University Of Edinburgh.

Studies on the Synthesis and Degradation of α-1:4-Glucosans by Yeast Enzymes.

Thesis presented for the Degree of Doctor of Philosophy

by

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Preface.

The experimental work described in this thesis was carried out under the supervision of Professor E. L. Hirst, F.R.S., and Dr. D. J. Manners from October 1953 to May 1956.

Some of the work has been incorporated into the following publications:-

Section II. Manners, D. J. and Khin Maung, J. Chem. Soc. (1955), 867.

Section IV. Manners, D. J. and Khin Maung, Chem. and Ind. (1955), 950.

Section V. Manners, D. J. and Khin Maung, A communication entitled "Yeast Branching Enzyme" presented at the 350th meeting of the Biochemical Society held in the University of Glasgow on 13th April, 1956.

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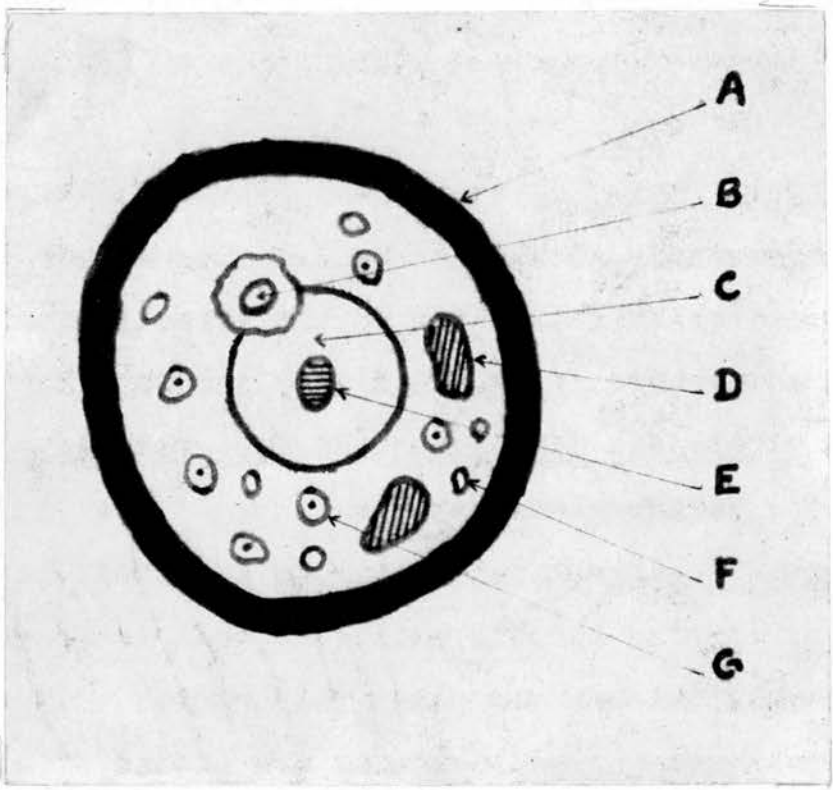
S E C T I O N I.

INTRODUCTION

The use of yeast for brewing and baking has been practised by man for many centuries without a knowledge of the nature of yeast itself. Originally the name was a mere description of its function; the English word "yeast" is derived from a Greek word meaning "I boil" and the French "levure" from the Latin "levere" - to raise. In 1680 Leeuwenhoek examined yeast under his newly invented microscope and found that it consisted of a colony of very small spherical or oval bodies. Later it was shown that these bodies (2-10μ) are isolated cells which reproduce by budding. In 1860 Pasteur(103) demonstrated that in the absence of living yeast, fermentation does not take place. Since that time it has been recognised that the yeast cell, with its thick wall, belongs to the plant kingdom; it is also a fungus because of its dependence on external food. Thus a conception of the yeast grew up, as a unicellular group of fungi, reproducing by budding, and characterised by its ability to cause fermentation.

1. The structure of yeast cells.

Like plant cells, yeast cells (Fig.1) have a well defined wall, quite thick in old cells, which encloses



Key:
- A, Cell wall.
- B, Nucleus.
- C, Vacuole.
- D, Glycogen deposits.
- E, Volutin granule in the vacuole.
- F, Fat globules.
- G, Basophilic granules.

Figure 1. The yeast cell.

a protoplast containing a "nucleus" and "vacuole" and many other visible inclusions. The whole may be embedded in a rather vaguely defined layer of capsular material.

(a) Capsular material:- Hansen[50] first observed that yeasts sometimes secrete a mucilaginous material in which the yeast cells themselves remain closely embedded. Aschner and co-workers[3] reported that the capsular material in Torulopsis neoformans and Tor. rodundata consisted of a pentosan and amylose.

(b) The cell wall:- Initially the cell wall is very thin and remains elastic while the cell is growing. Later it becomes thicker and relatively rigid. Chemical components recognised histologically are pectic substances, hemicelluloses, cellulose, yeast gum and chitin. Recent analytical studies by Northcote and Horne[100] show that it consists of two layers, believed to be glucan-lipid and mannan-protein with some glycogen granules adhering internally.

(c) The protoplast:- It is relatively devoid of structure in the young cells but becomes progressively filled with various visible inclusions as the cell ages.

(i) Although the cytoplasm of actively dividing cells appears homogeneous, inclusions are visible in resting cells.

(ii) Glycogen accumulates in old and well nourished

cells. This is the reserve carbohydrate of yeast, the normal form in which energy is stored and it disappears during starvation.(78)

(iii) Fat appears in the cytoplasm of cells fed with sugar in the form of refractile globules.

(iv) Pigments are responsible for the colour of yeast; in a pigmented strain of Saccharomyces cerevisiae, the pigment is a quinoid prosthetic group carried on a polypeptide.

(v) There is usually one large central vacuole, though there may be others near the poles of elongated cells.

(d) The nucleus:- The nucleus, first described by Guilliermond, is a small and relatively dense body situated at one side of the central vacuole. The internal structure of the nucleus is still unknown.

2. The chemical composition of yeast cells.

The chief elements present in yeast are carbon, hydrogen, oxygen and nitrogen which normally account for as much as 94% of the dry matter. They are mainly present in the form of carbohydrates and nitrogeneous substances although lipids, sterols and vitamins, especially members of the B group, are also present in yeast. In addition, various inorganic substances have been reported in yeast.

(a) Water:- The chief component of yeast is water

comprising 65-68% of the wet weight, which includes some water adhering to, and not inside, the cells. Yeast has been considered as a "solid phase" or mass of yeast cells distributed amongst a "liquid phase," which consists of water surrounding the cells. The solid phase being the actual yeast cells which is composed of the dry matter of the yeast and water enclosed within the outer cell membrane.(137)

(b) Nitrogen compounds:- The principal nitrogen containing substances present in yeast include proteins, amino acids, purine and pyrimidine bases (adenine, guanine, cytosine and uracil) together with very small quantities of materials possessing considerable physiological activity (respiratory pigments [e.g., cytochrome] and vitamins), lecithin and cephalin. Some 70% of the total nitrogen of the yeast is present as protein, 8-10% as purine bases and about 4% as pyrimidines. The remainder of the nitrogen is probably present as soluble compounds such as free amino acids and nucleotides.

(c) Carbohydrates:- A number of carbohydrate substances have been reported in yeast. Glycogen (up to 30% of dry weight) is present and is utilised by yeast cells as reserve carbohydrate. An investigation of the molecular structure of brewer's yeast glycogen will be described in Section II. Mannan or yeast gum (approximately 4%) is present in most yeasts. The yeast cell wall, which

according to Roelofsen[117] comprises 20% of the dry weight of the cell, has a high polysaccharide content (approximately 70%); it also contains about 6% protein. The cell wall polysaccharides consist of approximately equal amounts of glucan and mannan, together with a small quantity of polysaccharide which is believed to be chitin. Other carbohydrates reported to be present in yeast are trehalose, 2-desoxy-D-ribose and hexose phosphates.

(d) Fats:- The fat-soluble fraction of the yeast cell consists of mixtures of true fats (glycerides of fatty acids) together with phospholipids (lecithin and cephalin) and sterols. Fatty acids which have been reported to be present in yeast include palmitic, stearic, oleic, linoleic, myristic and hexadecanoic acids. The sterols of yeast include ergosterol, zymosterol and cervisterol, the ergosterol predominating.

(e) Inorganic constituents:- The chief component is potassium phosphate; other inorganic materials reported to be present in yeast are Na, Mg, Ca, S, Cl, Si, Fe, Cu, Mn and Co. Richards and Troutman[114] reported that B, Ba, Cr, Cu, Au, La, Pt, Mn, Na, Pb, Ag, Tl and Sn have been detected spectroscopically.

3. Alcoholic fermentation.

Prior to the work of Lavoisier, which started in 1784, the nature of alcoholic fermentation was completely unknown

and it was thought that only a sugar and a "ferment" were required for fermentation. Lavoisier[73] made the first detailed chemical analysis of the reactants and the final products, and found that there was a quantitative relationship between the sugar utilised and the amount of the terminal products.

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

In those days, yeast was regarded as an organic chemical compound of animal nature. Thenard[129] in 1803 noted that yeast itself underwent a change, since after fermentation, it lost weight and could exert no further action. In 1810 Gay-Lussac, working with preserved grape juice,[39] found that fermentation did not occur unless air was present, and in 1837 Cagniad-Latour,[22] Kützing[70] and Schwann,[120] working independently of each other, advanced the hypothesis that yeast was a living organism. This hypothesis was repudiated by such eminent chemists as Berzelius,[16] Wohler[138] and Liebig.[77]

It was the classical researches of Pasteur[103,104,105] which settled the dispute. In his studies he came to the following conclusions:- (a) alcohol and carbon dioxide are not the only products formed during fermentation; glycerol, succinic acid and other substances were also produced, (b) there was a definite correlation between sugar fermented and life of the yeast, and that fermentation was impossible without yeast, (c) decomposition of sugar

by yeast was an anaerobic process in which yeast obtained energy by the breakdown of intermediate substances, (d) yeast could be cultivated in a manner similar to bacteria.

For the next twenty years, a controversy prevailed as to whether a living substance, as Pasteur suggested, was absolutely essential for alcoholic fermentation. Traube[130] advanced the theory that definite chemical substances (enzymes), formed within the cells, were responsible for fermentation. Although no direct experimental proof was available, many investigators favoured this hypothesis. In 1897 E. Buchner[21] settled this question by preparing a yeast juice and showed that fermentation without intact yeast cells was possible. He named the substance which was responsible for fermentation "Zymase." Harden and Young[54] later found that yeast juice contained a dialysable and thermostable substance, and that zymase was not active in the absence of this substance; it was called "Cozymase." The chemical equation expressing the overall reaction, $C_6H_{12}O_6 \rightarrow 2C_2H_5OH + 2CO_2$ has been shown to be more complex than was originally realized.

The importance of inorganic phosphate in yeast fermentation was first recognised by Wroblewski[139] who found that the rate of fermentation was increased when sodium phosphate was added. Iwanow[66] in 1905 observed

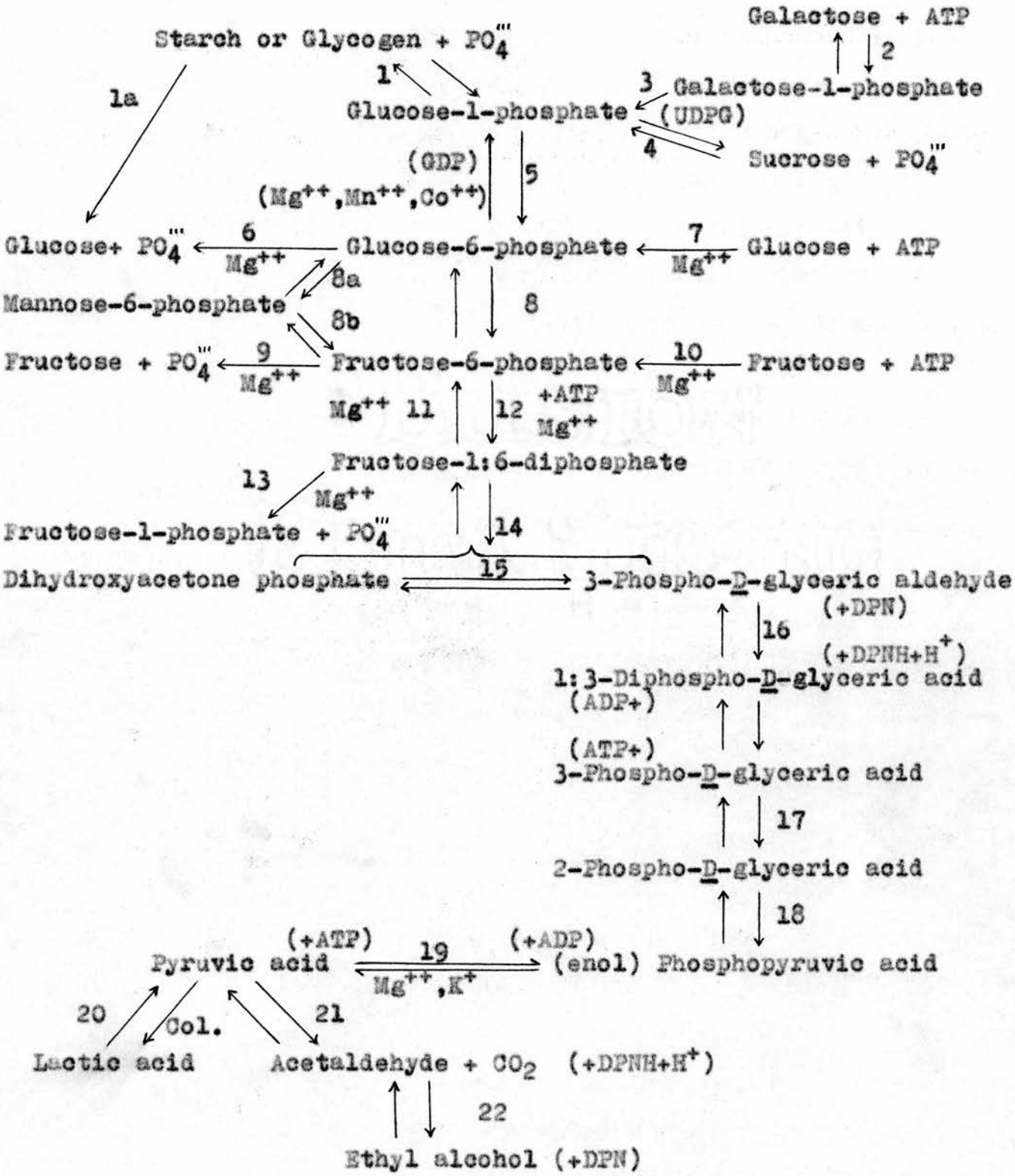
that living yeast could convert inorganic phosphate into organic phosphate. Harden and Young, in a series of investigations[53,54] found that if boiled yeast juice is added to a yeast extract supplemented by added phosphate, there is an increase in the rate of fermentation indicating that a definite chemical reaction takes place between the sugar present and the added phosphate. They found that for every molecule of glucose which formed carbon dioxide and alcohol, one molecule of sugar was esterified to hexose diphosphate as follows -

$$2C_6H_{12}O_6 + 2Na_2HPO_4 \longrightarrow 2CO_2 + 2C_2H_5OH + C_6H_{10}O_4(PO_4Na_2)_2 + 2H_2O.$$

This is referred to as the Harden and Young equation. Later Harden and Robinson[51] showed that there is also present in yeast juices, in addition to hexose diphosphate, a "hexose monophosphate" (an equilibrium mixture of glucose and fructose monophosphates). After further investigation, Robinson[116] suggested that "hexose monophosphate" was an intermediate in the formation of hexose diphosphate.

