STUDIES ON CARBOHYDRATE METABOLISING ENZYMES

-by-

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TO MY PARENTS
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SECTION I

GENERAL INTRODUCTION

The process of metabolism (katabolism - breakdown of biological materials, and anabolism - synthesis of biological substances) occurs naturally in yeast, a single-celled fungi belonging to the plant kingdom. A study of the metabolism of any living cell and in particular, a yeast cell, will show the different pathways by which the cell synthesises, maintains and degrades the complex organic molecules which form its structure. For a living cell to survive, it must be able to carry on its own metabolic processes; these consist of a series of distinct but often related chemical reactions. When examined in a laboratory, the same reactions often require a high temperature and strong reagents for them to proceed at a reasonable speed. In living cells none of the above conditions are met with and the cells must produce catalytic agents to accelerate the reactions under "physiological" conditions (at pH 7 and 30°C). These biological catalysts are known as "enzymes" or "ferments". Hence, the various physiological processes of living matter largely depend upon enzyme action. The word "enzyme" as suggested by Kühne (1) in 1878 has been derived from a Greek word which simply means "in yeast", because it was in yeast that enzymes were first discovered.

Much of the early history of enzymes is connected with
alcoholic fermentation by yeast. Many workers had seen a parallelism between their action and that of yeast. Prior to the work of Lavoisier (2) which began in 1784, the nature of alcoholic fermentation was completely unknown. Early workers such as Willis (1659), Stahl (1697) and Fabroni (1787) made attempts to explain the process of fermentation. They suggested that a body in a state of "internal action" communicated this motion to another body which was then fermented. About the same time, observations were made by various workers on the process of metabolism in higher plants and animals. Spallanzani (3) in 1785 observed that meat was dissolved in the stomach by gastric juices while the conversion of starch into sugar and dextrin by the glutinous component of wheat was discovered by Kirchoff (1814).

The work of Lavoisier (2) showed a correlation between the reactants and the end-products in the process of alcoholic fermentation as follows:

$$C_6H_{12}O_6 \rightarrow 2C_2H_5OH + CO_2$$

The first clear recognition of an enzyme was made by Payen and Persos (4) in 1885, when they observed that an alcohol precipitate of malt extract contained a thermolabile substance which converted starch into sugar. Since then, much controversy took place around the work of Leibig (5) who suggested that fermentation was caused by chemical substances and that of Pasteur (6) in 1857, who regarded fermentation as a physiological process which was inseparable from living cells. Prior to the work of
Leibig and Pasteur, in 1838, Caignard-Latour (7), Kützing (8) and Schwann (9) suggested that yeast cells were living organisms and fermentation was definitely caused by them. The idea of an enzyme as a catalyst was put forward by Berzelius (10) who explained fermentation as caused by a "catalytic force". Traube (11) regarded ferments as being closely related to proteins.

The Pasteur-Leibig controversy came to an end with the classical discovery of Buchner (12) made in 1897 when he obtained a cell free extract (zymase) from yeast which was able to ferment sugar to alcohol and carbon dioxide. This suggested that metabolic chemical reactions could be repeated in a test-tube under normal conditions in the presence of such biological extracts.

With the advent of this discovery, the existence and properties of other enzymes were studied in detail and a large number (at least 600) of enzymes have since been isolated. The idea of enzyme specificity and the close steric relationship between the enzyme and the substrate were studied by the eminent chemist Emil Fischer (13). Sørensen and Michaelis (14) have contributed largely to enzyme kinetics. Sørensen pointed out that enzyme action depended largely upon the hydrogen ion concentration of the medium and Michaelis showed the great importance of substrate concentration as a major factor in determining the velocity of an enzyme reaction.

Enzymes can now be described as catalytically active proteins (simple or conjugated) and specific in their mode of action.
towards the substrate depending upon the temperature and pH of the media. They may act together as systems each specifically catalysing a particular reaction in the system. The overall processes of metabolism are achieved by the combined action of various enzymes. The process of alcoholic fermentation and glycolysis (Scheme 1) as formulated by Embden, Meyerhof and Parnas (15) clearly shows that enzymes may act in systems, each catalysing a particular reaction.

Scope of the present work

Various enzymes from the animal and plant tissues, involved in the degradation and synthesis of starch-type polysaccharides have been extensively studied especially by G.T. and C.F. Cori, Hanes, Peat and their co-workers. However, very little is known about the related enzymes which are present in yeast. This thesis is mainly concerned with studies on starch and glycogen metabolising enzymes of yeast and with the metabolism of maltose. As a preliminary to the experimental work, a brief account of the structure of these substrates and the related enzymes of plant and animal tissues will be given.

General Structure of Starch and Glycogen

Starch

Starch, which is heterogeneous in nature (16, 17), occurs as a reserve polysaccharide in all photosynthetic plants. It consists of two major components, amylose and amylopectin, which
Key to Scheme 1

1. Phosphorylase, branching enzyme and debranching enzyme.
1a. Amylases
2. Phosphoglucomutase
3. Phosphohexose isomerase
4. Phosphohexokinase and hexose diphosphatase
5. Maltase
6. Phosphatases
7. Aldolase
8. Phosphotriose isomerase
9. Diphosphoglyceric aldehyde dehydrogenase
10. Phosphoglyceric phosphokinase
11. Phosphoglyceromutase
12. Enolase
13. Phospho enol transphophorase
14. Lactic dehydrogenase
15. Carboxylase
16. Alcohol dehydrogenase
Embeden-Meyerhof-Parnas Scheme (15)

Starch or Glycogen

Maltose

Glucose-1-phosphate

Glucose + $\text{PO}_4$ → Glucose-6-phosphate

Fructose-6-phosphate

Fructose-1:6-diphosphate

Dihydroxyacetone phosphate → Glyceraldehyde phosphate

1:3-Diphosphoglyceric acid

enol-Phosphopyruvic acid → 2-Phosphoglyceric acid → 3-Phosphoglyceric acid

Pyruvic acid

Lactic acid

Acetaldehyde + $\text{CO}_2$

Ethyl alcohol

Scheme 1: Alcoholic Fermentation and Glycolysis
occur together in characteristic granules.

Amylose, which has a linear structure, consists of several thousand α-1:4-linked D-glucose residues. It can be separated from amylopectin by selective precipitation with butanol or thymol. The presence of α-1:4-linked D-glucopyranose residues was shown by methylation and periodate oxidation studies together with exhaustive degradation by amylases and phosphorylase. Depending upon the source (18, 19) and the method of extraction (20), the degree of polymerisation (DP) of an amylose varies from Ca 50 to 6,000. Peat and co-workers (21, 22) in 1952 suggested that amylose contained a small number of linkages (anomalous) which were not susceptible to the action of pure soya bean β-amylase. Gilbert and co-workers (23) in 1956 postulated that these anomalous linkages could be introduced into an amylose molecule by oxidation during the fractionation of starch. Manners and Wright (24) have recently obtained similar results.

Amylopectin is a branched polysaccharide of high molecular weight (Ca $10^7$), composed of several hundred chains each consisting on the average of 20-25 α-1:4-linked D-glucopyranose residues; each chain is linked to an adjacent chain by an α-1:6-glucosidic linkage. The branched structure is shown by the high DP of amylopectin ($10^5 - 10^7$) compared with the chain length values obtained by methylation studies (25). The specific rotation and hydrolysis by amylases to maltose indicate an α-configuration. Amylopectin is readily attacked by phosphorylase, α and β amylases and α-amylase (36). Posternak (37) has reported
SOME MULTIPLE BRANCHED STRUCTURES.

FIG. 1
the presence of phosphate ester groups in acid hydrolysates of various starches.

Various structures for amylopectin have been put forward. Haworth and Hirst (25) from methylation studies, postulated a singly branched "laminated" structure. Later, Meyer (28) proposed a multiply branched "tree" structure. Peat and co-workers (29) described the presence of three types of chains in the above structures: (1) A-chains consisting of 7-20 glucose residues linked to the rest of the molecule only by the reducing group; (2) B-chains, to which one or more A-chains are attached, are linked to the neighbouring chains by an \( \alpha-1:6 \)-linkage from the reducing group; (3) C-chains, which carry the sole free reducing group in the molecule, are linked by other A and B chains (see Figure 1). The ratio of A to B chains for a fully developed "tree" structure is approximately 1:1 (30). Enzymic studies have led to the final acceptance of a "tree"-type structure. (16,17).

Glycogen

Glycogen, a highly branched polysaccharide found in animal tissues, consists of several thousand chains which are formed by 12-18 \( \alpha-1:4 \)-linked D-glucose residues and inter-linked by \( \alpha-1:6 \)-linkages as found in amylopectin. It is readily degraded by phosphorylase, \( \alpha \), \( \beta \) amylases and isomylase (31) and these enzymic studies indicate an overall structure represented by the multiply-branched formula of Meyer. It may be possible that small numbers of linkages other than \( \alpha-1:4 \) and \( \alpha-1:6 \) are present. Glycogen
differs markedly from amylopectin in hydrodynamic properties (32). This may be due to the variations in molecular shape (Figure 1) rather than to the difference in degree of multiple-
branching.

General Structure of Maltose

Maltose (O-α-D-glucopyranosyl-(1→4)-D-glucopyranose) is the lowest member of the maltodextrin series (33). It is a reducing disaccharide and can be obtained as an end product of amylase action on amylase, amylopectin and glycogen. Commercial maltose contains a considerable quantity of dextrins which can be removed by fractional precipitation of aqueous solutions with alcohol. Maltose exists in both α- and β-forms which show mutarotation in an aqueous solution, the specific rotation being 

$$[\alpha]_D^\circ + 112^\circ \rightarrow 150^\circ.$$  

It can be readily hydrolysed with mineral acid or with the specific enzyme maltase to give two molecules of D-glucose; this confirms the presence of an internal α-glucosidic link. On methylation, maltose gives a methyl hepta-O-methyl maltoside which by acid hydrolysis is converted into 2:3:4:6-tetra-O-methyl glucose and a tri-O-methyl glucose (34). Maltose is oxidised by bromine to maltobionic acid (35) which on subsequent methylation and hydrolysis gives tetra-O-
methyl glucose and 2:3:5:6-tetra-O-methyl gluconic acid, the latter in turn being converted to furano-lactone. From the experiments it was concluded that maltose has the structure of 

$$0-\alpha-D-glucopyranosyl-(1\rightarrow4)-D-glucopyranose.$$
Enzymes involved in Synthesis and Degradation of α-1:4-glucosans

Branching Enzymes

The presence of branched polysaccharides such as glycogen and amylopectin in animal and plant tissues implies that these tissues will contain enzymes capable of synthesising α-1:6-glucosidic linkages. Cori and Cori (36) in 1943, incubated glucose-1-phosphate with an enzyme from extracts of liver, heart and brain tissues together with muscle phosphorylase producing a branched polysaccharide, similar to glycogen in solubility and iodine staining power. This discovery aroused the interest of several workers to look for similar enzymes in other animal and plant tissues containing α-1:6-linked polysaccharides.

Haworth, Peat, Bourne and Macey (37, 38, 39) in 1944-46 reported the presence of a related enzyme in plant tissues and called it Q-enzyme. This has now been isolated in crystalline form from potatoes (40, 41) and its properties have been extensively studied by Peat and his co-workers. The presence of Q-enzyme in broad beans (25), wrinkled peas (25), the flagellated protozoon Polytonella Coeca (42) and green gram (43) has also been reported. Q-enzyme acts on amylose, and in combination with potato phosphorylase on glucose-1-phosphate, synthesising a polysaccharide similar to amylopectin in its solubility, iodine staining power and β-amylolysis limit.

A branching enzyme is a transglycosylase (systematic name amylo-1:4→1:6-transglucosidase). It performs a double function,
i.e. scission of α-1:4-linkages followed by the formation of an equal number of α-1:6-linkages. This infers that amylose acts both as a donor as well as an acceptor substrate. This fact cannot be restricted to linear chains as the end product would contain only one point of branching; therefore, branched molecules must also act as donor and acceptor substrates. The minimum length of a linear amylose chain which can function as a donor substrate for Q-enzyme action lies between DP 42 and DP 116 (44, 45).

Manners and Khin Maung (46) in 1956 reported the presence of a branching enzyme in brewer's yeast extracts. Unlike Q-enzyme from potatoes and Polypomella Coeca, yeast branching enzyme was found to act on amylopectin producing a more highly branched polysaccharide resembling glycogen in its superficial properties. This property of yeast branching enzyme will be described in detail in Section V.

Phosphorylase

Phosphorylases catalyse the degradation or synthesis of α-1:4-linkages according to the following reaction:

\[ [α-1:4-Glucosan]_n + n \text{Inorganic Phosphate} \rightleftharpoons n[α-Glucose-1-Phosphate] \]

The reversibility of phosphorylase activity was shown by Keisling (47) and Cori, Schmidt and Cori (48). During degradation, it attacks a chain of α-1:4-linked glucose residues by transferring a glucosyl residue from the chain to inorganic phosphate; in synthesis, it attaches the glucosyl residue of glucose-1-phosphate
to the chain by an α-1:4-linkage, thereby liberating one molecule of inorganic phosphate.

Phosphorylase affects the stepwise degradation of successive α-1:4-linkages from the non-reducing end and degrades a linear amylose completely. Branched α-1:4-glucans are degraded until an α-1:6-linkage is encountered; the main product is a high molecular weight limit dextrin containing all the original inter-chain linkages. The synthetic polysaccharide formed from glucose-1-phosphate by muscle phosphorylase was shown by Hassid, Cori and McCready (49) to resemble the amylose component of starch. A synthetic polysaccharide resembling the amylpectin component of starch has also been synthesised by the combined action of phosphorylase and Q-enzyme on glucose-1-phosphate (50).

Phosphorylase occurs in skeletal muscle, heart muscle, liver and brain of animals, yeasts and in higher plants such as potatoes, bananas, peas, beans and corn. The function of the plant and animal phosphorylase is identical but the enzyme proteins differ in properties, e.g. adenylic acid is a component of muscle phosphorylase but not potato phosphorylase.

Disproportionating Enzyme (D-enzyme)

D-enzyme acts on achoic maltodextrins with the production of iodine staining material. It was first isolated by Peat, Whelan and Rees (50) from potato juice and has recently been obtained in a purified form by Peat, Turvey and Jones (51). The smallest substrate required for its action is maltotriose, and
has no action on maltose. D-enzyme is a transglycosylase (52), catalysing the degradation as well as synthesis of maltodextrans. It does not function by a phosphorylase-type mechanism but simply disproportionates a maltodextrin substrate and transfers the glucosyl groups removed from one maltodextrin (donor) to an acceptor substrate, which may be any maltodextrin, glucose, maltose, mannose, methyl-α-D-glucoside or xylose. D-enzyme differs from the Bacillus macerans amylase (53) and amylomaltase from Escherichia coli (54), both of which catalyse the redistribution of α-1:4-glucosidic linkages in maltodextrans. The synthesis of amylose by D-enzyme from maltodextrans has been achieved by Walker and Whelan (55).

**Maltases**

Maltases catalyse the hydrolysis of α-glucosidic linkage in maltose, a number of maltose derivatives and some alkyl and aryl α-D-glucopyranosides. The common feature of all these compounds is a non substituted α-D-glucopyranosyl residue. Maltase is widely distributed throughout the vegetable and animal kingdoms; it usually occurs in association with amylase. It was first discovered by Brown and Heron (56) in extracts of pig's pancreas; since then maltase has been isolated from a great number of organisms, especially yeast, and from the digestive tracts and blood serum of animals and mammals. Maltases from different sources vary in their mode of action towards various maltase derivatives, e.g. taka maltase from Aspergillus oryzae differs markedly from
yeast maltase, some of the properties of which have been described in Section VI.

The enzymic hydrolysis of maltose is a reversible reaction as first shown by Hill (57). He demonstrated the synthesis of maltose from glucose in vitro for the first time. Bourquelot and co-workers (59) have also studied the synthesis of a series of allyl-α-D-glucosides from α-D-glucopyranose and various alcohols by the action of yeast maltase.

Amylases

Three types of amylolytic enzyme have been reported to be present in animal and plant tissues:

(1) α-Amylase

α-Amylases catalyse a random hydrolysis of α-1:4-linkages in starch, glycogen and maltodextrins with the production of maltose and α-dextrins consisting of 4-10 glucose residues. The enzyme action can be followed by measurement of the decrease in viscosity, turbidity and iodine staining power of the substrate. α-Dextrins from amylpectin and glycogen have branched structures, since α-amylase cannot hydrolyse the α-1:6-glucosidic linkages. α-Amylases have been isolated in purified form from many sources, e.g. barley malt, mammalian pancreatic and salivary secretions and several bacterial and fungal extracts (59, 60). The enzymes from different sources differ from each other in their mode of action (61). For example, malt α-
amylase and *B. subtilis* amylase, unlike salivary *α*-amylase, can hydrolyse maltotriose (62) and the linkage adjacent to a reducing group, thereby liberating glucose (62). Hence, the end-products of *α*-amylolysis are dependent on the enzyme source.

(2) **β-Amylase**

β-Amylases catalyse a stepwise hydrolysis of alternate *α*-1:4-glucosidic linkages from the non-reducing end with the liberation of β-maltose. The enzyme action is arrested by the presence in the chain of linkages other than *α*-1:4, i.e. interchain *α*-1:6-glucosidic linkages or ester phosphate groups. Linear amylase molecules are completely degraded by β-amylase, while branched *α*-1:4-glucans, e.g. amyllopectin and glycogen yield maltose and β-dextrin of high molecular weight and which differs from amyllopectin in that the exterior chains contain only 2–3 glucose residues (63, 59). β-Amylase occurs only in plant tissues, e.g. germinated and ungerminated cereals, rice, sweet potatoes and soya beans.

(3) **Glucose-producing amylases**

Glucose-producing amylases catalyse a stepwise hydrolysis of successive *α*-1:4-glucosidic linkages. This hydrolysis also begins at the terminal non-reducing ends and then proceeds towards internal *α*-1:4-linkages. One such amylase (glucosamylase) isolated by Phillips and Caldwell (64) from the mould *Rhizopus delemar*, liberates over 90% glucose from
amylose, amylopectin, glycogen and a β-dextrin. Glucamylase cannot hydrolyse α-1:6-linkages, but can by-pass them attacking the interior chains. Amyloglucosidase (or glucamylase (65)) has been isolated from Aspergillus niger (66, 65). In the mode of action, glucose producing amylases resemble β-amylase rather than α-amylase.

**Debranching enzymes**

Debranching enzymes catalyse the hydrolysis of α-1:6 inter-chain linkages. Five such debranching enzymes have been reported recently:

(a) **R-Enzyme**

Hobson, Whelan and Feat (67, 29) isolated an enzyme (R-enzyme) in a partly purified form from potato and broad bean. R-Enzyme catalyses the hydrolysis of inter-chain α-1:6-linkages in amylopectin and its β-dextrin as shown by the increase in both iodine staining power and β-amylolysis limit; it does not hydrolyse α-1:4-glucosidic linkages.

The original R-enzyme preparation does not hydrolyse terminal α-1:6-linkages (it has no action on isomaltose) but acts on branched α-dextrins giving a mixture of linear maltosaccharides. It has been suggested that the pentasaccharide product of salivary amylase on amylopectin and glycogen is the smallest substrate for R-enzyme action (68). Since it cannot hydrolyse the α-1:6-linkages of normal glycogen (chain length 12), it seems probable that since glycogen with its
short interior chains of only 3-4 glucose residues is an extremely compact molecule, the formation of an enzyme-substrate complex near the 1:6-linkages is sterically hindered. However, Fleming and Manners (69) have shown the limited action of R-enzyme on an 18-unit glycogen. Recently McWilliam (70) has shown the heterogeneous nature of R-enzyme obtained by the method of Hobson, Whelan and Peat, by column chromatography. One fraction attacked amylopectin but not α-limit dextrin, and a second fraction had no action on the polysaccharide but showed limit dextrinase activity.

(b) **Amylo-1:6-glucosidase**

Cori and Lerner (71) isolated from rabbit muscle an enzyme "amylo-1:6-glucosidase" which could only hydrolyse terminal α-1:6-linkages. It had no action on the non-terminal α-1:6-linkages in amylopectin or glycogen but could hydrolyse those α-1:6-linkages which are exposed by the action of muscle phosphorylase on these polysaccharides.

(c) **Oligo-1:6-glucosidase**

The discovery of an enzyme "oligo-1:6-glucosidase" which catalyses the hydrolysis of α-1:6-linkages in α-limit dextrans, isomaltose and panose was reported by Larner and McIlvickle (72). It had no action on muscle phosphorylase limit dextrans. Larner and Gillespie (73) have also shown that the same preparation could hydrolyse nigerose but had no action on gentio-biose.
Limit dextrinase

Hopkins and Weiner (74) reported that brewery malts contained an enzyme "limit dextrinase" which hydrolysed terminal α-1:6-linkages of limit dextrins producing glucose. The limit dextrinase of Aspergillus oryzae (75) has been crystallised but little is known of the nature of its action or even the action of any limit dextrinases from any source.

Isoamylase

Maruo and Kobayashi (76) reported the presence of an enzyme which hydrolysed the interchain linkages of glutinous rice starch producing linear low molecular weight α-1:4-glucosans from the autolysates of brewer's yeast. The presence of such a debranching enzyme in yeast was first reported by Meyer and Bernfeld (77), the mode of action of which was unknown until the investigations carried out by Maruo and Kobayashi. This enzyme previously known as "amylosynthase" and now renamed "isoamylase" is similar to B-enzyme since it hydrolyses non-terminal α-1:6-linkages in amylpectin and β-dextrin producing a polysaccharide which could further be degraded by β-amylase. Manners and Maung (31) have recently studied some of its properties and has shown that isoamylase has a wider specificity than B-enzyme and amylo-1:6-glucosidase. Further investigations of the properties of pure isoamylase will be described in Section IV.
Uses of Enzymes in Structural Studies

The mode of action of some of the above enzymes have been determined by using substrate of known molecular structure. It is then possible to use these enzymes to investigate the fine structure of starch-type polysaccharides, obtained either by enzymic synthesis (as described in Section V) or from a variety of biological sources.

\( \alpha \)-Amylase

The salivary \( \alpha \)-amylase has been used to detect \( \alpha-1:4 \)-glucosidic linkages in maltodextrins and linear and branched polysaccharides; resistance to its action implies that the substrate contains few if any, chains of 2 or 3 adjacent \( \alpha-1:4 \)-linkages. It can also be used to distinguish between linear and branched polysaccharides. Linear molecules are completely degraded with the production of reducing sugars of high EM (apparent percentage conversion to maltose) values whereas branched polysaccharides yield \( \alpha \)-dextrins and reducing sugars having low EM values. Paper chromatographic analysis of amylolytic digests which contain glucose, maltose, maltotriose and \( \alpha \)-dextrins provide qualitative evidence for branched polysaccharides.

\( \beta \)-Amylase

\( \beta \)-Amylase has been used for the following purposes:

(i) to detect the presence of anomalous linkages in amylases;
(ii) to distinguish qualitatively between linear and branched \( \alpha-1:4 \)-glucosans;
(iii) to determine the exterior chain lengths of branched
\( \alpha-1:4 \)-glucosans.

(a) The main product of \( \beta \)-amylolysis of amylose is maltose; amounts of between 57-100\% have been reported by many workers (61). The low limits are not due to the degradation during the preparation or retrogradation of the substance during enzyme action, but suggests the presence of linkages other than \( \alpha-1:4 \).

(b) Linear \( \alpha-1:4 \)-linked polysaccharides are completely degraded to give maltose whereas branched \( \alpha-1:4 \)-linked polysaccharides give 40-70\% maltose and a \( \beta \)-dextrin of high molecular weight. The determination of the \( \beta \)-amylolysis limit and the nature of the end-products serve as a useful method for distinction between linear and branched polysaccharides even though the chain length is unknown.

(c) The exterior and interior chain length of branched polysaccharides which cannot be determined by any chemical methods can be calculated from the \( \beta \)-amylolysis limit and the chain length, assuming that the outer chain 'stubs' in the \( \beta \)-dextrin contain only 2-3 glucose residues.

Amylases and R-enzyme

Whelan and Roberts (78, 35) by analysis of the products liberated by the successive action of salivary \( \alpha \)-amylase and R-enzyme, determined the chain length of rabbit liver glycogen and also obtained evidence for a multiply-branched structure. They found a chain length value of 12.5 which was in accordance with a value
of 15,6 obtained by periodate oxidation. They also observed that linear maltosaccharides obtained by debranching glycogen α-dextrins contained a small portion of hexa or hepta saccharides. The presence of these higher saccharides suggests that some of the α-dextrins contained two α-1:6-linkages, hence confirming the presence of multiple-branching in glycogen.

The successive action of α-amylase and β-amylase was employed by Peat, Whelan and Thomas (63, 79) to show multiply-branched structure for amylopectin. Treatment of β-dextrin of waxy maize starch with α-amylase gave a mixture (12.60%) of maltose and maltotriose. A singly branched structure as postulated by Haworth and Hirst would yield only 0.085% of maltose and maltotriose, whilst a multiply-branched structure of the type suggested by Meyer would yield 12.60%.

The combined action of β-amylase and α-amylase has also been used to determine the chain length of waxy maize starch (30, 79).

From this short account it is seen that enzymic methods provide a useful means of analysis of starch-type polysaccharides (with certain reservations). These methods have therefore been used to examine the polysaccharides isolated after incubation of glycogen and amylopectin with yeast branching enzyme (Section V) and isomylase (Section IV).
FIG. 2. - Calibration of Shaffer-Somogyi Reagent.

A - Glucose.
B - Maltose.

FIG. 3. - Calibration of Somogyi Reagent.

A - Glucose.
B - Maltose.
SECTION II

GENERAL METHODS

The following general methods were employed in the present investigations.

1. **Quantitative Estimations of Reducing Sugars**

   (a) The cuprimetric titration method of Shaffer and Somogyi (31) as modified by Hanes and Cattle (82) was employed in the earlier part of this work. In this method, the reagent was stored as four separate solutions. Reduction was obtained by heating a copper tartarate-bicarbonate mixture and sugar sample at 100°C for 20 minutes; after addition of iodate-iodide, the excess iodine was then titrated with sodium thiosulphate solution (0.005N). Calibration curves, (Figure 3) for glucose and maltose were obtained.

