch which are more resistant both to the hydrolytic action of acid and malt diastase. One fungal preparation was found able to
hydrolyze even the limit dextrins remaining after an extended hydrolysis of starch by malt diastase, materially increasing the glucose yield and decreasing the percentage of residual dextrins.

Similar activity of the alpha-glucosidase from Aspergillus niger enzyme preparation was reported by Weill and co-workers (163). Glucose was the only reducing sugar noted even in the early stages of the hydrolysis of starch by the enzyme preparation used. 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 amyloolastic 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 very little positive evidence for the presence of more than one enzyme.

Kerr and co-workers (71) later investigated the action of the amylglucosidase of Aspergillus niger on amyllose and amylopectin. The action of the enzyme was studied by comparing the wave length of minimum light transmission for the iodine hydrolysate complex with the extent of hydrolysis of amyllose as measured by ferricyanide oxidation.
From this it was concluded either that the very rapid production of reducing substance, calculated as glucose, is by random hydrolysis, perhaps in the manner shown by alpha-amylase, or that the glucosidase operates in an orderly fashion but was not entirely free of an alpha-amylase enzyme. The latter alternative was, however, eliminated when further investigations indicated that relatively large percentages of glucose were formed early in the hydrolysis. This effect is not typical of alpha-amylase activity since a large number of reports have established that glucose is produced in lesser yields, and then mostly in the later stages of alpha-amylase hydrolyses. Their results, therefore, suggest that glucose is liberated directly from the amyllose molecules. That glucose is the only lower sugar formed in measurable quantities, both in the early stages as well as the later phases of hydrolysis, was established by paper chromatography. By treatment of the enzyme preparation with acid at lower pH levels, the alpha-amylase function was inactivated more in proportion than the amyloglucosidase. At pH 2.2, substantially only amyloglucosidase activity remained, although a large part of the total enzyme activity was lost. This, however, appears to confirm that alpha-amylase and amyloglucosidase activities are two distinguishable functions. Amyloglucosidase
was found to produce glucose at the same rate, in weight per unit time, from equimolar solutions of amyloses differing widely in original chain length. At equal weight concentrations, the initial rate of hydrolysis for corn amylopectin was found to be 4.5 times the initial rate for corn amylose, but the rate for the amylopectin decreased abruptly to a lower value at a level of 60 per cent hydrolysis to glucose. The hydrolysis of a highly linear substrate, corn crystalline amylose, was found to be a first order reaction. The workers concluded that amyloligosidase action can be interpreted by assuming a terminal-wise hydrolysis of starch molecules to glucose, and in the case of the linear ones at least, according to the "single chain" mechanism. The enzyme appears to act in such a manner as to pick off successive glucose units on a molecule before proceeding to the next, similar to the action of beta-amylase as they had previously proposed.

A glucose-producing amylase from the mold *Rhizopne delemar*, already previously mentioned, was likewise investigated by Phillips and Caldwell (118). It was shown that the highly purified enzyme which was free of all detectable traces of alpha-amylase activity possessed maltase as well as amylase activities. At relatively low temperatures as 5°C and 10°C, the gluc amylase was less
sensitive than the alpha-amylase to exposure to relatively acid conditions. This was taken advantage of in the removal of traces of alpha-amylase from the enzyme preparations. Glucose was found to be responsible for practically all of the reducing value of the hydrolysates formed from starch until approximately 90 per cent of the possible glucose had been formed. The enzyme apparently was capable of splitting glucose from the nonreducing end of the glucosidic chain somewhat after the manner of the formation of maltose from its substrates through beta-amylase action. However, with gluc amylase there was no indication of the formation of limit dextrins from its action upon either the linear or 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 beta Schardinger dextrins, dextran or upon isomaltose. However, the extensive hydrolysis of the branched substrates seems to indicate that the gluc amylase must have either hydrolyzed the alpha-1,6-glucosidic linkages of the substrates or by-passed them in some manner. The failure of the enzyme to hydrolyze completely (actually 92 per cent for glycogen, 89 per cent for residual beta dextrins, etc.), together with the inactivity shown toward dextran and isomaltose suggested
that gluc amylase was inactive toward the alpha-1,6-
glucosidic linkages and that, therefore, the branching
points were by-passed. The Michaelis constants of gluc
amylase reported were $6.6 \times 10^{-3}$ M for maltose, $4.4 \times 10^{-5}$ M
for the linear substrate, and $4.1 \times 10^{-7}$ M for the branched
substrate. The values of the affinities (the reciprocal
of the Michaelis constants) 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.

Weill, Burch and Van Dyk (164) recently reported
another glucose-producing amylase from *Aspergillus niger*
NRRL 599 which seems to resemble the amylase system of
*Aspergillus niger* NRRL 330 and *Rhizopus delemar*. The
enzyme yielded large amounts of glucose from the hydrolysis
of both maltose and starch. Repeated fractionations of
the enzyme preparation increased the ratio of saccharogenic
activity to dextrinizing activity, but some dextrinizing
activity always remained in the most highly purified samples.
Glucose was the only reducing sugar obtained from the
hydrolysates of the enzyme. Phenyl mercuric chloride and
nitrites in acid solution, reagents which have been used by previous workers to inactivate amylases, did not show any effect on the amylase from *Aspergillus niger* NRRL 599. Perhaps the most interesting property of the enzyme reported is its ability to produce, from the hydrolysis of both starch and maltose, solutions which exhibited rising mutarotation. Results of other workers have indicated that the hydrolysates from some of the beta-amylases exhibit rising or beta mutarotation (82, 111), but the hydrolysates from the other amylases have been known generally to exhibit falling mutarotation. Mutarotation studies on the amylglucosidase indicated that the enzyme produces beta-glucose from maltose and starch. There was a large difference noted between the measured rotation and that calculated for glucose formed as alpha-glucose. It was not admitted, however, that there was a complete absence of alpha mutarotation, but it was demonstrated that rising mutarotation is predominant with the amylglucosidase investigated.

Another enzyme from *Aspergillus awamori* has been reported (75, 76) to produce large amounts of glucose in addition to maltose (a 4:1 ratio) from its action upon soluble starch. Kitahara and Kurushima consider the enzyme to be a distinctly new enzyme and have named it gamma-amylase.
Alpha-amylase has been successfully eliminated from enzyme preparations of the mold but all attempts to separate beta-amylase from the maltase have failed believed to be due to the identity in the physical properties of the two enzymes. A previous worker, Kitano, had reported them to be a single enzyme. In the case of enzymes from ordinary Aspergillus species it was claimed that alpha-amylase was usually isolated when the extract was treated at 50°C for 15 minutes at pH 7.0, while the fraction containing beta-amylase and maltase was obtained upon treatment at 50°C for 15 minutes at pH 3.5 (77). The enzymes of Aspergillus awamori, however, when treated similarly gave the reverse results. This behavior led the workers to the hypothesis that the enzyme carriers of alpha- and beta-amylase could be interchanged as follows:

In ordinary mold

\[
\begin{align*}
\text{alpha amylase} & = \text{coenzyme alpha} + \text{carrier A} \\
\text{beta amylase} & = \text{coenzyme beta} + \text{carrier B}
\end{align*}
\]

In Aspergillus awamori

\[
\begin{align*}
\text{alpha amylase} & = \text{coenzyme alpha} + \text{carrier B or A} \\
\text{beta amylase} & = \text{coenzyme beta} + \text{carrier B or A}
\end{align*}
\]
The workers feel that gamma-amylase and beta-amylase are alike in their modes of action on starch, and therefore, gamma-amylase has to be classified as one of the beta-amylases. However, it was claimed that gamma-amylase is different not only from the beta-amylase of malt, but also from other previously reported beta-type mold amylases as taka-maltase, gluc amylase and amyloglucosidase. While the last three mentioned amylases are known to be able to hydrolyze maltose as well as starch, gamma-amylase is reported to have no maltase activity. The enzyme is comparatively thermo-stable near pH 7, but easily inactivated at acidic reactions. These properties, believed to be entirely reversed to those of maltase, were taken advantage of in the separation of the two enzymes. It was reported that gamma-amylase has been obtained in a highly purified form and can hydrolyze starch directly to glucose without the cooperation of maltase.

The enzyme systems of molds, indeed, seem to be of greater complexity than has been suspected. Filter paper chromatography and filter paper electrophoresis together with the more recently developed modifications on them have helped disclose some of these complexities. Using filter paper electrophoresis technique, Guillepie, Jermyn and Woods (46) reported on the multiple nature of the enzymes
of *Aspergillus oryzae*. A later paper by the same group (65) 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 their previous work. The components were not only active in splitting simple glucosides but were also capable of depolymerizing sodium carboxymethyl-cellulose. The experimental evidence admitted neither the postulate for a single beta-glucosidase nor of a specific enzyme for each substrate. Presumably, the enzyme maltase has a similar multiple nature.

Although the various protein components in mold enzyme filtrates can easily be separated by chromatographic and electrophoretic procedures, the task of testing and localizing enzymes in a protein mixture still does not seem to be an easy one. A report on the detection of carbohydrases in paper electrophoresis was recently made by Wetter and Corrigan (165). Maltase, invertase and limit dextrinase from *Aspergillus niger* were detected by the procedure described. It was claimed that the enzymatic activity of 50 micrograms equivalent to 20 micrograms of protein of crude material could be detected quite easily by the technique. It is, however, limited to the detection
of carbohydases which yield glucose as one of the end products.

Another means of detecting and identifying enzymes developed by Rohdewald and Zechmeister (123) is by a chromatographic brush method. Various enzymes from a mixture have been conveniently located from a column chromatogram by brushing the column, along its main axis, with the corresponding substrate, and then, after a brief incubation period, with a suitable color reagent.

B. Other Carbohydrases Closely Related to Maltase and Amyloglucosidase

1. Alpha-amylase

The role of alpha-amylase as one of the carbohydases has already been mentioned. Alpha-amylase has often been designated as the dextrinizing enzyme due to its hydrolytic action of breaking the alpha-1,4-glucosidic linkage in starch to produce smaller dextrin fragments. The action of this enzyme causes a rapid decrease in the average chain length and complexity of the starch molecule, thus causing a rapid decrease in the viscosity of the solution with little increase in detectable reducing sugar. The dextrin
fragments formed from amylase are hydrolyzed to still smaller fragments which explains the diminishing and changing hue of the characteristic blue color with iodine during hydrolysis of starch by alpha-amylase. A second, much slower, action of alpha-amylase then occurs upon the fragments to produce a mixture of maltose and maltotriose which no longer gives a color with iodine.

Alpha-amylase is of frequent occurrence in nature. The enzyme is present in many animal and plant tissues and fluids, in germinated cereals such as wheat and barley, and many bacteria and molds such as Aspergillus oryzae and Aspergillus niger. Up to the present time, alpha-amylase from a number of sources has been prepared in crystalline form, among them human and swine pancreatic alpha-amylase (16, 17, 96), Bacillus subtilis amylase (97), Bacillus mesentericus amylase (47), malt alpha-amylase (132), Aspergillus oryzae alpha-amylase (3, 33, 137, 157), and Aspergillus candidus alpha-amylase (149).

An excellent summary of the literature on alpha-amylase is already available (12). However, additional information about alpha-amylase has appeared since that review was prepared.

A new procedure for the preparation of crystalline pancreatic amylase has been developed (16), claimed to give
higher yields of the highly active crystalline enzyme and to be less laborious and less time consuming. The crystalline enzyme was reported to be homogeneous electrophoretically as well as by sedimentation measurements in the ultracentrifuge. Other additional properties were reported; an interesting one among them is on the enzymic homogeneity. It has been previously reported by a number of other workers that pancreatic amylase exerts different kinds of enzymic activity: saccharogenic or sugar forming activity; amyloclastic or liquefying activity (4, 136); glucosidase or dextrinase activity (4, 19, 90). Studies with the highly purified amorphous preparations of pancreatic amylase have given evidence that all the above different activities are properties of the amylase itself and are not due to contaminating carbohydrases. This has now been confirmed and strengthened by data from crystalline pancreatic amylase. Perhaps, a more interesting property of the crystalline enzyme is its maltase activity. Earlier work by Bernfeld and Studer-Pecht (5), in an exhaustive study of the action of several crystalline alpha-amylases, has indicated 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. Now, it has been indicated
that three times crystallized pancreatic amylase also will have slight action on maltose if sufficiently high concentrations of amylase and substrate react for sufficiently long periods of time.

The influence of a number of factors upon the stability and upon the activity of pancreatic amylase has, likewise, been investigated (18). Previous evidence has shown that pancreatic amylase requires certain anions for its action and that chloride ions are outstanding in this respect. These findings have now been confirmed and extended.

The action of *Aspergillus oryzae* taka-amylase, believed to be free from all detectable traces of maltase activity and from any significant traces of contaminating dextrinase or other glucosidase activities, has recently been investigated (49). Comparisons have shown that the linear fraction from corn starch is hydrolyzed more rapidly and more extensively by taka-amylase than several branched substrates. When given sufficient time for the hydrolysis and sufficiently high concentrations of the enzyme, taka-amylase hydrolyzes the linear substrates completely to maltose and glucose. No evidence of other products was obtained in the final hydrolysates from the results of selective fermentation and chromatographic techniques. The results show that taka-amylase hydrolyzes trisaccharides and higher sugars
emphases action that of the exponential activity to the
investigations. While the ratio of two manure treatments of
end take-must be under different conditions have been
free take-emphases (50). The efficiencies of take-emphasis
investigation of dy or suggest that manure treatment can be removed by selective
enzymes among them do not, they have been shown
often been reported to be accompanied by a number of other
take-emphases in extenses of ambient oxygen.

Enzyme

and also besides dextrinase to dextrinase to manure and
the enzyme, however, were found to hydrolyse both the starch
points of bromine were not hydrolyzed by take-emphasis.
Fucose linked together water by alpha-1,4-D-glucosidic linkage at the
Fucose linked together water by alpha-1,4-D-glucosidic
substitutes increase, a bacterium dextrinase composed of D-
are the proportions of alpha-1,4-D-glucosidic linkage in the
hydrolysis of the branched substituents was found to decrease
freedom from corn stover. The rate and extent of the
by equivalent concentrations of take-emphasis than the treatment
readily and these experiments were under the same conditions and
substituents studied were observed to be hydrolyzed less
however, were not found to hydrolyze manure. The branched
composed of alpha-1,4-D-glucosidic linkage, take-emphasis,
saccharogenic activity remained substantially constant 
under the different conditions studied, the ratios of 
amylase activity (starch as substrate) to maltase activity 
(maltose as substrate) changed markedly, showing selective 
inactivation of maltase or of amylase. It has been observed 
that calcium ions do not protect taka-maltase as efficiently 
as they do taka-amylase from inactivation at higher tempera-
tures. It was, therefore, taken advantage of to free highly 
purified taka-amylase from the last traces of maltase 
impurities by selective inactivation at higher temperatures 
in the presence of calcium ions to protect the amylase. 
Dextrinase activity was, however, demonstrated to be a 
property of taka-amylase. In contrast to the evidence for 
a maltase impurity that could be removed, the highly purified 
maltase-free preparations of taka-amylase failed to show any 
evidence of the presence of extraneous dextrinase activity. 
It was shown that the ratio of amylase activity (starch as 
substrate) to dextrinase activity (dextrins as substrate) 
remained constant under a number of conditions that cause 
marked losses of amylase and of dextrinase activities which 
should have caused the selective inactivation of the amylase 
or of the dextrinase if the two activities were due to two 
separate enzymes.
Starch degradation by the action of salivary alpha amylase has recently been discussed by Myrback and Willstaedt (106), by Pazur (115), and by Nordin (109).

Evidences have been presented that alpha-amylases from different sources differ markedly in their action. The action of several alpha-amylases upon a linear fraction from corn starch has been investigated (154). An almost exhaustive comparison of the action of malt, bacterial, salivary, and fungal alpha-amylases on a number of different substrates has recently been published by Bird and Hopkins (7). A hypothesis was suggested to explain the actions of the alpha-amylases, and a general picture of the specific union of alpha-amylases with their substrates was presented.

2. Beta-amylase

Amyloglucosidase has been reported to be comparable with beta-amylase in certain aspects of its action and in certain of its properties. Amylose, the constituent of starch which consists essentially of unbranched glucosidic chains, is hydrolyzed by beta-amylase in a stepwise fashion starting from the nonreducing end. Unlike amyloglucosidase, however, the sole fission products of beta-amylase action are maltose and continually shortening amylose chains.
(103, 111, 127). 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 (82). This has suggested a Walden inversion during hydrolysis (147).

Maltose is, likewise, split from the molecule of amylpectin by beta-amylase but about 20 times as rapidly as from the molecule of amylose (64). Although amylose is hydrolyzed virtually completely to such simple saccharides as maltose and traces of higher saccharides (104, 105), a dextrin unreactive to beta-amylase action is another end product of the hydrolysis of amylpectin in addition to maltose (60). Approximately 60 per cent of the amylpectin is ultimately converted to maltose (48).

The beta-amylases that have been described in the literature are mostly of plant origin, among them wheat, barley, soybean and sweet potato beta-amylases (15, 107).