Contemporary knowledge of the understanding of the enzymic breakdown of monosaccharides commenced in 1902 when Magnus-Levy[80] suggested that acetaldehyde was an intermediate product in the fermentation of sugars. Neubauer[97] in 1910 observed that pyruvic acid was easily fermented by yeast and appeared to be an intermediate product in alcoholic fermentation. This finding was confirmed by Fernbach and Schoen who isolated pyruvic acid

Embden-Meyerhof-Parnas Scheme.(125)



Scheme 1. **Alcoholic Fermentation and Glycolysis.**

KEY

ATP = adenosine-triphosphate

UDPG = uridine diphosphoglucose

GDP = glucose-1:6-diphosphate

1. Phosphorylase, branching enzyme and debranching enzyme.

1a. Amylases and maltase.

2. Galactokinase.

3. Phosphogalactose isomerase.

4. Sucrose Phosphorylase.

5. Phosphoglucomutase.

6. Phosphatases.

7. Hexokinase.

8. Phosphohexose isomerase.

8a and 8b. The reactions are uncertain. G-6-P, F-6-P and M-6-P form an equilibrium mixture.

9. Phosphatases.

10. Fructokinase (Hexokinase).

11. Phosphatases.

12. Phosphofructokinase.

13. Phosphatases.

14. Aldolase.

15. Phosphotriose isomerase.

16. Diphosphoglyceric aldehyde dehydrogenase.

17. Phosphoglyceromutase.

18. Enolase.

19. Phospho enol transphosphorylase.

20. Lactic dehydrogenase.

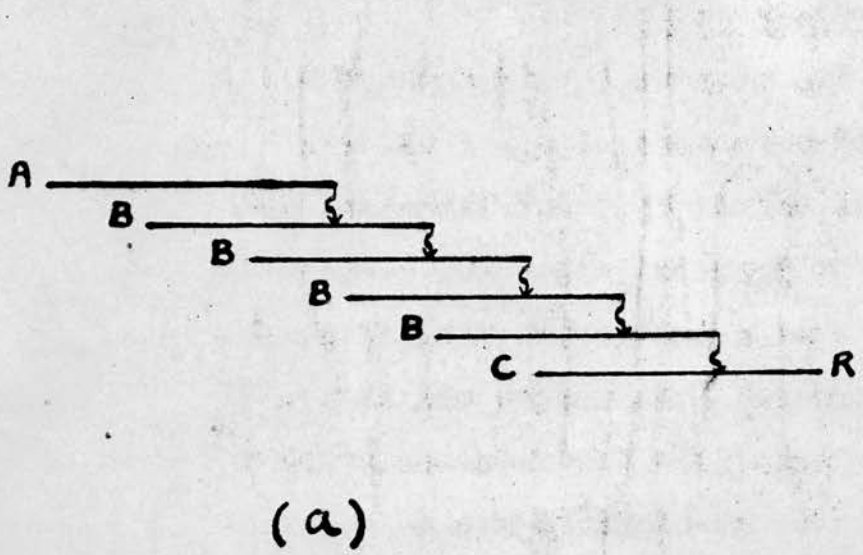
21. Carboxylase.

22. Alcohol Dehydrogenase.

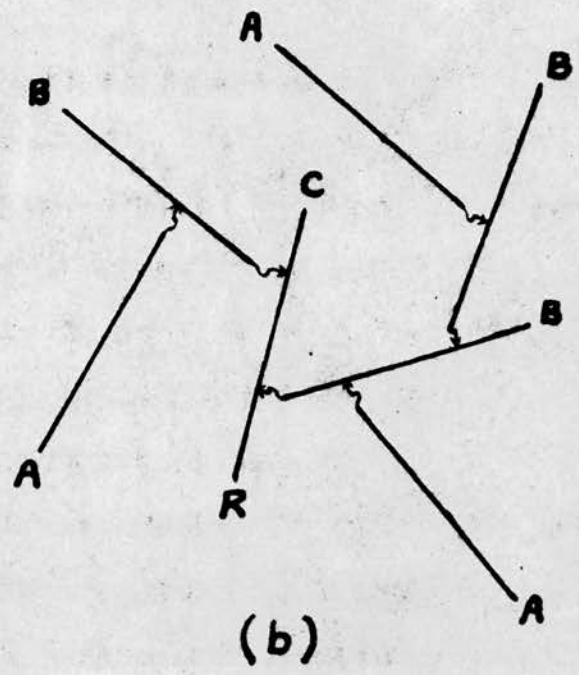
as the calcium salt from fermentation mashes containing living yeast. An important contribution was then made by Embden who pointed out that diphosphoglyceric acid and phosphoglyceric acid, which had been isolated originally from pig's blood[45] and muscle extract, were intermediates in alcoholic fermentation.

Thus the overall picture of the mechanism of yeast fermentation at this time was not too well understood. However, the further investigations of Embden, Meyerhof, Parnas and others have greatly clarified our knowledge of alcoholic fermentation. This progress has been greatly aided by parallel investigations of glycolysis in muscle. The two processes differ since in muscle, lactic acid is formed while in fermentation, alcohol is the terminal product.

Most of the enzymes involved in alcoholic fermentation have been extensively studied and some isolated in the crystalline form. Very little attention has however been paid to the enzymes involved in reaction 1 (Scheme 1) and this thesis is mainly concerned with studies of these enzymes. However, before considering these enzymes, a brief account of the structure of their substrates (starch and glycogen), and of related enzymes in plant and animal tissues, will be given.



(a)



(b)

Key: (a) Laminated structure.

(b) Multiply branched structure.

——— : Linear chain of α-1:4-linked glucose residues.

⌇ : α-1:6-glucosidic linkage.

A,B,C : Type of unit chain.

R : Free reducing group.

Figure 2. Structure of amylopectin.

4. Enzymes involved in degradation and synthesis of α-1:4-glucosans.

Starch consists of two component polysaccharides:- (i) amylose, a linear molecule consisting of several thousand glucose residues united by α-1:4-linkages, and (ii) amylopectin, a highly branched molecule, composed of several hundred unit chains each consisting of 20-25 α-1:4-linked glucose residues; the chains are interlinked by glucosidic linkages from the reducing group to $C_{(6)}$ of a glucose residue in an adjacent chain.

Methylation studies of amylopectin led Haworth and Hirst[57] to postulate a singly branched "laminated" structure. In contrast Meyer[90] proposed a multiply branched "tree" structure for the amylopectin molecule, to explain the results obtained from enzymic degradation studies. (Fig.2).

The two structures contain three types of unit-chains,[111] each of which is linear and composed of α-1:4-linked glucose residues. (i) A-chains are linear and are linked only by an α-1:6-linkage to an adjacent chain. (ii) B-chains are the chains to which one or more A-chains are attached and which is itself linked by an α-1:6-linkage from the reducing group to an adjacent chain. (iii) C-chains, to which other chains are attached, and carry the sole free reducing group in the molecule.

The "laminated" and the "tree" structure differ in

the ratio of A:B chains ($\overline{AB}$); in the former, the ratio is 1:(n-2) where n is the number of chains in the molecule, whereas in the "tree" structure there are approximately equal number of A and B chains.

Glycogen (animal "starch"), a highly branched molecule, consists of several thousand unit chains (12-18 α-1:4-linked glucose residues) interlinked by α-1:6-linkages. Thus the "interior chains" (that part of a unit chain between two branch points) and the "exterior chains" (those parts of a unit chain between the branch point and the non reducing terminal group) are shorter than those of amylopectin.

(a) Amylases.

Three types of amylolytic enzyme have been reported to be present in the plant and animal kingdom.

(i) α-Amylase:-

α-Amylases catalyse a random hydrolysis of α-1:4-linkages in starch and glycogen producing maltose and α-dextrins consisting of 6-10 glucose residues. The reaction can be followed by measurement of the rapid decrease in viscosity, turbidity or iodine staining power of the substrate. α-Dextrins from amylose are linear molecules, whereas those from amylopectin or glycogen have branched structures, since α-amylases cannot hydrolyse the α-1:6-glucosidic inter-chain 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.[81,89]

(ii) β-Amylase.

β-Amylases catalyse a stepwise hydrolysis of alternate α-1:4-glucosidic linkages, from the non reducing end of a chain of glucose residues, with the liberation of β-maltose. The enzyme action is arrested by the presence of anomalous linkages in the chain. Since β-amylase cannot by-pass such linkages, the interior chains in branched α-1:4-glucosans are not attacked. Linear amylose molecules are completely degraded by β-amylase but amylopectin and glycogen yield maltose and a β-dextrin of high molecular weight, which differs from the original polysaccharide in that the exterior chains contain only two or three glucose residues. β-Amylase occurs in germinated and ungerminated cereals and in rice, sweet potatoes and soya-beans.

(iii) "Glucose-producing" amylases.

Glucose producing amylases catalyse a stepwise hydrolysis of successive α-1:4-glucosidic linkages beginning at the non-reducing terminal linkage. Although these enzymes cannot hydrolyse the α-1:6-linkages, they can by-pass them and thereby attack the interior chains. Phillips and Caldwell[112] reported that such an amylase from the mould Rhizopus delemar liberated over 90% of the glucose from amylose, amylopectin, glycogen and a β-dextrin.

(b) Debranching enzymes:-

Five debranching enzymes which hydrolyse the α-1:6-interchain linkages had been reported recently.

(i) R-Enzyme.

Hobson, Whelan and Peat[63,110] isolated R-enzyme in purified form from potato and broad-bean. R-Enzyme hydrolyses the inter-chain linkages in amylopectin and β-dextrin. α-Dextrins are also attacked by R-enzyme giving a mixture of linear maltosaccharides. However, R-enzyme cannot hydrolyse the α-1:6-linkages of glycogen and the reason put forward by the authors is that glycogen, with its short interior chains, is an extremely compact molecule and hence, the formation of an enzyme-substrate complex near the 1:6-linkage is sterically hindered.

(ii) Amylo-1:6-glucosidase

Cori and Larner[33] 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.

(iii) Oligo-1:6-glucosidase

Larner and McNickle[72] discovered an enzyme "oligo-1:6-glucosidase" in intestinal mucosa which catalysed the hydrolysis of α-1:6-linkages in α-limit dextrins, iso-maltose and panose. It had no action on muscle phosphorylase limit dextrins.

(iv) Limit dextrinase

Hopkins and Wiener[64] reported that brewery malts contained an enzyme "limit dextrinase" which hydrolysed terminal α-1:6-linkages of "limit dextrins" producing glucose. Evidence to show that limit dextrinase hydrolysed the non-terminal α-1:6-linkages was not however obtained.

(v) Isoamylase

Maruo and Kobayashi[86] reported that brewer's yeast autolysates contained an enzyme which hydrolysed the inter-chain linkages in glutinous rice starch producing a more linear polysaccharide of lower molecular weight. This enzyme, provisionally named "isoamylase," and previously referred to as "amylosynthease," is similar to R-enzyme since it hydrolyses non-terminal α-1:6-linkages in amylopectin.

An investigation of the properties of this debranching enzyme will be described in Section IV; it is shown that isoamylase has a wider specificity than R-enzyme, amylo-1:6-glucosidase, oligo-1:6-glucosidase or limit dextrinase.

(C) Phosphorylase.

Phosphorylases catalyse the following reaction. $[\alpha\text{-}1{:}4\text{-Glucosan}]_n$ + Inorganic Phosphate $\rightleftharpoons$ $[\alpha\text{-}1{:}4\text{-Glucosan}]_{n-1}$ + α-Glucose-1-Phosphate. The reversibility of the phosphorylase activity was shown by Kiessling[69] and Cori,

Schmidt and Cori.[27] The enzyme action is specific in that it can only degrade or synthesise α-1:4-linkages. 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, liberating inorganic phosphate.

The synthetic polysaccharide formed from glucose-1-phosphate by muscle phosphorylase was shown by Hassid, Cori and McCready[56] to resemble the amylose component of starch. Phosphorylase degrades synthetic amylose completely. Like β-amylase, phosphorylase acts upon the branched α-1:4-glucosans attacking the non-reducing ends and splitting off successive glucose residues until it encounters an α-1:6-glucosidic linkage (the branching point) which acts as an obstruction.

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 animal and plant phosphorylases are identical but their properties are different. Some criteria which differentiate muscle and potato phosphorylase, and a description of the phosphorylase of brewer's yeast will be given in Section III.

(d) Branching enzymes.

Branching enzyme, which could convert amylose into a branched polysaccharide similar to glycogen, was first obtained from liver, heart and brain extracts by Cori and Cori.[31]

The existence of a branching enzyme in plant tissues was first demonstrated by Haworth, Peat, Bourne and Macey.[17,19,58] This enzyme is referred to as "Q-enzyme." Q-Enzyme performs a double function, namely the formation of α-1:6-linkages, which follows the scission of an equal number of α-1:4-linkages. Q-Enzyme thus acts on amylose producing branched polysaccharides which have structures similar to amylopectin.

The branching enzyme of brewer's yeast will be described in Section V.

5. Use of enzymes in structural studies.

The action patterns of the above enzymes have been determined using purified amylose, amylopectin and glycogen of known molecular structure. It follows therefore that these enzymes may be used to investigate the fine structure of starch-type polysaccharides from a variety of biological sources.

(a) α-Amylase

α-Amylase has been used to detect α-1:4-glucosidic linkages. Polysaccharides which contain few, if any,

sequences of two or three adjacent α-1:4-linkages are resistant to α-amylolysis.[83] It can also be used to determine the nature of the α-1:4-glucosans, i.e. branched or linear. α-Amylolysis of linear α-1:4-glucosans gives a high R_m value (apparent percentage conversion into maltose) whereas that of branched polysaccharides is lower. Paper chromatographic examination of α-amylolytic digests also provides qualitative evidence of branching in an α-1:4-glucosan since α-dextrins are easily distinguished from glucose, maltose and maltotriose.

(b) <u>β-Amylase</u>

β-Amylase has been used for the following purposes:-

(i) to detect the presence of anomalous linkages in amyloses,

(ii) to distinguish qualitatively between linear and branched α-1:4-glucosans,

(iii) to determine the exterior chain lengths of branched α-1:4-glucosans.