   (b) The improved Somogyi reagent (83) was also employed for the estimation of reducing sugars. Calibration curves for glucose and maltose are shown in Figure 3.

   (c) Estimation of glucose in the presence of maltose by the method of Phillips and Caldwell (84).

Reagents:

(i) **Copper sulphate** 69.23 Gm. of copper sulphate pentahydrate were dissolved in one litre of distilled water.

(ii) **Sodium acetate trihydrate** 500 Gm were dissolved in
FIG. 4. - Calibration of Philips & Caldwell Reagent.
500 ml of hot distilled water, cooled, and diluted to one litre. This gave a pH value of 8.7 to 8.8. It is important that the pH of this solution should not be lower than 8.7.

(iii) **Potassium iodide-iodate** 2.7 Gm of potassium iodate and 60.0 gm of potassium iodide were dissolved in 500 ml distilled water, together with 0.25 gm of sodium hydroxide. The final volume was made up to one litre.

(iv) **Sulphuric acid** 4N Sulphuric acid was obtained by diluting 114 ml of A.R. concentrated sulphuric acid to a final volume of one litre.

(v) **Potassium oxalate** 330 Gm of potassium oxalate were dissolved in one litre of hot distilled water to give a saturated solution.

(vi) **Sodium thiosulphate** Thiosulphate solution (0.01N) was obtained by diluting 0.1N stock solution, which in turn was prepared by dissolving 24.8 gm sodium thiosulphate pentahydrate and 3.0 gm of sodium tetraborate in one litre of water.

(vii) **Glucose and maltose solutions** 5% Standard solutions of both glucose and maltose were prepared, the exact concentrations of which were determined polarimetrically. A graph was obtained by plotting the corrected readings against the concentration of sugar (Figure 4).

2. Chromatographic Methods

(a) **Paper partition chromatography** The following solvents and spray reagents were used, for the separation and preliminary
identification of carbohydrates by paper partition chromatography. Separation of sugars was effected by the descending flow method on Whatman No. 1 paper at room temperature and at 23°C.

**Solvents**

(i) Benzene-butanol-pyridine-water. (10:50:30:30 by volume).

(ii) Ethylacetate-acetic acid-water (3:1:3 by volume).

(iii) Ethylacetate-pyridine-water (10:4:3).

**Spray reagents**

(i) *Ammoniacal silver nitrate* The dried paper was passed through a solution prepared by adding saturated aqueous silver nitrate solution (1 ml) to acetone (200 ml) and adding water dropwise with shaking until the precipitated silver nitrate re-dissolved. After drying, the paper was sprayed with 0.5N solution sodium hydroxide in aqueous ethanol. Excess of reagent was removed by washing in 6N-ammonium hydroxide solution and then water (85) or by washing in saturated solution of sodium thiosulphate and then water (86). After washing in water for one hour, the paper was allowed to dry.

(ii) *Aniline oxalate* The dried paper was sprayed with a saturated aqueous solution of aniline oxalate; the paper was then heated at 130°C until the development of spots was complete (87).

(b) *Column chromatography* Large scale quantitative separations of oligosaccharide were carried out on charcoal-celite columns (88), prepared as follows: equal weights of activated
B.D.H. charcoal and Celite "545" were mixed dry and poured in the form of a slurry, into a glass column containing a pad of Celite (1-2" thick) supported on a glass wool pad on top of a porous disc. The slurry was added a little at a time under gentle suction. The column so prepared, was washed thoroughly with distilled water. After the addition of the oligosaccharides, separation of the individual components of the mixture was effected by elution with increasing concentrations of aqueous alcohol.

(c) **Electrophoresis of sugars** The purity of oligosaccharides separated on charcoal-Celite column was checked by electrophoresis, which was carried out on apparatus similar to that described by Foster (89). Oligosaccharides were run on Whatman No. 1 paper against the standard tetra methyl glucose, in the form of borate complexes, in 0.1 M boric acid-sodium hydroxide buffer pH 10. The separation was effected at 750 volts, and final current density ca. 10 milliamperes for 4½ to 5 hrs.

**Spray reagent** The dried paper was sprayed with a saturated aqueous solution of aniline oxalate, acidified with acetic acid to effect the breakdown of borate complexes and heated at 100°C until the development of the spots was complete.

3. **Drying Polysaccharide Samples**

Prior to quantitative analyses, samples of polysaccharides were dried by heating for several hours at 60°C over phosphorus pentoxide under reduced pressure.
4. Acid Hydrolysis of Carbohydrates

(a) Complete acid hydrolysis. In the present investigations, the exact concentration of oligo- and polysaccharides was determined by estimation of the monosaccharides liberated on complete acid hydrolysis. A solution containing 1-5 mg. sugar/ml. was heated in 1.5 N sulphuric acid for 2 hrs. at 100°C. Complete acid hydrolysis of α-linked oligo- and polysaccharides occurred with the above conditions (90). The reducing sugar thus obtained was quantitatively determined by the Somogyi (91) method, after neutralisation with sodium hydroxide of the acid hydrolysate.

The expression:

\[ \text{wt. of oligo- or polysaccharide} = \frac{\text{wt. of glucose} \times 162}{180} \]

was then used.

In addition, hydrolysates of some carbohydrates, after neutralisation with barium carbonate followed by deionisation with Amberlite IR-120 and IR-4B resins were concentrated in vacuo at 55°C and examined by paper chromatography.

(b) Partial acid hydrolysis. Partial acid hydrolysis of oligo-saccharides was effected by heating with 0.25N sulphuric acid, at 100°C, for one hour. The resultant solution was neutralised, deionised with resins, concentrated in vacuo, and analysed by paper chromatography.

5. Deproteinisation

Enzymic digests were deproteinised, prior to reducing sugar estimation, according to Nelson (91) using zinc sulphate solution (5%
ZnSO₄·7H₂O) and barium hydroxide solution (approximately 0.3N).

The two reagents were adjusted so that 5 ml of zinc sulphate solution required 4.7 ml of barium hydroxide solution to produce a definite pink colour to phenolphthalein. 0.5 ml each of zinc sulphate and barium hydroxide solutions were used for a 5 ml sample of an enzymic digest. Zinc sulphate was added before the addition of barium hydroxide as recommended by Hers, Benfays and DeDuve (92).

6. **Determination of Ash Content**

Ash contents of various acetone and ethanol fractions of brewer's and baker's yeast, obtained during the preparation of various enzymes were estimated. Weighed samples of various fractions (40-70 mg) were ignited to constant weight in a silica crucible and the weight of the resultant ash determined. Ash contents of polysaccharide samples were determined in a similar manner, in a platinum crucible.

7. **Iodine Staining**

Iodine solution (1 ml, 0.2% iodine in 2% potassium iodide) was introduced into a graduated flask (100 ml in the case of amyllose and starch, and 50 ml in the case of amylopectin) and water added. A sample (1 ml) of a digest containing polysaccharide was added to the above solution and the volume made up to the mark. The absorption value (A.V.) of the polysaccharide-iodine complex was measured in a Unicam SP.500 spectrophotometer at 540 m\(\mu\) (Amylopectin), 630 m\(\mu\) (Amylose) and 600 m\(\mu\) (Starch), against an iodine-water blank.

8. **Examination of the Spectra in Aqueous Solution**

2.5 mg of polysaccharide (glycogen or amylopectin) was introduced into a standard flask (25 ml). One drop of 5N-hydrochloric
acid and iodine solution (2.5 ml; 0.2% iodine in 2% potassium iodide) were then added and the mixture diluted to 25 ml with water. The resulting coloured solution was then examined at room temperature against an iodine water blank in a Unicam SP.500 spectrophotometer over the range of 400-700 nm. The absorption spectra of glycogen-iodine complex showed a wide absorption curve, and λ max values which are reported represent the midpoint of such a curve.

9. **Estimation of Protein Nitrogen**

Protein nitrogen was determined by the biuret method of Robinson and Hogden (93). Yeast protein which had 12.0% of N determined by the Kjeldahl method was used as a standard.

**Reagents**

- 10% trichloroacetic acid solution
- 3% sodium hydroxide
- 20% copper sulphate pentahydrate

**Calibration** Standard yeast protein solution (1 mg nitrogen/ml) was prepared by dissolving 308.30 mg of yeast protein in 3% sodium hydroxide in a total volume of 25 ml.

To each of 0.5, 1.0, 1.5, 2.0, 2.5 and 3.0 ml of standard protein solution in graduated centrifuge tubes was added sodium hydroxide reagent to give a total volume of 9 ml. 0.25 ml Copper sulphate solution was added and the volume made up to 10 ml with the alkali. After shaking the mixture for one minute, they were allowed to stand for 15 minutes. After centrifugation, the colours of the supernatant solutions were compared on a Spelker Photoelectric Absorptiometer in 1 cm cells at 550 nm against a water blank.
FIG. 5. - Calibration of Biuret Reagent.

Absorption value at 550 m\(\mu\)  

Concentration of Formaldehyde (mg)

FIG. 6. - Calibration for Formaldehyde Reagent.

(A) - Unbuffered  
(B) - Buffered  

Concentration of Formaldehyde (mg)
The following results after correction for a reagent blank were obtained and are graphically represented in Figure 5:

Protein nitrogen (mg)  0.0  0.5  1.0  1.5  2.0  2.5  3.0
Absorption value (A.V.)  0.02  0.12  0.25  0.32  0.42  0.52  0.62

10. Periodate Oxidation

(a) Determination of formaldehyde The micro-method of formaldehyde estimation in the presence of periodate, as used by Hough, Powell and Woods (94) was employed.

Reagents
(i) 2 Volumes of aqueous 2% solution of A.R. potassium ferricyanide were mixed with 5 volumes of concentrated hydrochloric acid.
(ii) 1% Solution of purified phenylhydrazine hydrochloride in 0.2N-sodium acetate-acetic acid buffer, pH 5.5.
(iii) 4 Volumes of standard aqueous barium chloride solution were mixed with one volume of saturated aqueous sodium bicarbonate solution. The resulting precipitate was removed by centrifugation.

Reagents (i) and (iii) were prepared freshly each day as required.

Calibration The reagents were calibrated under the following conditions:
(a) Unbuffered conditions To each of 0, 1, 2, 3, 4 and 5 ml standard formaldehyde solution (191.6 μg/ml); concentration determined by means of standard iodine and sodium thiosulphate (95) was added sodium metaperiodate
solution (2 ml, 0.5 M) and the mixture made up to 25 ml with water. The solutions were stored in the dark at 35°C for 24 hrs.

Samples (2 ml) were withdrawn and mixed with reagent (3) (2 ml). After standing for 10 min., the mixtures were clarified by centrifugation. Clear supernatant (2 ml) was then treated with reagent (2) (2 ml) in a 50 ml graduated flask, and set aside in the dark for 30 mins.

Reagent (1) (7 ml) was then added and, after 3 mins., the mixtures were diluted to 50 ml with water. The solutions were thoroughly mixed, and the absorption values of the colours thus obtained were measured in 1 cm cells in a Unicam at 518 μ, against a reagent blank (solution containing no formaldehyde).

The results are recorded in the following table (Figure 6).

(b) **Buffered conditions** Mixtures of formaldehyde and sodium metaperiodate, similar to those set up for unbuffered conditions were prepared. Phosphate buffer (12.5 ml, 0.1 M; pH 8) was added to each in a total volume of 25 ml. The reagents were then calibrated as above. The following results were obtained (Figure 6).
(c) **Estimation of formic acid**  The formic acid, released during the periodate oxidation of carbohydrates carried out under various conditions, was determined by neutralisation of the acid with approximately 0.01 M standard, carbonate free sodium hydroxide and methyl red as indicator to pH 5.7.

Excess of periodate was removed by pretreatment of the sample; with neutral ethylene glycol (1-2 ml, depending on the amount of residual periodate) (96, 97), in the dark at room temperature. A blank without carbohydrate material was always set up.
SECTION III

FRACTIONATION OF YEAST ENZYMES

INTRODUCTION

Although the foundations of enzyme biochemistry were laid down long before any pure enzymes had been isolated, e.g., the work of Sørensen, Michaelis, Menton, Briggs, Haldane, many obscure and controversial enzyme reactions have only been elucidated after highly purified enzymes have become available. In order to study the specificity and mode of action of a particular enzyme, the aim of an enzymologist must be to obtain it in a pure state. The purification of an enzyme involves not only its isolation from other cell constituents but also its separation from other enzymes. This can be achieved by following some of the methods reviewed by Sumner and Myrbäck (98), Gunsalus (99), Schwimmer and Pardee (100) and Dixon and Webb (101).

The brewer's and baker's yeast obtained from the fermentation and food industries contain a complex mixture of intracellular enzymes and provide an easy and inexpensive material for their extraction. Many of the enzymes can be brought into solution from the starting material by rupture of the cell-membrane followed by extraction with water or dilute salt or buffer solution. The ease of release of enzymes from cells depends to a great extent on their previous
treatment. Drying of the cells at relatively low temperature serves to preserve enzyme activity. Dried cell preparations can be obtained by air-drying, slow vacuum drying, freeze-drying or dehydration with water miscible solvents, e.g. acetone. Rupture of cell-walls can be brought about mechanically by grinding the material with sand or shaking at high speed with fine glass beads, by ultrasonic and sonic oscillations, freezing followed by thawing, or chemically by autolysis (with toluene) or lysis with added cytolytic enzymes.

Some of the impurities from enzyme extracts may be eliminated by fractionation methods. Each fractionation step consists in the separation of the total protein into a series of fractions by precipitation with salt or organic solvent, by addition of varying amounts of adsorbents, or by changes in pH. In all fractionations, attention must be paid to the pH of the solution. The most widely used methods are (a) fractional precipitation by salts (usually ammonium sulphate), (b) fractional adsorption (on alumina or calcium phosphate gel), (c) by fractional precipitation with organic solvents (acetone or ethanol) or (d) chromatographic methods using cellulose-derivatives or ion-exchange resins as the supporting material.

Ammonium sulphate is most widely used as a precipitating agent due to its large solubility in water and absence of harmful effects on most enzymes. Fractional precipitation by salts can be effected either by increasing the salt concentration step by step or by decreasing the salt concentration as described by Zahn and Stahl (102). In the latter, the material is first completely precipitated by a
high concentration of salt and then the precipitate is extracted successively with solutions of gradually diminishing salt concentration. Cohn and co-workers (103) have used ethanol for protein fractionation while Askonsas (104) has used various organic solvents for separating enzymes of muscle extracts. She came to the conclusion that acetone gave the sharpest separations and lowest losses. Since most enzymes are inactivated by organic solvents at room temperature, it is advisable to carry out fractionation at low temperatures (between -10°C to 0°C). Fractional adsorption can be achieved either by adsorbing the enzyme and recovering it by selective elution from the adsorbent or if the enzyme is not adsorbed, removing the unwanted material by adsorption. The rapid fractionation of enzymes from malt and barley has been successfully effected by Harris and McWilliam (105). Cook and Phillips (106) have also studied the purification of yeast maltases by adsorption on alumina.

Chromatography, paper and column, is yet another method used for the isolation of enzymes. Paper chromatography of enzymes by which the solvent such as acetone, passing through the paper would preferentially elute a particular enzyme in the mixture, was carried out by Giri et al. (107) to achieve a separation of amylases, phosphorylases and phosphatases from sweet potato, saliva, rice, green gram, kidney and liver.

Electrophoresis of enzymes is a most satisfactory and modern method employed for small scale isolation of enzymes in their final stages of purification. In electrophoresis, the major factor employed in separating the components of a complex mixture is their
differing ionic mobilities in an electric field. Zone electrophoresis in starch gels has been studied by Smithies (108) while Porath (109) has used electrophoresis on cellulose columns. Continuous paper electrophoresis using an apparatus of the type described by Durrum (110) makes it possible to prolong the duration of the separations. In this a buffer solution is made to flow down a sheet of filter paper dripping from serrated edges at the bottom into a series of tubes. The electric current is passed at right angles to this flow, and as the enzyme is fed continuously onto the paper, the components having different mobilities in an electric field will travel down at different angles. This method has been used for the attempted separation of yeast enzymes and is described later in this section.

A number of yeast enzymes catalysing different reactions have been investigated, the most well-known of these being those concerned with the fermentation of glucose to ethanol (Scheme 1); and many of these yeast enzymes have since been obtained in the crystalline form, for example, phosphorylase, amylases, phosphoglucomutase, aldolase, diphosphoglyceric aldehyde dehydrogenase, enolase, phosphoglyceromutase and alcohol dehydrogenase.

The aim of the present work, therefore, was to attempt the purification of yeast enzymes involved in synthesis and degradation of glycogen. This includes the demonstration that isomylase, a branching enzyme and maltase were separate enzymes, i.e. that none of these enzymes showed dual specificity.
I. Brewer's yeast was supplied by Messrs William Younger and Company Ltd. The yeast was spread on shelves and dried by a current of warm air (35-40°C) in a "Mitchell Dryer". The dried yeast was stored at 0°C in air tight bottles.

(1) Acetone Fractionation of Extracts of Brewer's Yeast

The enzymes were extracted by treating dried brewer's yeast (200 gm) with 0.1 M sodium bicarbonate solution (1,000 ml) at room temperature for 2 hr, with constant stirring. The solution was centrifuged (2,500 r.p.m.) for 20 min. at -4°C. The supernatant solution (580 ml) was treated with 'Analar' acetone (580 ml) at -7°C with constant stirring. The precipitate was collected on the centrifuge (-4°C) and dissolved in 0.2 M citrate buffer (500 ml; pH 5.8). It was then triturated for several hours in the cold, and insoluble material was removed by centrifugation. The solution (480 ml) was further fractionated with 'Analar' acetone at -7°C.

The first fraction (protein D₁) was collected between 0-20% acetone concentration by adding 125 ml of acetone to 480 ml of the solution. The precipitate was collected on the centrifuge, washed with cold acetone and dried under vacuum over phosphorus pentoxide. Second and third fractions (protein D₂ and D₃) were obtained in a similar manner, when the acetone concentration was increased from 20-33% and 33-42% respectively. Protein D₂ and D₃ were collected on the centrifuge, washed with cold acetone and dried under vacuum over phosphorus pentoxide.
A second extraction of enzymes was carried out by treating the insoluble material collected from the first extraction with a further 500 ml of 0.1 M-sodium bicarbonate solution. The insoluble material was removed on the centrifuge at -4°C and the supernatant solution was treated with acetone to 50% concentration. The precipitate (protein $D_4$) was collected on the centrifuge, washed and dried.

The nitrogen content of the various fractions was estimated by the biuret method described in Section II. The percentage protein was calculated from the conversion factor (6.25) and percentage nitrogen. The yield, ash content, nitrogen, and protein contents have been tabulated in the Table I below.

**TABLE I**

**Properties of Acetone Fractions of Brewer's Yeast**

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Acetone concentration (%)</th>
<th>Yield (g)</th>
<th>Ash (%)</th>
<th>Nitrogen content (%)</th>
<th>Protein (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Protein $D_1$</td>
<td>0 - 20</td>
<td>5.0</td>
<td>33.8</td>
<td>6.04</td>
<td>38</td>
</tr>
<tr>
<td>$D_2$</td>
<td>20 - 33</td>
<td>11.7</td>
<td>31.4</td>
<td>5.85</td>
<td>37</td>
</tr>
<tr>
<td>$D_3$</td>
<td>33 - 42</td>
<td>5.0</td>
<td>10.0</td>
<td>9.05</td>
<td>57</td>
</tr>
<tr>
<td>$D_4$</td>
<td>second extraction</td>
<td>15.5</td>
<td>15.0</td>
<td>9.35</td>
<td>59</td>
</tr>
</tbody>
</table>

(2) Preliminary Experiments with Protein $D_1, D_2, D_3$ and $D_4$.

The following experiments were carried out to characterise some of the enzymes present in these fractions.

(a) Debranching (isoamylase) activity

Digests containing the following were prepared:—
The digests were incubated at room temperature overnight, and heated in a boiling water bath for 3 minutes to inactivate the enzyme. The solutions were centrifuged to remove coagulated protein. β-Amylase solution (2 ml, 7 mg/ml) and water (3 ml) were added to each digest which was then incubated at 35°C, and the β-amylolysis limit determined after 24 and 48 hr. The following results were obtained.

<table>
<thead>
<tr>
<th>Fractions</th>
<th>β-Amylalysis-limit (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>24 hr.</td>
</tr>
<tr>
<td>D_1</td>
<td>47.4</td>
</tr>
<tr>
<td>D_2</td>
<td>97.1</td>
</tr>
<tr>
<td>D_3</td>
<td>48.9</td>
</tr>
<tr>
<td>D_4</td>
<td>45.4</td>
</tr>
<tr>
<td>Control</td>
<td>39.4</td>
</tr>
</tbody>
</table>

The above results show the presence of a considerable amount of debranching activity in protein D_2, but not in any of the other fractions.

(b) Branching activity

The following digest was set up with various acetone fractions:
potato amylose (2 mg/ml) 3.5 ml
0.2 M-citrate buffer pH 7.0 1.5 ml
enzyme solution (30 mg/ml) 1.0 ml
distilled water 1.0 ml

The reaction was followed by iodine staining. The absorption values of amylose-iodine complexes were measured on a Spekker photoelectric absorptiometer at 680 m\(\mu\) in 4 cm cells against an iodine-water blank.

The following results were obtained:

<table>
<thead>
<tr>
<th>Fractions</th>
<th>20 min</th>
<th>30 min</th>
<th>1550 min</th>
</tr>
</thead>
<tbody>
<tr>
<td>Protein D(_1)</td>
<td>16.4</td>
<td>20.5</td>
<td>44.0</td>
</tr>
<tr>
<td>D(_2)</td>
<td>26.0</td>
<td>26.0</td>
<td>42.5</td>
</tr>
<tr>
<td>D(_3)</td>
<td>45.0</td>
<td>52.5</td>
<td>61.5</td>
</tr>
<tr>
<td>D(_4)</td>
<td>38.5</td>
<td>39.2</td>
<td>41.0</td>
</tr>
</tbody>
</table>

All the fractions show some branching enzyme activity, the greatest being found with fraction D\(_3\).

(b) Maltase activity

Maltose solution (2 ml, 2\%) was incubated with the various protein fractions at 35\(^\circ\)C under toluene. The reaction digests were examined by paper chromatography after 24 and 48 hr. and were found to contain glucose and maltose. Equal volume of digests were examined and from the intensity of the developed glucose spots, the following data were obtained.
Fractions & Inference, maltase activity

<table>
<thead>
<tr>
<th>Protein $D_1$</th>
<th>Trace only</th>
</tr>
</thead>
<tbody>
<tr>
<td>$D_2$</td>
<td>Medium</td>
</tr>
<tr>
<td>$D_3$</td>
<td>Strong</td>
</tr>
<tr>
<td>$D_4$</td>
<td>Trace only</td>
</tr>
</tbody>
</table>

(d) **Amylase activity**

Digests consisting of the following were set up with various acetone fractions:

- Glycogen (6 mg/ml) 5 ml
- 0.2 M-acetate buffer pH 4.54 5 ml
- Enzyme solution (30 mg) 4 ml
- Distilled water 1 ml

The digests were incubated at 35°C for 24 hr. Reducing sugars were estimated with the Somogyi 1933 reagent. The following results were obtained:

<table>
<thead>
<tr>
<th>Fractions</th>
<th>Maltose (mg)</th>
<th>Conversion into maltose (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>$D_1$</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>$D_2$</td>
<td>1.38</td>
<td>4.35</td>
</tr>
<tr>
<td>$D_3$</td>
<td>1.58</td>
<td>4.35</td>
</tr>
<tr>
<td>$D_4$</td>
<td>1.98</td>
<td>6.20</td>
</tr>
</tbody>
</table>

A trace of amylase activity can be detected after 24 hr. The enzymic digests were examined on paper chromatograms; maltose and glucose were detected.

The preliminary experiments carried out with protein $D_1$, $D_2$, $D_3$ and $D_4$ therefore show the presence of the following enzymes:
II (1) *Acetone Fractionation of Baker's Yeast*

Dried baker's yeast (200 g) was extracted with 0.1 M-sodium bicarbonate solution (1,000 ml) for 2 hr. Insoluble material was removed on the centrifuge (2,500 r.p.m. at -4°C). The supernatant solution (600 ml) was treated with an equal volume of 'Analar' acetone at -7°C with constant stirring. The precipitate was collected on the centrifuge (-4°C) and dissolved in 0.2 M-citrate buffer (pH 5.8, 500 ml). After allowing the precipitate to dissolve overnight at 0°C, insoluble material was removed by centrifugation. The supernatant solution (480 ml) was then fractionated with 'Analar' acetone at -7°C.

Four fractions (protein $Z_1$, $Z_2$, $Z_3$ and $Z_4$) were obtained by treating the supernatant solution (480 ml) with subsequent 125 ml of acetone to 0-20%, 20-33%, 33-43% and 42-50% acetone concentration. The precipitates were collected on the centrifuge at 0°C, washed with cold acetone and dried under vacuum over phosphorus pentoxide.

The protein nitrogen was determined by the biuret method. The percentage protein was calculated from the conversion factor (6.25) and percentage nitrogen. The yields, percentage nitrogen, percentage protein and ash content of various fractions have been tabulated in the Table II below.
TABLE II

Properties of acetone fractions of baker's yeast

<table>
<thead>
<tr>
<th>Fractions</th>
<th>Acetone concentration (%)</th>
<th>Yield (g)</th>
<th>Ash (%)</th>
<th>Nitrogen (%)</th>
<th>Protein (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Protein $D_1$</td>
<td>0 - 20</td>
<td>0.4</td>
<td>41.1</td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td>$D_2$</td>
<td>20 - 35</td>
<td>3.8</td>
<td>40.9</td>
<td>4.4</td>
<td>27.5</td>
</tr>
<tr>
<td>$D_3$</td>
<td>35 - 42</td>
<td>5.3</td>
<td>38.7</td>
<td>6.0</td>
<td>37.5</td>
</tr>
<tr>
<td>$D_4$</td>
<td>42 - 50</td>
<td>2.4</td>
<td>78.5</td>
<td>1.4</td>
<td>8.8</td>
</tr>
</tbody>
</table>

(2) Preliminary Experiments with Protein $Z_1$, $Z_2$, $Z_6$ and $Z_4$.