More interesting and recent reports on the enzyme are about its mechanism of action. It was earlier concluded by Swanson (148) that the enzyme having started the attack on one chain, completes its hydrolysis before beginning to attack another chain. Simultaneously, Kerr and Cleveland (69, 70) came to the same conclusion. The above hypothesis
of amylase action, which has been described as the single chain mechanism has received wide acceptance, but has been criticized by Hopkins and Jelinek (61, 62, 63), and by Bourne and Whelan (10), whose observations favor the multi-chain hypothesis. According to the latter mechanism, the enzyme attacks chains of all lengths at random and having completed a single fission, does not necessarily continue to attack the same chain. French, Levine, Pazur and Norberg (36), using amyloheptaose prepared from a Schardinger dextrin, obtained evidence that under conditions favorable to enzyme action, no pentaose is present in partly hydrolyzed reaction mixtures. This observation supports the single chain mechanism. On the other hand, French, Knapp and Pazur (35), found that if the enzyme was made to act under certain unfavorable conditions, pentaose was formed, thus suggesting that the two mechanisms are liable to operate according to the conditions involved. Lately, the mechanism of beta-amylase action on both amylose and amylase degradation products has been investigated farther by Hopkins and Jelinek (62), and by Bird and Hopkins (8). More evidence in favor of the multichain mechanism has been presented. The early disappearance of the short chains from a mixture of long and short chain polysaccharides is believed to be due to a multichain mode of attack by the enzyme. Paper
chromatographic analysis of the hydrolysis products of the enzyme and results obtained from measurements of absorption value of iodine color and reducing values were interpreted to be in favor of the multichain mode of action of beta-amylase.

3. Amylo-1,6-glucosidases

The group of enzymes designated as amylo-1,6-glucosidases includes the enzymes capable of attacking the alpha-1,6-glucosidic linkage between two glucose units either in the starch molecule or partially hydrolyzed starch. The size of the substrate molecule may vary from the disaccharide isomaltose to starch itself. Isomaltose differs from maltose in that the chemical linkage between the glucose residues is of the alpha-1,6 type in isomaltose, whereas it is of the alpha-1,4 type in maltose.

Whelan (166), is of the opinion that this group of enzymes hydrolyzing alpha-1,6 linkages can be subdivided into enzymes that can only hydrolyze terminal alpha-1,6 linkages such as those which exist in isomaltose and in limit muscle phosphorylase dextrins from amylopectin and glycogen, and another subgroup which includes enzymes capable only of severing a non-terminal linkage such as
those found in amylopectin and the smallest alpha dextrins from glycogen and amylopectin. The first subgroup includes amylol-1,6-glucosidase, first prepared from rabbit muscles (21, 22), Clostridium acetobutylicum maltase and Aspergillus niger amylglucosidase, already previously discussed under the sections on maltase and amylglucosidase respectively. The first named enzyme seems to be specific for the alpha-1,6-glucosidic linkage, while the latter two enzymes also hydrolyze alpha-1,4 linkages as discussed before. There appear to be no other specific requirements for these enzymes since they all hydrolyze isomaltose and also will hydrolyze glucose units attached by alpha-1,6 linkages to large starch-type molecules. The second subgroup includes only one enzyme, the specific requirements of which have been studied. This is R-enzyme, isolated from the broad bean and potato by Hobson, Whelan and Peat (55), and characterized by Roberts as mentioned by Whelan (166), who showed that the enzyme cannot sever a terminal alpha-1,6 linkage but will remove maltose or longer chains attached by these linkages, such as the unit chains in amylopectin. Although R-enzyme can attack amylopectin, it is of interest that the enzyme has been found to be entirely without action on glycogen or its beta limit dextrin (166), unless the glycogen was first degraded by alpha-amylase after which complete scission of
the branched linkages was observed to be effected by R-
enzyme (169). Isoamylase, from brewers' yeast and other
sources, is thought by Maruo and Kobayashi to be identical
with R-enzyme (92). These enzymes which are capable of
hydrolyzing the non-terminal alpha-1,6-glucosidic linkage
have been observed, in various animal tissues and higher
plants (22, 55), but as these are apart from our subject
matter will not be discussed further here.

The enzyme produced by molds which hydrolyzes malt
limit dextrins with the production of fermentable sugars
has been designated as limit dextrinase. The enzyme has
been isolated from barley malts by Kneen and co-workers
(80), and crystallized from Aspergillus oryzae by Under-
kofer and Roy (157). As the name implies, the enzyme acts
on the branched linkages present in the alpha limit dextrins
of starch, but not much more is known about the enzyme. It
is possible that it may hydrolyze the alpha-1,6-glucosidic
linkages at the branched points in the amylpectin fraction
of starch.

Tsuchiya, Montgomery, and Corman (153), found that over
90 per cent of isomaltose could be hydrolyzed by a culture
filtrate from Aspergillus niger 330. The mold filtrate
obviously contains an enzyme capable of hydrolyzing 1,6-
glucosidic linkages.
An isomaltase activity of an enzyme preparation from *Aspergillus oryzae* has recently been reported (66). Determination of the reducing power indicated that isomaltose was split into two molecules of glucose by the enzyme. With the exception of the pH optimum of the enzyme, not much more was reported about the specificity and other properties of the enzyme.

It is apparent that much additional investigation will be needed to learn more about the specific requirements, properties, and functions of these mold enzymes as well as the exact nature of their substrates.
III. METHODS AND MATERIALS

A. Preparation of Mold Enzyme Solutions

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. Underkofer. This culture was chosen since Corman and Langlykke (23) reported filtrates from this mold strain to be relatively low in amylase activity and to have the highest maltase activity of any culture tested.

1. Preparation of mold bran cultures

Mold bran cultures served as a convenient source of inoculum whenever needed for the preparation of mold enzyme solutions. The bran substrate was prepared using the following:

100 grams wheat bran
10 grams ground corn meal
60 milliliters 0.2 normal hydrochloric acid solution containing
0.62 parts per million ZnSO₄·7H₂O
0.62 parts per million FeSO₄·7H₂O
0.08 parts per million CuSO₄·5H₂O

A stock acid solution was made containing hydrochloric acid and the zinc, iron and copper sulfates in such concentrations that 5 milliliters of this when diluted to 60 milliliters would give the desired concentrations of the salts in the final medium. The stock acid solution contained:

8.5 milliliters concentrated HCl
0.0074 grams ZnSO₄·7H₂O
0.0074 grams FeSO₄·7H₂O
0.0009 grams CuSO₄·5H₂O
tap water to 1.0 liter.

Ten gram portions of the moist mixture of wheat bran, corn meal and acidic mineral solution were placed in 150-milliliter wide-mouth Erlenmeyer flasks which were then plugged with cotton. The flasks were sterilized for 15 minutes at 15 pounds steam pressure, cooled and inoculated with the mold from the dextrose-agar slants. This was done by removing a small portion of the mold growth from the agar
slants by means of a sterile wire and placing it in the sterilized bran preparation. The flasks were then shaken to distribute the mold spores throughout the moistened bran, after which they were laid horizontally in the incubator at 30°C. After about a week, during which mycelial growth, sporulation, and finally, drying of the material occurred, the bran cultures were ready for use.

The moisture content of the preparation is very critical. It was sometimes necessary to vary the amount of the acid-salt solution depending on the humidity conditions of the time so that the material was never so dry as to be unable to support good growth nor so moist that drying was delayed. If drying does not occur soon after sporulation, autolysis occurs and the culture spoils.

2. Production of mold enzyme filtrate

Three different substrates were tried for the production of the mold enzyme.

a. A modification of the method by Tsuchiya, Gorman and Koepeell. (152) Five hundred milliliters of medium in 2-liter Fernbach flasks containing 5% corn meal and 5% distillers' dried solubles were sterilized for 30 minutes at 25 pounds pressure. The flasks were cooled and
inoculated directly with the bran culture of the mold. A good dispersion of the mold bran spores was made by using 200 milliliters of sterile 0.05% Tween 80 for the mold bran culture in each Erlenmeyer flask prepared as described previously, and shaking well. Ten milliliters of this spore dispersion were used to inoculate the medium. The culture was aerated for 6 days at 30°C on a reciprocal shaker. The mold mycelium was filtered off and the filtrate stored in the refrigerator until needed for the purification and characterization studies of the enzyme.

b. A modification of the method by Adams, Balankura, Andresen and Stark. (2) The medium employed contained 2% ground yellow corn, 0.5% Difco yeast extract and 0.5% calcium carbonate in tap water. The medium was sterilized in 500 milliliter quantities in 2-liter Fernbach flasks for 30 minutes at 25 pounds steam pressure. After cooling, each flask was inoculated directly with the bran culture of the mold in a similar manner as described above under a. After 3 days of incubation at 30°C on a reciprocal shaker, the cultures were filtered and the combined filtrates stored in the refrigerator until needed.

c. A modification of the method by Shu and Blackwood. (139) The medium had the following composition:
<table>
<thead>
<tr>
<th>Material</th>
<th>Per cent</th>
</tr>
</thead>
<tbody>
<tr>
<td>Soluble starch</td>
<td>2.00</td>
</tr>
<tr>
<td>NH₄Cl</td>
<td>0.78</td>
</tr>
<tr>
<td>CaCO₃</td>
<td>0.50</td>
</tr>
<tr>
<td>KH₂PO₄</td>
<td>0.10</td>
</tr>
<tr>
<td>MgSO₄·7H₂O</td>
<td>0.05</td>
</tr>
<tr>
<td>Soybean oil</td>
<td>0.30</td>
</tr>
<tr>
<td>Hydrolyzed casein</td>
<td>0.10</td>
</tr>
<tr>
<td>ZnSO₄</td>
<td>13.2 ppm.</td>
</tr>
<tr>
<td>FeSO₄</td>
<td>13.2 ppm.</td>
</tr>
<tr>
<td>CuSO₄</td>
<td>6.6 ppm.</td>
</tr>
</tbody>
</table>

The zinc, iron and copper salts were added from a single stock solution. Five hundred milliliter quantities of the medium in 2-liter Fernbach flasks were cooked in the autoclave at atmospheric pressure with occasional shaking until all of the starch in the medium had completely formed a uniform thin gel. The soybean oil and the calcium carbonate were added and the solutions sterilized for 30 minutes at 25 pounds steam pressure. After cooling, the flasks were inoculated with the bran culture of the mold, and the enzyme filtrate collected and stored as described above under a.
B. Methods of Enzyme Analysis

1. Maltase activity

The method employed for determining maltase activity was developed by Tsuchiya, Corman and Koepsell (152). A unit of maltase activity was defined as that amount of enzyme which hydrolyzes 1 milligram of maltose monohydrate in 1 hour at 30°C. The method depends on the observation that an increase of 78% in reducing power is obtained when maltose monohydrate is hydrolyzed to glucose under conditions given below. There is a stoichiometric relationship between the amount of enzyme and hydrolysis rate, within limits, when the rate is calculated from the difference in maltose hydrolyzed at 15 and 120 minutes.

a. Reagents

(1) **Stock acetate buffer. 6.0 molar, pH 4.4.**
Prepared by diluting 217 milliliters of glacial acetic acid and 183 grams of anhydrous sodium acetate to one liter with water.

(2) **Maltose substrate.** The buffered maltose solution that was used as the substrate for maltase activity determination was prepared by dissolving 2.35 grams of maltose monohydrate (commercial C. P. grade, specific
rotation + 131°, produced by Pfistiehl 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.** One normal.

(4) **Sodium hydroxide.** One normal.

(5) **Phenolphthalein indicator.** One-tenth per cent.

(6) **Reagents for reducing sugar analysis.** The method developed by Somogyi was used (142).

b. **Procedure for the determination of maltase activity.**

Five milliliters of the mold enzyme solution and 10 milliliters of the buffered maltose substrate solution were both attempered to 30° C. They were then mixed in a test tube and held in the water bath at 30° C. After 15 minutes, a 3 milliliter aliquot of the enzyme-substrate mixture was transferred to a 100-milliliter volumetric flask containing 3 milliliters of 1 normal sulfuric acid to stop the maltose hydrolysis by acid inactivation of the enzyme. After 120 minutes, a second 3 milliliter aliquot of the reaction mixture was treated in a similar manner.

After the acid inactivation of the enzyme for 10 minutes, the acidified reaction mixtures were adjusted to the phenolphthalein end point with 1 normal sodium hydroxide solution and made up to 100 milliliters with water. Five
milliliter aliquots were taken for analysis for reducing value (R.V.) by the method of Somogyi using the 20 minute heating period. By this procedure, the reducing values of the reaction mixture after 15 and 120 minutes hydrolyses were obtained. The reducing values are measures of the glucose produced, the residual maltose, and the reducing sugars originally present in the enzyme preparation.

c. Calculation of maltase activity. The units of maltase activity of an enzyme solution were expressed in terms of milligrams of maltose hydrolyzed per milliliter of enzyme preparation per hour. The following expression gives the units of maltase activity where "a" is the reducing value in terms of milliliters of thiosulfate solution of the 15 minute reaction mixture, and "b" is the corresponding value for the 120 minute reaction mixture.

\[
\frac{(b-a)}{0.78} \times (\text{glucose equivalent of Na}_2\text{S}_2\text{O}_3 \text{ solution} \times 1.78) \times 20 \times \frac{60}{105}
\]

= milligrams maltose hydrolyzed per milliliter of enzyme preparation per hour

= units of maltase activity.
d. **Precautions observed.** Hydrolysis rate values were limited within the range between 2 and 10 milligrams maltose hydrolyzed per milliliter of enzyme preparation per hour. Values in the higher range were preferred. A pH of 4.4 prevailed in the reaction mixtures. Enzyme preparations highly buffered at pH values other than 4.4 were adjusted to approximately this point before the maltase activity determination. The copper reagent used in the sugar analysis was measured with a pipet rather than a buret.

2. **Alpha-amylase activity**

There are two general methods of analyzing for alpha-amylase activity, one dependent upon the viscometric influence of alpha-amylase upon starch solutions, and the other dependent upon the color produced by the enzyme substrate solution after addition of a certain amount of iodine as originally proposed by Wohl gemuth (173). The method used in this work was the Wohl gemuth procedure as modified by Sandstedt, Kene and Blish (128), together with the inorganic color standard recommended by Olson, Evans, and Dickson (113). The unit of alpha-amylase activity was defined as the amount of enzyme required to dextrinize one
gram of beta-amylase-treated soluble starch in one hour at 30°C.

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 was 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.

(3) **Stock iodine solution.** Eleven grams of resublimed iodine crystals and 22.0 grams of potassium iodide were dissolved in a 500-milliliter volumetric flask with distilled water and diluted to the mark.

(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 by Olson, Evans and Dickson.** Twenty five grams of CoCl₂·6H₂O and 3.89 grams of potassium dichromate were dissolved in 100.0 milliliters of 0.01 normal HCl.
(6) **Soluble starch.** The reagent grade soluble starch labeled "suitable for iodometry" was purchased from Merck and Co., Inc., Rahway, New Jersey.

b. **Procedure**

(1) **Preparation of the beta-amylase treated soluble starch solution.** A slurry of 5.0 grams of soluble starch in 20 milliliters of water was added to approximately 120 milliliters of vigorously boiling water with rinsing to insure complete transfer. The solution was cooled in running tap water until it could be conveniently handled and 12.5 milliliters of buffer solution were added. The solution was then transferred with rinsing into a 250-milliliter volumetric flask. The solution was cooled to room temperature, and 125 milligrams of the beta-amylase preparation slurried in a small volume of water were 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 more than 48 hours and not less than 24 hours.

(2) **Dextrinizing activity.** Twenty milliliters of the buffered beta-amylase treated starch solution and 5.0 milliliters of water were transferred by pipet to a 50-milliliter Erlenmeyer flask and the flask placed in the
water bath maintained at 30°C. After the flask temperature had attained equilibrium, 5.0 milliliters of the enzyme solution were added by pipet. The volumes of water and enzyme solution were altered to compensate for low or high activities, but the total volume of the two solutions were kept to 10.0 milliliters. After 10 minutes, one milliliter portions were withdrawn by pipet at appropriate time intervals and added to a series of 12-milliliter Pyrex centrifuge tubes, each of which contained 5.0 milliliters of the dilute iodine solution. The tubes were inverted to insure thorough mixing. The tube was then placed in the color comparator between two similar centrifuge tubes, each containing 6.0 milliliters of the cobaltous chloride color standard, and the solutions were compared. When the color of the sample from the enzyme substrate 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 colors of the sample and that of the standard to match was recorded. When the color of the solution from the sample was too light after 10 minutes reaction between the enzyme and substrate, 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 was used.

(c) Calculation of alpha-amylase activity. The unit of alpha amylase activity is the quantity of enzyme required to dextrinize one gram of beta-amylase-treated soluble starch in one hour at 30°C. to the point where the dextrin-iodine color matches that of the cobaltous chloride color standard. The concentration of the enzyme solution in terms of alpha-amylase activity was calculated by using the following formula.

\[
\text{Units of alpha-amylase activity} = \frac{0.4 \times 60}{V \times T}
\]

where \(V\) represents the original volume of mold filtrate in milliliters or fraction thereof, and \(T\) is the dextrinization time in minutes.

C. Determination of Reducing Sugars

1. Method of Somogyi

This method was used for the determination of reducing values involved in the measurement of maltase activity of
the enzyme solutions. It was likewise adopted for the reducing sugar analyses of the products of hydrolysis of the enzyme upon the various substrates tested. The method was designed originally for the determination of blood sugar (142), but has been adopted for the sugar determination of other biological materials. The copper-phosphate-tartrate reagent involved in the method is claimed by Somogyi to offer some definite advantages in the determination of blood sugar in that it is equally suitable for the iodometric and the colorimetric determination of cuprous oxide, that a single reagent is suitable for both macro and micro analysis, its useful range extending from 0.01 to 3.0 milligrams of glucose, and that in the lowest ranges of sugar concentrations the new reagent yields more consistent and more closely reproducible results than the copper-carbonate-tartrate reagent.

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. Two normal.