(i) The main product of the β-amylolysis of amylose is maltose. β-Amylolyses of various amyloses have been reported by many authors and the percentage conversion into maltose (R_m) varied from 57 to 100%.[83,98] The low limits were not due to retrogradation of amylose during enzyme action, or to the presence of contaminating branched glucosans. In some cases the addition of β-glucosidase to a β-amylolytic digest resulted in complete amylolysis,

suggesting that these amyloses contained one or more β-glucosidic linkages.[107,109]

(ii) The product of the β-amylolysis of a linear α-1:4-glucosan is maltose, whereas branched polysaccharides, e.g. amylopectin or glycogen, gives maltose (40-70%) and a β-dextrin of high molecular weight. The determination of the β-amylolysis limit and the nature of the end products of enzyme action serves as a convenient method for differentiating between linear and branched α-1:4-glucosans even though the chain length of the polysaccharide is unknown.

(iii) The exterior chain length of branched α-1:4-glucosans, which cannot be determined by any chemical method, can be calculated from the β-amylolysis limit and the chain length.

(c) Amylases and R-enzyme.

Whelan and Roberts[135,136] determined the chain length of rabbit liver glycogen by analysis of the products liberated by the successive action of salivary α-amylase and R-enzyme. They obtained the value 12·5, (periodate oxidation gave a chain length of 13·6). They also found 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, thus providing further evidence that glycogen

has a multiply branched structure.

Peat, Whelan and Thomas[106,111] employed β-amylase and R-enzyme to show that amylopectin has a multiply branched structure. Treatment of β-dextrin (from waxy maize starch) with R-enzyme gave a mixture of maltose and maltotriose in 12·8% yield. A singly branched molecule would yield only 0·083% of maltose and maltotriose, whilst a multiply branched structure with equal numbers of A- and B-chains (Fig.2, p. 10) would yield 12·5%.[61]

The combined action of β-amylase and R-enzyme has been used to determine the unit chain length of waxy maize starch[134] This enzymic method gave a chain length of 26; periodate oxidation gave a value of 24-25.

(d) Phosphorylase and amylo-1:6-glucosidase.

The combined action of phosphorylase and amylo-1:6-glucosidase, in the presence of inorganic phosphate, serves as an alternative method of enzymic end-group assay.[33,65] These two enzymes acting on amylopectin or glycogen produce glucose-1-phosphate and glucose; the latter arises from the hydrolysis of α-1:6-linkages by amylo-1:6-glucosidase. By estimating the molar percentage of glucose, the number of α-1:6-linkages and hence the chain length can be calculated. The results obtained with several amylopectins and glycogens are in good agreement with those obtained by methylation and periodate oxidation assays of the same samples.

The stepwise action of phosphorylase and amylo-1:6-

glucosidase on amylopectin and glycogen has provided further evidence that these molecules have multiply branched structures.[71]

SECTION II

Molecular Structure of Brewer's Yeast Glycogen.

1. Introduction

The presence of glycogen in yeast was first indicated by Errera in 1885[36] and in 1894, Cremer[34] succeeded in isolating glycogen from yeast and demonstrating its general resemblance to animal glycogen. Harden and Young[52,55] described a method for preparation of yeast glycogen (α_D + 198·3°), in which glycogen was extracted with water from disintegrated cells of pressed yeast, and separated from yeast gum by precipitation with ammonium sulphate.

In 1925, Ling, Nanji and Paton[79] found that dried yeast is a more suitable source for the extraction of glycogen than pressed yeast. By using 2% sodium hydroxide solution as solvent, they avoided the necessity of disintegrating the cells. However they reported that the glycogen extracted by this method generally contained 0·5-1·0% ash and about 0·25% phosphorus. Yokoyama[140] extracted glycogen from dried yeast with 50% potassium hydroxide solution and purified it by removing gum with Fehling's solution and precipitation of the glycogen (α_D + 192°) with alcohol. Later Daoud and Ling[35] modified the method of Ling, Nanji and Paton[79] by improving the

method of removing copper from the glycogen solution after the mannan has been precipitated by Fehling's solution. The sample they obtained, gave a brownish-red colour with iodine, was free from nitrogen, but contained traces of phosphorus; the ash content was 1·59% and α_D + 179°.

In the extraction of yeast glycogen, the first stage involves breaking the cells so that the glycogen can be brought into solution; there are three methods for this operation:-

(i) mechanical breakage of the cells by grinding with sand followed by extraction with water,[55,67]

(ii) breaking the cells by drying and extraction of the glycogen with (2-3%) sodium hydroxide solution,[35,79,140]

(iii) alkaline cytolysis using (2-3%) sodium hydroxide at 95° followed by the extraction of glycogen from the insoluble material, containing glycogen, with N hydrochloric acid[87] or with 0·5N acetic acid at 75°.[14,99]

Glycogen from baker's yeast had been studied by several workers and recently Northcote[99] has described the molecular structure of this glycogen. Since no structural examinations on brewer's yeast glycogen have been reported, the molecular structure of brewer's yeast glycogen has been investigated. In this study, alkaline cytolysis and extraction with 0·5N acetic acid was used for the preparation of the glycogen.

2. Analytical methods and materials.

(a) Determination of reducing sugars.

Reducing sugars were determined by the Shaffer-Somogyi reagent 60[121] as modified by Hanes and Cattle[49] or by the Somogyi 1945 reagent[123] which had been calibrated against glucose and maltose.

(b) Paper chromatography.

Descending chromatograms were carried out at room temperature with Whatman No.1 paper and benzene-pyridine-butanol-water (1:3:5:3) as solvent. Development was by spraying with aniline oxalate or with silver nitrate-sodium hydroxide reagent.[132]

(c) Iodine staining.

Polysaccharide solution (2 ml.; containing 2 mg. of glycogen) was added to iodine solution (1 ml.; containing 1 mg. of iodine and 10 mg. of potassium iodide per ml.) and water 2 ml.; the absorption value of the polysaccharide-iodine complex was measured on a Spekker Photoelectric Absorptiometer in 1 cm. cells, an Ilford filter No.603 being used, against iodine-water blank.

(d) Determination of unit-chain length.

Unit-chain length was determined by the oxidation of glycogen with potassium periodate[12,46] and measurement of the maximum amount of formic acid produced.

(e) Salivary α-amylase. (Prepared by Dr. D. J. Manners).

Salivary α-amylase solution was prepared by dissolving freeze-dried human saliva in distilled water, and removing insoluble materials by centrifugation. The amylase solution showed no maltase activity, but was contaminated with maltotriase. During the digestion of waxy maize starch, maltose, maltotriose and α-dextrins were the initial products of the reaction, and glucose, maltose and α-dextrins the end-products.(135,136)

(f) β-Amylase.

β-Amylase was prepared from soya beans by Bourne, Macey and Peat's method.(17) A solution of β-amylase was prepared by dissolving soya bean β-amylase (50 mg.) in 0·2M acetate buffer (pH 4·6; 20 ml.), and removing insoluble material by centrifugation; the supernatant liquid had an activity of 125 units per ml., estimated by Hobson, Whelan and Peat's method.(62) Preliminary experiments with maltose and starch showed that the enzyme solution was free from maltase and α-amylase.

3. Experimental.

(a) Preparation of brewer's yeast glycogen.

1·5 Kg. of washed brewer's yeast, dispersed in 3% sodium hydroxide solution (1 l.) was heated at 95° for 6 hrs.(100) The mixture was cooled, the cell-wall material

was collected on the centrifuge and treated again with hot 3% sodium hydroxide solution. The sodium hydroxide extracts did not contain any appreciable amount of glycogen. The glycogen was extracted from the cell-wall material by three successive treatments with 0·5N acetic acid (each 500 ml.) at 75° for 2 hr.[14,100] The combined acetic acid extracts were concentrated under reduced pressure to about 500 ml., and ethanol (6 vols.) was added. The crude precipitate of glycogen was purified by three precipitations from 80% acetic acid[15] and finally several precipitations from ethanol. The yield was 17·7 g.

(b) Properties of the glycogen.

(i) White amorphous powder. Found: Nitrogen, 0.05%; Phosphorus, nil; Ash, 0·10%. An aqueous solution was opalescent, and stained red-brown with iodine.

(ii) Specific rotation:-

In water $[\alpha]_D$ + 198° (C, 0·252; α = 2·00°; l = 4)

In N NaOH $[\alpha]_D$ + 175° (C, 0·250; α = 1·75°; l = 4).

(iii) Acid hydrolysis of the glycogen (10 mg.) in 1·5N sulphuric acid (2 ml.) at 100° for 2 hrs. gave glucose and no other sugar (paper chromatography). Quantitative acid hydrolysis of the glycogen was determined by Pirt and Whelan's method.[113] 0·5 ml. of glycogen solution (1·04 mg. glycogen) was hydrolysed for 2 hrs. in 1·5N sulphuric

acid at 100°. By the Somogyi 1945 reagent, the hydrolysate contained 1·10 mg. glucose, i.e. percentage hydrolysis 96.

(c) Potentiometric titration.

The iodine binding power of the glycogen was quantitatively determined by Mr. D. M. W. Anderson, using the potentiometric titration method described by Anderson and Greenwood.(2) It gave a typical glycogen curve, no coloration was produced in the titration flask and the uptake of iodine was very similar to that of mammalian glycogens.

(d) Molecular weight determination.

The sedimentation constant of the glycogen was determined by Dr. C. T. Greenwood using an ultracentrifuge (Spinco Model E). Although polymolecular, the glycogen sedimented as one component, the sedimentation constant (S_{20}) being 52 x 10^{-13} c.g.s. units equivalent to a molecular weight of ca. 2 x 10^6, the diffusion constant being assumed to be of the same order as that of other glycogens.(11)

(e) Salivary α-amylolysis.

An enzymic digest was set up containing glycogen (50·0 mg.), phosphate-citrate buffer (0·16M with respect to phosphate) of pH 7·0 (20 ml.), sodium chloride (25.0 mg.), salivary α-amylase solution (1 ml.), and water (29 ml.). Aliquot portions were analysed for iodine staining power, and for maltose, at intervals, with Shaffer-Somogyi

reagent 60, after incubation at 35°.

The decrease in iodine-staining power was as follows:

Time of incubation (mins.)	0	4	10	20
Absorption value	0·305	0·035	0·020	0·010

The apparent percentage conversion into maltose after 1, 2 and 48 hrs' incubation was 62, 68 and 95 respectively.

Paper chromatography of the amylolytic digest showed the presence of glucose (R_G = 1), maltose (R_G = 0·55), and a series of sugars of higher molecular weight (R_G < 0·09). Maltulose was absent.

(f) Potassium periodate oxidation.

(i) Glycogen (549·0 mg.) was dissolved in 5% potassium chloride solution (100 ml.) contained in a brown bottle; 10 ml. were withdrawn for a control determination. 8% (w/v) sodium periodate (20 ml.) was added to the bulk which was gently agitated (on rollers). 10 ml. samples were removed at intervals, excess periodate was decomposed by the addition of 1 ml. ethylene glycol and the formic acid produced was determined by titrating with 0·01N sodium hydroxide solution in a carbon dioxide free atmosphere, methyl red being used as indicator.[12,46]

The following results were obtained.

Time (hours)	96	168	266	386
Total formic acid produced (mg.)	8·9	10·2	10·6	10·5
Apparent chain-length (glucose residues)	15·6	13·6	13·2	13·2

A 12-unit or 14-unit glycogen would yield 11·6 or 10·0 mg. of formic acid, respectively. In a duplicate experiment, 302·3 mg. glycogen produced 6·5 mg. formic acid, corresponding to the apparent chain length of 13·1 glucose residues.

(ii) The remaining solution of periodate-oxidised glycogen was neutralised with ethylene glycol (5 ml.) and dialysed against running tap water for 36 hrs., and the non-diffusible material collected by freeze-drying. 50 mg. of the periodate-oxidised glycogen were hydrolysed by 2N-sulphuric acid (2 ml.) at 100° for 3 hrs. No glucose could be detected in the hydrolysate by paper chromatography; brewer's yeast glycogen does not therefore contain 1:2 or 1:3-glucosidic linkages.

(g) β-Amylolysis of the glycogen.

Glycogen (48·4 mg.) was incubated with 0·2M-acetate buffer (pH 4·6; 6 ml.), water (21 ml.), and β-amylase solution (3 ml.; 375 units) at 35°. Samples (3 ml.) were withdrawn at intervals and analysed for maltose with the Somogyi (1945) reagent.

The course of degradation was as follows:-

Time of incubation (hours)	1	2	20	44
% Conversion into maltose	30·7	38·4	43·5	44·0

In a duplicate experiment with 50·4 mg. of glycogen, the β-amylolysis limit was 43·8%.

Examination of the β-amylolytic digests by paper

chromatography showed the presence of maltose, and no other reducing sugar.

(h) Phosphorolysis of the glycogen.

The following experiment was carried out by Miss A. M. Liddle.[75] The phosphorolysis limit of the glycogen was determined with crystalline rabbit-muscle phosphorylase, prepared by Green and Cori's method.[43] Glycogen (46·0 mg.) was incubated at 35° with 0·5M-phosphate buffer (pH 6·8; 4·0 ml.), 0·01M-adenosine-5'-monophosphate (2·0 ml.), muscle phosphorylase solution (0·5 ml.; 3680 units) and water to a total volume of 20 ml. Glucose-1-phosphate produced was determined by a slight modification of Allen's method.[1,75]

The following results were obtained:-

Time of incubation	1 min.	1 hr.	4 hr.	24 h
% Conversion into glucose-1-phosphate	5·1	30·0	30·0	30·(

In a duplicate experiment with 47·0 mg. glycogen and 15,000 units of muscle phosphorylase, the phosphorolysis limit was 31%.

4. Discussion.

The experimental evidence shows that brewer's yeast glycogen is generally similar to mammalian glycogens. Brewer's yeast glycogen is dextro-rotatory (α_D + 198°) and is soluble in water giving an opalescent solution which is stained red brown with iodine. Potentiometric titration

shows the iodine binding power to be similar to that of other glycogens. It is composed solely of glucose residues since acid hydrolysis yields glucose (96%) and no other reducing sugar. Furthermore the absence of maltulose in the α-amylolytic digest shows that fructose is not a constituent of the glycogen.(108)

When brewer's yeast glycogen is subjected to salivary α-amylase action, a rapid loss of iodine staining power is observed and glucose, maltose and α-dextrins are obtained as final products. The glucosidic linkages in the glycogen are therefore predominantly of the α-1:4-type. Potassium periodate oxidation of the glycogen indicates a unit-chain length of thirteen glucose residues; the absence of 1:2 or 1:3-linkages in the glycogen is shown by the fact that glucose is absent from the hydrolysate of the periodate-oxidised glycogen.(13,60) Thus brewer's yeast glycogen contains only α-1:4 and 1:6-glucosidic linkages.