(a) Debranching (isoamylase) activity

The following digestes were prepared with various acetone fractions.

- glycogen (6 mg/ml)
- 0.2 M-acetate buffer pH 5.89
- enzyme
- distilled water

A control digest without the yeast protein was also set up. The digestes were incubated at room temperature overnight. The enzyme was inactivated by heating in boiling water bath for 3 min. After cooling to room temperature, the solutions were centrifuged to remove coagulated protein. $\beta$-Amylase (2 ml, 7 mg/ml) and water (3 ml) were added to each of the digestes. After incubation at 35°C for 24 hr., $\beta$-amylolysis limits were determined as tabulated below.

<table>
<thead>
<tr>
<th>Fractions</th>
<th>$\beta$-Amylolyis limit (%)</th>
<th>Increase in $\beta$-Amylolyis-limit (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Protein $Z_1$</td>
<td>46.4</td>
<td>5.0</td>
</tr>
<tr>
<td>$Z_2$</td>
<td>65.1</td>
<td>23.7</td>
</tr>
<tr>
<td>$Z_3$</td>
<td>64.5</td>
<td>23.1</td>
</tr>
<tr>
<td>$Z_4$</td>
<td>46.0</td>
<td>4.6</td>
</tr>
<tr>
<td>Control</td>
<td>41.4</td>
<td>-</td>
</tr>
</tbody>
</table>
(b) Maltase activity

Digests consisting of the following were set up with various acetone fractions:

- maltose solution 0.2% 10 ml
- 0.2 M-acetate buffer pH 5.89 4 ml
- enzyme solution (30 mg/ml) 2 ml
- distilled water 4 ml
- toluene 2 drops

A control digest without the enzyme was also set up. The digests were incubated at room temperature for 24 hr. Reducing sugars were estimated cuprimetrically; the following results were obtained.

<table>
<thead>
<tr>
<th>Fractions</th>
<th>Maltose (mg)</th>
<th>Apparent increase in maltose</th>
</tr>
</thead>
<tbody>
<tr>
<td>Z₁</td>
<td>21.2</td>
<td>1.3</td>
</tr>
<tr>
<td>Z₂</td>
<td>21.0</td>
<td>1.1</td>
</tr>
<tr>
<td>Z₃</td>
<td>20.5</td>
<td>0.4</td>
</tr>
<tr>
<td>Z₄</td>
<td>20.4</td>
<td>0.5</td>
</tr>
<tr>
<td>Control</td>
<td>19.9</td>
<td>-</td>
</tr>
</tbody>
</table>

A portion of the enzymic digests was examined by paper chromatography; maltose and small amounts of glucose were detected. Hence a trace of maltase is present in Z₁ and Z₂.

(c) Branching activity

Digests consisting of the following were set up with various fractions:

- potato amylase (2 mg/ml) 5.5 ml
- 0.2 M-citrate buffer pH 7.0 1.5 ml
- enzyme solution (30 mg/ml) 1.0 ml
- distilled water 1.0 ml

The reaction was followed by iodine staining. The absorption values of amylase-iodine complexes were measured on a Spekker photoelectric
absorptiometer at 680 mp in 4 cm cells against an iodine-water blank. The following data were obtained:

<table>
<thead>
<tr>
<th>Fractions</th>
<th>Fall in A.V. (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>30 min</td>
</tr>
<tr>
<td>$Z_1$</td>
<td>15.0</td>
</tr>
<tr>
<td>$Z_2$</td>
<td>16.2</td>
</tr>
<tr>
<td>$Z_3$</td>
<td>18.3</td>
</tr>
</tbody>
</table>

Fraction $Z_3$ contains a considerable amount of branching activity.

III Attempted Separation of Isoamylase from Branching Enzyme by Starch Adsorption

Protein $D_2$, which contained a considerable amount of debranching activity was contaminated with traces of branching enzyme, maltase and amylase. An attempt to remove the amylase and branching enzyme by starch adsorption was then carried out.

Protein $D_2$ (1 gm) was dissolved in 20% ethanol solution (50 ml) at -4°C. Anhydrous sodium sulphate (0.5 g) was added and the solution slowly stirred with cooling to -7°C. Waxy maize starch (10 gm) was added to the solution with constant stirring. Samples (12 ml) were removed after 30, 60, 90 and 120 min. and centrifuged at -4°C (5,000 r.p.m). The supernatant solution from each of the fractions was collected and analysed for branching and debranching activity.

(i) Branching activity

Branching activity was determined by incubating potato amylase with different fractions in the presence of citrate buffer. The reaction was followed by iodine staining after the time intervals of 10, 20, 30 and 40 min. The absorption values were read on a Spekker photoelectric absorptiometer in 4 cm cells at 680 mp. The following results were obtained.
The fraction after 120 min. (3 hr.) starch treatment showed only a trace of branching activity. However, the increase in A.V. after 30 min. can only be explained by the presence of debranching enzyme, the activity of which is "masked" in the earlier stages.

(ii) Isoamylase activity

Digests consisting of the following were set up with various fractions:

- glycogen (6 mg/ml) 5 ml
- enzyme solution 5 ml
- 0.2 M-acetate buffer pH 5.89 5 ml
- distilled water 5 ml

A control digest without isoamylase was also set up. After incubation at room temperature, overnight, the digests were inactivated by heating in boiling water bath for 5 min. The digests, after the addition of β-amylase (6 mg/ml, 2 ml) were incubated at 35°C and the β-amylolysis limit determined. The following data were obtained:

<table>
<thead>
<tr>
<th>Fractions</th>
<th>30 min</th>
<th>60 min</th>
<th>90 min</th>
<th>120 min</th>
<th>Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hydrolysis (%)</td>
<td>44.9</td>
<td>49.7</td>
<td>42.3</td>
<td>53.9</td>
<td>54.6</td>
</tr>
</tbody>
</table>

The greatest isoamylase activity was observed in 120 min. fraction which is still contaminated with a trace of branching enzyme. Hence the attempt at separation of isoamylase from branching enzyme by starch adsorption was not entirely successful.
IV Attempted Separation of Enzymes by Paper Chromatography

Since a mixture of amino acids and peptides can be very effectively separated by paper partition chromatography, this method has been applied to separation of a mixture of enzymes. The enzyme solution (13%; 1 ml) was spotted horizontally on Whatman No. 1 papers, which were developed with the solvent (water) in a chromatographic tank at 0°C. In 6 hr., the solvent front moved 24 cm from the starting line. The papers were dried at room temperature and then cut into 14 strips (2.5 cm in width) from the starting point to the solvent front. Each strip was incubated with the substrates, e.g. glycogen (0.2%) and maltose (0.4%) in the presence of an acetate buffer and at room temperature to determine the isomaltase and maltase activities. Both isomaltase and maltase travelled at the same rate in water as a solvent.

V Rapid Fractionation of Protein D₂ by Selective Elution from Alumina

A glass column (1.8 cm x 14 cm) was packed with Spence activated alumina and washed with a phosphate-citric acid buffer solution at pH 4.0 (McIlvain) until the effluent attained the same pH value. Protein D₂ (100 mg) containing isomaltase was dissolved in buffer solution (1 ml, pH 4.0) and this solution washed on to the alumina column. The enzymes were washed into the column with further 15 ml buffer solution and their elution was effected by slowly dripping 0.2 M-aqueous sodium phosphate into already stirred phosphate-citric acid buffer solution (100 ml, pH 4.0). The resultant solution, the pH of which was continuously changing was siphoned to the top of the column, so that the rate of change of pH was controlled by the rate of flow of the buffer solution through the column. The eluate was collected in fractions (5 ml) on a Towers fraction collector. The optical density of the eluate was measured at 280 mp in a Unicam
FIG. 7. Protein Distribution of Alumina Column Fractions

At 28°C

At 0 ± 2°C

Optical Density at 280 m\(\mu\) (x 10^y)

Fraction Number
absorptiometer against a sodium phosphate blank. The readings so obtained gave a provisional measure of the protein contents of the fractions (60 in all; Fig. 1).

Detection and measurement of enzyme activities of fractions

Various fractions which had high optical density values were measured for the enzyme activities. In this case, the tests were carried out for determining isomaltase, maltase, isomaltase and branching enzyme activities. The digests were set up with fractions 2-5 and 20-30 and substrates glycogen (2%), maltose (2%), isomaltose (1%) and starch (1%). Isoamylase activity was determined by increase in β-amylolysis limit. In the case of branching activity, the reaction was followed by iodine staining, and the colours of the polysaccharide-iodine complexes were measured on Unicam absorptiometer at 600 nm. Maltase and isomaltase activities were determined by paper chromatography.

It was found that, since the fractionation was carried out in a constant temperature room (28°C), the fractions so obtained although rich in protein content were enzymically inactive. This led to the conclusion that yeast enzymes lost their activity at 28°C during elution over the course of several hours.

Second attempt at fractionation by selective elution from alumina

The same procedure as described in the previous experiment was followed using a further purified fraction D₂ (see p. 60); the difference was that chromatographic separation was effected in a cold room (0° ± 2°). 50 fractions were collected. The protein content was obtained by determining the optical densities of the eluates (Figure 1).
Detection and measurement of enzyme activities of fractions

The fractions 15, 19, 24, 25 and 28 were analysed for isocyanlase, maltase, isomaltase and branching enzyme activities. Substrates, e.g. glycogen 0.2%, maltose 1%, isomaltose 1% and starch 1% were prepared. Portions (0.2 - 1 ml) of the substrate solutions were treated with aliquot parts (1 ml) of each of the above fractions from the column and incubated at room temperature. Isoamylase and branching enzyme activities were measured by following the reaction by iodine staining. The absorption values of the polysaccharide-iodine complexes were measured in a Unicam absorptionmeter at 420 mp (glycogen) and 600 mp (starch). Maltase and isomaltase activities were established by paper chromatography. The following data were obtained.

<table>
<thead>
<tr>
<th>Fractions</th>
<th>Isoamylase</th>
<th>Isomaltase</th>
<th>Maltase</th>
<th>Branching enzyme</th>
</tr>
</thead>
<tbody>
<tr>
<td>15</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>19</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>24</td>
<td>±</td>
<td>+</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>25</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>28</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

(+) indicates the presence of enzyme in the fractions;
(-) indicates the absence of enzyme in the fractions;
(+4) indicates the presence of a trace of enzyme in the fractions.

Thus, fractions 19 and 28 show the presence of only one particular enzyme. Also, isocyanlase and isomaltase appear to be separate enzymes.

A reasonable separation of enzymes can therefore be achieved by the above method, although the method is of analytical rather than preparative value.

VI Electrophoresis of Enzymes

(a) Micro-electrophoretic method

A rapid and preliminary fractionation of enzymes was carried out
qualitatively by a micro-electrophoretic technique (ill) using Shandon's apparatus. The technique is a combination of membrane filter electrophoresis (ill) and the use of a very sensitive stain Nigrosin (112).

Cellulose-acetate membrane filter strips (10-12 x 2.5 cm) with strip-holders lined with filter paper acting as wicks were placed over a bridge gap of the electrophoretic tank, containing 0.05 M-veronal-acetic acid buffer (pH 8.6 or 8.2). After obtaining an equilibrium in the tank, the protein D_{22} (see p. 60) solution (50 μg) was applied on the membrane by a capillary tube, 2 cm from the electrode. 120 V was applied for 2 hr, after which the strips were heated for 30 min in an oven at 100°C.

The dried strips were then stained with Nigrosin solution (0.1% Nigrosin in 1% acetic acid).

Three bands (blue) were observed on electrophoresis at both pH 8.6 and 8.2, thereby inferring the presence of three major protein components. The distance travelled by each of the bands from the starting point was measured. The distances were 2.4, 3.5 and 4.6 cm respectively.

(b) Continuous electrophoresis of enzymes

Continuous electrophoresis on paper was employed for separating a mixture of enzymes present in protein D_{22}. A Shandon continuous electrophoresis apparatus enclosed in a transparent Perspex cabinet was used.

According to this method, the separated components were eluted continuously, and this was accomplished by perfusing buffer at right angles to the electric field, at the same time feeding the mixture sample at a constant rate from a suitable position in the buffer stream. The electrolyte was 0.05 M-veronal-acetic acid buffer pH 8.6. The elution was carried out in the cold (0°C). The filter paper curtain (Whatman No: 54) with electrode tabs and serrated drip points was hung from the edge of the electrolyte reservoir containing 0.05 M-veronal-acetic acid buffer.
pH 8.6. The paper curtain was washed free of any contaminated proteins by allowing a certain amount of the buffer to drip down slowly from the reservoir into the collecting tubes. When the equilibrium had been established overnight, the reservoir containing protein solution (5%) was placed in the rack in front of the paper about 5 cm away from the cathode. The solution was continuously fed on to the paper curtain by capillary action from a narrow filter paper wick. A potential of 700 - 750 V was then applied. The samples from the receiving tubes (30 in all) were collected and stored at 0°C in separate flasks. The eluates were measured for the protein content at 280 mp and analysed for various enzyme activities. Fractions 1 and 23-50 possessed varying amounts of protein content.

The digests were set up with substrates such as glycogen, amylase, maltose and isomaltose to determine isomylase, branching enzyme, maltase and isomaltase activities. Portions (1 - 5 ml) of the substrates, in admixture with aliquot parts (2 ml) of the fractions were incubated at room temperature for 24 hr. Maltase and isomaltase activities were determined by paper chromatography using solvent 3 and spray 1. Isoamylase and branching enzyme activities were followed by iodine staining. The absorption values of glycogen-iodine complexes were measured in a Unicam SP 500 spectrophotometer at 550 mp, whilst those of amylase-iodine complexes were measured in a EEL colorimeter, using a red filter No: 609, against an iodine-water blank. The following data were obtained.

<table>
<thead>
<tr>
<th>Fractions</th>
<th>Isoamylase</th>
<th>Branching enzyme</th>
<th>Maltase</th>
<th>Isomaltase</th>
</tr>
</thead>
<tbody>
<tr>
<td>24</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>25</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>26</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>27</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>28</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>29</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>30</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
(-) indicates the absence of the enzyme;
(±) indicates the presence of a trace of the enzyme;
(+) indicates reasonable amount of enzyme;
(++) indicates considerable amount of enzyme.

A partial separation of the isomylase and isomaltase activities was achieved by continuous paper electrophoresis.

**DISCUSSION**

The main aim in this work, which was the purification of yeast isomylase, branching enzyme and maltase, has been partly achieved as small quantities of apparently enzymically homogeneous protein have been obtained in addition to larger amounts of partially purified preparations.

The starting material was air dried brewer's or baker's yeast which on extraction with sodium bicarbonate solution (pH 7.9), gave extracts showing satisfactory activity. The soluble enzymes were separated from insoluble cell-wall and particulate by centrifugation, and then fractionated with acetone; the acetone concentration being increased from 0-50% in four successive stages. All the fractions contained much inorganic material (15-40%) in addition to protein (0-15%).

One difficulty in the work was in the measurement of relative enzymic activities. Although isomylase action could in theory be followed by iodine staining, the presence of branching enzyme in the original extracts made this impossible. For this reason, the activities of fractions obtained at one particular stage of purification have been compared with each other, but specific activity values for the original and final enzyme preparations have not been observed.

In the case of maltase activity determination, a reducing power method based on the Phillips and Caldwell reagent (34) which measures glucose in the
presence of maltose was preferred to the methods of Willstätter and Bamann (113); according to these, the activity was determined from changes in optical rotation or in reducing power of the neutralised solution.

**Fractionation of brewer's yeast.** The protein precipitated between 20-33\% acetone concentration contains practically all the isoamylase activity of the extract. This fraction \( \text{D}_2 \) was therefore employed as the starting material for studies on the properties and mode of action of isoamylase (see Section IV). Since it was contaminated with amylace and yeast branching enzyme, an attempt was made to separate them from isoamylase by starch adsorption. It was hoped that the amylace and branching enzyme would be adsorbed on to the starch and isoamylase could be obtained in solution (cf. purification of \( R \)-enzyme (67)). The starch adsorption was carried out in the presence of sodium sulphate which acted as a buffer medium.

After removal of adsorbent by centrifugation, the supernatant solutions were analysed for branching and debranching activity. However, although the greatest debranching activity was observed after 2 hr. adsorption, some branching enzyme was still present.

Attempts to separate these enzymes by paper chromatography (cf. Giri et al. (107)) were not successful. All the yeast enzymes showed the same chromatographic mobility. The chromatographic separation of the enzymes in fraction \( \text{D}_2 \) using an alumina column was then employed. The enzymes were adsorbed on to Spence activated alumina and eluted with phosphate-citrate buffer solution, the pH of which was constantly increasing. Fractions of 5 ml were collected, and the apparent protein content measured from the A.V. at 280 \( \mu \text{m} \), this being the absorption peak for tyrosine and tryptophane which are assumed to be present in all protein. Those fractions containing protein material were then analysed for specific enzymic activities.

In the first experiment carried out at 28° over a period of 10 hr., no
enzymic activity could be detected in any of the fractions. Since the optimum temperature for XBE is 20° (46) and for isomaltase 20°C, it was concluded that thermal inactivation had occurred. A second experiment was carried out with the alumina column and fraction collector placed in a cold room (temperature 0 ± 2°). The distribution of protein material in the fractions is shown in Figure 7. Analysis of fractions 15, 19, 24, 25 and 28 so obtained showed a reasonable separation of various enzymes. For example, branching enzyme has been eliminated from fraction 28 which contained only isomaltase. The presence of isomaltase activity in fractions 24 and 25 suggests that isomaltase and isomaltase are two separate enzymes. This means that the suggestion by Manners and Khin Maung (31) that yeast isomaltase would hydrolyse terminal α-1,6-links as in isomaltose is incorrect. This chromatographic method showed a sharp separation of enzymes; yet, it cannot be employed for the preparation of pure enzymes on a large scale.

Micro-electrophoresis of protein D22 showed three distinct bands, thereby confirming the presence of three main types of protein constituents. This finding is in agreement with three different enzymes isolated by elution from alumina. In view of this fact, a continuous paper electrophoretic method was then employed. The isomaltase activity was observed in fractions 27 and 28, while branching enzyme was absent. As the original protein D22 was contaminated with branching enzyme, the disappearance of this activity can only be explained by inactivation during the electrophoresis. The presence of inorganic ions may also cause the incomplete separation of enzymes.

The protein precipitated between 33-42% acetone concentration (fraction D3) was rich in maltase and was used as a source of this enzyme in the experiments described in Section VI.
Fractionation of baker's yeast. The baker's yeast was fractionated with acetone in a similar manner. Investigations of protein Z2 and Z3 showed the presence of considerable amount of debranching activity. A trace of maltase was observed in all the fraction. An important difference is that compared to brewer's yeast, baker's yeast contains very little maltase activity.
SECTION IV
PROPERTIES OF YEAST ISOAMYLASE

INTRODUCTION

In 1951, Maruo and Kobayashi (76) reported that an enzyme from autolysates of brewer's yeast, hydrolysed the inter-chain linkages in glutinous rice starch producing a polysaccharide which could be further degraded by β-amylase. This enzyme, previously known as "amylosynthease", was renamed isoamylase by these workers, who also found that its action was optimum at pH 6.0-6.2, and at a temperature of 20 C; isoamylase did not require phosphate ions for its action. They claimed it to be similar to R-enzyme (67) from potatoes and broad beans, which also hydrolyses inter-chain linkages in amylopectin.

Evidence for the presence of a debranching enzyme in brewery yeasts has also been demonstrated by Hopkins (115) who observed an increase in the β-amylolysis limit of amylopectin β-dextrin after incubation at pH 6.0 with yeast extracts.

Manners and Khin Maung (31) obtained a protein preparation by acetone fractionation of brewer's yeast extracts, which catalysed the hydrolysis of a proportion of the α-1:6-linkages in glycogen, β-dextrin of waxy maize starch and α-maltodextrins. This fact was observed by an increase in β-amylolysis limit of the polysaccharides, and in the case (glycogen) by an increase in absorption value of the polysaccharide-iodine complexes. They referred to this enzyme preparation as "isoamylase"; this preparation also catalysed the hydrolysis of terminal α-1:6-glucosidic linkages as present in isomaltose. The action of isoamylase (contaminated with maltase) on glycogen was found to be incomplete as the percentage increase in β-amylolysis limit of brewer's yeast glycogen was only 20.
In contrast to the findings of Maruo and Kobayashi, Manners and Khin Maung found it to be different from R-enzyme due to its action on glycogen and isomaltose. In fact, they claimed it to have a wider specificity than any other debranching enzyme such as R-enzyme, amylo-1:6-glucosidase or limit dextrinase.

In this Section, the mode of action of isoamylase has been studied in greater detail. Since the isoamylase preparation devised by Manners and Khin Maung is contaminated with maltase, attempts have been made to effect their separation. The effect of inhibitors, pH, and its action on various substrates such as glycogen, amylpectin, β-dextrin, pustulan, isochichinin and maltodextrins have also been studied. The results have been correlated with those obtained by Peat and co-workers on the related plant enzyme (R-enzyme).

**EXPERIMENTAL**

The enzyme solution was centrifuged prior to its addition to various digests, especially in the case of experiments involved in measurements of absorption values of polysaccharide-iodine complexes.

The enzymic digests were deproteinised either by the method described in Section III or by heating and centrifugation of the coagulated protein prior to the quantitative estimation of reducing sugars.

Various oligo- and polysaccharide samples were kindly provided by Dr. D.J. Manners.

I Preparation of Isoamylase

(a) Preparation I. Isoamylase (protein D₂) was prepared according to the procedure described in Section III. On analysis, this isoamylase preparation was found to be contaminated with a trace of yeast branching enzyme and maltase. Inactivation of yeast maltase was then effected by acid denaturation (98).
Preliminary observations on the selective inactivation of yeast maltase

Protein D₉ (450 mg) was dissolved in 0.2 M-acetate buffer pH 4.54 (45 ml) and incubated at room temperature. The samples (5 ml) were removed at intervals of 4, 6, 7, 8, 10 hr and treated with the appropriate amount of 0.2 M-sodium hydroxide to obtain a pH value of 5.89. Any insoluble material formed was removed by centrifugation and these samples were then incubated with glycogen and maltose to determine isomylase and maltase activity.

Maltase activity

Digests consisting of the following were set up with the samples obtained after time intervals:

- maltose solution 0.4% 10.00 ml
- treated enzyme solution (50 mg) 5.45 ml
- 0.2 M-acetate buffer pH 5.85 3.55 ml
- distilled water 3.00 ml

The digests were incubated at room temperature for 24 hr., and reducing sugars were estimated by the method of Phillips and Caldwell.

Isomylase activity

<table>
<thead>
<tr>
<th>Glycogen (35 mg)</th>
<th>5.00 ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>treated protein solution (50 mg)</td>
<td>5.45 ml</td>
</tr>
<tr>
<td>distilled water</td>
<td>4.55 ml</td>
</tr>
</tbody>
</table>

After incubation at room temperature, overnight, the digests were heated in a boiling water bath to inactivate the enzyme. β-amylase solution (3 mg/ml; 3 ml, 7,200 units) was added to the digests after centrifugation of the coagulated protein and incubated at 35°C for 24 hr. The following data were obtained.
The above results indicate the denaturation of maltase activity after incubation for 10 hr. Isoamylase is still active, though part of it has also been denatured.

Large scale isolation of isomylase freed from maltase by acid inactivation

Protein $D_2$ (1 g) was dissolved in 0.2 M-acetate buffer (100 ml) pH 4.4 and incubated at room temperature for 10 hr. The insoluble material was removed by centrifugation and the pH of the supernatant solution was adjusted to 5.89 with 0.2 M-sodium hydroxide solution. This solution was treated with cold 'Analar' acetone at -7°C. The precipitate so formed was collected on the centrifuge, washed with cold acetone and dried under vacuum over phosphorous pentoxide. The yield obtained was 0.55 gm.

Maltase activity

Digest consisting of the following was set up:

- maltose solution 0.4% 10 ml
- treated enzyme solution (40 mg) 5 ml
- 0.2 M-acetate buffer pH 5.85 2 ml
- distilled water 5 ml

After incubation at room temperature, the reducing sugars were estimated by Phillips and Caldwell method and Somogyi method. The following data were obtained.

<table>
<thead>
<tr>
<th>Time (hr)</th>
<th>Hydrolysis (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td>4.0</td>
<td>0.0</td>
</tr>
<tr>
<td>22.5</td>
<td>0.0</td>
</tr>
</tbody>
</table>
The residual digest was deionised by treatment with Amberlite resins, evaporated to dryness under vacuum and examined by paper chromatography. No glucose could be detected. Hence maltase has been completely inactivated by the acetate treatment.

Debranching activity

Glycogen (5 ml, 25 mg), 0.2 M-acetate buffer pH 5.85 (3 ml), distilled water (3 ml) and acetate treated isoamylase (40 mg, 5 ml) were incubated at room temperature overnight.

Amylopectin β-dextrin (40 mg, 10 ml), 0.2 M-acetate buffer pH 5.85 (5 ml), water (2 ml) and acetate treated isoamylase (40 mg, 5 ml) were similarly incubated.