(3) Standard thiosulfate. A 0.005 normal sodium thiosulfate solution was made alkaline by addition
of 2.0 milliliters of 10 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) **The copper-phosphate-tartrate reagent.** One liter of this reagent contained the following:

<table>
<thead>
<tr>
<th>Compound</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Na$_2$HPO$_4$ anhydrous</td>
<td>28 grams</td>
</tr>
<tr>
<td>1 normal NaOH</td>
<td>100 ml</td>
</tr>
<tr>
<td>NaK[C$_4$H$_6$O$_6$].4H$_2$O (Rochelle salt)</td>
<td>40 grams</td>
</tr>
<tr>
<td>CuSO$_4$</td>
<td>8 grams</td>
</tr>
<tr>
<td>Na$_2$SO$_4$ anhydrous</td>
<td>180 grams</td>
</tr>
</tbody>
</table>

The phosphate and tartrate were dissolved in about 700 milliliters of water. The sodium hydroxide was added, and then with stirring, 80 milliliters of a 10 per cent copper sulfate solution were introduced. Finally, the sodium sulfate was added and, when dissolved, the solution was diluted to one liter, and allowed to stand for a day or two, during which time impurities separated out. The clear top part of the solution was decanted and then filtered through a good grade of filter paper. 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 normal iodate for 3 milligrams glucose
- 10 milliliters normal iodate for 1 milligram glucose
- 5 milliliters for 0.5 milligrams glucose.

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 through which a short piece of capillary glass tubing had been 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 added from a buret allowing it to flow down the side of the inclined test tube. The amount of potassium iodide added varied with the amount of potassium iodate added to the copper-phosphate-tartrate reagent as follows:

- 0.5 milliliter of 2.5 per cent potassium iodide solution for 5 milliliters of normal iodate per liter of the copper reagent.
1.0 milliliter of 2.5 per cent potassium iodide solution for 10 milliliters of 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 milliliters of the 2 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. The solution was then titrated with the 0.005 normal sodium thiosulfate solution. Two drops of the starch indicator were added toward the end of the titration.

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

\[ 2 \text{Cu}^{2+} + 2 I^- \rightarrow \text{Cu}_2I_2 + I_2 \]

Somogyi minimized this reaction through the use of minimum amount of potassium iodide, but the undesirable drift in the end point during the titration was still observable.
The oxalate ions eliminate 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 reagent solutions were calibrated by determining the volume of the thiosulfate solution needed to titrate a series of determinations conducted upon sugar solutions of known concentrations ranging from 0.1 to 2.0 milligrams of glucose (commercial anhydrous C. P. grade, specific rotation +52.5°, produced by Pfamstiehl Chemical Co., Waukegan, Illinois) per 5.0 milliliters of solution. 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 concentrations. 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 a month or once in two weeks.
2. Detection of reducing carbohydrates on filter paper chromatograms

Two color reagents were used for the detection of the presence of reducing sugars on paper chromatograms. One of them was an alkaline copper reagent used by Wild (170). This was prepared by dissolving 7.5 grams of copper sulfate in 100 milliliters of water which was then added with stirring to a solution of 25 grams of Rochelle salt and 40 grams of anhydrous sodium carbonate in 300 milliliters of water. To this mixture was added 500 milliliters of methanol, and the whole solution was diluted to one liter with water. The second color reagent was phosphomolybdic acid solution (150), which brought out as blue spots the areas where reduction of the copper by the sugars had occurred. The phosphomolybdic acid solution was prepared by mixing 150 grams of molybdic acid anhydride (Baker MoO₃; 99.5 per cent) with 75 grams of anhydrous sodium carbonate. The mixture was dissolved in 500 milliliters of distilled water and boiled until all of the molybdic acid had been dissolved. The solution was cooled and filtered and 300 milliliters of 85 per cent orthophosphoric acid was added. The solution was cooled to room temperature and diluted to one liter with distilled water. Oligosaccharides with a reducing terminal
unit yield spots with a blue or greenish blue color. The above combination of reagents can be used to detect as little as one microgram of glucose, two micrograms of amylotriose and about 5 micrograms of amylo tetraose.

D. Measurement of Blue Values

With the linear fractions from starches, the hydrolysis of the substrate can be followed quantitatively by determination of blue values. These determinations depend upon the fact that the linear components of starches absorb approximately 19 per cent of their weight of iodine to form complexes that can be determined quantitatively by spectrophotometric methods (118). Blue values of the starch hydrolysates were measured according to McCready and Hassid (93). The method was designed originally for the quantitative estimation of amylose and amylpectin in potato starch. Blue value is defined as follows: 

\[ E.V. = k \log \frac{100}{T} \]

where \( T \) is the per cent transmission of a solution of 5 milligrams of a linear fraction in 500 milliliters of a solution of 0.002 per cent iodine and 0.02 per cent potassium iodide, and where \( k = \frac{1}{\log 100/t} \) and \( t \) is the per cent transmission of a solution of a pure linear fraction of the same concentration.
For this determination, a 0.2 per cent iodine solution in 2 per cent potassium iodide was prepared. Aliquots were removed from the hydrolysates of the linear fraction of potato starch at suitable time intervals, and treated with the appropriate amount of iodine solution, carrying out the proper dilution to such quantities in agreement with those set forth by the definition of blue value with regards to the linear fraction as well as the iodine and potassium iodide. The color developed immediately to its full intensity and is supposed to remain stable for several days. The intensity of the blue color was measured, using a Klett-Summerson photoelectric colorimeter. The 20 millimeter glass cell was used in conjunction with the red K66 filter furnished with the instrument. The photocolorimeter was adjusted so that the blank which had a light yellow color, due to iodine, gave a reading of zero.

E. Filter Paper Chromatography

1. Filter paper

The paper used was Whatman No. 1, which was purchased in large sheets, 46.5 centimeters by 57 centimeters. The desired lengths were cut and used as such. To obtain
uniform chromatograms, sheets of 8 x 8.5 inches were used which fitted nicely in the containers which were employed.

2. **Containers and developing solution**

The ascending type chromatography was used consistently. The containers used were wide-mouthed gallon jars with screw lids. Eighty per cent normal propanol was used as the developing solution. The same solution was seldom used for more than four ascents, since its effectiveness in moving and resolving substances on the chromatogram was believed to be significantly reduced with repeated use.

3. **Procedure**

A line one inch from the bottom of the 8 x 8.5 inches filter paper was drawn, and marks were placed at about 3/4 inch intervals along the line. This allowed from eight to nine marks on a sheet. The sheet was labeled accordingly with the type of substrate tested as well as with the stage of purification of the enzyme used for the hydrolysis test. The marks were likewise labeled with the time intervals at which the hydrolysate samples were withdrawn. Samples of the solution at the indicated times were applied to the
spots by means of wood applicators (by Glasco, Chicago, Illinois). The last mark or two on the sheet were usually spotted with samples from a 1 per cent solution of glucose and maltose to serve as standards with which to compare the hydrolysis products. After the spots on every mark on the paper had been applied and allowed to dry, the paper was stapled to form a cylinder with the spots near the bottom edge. The stapled edges were not allowed to touch. The staples did not interfere with the development, since the staples were nearly 2 centimeters away from the path of the nearest spot. From 50 to 65 milliliters of the developing solution was used in each jar. The paper was allowed to stand in the solution in the closed jar until the solution had reached the top of the paper by capillary action. The chromatogram was then taken out, dried, and usually returned to the jar for another ascent. After drying, the paper was sprayed with the alkaline copper reagent described previously until uniformly moistened using an all glass atomizer with a continuous air stream. The chromatogram was then heated in an oven at 100-110°C. for approximately five minutes. After this, the chromatogram was sprayed with the phosphomolybdic acid solution. The resulting blue spots were then marked immediately with pencil since
these were not stable and tended to fade within a rather short period of time.

F. Determination of Protein Nitrogen

Samples of the enzyme solution at various stages of purification were analyzed for protein nitrogen, after extensive dialysis, by the micro-Kjeldahl technique (145).

1. Reagents

a. Boric acid. Four per cent aqueous solution of reagent grade of boric acid was used in the receiving flask during distillation.

b. Catalysts. A mixture containing 5 per cent powdered copper sulfate and 95 per cent powdered potassium sulfate furnished catalysts for the digestion.

c. Sulfuric acid. Reagent grade of concentrated sulfuric acid, specific gravity 1.84, was used for the digestion.

d. Indicator mixture. The indicator was prepared by mixing five parts of 0.2 per cent brom cresol green with one part of 0.2 per cent methyl red, both in 95 per cent ethanol.
e. **Standard solution of hydrochloric acid.** Hydrochloric acid of 0.01 normal concentration was used for titrating the distilled ammonia. This was standardized by titration against standard 0.01 normal sodium hydroxide.

f. **Sodium hydroxide solution.** Forty per cent sodium hydroxide solution was used for liberating ammonia during the distillation of the digested protein.

2. **Procedure**

One milliliter of the enzyme solution of such a concentration as would require several milliliters of 0.01 normal hydrochloric acid in the titration of the distilled ammonia was refluxed with one milliliter of concentrated sulfuric acid until the brown color disappeared and the solution was almost colorless. The mixture was boiled vigorously enough so that refluxing took place about one half way up the neck of the flask. A small amount of the catalyst was added to the digestion mixture to speed up the decomposition of the organic matter. After the completion of the digestion, the mixture was cooled, about two milliliters of distilled water were added to the cooled digest and transferred quantitatively to the filling funnel of the distillation apparatus. The distilling apparatus
used was the Pregl or Parnas-Wagner type. With the distilling flask cool and the pinch clamps on the filling funnel and on the steam trap open, steam was generated in the steam generator. With the apparatus so arranged most of the steam passed out through the bottom of the trap and a little passed out through the filling funnel. The diluted digest which was poured into the funnel passed into the distillation flask. About 10 to 12 milliliters of distilled water were used for quantitatively washing the contents of the Kjeldahl flask into the distillation flask. A 125-milliliter Erlenmeyer flask containing 5 milliliters of the boric acid solution and four drops of the indicator was then placed under the delivery tube of the condenser and slightly tilted so that the tip extended well below the liquid surface. Five milliliters of the 40 per cent sodium hydroxide solution were carefully added through the funnel so that two liquid layers were formed with the acid on top and the alkali on the bottom. The pinch clamps beneath the filling funnel and steam trap were closed in the respective order which caused the steam to enter the tube leading to the bottom of the distillation flask, mixing the two layers. The resulting strongly alkaline mixture was steam distilled for 5 minutes from the time condensation began in the condenser with the end of the delivery tube
below the surface of the boric acid and for an additional
two to three minutes after the flask had been lowered so
that the end of the tube was out of the liquid and up
towards the neck of the receiving flask. The tip of the
delivery tube was then washed with a few milliliters of
distilled water to transfer any boric acid adhering to the
outside into the flask. The boric acid solution of
ammonia was titrated with the standard hydrochloric acid
to the endpoint.

3. **Calculations**

Each milliliter of 0.01 normal hydrochloric acid is
equivalent to 0.14008 milligram of nitrogen. Therefore,

\[
\frac{\text{ml. of } 0.01 \text{ N HCl} \times 0.14008}{\text{ml. of sample}} = \text{mg. nitrogen per ml. enzyme solution}
\]
IV. EXPERIMENTAL RESULTS

A. Purification of the Glucosidase

1. Purification scheme of Roy and Underkofler

The method of Roy and Underkofler (125), already mentioned in the Review of Literature, was applied to the enzyme filtrate from *Aspergillus niger* NRRL 330 with the hope of obtaining a dry maltase concentrate, described by the above workers as the end product obtained. The method was tried twice on both the enzyme preparations prepared according to the modifications of the methods by Tsuchiya, Corman and Koepsell (152), and by Adams, Balankurz, Andreasen and Stark (2), details of which have been described in the Methods and Materials. Attempts to obtain the maltase concentrate by the method did not meet with success. No precipitate was obtained from the dialyzed eluate from Fuller's earth on addition of absolute alcohol. The enzyme appeared to have been lost at some previous step of the procedure. Work was, therefore, directed towards an evaluation of the effect of the various stages of the scheme by determination of the glucosidase activities of the different fractions. The analytical procedure used to
indicate glucosidase activity was the standard method for maltase determination, details of which have been described in the section on Methods and Materials. The effect of the oxalate-calcium carbonate adsorption of the enzyme was investigated, and the results obtained are given in Table 1.

Table 1. Analysis of some fractions obtained from the purification scheme of Roy and Underkofler

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Units of maltase activity per milliliter</th>
</tr>
</thead>
<tbody>
<tr>
<td>Original mold filtrate (2)</td>
<td>2.45</td>
</tr>
<tr>
<td>Supernatant after oxalate-calcium carbonate adsorption</td>
<td>0.87</td>
</tr>
<tr>
<td>Original mold filtrate (152)</td>
<td>10.48</td>
</tr>
<tr>
<td>Supernatant after oxalate-calcium carbonate adsorption</td>
<td>9.25</td>
</tr>
</tbody>
</table>

The oxalate-calcium carbonate did not appear to be a very promising adsorbent for the enzyme especially from the original mold enzyme filtrate which had the higher maltase activity. Further investigation of the succeeding steps of the purification scheme, therefore, did not seem desirable. However,
the adsorption with Fuller's earth and precipitation by absolute alcohol in the purification procedure were investigated later in this work.

2. Purification scheme of Phillips and Caldwell

It was mentioned earlier that Phillips and Caldwell (117) have reported a purification procedure for a glucose-forming amylase produced by the mold Rhizopus delemar. The enzyme was obtained in a highly purified form and freed from all detectable traces of alpha amylase which accompanied it in crude extracts. Since the purified enzyme was reported to exert maltase as well as amylase activity, it appeared interesting to determine how the purification scheme of the above-mentioned workers would work with the glucosidase from Aspergillus niger. The scheme was applied to the enzyme preparation prepared according to the modification of the method by Tsuchiya, Corman and Koepsell (152).

Three liters of the mold filtrate were made to 40 per cent saturation with solid ammonium sulfate. At this point, not much precipitate was obtained except a few very black, and sticky, gum-like particles floating on top of the dark brown solution. The dissolved precipitates were dialyzed until the dialysate gave a negative test for sulfate with
dilute barium chloride solution. The dialysis was carried out at room temperature in bags made of cellophane tubing against distilled water which was changed frequently. Cellulase, present in the crude enzyme preparation, destroyed the dialysis bags so that it became necessary to change the bags frequently. The same problem was encountered by Phillips and Caldwell with the enzyme preparation from *Rhizopus delemar*.

The final precipitate obtained, was still a dark brown color. It appeared that the enzyme had not been sufficiently purified. The effect of some of the steps of the purification on the activity of the enzyme was determined, and the results obtained in terms of units of maltase activity per milliliter of enzyme solution are shown in Table 2.

The results indicate a rather low glucosidase activity in the final enzyme solution obtained. The slight increase in the glucosidase activity of the enzyme solution and the dark brown color retained after all the various steps of the purification procedure indicated that the scheme did not work favorably with the glucosidase from *Aspergillus niger*. Apparently, the glucosidases produced by the two different molds differ in some of their physical and chemical properties, although they exhibit similar hydrolytic activity on maltose. Thus, other methods of purification
Table 2. Analysis of some fractions obtained from the purification scheme of Phillips and Caldwell

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Units of maltase activity per milliliter of dissolved precipitate</th>
</tr>
</thead>
<tbody>
<tr>
<td>Original enzyme solution</td>
<td>10.48</td>
</tr>
<tr>
<td>Precipitate from 1st 0 to 40 per cent ammonium sulfate fractionation</td>
<td>6.99</td>
</tr>
<tr>
<td>Precipitate from 40 to 55 per cent ammonium sulfate fractionation</td>
<td>12.41</td>
</tr>
<tr>
<td>Precipitate from 0 to 45 per cent ammonium sulfate refractionation</td>
<td>5.07</td>
</tr>
<tr>
<td>Precipitate from 45 to 60 per cent ammonium sulfate refractionation</td>
<td>19.57</td>
</tr>
<tr>
<td>Precipitate from 60 per cent saturation with ethanol</td>
<td>72.13</td>
</tr>
</tbody>
</table>

usually employed in the isolation and purification of enzymes had to be investigated. However, ammonium sulfate fractionation and precipitation by alcohol, employed by Phillips and Caldwell in their purification scheme, were investigated further to determine the conditions favorable for their use.
3. **Attempted steps of purification**

The methods which have proved most useful in the purification of many of the fungal carbohyd rases have involved precipitation by salts and organic solvents, and adsorption on a variety of solids. The effects of these methods on the glucosidase of *Aspergillus niger* were studied. The enzyme preparation used in this entire work was that prepared according to the modification of the method by Tsuchiya, Corman and Koepsell (152), unless otherwise stated.

a. **Precipitation by salts**

(1) **Fractional precipitation with ammonium sulfate.** To determine the optimum concentration of ammonium sulfate for the precipitation of the glucosidase component of the mold filtrate, a fractional precipitation was carried out. This was done as follows: A 200 milliliter portion of the original mold filtrate was used. Solid ammonium sulfate was added such that the salt concentration was increased in increments of 10 per cent, from 0 to 100 per cent. The salt was dissolved with occasional stirring and the solution was centrifuged 20 minutes after each addition of ammonium sulfate. The resulting precipitate brought about by each ten per cent increment of ammonium sulfate was dissolved in 20 milliliters of distilled water each time. Five milliliter
portions were withdrawn by pipet from the resulting enzyme solutions and analyzed for glucosidase activity. The results obtained are given in Table 3.

It is evident from the data of Table 3 that very little of the glucosidase was precipitated by ammonium sulfate up to 30 per cent saturation. The greatest portion of the glucosidase in the crude mold filtrate was precipitated by the ammonium sulfate from 30 to 70 per cent saturation. Very little more of the enzyme was precipitated beyond 70 per cent saturation.

That precipitation of the glucosidase from the crude enzyme preparation by ammonium sulfate holds little promise as a first step in the purification of the enzyme is indicated by a rather high loss of the glucosidase. Only 46.1 per cent of the total maltase activity was precipitated by ammonium sulfate up to 90 per cent saturation of an original enzyme solution containing 16.42 units of maltase activity per milliliter.