The action of β-amylase on the glycogen results in a 44% conversion into maltose, which is the only reducing sugar liberated. The exterior chains therefore comprise 8 glucose residues and the interior chains, on the average, contain 4 glucose residues. Brewer's yeast glycogen is attacked by crystalline muscle phosphorylase in the presence of excess of inorganic phosphate producing glucose-1-phosphate, the limit being 30%. The phosphorolysis limits of a 12-unit and 13-unit glycogen from various animal tissues vary from 29 to 36.(75,76) The glycogen has a molecular

weight of ca. 2×10^6 (obtained from the sedimentation constant).

Thus brewer's yeast glycogen consists of ca. 10^3 unit chains, each comprising, on the average, 13 α-1:4-linked glucose residues, which are randomly interlinked by 1:6-glucosidic linkages. It therefore resembles the majority of the glycogens from mammalian, invertebrate and protozoan tissues;[84] nevertheless, small but significant differences in degree and position of branching between baker's yeast and the brewer's yeast glycogen are revealed (Table 1).

Table 1.

A comparison of the properties of baker's yeast and brewer's yeast glycogens.

Properties	Baker's Yeast A	Baker's Yeast B	Brewer's yeast
$[\alpha]_D$ in water	+184°	+187°	+198°
Unit-chain length	11-12	-	13
β-Amylolysis limit	50	46-48·5	44
Exterior-chain length*	8	-	8
Interior-chain length†	2-3	-	4

A, Data from Northcote.[99]

B, Data from Jeanloz.[67]

* Number of glucose units removed on β-amylolysis plus 2·5.

† Unit-chain length - Exterior-chain length - 1.

It is not unexpected that the glycogen from baker's yeast and brewer's yeast show small differences in molecular structure, since the conditions of growth and fermenting properties of the two yeasts are different; furthermore the enzymic composition is not the same. The unit-chain length of the glycogen and the position of branching in the chains will depend upon the "balance" between the activities of phosphorylase and the branching and debranching enzymes. Thus the structure of the glycogen will be related to the metabolic condition of the organism at the time of isolation of the glycogen.

Section III

Studies on Brewer's Yeast Phosphorylase

1. Introduction

The reaction between glycogen and inorganic phosphate yielding hexose phosphate, was discovered by Parnas and Baranowski[101,102] who called this enzymic process "phosphorolysis" by analogy with the hydrolysis produced by amylases. Glucose-1-phosphate was isolated by Cori and Cori[25] and shown to be the product of phosphorolytic breakdown of glycogen in muscle.

In 1938, Schaffner and Specht[118,119] showed that glycogen was converted by fresh or dialysed yeast juice into hexose monophosphate. No cozymase was required, and the presence of arsenate or iodoacetate had no effect on enzyme action. They also reported that by adjusting the pH of a dialysed extract of dried yeast to 5·5, phosphorylase precipitated out. Cori and Cori[28] also reported that dialysed extracts of dried brewer's yeast formed glucose-1-phosphate from glycogen and inorganic phosphate under conditions in which glucose was not phosphorylated. They showed that at pH 4·5 more glucose-1-phosphate than 6-phosphate was formed; the reverse was found at pH 7·5. Activity determinations carried out by estimating both glucose-1-phosphate and glucose-6-phosphate gave maximum

activity at pH 7.5.

Kiessling[68,69] in 1939 prepared a protein fraction C from Lebedew's juice[74] by repeated precipitation with ammonium sulphate solution (0·30-0·35 saturation). C-Protein contained phosphorylase and catalysed the esterification of glycogen with added inorganic phosphate, 15% being converted to glucose-1-phosphate. He also showed that the reaction was reversible and that 85% of the glucose-1-phosphate was synthesised into glycogen, the reaction having an equilibrium constant K = 5·2 at 28° when the total free inorganic phosphate concentration was 2·4-22·2 x 10^{-2}M. By warming C-protein in aqueous ammonium sulphate (0·1 saturation) at 38-40° for 30 mins., and rejecting the precipitate, he obtained phosphorylase (free from glucose-1-phosphatase) which required no co-enzyme for its action.

The specificity of yeast and muscle phosphorylase for the α-1:4-glucosidic linkage was noted by Meyer and Bernfeld[92] and by Hassid, Cori and McCready[56] respectively. Although phosphorylases of muscle and potato have been isolated in highly purified and crystalline forms, very little attention has been paid to the phosphorylase of brewer's yeast. Attempts have therefore been made to purify this enzyme and to study its properties.

Methods which have proved effective in liberating enzymes from microbial cells, e.g. mechanical rupture of the cell, autolysis and chemical treatment, have been employed

to bring phosphorylase into solution. The following methods which gave active extracts will be described:-

(a) mechanical breakage of yeast cells by grinding with sand, and extraction with water,

(b) autolysis of pressed brewer's yeast,

(c) maceration juice or Lebedew's juice,(74)

(d) butanol-water extraction of dried brewer's yeast,

(e) sodium bicarbonate extraction of dried brewer's yeast.

Preliminary experiments showed that phosphoglucomutase, phosphatases, branching and debranching enzymes were present in all the above extracts. Phosphorylase activity tests have therefore been carried out in the presence of appropriate inhibitors (sodium fluoride, mercuric chloride and ammonium molybdate) which had no action on phosphorylase.

2. Methods and materials

Unless otherwise stated, reagents were used as aqueous solutions.

(a) Brewer's yeast.

Brewer's yeast, supplied by Messrs. William Younger and Company Limited, was thoroughly washed with water; any excess water was removed, using a Buchner funnel. The yeast was spread on shelves and dried by a current of warm air (35°) in a 'Mitchell Dryer.' The dried yeast was then stored in air tight bottles at 0°C.

(b) Reagents for phosphate estimations.

(i) Fiske and Subbarow's reagent.[37]

The original reagent was the filtrate obtained by dissolving 0·5 g. 1-amino-2-naphthol-4-sulphonic acid in 195 ml. 15% sodium bisulphite and 5 ml. 20% sodium sulphite. Since pure sodium bisulphite was not readily accessible, an attempt was made to use the stable sodium metabisulphite. When the reagent was prepared with the theoretical amount of sodium metabisulphite according to the above directions, a fine precipitate was observed in the final coloured solution which made accurate colorimetric determinations impossible. Varying concentrations of sodium metabisulphite and sodium sulphite were tried and the most promising results were obtained with a reagent prepared in the following manner:-

14 g. of sodium metabisulphite were dissolved in about 80 ml. of water in a 100 ml. standard flask. 250 mg. of 1-amino-2-naphthol-4-sulphonic acid and 1 g. of sodium sulphite were added and the solution made up to 100 ml. The solution was filtered and stored in the dark.

(ii) Acid molybdate.

2·5 g. of ammonium molybdate (AnalaR) was dissolved in 100 ml. 5N-sulphuric acid (AnalaR).

The reagent was calibrated against standard potassium dihydrogen phosphate solution (1 mg. inorganic phosphate per ml.) stored over chloroform at 0° C.

(c) Estimation of ester-phosphate.[47,48]

(i) Glucose-1-phosphate.

A solution (2 ml.) containing free inorganic phosphate and "ester phosphate" (ester-P) was treated with 2·5 ml. magnesia mixture (magnesium chloride dihydrate 55 g., ammonium chloride 135 g., concentrated ammonium hydroxide 350 ml., and water to a total volume of 1000 ml.) to remove free inorganic phosphate and the mixture diluted with water to 25 ml. The precipitate was filtered off. The traces of inorganic phosphate remaining in the filtrate were determined by treating 2 ml. with the Fiske and Subbarow reagent. A second portion (2 ml.) of the filtrate was hydrolysed with 0·25 ml. concentrated hydrochloric acid for 10 mins. at 100°. The free phosphate liberated was estimated with the Fiske and Subbarow reagent. The difference between the two estimations represents the ester phosphate.

(ii) Glucose-6-phosphate.

Glucose-6-phosphate was estimated in a similar manner, except that inorganic phosphate was liberated from glucose-6-phosphate by digesting the ester with 60% perchloric acid.

(d) Determination of protein nitrogen.

Protein nitrogen was determined by the biuret method of Robinson and Hogden,[115] yeast protein being used as the standard.

(i) Reagents:- 10% Trichloroacetic acid,
3% Sodium hydroxide,
20% Copper sulphate (penta hydrate).

(ii) Preparation of standard yeast protein.

10 g. dried brewer's yeast was incubated with 0·1M sodium bicarbonate solution (50 ml.) at 35° for 2 hours. The extract was centrifuged (5000 r.p.m.) at 0°C for 15 mins. and the supernatant collected. An equal volume of 10% trichloroacetic acid was added, and the precipitated protein washed several times with 5% trichloroacetic acid, AnalaR acetone, and dried under vacuum over phosphorus pentoxide. The yield was 1·28 g. The protein nitrogen content was determined by the Kjeldahl procedure - Found N, 12·0%. Standard yeast protein solution (1 mg. nitrogen per ml.) was prepared by dissolving 208·30 mg. yeast protein in 3% sodium hydroxide in a total volume of 25 ml.

(iii) Calibration curve.

0·5, 1·0, 1·5, 2·0, 2·5 and 3·0 ml. of the standard protein solution was introduced into 10 ml. graduated centrifuge tubes. The volume was brought to about 9 ml. with 3% sodium hydroxide, 0·25 ml. copper sulphate solution added and the volume made up to 10 ml. with the alkali. After shaking the contents for 1 min., the solutions are allowed to stand for 15 mins. The colour of the supernatant solutions, after centrifugation, are compared on the Spekker Photoelectric Absorptiometer in 1 cm. cells at 550 mμ against

a water blank.

Protein nitrogen (mg.)	0·0	0·5	1·0	1·5	2·0	2·5	3·0
Absorption value (A.V.)	0·04	0·12	0·21	0·30	0·39	0·48	0·57

(e) Determination of phosphorylase activity.

Phosphorylase activity was determined in terms of Green and Stumpf units[44] where one unit of phosphorylase activity is defined as the amount of enzyme which catalyses the liberation of 0·1 mg. of inorganic phosphate from glucose-1-phosphate in 3 mins. at 35° in the following digest.

5-10 units Enzyme solution,
0·5 ml. 0·5M-citrate buffer pH 6·0,
0·2 ml. 5% soluble starch,
1·0 ml. 0·1M-glucose-1-phosphate.
Final volume 3·5 ml.

Since yeast phosphorylase preparations contain phosphoglucomutase, phosphatase, branching and debranching enzyme as impurities, the phosphorylase activity tests were carried out in the presence of inhibitors as in the following digest:-

1·0 ml enzyme solution,
0·4 ml. 2% ammonium molybdate, (phosphatase inhibitor)
0·4 ml. 0·1M-sodium fluoride, (phosphoglucomutase inhibitor)
0·2 ml. 0·01M-mercuric chloride, (branching and debranching enzyme inhibitor)
0·5 ml. 0·2M-citrate buffer pH 6·6,
0·5 ml. 2% soluble starch,
1·0 ml. 0·1M-glucose-1-phosphate,
Final volume 4·0 ml.

Glucose-1-phosphate solution was added after temperature equilibration (35°). The reaction was stopped after 6 mins. by adding 10% trichloroacetic acid (4 ml.) and the volume made up to 10 ml. The solution was centrifuged and 1 ml. of the supernatant analysed for free inorganic phosphate using the Fiske and Subbarow reagent.

(f) Preparation of glucose-1-phosphate.

Glucose-1-phosphate was prepared by the action of potato phosphorylase on starch as described by Hanes[47,48] and purified by means of ion-exchange resins according to the method of McCready and Hassid.[88]

(i) 72 g. ammonium hydrogen phosphate were dissolved in 500 ml. of water,

(ii) 30 g. soluble starch were suspended in 1000 ml. of water and heated at 100° for 30 mins. and cooled,

(iii) 1245 g. skin peeled King Edward VII potatoes were minced and the potato juice squeezed out through two layers of muslin. 400 ml. of potato juice thus obtained were treated with 120 ml. dry Kieselguhr and filtered through a layer of Kieselguhr in a Buchner funnel. 300 ml. juice were obtained.

Solutions (i), (ii) and (iii) were mixed and water added to a final volume of 2·25 l. The pH of the solution was adjusted to 7·0 (bromo thymol blue as external indicator), 10 ml. of toluene was added and the mixture incubated at 35°. The reaction was followed by estimating the amount of

glucose-1-phosphate formed.

Time (hours)	1	2	19	20
Ester phosphate in digest (g.)	0·315	0·793	1·935	2·003

Thus 24·03 g. of glucose-1-phosphate were formed after 20 hours. The digest was boiled, cooled to room temperature, 2 mols. magnesium acetate added and the pH of the solution adjusted to 8·5 with concentrated ammonium hydroxide. The precipitated proteins and magnesium ammonium phosphate were filtered through a layer of Kieselguhr and a filter paper on a Buchner funnel. The filtrate was now ready for passage through ion-exchange columns, which were regenerated three times before use.

The solution was first passed through a column containing Amberlite resin IR-120(H) (40 x 4·5 cm. diameter) and then through a second column containing IR-4B(OH) (35 x 3 cm. diameter). The latter was washed with distilled water (3 l.) and the glucose-1-phosphate eluted with 4% ammonium hydroxide (500 ml.). The eluate was treated with potassium acetate (50 g.) and the pH adjusted to 12 with 10% potassium hydroxide. The volume was reduced under vacuum at 30° and the pH readjusted to 12. Methanol (1·5 volume) was added and the glucose-1-phosphate allowed to crystallise at 4° for 24 hours. The crystals of di potassium glucose-1-phosphate dihydrate were washed with methanol, ether and dried in vacuo at 50°; yield 16·36 g.

Theoretical ester-P as glucose-1-phosphate	8·33%
Found total-P	8·32%

(g) Preparation of alumina gel "C_r"[24]

A hot solution of aluminium ammonium sulphate (340 g. in 500 ml. water) was poured into 3·25 l. of ammonium sulphate and hydroxide (100 g. ammonium sulphate and 215 ml. 20% ammonium hydroxide) kept at 60°, with vigorous stirring. When the precipitate flocculated, the mixture was diluted to 20 l. with water and the supernatant fluid decanted as soon as the precipitate settled. Washing with water was repeated three times, and the precipitate was treated with 40 ml. 20% ammonium hydroxide to remove the last traces of basic aluminium sulphate. The precipitate was washed 20 times with a large volume of water and stored at 0°C for three months in a small volume of water. The pH of the solution was adjusted to 6·5 and the dry weight of alumina C_r in a known volume of the suspension was determined. Found 22 mg. per ml.