The above two digests were then heated in a boiling water bath for 3 minutes to inactivate isoamylase. β-amylase (1,500 units, 2 ml) and water (3 ml) were added to each digest and after incubation at 35°C for 24 hr, the β-amylolysis limits were determined. The following results were obtained:

<table>
<thead>
<tr>
<th>Digest</th>
<th>β-Amylolysis-limit (%)</th>
<th>Increase in β-amylolysis limit (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glycogen</td>
<td>65.0</td>
<td>24.9</td>
</tr>
<tr>
<td>β-Dextrin</td>
<td>25.8</td>
<td>25.0</td>
</tr>
</tbody>
</table>

Simultaneous action of acetate-treated isoamylase and βamylase on glycogen, amylopectin and β-dextrin of waxy maize starch

Polysaccharide, isoamylase preparation in 0.2 M-acetate buffer pH 5.85, water and β-amylase solution were incubated at room temperature for 24 hr. Samples were removed and estimated for reducing sugars cuprimetrically after deproteinisation. The detailed compositions of the digests are tabulated below.
<table>
<thead>
<tr>
<th>Digest</th>
<th>Substrate</th>
<th>Weight (mg)</th>
<th>Β-Amylase solution (units; ml)</th>
<th>Water (ml)</th>
<th>Isoamylase solution (mg; ml)</th>
<th>Total volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Glycogen</td>
<td>30</td>
<td>1,500</td>
<td>2</td>
<td>2</td>
<td>40; 5</td>
</tr>
<tr>
<td>2</td>
<td>Amylopectin (potato)</td>
<td>50</td>
<td>1,500</td>
<td>2</td>
<td>2</td>
<td>40; 5</td>
</tr>
<tr>
<td>3</td>
<td>β-Dextrin (waxy maize starch)</td>
<td>40</td>
<td>1,500</td>
<td>2</td>
<td>3</td>
<td>40; 5</td>
</tr>
</tbody>
</table>

The following results were obtained:

<table>
<thead>
<tr>
<th>Digest</th>
<th>Substrate</th>
<th>Conversion into maltose (%)</th>
<th>2 hr</th>
<th>4 hr</th>
<th>24 hr</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Glycogen</td>
<td></td>
<td>-</td>
<td>-</td>
<td>71.0</td>
</tr>
<tr>
<td>2</td>
<td>Potato amylpectin</td>
<td></td>
<td>61.1</td>
<td>67.5</td>
<td>76.2</td>
</tr>
<tr>
<td>3</td>
<td>β-Dextrin</td>
<td></td>
<td>10.9</td>
<td>23.2</td>
<td>28.1</td>
</tr>
</tbody>
</table>

(b) 1. Preparation II - (Improved method of preparation of isocyanase)

Dried brewer's yeast (100 gm) was extracted with 0.1 M-sodium bicarbonate solution (600 ml) with constant stirring for 2 hr. The insoluble material was removed by centrifugation (2,500 r.p.m.; 20 min) at 0°C. The supernatant solution was then treated with cold 'Analar' acetone to 50% acetone concentration. The precipitate was collected on the centrifuge (0°C), and dissolved in 0.2 M-citrate buffer (400 ml) pH 6.0. After triturating for several hours, the insoluble material was removed on the centrifuge and discarded.

The supernatant solution was again treated with cold 'Analar' acetone to 0-50% acetone concentration at -7°C. The precipitate was collected on the centrifuge (0°C) and dissolved once again in 0.2 M-citrate buffer pH 6.0 (100 ml). The insoluble material was removed by centrifugation and the supernatant solution was then fractionated with cold acetone at -7°C.
The precipitate (protein D_{22}) obtained between 35-50% acetone concentration, was collected on the centrifuge, washed with cold acetone and dried under vacuum over phosphorus pentoxide. The yield of the protein obtained was 8.0 mg.

2. Experiments with isoamylase (Preparation II)

(a) Debranching (isoamylase) activity

The following digest was prepared:

- glycogen (25 mg) 5 ml
- 0.2 M-acetate buffer, pH 6.0 5 ml
- enzyme solution (55 mg) 2 ml

The volume of the digest was made up to 25 ml. Samples (2.5 ml) were removed at intervals and stained with iodine solution (2.5 ml; 0.2% iodine in 2% potassium iodide). The absorption values were measured on a Unicam SP.500 spectrophotometer at 440 nm, when the following results were obtained:

<table>
<thead>
<tr>
<th>Time (hr)</th>
<th>0.0</th>
<th>2.0</th>
<th>4.0</th>
<th>24.0</th>
</tr>
</thead>
<tbody>
<tr>
<td>A.V.</td>
<td>0.270</td>
<td>0.305</td>
<td>0.345</td>
<td>0.375</td>
</tr>
<tr>
<td>Increase in A.V. (%)</td>
<td>0.0</td>
<td>15.0</td>
<td>27.8</td>
<td>39.0</td>
</tr>
</tbody>
</table>

(b) Branching activity

The following digest was made up:

- potato amylose (2 mg/ml) 5 ml
- 0.2 M-citrate buffer pH 7.0 2 ml
- isoamylase (50 mg) 1 ml

The reaction was followed by iodine staining and absorption values of iodine complexes were measured against an iodine blank on an EEL colorimeter using a red filter. The following data were obtained:
<table>
<thead>
<tr>
<th>Time (hr)</th>
<th>0.0</th>
<th>3.0</th>
<th>24.0</th>
</tr>
</thead>
<tbody>
<tr>
<td>A.V.</td>
<td>2.7</td>
<td>1.8</td>
<td>1.0</td>
</tr>
<tr>
<td>Fall in A.V. (%)</td>
<td>0.0</td>
<td>33.3</td>
<td>63.0</td>
</tr>
</tbody>
</table>

(c) **Isomaltase activity**

Isomaltose (10 mg) was incubated with isomylase (prep: II, 20 mg) in 0.2 ml of 0.02 M-acetate buffer pH 5.89 for 24 hr at room temperature. The enzymic digest was examined by paper chromatography. Isomaltose and glucose were detected. Therefore, the isomylase preparation hydrolyses terminal α-1:6-linkages.

(d) **Maltotriase activity**

Maltotriose (10 mg) was incubated with isomylase (prep: II, 20 mg) in 0.2 ml of 0.02 M-acetate buffer pH 6.0 for 24 hr at room temperature. On examination of the enzymic digest by paper chromatography, no glucose was detected.

(e) **Maltase activity**

Digest consisting of the following was set up:-

- maltose solution (approx. 1.5%) 12.5 ml
- 0.2 M-acetate buffer, pH 6.0 5.0 ml
- enzyme solution (50 mg) 2.0 ml

The volume of the digest was made up to 25 ml. After incubation at room temperature, samples were removed at intervals to determine the reducing sugars by the method of Somogyi. The following data were obtained:-

<table>
<thead>
<tr>
<th>Time (hr)</th>
<th>0.0</th>
<th>24.0</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total maltose (mg)</td>
<td>40.5</td>
<td>41.0</td>
</tr>
</tbody>
</table>

The above digest was examined by paper chromatography; no glucose could be detected. Hence maltase is absent from the preparation.

The preparation II is contaminated with a trace of yeast branching.
enzyme and isomaltase. Maltase has been inactivated during further fractionation.

II Properties of Isomaltase

(a) Time curve

Glycogen. The digest consisting of the following was set up:

- glycogen (2 mg/ml) 4 ml
- 0.2 M-acetate buffer pH 6.0 4 ml
- enzyme solution (prep: I) 4 mg/ml 4 ml

After incubation of the digest at room temperature, the reaction was followed at intervals by iodine staining, and the following results were obtained.

<table>
<thead>
<tr>
<th>Time (hr)</th>
<th>0.0</th>
<th>2.0</th>
<th>4.0</th>
<th>6.0</th>
<th>24.0</th>
</tr>
</thead>
<tbody>
<tr>
<td>A.V.</td>
<td>0.094</td>
<td>0.124</td>
<td>0.138</td>
<td>0.145</td>
<td>0.16</td>
</tr>
<tr>
<td>Increase in A.V. (%)</td>
<td>32.0</td>
<td>45.5</td>
<td>55.0</td>
<td>71.0</td>
<td></td>
</tr>
</tbody>
</table>

A time curve was obtained (Figure 3).

β-Dextrin of waxy maize starch

The following digest was prepared:

- β-dextrin solution (40 mg) 5 ml
- 0.2 M-acetate buffer pH 5.89 5 ml
- enzyme solution (prep: I) 30 mg 8 ml
- distilled water 7 ml

The digest was incubated at room temperature. Samples (4 ml) were removed after time intervals and heated in a boiling water bath for 5 min. to inactivate the enzyme. After centrifugation of coagulated protein, β-amylase (5 mg/ml; 2 ml) was added to each of the samples. After incubation at 35°C, the reducing sugars thus obtained were estimated. The following data were obtained:
FIG. 8. - Time Activity Curve for Isoamylase.

FIG. 9. - THE Effect of Enzyme Concentration (Isoamylase)
<table>
<thead>
<tr>
<th>Time (hr)</th>
<th>0.5</th>
<th>1.0</th>
<th>2.0</th>
<th>4.0</th>
<th>20.0</th>
</tr>
</thead>
<tbody>
<tr>
<td>β-Amylolysis-limit (%)</td>
<td>13.3</td>
<td>24.2</td>
<td>33.9</td>
<td>44.0</td>
<td>70.0</td>
</tr>
</tbody>
</table>

The results were plotted on the graph, the reaction proceeds with time (Figure 3).

Attempts to follow the reaction by iodine staining in the case of β-dextrin and amylopectin were not successful. Hence a unit of activity could not be defined using the method described by Hobson, Whelan and Peat (67) for R-enzyme. Isoamylase activity has therefore been expressed in terms of the increase in β-amylolysis limit in the case of β-dextrin and amylopectin, and both increase in iodine staining power and β-amylolysis limit in the case of glycogens.

(b) Effect of enzyme concentration

(i) By iodine staining

Digests consisting of the following were prepared:

- glycogen solution (2 mg/ml) 4 ml
- isomylase (prep II) solution (1.2, 3, 4 & 5 mg/ml) 4 ml
- 0.2 M-acetate buffer pH 6.0 4 ml

The digests were incubated at room temperature and samples (2.5 ml) from each digest were stained with iodine solution (2.0 ml) after time intervals. The absorption values of the glycogen-iodine complexes were measured on a Unicam spectrophotometer against an iodine-water blank at 425 mp. The following results were obtained: (Figure 9)

<table>
<thead>
<tr>
<th>Enzyme concentration (mg/ml)</th>
<th>Increase in A.V. (%)</th>
<th>2 hr</th>
<th>6 hr</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td></td>
<td>2.5</td>
<td>5.0</td>
</tr>
<tr>
<td>2</td>
<td></td>
<td>3.0</td>
<td>13.5</td>
</tr>
<tr>
<td>3</td>
<td></td>
<td>12.5</td>
<td>22.5</td>
</tr>
<tr>
<td>4</td>
<td></td>
<td>13.0</td>
<td>31.5</td>
</tr>
<tr>
<td>5</td>
<td></td>
<td>23.0</td>
<td>40.0</td>
</tr>
</tbody>
</table>

A straight line passing through 0.5 mg was obtained (116).
(ii) **Hydrolysis (%)**

A series of digests consisting of the following were set up with 0, 10, 20, 30, 40 and 50 mg of isoamylase together with a constant excess amount of β-amylase.

- glycogen solution (5 mg/ml) 6 ml
- isoamylase (prep I) in 5 ml
- 0.2 M-acetate buffer pH 5.85 0-50 mg
- β-amylase (1,800 units) 5 ml
- distilled water 1 ml

After incubation at room temperature, samples were removed after 5 and 24 hr to determine the reducing sugars quantitatively. Since the enzyme preparation used is contaminated with maltase, percentage hydrolysis was obtained in terms of glucose. The following data were obtained:— (Figure).

<table>
<thead>
<tr>
<th>Enzyme concentration (mg)</th>
<th>Hydrolysis (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>5 hr</td>
</tr>
<tr>
<td>0</td>
<td>16.4 (34.0)</td>
</tr>
<tr>
<td>10</td>
<td>28.4</td>
</tr>
<tr>
<td>20</td>
<td>37.6</td>
</tr>
<tr>
<td>30</td>
<td>45.0</td>
</tr>
<tr>
<td>40</td>
<td>53.0</td>
</tr>
<tr>
<td>50</td>
<td>57.0</td>
</tr>
</tbody>
</table>

The above results indicate a proportionality between enzyme concentration and the activity as determined by quantitative A.V. and hydrolysis measurements.

(c) **Effect of heat on isoamylase**

4.0 ml aliquot portions of isoamylase solution (10 mg/ml; in acetate buffer pH 5.89) were heated at 35°, 40°, 45°, 50° and 55° for one hour and incubated with glycogen solutions as follows:-

- glycogen solution (5 mg/ml) 5 ml
- enzyme solution 4 ml
0.2 M-acetate buffer pH 5.69 3 ml
distilled water 3 ml

The digests were incubated at room temperature overnight and the enzyme inactivated by heating in a boiling water bath for 3 min. β-Amylase solution (2 ml; 1,400 units) together with water (1 ml) were added to the digests which were incubated at 35°C. β-Amylolysis limits were determined and the following data were obtained.

<table>
<thead>
<tr>
<th>Temperature °C</th>
<th>35</th>
<th>40</th>
<th>45</th>
<th>50</th>
<th>55</th>
</tr>
</thead>
<tbody>
<tr>
<td>Isoamylase activity (as increase in β-amylolysis limit)</td>
<td>15.0</td>
<td>10.3</td>
<td>4.5</td>
<td>4.5</td>
<td>1.5</td>
</tr>
</tbody>
</table>

Isoamylase activity is completely destroyed when heated to 55°C, and is partly inactivated even at 40°C.

(d) pH Optimum of isoamylase

(i) Acetate buffer. The isoamylase solution (2 ml; 60 mg) was incubated with glycogen solution (3 ml; 10 mg/ml) and 0.2 M-acetate buffer of pH values ranging from 5.0 - 6.8 (0.2 interval; 3 ml) together with distilled water (4 ml).

The reactions were followed by iodine staining and also by increase in β-amylolysis limit; in the case of the latter, a blank digest without isoamylase was also prepared. The digests were incubated at room temperature. After inactivation of isoamylase, β-amylase solution (1,400 units) was added and the β-amylolysis limit determined. The absorption values of polysaccharide-iodine complexes were measured on a Unicam spectrophotometer at 425 mp. The following results were obtained.
FIG. 10.- Effect of pH

Hydrolysis (%) vs. pH

Absorption value at 425 m\(\mu\)
Thus the pH optimum of the isocarminase is extremely marked at 6.0 (Figure 10) in acetate buffer.

(ii) HDH Universal buffer. Isoamylase activity tests were carried out in HDH universal buffer solution, the pH values of which ranged from 5.0 - 7.0. The reaction was followed by iodine staining by removing samples after 0, 4 and 24 hr. The following results were obtained.

<table>
<thead>
<tr>
<th>pH</th>
<th>3 hr</th>
<th>22 hr</th>
</tr>
</thead>
<tbody>
<tr>
<td>5.0</td>
<td>39.6</td>
<td>-</td>
</tr>
<tr>
<td>5.5</td>
<td>45.0</td>
<td>0.370 0.395</td>
</tr>
<tr>
<td>5.8</td>
<td>50.2</td>
<td>0.330 0.420</td>
</tr>
<tr>
<td>6.0</td>
<td>53.3</td>
<td>0.445 0.495</td>
</tr>
<tr>
<td>6.3</td>
<td>47.5</td>
<td>0.580 0.395</td>
</tr>
<tr>
<td>6.6</td>
<td>46.5</td>
<td>0.390 0.390</td>
</tr>
<tr>
<td>6.8</td>
<td>45.5</td>
<td>-</td>
</tr>
</tbody>
</table>

Thus the pH optimum of the isocarminase is extremely marked at 6.0 (Figure 10) in acetate buffer.

(ii) HDH Universal buffer. Isoamylase activity tests were carried out in HDH universal buffer solution, the pH values of which ranged from 5.0 - 7.0. The reaction was followed by iodine staining by removing samples after 0, 4 and 24 hr. The following results were obtained.

<table>
<thead>
<tr>
<th>pH</th>
<th>A.V.</th>
</tr>
</thead>
<tbody>
<tr>
<td>5.0</td>
<td>0.510 0.510 0.515</td>
</tr>
<tr>
<td>5.5</td>
<td>0.515 0.515 0.520</td>
</tr>
<tr>
<td>6.0</td>
<td>0.505 0.505 0.520</td>
</tr>
<tr>
<td>6.5</td>
<td>0.510 0.510 0.515</td>
</tr>
<tr>
<td>7.0</td>
<td>0.510 0.510 0.515</td>
</tr>
</tbody>
</table>

It is clear that some component of HDH universal buffer (phosphate, phenylacetate or borate) is an isocarminase inhibitor.

(e) Inhibition of isocarminase

(i) Effect of ammonium molybdate. The effect of various concentrations of ammonium molybdate, which inhibits the related plant enzyme (R-enzyme), on isocarminase was studied. Digests consisting of the following were set up,
with either 0.2% or 2% concentration of ammonium molybdate:

- glycogen (2 mg/ml) 3 ml
- isoenzyme solution (5 mg/ml) 3 ml
- 0.2 M-acetate buffer pH 5.89 2 ml
- ammonium molybdate (12.0%) 2 ml or 1.67 + 0.33 ml

A control digest without ammonium molybdate was also set up and the reactions were followed by iodine staining at 480 mp. The following data were obtained:

<table>
<thead>
<tr>
<th>Digest</th>
<th>A.V.</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0 hr</td>
</tr>
<tr>
<td>Control</td>
<td>0.115</td>
</tr>
<tr>
<td>Ammonium molybdate 0.2%</td>
<td>0.135</td>
</tr>
<tr>
<td>&quot;</td>
<td>2.0%</td>
</tr>
</tbody>
</table>

No appreciable increase in A.V. was observed, when the concentration of ammonium molybdate is 2.0%. Hence 2% ammonium molybdate inhibits isoenzyme.

(ii) Gluconic acid. \((\text{CH}_2\text{OH.CH}_2\text{OH.CH}_2\text{OH.CH}_2\text{OH.CO.O})\)

Conchie and Levy (117) have studied the inhibitory action of aldono-lactones on carbohydrases obtained from various sources. The effect of gluconic acid on isoenzyme has therefore been studied; two concentrations of gluconic acid (5 and 10 ml) being used. The following digest was prepared:

- glycogen (5 mg/ml) 5 ml
- 0.2 N-acetate buffer pH 6.0 5 ml
- gluconic acid solution (4.45%) 1 ml and 0.5 ml
- isoenzyme solution (50 mg in water) 2 ml

A control digest without gluconic acid was also set up and the volume of the digest was made up to 25 ml. The reaction was followed by iodine staining, when 2.5 ml samples were stained with 2.5 ml of iodine solution in
a total volume of 25 ml. The absorption values were read on a Unicam absorption
meter at 425 mp. The following results were obtained.

<table>
<thead>
<tr>
<th>Digest</th>
<th>Control</th>
<th>Gluconic acid (5 mM)</th>
<th>Gluconic acid (10 mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Increase in A.V. (%)</td>
<td>52.5</td>
<td>26.0</td>
<td>1.0</td>
</tr>
<tr>
<td>Inhibition (%)</td>
<td>0.0</td>
<td>55.5</td>
<td>99.5</td>
</tr>
</tbody>
</table>

10 mM Gluconic acid thus completely inhibits isocamylase whilst 5 mM shows 56% inhibition.

(iii) Inorganic salts. The effect of (a) mercuric chloride, (b) sodium
fluoride, (c) borate, (d) magnesium chloride and (e) sodium chloride in molar
concentrations of $1 \times 10^{-4}$ M, $1 \times 10^{-2}$ M, $1 \times 10^{-2}$ M, $2 \times 10^{-3}$ M and $2 \times 10^{-2}$ M respectively on isocamylase was examined, since the above salts have a marked
effect on other plant enzymes such as Q-enzyme, a-amylase and b-amylase.

Borate ($1 \times 10^{-2}$ M) was employed to investigate the possible inhibitory action
of BH universal buffer on isocamylase.

Digests consisting of the following were set up with various inorganic
salts:

- glycogen (25 mg)
- 0.2 M-acetate buffer pH 6.0
- isocamylase solution (50 mg)
- inhibitors (M concn. corr. to final conc.) 1 ml

A control digest without any inorganic salts was also made up and the
volume of the digests made up to 25 ml. After incubation at room temperature,
the reactions were followed by iodine staining when following data were ob-
tained.

<table>
<thead>
<tr>
<th>Digest</th>
<th>Control</th>
<th>Mercuric chloride (1x10^{-4} M)</th>
<th>Magnesium chloride (2x10^{-4} M)</th>
<th>Sodium fluoride (1x10^{-4} M)</th>
<th>Sodium chloride (2x10^{-4} M)</th>
<th>Borate (1x10^{-8} M)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Increase in A.V. (%)</td>
<td>55.0</td>
<td>1.0</td>
<td>19.8</td>
<td>32.5</td>
<td>16.7</td>
<td>0.5</td>
</tr>
</tbody>
</table>
Mercuric chloride \((1 \times 10^{-4} \text{ M})\) and borate \((1 \times 10^{-2} \text{ M})\) completely inhibit isoamylase, while sodium chloride, sodium fluoride and magnesium chloride (at \(2 \times 10^{-2} - 2 \times 10^{-3} \text{ M}\)) are partial inhibitors.

(f) Inhibition of isomaltase

The effect of gluconic acid on isomaltase activity was studied with reference to its inhibitory effect on isoamylase. The same concentrations (i.e. 5 and 10 mM) were employed.

Digests consisting of the following were made up:

- isomaltose \((7.7 \text{ mg})\) \(1 \text{ ml}\)
- 0.2 M-acetate buffer pH 6.0 \(5 \text{ ml}\)
- gluconic acid \((4.445%)\) 0.5 or 1.0 ml
- isoamylase solution \((25 \text{ mg})\) \(2.5 \text{ ml}\)

A control experiment without gluconic acid was also set up. The volume of the digests was made up to 25 ml. After incubation at room temperature, samples were removed at intervals to determine the reducing sugars cuprimetrically; the following data were obtained.

<table>
<thead>
<tr>
<th>Time (hr)</th>
<th>Total maltose equivalent (5 \text{ mM})</th>
<th>Total maltose equivalent (10 \text{ mM})</th>
<th>Total maltose equivalent Control</th>
<th>Increase in maltose equivalent (5 \text{ mM})</th>
<th>Increase in maltose equivalent (10 \text{ mM})</th>
<th>Increase in maltose equivalent Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>7.3</td>
<td>9.9</td>
<td>7.3</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td>24</td>
<td>8.3</td>
<td>9.9</td>
<td>8.5</td>
<td>1.0</td>
<td>0.0</td>
<td>1.2</td>
</tr>
<tr>
<td>48</td>
<td>9.2</td>
<td>9.9</td>
<td>9.2</td>
<td>1.9</td>
<td>0.0</td>
<td>1.9</td>
</tr>
<tr>
<td>72</td>
<td>9.1</td>
<td>9.9</td>
<td>9.2</td>
<td>1.8</td>
<td>0.0</td>
<td>1.9</td>
</tr>
</tbody>
</table>

Like isoamylase, the isomaltase activity is inhibited by 10 mM of gluconic acid, but is little altered in the presence of 5 mM inhibitor. Since isoamylase shows 56% inhibition under these conditions, the two activities are unlikely to be due to the same enzyme (see later).

III Specificity of Isoamylase

(a) Isolichenin and isoamylase

Isolichenin \((118)\), a linear polysaccharide consisting of 40 - 50 glucose
residues linked by α-1:4- and α-1:3-linkages (47.5 mg), and α-amylase (5 mg) and 0.1 M-sodium chloride (5 ml), was incubated in a total volume of 25 ml at 35°C for 24 hr. The reducing sugars so produced were then estimated cuprimetrically when 9.7% apparent conversion into glucose was observed.

The above residual solution (15 ml) was then acted upon by isomylase in the following manner, in a total volume of 20 ml:–

- isomylase solution (60 mg) 1 ml
- 0.2 M-acetate buffer pH 5.8 4 ml
- treated isolichenin solution 15 ml

The apparent percentage conversion into glucose was measured cuprimetrically (deproteinised samples) before and after incubation at room temperature for 24 hr. The following results were obtained:

<table>
<thead>
<tr>
<th>Time (hr)</th>
<th>Conversion into glucose (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>9.7</td>
</tr>
<tr>
<td>24</td>
<td>9.7</td>
</tr>
</tbody>
</table>

There was no increase in hydrolysis thereby indicating that isomylase does not split α-1:3-glucosidic linkages.

(b) Pustulan and isomylase

Pustulan, an insoluble linear glucan containing β-1:6-linkages, was partially hydrolysed (119) prior to its incubation with isomylase in the following manner:

- pustulan hydrolysate (40 mg) 5 ml
- 0.2 M-acetate buffer pH 5.89 3 ml
- isomylase solution (60 mg) 2 ml
- distilled water up to 25 ml

The reducing power of a sample of the digest was determined before and after incubation at room temperature for 24 hr. No increase in reducing power was observed, hence isomylase does not hydrolyse β-1:6-glucosidic
linkages as present in oligosaccharides derived from pustulan.

(c) 6-1:4-Glucosans and isomylase

Substrate (10 mg) was incubated with isomylase solution (20 mg in acetate buffer pH 6.0; 0.5 ml) for 24 hr and the enzymic digest then examined by paper chromatography. The following results were obtained.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Isomylase preparation</th>
<th>Reducing sugar present</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amylopectin β-dextrin</td>
<td>Acetate treated</td>
<td>maltose, maltotriose</td>
</tr>
<tr>
<td>Von Gierke glycogen</td>
<td>D₂*</td>
<td>glucose</td>
</tr>
<tr>
<td>Amylopectin β-dextrin</td>
<td>D₂*</td>
<td>glucose, maltose</td>
</tr>
<tr>
<td>α-Limit dextrin</td>
<td>D₂*</td>
<td>glucose, maltose,</td>
</tr>
<tr>
<td></td>
<td></td>
<td>maltotriose</td>
</tr>
</tbody>
</table>

* trace of maltase present

Thus, isomylase hydrolyses 6-1:6-glucosidic linkages.

IV Studies on the Mode of Action of Isomylase

(a) Simultaneous action of isomylase and β-amylase

Substrates: (1) Potato amylopectin
            (2) Waxy maize starch
            (3) Glycogen (cyster)
            (4) Synthetic glycogen (see Section V)
            (5) Maltose

Enzyme preparation: The isomylase preparation II (protein D₂₂) which is free from maltase was used.