The enzyme may possibly have been denatured by the salt, or other foreign proteins may have been selectively precipitated by the ammonium sulfate in preference to the mold glucosidase under the conditions for the precipitation employed. Accordingly, the effect of certain variations in
Table 3. Precipitation of the glucosidase from the crude mold filtrate at various ammonium sulfate concentrations

<table>
<thead>
<tr>
<th>Per cent saturation with ammonium sulfate</th>
<th>Units of maltase activity per milliliter of concentrated solution</th>
<th>Units of maltase activity precipitated per milliliter in terms of original solution</th>
<th>Total units of maltase activity precipitated per milliliter in terms of original solution from 0 per cent</th>
<th>Total per cent maltase activity precipitated from 0 per cent</th>
</tr>
</thead>
<tbody>
<tr>
<td>0-10</td>
<td>no precipitate</td>
<td>---</td>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td>10-20</td>
<td>no precipitate</td>
<td>---</td>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td>20-30</td>
<td>5.59</td>
<td>0.559</td>
<td>0.559</td>
<td>3.40</td>
</tr>
<tr>
<td>30-40</td>
<td>19.04</td>
<td>1.904</td>
<td>2.463</td>
<td>15.25</td>
</tr>
<tr>
<td>40-50</td>
<td>16.60</td>
<td>1.660</td>
<td>4.163</td>
<td>25.48</td>
</tr>
<tr>
<td>50-60</td>
<td>16.25</td>
<td>1.625</td>
<td>5.788</td>
<td>35.21</td>
</tr>
<tr>
<td>60-70</td>
<td>12.23</td>
<td>1.223</td>
<td>7.011</td>
<td>42.7</td>
</tr>
<tr>
<td>70-80</td>
<td>3.67</td>
<td>0.367</td>
<td>7.378</td>
<td>44.98</td>
</tr>
<tr>
<td>80-90</td>
<td>1.92</td>
<td>0.192</td>
<td>7.570</td>
<td>46.1</td>
</tr>
<tr>
<td>90-100</td>
<td>ammonium sulfate added would no longer completely dissolve</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

93
the conditions of precipitation such as pH and temperature appeared worth investigating.

(2) Effect of the pH of the enzyme solution on the ammonium sulfate precipitation. The solubility of the protein fraction possessing the glucosidase activity was determined at various pH levels and concentrations of ammonium sulfate.

Fifty milliliters of the mold filtrate were placed in each of 8 beakers. The pH levels of the solutions were adjusted using the Beckman pH meter from pH 4.0 to pH 7.5 in increments of 0.5 with either 0.1 normal sodium hydroxide or sulfuric acid. The desired quantity of solid ammonium sulfate was added such that the salt concentration was increased in increments of 10 per cent after each addition into each of the solutions. The pH was readjusted whenever necessary before the addition of the next fraction of ammonium sulfate, in order to keep the pH of the solutions at the same levels as when the precipitation was started. The precipitation was done in an ice bath, with occasional stirring, within 20 minutes, after which time the resulting precipitate was centrifuged off. The precipitate from each fraction was dissolved in 10 milliliters of distilled water. Five milliliter portions were withdrawn by pipet from each
of the resulting enzyme solutions and analyzed for glucosidase activity. The results obtained are given in Table 4.

From Table 4 it is apparent that the optimum pH for the precipitation of the glucosidase from the crude mold filtrate was near pH 4.5, and that the bulk of the glucosidase activity was precipitated at that pH by the ammonium sulfate between the 30 to 60 per cent saturation.

The data obtained from the precipitation at pH 4.5 was calculated further to determine the total per cent of glucosidase activity in the precipitate obtained at that pH which appeared optimum for the precipitation. The results are given in Table 5.

Of the total glucosidase activity in the original mold filtrate used, 63.8 per cent was precipitated by the ammonium sulfate from 0 to 70 saturation at pH 4.5. The original mold filtrate used contained 15.56 units of glucosidase activity per milliliter.

The fractional precipitation of the glucosidase with ammonium sulfate indicated that the greatest portion of the total activity was precipitated between 30 to 60 per cent saturation with the salt. The effect of the addition of ammonium sulfate in greater increments than 10 per cent each time was investigated.
Table 4. Precipitation of the glucosidase from the original mold filtrate with ammonium sulfate at various pH levels

(Units of maltase activity per milliliter of solution containing the dissolved precipitate)

<table>
<thead>
<tr>
<th>Per cent of ammonium sulfate saturation</th>
<th>pH Levels</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>4.0</td>
</tr>
<tr>
<td>0-10 prec. no prec. no prec. no prec.</td>
<td>1.09</td>
</tr>
<tr>
<td>10-20 2.32</td>
<td>1.56</td>
</tr>
<tr>
<td>20-30 0.93</td>
<td>1.09</td>
</tr>
<tr>
<td>30-40 6.84</td>
<td>7.46</td>
</tr>
<tr>
<td>40-50 21.09</td>
<td>11.20</td>
</tr>
<tr>
<td>50-60 11.05</td>
<td>24.9</td>
</tr>
<tr>
<td>60-70 3.72</td>
<td>3.41</td>
</tr>
<tr>
<td>70-80 ammonium sulfate would not completely dissolve in all the solutions</td>
<td></td>
</tr>
<tr>
<td>Total 45.95</td>
<td>49.62</td>
</tr>
</tbody>
</table>


Table 5. Precipitation of the glucosidase from the crude mold filtrate by ammonium sulfate at pH 4.5

<table>
<thead>
<tr>
<th>Per cent saturation with ammonium sulfate</th>
<th>Units of maltase activity per milliliter of concentrated solution</th>
<th>Units of maltase activity precipitated per milliliter in terms of original solution</th>
<th>Total units of maltase activity precipitated per milliliter in terms of original solution from 0 per cent</th>
<th>Total per cent maltase activity precipitated from 0 per cent</th>
</tr>
</thead>
<tbody>
<tr>
<td>0-10</td>
<td>no precipitate</td>
<td>---</td>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td>10-20</td>
<td>1.56</td>
<td>0.31</td>
<td>0.31</td>
<td>1.99</td>
</tr>
<tr>
<td>20-30</td>
<td>1.09</td>
<td>0.22</td>
<td>0.53</td>
<td>3.42</td>
</tr>
<tr>
<td>30-40</td>
<td>7.46</td>
<td>1.49</td>
<td>2.02</td>
<td>12.89</td>
</tr>
<tr>
<td>40-50</td>
<td>11.20</td>
<td>2.24</td>
<td>4.26</td>
<td>27.40</td>
</tr>
<tr>
<td>50-60</td>
<td>24.9</td>
<td>4.99</td>
<td>9.25</td>
<td>59.5</td>
</tr>
<tr>
<td>60-70</td>
<td>3.41</td>
<td>0.68</td>
<td>9.93</td>
<td>63.8</td>
</tr>
</tbody>
</table>
Fifty milliliters of the mold filtrate were placed in separate beakers. Precipitation by ammonium sulfate was done at various pH levels in the same manner as previously described, except that the salt was added in different increments. One group of solutions was made between 0 to 40 per cent saturated, and another group was made between 0 to 60 per cent saturated with ammonium sulfate. The glucosidase activity of the dissolved precipitates was determined and the results are given in Table 6.

The data of Table 6 indicate an interesting property of the glucosidase. It appears that there may be two protein fractions possessing the glucosidase activity. One

Table 6. Precipitation of the glucosidase from the original mold filtrate at various pH levels with ammonium sulfate fractionation

(Units of maltase activity per milliliter of solution containing the dissolved precipitates)

<table>
<thead>
<tr>
<th>Per cent of ammonium sulfate saturation</th>
<th>pH Levels</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>4.0</td>
</tr>
<tr>
<td>0-40</td>
<td>11.82</td>
</tr>
<tr>
<td>0-60</td>
<td>9.95</td>
</tr>
</tbody>
</table>
fraction seems to be precipitated best near pH 4.5, and another fraction near pH 6.0.

Similar results have been reported by Stinson (146) in his work on the purification of maltase from *Aspergillus niger* grown in a synthetic medium described by Shu and Blackwood (139). To investigate further the above observation, the precipitation of the glucosidase at various pH levels with ammonium sulfate was performed simultaneously on two mold filtrates prepared differently. One mold filtrate (I) was prepared according to a modification of the method by Shu and Blackwood (139), and the other (II) prepared according to a modification of the method by Tsuchiya, Corman and Koepsell (152), details of both procedures having been described in the section on Methods and Materials.

The fractional precipitation of the glucosidase from the two different enzyme preparations at various pH levels was carried out in the same manner as previously described. Ammonium sulfate was added to the enzyme solutions in increments from 0 to 30 per cent; 30 to 60 per cent; and 60 to 90 per cent. No precipitate was obtained from the mold filtrate between the 0 to 30 per cent saturation with ammonium sulfate. A slight turbidity of the enzyme solution (I) was observed upon addition of 0.1 normal sodium
hydroxide which was used for the adjustment of pH at various levels. The ammonium sulfate added to make the solutions saturated from 60 to 90 per cent did not dissolve completely in both enzyme preparations. The glucosidase activity of the dissolved precipitates were determined and the results are given in Table 7.

Table 7. Precipitation of the glucosidase from two different enzyme preparations at various pH levels with ammonium sulfate

<table>
<thead>
<tr>
<th>Per cent of ammonium sulfate saturation</th>
<th>pH Levels</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>4.5</td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Units of maltase activity per milliliter of solution containing the dissolved precipitates from the mold filtrate (I)

<table>
<thead>
<tr>
<th></th>
<th>0-30</th>
<th>30-60</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>no prec.</td>
<td>1.92</td>
<td>8.74</td>
<td>20.61</td>
</tr>
<tr>
<td>no prec.</td>
<td>0.0</td>
<td>5.59</td>
<td>13.44</td>
</tr>
<tr>
<td>no prec.</td>
<td>0.87</td>
<td>8.39</td>
<td>17.46</td>
</tr>
<tr>
<td>no prec.</td>
<td>1.75</td>
<td>6.12</td>
<td>21.83</td>
</tr>
<tr>
<td>no prec.</td>
<td>0.18</td>
<td>1.92</td>
<td>15.88</td>
</tr>
<tr>
<td>no prec.</td>
<td>0.0</td>
<td>8.74</td>
<td>16.92</td>
</tr>
</tbody>
</table>

Units of maltase activity per milliliter of solution containing the dissolved precipitates from the mold filtrate (II)

<table>
<thead>
<tr>
<th></th>
<th>0-30</th>
<th>30-60</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.0</td>
<td>18.69</td>
<td>13.44</td>
<td>20.61</td>
</tr>
<tr>
<td>0.0</td>
<td>15.7</td>
<td>16.92</td>
<td>32.63</td>
</tr>
<tr>
<td>0.0</td>
<td>15.88</td>
<td>16.92</td>
<td>32.80</td>
</tr>
</tbody>
</table>
The data of Table 7 indicate two possible pH optima for the precipitation of the glucosidase from each of the two different enzyme preparations used. Maximum precipitation of the enzyme from the mold filtrate (I) was obtained near pH 4.5 and pH 7.0. Near pH 4.5 and pH 6.0, the maximum precipitation of the glucosidase from the mold filtrate (II) was obtained. This result suggests a disadvantage of ammonium sulfate as a precipitant of the glucosidase for the purification of the enzyme. If there are two protein fractions possessing the glucosidase activity, the ammonium sulfate precipitation would have to be worked out at the two pH optima. Otherwise, one fraction would have to be eliminated which would result in a low recovery of the enzyme. However, the two pH optima may be due, not to two protein fractions possessing the glucosidase activity, but to a possibility that at one pH level, one of the protein components of the mold filtrate was precipitated by the ammonium sulfate. This precipitated protein could have acted as an adsorbent for the glucosidase, which suggests a poor purification step.

(3) Effect of heating the enzyme solution prior to ammonium sulfate precipitation. A first step common to many enzyme purifications is a selective denaturation of inert protein. By careful heating of extracts, much of the


inert protein material may be denatured, precipitated and removed without destruction of the desired enzyme (134). The effect of heating the crude mold filtrate prior to precipitation with ammonium sulfate was determined, and this was done as follows.

Fifty milliliters of the mold filtrate were placed in separate beakers. The solutions were heated at various temperatures, such as 50°, 60°, 70°, 75° and 80°C., and maintained at one temperature in a water bath for 15 minutes. The solutions were cooled in an ice bath immediately after heating. The precipitates that were formed after heating were centrifuged off, and the supernatants were fractionated with ammonium sulfate, in the same manner as described previously. Precipitation was done with the salt from 0 to 40 per cent saturation, and then from 40 to 60 per cent saturation. The precipitates that were formed still had the usual dark brown color that had been obtained from previous ammonium sulfate precipitations. However, the precipitates obtained from the heated enzyme solutions were different in that they were flocculent and mostly floating on the surface of the solutions. The precipitates from each fraction were centrifuged off and dissolved in 10 milliliters of distilled water. Five-milliliter portions were used for the determination of maltase activity, and the results are
given in Table 8. The original enzyme solution used for this determination contained 11.19 units of maltase activity per milliliter.

It is apparent from the data of Table 8 that the glucosidase, like most other enzymes, is affected by heat. The glucosidase appears to be completely inactivated after heating at 75° and at 80°C., and partially inactivated after heating at 70°C. However, the heat treatment for 15 minutes at 50°C. prior to the ammonium sulfate precipitation indicates an increase in the total glucosidase activity recovered from the original enzyme solution by ammonium sulfate precipitation. Only 46.1 per cent of the total glucosidase activity was precipitated with ammonium sulfate from 0 to 90 per cent saturation, as given in Table 3. The total glucosidase activity precipitated with ammonium sulfate from 0 to 60 per cent saturation of the enzyme solution which had been previously heated at 50°C. was 59.4 per cent. This figure, compares very well with the total glucosidase activity precipitated with ammonium sulfate from 0 to 60 per cent saturation at pH 4.5 which is 59.5 per cent of the total as can be seen from Table 5.

From the results so far obtained, it still seems that precipitation of the glucosidase with ammonium sulfate holds little promise as a first step in the purification of the
<table>
<thead>
<tr>
<th>Per cent saturation with ammonium sulfate</th>
<th>Units of maltase activity per milliliter of concentrated solution</th>
<th>Units of maltase activity precipitated per milliliter in terms of original solution</th>
<th>Total units of maltase activity precipitated per milliliter in terms of original solution from 0 per cent</th>
</tr>
</thead>
<tbody>
<tr>
<td>(1) Enzyme solution heated at 50°C.</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0-40</td>
<td>15.72</td>
<td>3.14</td>
<td>3.14</td>
</tr>
<tr>
<td>40-60</td>
<td>17.50</td>
<td>3.50</td>
<td>6.64</td>
</tr>
<tr>
<td>(2) Enzyme solution heated at 60°C.</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0-40</td>
<td>13.48</td>
<td>2.70</td>
<td>2.70</td>
</tr>
<tr>
<td>40-60</td>
<td>17.58</td>
<td>3.52</td>
<td>6.21</td>
</tr>
<tr>
<td>(3) Enzyme solution heated at 70°C.</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0-40</td>
<td>7.51</td>
<td>1.50</td>
<td>1.50</td>
</tr>
<tr>
<td>40-60</td>
<td>5.41</td>
<td>1.08</td>
<td>2.58</td>
</tr>
<tr>
<td>(4) Enzyme solution heated at 75°C.</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0-40</td>
<td>0.0</td>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td>40-60</td>
<td>0.0</td>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td>(5) Enzyme solution heated at 80°C.</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0-40</td>
<td>0.0</td>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td>40-60</td>
<td>0.0</td>
<td>---</td>
<td>---</td>
</tr>
</tbody>
</table>
enzyme. It is obvious that if the loss of the enzyme at each step of purification is great, very little of the material would be present by the time high purity is achieved. Therefore, the use of other salts that have been successfully employed for enzyme purification was tried.

(4) **Precipitation by salts other than ammonium sulfate including heavy metal salts.** Attempts to precipitate the protein fraction possessing the glucosidase activity by means of sodium chloride, magnesium sulfate, and potassium dihydrogen phosphate were made. Fifty milliliters of the mold filtrate were placed in separate beakers. The solid salt was added slowly in portions sufficient to increase the saturation by 20 per cent each time up to 100 per cent saturation. The precipitation was done in an ice bath, and the salts were dissolved by stirring before the addition of the next fraction. No detectable quantities of material were precipitated by any of the above-mentioned salts. These results indicate that neither sodium chloride nor magnesium sulfate nor potassium dihydrogen phosphate is suitable for precipitating the glucosidase.

Attention was next directed towards the use of heavy metal salts. Some of the heavy metal salts have been successfully employed by other workers to precipitate inert protein or inactivate an unwanted enzyme from enzymes and other protein mixtures (134).
A preliminary precipitation with zinc acetate was done as follows. The mold filtrate was distributed in 10-milliliter portions in different test tubes which were placed in an ice bath. A 0.05 molar solution of zinc acetate was added in 3, 4, 5, 6, 7, 8, and 9-milliliter portions into each of the different test tubes containing the mold enzyme solution. The solutions were mixed by stirring, and chilled overnight for complete precipitation. The resulting precipitates were centrifuged off and dissolved in 10 milliliters of distilled water. The supernatants and the solutions containing the dissolved precipitates were analyzed for glucosidase activity. The results obtained indicated the possibility of the use of heavy metal salts for the purification of the mold glucosidase. A good quantity of brown precipitate was obtained from the precipitation of the crude enzyme solution by zinc acetate at each of the various concentrations of salt tested. The glucosidase activity determinations indicated that practically all of the activity remained in the supernatant, and that the precipitates obtained were mostly all foreign materials. This finding led to an investigation of other heavy metal salts which might give similar or better results. The list of heavy metal salts investigated includes barium acetate, calcium acetate, copper acetate, ferric chloride, mercuric acetate,
lead acetate and lead chloride, manganese sulfate, and zinc acetate. The precipitation was done in a manner similar to the preliminary precipitation with zinc acetate, except that 5 milliliter and 10 milliliter portions of 0.01 molar solutions of the different salts were used for the precipitation. The amounts of precipitates formed by each of the salts as observed were compared as given in Table 9.