(h) Preparation of calcium phosphate gel.[24]

150 ml. calcium chloride solution (132 g. $CaCl_2,6H_2O$ per litre) was diluted to about 1600 ml. with tap water and shaken with 150 ml. trisodium phosphate solution (152 g. $Na_3PO_4,12H_2O$ per litre). The mixture was brought to pH 7·4 with dilute acetic acid and the precipitate washed four times by decantation with large volumes of water (15-20 l.). The precipitate was finally washed with distilled water, and suspended in a known volume of water. The pH of the solution was adjusted to 6·8 and stored at 0°C.

Calcium phosphate content - 20 mg. dry weight per ml.

3. Phosphorylase activity of aqueous extract of pressed brewer's yeast.

Pressed brewer's yeast (1000 g.) was disrupted by grinding with sand (500 g.) in a mechanical mortar, and the mixture extracted with water (2·5 l.). Insoluble material was removed on the centrifuge, and the pH of the supernatant (2 l.) adjusted to 7·8. A small precipitate was removed, and the pH readjusted to 6·5 with dilute acetic acid. An equal volume of saturated ammonium sulphate (pH 6·5) was added, the precipitate being collected on the centrifuge, and dissolved in water; total volume 100 ml.

Phosphorylase activity;- 0·58 units per ml.;
total 58 units.

4. Phosphorylase activity of autolysate.

Pressed brewer's yeast (2·5 Kg.) was washed with excess water, collected on the refrigerated centrifuge at 0° and the adhering water removed by filtration, using a Buchner funnel. It was incubated with toluene (300 ml.) and a little water at 35° for 5 hours. Citrate buffer (0·2M-pH 6·8; 500 ml.) and water (1500 ml.) were added and the mixture allowed to stand for 15 hours. The solution was centrifuged at 0°, and the supernatant (2800 ml.) collected. Solid ammonium sulphate was added to 0·75 saturation; the precipitate was collected by filtration, using a Buchner

funnel, and dissolved in 0·1M-citrate buffer pH 6·8 (100 ml.).

Phosphorylase activity:- 1·80 units per ml.; total 180 units.

5. Phosphorylase activity of Lebedew's juice.(74)

Dried brewer's yeast (100 g.), crushed in a mechanical mortar for 1·5 hours, was incubated with water (300 ml.) for 3 hours at 35°. The solution was centrifuged (3000 r.p.m. for 45 mins. at 0°C); the supernatant was collected and treated with an equal volume of saturated ammonium sulphate at 0°. The precipitate was collected on the centrifuge and dissolved in 0·1M-citrate buffer pH 6·8 (100 ml.).

Phosphorylase activity;- 4·20 units per ml.; total 420 units.

6. Phosphorylase activity of butanol extract of dried brewer's yeast.(93)

Dried brewer's yeast (crushed in a mechanical mortar, 100 g.) was suspended in water (300 ml.) and 0·2M-citrate buffer pH 6·8 (100 ml.). n-Butanol (200 ml.) was added slowly with constant stirring and incubated at 35° for 30 mins. The solution was centrifuged at 0° and the water layer collected. An equal volume of saturated ammonium sulphate (at 0°) was added, the precipitate collected on the centrifuge and dissolved in 0·1M-citrate buffer pH 6·8 (100 ml.).

Phosphorylase activity:- 0·40 units per ml.; total 40 units.

7. Phosphorylase activity of sodium bicarbonate extract of dried brewer's yeast.[131]

Powdered dried yeast (100 g.) was incubated with 0·1M-sodium bicarbonate (500 ml.) at 35° for 2 hours. The solution was centrifuged (5000 r.p.m. for 15 mins. at 0° C), and the supernatant collected (350 ml.) and treated with an equal volume of ammonium sulphate solution (saturated at 0° C). The precipitate was collected on the centrifuge and dissolved in 0·1M-citrate buffer pH 6·8 (100 ml.).

Phosphorylase activity:- 5·25 units per ml.; total 525 units.

8. Preliminary experiments with crude phosphorylase preparation.

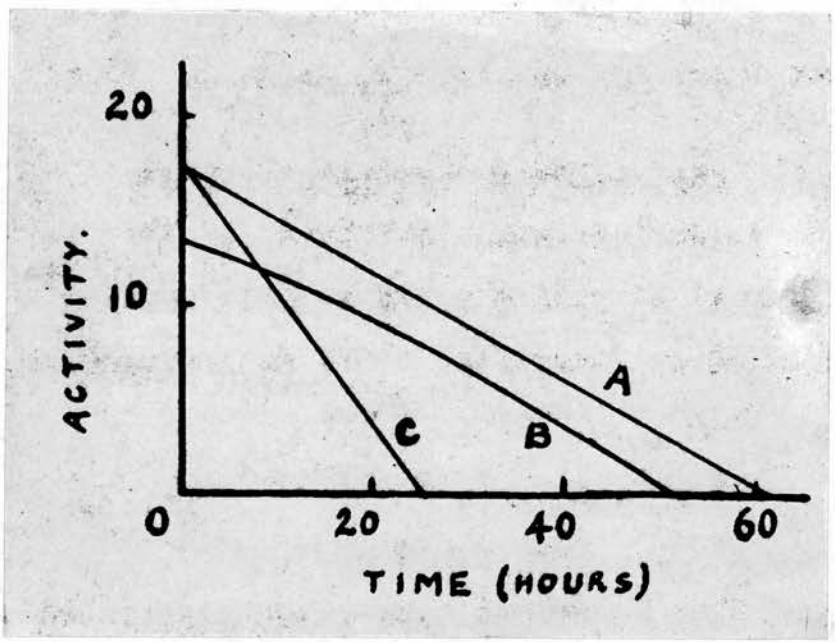
The sodium bicarbonate extract of dried brewer's yeast showed the highest phosphorylase activity and hence this enzyme solution was used for the following experiments.

(a) Stability of the enzyme in solution.

(i) Yeast phosphorylase solution in 0·1M-citrate buffer pH 6·8 was stored at 0° C and its activity determined at intervals. The following results were obtained.

Time (hours)	0	25	48	68
Units in 10 ml.	17·5	10·5	3·7	0·0

(ii) Yeast phosphorylase solution in 0·1M-citrate buffer (pH 6·8; 8 ml.) and 0·2M-cysteine hydrochloride solution (pH 6·8; 2 ml.) was kept at 0° and the activity determined at intervals.



Key: A, Citrate buffer pH 6·8.

B, Cysteine buffer pH 6·8.

C, Dialysis.

Figure 3. Stability of yeast phosphorylase in solution.

Time (hours)	0	25	48	68
Units in 10 ml.	13·5	8·4	1·6	0·0

Thus yeast phosphorylase is very unstable (Figure 3).

(b) Effect of dialysis on phosphorylase activity.

(i) Yeast phosphorylase solution (10 ml.; 17·5 units) was dialysed against distilled water at 0°C, and its activity determined at intervals. The following results were obtained.

Time (hours)	0	22	48
Units (total)	17·5	2·6	0·0

(ii) The distilled water was recovered, evaporated to dryness at 40° under vacuum, and the activity of the above solution redetermined in the presence of the substance obtained. No activity was found.

(iii) Yeast phosphorylase solution (10 ml.) was dialysed against 0·2M-citrate buffer pH 6·8 at 0°C for 18 hours. The phosphorylase activity of the original solution and the dialysed solution was determined. The following results were obtained.

Phosphorylase activity of original solution	21·4 units
Phosphorylase activity of the dialysed solution	12·4 units.

Yeast phosphorylase is therefore deactivated on dialysis against water. (Figure 3).

(c) Freeze drying.

Phosphorylase solution (25 ml.) was freeze dried in 0·2M-

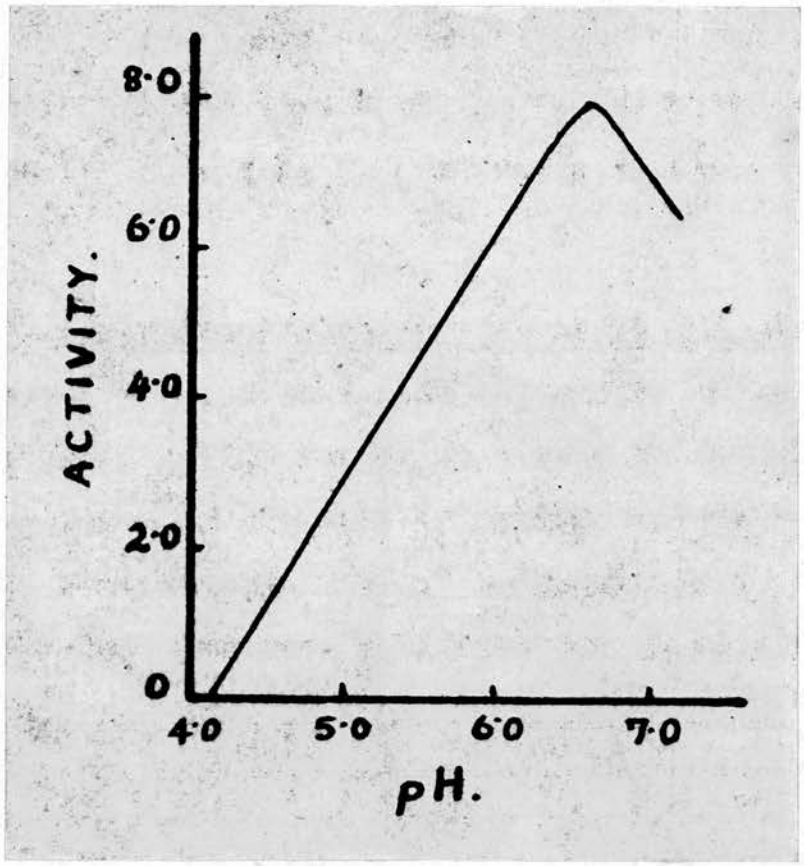


Figure 4. Optimum pH of yeast phosphorylase.

citrate buffer pH 6·8. The protein obtained was redissolved in water and the volume made up to 25 ml. The phosphorylase activity of the solution was determined together with the original solution.

Phosphorylase activity of the original solution 21·4 units
Phosphorylase activity of the freeze dried protein 26·0 units.

Thus yeast phosphorylase is stable in the freeze dried state.

(d) Optimum pH of brewer's yeast phosphorylase.

Enzyme solutions (10 ml.) were kept at various pH values, measured on glass electrode pH meter, for 20 hours at +1°C. Precipitates which formed in some of the solutions were removed by centrifugation and the supernatants collected. The activities of the solutions were then determined:-

pH	3·4	3·8	4·2	4·6	5·0	5·4	5·8	6·2	6·6	7·0
Total Units	0·0	0·0	0·0	2·7	3·1	3·7	5·5	7·0	8·0	7·0

The optimum pH of brewer's yeast phosphorylase is approximately 6·6. (Figure 4).

(e) Temperature effects.

(i) Rate of enzyme action:- phosphorylase activity tests were carried out at 25 , 30 , 35 , 40 , 50 and 60°C. The following results were obtained:-

Temperature °C.	25	30	35	40	50	60
Units per ml.	5·2	6·1	6·3	4·0	0·0	0·0

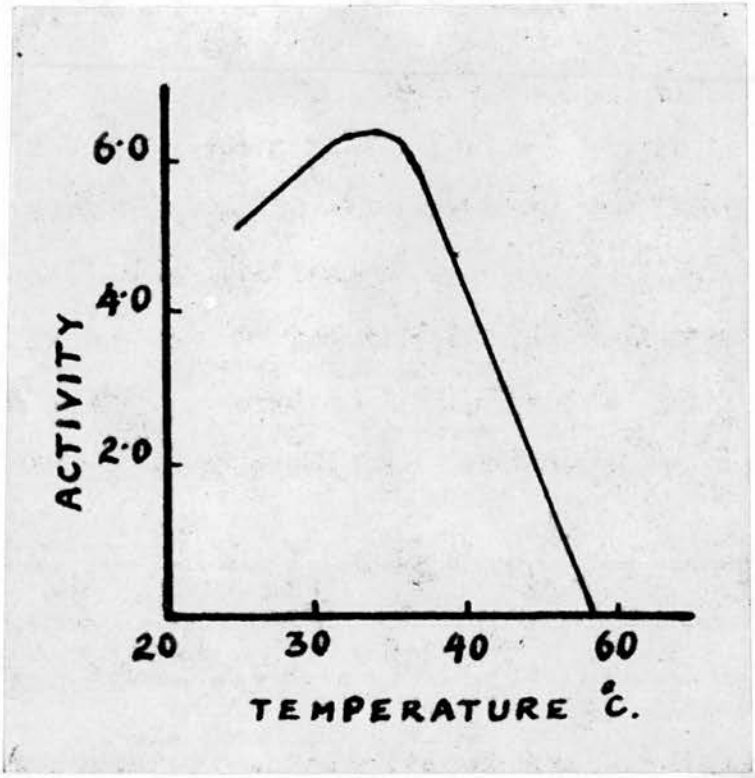


Figure 5. Optimum temperature of yeast phosphorylase.
(Time of incubation 15 mins.)

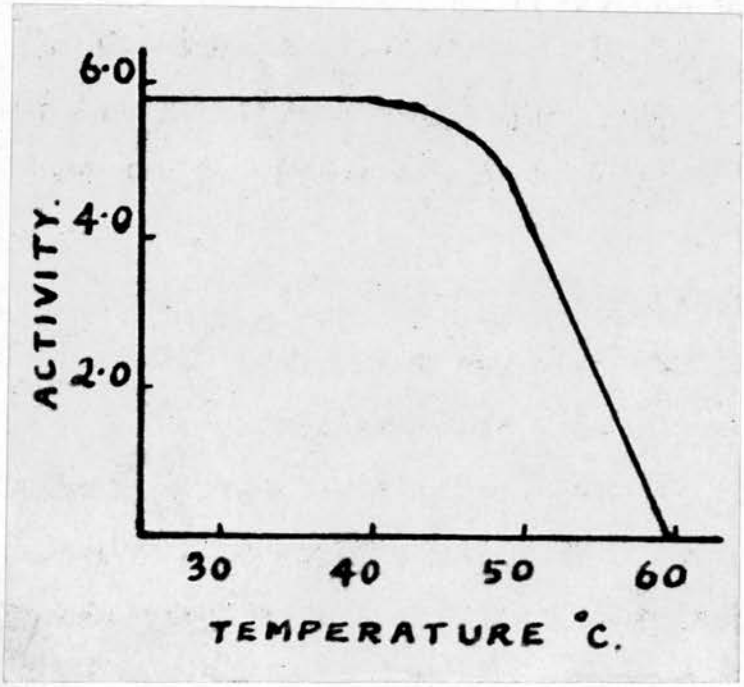


Figure 6. Effect of temperature on yeast phosphorylase.
(Time of incubation 20 mins.)

The rate of brewer's yeast phosphorylase action decreases at temperatures greater than 35°. (Figure 5).

(ii) Enzyme inactivation:- phosphorylase solutions (1 ml.) was incubated with 0·2M-citrate buffer pH 6·6 (0·5 ml.) at 25, 30, 35, 40, 50, and 60°C for 20 mins. The solutions were cooled to room temperature, and their activities determined at 35°.