Digests consisting of the following were made up:

- Polysaccharide solution (15-25 mg) 5 ml
- 0.2 M-acetate buffer pH 6.0 4 ml
- Isomylase solution (30 mg/ml) 2 ml
- β-amylase solution (10 mg) 2 ml

Digests, the volumes of which were made up to 25 ml, were incubated at room temperature for 24 hr. After deproteinisation, the percentage conversion into maltose was determined. The exact concentration of the substrates was
obtained by acid hydrolysis of a sample of each digest. The results are tabulated below:

<table>
<thead>
<tr>
<th>Digest</th>
<th>Substrate</th>
<th>Conversion into maltose (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Potato amylopectin</td>
<td>83.5</td>
</tr>
<tr>
<td>2</td>
<td>Waxy maize starch</td>
<td>82.5</td>
</tr>
<tr>
<td>3</td>
<td>Glycogen (oyster)</td>
<td>94.5</td>
</tr>
<tr>
<td>4</td>
<td>Glycogen (synthetic)</td>
<td>89.6</td>
</tr>
<tr>
<td>5</td>
<td>Maltose</td>
<td>0.0</td>
</tr>
</tbody>
</table>

(b) Action of isoamylase on some branched α-1:4-glucosans

The effect of isoamylase on the β-amylolysis limit of some branched α-1:4-glucosans was investigated as follows:

A polysaccharide, 0.2 M-acetate buffer pH 6.0, water and isoamylase solution were incubated at room temperature for 24 hr. The digests were then heated at 100° for 3 min. to deactivate the enzyme, and after cooling, β-amylase solution was added. After 24 hr incubation at 35°C, the percentage conversion into maltose was determined.

The detailed composition of the digests are tabulated below.

<table>
<thead>
<tr>
<th>Digest No</th>
<th>Substrate</th>
<th>Weight (mg)</th>
<th>Buffer (ml)</th>
<th>Water (ml)</th>
<th>Isoamylase solution mg; ml</th>
<th>Total volume (ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Maltodextrin</td>
<td>15.0</td>
<td>1.5</td>
<td>2.5</td>
<td>30; 1</td>
<td>10</td>
</tr>
<tr>
<td>2</td>
<td><em>Dunaliella bioculata</em> amylopectin</td>
<td>30.1</td>
<td>10.0</td>
<td></td>
<td>50; 5</td>
<td>18</td>
</tr>
<tr>
<td>3</td>
<td>Rabbit liver glycogen IV</td>
<td>22.7</td>
<td>4.0</td>
<td>5.0</td>
<td>40; 2</td>
<td>14</td>
</tr>
<tr>
<td>4</td>
<td>Alkali-treated rabbit liver glycogen IV</td>
<td>21.6</td>
<td>4.0</td>
<td>5.0</td>
<td>40; 2</td>
<td>14</td>
</tr>
<tr>
<td>5</td>
<td>β-Dextrin of waxy maize starch</td>
<td>40.0</td>
<td>5.0</td>
<td>12</td>
<td>80; 8</td>
<td>28</td>
</tr>
<tr>
<td>6</td>
<td>Potato amylopectin</td>
<td>24.5</td>
<td>4.0</td>
<td>14</td>
<td>50; 2</td>
<td>25</td>
</tr>
</tbody>
</table>

The following results were obtained:
<table>
<thead>
<tr>
<th>Digest</th>
<th>Substrate</th>
<th>β-amylolysis limit%</th>
<th>β-Amylolysis limit after treatment with isoamylase (%)</th>
<th>Increase in β-amylolysis limit%</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Maltodextrin</td>
<td>67</td>
<td>86</td>
<td>19</td>
</tr>
<tr>
<td>2</td>
<td>Dunaliella bioculata amylopectin</td>
<td>61</td>
<td>73</td>
<td>15</td>
</tr>
<tr>
<td>3</td>
<td>Rabbit liver glycogen IV</td>
<td>46</td>
<td>73</td>
<td>22</td>
</tr>
<tr>
<td>4</td>
<td>Rabbit liver glycogen alkali treated</td>
<td>45</td>
<td>75</td>
<td>29</td>
</tr>
<tr>
<td>5</td>
<td>β-Dextrin of waxy maize starch</td>
<td>0</td>
<td>71</td>
<td>71</td>
</tr>
<tr>
<td>6</td>
<td>Potato amylopectin</td>
<td>54</td>
<td>80</td>
<td>26</td>
</tr>
</tbody>
</table>

* Determined in a control experiment, without isoamylase.

(c) Characterisation of isoamylase treated polysaccharides

Glycogen and potato amylopectin were incubated with isoamylase under standard conditions, and the residual dextrins thus produced (for convenience termed iso-dextrins) have been characterised.

Large scale preparation of iso-dextrins of glycogen and amylopectin

Glycogen and potato amylopectin (1 g, each) were incubated separately with isoamylase solution (30 ml; 100 mg) and 10 ml of 0.2 M-acetate buffer pH 5.89 at 21°C for 48 hr. The digestes were heated in a boiling water bath for 15 min. to inactivate isoamylase. After filtration through a 8.5 x 3 sintered glass funnel to remove coagulated protein, the resultant solutions were dialysed against distilled water for 48 hr. The undialysed material (iso-dextrin) of glycogen and amylopectin was isolated by freeze drying.

The yields obtained were glycogen iso-dextrin, 850 mg and amylopectin iso-dextrin, 600 mg.

Comparison of the original glycogen and its iso-dextrin

(i) Action of β-amylase
Digests consisting of the following were made up:

- glycogen or iso-dextrin solution (40 mg) 10 ml
- 0.2 M-acetate buffer pH 5.89 10 ml
- β-amylase (20 mg) 5 ml

The digestes were incubated at 35°C and reducing sugars were determined cuprimetrically after 4 and 24 hr. The following results were obtained.

<table>
<thead>
<tr>
<th>Digest</th>
<th>β-Amylolyis limit (%)</th>
<th>4 hr</th>
<th>24 hr</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glycogen</td>
<td></td>
<td>37.1</td>
<td>40.2</td>
</tr>
<tr>
<td>Iso-dextrin</td>
<td></td>
<td>42.5</td>
<td>49.0</td>
</tr>
</tbody>
</table>

The results indicate an increase in β-amylolysis limit of iso-dextrin.

(ii) Action of isoamylase

The following digest was prepared:

- glycogen or iso-dextrin (25-26 mg) 5 ml
- isoamylase solution (50 mg 5 ml
- 0.2 M-acetate buffer pH 5.89 4 ml
- distilled water 1 ml

The digestes were incubated at room temperature overnight and the enzyme inactivated by heating in a boiling water bath for 5 min. β-Amylase solution (2 ml; 7 mg/ml) and water (3 ml) were added. After incubation at 35°C for 24 hr., the reducing sugars were estimated cuprimetrically. The following results were obtained.

<table>
<thead>
<tr>
<th>Digest</th>
<th>β-Amylolyis limit (%)</th>
<th>Increase in β-amylolysis limit (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glycogen</td>
<td>86.2</td>
<td>46.0</td>
</tr>
<tr>
<td>Iso-dextrin</td>
<td>99.0</td>
<td>50.0</td>
</tr>
</tbody>
</table>

(iii) Absorption spectra of the iodine complex

2.5 Mg of polysaccharide were stained with 2.5 ml of iodine solution.
FIG. 11. - The Absorption Spectrum of the Iodine Complex of (A) Glycogen
(B) Glycogen Iso-dextrin
(C) Potato Amylopectin
(D) Amylopectin Iso-dextrin
The absorption values of the polysaccharide-iodine complexes were measured against an iodine-water blank in a Unicam absorption meter over the range of 400 - 650 μ (Figure II).

Found: λ max (iso-dextrin), 435-440 μ; and λ max (original glycogen), 420 μ.

(iv) Potassium periodate oxidation

Potassium periodate oxidation of the original glycogen and iso-dextrin was carried out according to the method described in Section V (p. 98). The weights of the polysaccharides were iso-dextrin, 147.4 mg, and original glycogen, 255.0 mg. A constant amount of formic acid was obtained after 14 days oxidation in each case. The following results were obtained.

<table>
<thead>
<tr>
<th></th>
<th>Glycogen</th>
<th>Iso-dextrin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Formic acid production (mg)</td>
<td>2.80</td>
<td>2.33</td>
</tr>
<tr>
<td>Chain length (glucose residues)</td>
<td>10.0</td>
<td>12.0</td>
</tr>
</tbody>
</table>

Comparison of chemical analysis of original amylopectin and its iso-dextrin

(i) Action of β-amylase and isoamylase

Digests consisting of the following were made up:-

- potato amylopectin or iso-dextrin (25-26 mg) 5 ml
- isoamylase solution (50 mg) 5 ml
- 0.2 M-acetate buffer 4 ml
- distilled water 1 ml

Similar digests without isoamylase were also set up for β-amylase action. The digests after incubation at room temperature, overnight, were heated to inactivate the enzyme. β-Amylase solution (7 mg/ml; 2 ml) and water (5 ml) were added to all the digests. After incubation at 35°C for 24 hr, the reducing sugars were estimated cuprimetrically. The following results were obtained.
THE MODIFIED URBULOIDE VISCOSIMETER

FIG. 12.
Digest  \( \beta \)-Amylolyis-limit (%)  Increase in \( \beta \)-amyloolysis limit (%)
\( \text{b) (a)} \)

<table>
<thead>
<tr>
<th></th>
<th>( \text{b) (a)} )</th>
</tr>
</thead>
<tbody>
<tr>
<td>potato amylopectin</td>
<td>55.0 80.9</td>
</tr>
<tr>
<td>iso-dextrin</td>
<td>44.5 80.3</td>
</tr>
</tbody>
</table>

Isoamylase clearly has further action on the \( \alpha \)-1:6-linkages of iso-dextrin of amylopectin.

(ii) Absorption spectra of iodine complexes

Iso-dextrin (2.5 mg) was treated with iodine solution (2.5 ml) and the absorption values of the polysaccharide-iodine complex were measured in a Unicam absorptiometer over the range of 395-620 nm (Figure 11).

(iii) Potassium periodate oxidation

The original amylopectin (226.6 mg) and iso-dextrin (163.5 mg) were oxidised with potassium periodate in a similar manner, described in Section V (p. 95). The formic acid release in each case which remained constant after 14 days, was estimated by titration against a standard alkali. The following results were obtained:

<table>
<thead>
<tr>
<th></th>
<th>Original amylopectin</th>
<th>Iso-dextrin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Formic acid release (mg)</td>
<td>1.38</td>
<td>2.34</td>
</tr>
<tr>
<td>Chain length (glucose residues)</td>
<td>20.6</td>
<td>10.0</td>
</tr>
</tbody>
</table>

(iv) Limiting viscosity number

The original amylopectin and iso-dextrin (60-70 mg) were each dissolved in about 18 ml of M-potassium hydroxide solution. The above solutions and M-potassium hydroxide were filtered through a G.3 x 4 sintered funnel to obtain the solutions completely free of any aggregates or dust particles. A modified Ubbelhode viscometer (Figure 12) which was thoroughly washed with chromic acid and distilled water and dried with 'Analar' acetone, was clamped firmly in a vertical position on a brass stand and placed in a bath thermo-
FIG. 13. - THE RELATIONSHIP BETWEEN THE SPECIFIC VISCOSITY AND THE CONCENTRATION OF

(A) POTATO AMYLOPECTIN

(B) ISO-DEXTREN

\[ C \times 10^3 \text{ (g/ml)} \]
statically controlled at 25°C ± 0.01°C. The above solutions (15 ml) in turn were introduced into the viscometer by a pipette down tube B. After allowing the solution to equilibrate to the bath temperature (20 min), tube B was closed with a 'Quick fit' glass stopper and pressure was applied at the top of C, thus transferring a quantity of liquid to the bulbs above capillary tube A. After releasing the pressure, the stopper was removed and the time flow for the liquid level to pass two marks (a to b) was measured. A stop watch reading to 0.02 sec, was used and the flow time of the solution taken as the mean of two such observations. Successive dilutions (4-5; 5 ml each) were made by pipette down tube B and the solution mixed by blowing gently down tube B a number of times. The flow time for the pure solvent was also determined. The concentration of the polysaccharide solutions was determined by acid hydrolysis of the diluted solution. The following results were obtained (Figure 13).

**Original amylpectin**

<table>
<thead>
<tr>
<th>C</th>
<th>T</th>
<th>T₀</th>
<th>T-TO/T₀</th>
<th>η sp x ¹/C</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.00221</td>
<td>1174.60</td>
<td>680.7</td>
<td>0.725</td>
<td>328</td>
</tr>
<tr>
<td>0.00166</td>
<td>114.25</td>
<td>680.7</td>
<td>0.489</td>
<td>295</td>
</tr>
<tr>
<td>0.00153</td>
<td>933.80</td>
<td>680.7</td>
<td>0.371</td>
<td>279</td>
</tr>
<tr>
<td>0.00110</td>
<td>830.00</td>
<td>680.7</td>
<td>0.293</td>
<td>254</td>
</tr>
</tbody>
</table>

**Iso-dextrin**

<table>
<thead>
<tr>
<th>C</th>
<th>T</th>
<th>T₀</th>
<th>T-TO/T₀</th>
<th>η sp x ¹/C</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.00226</td>
<td>761.70</td>
<td>680.7</td>
<td>0.1190</td>
<td>52.7</td>
</tr>
<tr>
<td>0.00170</td>
<td>733.20</td>
<td>680.7</td>
<td>0.0845</td>
<td>49.7</td>
</tr>
<tr>
<td>0.00133</td>
<td>725.10</td>
<td>680.7</td>
<td>0.0653</td>
<td>46.0</td>
</tr>
</tbody>
</table>

C is concentration of polysaccharide (g/ml).
T is flow time of solution (secs).
T₀ is flow time of solvent (secs).
The specific viscosity, η sp., is given by T-TO/T₀.
\[ \eta_{sp/C} \] is known as the Viscosity Number and by extrapolation of the graph \[ \eta_{sp/C} \] against \( C \), to zero concentration, the limiting viscosity number \( [\eta] \) is obtained.

From the graph (Figure 13),

\[
[\eta] = 205 \text{ for original amylpectin} \\
[\eta] = 41 \text{ for iso-dextrin.}
\]

This suggests that isoamylase action on parent polysaccharide causes an 80% decrease in the limiting viscosity number.

**DISCUSSION**

**Preparation and Properties of Isoamylase**

The earlier investigations on the mode of action of isoamylase carried out by Manners and Maung were made on a protein preparation obtained by acetone fractionation of brewer's yeast extracts 0 to \(-10^\circ\)C. This preparation, with a slight modification in the procedure prior to its acetone fractionation, has been used for a part of the present investigations on the properties and action pattern of isoamylase. On analysis, as reported in Section III, this preparation was found to be contaminated with a trace of maltase and yeast branching enzyme. The presence of this maltase, however, should not theoretically interfere with degradation studies on polysaccharides, and \( \beta \)-amylase would be added only after the inactivation of the isoamylase preparation. But, in special cases, for example, the simultaneous degradation of branched polysaccharides by isoamylase and \( \beta \)-amylase, the effect of maltase present cannot be eliminated. In view of this fact, attempts to inactivate maltase in the isoamylase preparation have been made. One such attempt to inactivate maltase by acetate treatment of the protein preparation at pH 4.0 - 4.5 was carried out. It was observed from the experimental data that maltase was
inactivated after 10 hr incubation in acetate buffer pH 4.45, although at the same time part of the isoamylase had also been denatured. However, it served as a satisfactory method for inactivating maltase, and thus helped in further investigations.

Many preparations of isoamylase were necessary for the entire work and it was observed in one such preparation that the repeated fractionation of the above protein fraction helped in the inactivation of maltase, which could not be detected either by paper chromatography or by quantitative cuprimetric estimations. However, this preparation was found to be contaminated with yeast branching enzyme and isomaltase since the enzyme preparation acted on amylose causing a decrease in iodine staining power and also hydrolysed the terminal α-1:6-linkage of isomaltose producing glucose. It had no action on maltotriose. Therefore, regarding the method of preparation of isoamylase, the repeated fractionation of the protein obtained between 20-30% acetone concentration served as a satisfactory preliminary method of preparation. The contaminating yeast branching enzyme could then be eliminated from the preparation by starch adsorption (this method is satisfactory in the case of the related plant β-enzyme (67)) or by electrophoretic separation.

In all cases, the preparations were found to be rich in debranching activity. This was observed by incubating the isoamylase preparation with glycogen, amylpectin and β-dextrin. These polysaccharides, after incubation with isoamylase were further degraded by β-amylase as an increase in β-amylolysis limit was always observed. The increase in iodine staining power, however, was achieved only in one case (glycogen). These observations served as standard methods for the estimation of enzyme activity in the investigation of the properties and mode of action of isoamylase.

Isomylase activity was found to progress with time and also to be
proportional to enzyme concentration in the normal way. However, attempts to define a unit of activity by iodine staining method to that used for R-enzyme activity determinations was not possible, since the iodine staining method was not applicable to amylpectin and its β-dextrin. This can probably be explained by interference due to contaminating yeast branching enzyme which is capable of acting on both substrates (see Section V). In fact, with amylpectin or β-dextrin, a slight decrease in iodine staining power could be observed after 4 hr incubation during the investigations (unreported). As yeast branching enzyme has no action on glycogen (Section V), the iodine staining method could be used with this substrate although glycogen is not an ideal substrate on account of its relatively poor iodine staining properties.

Activity measurements for amylpectin and β-dextrin were obtained by measurement of the increase in β-amylolysis limit after 24 hr incubation of polysaccharides with isoaamylase. This increase was not due to degradation by α- or β-amylases, as its action on glycogen caused an increase rather than a decrease in iodine staining power.

Isoamylase action was found to be optimum at pH 6.0 in acetate buffer. This was the most satisfactory buffer media as BDH universal buffer had an inhibitory effect on the enzyme. Isoamylase is very sensitive to temperature; one-third of the activity is lost at 40° and two-thirds at 45°. Above 55° only a trace of activity remains.

No specific activators for isoaamylase could be detected. The enzyme is partly inhibited by sodium fluoride (1 x 10⁻² M), sodium chloride (1 x 10⁻² M) and magnesium chloride (2 x 10⁻² M) and is completely inhibited by mercuric chloride (1 x 10⁻⁴ M) and borate (1 x 10⁻³ M). These observations distinguish isoaamylase from certain α-amylases which are activated by chloride ions, and
are not completely inhibited by borate. Ammonium molybdate which inhibits R-enzyme (16) is a strong inhibitor at 2% concentration and has only a slight effect at 0.2%.

The effect of gluconic acid on the enzyme preparation suggests that isocamylase and isomaltase activities are due to different enzymes. At 5 mL concentration, 56% of the isocamylase activity was lost while little alteration in isomaltase activity was observed. This conclusion on dual enzymes is supported by the evidence obtained in Section III.

Specificity and Mode of Action of Isocamylase

The experimental evidence showed that the hydrolytic activity of isocamylase is directed only towards the hydrolysis of α-1:6-glucosidic linkages of branched α-1:4-glucosans. It was found to be incapable of hydrolysing β-1:6 or α-1:3-glucosidic linkages as on incubation with oligosaccharides prepared from pustulan and isochelin, no increase in reducing power of the resultant solution was observed. The increase in β-amylolysis limit of amyllopectin, glycogen and β-dextrin clearly showed that this can only be due to the scission of the outermost branch links (α-1:6-glucosidic) in these polysaccharides. The enzyme does not hydrolyse α-1:4-glucosidic linkages.

Isocamylase action (cf. R-enzyme) on glycogens, amyllopectins and β-dextrin was incomplete, since the β-amylolysis limit of the isocamylase treated polysaccharide was not 100%; this was not due to denaturation of the enzyme in the digest (120). It may be possible that isocamylase action was confined to the hydrolysis of the 1:6-linkages of A-chains. In the case of potato amyllopectin, the incomplete action may also be due to the presence of ester phosphate groupings. In view of this fact, β-dextrin of waxy maize starch and glycogen which contain none or very little phosphate groupings might be expected to be completely hydrolysed. However, this was not so; hence, some
α-1:6-linkages in these polysaccharides must be immune to isoamylase action.

The increase in β-amylolysis limit of amylopectin (36%) was found to be lower than the increase in the β-amylolysis limit of β-dextrin of waxy maize starch (71% on 47% of the original polysaccharide). This indicates that isoamylase had more action on β-dextrin (shorter outer chains) and that the long outer chains of amylopectin constitute a hindrance to enzyme action. A similar situation exists with regard to R-enzyme as the β-amylolysis limits of β-dextrin and amylopectin after treatment with R-enzyme are 73 and 74% respectively (67).

The simultaneous action of isoamylase and β-amylase on glycogen and amylopectin showed slightly higher values for the increase in β-amylolysis limit than the successive action of both. It may be possible that in the latter case the linear chains obtained by hydrolysis of some outer α-1:6-linkages and newly exposed chains in the interior may as already suggested have a hindering effect. During the simultaneous action of the two enzymes, these chains will be degraded to maltose and hence the inner α-1:6-linkages will become more accessible to isoamylase action. This could result in further functioning of the enzyme.

The observation that isoamylase hydrolyses some of the α-1:6-linkages in both amylopectin and glycogen was confirmed by the chemical and enzymic analysis of isoamylase treated dextrins, conveniently termed as iso-dextrins. These have been summarised in Table III below.
### TABLE III

A comparison of the properties of isoamylase treated amylopectin-glycogen type polysaccharides

<table>
<thead>
<tr>
<th>Property</th>
<th>Isodextrin of amylopectin</th>
<th>Amylopectin</th>
<th>Isodextrin of glycogen</th>
<th>Glycogen</th>
</tr>
</thead>
<tbody>
<tr>
<td>β-Amylolysis limit (%)</td>
<td>44.5</td>
<td>55.0</td>
<td>49.0</td>
<td>40.2</td>
</tr>
<tr>
<td>(a) before isoamylase</td>
<td>79.8</td>
<td>80.9</td>
<td>99.0</td>
<td>86.2</td>
</tr>
<tr>
<td>Limiting viscosity number</td>
<td>41</td>
<td>205</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>in (N-KOH)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Chain length (glucose residues)</td>
<td>10.0</td>
<td>20.0</td>
<td>12.0</td>
<td>10.0</td>
</tr>
<tr>
<td>Iodine complex</td>
<td>550</td>
<td>540</td>
<td>440-445</td>
<td>420</td>
</tr>
<tr>
<td>λ max μm</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

These observations also indicated that isoamylase did not function by a reverse of yeast branching enzyme action, i.e., did not catalyse the scission of 1:6-linkages and re-synthesis of chain-forming 1:4-links. The large decrease in viscosity of amylopectin (from 305-41) shows that extensive degradation had occurred. This also shows that amyllose synthesis is not taking place. B-enzyme action on amylopectin is also characterised by a large (approx. 50%) decrease in specific viscosity. Since the viscosity of glycogen is so low, the viscosity of glycogen iso-dextrin could not be measured.

A shift in the wavelength of maximum absorption of the polysaccharide-iodine complex from 430 μm to 435-440 μm was observed in the cases of glycogen and its iso-dextrin respectively. There is evidence that the iodine staining power of glycogen is dependent largely on the B-chains (unpublished work). The hydrolysis of some of the A-chains would increase the length of unbranched B-chains available for combination with iodine. A change in iodine
staining power is therefore to be expected. The A-chains in glycogen are less than 18 glucose residues which is now regarded as the minimum chain length to effect light absorption of the iodine complex (Whelan and Bailey, unpublished work). In the case of amyllopectin, some of the linear A-chains might affect the absorption-spectra of the iodine complex and the change in spectra in the region (395 nm) is in accordance with this suggestion.

The periodate oxidation results show that the glycogen iso-dextrin has a significantly longer average chain length (12) than the original glycogen (10). If isoamylase selectively removes A-chains, the proportion of non-reducing end-groups in the residual iso-dextrin will decrease, i.e. the apparent chain length will increase. This follows from the following argument: in a glycogen containing equal numbers of A and B chains, and with a β-amyolysis limit of 50% and a chain length of 12, the average length of the A-chains is \(\frac{12 \times 50}{100} + 2.5 = 8.5\) and that of the B-chains is \((2 \times 12) - 3.5 = 15.5\).

In the case of amyllopectin, the average chain length of the iso-dextrin is smaller than the original polysaccharide. This may be due to the fact that the released A-chains (ca. 15 glucose residues compared to 8-9 for glycogen) have not been completely removed by dialysis. Hence, in view of this fact, periodate oxidation of each A-chain will give three moles of formic acid, including two per free reducing group. The combined production of formic acid from the reducing A-chains may therefore exceed that from the non-reducing end groups in the iso-dextrin.

In conclusion, yeast isoamylase differs from the plant R-enzyme in being able to attack both glycogen and amyllopectin, and also apparently differs from the animal amyl-1:6-glucosidase, since this has no action on the intact polysaccharides. Isomaltose is not a substrate for purified isoamylase and R-enzyme.
SECTION V
PROPERTIES OF YEAST BRANCHING ENZYME

INTRODUCTION

The existence of yeast branching enzyme (YBE) was first reported by Manners and Khin Maung (46). They observed that certain protein fractions, isolated during the purification of yeast phosphorylase, acted on amylase producing a marked decrease in its iodine staining power and β-amylolysis-limit. The enzyme preparations were obtained by ethanol-citrate fractionation of brewer's yeast extracts at -5°C. The YBE preparation was contaminated with traces of maltase, isoamylase and maltotriase; however, it was free from α-amylase, β-amylase and glucamylase (46). Like the plant Q-enzymes (121), the activity of YBE was optimum at pH 7.0 and 20°C and was completely inhibited by mercuric chloride (5 x 10^{-4} M). It was not activated by maltosaccharides and hence differed from the Q-enzyme of Polytomella Coccia (42).

The presence of an enzyme in yeast culture exhibiting the properties of a branching enzyme, i.e. decrease in iodine staining power and β-amylolysis limit has also been reported by Hopkins (115).