Table 9. A rough comparative estimate of the relative amounts of precipitates formed by each of the heavy metal salts tested

<table>
<thead>
<tr>
<th>Salt</th>
<th>Precipitate formed by 5.0 milliliters of 0.01 molar salt solution</th>
<th>Precipitate formed by 10.0 milliliters of 0.01 molar salt solution</th>
</tr>
</thead>
<tbody>
<tr>
<td>Barium acetate</td>
<td>++</td>
<td>+</td>
</tr>
<tr>
<td>Calcium acetate</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Copper acetate</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Ferric chloride</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Lead acetate</td>
<td>+++</td>
<td>+++</td>
</tr>
<tr>
<td>Lead chloride</td>
<td>+++</td>
<td>+++</td>
</tr>
<tr>
<td>Manganese sulfate</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Mercuric acetate</td>
<td>+++</td>
<td>+++</td>
</tr>
<tr>
<td>Zinc acetate</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>
The precipitates were dark brown and gummy and mostly insoluble in water. It can be noted from Table 9 that lead acetate, lead chloride, and mercuric acetate formed the most precipitate from the crude mold enzyme solution. Copper acetate and manganese sulfate did not yield any precipitate. The precipitates were centrifuged off and the supernatant solutions were tested for the glucosidase activity. The results obtained are given in Table 10.

The combined data of Table 9 and Table 10 suggested that mercuric acetate precipitation should serve as a good step towards the purification of the mold glucosidase. Of the different heavy metal salts tested, mercuric acetate appeared to have precipitated much of the unwanted materials in the crude mold filtrate leaving most of the glucosidase activity in the supernatant.

It can be noted from Table 10 that the supernatants, obtained after precipitation with 10.0 milliliters of 0.01 molar solutions of some of the heavy metal salts, showed an increased glucosidase activity. This may be attributed to the removal of some foreign material from the solution which if present may have some inhibitory action on the glucosidase, or may have tended to compete for the same substrate.

The above results point out that the glucosidase of Aspergillus niger is not inhibited nor inactivated by heavy
Table 10. Glucosidase activity in the supernatant solutions after precipitation with various heavy metal salts

| Salts         | Units of maltase activity per milliliter of the supernatant solutions
<table>
<thead>
<tr>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>5.0 milliliters of 0.01 molar salt solution added</td>
</tr>
<tr>
<td>Barium acetate</td>
<td>9.18</td>
</tr>
<tr>
<td>Calcium acetate</td>
<td>10.47</td>
</tr>
<tr>
<td>Copper acetate</td>
<td>9.98</td>
</tr>
<tr>
<td>Ferric chloride</td>
<td>11.53</td>
</tr>
<tr>
<td>Lead acetate</td>
<td>9.44</td>
</tr>
<tr>
<td>Lead chloride</td>
<td>9.69</td>
</tr>
<tr>
<td>Manganese sulfate</td>
<td>9.22</td>
</tr>
<tr>
<td>Mercuric acetate</td>
<td>9.44</td>
</tr>
<tr>
<td>Zinc acetate</td>
<td>8.43</td>
</tr>
</tbody>
</table>

*The original enzyme solution used contained 10.62 units of maltase activity per milliliter.*
metal salts at the concentrations used. This was very encouraging, and it was hoped that a preliminary precipitation with mercuric acetate would lead to a marked purification of the mold glucosidase with destruction or inactivation of the other carbohydrases. Underkofler and Roy previously reported the destruction and denaturation of alpha-amylase from *Aspergillus oryzae* with addition of mercuric salts, and it was hoped that the alpha-amylase of *Aspergillus niger* would be affected similarly. This was investigated and will be discussed later.

Further studies were made on the use of mercuric acetate. The concentration of mercuric acetate necessary to bring about the precipitation of the greatest amount of inactive material, but at the same time leaving the maximum amount of glucosidase in the supernatant was determined. Mercuric acetate solution was prepared in 0.01 molar, 0.02 molar and 0.05 molar concentrations. Ten milliliters of the mold filtrate were measured out in separate test tubes which were placed in an ice bath. The mercuric acetate solution was added in varying quantities such as in 2, 3, 4, 5, 6, 8, 10, and 15 milliliter portions of the 0.01 molar solution; 2, 4, and 6 milliliter portions of the 0.02 molar and 0.05 molar solutions. The precipitation was carried out in the same manner as described previously.
using the heavy metal salts as precipitants. The amounts of precipitates formed by the different concentrations of mercuric acetate were noted. The precipitates were centrifuged off and the glucosidase activities in the supernatants were determined.

From the amounts of inactive material precipitated and the glucosidase activity remaining in the supernatant, it was found that the 6 milliliter portion of 0.01 molar mercuric acetate added to the 10 milliliters of enzyme solution yielded the most glucosidase activity in the supernatant relative to the amount of foreign materials precipitated out of solution.

A comparison between mercuric acetate and mercuric chloride was made, and it was found that the two mercuric salts behaved essentially alike towards the crude enzyme solution.

The effect of the use of the solid salt as a precipitating agent as compared to that of an aqueous solution of the salt was investigated. The results indicated that the solid mercuric acetate could be added to the enzyme solution without any disadvantage over that of the aqueous solution of the salt. This eliminates the necessity of preparing the salt solution before precipitation as well as prevents the unnecessary dilution of the enzyme solution.
The above findings indicated that the precipitation with solid mercuric acetate should serve well as a first step in the purification of the mold glucosidase. The best results were obtained when solid mercuric acetate was added to the enzyme solution until it was $3.75 \times 10^{-3}$ molar. The salt was dissolved with stirring, and the solution kept in the refrigerator overnight for complete precipitation. The precipitate was centrifuged off, and the supernatant containing the glucosidase was retained for the second step of the purification, which will be discussed later. This solution was designated as "mercuric supernatant" for future reference.

b. Precipitation of fractions having glucosidase activity by organic solvents

(1) Comparison of acetone, ethanol and other solvents as precipitants. A second group of precipitants commonly used for the isolation and purification of enzymes are organic solvents miscible in water. The most commonly used of these are acetone and ethanol; others include methanol and dioxane. Most enzymes are very readily denatured by organic solvents at room temperature, but are much more stable in the cold. For this reason, precipitation with organic solvents was mostly done in the cold room.
Acetone, ethanol, methanol, and dioxane were compared as precipitants of the glucosidase from the original mold filtrate. One hundred milliliters of the crude enzyme solution were measured out in separate beakers and placed in an ice bath. The previously chilled organic solvent was added to the cold enzyme solution slowly from a buret, and with stirring to prevent local high concentration of the solvent. The solvent was added to 100 milliliter portions of the enzyme solution in increments of 10 per cent from 0 to 70 per cent concentration of solvent in the solution as follows.

<table>
<thead>
<tr>
<th>Percentage</th>
<th>Organic Solvent Added</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 per cent</td>
<td>No organic solvent added</td>
</tr>
<tr>
<td>10 per cent</td>
<td>11 milliliters of organic solvent added</td>
</tr>
<tr>
<td>20 per cent</td>
<td>14 milliliters of organic solvent added to preceding solution</td>
</tr>
<tr>
<td>30 per cent</td>
<td>18 milliliters of organic solvent added to preceding solution</td>
</tr>
<tr>
<td>40 per cent</td>
<td>24 milliliters of organic solvent added to preceding solution</td>
</tr>
<tr>
<td>50 per cent</td>
<td>33 milliliters of organic solvent added to preceding solution</td>
</tr>
<tr>
<td>60 per cent</td>
<td>50 milliliters of organic solvent added to preceding solution</td>
</tr>
</tbody>
</table>
70 per cent  83 milliliters of organic solvent added to preceding solution

The precipitate from each added increment was centrifuged off, and dissolved in 10 milliliters of water for analysis of its glucosidase activity.

The results obtained were very far from encouraging. No precipitate was obtained up to 30 per cent ethanol by volume. Only 18.61 per cent of the glucosidase activity in the original mold filtrate was precipitated by ethanol from 40 to 70 per cent ethanol by volume.

In the case of acetone, methanol and dioxane, no precipitation was achieved at less than 50 per cent organic solvent by volume. Analysis of the glucosidase activity in the precipitates formed from 50 to 70 per cent solvent by volume indicated that almost negligible amounts of the glucosidase were precipitated by acetone, methanol or dioxane from the original mold filtrate.

Further studies on the purification of the enzyme revealed the possibility that precipitation of the glucosidase by organic solvents require the presence of some inorganic ions. An observation made later in this work strongly indicates the above possibility. When a partially purified solution of the mold glucosidase, which previous
to dialysis could be precipitated by acetone, was dialyzed, no precipitation could be achieved by the addition of any amount of acetone to the dialyzed solution. That the glucosidase was not lost or denatured by dialysis was indicated by its ability to precipitate again with acetone after addition of ammonium sulfate to the dialyzed solution.

Acetone has appeared to be a valuable precipitant for the glucosidase if it could be made to precipitate the enzyme from its solution. The work of Bovard (12) upon the precipitation of alpha-amylase with acetone suggested that fungal alpha-amylase was unstable in acetone solutions at concentrations above 40 per cent. Acetone precipitation, therefore, seemed to offer a good step towards the purification of the glucosidase to free it, even partially if not entirely, from alpha-amylase.

Precipitation of the glucosidase with acetone was then tried on the mold enzyme solution that had previously been precipitated with mercuric acetate as has been described earlier and designated as mercuric supernatant. Ethanol was, likewise, tried again since this was used by Phillips and Caldwell (117) in their purification of the gluamylase from *Rhizopus delemar*, and by Roy and Underkofler (126) in their purification of the maltase from *Aspergillus oryzae*. A mixture of solvents used by the latter workers was also
investigated. The mixture was made up of a 3:1 ratio of ethanol and anhydrous ether.

Ten milliliter portions of the mercuric supernatant were placed in separate test tubes which were placed in an ice bath. Precipitation was done in a manner similar to that previously described for the precipitation of the glucosidase from the original mold filtrate using organic solvents. Acetone, ethanol and the 3:1 mixture of ethanol and anhydrous ether were added in quantities sufficient to make the solvent concentrations up to 40 per cent by volume and then from 40 up to 70 per cent by volume. The precipitates formed from each added increment of solvent were centrifuged off, dissolved in 10 milliliters of distilled water and analyzed for glucosidase activity. Of the three solvents, acetone gave almost quantitative recovery of the enzyme. The ethanol-ether mixture gave the lowest recovery, while ethanol alone indicated a slight loss of the enzyme. Very little of the glucosidase was precipitated by the 0 to 40 per cent concentration of solvent added. The greater bulk of the glucosidase was obtained from the fraction precipitated between 40 and 70 per cent solvent.

(2) The effect of pH on the precipitation by acetone of the fraction containing the glucosidase activity. Since the highest recovery possible of the enzyme is desirable
after every step in a purification procedure, the effect of
pH on the amount of glucosidase that could be recovered
after precipitation by acetone was determined.

One hundred milliliter portions of the mercuric super-
natant were measured out in separate beakers and placed in
an ice bath. The pH of the enzyme solutions was adjusted
to different levels with addition of 1 normal ammonium
hydroxide. Precipitation of the fraction containing the
glucosidase by acetone was done in a manner similar to that
previously described for the precipitation using organic
solvents. The acetone was added in increments to make the
solution up to 30 per cent acetone by volume, then 30 to
50 per cent acetone by volume and finally 50 to 60 per cent
acetone by volume. The precipitate obtained after each
added increment of acetone was centrifuged off, dissolved
in 20 milliliters of water, and the glucosidase activity
of the dissolved precipitate was determined. The results
obtained are given in Table 11.

It is evident from the data of Table 11 that the mold
glucosidase was best precipitated by acetone when the enzyme
solution was at pH 6.0. Preliminary experiments on the
effect of pH on the precipitation of the glucosidase by
acetone indicated that the enzyme was least precipitated
at pH 5.0 and 5.5 and at pH levels below 4.0 and pH levels
Table 11. Precipitation of the glucoamylase from the mercuric supernatant with acetone at various pH levels

<table>
<thead>
<tr>
<th>Per cent acetone by volume</th>
<th>Units of maltase activity per milliliter of solution containing dissolved precipitate</th>
<th>Total units of maltase activity precipitated</th>
<th>Per cent recovery</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(1) pH 4.0</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0-30</td>
<td>2.24</td>
<td>44.8</td>
<td>2.84</td>
</tr>
<tr>
<td>30-50</td>
<td>64.8</td>
<td>1296.0</td>
<td>82.4</td>
</tr>
<tr>
<td>50-60</td>
<td>5.25</td>
<td>105.0</td>
<td>6.66</td>
</tr>
<tr>
<td>Total</td>
<td></td>
<td></td>
<td>91.90</td>
</tr>
<tr>
<td>(2) pH 4.5</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0-30</td>
<td>4.25</td>
<td>85.0</td>
<td>5.4</td>
</tr>
<tr>
<td>30-50</td>
<td>69.4</td>
<td>1388.0</td>
<td>88.1</td>
</tr>
<tr>
<td>50-60</td>
<td>4.38</td>
<td>87.6</td>
<td>5.6</td>
</tr>
<tr>
<td>Total</td>
<td></td>
<td></td>
<td>99.1</td>
</tr>
<tr>
<td>(3) pH 6.0</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0-30</td>
<td>1.61</td>
<td>32.2</td>
<td>2.04</td>
</tr>
<tr>
<td>30-50</td>
<td>73.2</td>
<td>1484.0</td>
<td>93.0</td>
</tr>
<tr>
<td>50-60</td>
<td>4.53</td>
<td>90.6</td>
<td>5.76</td>
</tr>
<tr>
<td>Total</td>
<td></td>
<td></td>
<td>100.80</td>
</tr>
<tr>
<td>(4) pH 7.0</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0-30</td>
<td>2.48</td>
<td>49.6</td>
<td>3.15</td>
</tr>
<tr>
<td>30-50</td>
<td>67.9</td>
<td>1358.0</td>
<td>86.8</td>
</tr>
<tr>
<td>50-60</td>
<td>6.42</td>
<td>128.4</td>
<td>8.16</td>
</tr>
<tr>
<td>Total</td>
<td></td>
<td></td>
<td>98.11</td>
</tr>
</tbody>
</table>

*The mercuric supernatant used contained 1.75 units of maltase activity per milliliter of solution.*
above 7.0. At the pH levels included in Table 11, it can be noted that the amounts of glucosidase precipitated by acetone do not differ significantly. However, the results are comparable with those obtained from the precipitation of the glucosidase with ammonium sulfate as given in Tables 6 and 7. At both pH levels 4.5 and 6.0, the mold glucosidase appears to precipitate best with either acetone or ammonium sulfate.

The per cent recovery of the glucosidase after acetone precipitation was very gratifying. This step, however, presented one problem. The precipitate obtained from all the acetone fractions and at all the different pH levels tested contained a water-insoluble fraction together with the protein fraction containing the glucosidase activity. A water-insoluble fraction is undesirable in the purification of an enzyme, since it is a common practice in enzyme purification to dissolve the precipitate from a purification step in the minimum amount of water in order to bring up the enzyme concentration higher after every step. The formation of a water-insoluble fraction, however, was eliminated with the addition of 5 per cent dipotassium hydrogen phosphate to the mercuric supernatant prior to precipitation with acetone. The addition of the dipotassium hydrogen phosphate brought the pH of the enzyme solution near the desirable pH 6, and
at the same time brought down a precipitate of inactive and dark brown materials which were centrifuged off before precipitation of the glucosidase with acetone. The precipitate thus obtained from the acetone was freed from the water-insoluble fraction, and was less dark-colored and much less gummy or sticky.

c. Adsorbents for the fraction containing the glucosidase activity or for impurities. The first attempt at adsorption in this work was that using oxalate-calcium carbonate. As has been mentioned earlier, the oxalate-calcium carbonate failed to adsorb the glucosidase of *Aspergillus niger* NRRL 330 under the conditions in which it was used. Fuller's earth, the second adsorbent used by Roy and Underkofler (126) in the purification of maltase, did not show any affinity for the glucosidase either, even under conditions similar to those employed by the above-mentioned workers. When adsorption was done by the continuous flow technique or chromatography through a column, there was likewise no appreciable increase in its ability to adsorb the enzyme. No work was done to determine if the above adsorbents adsorbed other impurities present in the enzyme solution in preference to the glucosidase. The dark color retained in the enzyme solution after treatment with
the above adsorbents, however, did not make further investigations on them appear worthwhile.

The work of Lipps, Roy, Andreasen, Vernon and Kolachov (89) with the amylase of *Aspergillus niger* NRRL 337, and of Underkofler and Roy (157) with concentrates of submerged cultures of *Aspergillus oryzae* made use of the selective adsorption of impurities from alpha-amylase solutions by bentonite at controlled hydrogen ion concentrations. The addition of 2 to 4 per cent bentonite to a solution of the partially purified enzyme resulted in only very slight adsorption of alpha-amylase but complete removal of the maltase and limit dextrinase activities. Bentonite was, therefore, investigated as a possible adsorbent for the glucosidase of *Aspergillus niger* NRRL 330.

Twenty milliliter portions of the partially purified enzyme solution after treatment with mercuric acetate, dipotassium hydrogen phosphate, and acetone precipitation as previously described, were placed in separate test tubes and placed in an ice bath. The pH of the solution was adjusted to different levels with 1 normal acetic acid or 1 normal ammonium hydroxide. The solutions were all diluted to 25 milliliters. One-gram portions of bentonite were added to the solutions at various pH levels with stirring and occasional shaking for 20 minutes after which the
bentonite was centrifuged off. The supernatants were analyzed for their glucosidase activities. The results indicated that no significant amount of the glucosidase was adsorbed by the bentonite at any of the different pH levels tested. There was no quantitative difference in the amounts of glucosidase adsorbed by the bentonite from pH 4.0 to 7.5 varied in increments of 0.5 pH unit.