Temperature °C	25	30	35	40	50	60
Units	5·7	5·8	5·8	5·7	4·6	0·0

The enzyme is therefore deactivated at temperatures above 40°C. (Figure 6).

(f) Effect of time on the extraction of phosphorylase from yeast.

Dried yeast (50 g.) was incubated with 0·1M-sodium bicarbonate (250 ml.) at +1°C. Samples were removed after 1, 2, 4 and 8 hours and centrifuged at 0°C. The free inorganic phosphate content of the solutions was then determined.

Time of extraction (hours)	1	2	4	8
Free phosphate content (mg. per ml.)	0·96	1·05	1·18	1·32

The solutions (20 ml.) were precipitated twice with saturated ammonium sulphate, at 0·5 saturation, under identical conditions. The precipitates were dissolved in 0·1M-citrate buffer pH 6·6 (5 ml.) and the free phosphate content and phosphorylase activities of the solutions were

determined. The following results were obtained.

Time of Extraction (hours)	Total free phosphate (mg.)	Total Units
1	0·05	12·5
2	0·05	13·5
4	0·05	12·0
8	0·05	9·0

Thus the free phosphate content of the extract increases with time, but this can be removed by ammonium sulphate precipitation. The optimum time for the extraction of phosphorylase from dried brewer's yeast with 0·1M-sodium bicarbonate solution is 2 hours.

(g) Temperature of extraction.

Dried brewer's yeast (20 g.) were extracted with 0·1M-sodium bicarbonate (100 ml.) at +1°C and 35°C for 2 hours. The solutions were centrifuged at 0°C (5000 r.p.m. for 15 mins.) and 65 ml. of each supernatant collected. Phosphorylase was precipitated twice with saturated ammonium sulphate (0.5 saturation at 0°C) and the final precipitate dissolved in 0·1M-citrate buffer pH 6·6 (16 ml.). The phosphorylase activities of the two solutions were determined:-

Temperature of extraction °C	1	35
Total units	14·4	112·0

Extraction of the yeast at 35°C is therefore desirable.

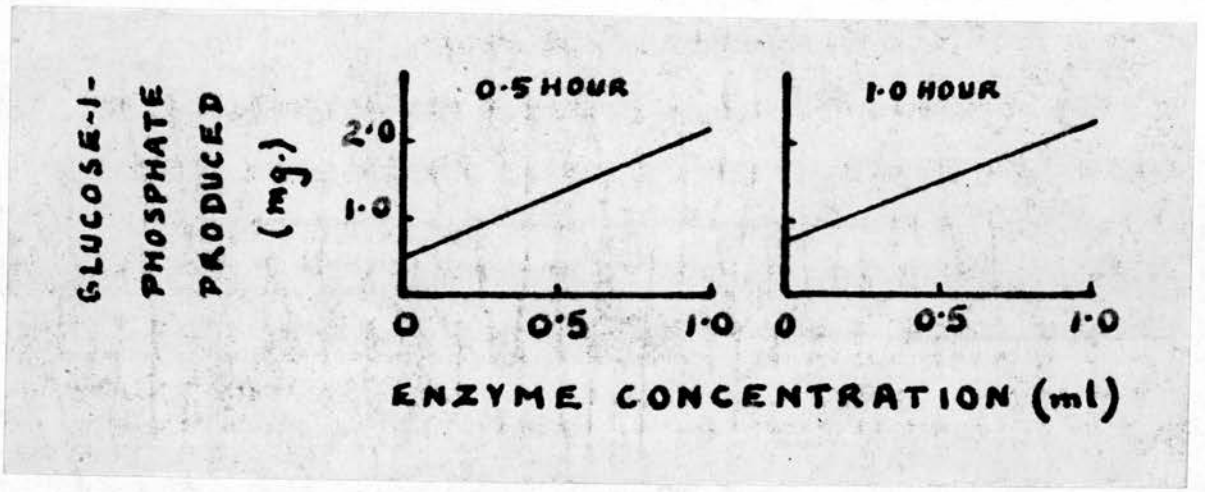


Figure 7. The relationship between phosphorylase activity and enzyme concentration.

(h) Degradation of starch with varying concentrations of the enzyme.

Digests containing 0·2, 0·5, 0·8 and 1·0 ml. of the enzyme solution were set up as follows.

2·5 ml. 2% starch,

0·5 ml. 2% ammonium molybdate,

1·0 ml. phosphate buffer (pH 6·7; 0·5M),

1·0 ml. enzyme solution and water.

The glucose-1-phosphate produced was estimated after 30 mins. and 1 hour. The following results were obtained.

Time (hours)	Glucose-1-phosphate produced in the digest (mg.)			
	0·2ml. enzyme	0·5ml.enzyme	0·8ml.enzyme	1·0ml.enzyme
0·5	0·84	1·44	1·92	2·16
1·0	1·08	1·46	2·04	2·28

The activity of brewer's yeast phosphorylase is therefore proportional to the concentration of the enzyme (Figure 7). The lag in the graphs may be due to the presence of 10 mins. hydrolysable (N.hydrochloric acid; 100°C) ester phosphate in the enzyme solution.

(i) Phosphatase activity in phosphorylase preparations.

Phosphatase activity was determined using sodium β-glycero-phosphate (12·5 mg. per ml.) as the substrate. The following digests were set up and the inorganic phosphate content estimated after 21 hours.

Digest	Composition of the digests				
	Enzyme solution (ml.)	Substrate (ml.)	Cysteine buffer pH7·0(ml.)	Water (ml.)	Free phosphate in the digest (mg.)
(i)	10	1	5	9	1·25
(ii)	-	1	5	19	0·00
(iii)	10	-	5	10	0·31

Thus 76% phosphate was liberated by the enzyme; phosphatase is therefore present.

(j) Inhibition of phosphatase.

The following digest containing 0·02% ammonium molybdate was set up:-

1·0 ml. glucose-1-phosphate (6·91 mg. per ml.),

2·0 ml. 0·2M-citrate buffer pH 6·6,

0·5 ml. 0·2% ammonium molybdate,

1·0 ml. water,

0·5 ml. enzyme solution.

1 ml. samples were removed at intervals and inorganic phosphate (free-P), ester-P (10 mins. hydrolysable in N HCl), and the total phosphate (total-P) concentrations determined.

Time (hours)	Free-P(mg.)	Ester-P(mg.)	Total-P(mg.)
0·5	0·48	0·73	1·83
3·0	0·55	0·65	1·83
24·0	1·38	0·13	1·83

Since the concentration of glucose-1-phosphate decreased and the free phosphate increased, the phosphatase was not

inhibited by 0·02% ammonium molybdate.

The experiment was repeated in the presence of 0·2% ammonium molybdate when the following results were obtained.

Time (hours)	Free-P(mg.)	Ester-P(mg.)	Total-P(mg.)
1	0·13	0·75	1·82
4	0·13	0·80	1·82
24	0·13	0·75	1·82

Thus phosphatase activity is inhibited by 0·2% ammonium molybdate.

(k) Phosphoglucomutase activity of phosphorylase preparations.

The phosphoglucomutase activity was determined according to Najjar.[95] The following digest was set up:-

1 ml. glucose-1-phosphate (74·4 mg. glucose-1-phosphate and 14·76 mg. $MgSO_4,7H_2O$ in 10 ml. at pH 7·5),
2 ml. cysteine hydrochloride (78·8 mg. in 10 ml.; pH 7·5),
1 ml. enzyme solution.

After 4 hours, 0·04 mg. inorganic phosphate and 0·48 mg. glucose-1-phosphate were present, corresponding to 93% conversion into glucose-6-phosphate.

Thus phosphoglucomutase was present in the enzyme preparations.

(l) Inhibition of phosphoglucomutase.

(i) Sutherland[127] reported that phosphoglucomutase from skeletal muscle, heart or yeast is inhibited by zinc, copper, mercury and silver ions. The following digest was set up

in the presence of zinc sulphate (10^{-4}M):-

1·0 ml. glucose-1-phosphate (74.4 mg. glucose-1-phosphate and 14·7 mg. $MgSO_4,7H_2O$ in 10 ml. at pH 7·5),

1·5 ml. cysteine hydrochloride (78·8 mg. in 10 ml. at pH 7·5),

1·0 ml. zinc sulphate (11·48 mg. $ZnSO_4$, $7H_2O$ in 50 ml.water),

1·0 ml. enzyme solution.

The amount of inorganic phosphate and glucose-1-phosphate was estimated after 4 hours. 0·04 mg. inorganic phosphate and 0·48 mg. glucose-1-phosphate were found in the digest corresponding to 93% conversion into glucose-6-phosphate. Thus 10^{-4}M zinc sulphate had no action on brewer's yeast phosphoglucomutase.

(ii) Sodium fluoride.

The use of sodium fluoride as a phosphoglucomutase inhibitor was suggested by Chung and Nickerson[23] during studies of polysaccharide synthesis in growing yeast. Three digests were set up containing 0·01M, 0·02M and 0·04M sodium fluoride as follows:-

1·0 ml. glucose-1-phosphate (19·8 mg. per ml.),

0·5 ml. sodium fluoride (0·1M and water),

0·5 ml. 2% ammonium molybdate,

2·0 ml. 0·2M-citrate buffer pH 6·8,

1·0 ml. enzyme solution.

The glucose-1-phosphate content was estimated after 2 and 24 hours. The following results were obtained.

Time (hours)	Glucose-1-phosphate content (mg.)		
	0·01M.NaF.	0·02M.NaF.	0·04M.NaF
2	19.80	19.80	19.80
24	19·80	19·82	19·74

The phosphoglucomutase activity of brewer's yeast is inhibited by 0·01M-sodium fluoride.

(m) Glucose-6-phosphatase activity.

Glucose-6-phosphatase activity was determined in the presence of 0·01M-sodium fluoride solution. The following digest was set up:-

2·0 ml. glucose-6-phosphate (12·5 mg. per ml.),

1·5 ml. 0·2M-citrate buffer pH 6·8,

0·5 ml. 0·1M-sodium fluoride,

1·0 ml. enzyme solution.

The inorganic phosphate in the digest was determined at intervals and the following results were obtained.

Time (hours)	2	4	24
Inorganic phosphate liberated (mg.)	0·0	0·0	0·0

Glucose-6-phosphatase is absent from the enzyme preparation.

(n) Test for primers.

Digests containing maltose, maltotriose, amylose, soluble starch and yeast glycogen (10 mg.) and a control

digest without primer, were set up as follows:-

1·0 ml. 0·1M-glucose-1-phosphate,

0·5 ml. 0·2M-citrate buffer pH 6·6,

0·4 ml. 2% ammonium molybdate,

0·4 ml. 0·1M-sodium fluoride,

0·2 ml. water,

0·5 ml. primer (10 mg.),

1·0 ml. enzyme solution.

The inorganic phosphate liberated was determined and the following results were obtained.

Primers	Nil	Maltose	Maltotriose	Amylose	Soluble starch	Yeast glycogen
Free-P liberated (mg.)	1·02	1·02	1·01	1·24	1·40	1·59

Yeast glycogen is the most effective primer for brewer's yeast phosphorylase.

(o) Activators.

The phosphorylase activity tests of the enzyme solution (10 ml.) were determined:- (i) without any activators, (ii) in presence of adenosine-3-phosphoric acid (yeast adenylic acid; 1 mg.), and (iii) in presence of cysteine hydrochloride (1 mg.). The following results were obtained.

Activators	Nil	Yeast adenylic acid	Cysteine hydrochloride
Total units	7·5	7·5	7·5

9. Purification of yeast phosphorylase.

(a) Precipitation of phosphorylase at pH 5·3.

Meyer and Bernfeld[91] described a method of preparing phosphorylase from dried baker's yeast in which phosphorylase was precipitated from solution by adjusting the pH to 5·3. An attempt has been made to use this procedure in the purification of brewer's yeast phosphorylase.

Enzyme extract (100 ml.; 180 units) was treated with dilute acetic acid to pH 5·3 and allowed to stand for 2 hours at 0°C. The solution was centrifuged at 0°C and the precipitate and the supernatant were collected. The precipitate was dissolved in 0·1M-citrate buffer pH 6·8 (10 ml.) and insoluble materials removed on the centrifuge. The phosphorylase activities of the solution and the supernatant (pH adjusted to 6·8) were determined. The solutions had no activity.

(b) Ammonium sulphate fractionation.

(i) Dried yeast (200 g.) was incubated with 0.1M-sodium bicarbonate (1000 ml.) at 35° for 2 hours. The solution was centrifuged at 5000 r.p.m. for 15 mins. at 0°C and the clear supernatant collected (700 ml.). Ammonium sulphate solution (700 ml.; saturated at 0°C) was added slowly with constant stirring at 0°C and the precipitate collected on the centrifuge. It was then dissolved in 0·1M-citrate buffer pH 6·6 (200 ml.) and insoluble material removed on the centrifuge.

The solution obtained was then fractionated with saturated ammonium sulphate at 0°, the precipitates being dissolved in 0·1M-citrate buffer pH 6·6 (100 ml.) and freeze dried. The phosphorylase activity of the protein fractions was determined and the following results were obtained.

Fraction	Concentration of ammonium sulphate (saturation)	Yield in g.	Units per g.
-	0·00 - 0·20	nil	-
A_1	0·20 - 0·35	5·80	110
A_2	0·35 - 0·50	8·05	78
-	0·50 - 0·65	nil	-

(ii) Fraction A_2 (7 g.; 546 units) was dissolved in 0·1M-citrate buffer pH 6·6 (100 ml.) and a further ammonium sulphate fractionation carried out at 0°C. The precipitates obtained were dissolved in 0·1M-citrate buffer pH 6·6 (50 ml.) and freeze dried. The phosphorylase activity of the protein fractions was determined.

Fraction	Concentration of ammonium sulphate (saturation)	Yield in g.	Units per g.
-	0·00 - 0·20	-	-
B_1	0·20 - 0·40	3·50	92
B_2	0·40 - 0·50	1·50	32
-	0·50 - 0·60	-	

Thus 176 units of phosphorylase (32%) were lost during refractionation.

(c) Protein nitrogen determinations of ammonium sulphate fractions.

Dried brewer's yeast (50 g.) was incubated with 0·1M-sodium bicarbonate (250 ml.) at 35° for 2 hours. The solution was centrifuged at 5000 r.p.m. for 15 mins. at 0°C. and the supernatant (175 ml.) collected. Yeast protein was precipitated with saturated ammonium sulphate at 0·5 saturation, collected on the centrifuge, and dissolved in 0·1M-citrate buffer pH 6·6 (protein fraction A; 60 ml.). The phosphorylase activity and protein nitrogen content of solution A were then determined.

Protein	Vol(ml.)	Units per ml.	Protein nitrogen per ml.(mg.)	Total units	Units per mg. nitrogen
A	60	4·0	3·16	240	1·26

The solution was fractionated with saturated ammonium sulphate at 0°C, and the precipitates collected on the centrifuge, and dissolved in 0·1M-citrate buffer pH 6·6. The phosphorylase activity of the solutions and their protein nitrogen contents were determined.