Previous workers have reported the synthesis by plant enzymes of branched polysaccharides from amylase, a linear molecule consisting of several thousand glucose residues united by α-1,4-linkages. However, Cori and Cori (36) have reported that heart and liver preparations acting alone do not convert amylase into glycogen, although the combined action of muscle phosphorylase and a liver
preparation on glucose-1-phosphate produces a glycogen-type polysaccharide.

The aim of the present work was therefore to examine the action of YBE on amylase and also, since amylopectin contains fewer branch points than glycogen, to attempt a synthesis of a highly branched polysaccharide similar in structure to that of glycogen from amylopectin. The action of YBE on various other polysaccharides has also been investigated.

EXPERIMENTAL

I 1. Preparation of YBE

Dried brewer's yeast (100 gm) was extracted with 0.1 M sodium bicarbonate solution (500 ml) at 35°C for 2 hr. The mixture was centrifuged at 0°C (5,000 r.p.m. for 20 min) and the supernatant solution (340 ml) collected. Cold saturated ammonium sulphate solution was added to the above solution (at 0°C) to 0.5 saturation. The precipitate was collected on the centrifuge (0°C) and dissolved in 0.1 M-citrate buffer pH 6.8 (100 ml). To this was then added 50% (V/V) ethanol-citrate solution containing 0.01 M-citrate buffer pH 6.8 at -4°C to 25% alcohol concentration. The precipitate was removed on the centrifuge and discarded; the alcohol concentration was then increased to 50%. Once again, the precipitate was collected and triturated with 0.1 M-citrate buffer pH 6.8 (40 ml) for one hr. Insoluble material was removed by centrifugation and the supernatant solution treated with saturated ammonium sulphate solution (0°C) to 0.5 saturation. The precipitate thus obtained was collected on the centrifuge, dissolved in 0.1 M-citrate buffer pH 6.8 (25 ml) and freeze-dried. The yield obtained was 2.0 gm. The nitrogen content of the protein thus obtained was determined by the biuret method, and found to be 1.6%.
2. XBE activity determination

Substrates: (a) amylose
(b) starch
(c) amylopectin

(a) Amylose  The following digest was set up:
- potato amylose (1.48 mg/ml) 2 ml
- 0.2 M-citrate buffer pH 6.8 2 ml
- XBE solution (25 mg in water) 1 ml
- water 1 ml

The digest was incubated at room temperature and the reaction followed by determination of the absorption value (A.V.) of the polysaccharide-iodine complex, obtained by staining samples (1 ml) with iodine solution (1 ml; 0.2% iodine in 2% potassium iodide), using a Unicam SP.500 spectrophotometer. The following results were obtained:

<table>
<thead>
<tr>
<th>Time (hr)</th>
<th>0.0</th>
<th>0.5</th>
<th>2.0</th>
<th>21.0</th>
</tr>
</thead>
<tbody>
<tr>
<td>A.V.</td>
<td>0.535</td>
<td>0.495</td>
<td>0.380</td>
<td>0.140</td>
</tr>
<tr>
<td>Fall in A.V. (%)</td>
<td>0.0</td>
<td>7.5</td>
<td>23.9</td>
<td>73.7</td>
</tr>
</tbody>
</table>

(b) Starch  The following digest was prepared:
- 'Analar' soluble starch (2%) 0.5 ml
- 0.2 M-citrate buffer pH 6.8 2.0 ml
- XBE (25 mg in water) 2.5 ml

The reaction was followed by iodine staining, when the following results were obtained:

<table>
<thead>
<tr>
<th>Time (hr)</th>
<th>0.0</th>
<th>0.5</th>
<th>2.0</th>
<th>21.0</th>
</tr>
</thead>
<tbody>
<tr>
<td>A.V.</td>
<td>0.535</td>
<td>0.555</td>
<td>0.450</td>
<td>0.220</td>
</tr>
<tr>
<td>Fall in A.V. (%)</td>
<td>-</td>
<td>5.0</td>
<td>23.1</td>
<td>62.2</td>
</tr>
</tbody>
</table>

(c) Amylopectin  The following digest was set up:
- potato amylopectin (2 mg/ml) 1.5 ml
- 0.2 M-citrate buffer pH 6.8 1.0 ml
- XBE solution (25 mg in water) 2.5 ml
The reaction was followed as above. The following results were obtained:

<table>
<thead>
<tr>
<th>Time (hr)</th>
<th>0.0</th>
<th>1.0</th>
<th>2.5</th>
</tr>
</thead>
<tbody>
<tr>
<td>A.V.</td>
<td>0.53</td>
<td>0.23</td>
<td>0.19</td>
</tr>
<tr>
<td>Fall in A.V. (%)</td>
<td>-</td>
<td>39.5</td>
<td>50.0</td>
</tr>
</tbody>
</table>

3. Test for contaminating enzymes

(i) \(\alpha\)-Amylase

The test for \(\alpha\)-amylase was carried out as described by Hobson, Whelan and Peat (26). The following digest was set up:

\[ \beta\text{-dextrin of yeast glycogen (5 mg/ml)} \oplus \]
\[ 0.2 \text{ M-citrate buffer pH 6.8} \oplus \]
\[ \text{XBE solution (60 mg in water)} \oplus \]

The digest was incubated at room temperature. Samples (1 ml) were removed at intervals and were stained with iodine solution (0.5 ml). The absorption values were measured on a Spekker photoelectric absorption meter in 4 cm cells at 480 m\(\lambda\) against an iodine-water blank. The following results were obtained:

<table>
<thead>
<tr>
<th>Time (hr)</th>
<th>0.0</th>
<th>0.5</th>
<th>1.0</th>
<th>19.0</th>
<th>30.0</th>
</tr>
</thead>
<tbody>
<tr>
<td>A.V.</td>
<td>0.035</td>
<td>0.035</td>
<td>0.035</td>
<td>0.035</td>
<td>0.035</td>
</tr>
</tbody>
</table>

Since the iodine staining power of glycogen \(\beta\)-dextrin remained constant, \(\alpha\)-amylase (and also isoamylase) is absent from the branching enzyme preparation.

(ii) Isoamylase

The following digest was prepared:

\[ \text{glycogen (2 mg/ml)} \oplus \]
\[ 0.2 \text{ M-citrate buffer pH 6.8} \oplus \]
\[ \text{XBE solution (25 mg in water)} \oplus \]
\[ \text{water} \oplus \]

The reaction was followed by iodine staining, when the following
FIG. 14. - Effect of enzyme concentration.

Fall in absorption value (%)

Enzyme concentration (mg)

Time (min.)
results were obtained:

<table>
<thead>
<tr>
<th>Time (hr)</th>
<th>0.0</th>
<th>2.0</th>
<th>4.0</th>
<th>6.0</th>
<th>24.0</th>
</tr>
</thead>
<tbody>
<tr>
<td>A.V. (at 425 mp)</td>
<td>0.049</td>
<td>0.062</td>
<td>0.062</td>
<td>0.062</td>
<td>0.062</td>
</tr>
</tbody>
</table>

The above results indicate that the enzyme preparation may be contaminated with a trace of isomaltase, the presence of which does not interfere with the branching activity determination. This fact was further confirmed by setting up a digest at the same time with amylose, when the fall in A.V. was from 0.0 to 72.5%.

4. Effect of enzyme concentration

YBE activity was determined by the method of Gilbert and Patrick (40). The following digest containing varying concentrations of YBE was set up:

- 'Analar' soluble starch (1%) 1.0 ml
- 0.2 M-citrate buffer pH 6.8 1.0 ml
- YBE solution and water (10 mg/ml) 3.0 ml

The digests were incubated at 20°C. After 20 and 50 min., the following results were obtained, where D is the A.V. at time t (min).

<table>
<thead>
<tr>
<th>Concentration of branching enzyme (mg)</th>
<th>20 min</th>
<th>50 min</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Fall in A.V. (%)</td>
<td>log(D-Da)</td>
</tr>
<tr>
<td>15</td>
<td>3.25</td>
<td>1.5556</td>
</tr>
<tr>
<td>20</td>
<td>12.50</td>
<td>1.5416</td>
</tr>
<tr>
<td>25</td>
<td>13.70</td>
<td>1.5159</td>
</tr>
<tr>
<td>50</td>
<td>20.60</td>
<td>1.4886</td>
</tr>
</tbody>
</table>

The above results indicate proportionality between enzyme concentration and the activity as determined by quantitative A.V. measurements.

5. Inhibition of YBE

Ammonium molybdate. The following digest was set up with either 0, 1 or 2% ammonium molybdate:
potato amylose (1.43 mg/ml) 3.0 ml
0.2 M-citrate buffer pH 6.8 1.0 ml
ammonium molybdate solution (12%) 0.5 or 1.0 ml
YBE solution (25 mg in water) 1.0 ml

A control digest without ammonium molybdate was also examined and the absorption values of the amylose-iodine complex were measured in a Unicam SP,500 spectrophotometer at 630 mp. The following results were obtained:

<table>
<thead>
<tr>
<th>Digest</th>
<th>Fall in A.V. (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>26.5 40.8 53.0</td>
</tr>
<tr>
<td>Ammonium molybdate 1%</td>
<td>21.0 31.6 50.0</td>
</tr>
<tr>
<td>2%</td>
<td>15.9 22.4 29.0</td>
</tr>
</tbody>
</table>

The above results infer that 1% ammonium molybdate has very little effect on YBE, while 2% ammonium molybdate shows 50% inhibition.

II Action of YBE on Amylose

Prior to the large scale preparation of synthetic amylpectin, a preliminary study of the action of β-amylase on it was carried out. The following digest was set up:

<p>| | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>potato amylpectin</td>
<td>(2.2 mg/ml) 16 ml</td>
</tr>
<tr>
<td>0.2 M-citrate buffer pH 6.8</td>
<td>5 ml</td>
</tr>
<tr>
<td>YBE solution (70 mg in water)</td>
<td>4 ml</td>
</tr>
</tbody>
</table>

The digest was incubated at room temperature. Samples (5 ml) were withdrawn and the YBE inactivated by heating in a boiling water bath for 5 min., after which the solutions were cooled to room temperature. β-Amylase solution (5 ml; 1,200 units) was added to each digest. After incubation at 35°C for 24 hr., the reducing sugars were estimated. The following results were obtained:
Thus, 40.1% decrease in β-amylolysis limit was obtained. This confirms the action of branching enzyme on amylose. A large scale digest was then carried out for the rigid characterisation of the synthetic amylpectin.

**Preparation of synthetic amylpectin**

Butanol-potato amylose complex which was concentrated by centrifugation (ca. 500-600 mg) was dissolved by heating to 60°C in 250 ml of water. A digest was prepared with this amylose solution, 40 ml of 0.2 M-citrate buffer pH 6.8 and 900 mg of branching enzyme in 10 ml of water, and incubated at 20°C. Samples (5 ml) were withdrawn after intervals to follow the decrease in iodine staining power and β-amylolysis limit of the end-products.

**Iodine staining** Samples (0.5 ml) thus withdrawn were treated with iodine solution (1 ml) in 100 ml graduated flasks. The absorption values of the resulting polysaccharide-iodine complex were measured over the range 500-700 nm to determine \( \lambda_{\text{max}} \), and at 650 nm in a SP.500 Unicam spectrophotometer against an iodine-water blank.

**β-Amylolysis limit** Samples (4.5 ml) were heated in a boiling water bath for 5 min to inactivate ΧΕ. β-Amylase solution (5 ml, pH 4.6) was added to the above solution in 50 ml graduated flasks. After addition of water to a final volume of 50 ml and incubation at 55°C for 24 hr, the reducing sugars thus obtained were estimated by the cuprimetric method. The following results were obtained:

<table>
<thead>
<tr>
<th>Time</th>
<th>0</th>
<th>1</th>
<th>2</th>
<th>3</th>
</tr>
</thead>
<tbody>
<tr>
<td>β-Amylolysis limit (%)</td>
<td>97.1</td>
<td>80.0</td>
<td>68.5</td>
<td>57.0</td>
</tr>
</tbody>
</table>

...
FIG. - 15. EFFECT OF BRANCHING ENZYME ON POTATO AMYLOSE.
The above results show the steady fall in both $\beta$-amyolysis limit and iodine staining power (Figure 15).

**Purification of synthetic amylopectin**

After incubation of the above digest for 48 hr, it was heated in a boiling water bath for 15 min to inactivate YBE. As the polysaccharide was slightly insoluble in cold water, the coagulated protein could not be removed by either filtration or by centrifugation. The solution was dialysed against distilled water for 48 hr, after which it was concentrated in vacuo at 35°C to 1/5 of its volume. De-proteinisation was carried out according to Anderson and Greenwood (122). Solid sodium chloride was added to give 0.1 M concentration and this was treated with 1/3 volume of freshly distilled toluene. The mixture was shaken mechanically in a stoppered glass bottle overnight and denatured protein collected at the water-toluene interface. The aqueous solution was separated in a separating funnel. The deproteinisation process was repeated three times. The aqueous solution containing polysaccharide was then dialysed for 24 hr and freeze dried.

**Purity** The sample was estimated by acid hydrolysis to have a glucose content of 81.0%.

<table>
<thead>
<tr>
<th>Time (hr)</th>
<th>Fall in A.V. * (°)</th>
<th>$\beta$-Amyolysis limit (%)</th>
<th>max (μg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>-</td>
<td>97.1</td>
<td>630</td>
</tr>
<tr>
<td>2</td>
<td>20.0</td>
<td>78.8</td>
<td>-</td>
</tr>
<tr>
<td>6</td>
<td>29.8</td>
<td>73.8</td>
<td>-</td>
</tr>
<tr>
<td>10</td>
<td>38.5</td>
<td>70.2</td>
<td>585</td>
</tr>
<tr>
<td>20</td>
<td>61.6</td>
<td>60.3</td>
<td>570</td>
</tr>
<tr>
<td>25</td>
<td>65.1</td>
<td>59.0</td>
<td>570</td>
</tr>
<tr>
<td>30</td>
<td>67.7</td>
<td>58.3</td>
<td>570</td>
</tr>
<tr>
<td>48</td>
<td>77.7</td>
<td>55.1</td>
<td>550-555</td>
</tr>
</tbody>
</table>

* at 630 μg
Characterisation of synthetic amylpectin

(a) Action of α-amylase  The following digest was set up:

- synthetic amylpectin (3.1 mg/ml) 10 ml
- 0.1 M-sodium chloride 10 ml
- α-amylase (freeze dried) 2.5 mg

The α-amylase was prepared from saliva by Dr. A.M. Liddle (123). The volume of the digest was made up to 50 ml and incubated at 55°C for 24 hr. Samples (5 ml) were removed to determine the reducing sugars produced cuprimetrically

<table>
<thead>
<tr>
<th>Total apparent maltose (mg)</th>
<th>Theoretical maltose (mg)</th>
<th>Hydrolysis (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>29.2</td>
<td>32.8</td>
<td>89.0</td>
</tr>
</tbody>
</table>

(b) Action of β-amylase  The following digest was set up in a standard flask (25 ml):

- synthetic amylpectin (3.1 mg/ml) 8 ml
- 0.2 M-acetate buffer pH 4.6 4 ml
- β-amylase 12.5 mg

After incubation at 35°C for 24 hr, the maltose was estimated cuprimetrically; the following results were obtained:

<table>
<thead>
<tr>
<th>Total maltose (mg)</th>
<th>Theoretical maltose (mg)</th>
<th>β-amylolysis limit (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>14.8</td>
<td>26.1</td>
<td>56.6</td>
</tr>
</tbody>
</table>

(c) Action of isoamylase

- synthetic amylpectin (3.1 mg/ml) 8 ml
- 0.2 M-acetate buffer pH 6.0 5 ml
- isoamylase (50 mg in water) 2 ml

The digest was incubated at room temperature for 24 hr, and then heated in a boiling water bath for 5 min to inactivate the isoamylase. After cooling
FIG. 16.- The Absorption Spectrum of the Iodine Complex of (A) Potato Amyl (B) Synthetic Amylo (C) Synthetic Glyco (D) Rabbit Liver Glyc
FIG. 17. -- THE RELATIONSHIP BETWEEN THE SPECIFIC VISCOSITY AND THE CONCENTRATION OF (A) Potato Amylopectin (B) Synthetic Amylopectin (C) Synthetic Glycogen
to room temperature, β-amylase (12 mg, 960 units) was added and the volume made up to 25 ml. The reducing sugars produced were determined after incubation at 35°C for 24 hr.

<table>
<thead>
<tr>
<th>Total maltose (mg)</th>
<th>Theoretical maltose (mg)</th>
<th>β-amylolysis limit (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>24.1</td>
<td>26.1</td>
<td>82.0</td>
</tr>
</tbody>
</table>

(a) **Absorption spectra of the iodine complex**

Synthetic amyllopectin (2.5 mg, corrected weight) was stained with iodine solution (2.5 ml). The absorption spectra of the resultant purple-coloured solution was measured over the range of 400-600 nm (Figure 16).

Found max., 550-555 nm; O.D. max., 1.3

(e) **Limiting viscosity number**

Viscosity measurements were carried out according to the method described in Section IV.

Synthetic amyllopectin (corrected weight 69.5 mg) was dissolved in M-potassium hydroxide solution (ca. 18 ml), 15 ml being used for viscosity measurements. The exact concentration of the polysaccharide was determined by acid hydrolysis. The time flow was measured at three successive dilutions. The following data were obtained (Figure 17):

<table>
<thead>
<tr>
<th>C (g/ml)</th>
<th>T (To)</th>
<th>T-To</th>
<th>η sp x 1/C</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.00586</td>
<td>904.06</td>
<td>721.92</td>
<td>0.2523</td>
</tr>
<tr>
<td>0.00290</td>
<td>852.62</td>
<td>721.92</td>
<td>0.1810</td>
</tr>
<tr>
<td>0.00232</td>
<td>825.50</td>
<td>721.92</td>
<td>0.1435</td>
</tr>
</tbody>
</table>

where C is concentration of synthetic amyllopectin (g/ml)

T is flow time of solution (secs)

To is flow time of solvent (secs)

The specific viscosity, sp, is given by $\frac{T-To}{To}$

The limiting viscosity number obtained from the graph of $\eta sp/C$ against C is 55.0.
III Action of YBE on Amylopectin

Potato amylopectin was kindly prepared by Mr A. Wright by the thymol fractionation of potato starch. Two different samples were provided.

Prior to the large scale preparation of a "synthetic glycogen", a preliminary study of the action of β-amylase was carried out. The following digest was made up:

- Potato amylopectin sample I (2.65 mg/ml) 16 ml
- 0.2 M-citrate buffer pH 6.8 5 ml
- YBE solution (70 mg in water) 4 ml

The digest was incubated at room temperature. Samples (5 ml) were removed and the branching enzyme was inactivated by heating in a boiling water bath for 3 min., after which the solutions were cooled to room temperature. β-Amylase solution (3 ml, 1,200 units) was added to each digest and incubated at 35°C for 24 hr. The reducing sugars produced were estimated by the cuprimetric method. The following results were obtained:

<table>
<thead>
<tr>
<th>Time (hr)</th>
<th>0</th>
<th>1</th>
<th>2</th>
<th>3</th>
</tr>
</thead>
<tbody>
<tr>
<td>β-Amylolytic limit (%)</td>
<td>59.6</td>
<td>53.0</td>
<td>52.2</td>
<td>49.5</td>
</tr>
</tbody>
</table>

The β-amylolytic limit has decreased by 10%, thereby confirming the action of YBE on amylopectin. Since it is probable that the branched polysaccharide so obtained resembles glycogen in its properties, a large scale preparation of synthetic glycogen was carried out for its rigid characterisation.

Preparation of synthetic glycogen

1.2 Gm of freeze-dried potato amylopectin (sample II) was dissolved and filtered through a G. 3 x 4 sinter funnel to obtain a homogeneous solution which was then diluted to 100 ml. A digest was prepared with this potato amylopectin solution, 30 ml of 0.2 M-citrate buffer pH 6.8 and 900 mg of YBE in 20 ml of water, and incubated at 20°C. Samples (0.5 ml and 1 ml) were withdrawn
FIG. 18.—EFFECT OF BRANCHING ENZYME ON
POTATO AMYLOPECTIN
after time intervals to follow the decrease in iodine staining and β-amylo-
lysis limit of the end products.

**Iodine staining** Samples (0.5 ml) thus removed, were stained with iodine
solution (1 ml, 0.2% iodine in 2% potassium iodide) in 50 ml standard flasks.
The absorption values of polysaccharide-iodine complexes were measured at
540 nm in a Unicam SP.500 spectrophotometer against an iodine-water blank.

**β-Amylolysis limit.** Samples (0.5 ml) were introduced into 50 ml stan-
dard flasks and XBE inactivated by heating in a boiling water bath for 5 min.
0.2 M-acetate buffer (5 ml, pH 4.6) together with β-amylase (800 units) was
added and the volume made up to 50 ml. After incubation at 35°C for 24 hr,
the reducing sugars were estimated cuprimetrically. The following results
were obtained:

<table>
<thead>
<tr>
<th>Time (hr)</th>
<th>Fall in A.V. (%)</th>
<th>β-Amylolysis limit (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.0</td>
<td>-</td>
<td>53.2</td>
</tr>
<tr>
<td>2.0</td>
<td>29.6</td>
<td>52.0</td>
</tr>
<tr>
<td>5.0</td>
<td>39.8</td>
<td>51.5</td>
</tr>
<tr>
<td>6.5</td>
<td>47.0</td>
<td>50.5</td>
</tr>
<tr>
<td>8.0</td>
<td>56.0</td>
<td>49.5</td>
</tr>
<tr>
<td>18.0</td>
<td>92.0</td>
<td>47.2</td>
</tr>
</tbody>
</table>

Thus, there is a steady fall both in the iodine staining power and β-
amylolysis limit of amylopectin (Figure 13).

**Purification of synthetic glycogen**

The above digest was heated in a boiling water bath for 15 min. to inacti-
vate XBE. After cooling to room temperature, the coagulated protein was re-
moved by filtering the solution through a G. 3 x 4 sinter funnel. Inorganic
material was removed by dialysis against distilled water for 24 hr. The
dialysed solution was then concentrated in vacuo at 35°C to ca. 90 ml. Any
soluble protein was removed by treatment with trichloracetic acid (10 ml, 40%)
which was very carefully added with constant stirring in the cold (at 0°C). The precipitated protein was removed on the centrifuge (0°C), and polysaccharide precipitated by the addition of ethanol (2 vols.). It was reprecipitated (twice) from the solution with ethanol, the white precipitate collected and dried over phosphorus pentoxide. The yield obtained was about 900 mg.

**Purity** Acid hydrolysis of the dried synthetic glycogen indicated a glucose content of 94.5%.

**Rigid characterisation of synthetic glycogen**

(a) **Action of α-amylase** The following digest was set up:

- synthetic glycogen (5 mg/ml) 10 ml
- 0.1 N-sodium chloride solution 10 ml
- α-amylase (freeze-dried) 5 mg

The α-amylase was prepared from saliva by Dr. A.M. Liddle (123). The volume was then made up to 100 ml, and the digest was incubated at 35°C for 24 hr. The reducing sugars so obtained were estimated by the cuprimetric method.

<table>
<thead>
<tr>
<th>Apparent maltose (mg)</th>
<th>Theoretical maltose (mg)</th>
<th>Hydrolysis (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>39.38</td>
<td>50.10</td>
<td>78.6</td>
</tr>
</tbody>
</table>

(b) **Action of β-amylase**

- synthetic glycogen (5 mg/ml) 5 ml
- 0.2 N-acetate buffer pH 4.6 4 ml
- β-amylase 12.5 mg

The volume was made up to 25 ml. After incubation at 35°C for 24 hr, the following results were obtained:

<table>
<thead>
<tr>
<th>Observed maltose (mg)</th>
<th>Theoretical maltose (mg)</th>
<th>β-Amylolytic limit</th>
</tr>
</thead>
<tbody>
<tr>
<td>11.7</td>
<td>25.1</td>
<td>46.6</td>
</tr>
</tbody>
</table>

(c) **Action of isomylase** The following digest was set up in a 25 ml standard flask:
synthetic glycogen (5 mg/ml) 5 ml
0.2 N-acetate buffer pH 6.0 4 ml
isoamylase (50 mg in water) 2 ml

After incubation at room temperature for 24 hr, the digest was heated in a boiling water bath for 5 minutes to inactivate the isoamylase. After cooling to room temperature, β-amylase solution (12.5 mg; 1,000 units) was added and the volume made up to 25 ml. This digest was incubated at 35°C for 24 hr, and the reducing sugars were estimated cuprimetrically. The following results were obtained:

<table>
<thead>
<tr>
<th>Total maltose (mg)</th>
<th>Theoretical maltose (mg)</th>
<th>β-Amylolysis limit (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>13.8</td>
<td>25.1</td>
<td>71.0</td>
</tr>
</tbody>
</table>

The increase in β-amylolysis limit, therefore, is 25%.

(a) Potassium periodate oxidation

(i) The synthetic glycogen (255 mg) was dissolved in 5% potassium chloride (110 ml) contained in a brown glass bottle to avoid sunlight; 10 ml were withdrawn to determine the acidity of the polysaccharide solution. Sodium metaperiodate (5%, W/V; 20 ml) was added to the bulk which was then gently agitated on rollers. Samples (10 ml) were removed at intervals, excess periodate was neutralised by the addition of ethylene glycol (1 ml) and the formic acid produced was determined by titration with sodium hydroxide (approx: 0.01 N) solution and methyl red as indicator (pH 5.7) (97).

A blank experiment without polysaccharide was also set up for a reagent blank. The exact concentration of the polysaccharide was determined by complete acid hydrolysis. The following results were obtained:

<table>
<thead>
<tr>
<th>Time (hr)</th>
<th>240</th>
<th>288</th>
<th>320</th>
<th>398</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total formic acid produced (mg)</td>
<td>3.58</td>
<td>4.33</td>
<td>4.59</td>
<td>4.59</td>
</tr>
<tr>
<td>Chain length (glucose residues)</td>
<td>17.6</td>
<td>14.6</td>
<td>15.5</td>
<td>15.5</td>
</tr>
</tbody>
</table>
(ii) The residual periodate oxidised solution was neutralised with ethylene glycol (5 ml), and dialysed against running tap water for 48 hr; undialysed material was concentrated in vacuo at 35°C to ca. 5 ml.