Adsorption by the continuous flow technique, or column chromatography, did not meet with success. The bentonite at the upper portion of the column became glue-like and sticky upon getting wet, and prevented the further continuous flow of the enzyme solution through the column.

Increase in the amount of bentonite used from 4 to 5 to 6 to 7 per cent, as well as increase in the time allowed for adsorption which was done either by stirring with a mechanical stirrer or shaking with a mechanical shaker did not cause more than 50 per cent of the total glucosidase activity in the enzyme solution to be adsorbed by the bentonite.

Attention was next directed to other adsorbents which may be poor adsorbents for the glucosidase but efficient adsorbents for impurities in the enzyme solution. It has appeared from the literature that adsorption techniques have been most successful when used to remove undesired
proteins and other substances (83, 110, 135) when elution problems need not be considered.

Starches from different sources have been reported as good adsorbents for alpha-amylase (133). One of the final steps used by Schwimmer and Balle (132) in the purification of malt alpha-amylase was adsorption of the enzyme on wheat starch from cold 40 per cent ethanol solution. French and Knapp (34), separated the maltase fraction from any detectable trace of alpha-amylase activity in an enzyme preparation from *Clostridium acetobutylicum* by adsorbing the fraction carrying the alpha-amylase activity upon acetone-extracted potato starch from an acetone-water solution of the enzyme. Bovard (12) in his work on the purification of alpha-amylase from taka-diastase reported rice starch to be the most effective adsorbent for the amylase from an enzyme solution containing 5 units of alpha-amylase per milliliter. When the enzyme solution was of higher alpha-amylase content, no difference in the alpha-amylase adsorbing power of corn, potato, rice or arrowroot starch was noted.

In the hope that the alpha-amylase of the enzyme solution could be selectively adsorbed by starch, thereby yielding a glucosidase free from alpha-amylase, corn starch, potato starch, rice starch and commercial soluble starch
were tested as adsorbents. The conditions included ice bath temperature, 20 per cent acetone-water enzyme solution and 20 grams of solid starch per 100 milliliters of solution. The mixtures were agitated in the mechanical shaker for 20 minutes.

The enzyme solutions obtained after the adsorbents were centrifuged off were analyzed for residual glucosidase and alpha-amylase activities. The results indicated that the different starches did not selectively adsorb the mold glucosidase under the conditions of the experiment. Alpha-amylase activity was still detected in the solutions as measured by the method for the determination of alpha-amylase described under the section on Methods and Materials. Of the four starches tested, soluble starch appeared to be the best adsorbent for the alpha-amylase. However, from Figure 2 (page 144), it can be noted that no clean cut separation of the mold glucosidase from alpha-amylase was accomplished after adsorption even with soluble starch. The criterion for this will be discussed later.

Although a thorough investigation of the possible conditions favorable for the use of starches as adsorbents for the unwanted materials in the mold enzyme solution was not made, it appeared that very little purification of the
glucosidase from alpha-amylase could be achieved by starch adsorption.

Another adsorbent that was investigated was activated carbon. Zittle (175), in his review on adsorption studies of enzymes and other proteins stated that charcoal can be used to remove contaminating proteins, but is not recommended for the isolation of proteins since there is no satisfactory eluant yet known. In the hope of adsorbing the undesirable proteins from the mold enzyme solution, Norit-A was tested as an adsorbent. Preliminary experiments indicated that treatment of the partially purified enzyme solution with from 4 to 6 per cent Norit-A, obtained from Fisher Scientific Company, either with stirring for 20 minutes in the cold or letting stand in the cold overnight removed much of the colored materials from the enzyme solution but did not adsorb the glucosidase. The charcoal, however, could not be completely eliminated from the enzyme solution either by the centrifuge or by any grade of filter paper available. Adsorption by the continuous flow technique using a glass column was, therefore, tried. An almost completely decolorized solution was collected from the column, which, however, was completely devoid of glucosidase activity. A series of buffer solutions from pH 3.0 to pH 11 prepared according to MacIlvain and Clark
and Lube (57), were tried to elute the enzyme from the charcoal. Analysis of the eluates obtained from the buffer solutions of different pH did not indicate any glucosidase activity.

Since much of the dark brown color of the enzyme solution was observed to have been removed by the Norit treatment, it was of interest to see whether Norit could be used as an adsorbent for the impurities present in the enzyme solution rather than as an adsorbent for the glucosidase, thereby eliminating, too, the problem of elution. To obtain a solution that would not contain residual charcoal, which as has been previously mentioned could not be completely eliminated either by the centrifuge or by filter paper, filter aids were tested. Filter pads manufactured by Hormann and Company, New Jersey, and of the D-0 Clariflow grade were found to retain all the charcoal and not absorb the glucosidase. A pale yellow solution was thus obtained after the Norit treatment, free from residual charcoal and still containing the same glucosidase activity as before adsorption. More than 6 per cent Norit used for adsorption resulted in a more decolorized solution but also an accompanying greater loss in glucosidase activity. Variations in the pH of the enzyme solution from 4.0 to 7.0 did not indicate marked effects on the adsorption of the
glucosidase by activated carbon. Some criteria for the extent of purification achieved after Norit treatment will be discussed later.

Other adsorbents tried in this work include gels such as calcium phosphate gel prepared according to Singer (140); zinc hydroxide gel prepared according to Green (45); and alumina gel prepared according to Hawk, Oser and Summerson (52). Such gels have been successfully employed by other workers for the purification of other enzymes. Zinc hydroxide gel has been used successfully to remove all red and brown proteins for the purification of a dehydrogenase (45). The same result, however, was not obtained in this work. Some criteria for the extent of purification achieved by adsorption with calcium phosphate and alumina gels will be given later.

It is a well known fact that the use of adsorption techniques for the purification of enzymes involves a number of factors which must be carefully considered to obtain the most purification and the least loss of the desired enzyme. Such variables as pH, temperature, rate of adsorption, concentration of the adsorbent, concentration of salts in the solution to be treated with the adsorbent, and concentration of the substance to be adsorbed play an important part in the efficiency of the adsorbent being used. A thorough
investigation of all these factors, however, was not under-
taken in this work.

4. Purification scheme developed and some criteria of
   purity for several of the various steps

   From the results obtained from the various attempts
   at purification, as have already been described, a purifica-
   tion scheme was developed which may be outlined as follows.

   (A) Original mold filtrate (2 liters)
       Solid mercuric acetate added to
       make solution $3.75 \times 10^{-3}$ molar.
       Stirred with mechanical stirrer
       for 30 minutes. Allowed to stand
       in the cold overnight for complete
       precipitation. Centrifuged.

   Precipitate
   (Discarded)

   (B) Supernatant
       Solid dipotassium hy-
       drogen phosphate added
       using 5 grams per 100
       milliliters solution.
       Stirred and allowed
to precipitate for 20 minutes. Centrifuged.

Supernatant    Precipitate

Previously chilled  (Discarded)
acetone added from a
buret with stirring
using a mechanical
stirrer until 30 per
cent acetone by
volume. Centrifuged.

Precipitate    Supernatant
(Discarded)    Acetone added to make
solution to 55 per cent
acetone by volume.
Centrifuged.

(C) Precipitate    Supernatant
Dissolved in 250 to 350  (Discarded)
milliliters cold water.
Norit-A added with stir-
ring using 4.0 grams per
100 milliliters solution.
Filtered in the cold using filter pads of the D-0 Clariflow grade.

**Precipitate**
- Discarded

**(D) Filtrate**
- pH adjusted to pH 6.0 and previously cooled acetone added as before until 30 per cent acetone by volume.
- Centrifuged.

**Supernatant**
- Cold acetone added to make solution to 50 per cent acetone by volume. Centrifuged.

**Precipitate**
- Discarded

**Supernatant**
- Discarded

**(E) Precipitate**
- Dissolved in minimum amount of cold water and dialyzed against distilled water in the cold for 24
hours, with frequent changes of the water.

Dialyzed solution
Solid ammonium sulfate added with stirring until 40 per cent saturated. Centrifuged.

Precipitate
Solid ammonium sulfate added to make solution to 60 per cent saturated. Allowed to stand in the cold overnight for complete precipitation. Centrifuged.

(F) Precipitate
Dissolved in minimum amount of cold water. Cold acetone added to make solution 30 per
cent acetone by volume. Centrifuged.

Precipitate      Supernatant
(Discarded) Cold acetone added to
make solution to 50 per
cent acetone by volume.
Centrifuged.

(G) Precipitate      Supernatant
Dissolved in minimum (Discarded)
amount of cold water to
give the purified en-
zyme solution.

Steps from the start to that point labeled (E) in the
scheme outlined above were carried out several times and
the products combined to give quantities large enough to
work with. The products obtained at some of those points
labeled with letters in the scheme above were analyzed for
glucosidase activity, alpha-amylase activity, and protein
nitrogen content per milliliter of solution by the methods
described in the section on Methods and Materials. This
was done to follow the extent of purification achieved,
and also to determine the extent of recovery or loss of the glucosidase activity after some of the purification steps. The results obtained are given in Table 12.

From Table 12, it can be noted that the glucosidase activity of the enzyme solution had been raised from 15.48 to 1,332.5 units per milliliter, an increase of less than a hundred-fold. Analysis of the nitrogen content of the various solutions indicates an increase of glucosidase activity per milligram of nitrogen from 11.09 to 362.63 units, or an increase of about 30-fold. More than 50 percent of the glucosidase activity in the original mold filtrate was lost through the various steps of the purification scheme. It is obvious that there is need for more refinement in the various procedures adopted. It can be noted from Table 12 that the greater loss of the glucosidase activity occurred after precipitation with ammonium sulfate. This can probably be attributed to incomplete precipitation, or loss of the enzyme into the supernatant. The precipitate formed after ammonium sulfate precipitation was very flocculent which tended to remain in suspension even after an hour in the centrifuge. Another probable cause may be the technique of handling bigger volumes. A preliminary experiment with ammonium sulfate precipitation using only 20 milliliters of the partially purified enzyme solution
### Table 12. Analytical results obtained for the products from some of the steps in the purification of the glucosidase from *Aspergillus niger* NRRL 330

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Units of glucosidase activity per milliliter solution</th>
<th>Per cent recovery of glucosidase activity</th>
<th>Units of glucosidase activity per milligram nitrogen</th>
<th>Units of alpha-amylase activity per milliliter solution</th>
</tr>
</thead>
<tbody>
<tr>
<td>A. Original mold filtrate</td>
<td>15.48</td>
<td>11.09</td>
<td>0.93</td>
<td></td>
</tr>
<tr>
<td>B. Supernatant after treatment with mercuric acetate</td>
<td>15.48</td>
<td>100</td>
<td>---</td>
<td>0.37</td>
</tr>
<tr>
<td>C. First acetone precipitate</td>
<td>78.84</td>
<td>89.5</td>
<td>114.26</td>
<td>0.04</td>
</tr>
<tr>
<td>D. Norit filtrate</td>
<td>73.73</td>
<td>83.5</td>
<td>121.06</td>
<td>---</td>
</tr>
<tr>
<td>E. Second acetone precipitate</td>
<td>208.05</td>
<td>67.3</td>
<td>243.64</td>
<td>---</td>
</tr>
<tr>
<td>F. Precipitate from 40 to 60 per cent ammonium sulfate fractionation</td>
<td>892.42</td>
<td>49.0</td>
<td>---</td>
<td>1.6</td>
</tr>
<tr>
<td>G. Third acetone precipitate</td>
<td>1,332.5</td>
<td>45.0</td>
<td>362.63</td>
<td>---</td>
</tr>
</tbody>
</table>
indicated more than 80 per cent recovery. It was a general observation that work with bigger volumes always has resulted in poorer recovery or greater loss of the enzyme from one step to the next. At that step labeled (F) in the purification scheme, lithium sulfate was compared with ammonium sulfate as a precipitant for the mold glucosidase, and it was observed that ammonium sulfate afforded higher recovery of the enzyme.

The alpha-amylase activity of the solution was decreased from 0.93 to 0.04 unit after the first acetone precipitation. However, as can be seen in Table 12, there was a noted increase in the alpha-amylase activity after the ammonium sulfate fractionation to 1.6 units per milliliter of solution. This increase in alpha-amylase activity, as measured by its action on starch by the method described in the section on Methods and Materials, is probably to be expected from a solution with an appreciable increase in glucosidase activity if the glucosidase is not specific for maltose as substrate but also has the ability to hydrolyze starch.

Preliminary experiments using the paper chromatography technique, described in the section on Methods and Materials, indicated that the most purified glucosidase so far obtained in this work hydrolyzes both the straight-chain as well as the branched fractions of starch yielding only
glucose. Whether the alpha-amylase activity detected by the method of Sandstedt, Kneen and Blish (128) was due to contamination by alpha-amylase or due to the glucosidase having alpha-amylase as well as maltase activity was investigated. This was done by a comparison of the blue value with the reducing value of the hydrolysate at certain times during the hydrolysis of starch by the glucosidase that had been through different stages of purification.

Fifty milliliters of a 0.1 per cent solution of potato amylose containing 2.5 milliliters of acetic buffer pH 4.4 were used as substrate. One milliliter portions of enzyme solutions of various degrees of purity of the proper dilution were added to 50 milliliters of the substrate after both enzyme and substrate temperatures had attained equilibrium in a water bath maintained at 30°C. Separate aliquot portions of the hydrolysates were withdrawn at various suitable time intervals and analyzed for blue values according to McCready and Hassid (93), and reducing values according to Somogyi (142), details of which have been given in the section on Methods and Materials. The original mold filtrate, the Norit filtrate obtained from the purification scheme as described earlier, a Norit filtrate that had been heated to 60°C., and an enzyme solution that had been purified by adsorption on Celite and on Silica gel in addition
to all the steps in the purification scheme were tested and compared. The results are given in Table 13 and the comparisons can be seen in Figure 1. Data reported by Tung-Kung, Hanrahan, and Caldwell (154) for similar measurements of the action of recrystallized maltase-free *Bacillus subtilis* and taka alpha-amylases upon the linear fraction from corn starch are included in Figure 1 to compare with the hydrolytic action of the glucosidase from *Aspergillus niger* at different stages of purification. Figure 1 indicates the relationship between the disappearance of the starch substrate as measured by blue values and the increase in the reducing values of the hydrolysates measured by determination of reducing sugars formed during the course of the hydrolysies. Tung-Kung and his co-workers in their studies on the action of alpha-amylases from different sources upon a linear fraction from corn starch found that the above relationship is independent of the concentration of the amylase but is characteristic of the amylase itself depending on its source. From Figure 1, it can be seen that the maltase-free alpha-amylases studied by Tung-Kung and co-workers show marked differences from the glucosidase of *Aspergillus niger* in their action towards the linear fraction of starch. Furthermore, the figure indicates the extent of purification of the mold glucosidase from
Table 13. Hydrolyses of the linear fraction of potato starch by the glucosidase from *Aspergillus niger* at different stages of purification measured by the blue values and reducing values of the hydrolysates, together with similar measurements by Tung-Kung and co-workers on the hydrolysis of the linear fraction of corn starch by maltase-free alpha-amylases.

<table>
<thead>
<tr>
<th>Original mold filtrate</th>
<th>Norit filtrate</th>
<th>Purified glucosidase</th>
<th>Norit filtrate heated to 60°C.</th>
<th>Purified <em>Bacillus subtilis</em> amylase</th>
<th>Purified taka amylase</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blue value glucose</td>
<td>Blue % glucose</td>
<td>Blue value glucose</td>
<td>Blue % glucose</td>
<td>Blue value glucose</td>
<td>Blue % glucose</td>
</tr>
<tr>
<td>95</td>
<td>11.2</td>
<td>59</td>
<td>32.8</td>
<td>138</td>
<td>10.1</td>
</tr>
<tr>
<td>59</td>
<td>21.8</td>
<td>5</td>
<td>46.5</td>
<td>128</td>
<td>18.1</td>
</tr>
<tr>
<td>10.2</td>
<td>26.6</td>
<td>5</td>
<td>89.5</td>
<td>111</td>
<td>22.8</td>
</tr>
<tr>
<td>8.8</td>
<td>41.5</td>
<td>0</td>
<td>90.6</td>
<td>90</td>
<td>35.1</td>
</tr>
<tr>
<td>0</td>
<td>61.6</td>
<td>0</td>
<td>96.3</td>
<td>46</td>
<td>36.7</td>
</tr>
<tr>
<td>0</td>
<td>63.4</td>
<td>0</td>
<td>96.3</td>
<td>22</td>
<td>68</td>
</tr>
<tr>
<td>0</td>
<td>76.6</td>
<td>0</td>
<td>96.3</td>
<td>8.0</td>
<td>79.2</td>
</tr>
<tr>
<td>0</td>
<td>82.6</td>
<td>0</td>
<td>96.3</td>
<td>3.5</td>
<td>84.5</td>
</tr>
<tr>
<td>9.0</td>
<td>90.2</td>
<td>5.6</td>
<td>5.6</td>
<td>3.0</td>
<td>10.0</td>
</tr>
<tr>
<td>1.0</td>
<td>96.3</td>
<td>3.0</td>
<td>3.0</td>
<td>10</td>
<td>10</td>
</tr>
</tbody>
</table>


Figure 1. Hydrolysis of potato amylase by the glucosidase from Aspergillus niger at different stages of purification and similar measurements on the hydrolysis of corn amylase by maltase-free alpha-amylases reported by Tung-Kung and co-workers.
alpha-amylase achieved through the various steps of the purification scheme. Curve 4 which represents the enzyme action of the original mold filtrate approaches curves 5 and 6 of the maltase-free alpha-amylases which, therefore, is indicative of the alpha-amylase content of the original mold filtrate. The alpha-amylase action on starch seems to be characterized by a rapid breakdown of the starch substrate to small dextrins indicated by the rapid disappearance of the blue color of the starch-iodine complex, accompanied by a slight increase in reducing value. On the other hand, the glucosidase from *Aspergillus niger* seems to hydrolyze the starch substrate in a different fashion characteristic of the enzyme and getting more pronounced as it increased in purity as can be noted from Curves 1 and 3. It is evident that the glucosidase action upon the linear fraction of starch is characterized by the slow disappearance of long chain fragments indicated by the slow disappearance of the blue starch-iodine complex color accompanied by a correspondingly big increase in reducing value.