Fraction	Concentration of ammonium sulphate (saturation)	Volume (ml.)	Units per ml.	Protein nitrogen (mg. per ml.)	Total units	Units per mg. nitrogen
C_1	0·00 - 0·27	25	2·10	2·24	52·5	0·94
C_2	0·27 - 0·36	12	6·80	3·52	81·6	1·93
C_3	0·36 - 0·45	11	4·40	3·74	48·4	1·18
C_4	0·45 - 0·60	8	0·75	3·36	6·0	0·22

Loss of activity 45·5 units (19%).

Protein solutions C_2 and C_3 were combined (fraction C) and refractionated with saturated ammonium sulphate at 0° C. The precipitates obtained were dissolved in 0·1M-citrate buffer pH 6·6, and their activities and nitrogen content determined.

Fraction	Concentration of ammonium sulphate (saturation)	Volume (ml.)	Units per ml.	Protein nitrogen (mg.per ml.)	Total units	Units per mg. nitrogen
C^1	0·00 - 0·27	5	4·2	2·52	21	1·68
C^2	0·27 - 0·36	6	8·5	4·00	51	2·12
C^3	0·36 - 0·45	5	3·2	3·50	16	0·91

Loss of phosphorylase activity 25 units (22%).

(d) Protamine sulphate treatment.

An attempt has been made to selectively remove nucleic acids from yeast extracts with protamine sulphate[42] The following experiments were conducted at 0° C.

(i) Dried yeast (50 g.) was extracted with 0·1M-sodium bicarbonate (250 ml.) and centrifuged at 5000 r.p.m. for 15 mins. The supernatant (170 ml.) was treated with 2% protamine sulphate solution (85 ml.) and kept in the refrigerator for 12 hours. The precipitate was removed on the centrifuge and the supernatant (250 ml.) treated with saturated ammonium sulphate solution to 0·5 saturation. The yeast protein was then dissolved in 0·1M-citrate buffer pH 6·6 (20 ml.) and the phosphorylase activity and protein nitrogen content determined.

Volume of the solution (ml.)	Units per ml.	Protein nitrogen per ml.	Total units	Units per mg.nitrogen
20	1·0	3·2	20	0·31

In the previous experiment, without protamine sulphate treatment, the solution had an activity of 1·26 units per mg. nitrogen (p. 58).

(ii) The experiment was repeated in the following way.

Yeast extract (170 ml.) was divided into three portions (50 ml.; 250 units) and treated with 50 ml., 25 ml. and 5 ml. 1% protamine sulphate solution. The precipitates were removed on the centrifuge, and the supernatants treated with saturated ammonium sulphate solution to 0·5 saturation; the precipitated protein was dissolved in 0·1M-citrate buffer pH 6·6. The phosphorylase activities were determined.

Protamine sulphate concentration (%)	Volume of the solution (ml.)	Total phosphorylase units
0·50	6	6·6
0·33	9	10·8
0·09	15	87·0

The protamine sulphate treatment thus deactivates brewer's yeast phosphorylase.

(e) Acetone fractionation.

The sodium bicarbonate extract of dried brewer's yeast (700 ml.; 3675 units) was fractionated with AnalaR acetone at -7°C. Acetone was added very slowly with constant stirring.

The precipitates obtained were washed with cold acetone and dried under vacuum. The phosphorylase activity of the acetone powders were determined and the following results were obtained.

Fraction	Acetone concentration (%)	Yield in g.	Units per g.
D_1	0 - 24	17·6	20
D_2	24 - 36	5·6	0
D_3	36 - 50	gum	0

The majority of the phosphorylase activity (90%) was lost during acetone fractionation.

(f) Alcohol fractionation.[41]

Yeast phosphorylase solution (50 ml.; 245 units) was fractionated with 50% (w/v) ethanol-citrate solution (127 ml. absolute alcohol; 10 ml. of 0.2M-citrate buffer pH 6.6, 63 ml. of water) at -4° C. The precipitates obtained were collected on the centrifuge, dissolved in 0·1M-citrate buffer pH 6.6 and the insoluble material removed. The following fractions were obtained.

Fraction	Alcohol concentration (%)	Volume (ml.)
E_1	0 - 11	2
E_2	11 - 17	6
E_3	17 - 23	8
E_4	23 - 30	11

The phosphorylase activity of the solutions were determined; none of the fractions showed appreciable phosphorylase activity.

Brewer's yeast phosphorylase is therefore deactivated by ethanol under conditions in which phosphorylases from other sources are stable.

(g) Alumina adsorption.

Alumina has been used by Cori, Cori and Schmidt[32] and Shapiro and Wertheimer[122] in the purification of phosphorylases. The following experiments were carried out at 0°C.

(i) Yeast phosphorylase solution (3 ml.; in water) was treated with alumina C_γ (22 mg. dry weight per ml.; pH 6·3) at various alumina concentrations for 15 mins. The solutions were centrifuged, and the phosphorylase activity of the supernatant liquids determined. The protein nitrogen content of these solutions was also determined and the following results were obtained.

Concentration of alumina C_γ mg.(dry weight) per ml.	Total volume (ml.)	Total units	Total protein nitrogen (mg.)	Units per mg. nitrogen
0	3·00	21·30	13·74	1·55
2	3·30	20·13	13·38	1·51
4	3·66	18·85	12·74	1·49
6	4·13	18·38	12·22	1·51
8	4·70	16·61	11·28	1·38
10	5·50	13·75	9·90	1·39
12	6·60	6·27	8·45	0·74
14	8·25	0·00	4·62	0·00
16	11·00	0·00	1·72	0·00

(ii) The experiment was repeated with protein C_2 (3 ml.; in 0·1M-citrate buffer pH 6·6) and the following results were obtained.

Concentration of alumina C_γ mg.(dry weight) per ml.	Total volume (ml.)	Total units	Total protein nitrogen (mg.)	Units per mg. nitrogen
0	3·00	15·75	8·04	1·96
2	3·30	14·36	6·80	2·11
4	3·66	13·36	6·73	1·98
6	4·13	11·98	6·20	1·93
8	4·70	10·11	5·64	1·79
10	5·50	6·88	4·95	1·39
12	6·60	2·64	2·90	0·91
14	8·25	0·83	2·31	0·36
16	11·00	0·00	0·00	0·00

Thus phosphorylase is completely adsorbed by alumina C_Y at a concentration of 16 mg. (dry weight) per ml. An attempt has been made to adsorb the phosphorylase on alumina followed by elution with citrate buffer. The following experiment was carried out at 0°C.

(iii) Protein solution C_2 (4·5 ml.; 23·6 units) was treated with alumina suspension to 4 mg. per ml. The solution was centrifuged and the supernatant treated with alumina to 16 mg. per ml. The alumina-protein complex was collected on the centrifuge and eluted with 0·2M-citrate buffer pH 6·6 (6 ml.). The eluate was found to have no phosphorylase activity.

(h) Calcium phosphate adsorption.

(i) Calcium phosphate adsorption studies were carried out in a similar manner; however, due to the high concentration of inorganic phosphate ions, measurement of phosphorylase activity according to Green and Stumpf[44] was impossible. Since the degradative activity of phosphorylase is directly proportional to the enzyme concentration (p. 50; Figure 7) a method was devised to determine phosphorylase activity by degradation.

A digest containing a known amount of phosphorylase (4 units) and the following solutions was set up:-

1·0 ml. 2% soluble starch,

0·5 ml. 0·5M-phosphate buffer pH 6·7,

0·3 ml. 2% ammonium molybdate,

0·3 ml. 0·1M-sodium fluoride,

0·15 ml. 0·01M-mercuric chloride,

0·25 ml. water,

0·5 ml. enzyme solution (4 units).

The enzyme solution was added after temperature equilibration (35°C) and the reaction allowed to proceed for 30 mins. It was stopped by adding 10% trichloroacetic acid (3 ml.), excess inorganic phosphate precipitated by magnesia mixture (4 ml.), the solution centrifuged and 2 ml. of the supernatant withdrawn for glucose-1-phosphate determination.

0·85 mg. of glucose-1-phosphate was present; 1 unit of phosphorylase thus produced 0·21 mg. of glucose-1-phosphate under the above conditions.

(ii) Protein solution C_2 (2 ml.; 16 units) was treated with calcium phosphate gel suspension (20 mg. dry weight per ml.; pH 6·8) at various concentrations and kept at 0° for 30 mins. The solutions were centrifuged at 0°, and the phosphorylase activity of the supernatants determined by the degradative method. The protein nitrogen contents of the solutions were also estimated, by the Biuret reaction.

Concentration of calcium phosphate gel mg.(dry weight) /ml.	Total volume (ml.)	Total units	Total protein nitrogen (mg.)	Units per mg. nitrogen
0	2·00	16·00	7·60	2·10
2	2·22	14·20	7·29	1·98
4	2·50	14·40	7·20	2·00
6	2·85	15·06	7·01	2·15
8	3·33	17·31	7·06	2·45
10	4·00	17·33	6·88	2·52
12	5·00	17·62	6·70	2·63
14	6·66	11·73	5·86	2·00
16	10·00	0·00	5·60	0·00

Up to a calcium phosphate concentration of 12 mg. per ml., the phosphorylase remains in the solution; it was totally adsorbed when the concentration of calcium phosphate was increased to 16 mg. per ml. An attempt was made to make use of this fact in the purification of yeast phosphorylase. The following experiments were carried out at 0°C.

(iii) Protein solution C_2 (6.5 ml.; 52 units) was treated with calcium phosphate gel suspension to 12 mg. per ml. for 30 mins. The solution was centrifuged, and the supernatant collected and treated with calcium phosphate suspension to 16 mg. per ml. for 30 mins. The mixture was centrifuged and the supernatant and adsorbent collected. The supernatant contained 8·3 units of phosphorylase activity. The adsorbent

was eluted with 0·25M-phosphate buffer pH 6·7 (5 ml.) for 12 hours at 0°C. The phosphorylase activity of the eluate was 1·1 units.

(iv) The experiment was repeated in the following way. Protein solution C_2 (30 ml.; 160 units) was treated with calcium phosphate suspension to 12 mg. per ml. for 30 mins. The solution was centrifuged and both the supernatant and adsorbent collected. The supernatant contained 41 units of phosphorylase activity. 119 units of phosphorylase was therefore adsorbed by the calcium phosphate. The latter was eluted with 0·5M-phosphate buffer pH 6·7 (10 ml.) and the phosphorylase activity of the eluate was 2·6 units.

The above experiments (iii and iv) show that experiment (ii) is not reproducible on a large scale and that yeast phosphorylase is very difficult to elute from its adsorbents.

10. "Debranching" and "branching" enzymes in yeast phosphorylase preparations.

(a) The presence of a "debranching" enzyme in yeast proteins was recognised when the following experiments were carried out.

(i) Degradation of waxy maize starch with yeast phosphorylase (protein A_1).

The following digest was set up.

5 ml. waxy maize starch (52·6 mg. in water),

1 ml. 0·1M-sodium fluoride,

1 ml. 2% ammonium molybdate,

2 ml. 0.5M-phosphate buffer pH 6.7,

1 ml. enzyme solution A_1 (100 mg. per ml.; 11 units).

2 ml. samples were withdrawn at intervals and the glucose-1-phosphate content determined.

Time (hours)	1.5	4	24	72
Phosphorolysis (%)	36	43	45	44

A yeast phosphorylase limit dextrin of waxy maize starch was prepared by treating waxy maize starch (4.088 g.) with protein A_1 as in the above digest. The phosphorolysis limit, determined after 24 hours incubation was 44%. The digest was boiled and deactivated proteins removed on the centrifuge. The solution was dialysed against running tap water for 48 hours, the volume reduced to about 100 ml. by distillation under vacuum, filtered and freeze dried. The yield was 2.14 g., (93%).

(ii) <u>β-Amylolysis of waxy maize starch phosphorylase limit dextrin</u>.

The following digest was set up:-

20 ml. waxy maize starch dextrin (52.4 mg. in water),

7 ml. 0.2M-acetate buffer pH 4.6,

3 ml. soya bean β-amylase solution (375 units).

3 ml. samples were withdrawn at intervals for estimation of maltose by the Somogyi reagent.(123)

Time (hours)	1	2	4	24
Amylolysis (%)	27.5	27.7	28.6	28.8

i.e. β-Amylolysis limit 29%.

Since 44% of the waxy maize starch had been degraded by yeast phosphorylase (as shown in experiment (i)), 29% degradation of the residual dextrin by β-amylase represents 60% total degradation of the original polysaccharide. The β-amylolysis limit of waxy maize starch is 50% and therefore small amounts of a debranching enzyme must be present in protein A_1.

Similar debranching activity has also been detected in protein fractions D_1, D_2, E_3 and E_4; the enzyme responsible for this activity, "isoamylase", will be described in Section IV.

(b) A "branching" enzyme was shown to be present in yeast phosphorylase preparations when the following experiments were conducted.

(i) Action of protein A_1 on amylose.

The following digest was set up:-

5.0 ml. amylose (2 mg. per ml.),
2.0 ml. 0.2M-citrate buffer pH 6.8,
1.0 ml. water,
2.0 ml. protein A_1 solution (10 mg. per ml.).

The reaction was followed by measurement of changes in the iodine staining power of amylose. 1 ml. samples were withdrawn at intervals and treated with 1 ml. iodine solution (2 mg. iodine and 20 mg. potassium iodide per ml.) the volume being made up to 100 ml. The absorption value (A.V.) of the amylose-iodine complex was then measured on a Spekker photo-electric absorptiometer in 4 cm. cells, an Ilford filter

No.608 being used, against an iodine blank.

Time (mins.)	0	15	30	60
A.V.	1·040	0·395	0·275	0·230

The reaction digest was reduced to a small volume and examined on a paper chromatogram. Glucose, maltose and reducing sugars were absent; the fall in absorption value of the amylose-iodine complex was not therefore due to hydrolytic enzymes.

(ii) β-Amylolysis of amylose treated with protein A_1.

The following digest was set up:-

5 ml. amylose (2 mg. per ml.),

2 ml. 0·2M-acetate buffer pH 5·89,

1 ml. protein A_1 solution (10 mg. per ml.).

A control digest without enzyme was also set up. The reaction mixture was incubated at 35°C for 1 hour when protein A_1 was destroyed by heating in a boiling water bath for 3 mins. The solution was cooled and the β-amylolysis limit determined by adding 2 ml. soya bean β-amylase solution (250 units) and estimation of the amount of maltose produced.

	Amylolysis (%)
Amylose treated with A_1	54
Amylose (control)	79

Since the β-amylolysis limit of amylose decreased, the fall in absorption value in experiment (i) was due to the presence of a branching enzyme in protein A_1. Similar branching activity has also been detected in other yeast

protein fractions; the branching enzyme of brewer's yeast will be described in Section V.