(a) One ml of the above solution was hydrolysed in 2 N-sulphuric acid for 2 hr, after which the hydrolysate was neutralised with barium carbonate deionised, concentrated in vacuo and examined by paper chromatography for reducing sugar. No glucose could be detected.

(b) The residual solution (4 ml) was treated with potassium borohydride (50 mg). After 3 days, an insoluble precipitate of poly-alcohol formed, which was washed several times with ethanol. The precipitate was hydrolysed, neutralised, deionised, and examined by paper chromatography. No glucose was detected; hence, branching enzyme does not introduce either 1:2 or 1:3-glucosidic linkages.

(e) Absorption spectra of the iodine complex. Synthetic glycogen (2.5 mg, corrected weight) was treated with 2.5 ml of iodine solution. A reddish brown coloured solution was produced. The absorption spectra of the coloured solution obtained over the range 400-600 nm was measured (Figure 16).

Found: λ max, 480 nm; O:D max, 0.56.

(f) Specific rotation. The synthetic glycogen (53.5 mg) was dissolved in water (25 ml). The optical rotation was measured polarimetrically and found to be 0.80° equivalent to [α]D + 138°.

(g) Limiting viscosity number. Viscosity measurements were carried out according to the method described in Section IV. Dry synthetic glycogen (302 mg) was dissolved in 18 ml of 0.1 M-sodium chloride solution, 15 ml being used for viscosity measurements. The exact concentration of the polysaccharide was determined by acid hydrolysis. The time flow was measured at four successive dilutions. The following results were obtained (Figure 17):
<table>
<thead>
<tr>
<th>C</th>
<th>T</th>
<th>To</th>
<th>( \frac{T - T_0}{T_0} )</th>
<th>( \eta_{sp} \times \frac{1}{C} )</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.01535</td>
<td>737.25</td>
<td>646.6</td>
<td>0.2173</td>
<td>13.80</td>
</tr>
<tr>
<td>0.01190</td>
<td>746.90</td>
<td>646.6</td>
<td>0.1554</td>
<td>13.04</td>
</tr>
<tr>
<td>0.00952</td>
<td>725.50</td>
<td>646.6</td>
<td>0.1220</td>
<td>12.80</td>
</tr>
<tr>
<td>0.00794</td>
<td>711.20</td>
<td>646.6</td>
<td>0.1000</td>
<td>12.60</td>
</tr>
</tbody>
</table>

where \( C \) is concentration of synthetic glycogen (g/ml),
\( T \) is flow time of solution (secs),
\( T_0 \) is flow time of solvent (secs)
The specific viscosity, \( \eta_{sp} \), is given by \( \frac{T - T_0}{T_0} \)
The limiting viscosity number obtained from the graph of \( \eta_{sp}/C \) against \( C \) is 11.6.

Characterisation of original potato amylopectin

The potato amylopectin was characterised in a similar manner. Experiments to determine the \( \alpha \)-amylolysis limit, \( \beta \)-amylolysis limit, action of isoamylase, potassium periodate oxidation, absorption spectra of amylopectin-iodine complex (Figure 16) and limiting viscosity number were carried out, when the following results were obtained:

- \( \alpha \)-Amylolysis limit 89.0%
- \( \beta \)-Amylolysis limit 55.8%
- \( \beta \)-Amylolysis limit after treatment with isoamylase 80.1% (26.3 increase)

Iodine complex -

\[ \lambda_{max} = 545 \text{ m} \mu \]

\[ \text{O.D. max} = 1.5 \]

- Apparent chain length 22.0 glucose residues
- Limiting viscosity number 205 (in M-potassium hydroxide)

IV Action of XBE on other Polysaccharide

Various branched polysaccharides which were kindly supplied by Dr. D.J. Manners, were incubated with XBE.
Branched polysaccharides

(1) Floridean starch Sample II, (124),
(2) rabbit-muscle glycogen,
(3) horse-muscle glycogen (chain length -17, (125)),
(4) malt amylopectin (126),
(5) β-dextrin of waxy maize starch,
(6) synthetic amylopectin.

The following digests were set up:

<table>
<thead>
<tr>
<th>Branched polysaccharide (30-35 mg)</th>
<th>5 ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.2 M-citrate buffer pH 6.8</td>
<td>5 ml</td>
</tr>
<tr>
<td>YBE</td>
<td>20 mg</td>
</tr>
</tbody>
</table>

The volume was made up to 25 ml. The digests were incubated at room temperature. Samples (5 ml) were withdrawn from each after time intervals, and the enzyme inactivated by heating. After cooling to room temperature, samples (1 ml) were removed for iodine staining and (3 ml) for β-amylolysis limits.

Iodine staining The absorption values of polysaccharide-iodine complexes were measured at or near the wavelengths of maximum absorption.

β-Amylolysis limit Samples (3 ml) were treated with β-amylase (2 ml, 140 units) solution and water (1 ml). The digests were incubated at 35°C for 24 hr, and the reducing sugars thus obtained were estimated cuprimetrically (3 ml samples).

The following results were obtained:

<table>
<thead>
<tr>
<th>Time (hr)</th>
<th>Rabbit muscle glycogen</th>
<th>Horse muscle glycogen</th>
<th>Floridean starch</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>A.V. 460 μ</td>
<td>β-limit (%)</td>
<td>A.V. 480 μ</td>
</tr>
<tr>
<td>0</td>
<td>0.105</td>
<td>40.0</td>
<td>0.119</td>
</tr>
<tr>
<td>3</td>
<td>0.098</td>
<td>40.7</td>
<td>-</td>
</tr>
<tr>
<td>24</td>
<td>0.101</td>
<td>41.4</td>
<td>0.117</td>
</tr>
<tr>
<td>48</td>
<td>0.106</td>
<td>-</td>
<td>0.113</td>
</tr>
</tbody>
</table>
The &-amylolysis limits of the amylopectin samples could not be accurately determined as barley &-amylase was used. This preparation also contains a weak a-amylase which is optimum at about pH 5.8; therefore the apparent &-amylolysis limits were rather higher than the original quoted values. The results show that YBE has no significant effect on the &-limit and iodine staining power of glycogen, in contrast to the marked action on amylopectin and its &-limit dextrin.

DISCUSSION

Preparation of YBE

The method devised by Khin Maung (120) gave a preparation of satisfactory activity. This involves the ethanol-citrate fractionation of brewer's yeast at -5°C, the protein precipitating between 25 - 30% ethanol being used. The freeze-dried protein preparation finally isolated, contained 1.6% nitrogen. In all, four different YBE preparations were necessary for the present work; the method of preparation was found to be quite reproducible.

The enzyme preparation was free from a-amylase, as no decrease in iodine staining power of glycogen, Floridean starch or glycogen &-dextrin was observed. This was further checked by determining the reducing power after deproteinisation of these digests. The YBE preparation was contaminated with an insignificant trace of isoamylase. There was a slight increase observed in iodine staining power of glycogen after 2 hr in one experiment but this remained constant up to 24 hr.
XBE acted on both the starch components, amyllose and amylopectin, and not on glycogen without the production of free reducing groups. The activity was measured by the method of Gilbert and Patrick (40), whereby it was found that the enzyme action was directly proportional to the concentration.

The effect of ammonium molybdate on XBE, with reference to R-enzyme and isoamylase was studied. Inhibition of R-enzyme (16) and isoamylase (22) by ammonium molybdate has been reported. 1% Ammonium molybdate had no action on XBE, while 2% showed 50% inhibition.

**Action on amyllose**

Plant Q-enzymes act directly on amyllose to give amylopectin-like polysaccharide. However, Cori and Cori (36) noted that the animal branching enzyme had no action on either amyllose prepared by fractionation of starch, or on a synthetic amyllose. The percentage fall in iodine staining power of amyllose on incubation with XBE in one experiment was 74, after 21 hr. In this time, a 40% decrease in β-amylolysis limit was also found. These results show that branching had occurred; hence, XBE reacts readily with amyllose to give an amylopectin-type polysaccharide.

A large-scale preparation of synthetic amylopectin was then carried out. Potato amyllose was incubated with XBE for 48 hr. A steady fall both in iodine staining power and β-amylolysis limit was again observed. After inactivating the enzyme, the polysaccharide was purified by the toluene-sodium chloride method. The final freeze-dried product had a glucose content of 81%.

The action of α- and β-amylase on the synthetic amylopectin produced 89 and 57% conversion into maltose. This indicates that the majority of the linkages are of the α-1,4-glucosidic type. The β-amylolysis limit of 57% in contrast to the parent one (97%), clearly shows the formation of branch points which are not susceptible to β-amylase action, and also the formation of shorter
outer chains. A 25% increase in β-amylolysis limit was obtained, when the synthetic amylopectin was treated with isoamylase, indicating the formation of α-1:6-glucosidic linkages, since isoamylase is particularly specific for the hydrolysis of such α-1:6 linkages (51). An attempt was made to determine the apparent chain length by periodate oxidation but due to a certain amount of protein or other impurities present, satisfactory results were not obtained. Other workers have shown that protein will interfere with periodate oxidation (122).

When treated with iodine, a purple coloured solution having a maximum absorption at a wavelength of 550-555 μm was obtained. Thus, a fall in the wavelength of maximum absorption from 630 μm (amylose) to 555 μm (synthetic amylopectin) had occurred. The maximum absorption values for the synthetic polysaccharide was 1.3, which is in agreement with that found with potato amylopectin. The limiting viscosity number obtained by viscosity measurements was 56, which is low compared to the limiting viscosity number of potato amylopectin (156-205). As the ultimate product of YBE action is a glycogen-type polysaccharide, the intermediate product (synthetic amylopectin) may tend to have the molecular shape of glycogen and low viscosity number. Unfortunately, further characterisation of the synthetic amylopectin was not possible.

However, on comparing the above results with a typical amylopectin (Table IV) indicates that the synthetic polysaccharide produced by YBE on amylose closely resembles amylopectin in many respects.

Therefore, YBE would appear to differ from animal branching enzyme in its action on amylose. The synthetic amylopectin does not represent the final end product of YBE action, since on incubation with a further sample of YBE, more branch points were introduced as shown by changes in iodine absorption values and β-amylolysis limit. YBE acts slowly on malt amylopectin too. The degree
TABLE IV

A comparison of the properties of amyllopectin type polysaccharides

<table>
<thead>
<tr>
<th>Property</th>
<th>Synthetic amyllopectin</th>
<th>Potato amyllopectin</th>
</tr>
</thead>
<tbody>
<tr>
<td>α-Amylolytic limit (%)</td>
<td>39</td>
<td>39</td>
</tr>
<tr>
<td>β-Amylolytic limit (%)</td>
<td>57</td>
<td>55</td>
</tr>
<tr>
<td>(a) before</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(b) after isomylase</td>
<td>52</td>
<td>80</td>
</tr>
<tr>
<td>Iodine complex</td>
<td></td>
<td></td>
</tr>
<tr>
<td>λ max (µm)</td>
<td>550-555</td>
<td>545</td>
</tr>
<tr>
<td>A.V. max</td>
<td>1.3</td>
<td>1.3</td>
</tr>
<tr>
<td>Colour of the solution</td>
<td>purple</td>
<td>purple</td>
</tr>
<tr>
<td>Limiting viscosity</td>
<td>56</td>
<td>205</td>
</tr>
</tbody>
</table>

of branching in the synthetic amyllopectin is therefore dependent on the relative concentration of YBE. This finding recalls the studies of Peat et al. (39) who prepared a range of synthetic polysaccharides by the use of varying ratios of phosphorylase and Q-enzyme.

Action on amyllopectin

The animal branching enzyme acts on waxy maize starch (127), while plant Q-enzymes have no action on amyllopectin (128). YBE acts on amyllopectin producing a marked decrease in iodine staining power (at 540 µm), which fell rapidly with time. Within 2.5 hr, a 50% fall in absorption value was obtained. A preliminary study of the action of β-amylase on amyllopectin (sample I) treated with YBE also showed a small but significant decrease of 10% in β-amylolysis limit. This suggested that unlike the Q-enzyme from potatoes, YBE is capable of synthesizing a more highly branched polysaccharide similar in structure to that of glycogen. Therefore, a large-scale preparation of branched amyllopectin or synthetic glycogen was carried out to characterise the end product more rigidly.
The sample II of potato amylopectin was incubated with branching enzyme at pH 6.8 and 20°C for 24 hr, and the relationship between time and iodine staining power and β-amylose limit was studied. The synthetic glycogen thus obtained was freed from any protein impurities by treatment with trichloroacetic acid and precipitated several times with ethanol. The final product had a glucose content of 94.5%.

Chemical and enzymic analysis of the synthetic glycogen as well as the parent amylopectin were then carried out. The synthetic glycogen was readily soluble in cold water and had a specific rotation of \([\alpha]_D + 138^\circ\). With iodine, the synthetic glycogen formed a reddish-brown coloured solution, which had a maximum absorption at a wavelength of 490 mp. The parent polysaccharide showed maximum absorption at 545 mp. A graphical comparison of both indicated that a more highly branched polysaccharide was formed. There was also a marked decrease in maximum optical density values (synthetic glycogen, 0.56 and original amylopectin, 1.3).

When the synthetic glycogen was treated with β-amylase, 47% conversion into maltose was achieved. This shows that outer branches of synthetic glycogen are relatively short and are comparable to those of a typical glycogen. For example, the majority of glycogens have β-amylolysis limits of 45 ± 5% (123). On treatment with salivary α-amylase, in the presence of chloride ions, an α-amylolysis limit of 79% was observed. Thus, the rapid and extensive degradation by α-amylase (79%) and 47% degradation by β-amylase shows that the majority of the linkages are of the α-1:4 glucosidic type. Isoamylase action on the synthetic polysaccharide produced a 28% increase in β-amylolysis limit. Hence, the outermost branch points formed by the enzyme are α-1:6 linkages. This fact could be further confirmed from the result of isoamylase action on the synthetic polysaccharide produced from amylose. The synthetic glycogen is
therefore similar to an animal glycogen with respect to degradation by β-amylase, α-amylase and isomylase, e.g. rabbit liver glycogen has a β-amylolysis limit of 46%, an α-amylolysis limit of 76% and incubation with isomylase gives a 28% increase in β-amylolysis limit.

End-group determination was carried out by potassium periodate oxidation, when the average number of glucose residues per end group for the synthetic glycogen was found to be 15.5. The apparent chain length of the original potato amylopectin by this method was 22.0. This provides clear evidence for the introduction of further branch points into the amylopectin. The periodate oxidised polysaccharide was then treated with potassium borohydride and the insoluble polyalcohol thus formed was hydrolysed to liberate any free sugars present. Since no glucose was detected, XBE would appear to form only α-1:6 linkages and not α-1:2 or α-1:3 linkages.

Viscosity measurements showed a marked fall in limiting viscosity number from 205 to 12. This suggests that the synthetic polysaccharide, like normal glycogen, is an extremely compact molecule probably spherical in shape. The results of chemical and enzymic analysis are compared in Table V with those of the original amylopectin and a typical animal glycogen, thereby confirming that it is a glycogen type polysaccharide.

**Specificity of XBE**

The results show no action of XBE on rabbit muscle or horse muscle glycogen and also on Floridean starch. In these polysaccharides, the content of 1:6 links is probably so large and the resulting molecule so compact as to prevent further enzyme action. Incubation of XBE with β-dextrin of waxy maize starch caused a slow but significant change in absorption value and β-limit. This means that 1:6 links are being introduced into the inner chains
A comparison of the properties of amylopectin-glycogen type polysaccharides

<table>
<thead>
<tr>
<th>Property</th>
<th>Potato amylopectin</th>
<th>Synthetic glycogen</th>
<th>Rabbit liver glycogen</th>
</tr>
</thead>
<tbody>
<tr>
<td>Average chain length (glucose residues)</td>
<td>22.0</td>
<td>13.5</td>
<td>12.6</td>
</tr>
<tr>
<td>Proportion of 1:6-linkages (%)</td>
<td>4.5</td>
<td>7.4</td>
<td>8.0</td>
</tr>
<tr>
<td>Iodine complex:</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$\lambda_{\text{max}}$ (mp)</td>
<td>545</td>
<td>430</td>
<td>430</td>
</tr>
<tr>
<td>A.V. max</td>
<td>1.30</td>
<td>0.56</td>
<td>0.41</td>
</tr>
<tr>
<td>$\alpha$-Amylolysis limit (%)</td>
<td>39</td>
<td>79</td>
<td>78</td>
</tr>
<tr>
<td>$\beta$-Amylolysis limit (%)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(a) before,</td>
<td>55</td>
<td>47</td>
<td>46</td>
</tr>
<tr>
<td>(b) after, treatment with isoamylase</td>
<td>80</td>
<td>75</td>
<td>78</td>
</tr>
<tr>
<td>Exterior chain length (glucose residues)</td>
<td>14.2</td>
<td>8.9</td>
<td>8.3</td>
</tr>
<tr>
<td>Interior chain length (glucose residues)</td>
<td>6.8</td>
<td>5.6</td>
<td>5.5</td>
</tr>
<tr>
<td>Limiting viscosity number</td>
<td>205</td>
<td>12</td>
<td>10</td>
</tr>
</tbody>
</table>

Of the molecule. As the $\beta$-amylolysis limit increased slightly, it is probable that the outer chains containing short branch stubs of 2-3 glucose residues are being transferred.

In general, YBE appears to have a wider specificity than the plant Q-enzymes or the animal branching enzyme, although the reason for the lack of action of animal branching enzyme on amylose is not known and this point requires further investigation.
SECTION VI

PROPERTIES OF YEAST MALTASE

INTRODUCTION

Yeast maltase ((98), often termed α-glucosidase, was first discovered by Emil Fischer (13) in 1894 when he observed that liquefied (toluene or chloroform) extracts of brewer's yeast catalysed the hydrolysis of not only maltose but also of methyl α-D-glucoside to glucose. Since then yeast maltase has been the subject of study by various workers, for example, Hill, Willstätter, Armstrong, Gottschalk, Leibowitz, Hestrin and Cook and Phillips particularly in connection with alcoholic fermentation. Attempts to purify it have been made by Willstätter and co-workers (113) and later by Weidenhagen. Recently Cook and Phillips have been successful in purifying yeast maltase by selective elution from alumina columns (129).

Yeast maltase, like all other maltases, catalyses the hydrolysis of maltose (0-α-D-glucopyranosyl-(1→4)-D-glucose). In addition, it hydrolysles a number of maltose derivatives and some alkyl and aryl-α-D-glucopyranosides, but has no action on poly-α-glucosides of large molecular size such as glycogen and starch. The main feature of all these compounds is a non-substituted α-D-glucopyranosyl residue. The aglycone group may be aryl or alkyl but must be attached to the D-glucose residue by an α-linkage. Hence, yeast maltase shows a high degree of tolerance towards structural changes in the aglycone part of the substrate. Substrates such as maltose (13), methyl α-D-glucoside (13), maltosone (130), maltosazone (131), allyl α-D-glucoside (132), phenyl α-D-glucoside (133),
maltobionic acid (134), D-mannitol mono-α-D-glucoside (135), hydroquinone mono-
α-D-glucoside (136), and maltotriose (108) are amongst the substrates tested
against yeast maltase by various workers.

Brewer's yeast provides a rich source of maltase whereas baker's yeast
contains a much smaller amount of this enzyme (see Section III). Yeast mal-
tases vary in their properties depending upon the strain and species of the
yeast; the pH optimum varies from 4.5 to 8.5 for different strains of brewer's
bottom yeast, and the temperature optimum between 25 - 35°C. Aqueous solutions
of yeast maltase are unstable; addition of glycerol has a protective effect (137).
Yeast maltase is inhibited by weak acids and alkali (138) and alcohol (137)
whereas acetone, toluene (137) and concentrations of sodium fluoride above 1%
cause partial inactivation of it; the enzyme is completely inactivated when
heated to 55°C (130). Jsaiev (137) has deduced from experimental data that
yeast maltase combined with both the substrate and the reaction product; the
enzyme is therefore progressively inhibited by the production of glucose (re-
action product).

Yeast maltase differs markedly from maltases obtained from various sources.
For example, in contrast to yeast maltase, taka-maltase from Aspergillus oryzae
(159) and malt maltase do not show any tolerance towards changes in the aglycone
portion of its substrate. It also differs from other maltases such as mould
maltase in its chemical behaviour (pH optima and the different stabilities to-
ward acid and heat).

Hill in 1898 (57) observed that the hydrolysis of maltose by yeast maltase
is a reversible reaction and effected the enzymic synthesis of a disaccharide
(maltose) from concentrated solutions of glucose. Pringsheim and Leibowitz
(140) have isolated the enzymically-synthesised product (maltose) in a crystalline
state. Yeast maltase shows the same specificity towards the synthesis of maltose
derivatives and various heterosides as demonstrated by Bourquelot and co-workers (132, 58, 141).

The ability of maltases to effect the synthesis of oligosaccharides by transglycosylation has been widely examined. This property of yeast maltase has not yet been reported.

During the fractionation of brewer's yeast enzymes, the protein preparation obtained between 30 - 40% acetone concentration was found to contain an enzyme capable of hydrolysing maltose into glucose. Some of the chemical properties of this enzyme (yeast maltase) have been studied and the results are reported in this Section. This protein preparation on incubation with maltose for a long period (up to 15 days) also catalysed the synthesis of α-1:4- and α-1:6-linked oligosaccharides. Their separation and characterisation is also described in this Section.

EXPERIMENTAL

1. Preparation of Yeast Maltase

The protein preparation (protein D₃) was obtained according to the method described in Section III. On analysis, protein D₃ showed a considerable amount of hydrolytic activity towards maltose. The yield and nitrogen content of the protein preparation was 5.0 gm and 9.0% respectively, from 200 gm air-dried yeast.

2. Maltase Activity

The following digest was set up:

maltose solution (0.4%) 10 ml
0.2 M-acetate buffer pH 5.8 4 ml
enzyme solution (20 mg/ml) 2 ml
distilled water 6 ml
toluene 2-3 drops
FIG. 20. - Effect of Enzyme Concentration (Maltase)

FIG. 21. Effect of BDH Universal Buffer
The digest was incubated at 35°C and the glucose so obtained was estimated at intervals by Phillips and Caldwell method. A portion of the enzymic digest after deionisation was examined by paper chromatography. Maltose and glucose were detected.

<table>
<thead>
<tr>
<th>Time (hr)</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>24</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hydrolysis (%)</td>
<td>21.2</td>
<td>26.2</td>
<td>29.8</td>
<td>34.8</td>
<td>59.2</td>
</tr>
</tbody>
</table>

3. pH Optimum

Yeast maltase (15 mg/ml; 2 ml) was incubated with maltose solution (10 mg/ml; 5 ml) and BHI Universal buffer of pH values ranging from 4.45 to 6.05 (3 ml). The digests were incubated at 35°C in the presence of toluene for 24 hr. After deproteinisation, the liberated glucose was estimated by Phillips and Caldwell's method and the following results were obtained:

<table>
<thead>
<tr>
<th>pH</th>
<th>4.45</th>
<th>5.00</th>
<th>5.55</th>
<th>5.80</th>
<th>6.05</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hydrolysis (%)</td>
<td>27.0</td>
<td>29.6</td>
<td>30.8</td>
<td>34.0</td>
<td>29.3</td>
</tr>
</tbody>
</table>

The optimum pH of brewer's yeast maltase is about 5.8 (Figure 2.1).

4. Effect of Enzyme Concentration

The following digest containing varying concentration of maltase preparation (protein D₃) was set up:

- maltose solution (1%) 10 ml
- BHI buffer pH 5.8 5 ml
- enzyme solution (5-35 mg/ml) 2 ml
- toluene 2 drops

A control digest without the enzyme solution was also set up. After incubation at 35°C for 3 hr, the samples from each digest were deproteinised and estimated for reducing sugars by the method of Phillips and Caldwell. The following data were obtained:
Enzyme concentration  0  5  10  15  20  25
Total glucose (mg)   6.8 10.2 13.5 16.6 19.8 22.9
Hydrolysis (%)      6.0 9.7 12.8 15.8 18.8 21.8

Graphical representation of the results (Figure 2.0) gave a straight line passing through the origin.

5. Specificity of Yeast Maltase

It has been reported in the literature that the specificity of yeast maltase is directed not only towards the hydrolysis of maltose but also towards maltose derivatives and many alkyl and aryl α-glucosides. Hence, a series of experiments were set up with various substrates and yeast maltase (protein D₂).

Autolysis of the enzyme preparation

As a preliminary to the above, the enzyme powder (10 mg) was dissolved in 0.02 M acetate buffer pH 5.54 (0.2 ml) and incubated at 35°C for several days. Samples were examined chromatographically (solvent iii, spray 1) at intervals. A faint trace of glucose was the only reducing sugar which could be detected; the amount present after 7 days was not greater than the amount present at zero time.

Action on various α-glucosides

All the samples were kindly given by Dr. D.J. Manners except maltobionic acid, which was prepared in the following manner:—

Recrystallised maltose (50 mg), iodine (50 mg) and potassium hydroxide (0.2 ml, 1.0 M) were shaken before the addition of a further portion of alkali (0.1 M). After standing overnight, the solution was deionised with Amberlite resins and a portion of it used for enzymic analysis.

Digests

Sugars (5-7 mg), 0.02 M acetate buffer pH 5.54 (0.2 ml) and enzyme solution (10 mg; 0.2 ml) were incubated at 35°C; samples were examined chromatographi-
ally (solvent iii, spray i) at intervals when the following data were obtained:

**TABLE VI**

**Hydrolysis of α-D-glucosides by yeast maltase**

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Reducing sugars produced</th>
<th>1 day</th>
<th>5 days</th>
<th>7 days</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Sucrose</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2. Methyl α-D-glucopyranoside</td>
<td>G+++</td>
<td>G+++</td>
<td>G+++</td>
<td></td>
</tr>
<tr>
<td>3. Phenyl α-D-glucopyranoside</td>
<td>G+++</td>
<td>G+++</td>
<td>G+++</td>
<td></td>
</tr>
<tr>
<td>4. Maltobionic acid (0-α-D-glucopyranosyl-</td>
<td>G+++</td>
<td>G+++</td>
<td>G+++</td>
<td></td>
</tr>
<tr>
<td>(1→4)-D-gluconic acid)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5. Trehalose (0-α-D-glucopyranosyl-</td>
<td>G*</td>
<td>G*</td>
<td>G*</td>
<td></td>
</tr>
<tr>
<td>(1→1)-D-glucopyranoside</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(1→4)-D-fructose</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* represents detected under ultra-violet lamp.
+ represents intensity of spots indicating the relative amount of reducing sugars produced.