Figure 1 indicates that heating the enzyme solution to 60°C does not remove the alpha-amylase in the solution as well as adsorption of the enzyme solution on Celite and on silica gel used as adsorbents. This was done as follows: The original mold filtrate was treated in a manner similar
to that described in the purification scheme outlined previously up to step labeled (D). Celite analytical filter-aid manufactured by Johns-Manville was added in 20 gram portions per 100 milliliters of the Norit filtrate and the mixture was allowed to shake in the mechanical shaker for 15 minutes. The adsorbent was centrifuged off and the supernatant treated in exactly the same manner again as in the purification scheme until that step labeled (F). The precipitate after dissolving in a minimum amount of water was treated with silica gel (source unknown) using 20 grams of the gel per 100 milliliters of enzyme solution, and allowed to shake in the shaker for 15 minutes. The adsorbent was centrifuged off and the supernatant precipitated with acetone as described in the scheme. The glucosidase solution, thus obtained, was the solution which appeared to have been freed the most from alpha-amylase and all of the dark colored compounds in the original mold filtrate. The cellulase present in the original mold filtrate, which gave trouble during dialysis in the early attempts of purification, seemed to have been removed, also, by the above steps of purification since the cellophane bags easily stood dialysis for 24 hours without necessity of changing them.

Since the Celite and silica gel adsorption successfully removed most of the alpha-amylase in the partially purified
enzyme solution, other adsorbents were tried to replace the Celite and silica gel in the hope of finding a more efficient adsorbent for the alpha-amylase and other impurities. The other adsorbents tried were alumina gel, soluble starch, and calcium phosphate gel. None of the various combinations of the adsorbents tried worked as efficiently as the Celite and silica gel as can be seen from Table 14 and Figure 2. The efficiency of the different adsorbents could possibly be improved by proper control of the different variables that affect adsorption of proteins but this was not undertaken in this work.

B. Studies on Some of the Properties of the Glucosidase

1. Activity of the glucosidase on various substrates

For the studies on some of the properties of the glucosidase, the enzyme solution obtained after purification by the scheme outlined on pages 128 to 132 with addition of the adsorption by Celite and silica gel as described on page 141 was used.

Studies on the substrate specificity of the mold glucosidase were done by paper chromatography. Details
Table 14. Hydrolyses of the linear fraction of potato starch by the glucosidase purified by the various steps of the purification scheme and adsorption on different adsorbents

<table>
<thead>
<tr>
<th>Twice adsorbed on alumina gel</th>
<th>Twice adsorbed on calcium phosphate gel</th>
<th>Twice adsorbed on soluble starch</th>
<th>Adsorbed on soluble starch and on alumina gel</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blue Per cent value glucose</td>
<td>Blue Per cent value glucose</td>
<td>Blue Per cent value glucose</td>
<td>Blue Per cent value glucose</td>
</tr>
<tr>
<td>101 25.9</td>
<td>118 3.72</td>
<td>97.0 21.8</td>
<td>98.0 22.9</td>
</tr>
<tr>
<td>65.0 37.2</td>
<td>96.0 7.98</td>
<td>90.0 24.0</td>
<td>81.0 24.0</td>
</tr>
<tr>
<td>21.0 43.6</td>
<td>86.0 12.21</td>
<td>32 50.5</td>
<td>32.0 55.9</td>
</tr>
<tr>
<td>7.0 71.9</td>
<td>85.0 17.04</td>
<td>8.0 63.4</td>
<td>11.0 66.0</td>
</tr>
<tr>
<td>7.0 77.1</td>
<td>65.0 21.1</td>
<td>0 77.0</td>
<td>7.0 78.6</td>
</tr>
<tr>
<td>2.0 77.6</td>
<td>54.0 26.1</td>
<td>0 77.0</td>
<td>0 78.8</td>
</tr>
<tr>
<td>0 78.6</td>
<td>53.0 31.4</td>
<td>0 78.6</td>
<td>0 79.8</td>
</tr>
<tr>
<td>32.5 38.3</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>18.0 43.6</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>7.5 65.0</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2.0 76.6</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Figure 2. Hydrolysis of potato amylose by the glucosidase purified by the various steps of the purification scheme and adsorption on different adsorbents.
of this technique were given in the section on Methods and Materials. The substrates investigated included: a linear fraction from potato starch, a branched fraction from corn starch, glycogen, a heptasaccharide, beta and gamma Schardinger dextrins, panose, gentiobiose, methyl alpha-glucoside, and maltose, samples of which were provided by Dr. Dexter French. Five milliliters of a 1.0 per cent solution of the various substrates containing acetate buffer of pH 4.4 were used for the hydrolysis. One milliliter of the purified glucosidase solution of the proper dilution was added to each 5 milliliter portion of 1.0 per cent substrate. Hydrolysis was carried out at 30°C. Of the substrates tested, methyl alpha-glucoside and beta Schardinger dextrin failed to be hydrolyzed by the glucosidase as indicated by the resulting paper chromatogram which did not show any hydrolysis product even after an hour of enzyme-substrate reaction. The beta and gamma Schardinger dextrins are cyclic alpha-D-glucopyranosides having 7 and 8 glucose units, respectively, joined by 1,4-alpha-D-glucosidic linkages (37). The gamma Schardinger dextrin was hydrolyzed by the glucosidase as indicated by the appearance of glucose in the chromatogram even within the first five minutes of the enzyme-substrate reaction. The difference in the activity of the purified glucosidase
towards beta and gamma dextrins may probably be due to the
difference in size between the two substrates which possibly
plays an important role in the steric requirements of the
enzyme-substrate reaction. Glucose appeared to be the sole
hydrolysis product of the hydrolyses of the linear and
branched fractions of starches and of glycogen from the
start until the very late stages of the hydrolyses. No
reducing sugar other than glucose was shown by the paper
chromatograms obtained. From the hydrolysis of the hepta-
saccharide tested, glucose was obtained within the first 5
minutes of the enzyme-substrate reaction up to the late
stages of the hydrolysis. Other reducing sugars of more
than one glucose unit were noted in addition to the glucose
until up to 20 minutes of the enzyme-substrate reaction,
after which time only glucose was evident. Gentiobiose and
panose were also hydrolyzed by the mold glucosidase only to
glucose which appeared within the first 10 minutes of the
enzyme-substrate reaction. Unreacted panose and unreacted
gentiobiose were noted in the paper chromatograms for 70
minutes of the enzyme-substrate reaction, after which time
only glucose was noted. Panose is a trisaccharide com-
posed of three glucose residues, joined by one alpha-1,4-
and one alpha-1,6-glucosidic linkage. Gentiobiose is a
disaccharide the 2 glucose units of which are joined by a
beta-1,6-glucosidic linkage. The above results indicate that the glucosidase is not specific for the alpha-1,4-glucosidic linkage in maltose, but is capable of hydrolyzing starch, its components and its hydrolysis products. The purified glucosidase also showed its ability to hydrolyze both the alpha- and the beta-1,6-glucosidic linkages in its substrates. The above results, however, are not entirely conclusive since many factors, in addition to the kind of linkage, influence the action and affinity of an enzyme. It may be very possible that other enzymes responsible for some of the hydrolytic actions observed have not been entirely removed from the mold enzyme solution in spite of all the various steps of purification. Attempts are still being continued to obtain the glucosidase in crystalline form, or at least to determine the homogeneity of the most purified enzyme solution so far obtained by electrophoretic technique.

The ability of the glucosidase to hydrolyze starch and its components, however, is evident. Studies on the rates of hydrolysis of maltose and of potato amylose by the partially purified and the most purified glucosidase so far obtained were made. The results obtained indicate that the rates of hydrolysis of the two substrates parallel each other when effected by either the less purified or the most
purified glucosidase so far obtained, as can be noted from the data in Table 15 and Figure 3. Fifty milliliters of 0.1 per cent solutions of the substrates containing 2.5 milliliters of acetate buffer, pH 4.4, were employed. One milliliter of normal potassium hydroxide was used to dissolve

Table 15. Rates of hydrolysis of maltose and the linear fraction from potato starch by the glucosidase from the Norit filtrate and the most purified glucosidase so far obtained

<table>
<thead>
<tr>
<th>Time in minutes</th>
<th>100 minus per cent hydrolysis by the crude glucosidase of Maltose</th>
<th>100 minus per cent hydrolysis by the purified glucosidase of Maltose</th>
<th>100 minus per cent hydrolysis by the crude glucosidase of Potato amylose</th>
<th>100 minus per cent hydrolysis by the purified glucosidase of Potato amylose</th>
</tr>
</thead>
<tbody>
<tr>
<td>5</td>
<td>44.1</td>
<td>81.9</td>
<td>59.5</td>
<td>93.61</td>
</tr>
<tr>
<td>10</td>
<td>40.3</td>
<td>79.0</td>
<td>45.8</td>
<td>91.5</td>
</tr>
<tr>
<td>30</td>
<td>30.9</td>
<td>54.3</td>
<td>43.1</td>
<td>86.15</td>
</tr>
<tr>
<td>45</td>
<td>24.2</td>
<td>35.1</td>
<td>41.0</td>
<td>81.39</td>
</tr>
<tr>
<td>60</td>
<td>19.1</td>
<td>28.7</td>
<td>37.8</td>
<td>80.8</td>
</tr>
<tr>
<td>90</td>
<td>2.1</td>
<td>4.5</td>
<td>35.1</td>
<td>72.6</td>
</tr>
<tr>
<td>120</td>
<td>0</td>
<td>4.0</td>
<td>34.0</td>
<td>68.6</td>
</tr>
<tr>
<td>150</td>
<td>0</td>
<td>0</td>
<td>26.1</td>
<td>62.2</td>
</tr>
<tr>
<td>180</td>
<td>21.4</td>
<td>50.5</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Figure 3. Rates of hydrolysis of maltose and potato amylose by crude glucosidase and purified glucosidase
50 milligrams of the potato amylase. One milliliter portions of the enzyme solutions of appropriate dilution were used for the hydrolysis, and one milliliter portions of the hydrolysates were withdrawn at certain time intervals. The reducing values of the hydrolysates were determined by the method of Somogyi (142), and the results expressed as per cent hydrolysis. The curves obtained, shown in Figure 3, seem to indicate that the hydrolytic activity of the glucosidase on starch is a property of the glucosidase itself and not due to the presence of another enzyme in the enzyme solutions used.

2. **Thermal stability of the glucosidase**

A preliminary experiment on the thermal stability of the glucosidase in the original mold filtrate was described on page 13 as an attempt towards the purification of the enzyme. The glucosidase was found to be completely inactivated after heating the crude enzyme solution at 75° and 80°C, and partially inactivated after heating at 70°C. The glucosidase appeared stable after heating at 50° to 60°C for 15 minutes followed by immediate cooling in an ice bath, as indicated by its glucosidase activity measured as maltase. A similar heat treatment was done on the
partially purified enzyme solution and the rates of hydrolysis of the different substrates by the heated enzyme solutions were studied by the paper chromatography technique. The enzyme solution that had been heated to 70°C showed no hydrolysis of all the substrates tested even after an hour of enzyme-substrate reaction. The enzyme solution that had been heated to 60°C showed decreased rates of hydrolysis of maltose as well as of all the other substrates tested. This was indicated by the slow disappearance of the unhydrolyzed substrates which have reducing groups accompanied by the faint appearance of glucose during the early stages of the hydrolysis. A comparison of the rates of hydrolysis of maltose and of the linear fraction of potato starch by the unheated and heated enzyme solution is given on Table 16 and Figure 4. In Figure 4, the relationship between the time of hydrolysis in minutes to the per cent of hydrolysis of the substrate is shown. It can be noted that the hydrolysis curves for maltose seem to parallel each other as do also the curves for the linear fraction of potato starch when effected by either the heated or unheated enzyme. This may be taken as a further indication of the ability of the glucosidase from Aspergillus niger to hydrolyze starches in addition to the simple disaccharide maltose.
Figure 4. Rates of hydrolysis of maltose and of potato amylose by the heated and unheated glucosidase in the Norit filtrate.
Table 16. Rates of hydrolyses of maltose and the linear fraction from potato starch by the heated and unheated mold glucosidase from the Norit filtrate

<table>
<thead>
<tr>
<th>Time in minutes</th>
<th>100 minus per cent hydrolyses by the unheated glucosidase of Maltose</th>
<th>Potato amylose</th>
<th>100 minus per cent hydrolyses by the glucosidase heated to 60°C. of Maltose</th>
<th>Potato amylose</th>
</tr>
</thead>
<tbody>
<tr>
<td>10</td>
<td>23.9</td>
<td>61.7</td>
<td>34.5</td>
<td>81.39</td>
</tr>
<tr>
<td>30</td>
<td>5.8</td>
<td>30.3</td>
<td>33.5</td>
<td>81.39</td>
</tr>
<tr>
<td>45</td>
<td>3.6</td>
<td>27.6</td>
<td>21.9</td>
<td>57.0</td>
</tr>
<tr>
<td>60</td>
<td>0</td>
<td>20.2</td>
<td>14.4</td>
<td>35.1</td>
</tr>
<tr>
<td>90</td>
<td>0</td>
<td>8.5</td>
<td>5.4</td>
<td>15.5</td>
</tr>
<tr>
<td>120</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>12.0</td>
</tr>
</tbody>
</table>

3. **Effect of hydrogen ion concentration on the glucosidase activity**

The optimum pH for the action of the purified glucosidase on maltose and on the linear fraction of potato starch was determined.

Buffer solutions of varying pH levels from pH 3.0 to pH 6.5, with ionic strength of 0.1, were prepared from acetic acid and sodium acetate solutions. Nine milliliter
portions of the buffers of known pH were added to 10 milli-
liter portions of 0.2 per cent solutions of maltose and of
potato amyllose and the temperature allowed to reach equilib-
rium in a water bath at 30°C. One milliliter portions of
the purified enzyme solution of the proper dilution, at-
tempered at the same temperature, were added to the sub-
strates and one milliliter aliquots were withdrawn after
the appropriate times. The hydrolysates were analyzed for
reducing sugar and the results obtained, expressed as
milligrams of glucose, are given on Table 17. It can be
seen that the pH optimum for the mold glucosidase action on
both maltose and potato amyllose is somewhere between pH 4.0
and pH 4.4.

4. Michaelis constants

The Michaelis or affinity constants (98), were deter-
mined for the action of the purified glucosidase upon the
linear and branched fractions of corn starch and upon
maltose at 30°C. One milliliter of a properly diluted en-
zyme solution was introduced into each of several test
tubes containing the substrates at varying concentrations.
Two milliliters of an acetate buffer of pH 4.2 were used
for each 10 milliliters of the enzyme-substrate mixtures
Table 17. The optimum pH for the mold glucosidase action on maltose and the linear fraction of potato starch

<table>
<thead>
<tr>
<th>Hydrolysis of maltose</th>
<th>Hydrolysis of potato amylase</th>
</tr>
</thead>
<tbody>
<tr>
<td>pH</td>
<td>Milligrams glucose in the hydrolysate after 30 minutes hydrolysis</td>
</tr>
<tr>
<td>3.3</td>
<td>4.12</td>
</tr>
<tr>
<td>3.6</td>
<td>4.65</td>
</tr>
<tr>
<td>3.8</td>
<td>4.98</td>
</tr>
<tr>
<td>4.0</td>
<td>5.07</td>
</tr>
<tr>
<td>4.2</td>
<td>5.17</td>
</tr>
<tr>
<td>4.4</td>
<td>5.07</td>
</tr>
<tr>
<td>4.6</td>
<td>4.55</td>
</tr>
<tr>
<td>4.8</td>
<td>4.44</td>
</tr>
<tr>
<td>5.0</td>
<td>4.22</td>
</tr>
</tbody>
</table>

in the tubes. One milliliter portions were withdrawn at zero and other time intervals after the addition of the enzyme to the substrate, and the glucose produced determined. Glucose produced from the hydrolysis of the corn starch fractions was measured by the method of Somogyi (142), and that from maltose was measured by the method of Tauber and Kleiner (150).
Amounts of glucose produced by the action of the glucosidase upon each of several concentrations of the three substrates after various digestion times are shown in Table 18. From the plots of the values given in Table 18, the initial velocities were calculated for each of the substrate concentrations. Values for the Michaelis constants were determined by the graphical method of Lineweaver and Burk (88), the curves for which are shown in Figure 5. The molar concentrations for the starch substrates were calculated from the molecular weights used by Phillips and Caldwell (118). The molecular weight for the linear fraction of corn starch was taken as 150,000, and for the branched fraction as 1,500,000. The Michaelis constants obtained for the three substrates were 0.455 per cent or 1.33 x 10^{-2} M for maltose, 0.0216 per cent or 1.44 x 10^{-6} M for the linear fraction of corn starch, and 0.0191 per cent or 1.27 x 10^{-7} M for the branched fraction.

The Michaelis constants for the action at 40°C of purified gluc amylase from Rhizopus delemar reported by Phillips and Caldwell (118) were 0.23 per cent or 6.6 x 10^{-3} M for maltose, 0.575 per cent or 4.4 x 10^{-5} M for the linear fraction of corn starch, and 0.062 per cent or 4.1 x 10^{-7} M for the branched fraction.
Table 18. Glucose produced by the action of the purified glucosidase upon different concentrations of three different substrates at various times during the hydrolysis

<table>
<thead>
<tr>
<th>Milligrams substrate per milliliter</th>
<th>Milligram glucose produced per milliliter after hydrolysis in 3 minutes</th>
<th>Milligram glucose produced per milliliter after hydrolysis in 6 minutes</th>
<th>Milligram glucose produced per milliliter after hydrolysis in 10 minutes</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Corn amylopectin</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.5</td>
<td>0.160</td>
<td>0.278</td>
<td>0.354</td>
</tr>
<tr>
<td>1.0</td>
<td>0.149</td>
<td>0.323</td>
<td>0.523</td>
</tr>
<tr>
<td>2.0</td>
<td>0.142</td>
<td>0.345</td>
<td>0.541</td>
</tr>
<tr>
<td>4.0</td>
<td>0.146</td>
<td>0.302</td>
<td>0.565</td>
</tr>
<tr>
<td>6.0</td>
<td>0.151</td>
<td>0.331</td>
<td>0.547</td>
</tr>
<tr>
<td>7.0</td>
<td>0.120</td>
<td>0.300</td>
<td>0.491</td>
</tr>
<tr>
<td><strong>Corn amylose</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.5</td>
<td></td>
<td>0.198</td>
<td>0.358</td>
</tr>
<tr>
<td>1.0</td>
<td></td>
<td>0.240</td>
<td>0.396</td>
</tr>
<tr>
<td>2.0</td>
<td></td>
<td>0.280</td>
<td>0.453</td>
</tr>
<tr>
<td>4.0</td>
<td></td>
<td>0.269</td>
<td>0.364</td>
</tr>
<tr>
<td>6.0</td>
<td></td>
<td>0.246</td>
<td>0.400</td>
</tr>
<tr>
<td>7.0</td>
<td></td>
<td>0.246</td>
<td>0.41</td>
</tr>
<tr>
<td><strong>Maltose</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.5</td>
<td>0.024</td>
<td></td>
<td>0.032</td>
</tr>
<tr>
<td>1.0</td>
<td>0.056</td>
<td></td>
<td>0.089</td>
</tr>
<tr>
<td>2.0</td>
<td>0.068</td>
<td></td>
<td>0.09</td>
</tr>
<tr>
<td>4.0</td>
<td>---</td>
<td></td>
<td>0.096</td>
</tr>
<tr>
<td>6.0</td>
<td>0.13</td>
<td></td>
<td>---</td>
</tr>
<tr>
<td>7.0</td>
<td>0.092</td>
<td></td>
<td>0.144</td>
</tr>
</tbody>
</table>
Figure 5. Lineweaver-Burk plots for the determination of Michaelis constants of the purified glucosidase action upon maltose and the linear and branched fractions of corn starch.
V. DISCUSSION

Three media were tested for growth of *Aspergillus niger* NRRL 330 in submerged culture. The highest yield of glucosidase was obtained from a medium described by Tsuchiya, Cormann and Koepsell (152). Submerged cultures in this medium were therefore used as the source of the enzyme, the purification and characterization of which were attempted in this work.

Several attempts to obtain the mold glucosidase in a highly purified form free from detectable traces of other carbohydrates, especially of alpha-amylase, were carried out. Roy and Underkofler (126) reported a purification of fungal maltase by which a dry maltase concentrate was obtained from a submerged culture filtrate of *Aspergillus niger* NRRL 330. Phillips and Caldwell (117), reported the purification of a glucose-forming amylase from another mold, *Rhizopus delemar*, which enzyme showed both maltase and amylase activities. The purification schemes developed by the above-mentioned workers were tried separately on the crude enzyme preparations used in this work but did not meet with success. Bovard (12), in his review of the fungal amylases, cited a number of investigators who have pointed out the vast differences which occur in the ability of
various strains and substrains of microorganisms to elaborate the amylases. It is quite probable that these enzymes produced in varying amounts are indeed different in their physical and chemical makeup depending on a wide variety of factors, and, therefore, would require different purification methods. Since it is a well-accepted fact that enzymes are largely made up of proteinaceous materials, it appears probable that different protein molecules may possess similar active centers responsible for similar enzyme activity, while the rest of the molecules may vary, causing differences in behavior towards a purification scheme.

Ammonium sulfate is the salt that has been most successfully used for the purification of enzymes. However, only 46.1 per cent of the total maltase activity in the original enzyme solution was precipitated by this salt from 0 to 90 per cent saturation. The glucosidase may have been denatured by the salt, or other foreign proteins could have been precipitated by the ammonium sulfate in preference to the mold glucosidase under the conditions for the precipitation employed.

The solubility of the protein fraction possessing the glucosidase activity was determined at various pH levels and concentrations of ammonium sulfate. The results obtained indicated an interesting property of the mold
glucosidase; two protein fractions possessed the glucosidase activity. One fraction seemed to be precipitated best at pH 4.5, and another fraction near pH 6.0. Similar results have been reported by Stinson (146), in his work on the purification of maltase from Aspergillus niger NRRL 330 grown in a synthetic medium. The two pH optima for the ammonium sulfate precipitation of the glucosidase, however, need not necessarily be due to two protein fractions possessing the glucosidase activity. It is possible that at one pH level one of the inactive protein components of the mold filtrate was precipitated by the ammonium sulfate and acted as an adsorbent for the glucosidase. It was concluded that precipitation of the glucosidase with ammonium sulfate as a first step in the purification of the enzyme was not very advantageous.

A first step common to many enzyme purifications is a selective denaturation of inert protein. By careful heating of extracts, much of the inert protein materials may be denatured, precipitated and removed without destruction of the desired enzyme (134). The glucosidase appeared to be completely inactivated after heating at 70°C. However, the heat treatment for 15 minutes at 50°C, prior to the ammonium sulfate precipitation indicated a slight increase in the total glucosidase activity recovered from the original
enzyme solution by the precipitation with the salt. The total glucosidase activity precipitated with ammonium sulfate from 0 to 60 per cent saturation of the enzyme solution which had been previously heated at 50°C. was 59.4 per cent. This figure still pointed out that precipitation of the glucosidase with ammonium sulfate holds little promise as a first step in the purification of the enzyme. It is obvious that if the loss of the enzyme at each step of the purification is great, very little of the material will be present by the time high purity is achieved.

An attempt to precipitate the protein fraction possessing the glucosidase activity by sodium chloride, magnesium sulfate, and potassium dihydrogen phosphate indicated that none of the above-mentioned salts is suitable for precipitating the enzyme.

Some of the heavy metal salts have been successfully employed by other workers to precipitate proteins or inactivate an unwanted enzyme from enzyme and other protein mixtures (134). A number of heavy metal salts were investigated. The precipitates that were obtained were dark brown, gummy and mostly insoluble in water. Lead acetate, lead chloride, and mercuric acetate formed the most precipitate from the crude mold enzyme solution. Copper acetate and manganese sulfate did not yield any precipitate. Of the
different heavy metal salts tested, mercuric acetate appeared to have precipitated much of the unwanted materials in the crude mold filtrate leaving most of the glucosidase activity in the supernatant.

It appears that the glucosidase of Aspergillus niger NRRL 330 is not inactivated nor inhibited by heavy metal salts under the conditions in which precipitation was done in this work. It was hoped that preliminary precipitation with mercuric acetate would lead to a marked purification of the mold glucosidase with destruction or inactivation of the other carbohydrases. This was investigated and it was observed that the alpha-amylase content of the enzyme solution was decreased by 60 per cent after the mercuric acetate treatment. The best result was obtained when solid mercuric acetate was added to the enzyme solution until it was $3.75 \times 10^{-3}$ molar.

Further work on the purification of the glucosidase revealed that precipitation of the glucosidase by organic solvents seems to require the presence of some inorganic ions. When a partially purified solution of the mold glucosidase, which previous to dialysis could be precipitated by acetone, was dialyzed, no precipitation could be achieved by the addition of any amount of acetone. That the glucosidase was not lost or denatured by dialysis was indicated by
its ability to precipitate with acetone after addition of ammonium sulfate to the dialyzed solution.

The work of Bovard (12) upon the precipitation of alpha-amylase with acetone suggested that fungal alpha-amylase was unstable in acetone solutions at concentrations above 40 per cent. Acetone precipitation, therefore, appeared to offer a good step towards the purification of the glucoamylase to free it, even partially if not entirely, from alpha-amylase.

Precipitation with acetone of the glucoamylase in the mold enzyme solution that had previously been precipitated with mercuric acetate gave almost quantitative recovery of the enzyme. The great bulk of the glucoamylase was precipitated in the 30 to 55 per cent acetone fraction.

Experiments on the effect of pH on the precipitation of the glucoamylase by acetone indicated that the enzyme was least precipitated at pH 5.0 and 5.5 and at pH levels below 4 and above 7. Results comparable with those from the precipitation of the glucoamylase with ammonium sulfate were obtained. At both pH levels 4.5 and 6.0, the mold glucoamylase appeared to precipitate best with either acetone or ammonium sulfate. Two protein fractions possessing the glucoamylase activity may be probable, although it can equally be probable that the protein fraction having the
glucosidase activity precipitates equally well at the two pH levels.

Several adsorbents commonly employed in the purification of enzymes were tried but did not meet with much success. Starches from different sources have been reported as good adsorbents for alpha-amylases. Although a thorough investigation of the possible conditions favorable for the use of starches as adsorbents for the unwanted materials in the mold enzyme solution was not made, it appeared that very little purification of the glucosidase from alpha-amylase could be achieved with starch adsorption.

Activated carbon was investigated as an adsorbent for either the glucosidase or other impurities in the enzyme solution. Preliminary experiments indicated that treatment of the partially purified enzyme solution with from 4 to 6 per cent Norit removed much of the colored materials from the enzyme solution but did not adsorb the glucosidase. Adsorption by the continuous flow technique using a column yielded an almost completely decolorized solution which, however, was completely devoid of glucosidase activity. Elution of the enzyme by a series of buffer solutions of different pH was not achieved. It appeared that the glucosidase had been adsorbed by the carbon and was not capable of being eluted by aqueous buffered solutions. It may be probable that the enzyme had been denatured, and resulted
in the total loss of the glucosidase activity. No further investigations were done along this line. Zittle (175), in his review on adsorption studies of enzymes and other proteins, stated that charcoal can be used to remove contaminating proteins, but is not recommended for the isolation of protein since there is no satisfactory eluant yet known.

It is a well known fact that the use of adsorption techniques for the purification of enzymes involves a number of factors which must be carefully considered to obtain the most purification and the least loss of the desired enzyme. Such variables as pH, rate of adsorption, temperature, and concentration of salts in the solution to be treated with the adsorbent play important parts in the efficiency of the adsorbent being used. A thorough investigation of all these factors, however, was not undertaken in this work.

A procedure for the purification of the mold glucosidase has been developed, the scheme of which has been outlined on pages 128 to 132. Analytical data of the enzyme solutions obtained after some of the steps of the procedure are given in Table 12, page 134. The glucosidase activity of the enzyme solution had been raised from 15.48 to 1,332.5 units per milliliter, an increase of less than a hundred-fold. Analysis of the nitrogen content of
the solutions indicated an increase of glucosidase activity per milligram of nitrogen from 11.09 to 362.63 units or an increase of about 30-fold. More than 50 per cent of the glucosidase activity in the original mold filtrate was lost through the various steps of the procedure. It is obvious that refinement in the various steps of the purification is wanting.

In spite of the low recovery of the glucosidase obtained, it was felt that the enzyme had been sufficiently purified from other carbohydrases, especially from alpha-amylase. This seemed evident after a study of the action of the purified glucosidase on starch. A comparison of the blue values with the reducing values of the hydrolysate, at certain time intervals during the hydrolysis of starch by the glucosidase that had been through the different stages of the purification, are presented in Table 13 and Figure 1, pages 138 and 139. Figure 1 indicates the relationship between the disappearance of the starch substrate as measured by blue values and the increase in the reducing values of the hydrolysates measured by determination of the reducing sugars formed during the course of the hydrolysis. It can be noted in Figure 1 that the maltase-free alpha-amylases studied by Tung-Kung, Hanrahan, and Caldwell (154) show marked differences from the glucosidase of Aspergillus niger in their
action towards the linear fraction of starch. The alpha-
amylase action on starch seems to be characterized by a
rapid breakdown of the starch substrate to small dextrins
indicated by the rapid disappearance of the blue color of
the starch-iodine complex, accompanied by a slight increase
in reducing value. On the other hand, the glucosidase from
Aspergillus niger seems to hydrolyze the starch in a dif-
f erent fashion characterized by the slow disappearance of
long chain fragments. This is indicated by the slow dis-
appearance of the blue starch-iodine complex accompanied by
a correspondingly large increase in reducing value. It
seems evident that the mold glucosidase hydrolyzes the
linear fraction of starch in a stepwise fashion breaking
the alpha-1,4 links between the glucose units of starch.
That glucose was the sole reducing product of the hydroly-
sis of starch was indicated by paper chromatography of the
hydrolysates during various time intervals of the hydrolysis.

Studies on the substrate specificity of the purified
mold glucosidase was done by paper chromatography. The sub-
strates investigated included: a linear fraction from
potato starch, a branched fraction from corn starch, glyco-
gen, a heptasaccharide, beta and gamma Schardinger dextrins,
panose, gentiobiose, cellobiose, methyl alpha-glucoside,
and maltose. Of the substrates tested, methyl alpha-glucoside
and beta Schardinger dextrin failed to be hydrolyzed by the glucosidase. The beta and gamma Schardinger dextrins are cyclic alpha-D-glucopyranosides having 7 and 8 glucose units, respectively, joined by 1,4-alpha-D-glucosidic linkages. The gamma Schardinger dextrin was hydrolyzed by the glucosidase to glucose and no other reducing sugar. The difference in the activity of the purified glucosidase towards the beta and gamma dextrins may probably be due to the difference in size between the two substrates which possibly plays a significant role in the steric requirements of the enzyme-substrate reaction. Glucose appeared to be the sole hydrolysis product of the hydrolyses of the various substrates that were hydrolyzed by the purified glucosidase until the very late stages of the hydrolyses. Both panose and gentiobiose were hydrolyzed by the glucosidase. Panose is a trisaccharide composed of three glucose units joined by one alpha-1,4- and one alpha-1,6-glucosidic linkage. Gentiobiose is a disaccharide the two glucose units of which are joined by a beta-1,6-glucosidic linkage. It appears that the glucosidase of *Aspergillus niger* is not specific for the alpha-1,4-glucosidic linkage in maltose, but is capable of hydrolyzing starches, their components, and their hydrolysis products. The purified glucosidase also showed its ability to hydrolyze both the alpha- and
the beta-1,6 glucosidic linkages in its substrates. These results, however, may not be entirely conclusive since many factors in addition to the kind of linkage influence the action and affinity of an enzyme. It may be possible that other enzymes responsible for some of the hydrolytic actions that were observed in this work have not been entirely removed from the mold enzyme solution. Attempts are still being continued to obtain the glucosidase in crystalline form, or at least to determine the homogeneity of the most purified enzyme solution, so far obtained, by electrophoretic techniques.
VI. SUMMARY

1. Mercuric acetate was found to precipitate much of the unwanted materials in the crude mold filtrate. The glucosidase in the original mold filtrate was not inactivated nor inhibited by various heavy metal salts up to $3.75 \times 10^{-3}$ molar concentrations. The alpha-amylase content of the mold filtrate was decreased by 60 per cent after the mercuric acetate treatment.

2. Precipitation of the glucosidase by organic solvents appeared to require the presence of some inorganic ions. Dialyzed solutions of the enzyme did not precipitate with acetone. After addition of ammonium sulfate to the dialyzed solution, the enzyme was precipitated by acetone.

3. Precipitation with acetone of the glucosidase in the enzyme solution that had previously been precipitated with mercuric acetate gave almost quantitative recovery of the enzyme. The great bulk of the glucosidase was precipitated by the 30 to 55 per cent acetone fraction.

4. At both pH levels 4.5 and 6.0, the glucosidase appeared to precipitate best with either acetone
or ammonium sulfate. There may be two protein fractions possessing the glucosidase activity.

5. Sodium chloride, magnesium sulfate, and potassium dihydrogen phosphate were found not suitable for precipitating the mold glucosidase.

6. Treatment of the partially purified enzyme solution with from 4 to 6 per cent activated carbon removed much of the colored materials from the enzyme solution but did not adsorb the glucosidase.

7. Celite and silica gel were observed to remove much of the alpha-amylase. Very little purification of the glucosidase from alpha-amylase was achieved with starch adsorption.

8. The purified glucosidase was observed to hydrolyze not only maltose but also starches, their components, and their hydrolysis products. Glucose, and no other reducing sugar, was the sole hydrolysis product as indicated by paper chromatography. The hydrolytic action of the purified glucosidase on starch appeared to be characterized by the slow disappearance of long chain fragments accompanied by a correspondingly large increase in reducing value, and differed markedly from the hydrolytic action of alpha-amylase on starch.
9. Methyl alpha-glucoside and beta Schardinger dextrin were not hydrolyzed by the purified glucosidase.

10. Glucose was the sole hydrolysis product of the hydrolysis of gamma Schardinger dextrin, glycogen, panose, celllobiose, and gentiobiose by the purified mold glucosidase.

11. The purified glucosidase appeared stable at 50°C., showed decreased rate of hydrolysis after heating at 60°C. and was completely inactivated by heating at 70°C.

12. The pH optimum for the glucosidase action on maltose and the linear fraction of potato starch is somewhere between pH 4.0 and 4.4.

13. The Michaelis constants obtained for the action of the purified glucosidase were 0.455 per cent or $1.33 \times 10^{-2}$ M for maltose, 0.0216 per cent or $1.44 \times 10^{-6}$ M for the linear fraction of corn starch, and 0.0191 per cent or $1.27 \times 10^{-7}$ M for the branched fraction of corn starch.
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