6. **Inhibition and the measurement of the isoelectric point of yeast maltase by use of Suramin**

Suramin, commercial name "Antrypol", is the sodium salt of a symmetrical urea substituted with m-benzoyl-m-amino-p-methyl benzoyl-1-aminonaphthalene-4:6:8-trisulphonic acid (Figure below); it is a drug which is freely soluble in water and normal saline and bitter in taste.
Suramin.

Its commercial use is for the prevention of sleeping sickness, a fatal disease which is prevalent in Central Africa. This action could be explained by the retention of the drug in the blood plasma and tissues as it combines firmly with body proteins. This observation on the ability of Suramin to combine with proteins led Willis et al. (142) to study its effects on various enzymes. Willis (143) has used Suramin as an enzyme inhibitor for enzymes involved in carbohydrate metabolism and also to determine the isoelectric point of some enzymes, since the inhibitory effect on the drug on enzymes was found to be dependent on pH of the media. It has been observed further by Willis (143) that a sharp drop in the inhibition-pH curve occurs at or near the isoelectric point of the enzyme, thereby making the determination of the isoelectric point of enzymes possible. In addition, the fact that crude enzyme preparations show a gradual slope in the inhibition-pH curve, it is possible to tell from the nature of the curve the purity of the enzyme preparation. Suramin does not inhibit all enzymes and this can also help in inactivating some unwanted enzymes. Hence, a study of the action of Suramin on yeast maltase was carried out.
FIG. 22.- EFFECT OF pH ON INHIBITION (SURAMIN)
Method. The procedure described by Willis (143) was followed. The enzyme solution (protein $D_3$, 15 mg/ml, 2 ml) was incubated with Suramin solution and water (7 ml, 12.97 mg ($1 \times 10^{-3}$ M)) and BDH Universal buffer, the pH values of which ranged from 5.0 - 8.0 (3 ml) at 35° for 30 min. so as to bring about a firm combination of enzyme protein and Suramin. Blank digests without Suramin at different pH values (5.0 - 8.0) were also prepared. To each digest was then added maltose solution (3 ml; 1%) and the mixtures incubated again at 35° for 24 hr; the percentage hydrolysis was then obtained after estimation of reducing sugars by Phillips and Caldwell's method. The following results were obtained:

<table>
<thead>
<tr>
<th>pH</th>
<th>Hydrolysis (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
</tr>
<tr>
<td>5.00</td>
<td>29.3</td>
</tr>
<tr>
<td>5.55</td>
<td>30.8</td>
</tr>
<tr>
<td>5.80</td>
<td>39.0</td>
</tr>
<tr>
<td>6.05</td>
<td>36.1</td>
</tr>
<tr>
<td>6.50</td>
<td>28.0</td>
</tr>
<tr>
<td>7.00</td>
<td>27.0</td>
</tr>
<tr>
<td>7.40</td>
<td>28.0</td>
</tr>
<tr>
<td>8.00</td>
<td>17.4</td>
</tr>
</tbody>
</table>

From the above results, the percentage inhibition was calculated and recorded as the difference between the degree of hydrolysis of the untreated and treated preparation divided by the percentage hydrolysis of the former.

<table>
<thead>
<tr>
<th>pH</th>
<th>Inhibition (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>5.00</td>
<td>61.9</td>
</tr>
<tr>
<td>5.55</td>
<td>64.9</td>
</tr>
<tr>
<td>5.80</td>
<td>88.0</td>
</tr>
<tr>
<td>6.05</td>
<td>80.2</td>
</tr>
<tr>
<td>6.50</td>
<td>48.5</td>
</tr>
<tr>
<td>7.00</td>
<td>38.0</td>
</tr>
<tr>
<td>7.40</td>
<td>25.0</td>
</tr>
<tr>
<td>8.00</td>
<td>15.0</td>
</tr>
</tbody>
</table>

The above results have been graphically represented when a curve falling from 88% to 15% values has been obtained. The isoelectric point is the mid-point of such a curve, or more correctly, the pH value corresponding to 50% inhibition; it is 6.5 for the maltase of brewer's yeast.
7. **Enzymic synthesis of oligosaccharides**

Recent discoveries have shown that enzymes which normally catalyse the hydrolytic break-down of carbohydrates can also, under certain conditions, catalyse the formation of short chain saccharides although without the production of polysaccharides. The action of yeast maltase on a concentrated solution of maltose was therefore studied.

**Enzymic synthesis of oligosaccharides from a concentrated solution of maltose**

(i) A digest containing maltose (1 gm), 0.2 M-acetate buffer pH 5.0 and enzyme powder (50 mg) was prepared; toluene was added to maintain aseptic conditions, and the digest was incubated at 35°C. Samples of the digest were examined at intervals, by paper chromatography. The enzyme digest was found to contain several reducing sugars as shown below.

**TABLE VII**

<table>
<thead>
<tr>
<th>Time of incubation (days)</th>
<th>Glucose</th>
<th>Maltose</th>
<th>Isomaltose</th>
<th>Panose</th>
<th>Maltotriose</th>
<th>Higher saccharides</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>-</td>
<td>+++</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>1</td>
<td>++</td>
<td>+++</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>3</td>
<td>+++</td>
<td>+++</td>
<td>±</td>
<td>-</td>
<td>±</td>
<td>-</td>
</tr>
<tr>
<td>5</td>
<td>++</td>
<td>+++</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>7</td>
<td>++</td>
<td>+++</td>
<td>++</td>
<td>++</td>
<td>++</td>
<td>+</td>
</tr>
<tr>
<td>10</td>
<td>++</td>
<td>+++</td>
<td>++</td>
<td>++</td>
<td>++</td>
<td>+</td>
</tr>
<tr>
<td>15</td>
<td>++</td>
<td>+++</td>
<td>++</td>
<td>++</td>
<td>++</td>
<td>+</td>
</tr>
</tbody>
</table>

+++ , ++ , + , and ± indicate intense, strong, moderate and weak "spots" respectively.

(ii) **Large scale digest**

Commercial maltose was freed from a small amount of higher oligosaccharides by re-crystallisation (three times) from 80% ethanol.
A digest consisting of maltose (30 gm), 0.2 M-acetate buffer pH 5.0 (100 ml) and enzyme solution (protein Dg; 1.5 gm in 100 ml water); toluene was added and the digest was incubated at 35°C for several days.

Samples were examined chromatographically at intervals, when sugars with the Rf values of glucose, maltose, isomaltose, panose, maltotriose and other higher oligosaccharides were seen. The reaction was terminated after 16 days incubation by heating the digest at 100°C for 15 min. The coagulated protein was removed by filtration and the resultant solution was concentrated in vacuo at 55°C to ca. 10 ml.

Preparation of a charcoal-Celite column

A charcoal-Celite column was prepared according to the procedure described in Section II. 700 Gm each of charcoal and Celite were used to pack a column (size, 155 x 7 cm).

Separation of oligosaccharides

The concentrated solution (10 ml) was run onto the column and mercuric chloride (ca. 300 mg) was added to maintain aseptic conditions. A step-wise elution of the component sugars was effected first with water and then with increasing concentrations of aqueous alcohol. The eluates (2-3 litres) were evaporated to a small volume (ca. 2-5 ml) at 35-40°C under reduced pressure; solutions wherever necessary were neutralised with dilute alkali to ensure the neutrality of the solution prior to its evaporation. Traces of charcoal and Celite were removed by filtration and the sugars examined chromatographically.

The fractions containing like components were combined and the sugars isolated in some cases in crystalline form or, where crystallisation was not possible, as freeze-dried powders. Table VIII gives the details of the nature and volume of eluant used, and approximate yields of the fractions; the numbered fractions have been subsequently characterised.
TABLE VIII

Chromatographic separation of oligosaccharides

<table>
<thead>
<tr>
<th>Eluant used</th>
<th>Vol. of eluant (litres)</th>
<th>$R_\text{G}$ values of component sugars</th>
<th>Yield (mg)</th>
<th>Fraction no</th>
</tr>
</thead>
<tbody>
<tr>
<td>Water</td>
<td>6.1</td>
<td>1.0</td>
<td>128</td>
<td>1</td>
</tr>
<tr>
<td>Water</td>
<td>9.1</td>
<td>0.49</td>
<td>160</td>
<td>2</td>
</tr>
<tr>
<td>Water</td>
<td>6.6</td>
<td>0.49 + 1.0</td>
<td>-</td>
<td>1 + 2</td>
</tr>
<tr>
<td>Ethanol (1%)</td>
<td>24.0</td>
<td>0.61</td>
<td>8,800</td>
<td>3</td>
</tr>
<tr>
<td>(2%)</td>
<td>24.7</td>
<td>0.61</td>
<td>-</td>
<td>3</td>
</tr>
<tr>
<td>(5%)</td>
<td>19.0</td>
<td>0.61</td>
<td>1,320</td>
<td>3</td>
</tr>
<tr>
<td>(10%)</td>
<td>28.0</td>
<td>0.61</td>
<td>989</td>
<td>3</td>
</tr>
<tr>
<td>(15%)</td>
<td>10.0</td>
<td>0.31</td>
<td>250</td>
<td>4</td>
</tr>
<tr>
<td>(20%)</td>
<td>15.0</td>
<td>0.61</td>
<td>58</td>
<td>5</td>
</tr>
<tr>
<td>(1%)</td>
<td>8.5</td>
<td>0.39 + 1.0</td>
<td>-</td>
<td>1 + 5</td>
</tr>
<tr>
<td>(11%)</td>
<td>12.0</td>
<td>0.39 + 1.0</td>
<td>68</td>
<td>1 + 5</td>
</tr>
<tr>
<td>(12%)</td>
<td>8.0</td>
<td>0.39</td>
<td>195</td>
<td>5</td>
</tr>
<tr>
<td>(13%)</td>
<td>8.5</td>
<td>0.59</td>
<td>90</td>
<td>5</td>
</tr>
<tr>
<td>(14%)</td>
<td>9.5</td>
<td>0.32 + 0.61 + 1.0</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>(25%)</td>
<td>10.0</td>
<td>higher saccharides</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

Characterisation of oligosaccharides

Paper partition chromatography

The $R_\text{G}$ values were obtained using solvent iii, and spray 1.

$R_\text{G}$ values of the fractions:

<table>
<thead>
<tr>
<th>Fraction no</th>
<th>1</th>
<th>1</th>
<th>3</th>
<th>4</th>
<th>5</th>
</tr>
</thead>
<tbody>
<tr>
<td>$R_\text{G}$ value</td>
<td>1.0</td>
<td>0.49</td>
<td>0.61</td>
<td>0.51</td>
<td>0.39</td>
</tr>
</tbody>
</table>

$R_\text{G}$ values of the reference sugars:

Sugar: Glucose Maltose Isomaltose Maltotriose Panose

<table>
<thead>
<tr>
<th>Sugar</th>
<th>Glucose</th>
<th>Maltose</th>
<th>Isomaltose</th>
<th>Maltotriose</th>
<th>Panose</th>
</tr>
</thead>
<tbody>
<tr>
<td>$R_\text{G}$ value</td>
<td>1.0</td>
<td>0.61</td>
<td>0.49</td>
<td>0.59</td>
<td>0.31</td>
</tr>
</tbody>
</table>

The authentic samples of maltotriose and panose were kindly given by
Dr. D.J. Manners and were run on the same chromatograms as the appropriate column fraction.

**Estimation of degree of polymerisation**

The degree of polymerisation of the component sugars was obtained by determining the reducing power of the sugars before and after acid hydrolysis in the following manner:

The sugars (2-5 mg) were dissolved in water (5 ml) and the reducing power of the samples (1 ml) estimated using the Somogyi reagent. The reducing powers were calculated as equivalents of maltose. Samples (1 ml) were hydrolysed with sulphuric acid (0.12 ml, 36 N) at 100° for 2 hr, neutralised with sodium hydroxide, using phenolphthalein as indicator and the glucose liberated was estimated by Somogyi reagent. The degree of polymerisation was calculated as follows:

\[
\text{Weight of oligosaccharide/2 ml} = \frac{\text{wt. of glucose in hydrolysate} \times 162}{\text{equivalent weight of maltose}}
\]

\[
\text{Degree of polymerisation} = \frac{\text{weight of oligosaccharide} \times 542}{\text{equivalent weight of maltose}}
\]

**Results:**

<table>
<thead>
<tr>
<th>Fraction No</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
</tr>
</thead>
<tbody>
<tr>
<td>Degree of polymerisation</td>
<td>1.0</td>
<td>2.1</td>
<td>2.2</td>
<td>3.2</td>
<td>2.9</td>
</tr>
</tbody>
</table>

**Periodate oxidation**

The fractions (1-5 mg) were oxidised with sodium metaperiodate solution buffered at pH 8 and the formaldehyde release determined (Section II).

**Results:**

<table>
<thead>
<tr>
<th>Fraction No</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
</tr>
</thead>
<tbody>
<tr>
<td>Formaldehyde release (moles)</td>
<td>1.0</td>
<td>0.1</td>
<td>2.0</td>
<td>1.0</td>
<td>2.9</td>
</tr>
</tbody>
</table>

**Action of α- and β-amylase**

Fractions (10 mg) were dissolved in a solution of salivary α-amylase (0.2 ml) or β-amylase (5 mg, 0.2 ml) and sodium chloride (2 mg) or 0.1 M-acetate
buffer pH 4.5 (0.2 ml). After incubation at 55°C for 24 hr, the enzymic digests were analysed chromatographically for the detection of reducing sugars.

**Characterisation of fraction 1 as glucose**

This fraction, which had the same chromatographic mobility as authentic glucose, had the same reducing power value. Overoxidation with sodium metaperiodate yielded the theoretical 1 mole of formaldehyde per mole of sugar. α- and β-amylase had no action on it.

**Characterisation of fraction 2 as isomaltose**

This fraction had the same chromatographic mobility as the authentic isomaltose and had a DP of 2. On overoxidation with sodium metaperiodate, a negligible amount of formaldehyde was liberated. The specific rotation \( \alpha_D + 122^\circ \) in water was similar to the values previously reported for isomaltose \( (\alpha)_D + 122^\circ (144) \). The partial acid hydrolysis (Section II) and chromatographic analysis showed that fraction 2 and glucose were the only reducing sugars present. Electrophoretic analysis showed the same mobility as authentic isomaltose; no other reducing sugars could be detected. α- and β-Amylase had no action. The action of maltase-free isomylase on fraction 2 yielded glucose (chromatographic analysis of enzymic digest).

**Characterisation of fraction 3 as maltose**

This fraction which had the same chromatographic mobility as an authentic maltose sample, had the same DP and reducing power value. Overoxidation with periodate gave 2 moles of formaldehyde per mole of disaccharide. The specific rotation \( \alpha_D + 131^\circ \) in water was similar to the values previously reported. Chromatographic analysis of a partial acid hydrolysate showed the presence of glucose and unchanged fraction 3; that of fraction 3 and α- and β-amylase in buffer solution did not show the presence of any additional reducing sugars.
Characterisation of fraction 4 as panose

This fraction, which had the same chromatographic mobility as an authentic sample of panose, had the DP value of a trisaccharide. The specific rotation $[\alpha]_D + 151^\circ$ in water was similar to the values reported by Pasur and French (145) $[\alpha]_D + 150^\circ$. Chromatographic analysis of partial acid hydrolysates showed the presence of isomaltose, maltose and glucose. This confirmed the presence of $\alpha$-1:6- and $\alpha$-1:4-linkages. Overoxidation with periodate yielded one mole of formaldehyde per one mole of sugar. $\alpha$-Amylase had no action on fraction 4.

Characterisation of fraction 5 as maltotriose

This fraction, which had the same chromatographic mobility as an authentic sample of maltotriose, had a DP value of a trisaccharide. The specific rotation was $[\alpha]_D + 165^\circ$ in water; cf. $[\alpha]_D + 160^\circ$ reported by Whelan et al. (1955) (33), for maltotriose. Chromatographic analysis of a partial acid hydrolysate showed the presence of glucose, maltose and unchanged fraction 5. It was slowly hydrolysed by barley $\beta$-amylase giving glucose and maltose as shown by paper chromatography. $\alpha$-Amylase had no action on it. On overoxidation with sodium metaperiodate, 3 moles of formaldehyde were obtained per mole of trisaccharide.

Enzymic action with glucose as the sole substrate

A digest consisting of glucose (1,000 mg) and enzyme preparation (70 mg) dissolved in 0.2 M-acetate buffer pH 5.54 (10 ml) was incubated at 35°C. Samples were examined chromatographically at intervals. It was observed that other sugars besides glucose were detected. The enzymic digest was then analysed at intervals against reference sugars; the following data were obtained:
TABLE IX

Oligosaccharide synthesis from glucose

<table>
<thead>
<tr>
<th>Time of incubation (days)</th>
<th>Glucose</th>
<th>Maltose</th>
<th>Isomaltose</th>
<th>Panose</th>
<th>Maltotriose</th>
<th>Higher saccharides</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>+++</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
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+ represents intensity of spots indicating the relative amount of reducing sugars produced.

This suggested that oligosaccharides were synthesised from glucose and that in the case of maltose, the synthesis of oligosaccharides was not entirely due to transglycosylation.

**DISCUSSION**

Evidence has been obtained for the presence of an enzyme-system in brewer's yeast extracts capable of hydrolysing maltose and its derivatives to glucose and of synthesising oligosaccharides from concentrated solutions of maltose and glucose.

α-Glucosidase (maltase) activity The protein preparation, obtained by the acetone fractionation of brewer’s yeast extracts, on incubation with maltose (dilute solution) at 35°C in acetate or HE buffer pH 5.8 was found to catalyse the hydrolysis of maltose to glucose. In addition, the hydrolysis of various
substrates such as methyl α-D-glucoside, phenyl α-D-glucoside, sucrose, malto- 
bionic acid, maltulose and maltotetraose was also observed. This enzyme is 
therefore of the type (yeast maltase) reported in the literature by various 
workers (Hill, Willstätter, Gottschalk). Hence, this maltase shows a marked 
tolerance towards structural changes in the aglycone part of the substrate. 
The action of yeast maltase on maltulose or maltotetraose has not been pre-
viously reported, although recently Cook and Phillips (106) have studied its 
action on maltotriose. No significant trehalase activity was observed in this 
preparation, although this enzyme can be obtained from other yeasts, e.g. 
Candida tropicalis (146).

Sucrose is the biological substrate for two different glycosidases, one 
of which, maltase (α-glucosidase), attacks the molecule through the glucose 
moiety, while the other, β-D-fructosidase attacks the fructose part of the 
molecule. Ishizawa and Miwa (147) have separated these two enzymes from yeast 
juice by adsorption on alumina gels and have established the heterogeneity by 
experimental data, i.e. the two fractions on incubation with sucrose in the 
presence of methyl alcohol gave methyl-β-glucopyranoside and methyl-β-fructo-
furanoside respectively. Thus, the hydrolysis of sucrose by protein D₃ (malt-
tase) may be due to the dual role of the α-glucosidase system. In the present 
investigations, the estimation of glucose (the resultant product) by the method 
of Phillips and Caldwell was found to be most satisfactory, as according to 
this method, glucose can be estimated quantitatively in the presence of maltose. 
The enzymic hydrolysis of maltose was found to progress with time in a 
normal manner and also to be proportional to enzyme concentration. The pH 
optimum of yeast maltase was about 5.8 in BSH Universal buffer. Since the 
borate component of this buffer inhibits isomylase (Section IV), this result 
provides further evidence for the non-identity of yeast isomylase and maltase.
The effect of the drug Suramin on yeast maltase has been studied to determine the isoelectric point and also the purity of the enzyme preparation. An inhibition-pH curve was obtained between pH 5.8 and 8.0, and the iso-electric point which is the mid-point of such a curve or rather, the pH value at 50% inhibition was 6.5 for yeast maltase. The iso-electric point values for yeast maltase obtained by normal electrophoretic methods have not yet been reported in the literature. As suggested by Willis et al. (142), the nature of the inhibition-pH curve should give information on the purity of the enzyme preparation. A crude preparation will give a normal slope while a purified enzyme preparation gives a sharp slope. In the case of yeast maltase, a sharp slope of the type obtained by Willis et al. for various purified carbohydrases was observed. The control results obtained in this experiment, without Suramin, also confirm the optimum pH of yeast maltase at 5.8 recorded on p. 110.

Enzyme synthesis of oligosaccharides

In view of the considerable amount of α-glucosidase activity of protein preparation D3, it was considered probable that this preparation would catalyse the transfer of glucose from α-linked glucoside (maltose) to form a series of higher saccharides by transglucosylation. Maltases from various sources (148), (54), (149), (150), (151), (152), (155) have effectively catalysed the synthesis of oligosaccharides by transglucosylation. Hence, a preliminary digest containing maltose (1 gm), enzyme powder (50 mg) and acetate buffer pH 5.0 was incubated at 35°C in the presence of toluene for a period of 0-15 days; samples were removed at intervals and the reducing sugars so produced were examined by paper chromatography. Over a set period of 15 days, sugars other than maltose and glucose were detected; these were isomaltose, maltotriose, panose and higher saccharides of low $R_F$ values. Hence, the synthesis of both α-1:4- and α-1:6-linked saccharides had occurred. In order to identify the products
of enzyme action, a large scale digest containing ca. 30 gm of maltose (15% solution) was prepared.

After termination of enzyme action (15 days), the sugars were separated by the use of a charcoal-Celite column. The estimation of degree of polymerisation indicated that one disaccharide and 2 trisaccharides besides glucose and maltose had been formed; no oligosaccharides were present in the original substrate as the maltose had been re-crystallised three times with ethanol. The detailed chemical and enzymic analysis of individual components have confirmed the identity of the newly formed oligosaccharides as isomaltose, panose and maltotriose. Characterisation of the higher saccharides was not possible due to very low yields. These experimental data would suggest the formation of oligosaccharides by the process of transglucosylation.

However, on incubation of glucose (1 gm) with enzyme powder under the same conditions, it was observed that the formation of oligosaccharides had occurred. This suggested that oligosaccharides could also be synthesised by the enzymic polymerisation of glucose and therefore, in the case of maltose, the synthesis was not entirely due to transglucosylation.

It is possible that the synthesis of oligosaccharides may be due to the action of two separate enzymes, i.e. the enzymic formation of disaccharides by one enzyme and subsequent transglucosylation of disaccharides to yield higher saccharides. The ability of yeast maltase to synthesise the disaccharide maltose from concentrations of glucose has been reported by Hill, Leibowitz and Pringsheim and may occur according to the following reaction.

\[ \text{glucose} + \text{glucose} \rightleftharpoons \text{maltose} \oplus \text{water} \]

Analysis of the enzymic digest with glucose showed the formation of isomaltose after only 2 days, i.e. enzymic polymerisation of two glucose radicals
had occurred at C₆ of a non-reducing glucose residue. This may also be the case with the formation of isomaltose obtained from the enzymic digest containing maltose.

The formation of panose can be attributed to either the transfer of a glucosyl residue to the reducing group of isomaltose or by the transfer of an α-glucosyl residue to the C₆ position of maltose. The latter reaction has been shown for maltase preparations from other biological sources.

The enzymic polymerisation of glucose by Aspergillus niger NRRL 530, has been reported by Peat, Whelan and Hinson (154) who obtained isomaltose, maltose and α-trehalose from a concentrated solution of glucose. They have also reported the formation of panose, isomaltose by transfer of glucosyl units from maltose by the aforesaid preparation.

This suggests that the synthetic action of the enzyme system from brewer’s yeast closely resembles that from Aspergillus niger NRRL 530. In this respect, the yeast maltase preparation differs markedly from maltases from other biological sources.
The enzymes present in brewer's yeast involved in the degradation and synthesis of amylopectin and glycogen-type polysaccharides, and maltose have been investigated.

The various protein preparations obtained by acetone fractionation of sodium bicarbonate extracts of brewer's yeast were found to be rich in isomylase, branching enzyme and maltase. These enzymes catalyse respectively the hydrolysis of α-1:6-glucosidic linkages, the synthesis of α-1:6-glucosidic linkages, and the hydrolysis of maltose and higher related saccharides.

The acetone fractionation of baker's yeast extracts has also been carried out.

Brewer's yeast isomylase which catalyses the hydrolysis of non-terminal α-1:6-glucosidic linkages has been studied in detail. Since the original enzyme preparations were contaminated with traces of maltase, isomaltase and branching enzyme, attempts have been made to effect further purification by various methods. The effect of pH and various organic and inorganic inhibitors on the activity of isomylase has been studied together with its action on certain branched polysaccharides. The chemical and enzymic analysis of isomylase-treated glycogen and amylopectin have further confirmed the mode of action of the enzyme in hydrolysing the outermost α-1:6-inter-chain linkages in these polysaccharides. The experimental data have established the non-identity of isomylase, branching enzyme and maltase.

A study of the branching enzyme of brewer's yeast responsible for the introduction of α-1:6-linkages into both linear and branched α-1:4-glucosans was carried out. This enzyme acted on both amylose and amylopectin producing
more highly branched polysaccharides having structures closely resembling those of amylopectin and glycogen respectively. The rigid characterisation of the synthetic polysaccharides by chemical and enzymic methods was achieved.

The hydrolytic and synthetic function of brewer’s yeast α-glucosidase (maltase) has also been studied. The enzyme hydrolyses maltulose and maltotetraose in addition to the various α-glucosides reported in the literature. On incubation with a concentrated solution of maltose, oligosaccharides are synthesised. These have been separated, and characterised as isomaltose, panose and maltotriose. The same enzyme preparation also catalyses oligosaccharide synthesis from glucose.
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