11. Synthesis of polysaccharides from glucose-1-phosphate

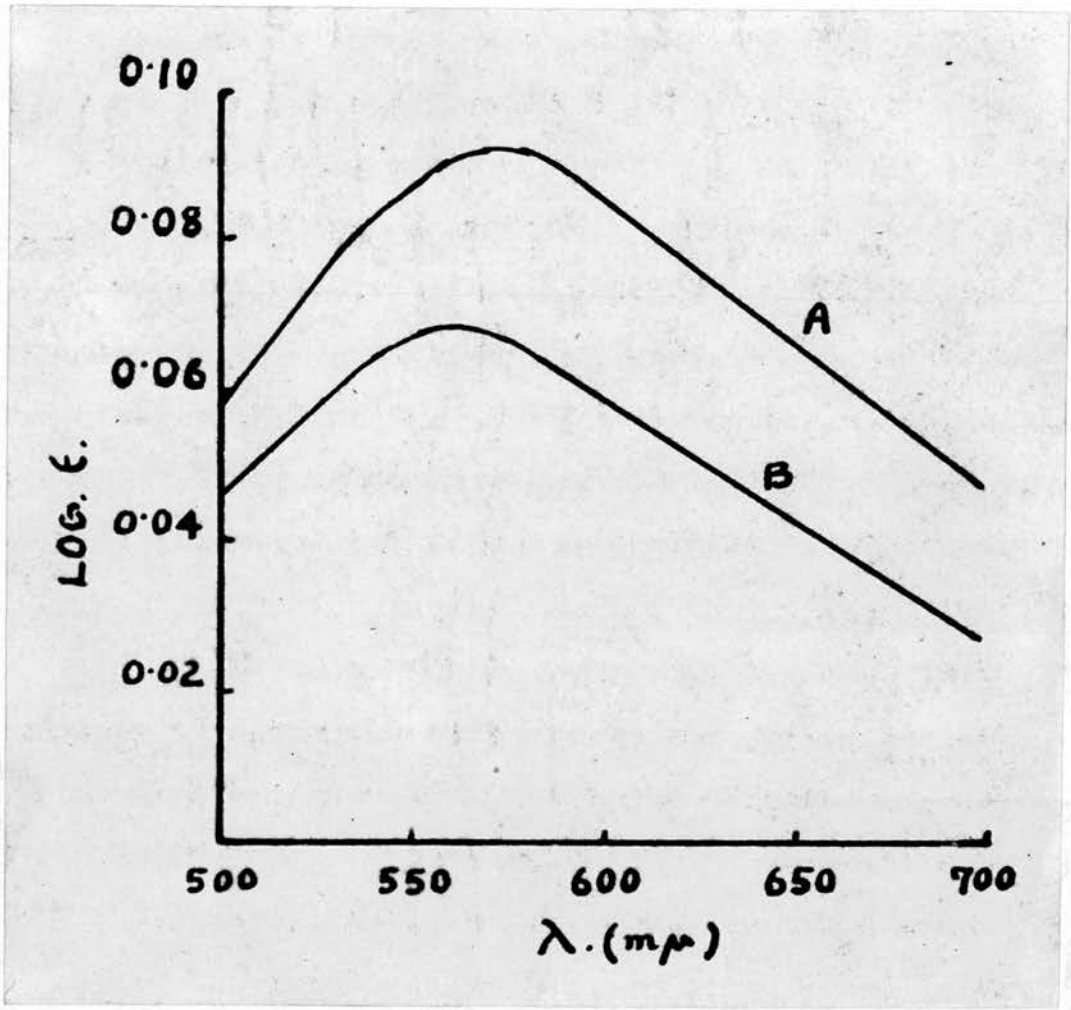
Phosphorylase catalyses the synthesis of amylose from glucose-1-phosphate in the presence of a suitable primer. The following experiments were carried out at 35°C.

(a) A digest was set up as follows:-

400 mg. glucose-1-phosphate,
1000 mg. yeast protein B_2 (32 units),
10 mg. glycogen (primer),
40 ml. 0·5M-citrate buffer pH 6·6,
2·5 ml. 0·1M-sodium fluoride,
2·5 ml. 2% ammonium molybdate,
and water to 25 ml.

The reaction was followed by (i) iodine staining, (ii) determination of the free inorganic phosphate liberated.

Iodine staining:- A 0·5 ml. sample was diluted to 5 ml. with water, heated to deactivate the enzyme and filtered. 3 ml. of the filtrate was treated with iodine (1 ml.) and the volume made up to 10 ml. The absorption value (A.V.) of the iodine-polysaccharide complex was then measured on the Spekker photoelectric adsorptiometer in 1 cm. cells at 680 mμ.



Key: A, 2 hours sample.
B, 24 hours sample.

Figure 8. The absorption spectra of synthetic polysaccharide-iodine complex.

Time (hours)	0·033	1	2	24
A.V.	0·130	0·370	0·695	0·610

To show that the fall in absorption value of the 24 hour sample was due to the branching enzyme, the absorption spectra of the 2 and 24 hour samples were determined on a Unicam spectrophotometer. The wavelengths of maximum absorption were 580 and 560 mμ respectively (Figure 8).

Determination of inorganic phosphate:- 1 ml. sample of the digest was diluted to 5 ml. and heated to deactivate the enzymes. The solution was made up to 15 ml. with water, filtered and 2 ml. filtrate analysed for inorganic phosphate.

Time (hours)	2	24
% Synthesis	68·1	83·8

After 24 hours, the molar ratio of inorganic phosphate to glucose-1-phosphate was 5·16. The whole digest was boiled and denatured proteins removed by filtration. The solution was dialysed for 36 hours and freeze-dried. The yield of polysaccharide was 97·7 mg.; ash content 14%.

(b) In order to obtain sufficient polysaccharide for purification, a large scale digest was set up as follows:-

4·00 g. glucose-1-phosphate,
2·00 g. yeast protein D_1 (40 units),
40·0 mg. yeast glycogen (primer),
15·0 ml. 0·1M-sodium fluoride,
15·0 ml. 2% ammonium molybdate,
7·5 ml. 0·01M-mercuric chloride,

50·0 ml. 0·2M-citrate buffer pH 6·6,

and water to 150 ml.

The reaction digest was heated after 8 hours and the mixture filtered. The filtrate was dialysed against running water for 4 days and freeze dried (941·3 mg.). The crude polysaccharide was dissolved in water (100 ml.) and treated with 40% trichloroacetic acid (20 ml.); a precipitate was removed on the centrifuge, and the supernatant solution dialysed against running water for 2 days. The polysaccharide was precipitated three times with glacial acetic acid and further purified by several precipitations with ethanol. The yield was 373·3 mg.

The polysaccharide contained 1·3% inorganic material, and by acid hydrolysis, had a glucose content of 90%. It had α_D + 170°, corrected as glucosan, and stained red-brown with iodine. To determine the β-amylolysis limit the following digest was set up:-

10 ml. synthetic polysaccharide (2 mg. per ml.),

2 ml. 0·2M-acetate buffer pH 4·6,

3 ml. β-amylase solution (375 units).

3 ml. aliquots were withdrawn for determination of the reducing power. The following results were obtained.

Time (hours)	2	4	24
Conversion into maltose (%)	36·5	40·7	44·1

The β-amylolysis of the polysaccharide was similar to that of yeast glycogen.

In this experiment polysaccharide containing α-1:4-linked glucose residues was synthesised from glucose-1-phosphate; since however the enzyme preparation contained traces of branching enzyme, the polysaccharide is branched rather than linear. The latter enzyme was not entirely inhibited by 0·0005M-mercuric chloride, as was the case in small pilot experiments.

2. Degradation of polysaccharides.

Phosphorylases degrade α-1:4-glucosans in the presence of inorganic phosphate producing glucose-1-phosphate. Polysaccharides of known structure were used to study the mode of yeast phosphorylase action.

(a) Digests containing yeast glycogen (50·3 mg.), cat liver glycogen (50·4 mg.) and waxy maize starch (50·0 mg.) were set up as follows:-

5 ml. polysaccharide,
2 ml. 0.5M-phosphate buffer pH 6.7,
1 ml. 0·1M-sodium fluoride,
2 ml. protein A_1 solution (9·8 units).

Glucose-1-phosphate produced was estimated and the following results were obtained.

Polysaccharides	% Conversion to glucose-1-phosphate		
	1 hour	3 hours	22 hours
Yeast glycogen	35	48	46
Cat liver glycogen	36	48	43
Waxy maize starch	38	49	44

Since the results indicate that phosphatase is hydrolysing the glucose-1-phosphate formed, the degradation of waxy maize starch was repeated in the presence of 0·2% ammonium molybdate.

(b) The following digest was set up:-

5 ml. waxy maize starch (52·6 mg. in water),
1 ml. 2% ammonium molybdate,
1 ml. 0·1M-sodium fluoride,
2 ml. 0·5M-phosphate buffer pH 6·7,
1 ml. protein A_1 solution (100 mg.; 11 units).

2 ml. samples were withdrawn for glucose-1-phosphate estimations.

Time (hours)	1·5	4	24	72
% Phosphorolysis	36	43	45	44

The studies on the degradation of polysaccharide by brewer's yeast phosphorylase indicate its general resemblance to other phosphorylases, in that its degradative action is limited to the phosphorolysis of exterior chains in glycogen and amylopectin.

13. Discussion.

The amount of phosphorylase brought into solution by the various methods described are summarised in Table 2.

Table 2.

Extraction of phosphorylase from 100 g. (dry weight) of brewer's yeast.

Method of extraction		Phosphorylase units (total)
Pressed brewer's yeast,	aqueous extraction after grinding with sand	39
	autolysis with toluene	48
Dried brewer's yeast,	Lebedew's juice	420
	butanol-water extract	40
	sodium bicarbonate extract	525

Dried yeast is preferable to pressed yeast as a source of phosphorylase. Although butanol is useful in the purification of alkaline phosphatase[94] and other enzymes, it apparently denatures yeast phosphorylase. Extraction of dried yeast with sodium bicarbonate is the most effective method and hence has been used for the preparation of yeast phosphorylase, the optimum extraction temperature and time being 35° and 2 hours respectively.

In solution, brewer's yeast phosphorylase is extremely unstable, even if stored under the conditions in which potato and muscle phosphorylase are stable. The rate of inactivation of yeast phosphorylase is especially rapid if the solution is dialysed, but the inactivation was not due to the fact that brewer's yeast phosphorylase required a dialysable co-enzyme such as yeast adenylic acid. The

presence of added yeast adenylic acid or cysteine hydrochloride, which activates many enzymes requiring free -SH groups for activity, had no effect on enzyme action. Freeze drying in citrate buffer had no effect on the enzyme and the freeze dried powder could be stored at 0° without loss of activity.

The optimum pH of yeast phosphorylase is 6·6; the rate of the enzyme action is maximum at 35°, and the enzyme is denatured if heated above 40° at pH 6·6 for 20 mins. Yeast phosphorylase, like potato phosphorylase is sensitive to heat; the yeast enzyme is completely denatured at 50° for 20 mins. (Figure 6), whilst the latter loses 61% of the activity at 58° for 3 mins.[44] As expected from the Michaelis-Menten theory, the rate of the enzyme action is proportional to its concentration. Like muscle phosphorylase[128] the synthetic activity of yeast phosphorylase is primed more effectively by glycogen than amylopectin. Potato phosphorylase has been shown to be primed more effectively by amylopectin than glycogen.[128] A comparison of some of the properties of muscle, potato and brewer's yeast phosphorylase is shown in Table 3.

Table 3

A comparison of the properties of muscle, potato and yeast phosphorylases.

Property	Muscle phosphorylase	Potato phosphorylase	Yeast phosphorylase
Optimum pH	6·8	5·9 - 6·3	6·6
Optimum temperature	35°	35°	35°
Activator	Adenylic acid; cysteine hydrochloride	nil	nil
Effective primer	Glycogen	Amylopectin, maltotetraose and higher maltosaccharides	Glycogen

During the purification studies, several attempts were made to precipitate phosphorylase at pH 5·3 as reported by other workers.(92,118) A suspension of the precipitate, the bulk of which was insoluble, showed no phosphorylase activity. The pH-phosphorylase activity curve (Figure 4) shows that activity is considerably lowered at pH 5.4. Since no phosphorylase could be detected in the precipitate the enzyme must have been irreversibly denatured.

The phosphorylase preparations contained phosphatase, phosphoglucomutase, isoamylase and branching enzymes as undesirable impurities. However, no means has yet been found of removing these impurities without denaturing the phosphorylase, and attempts have been made to inhibit these

enzymes with suitable inhibitors. Phosphatases are effectively inhibited by 0·2% ammonium molybdate and phosphoglucomutase by 0·01M-sodium fluoride. Branching enzyme and isoamylase have no action in the presence of 0·001M mercuric chloride and the inhibitors mentioned have no effect on phosphorylase action. The activity tests were therefore carried out in the presence of these inhibitors.

Ammonium sulphate fractionation appears to be the only method suitable for the purification of yeast phosphorylase. However the enzyme impurities, mentioned above, were always detected in the ammonium sulphate fractions and isolation of yeast phosphorylase cannot therefore be achieved by repeated precipitation with ammonium sulphate. Unlike potato phosphorylase, yeast phosphorylase is very sensitive towards organic solvents. When acetone was used for fractionation 90% of the activity was destroyed, whilst yeast phosphorylase is almost completely denatured when the alcohol-citrate reagent, used by Gilbert and his co-workers[9,41] in the purification of potato phosphorylase, was used. The activities of isoamylase and branching enzyme are greater in fractions prepared by use of organic solvents than the ammonium sulphate fractions and acetone and alcohol have therefore been used for the preparation of these enzymes (Section IV and V). An attempt to remove other impurities (.e.g. nucleic acids) with protamine sulphate, without effecting the phosphorylase activity, failed. In the experiments on the adsorption of yeast phosphorylase on alumina C_γ

and calcium phosphate gel, the phosphorylase was easily adsorbed, but it was difficult to elute the enzyme from the adsorbate. Citrate and phosphate buffers (0·1-0·5M) have little or no effect in bringing phosphorylase into solution, although this type of purification has been employed in the preparation of other phosphorylases.

In mode of action, yeast phosphorylase generally resembles the phosphorylases from other sources in that it catalyses the synthesis of α-1:4-glucosans from glucose-1-phosphate. The degradative action is also similar since it appears to be confined to the exterior chains. It does differ in fine detail, from muscle phosphorylase since the yeast phosphorolysis limits are significantly greater than the corresponding limits[75] with the muscle enzyme (Table 4).

Table 4.

Phosphorolysis of polysaccharides with muscle and yeast phosphorylase.

Polysaccharides	Yeast phosphorylase limit (3 hrs.) (%)	Muscle phosphorylase limit (%)	β-Amylolysis limit (%)
Yeast glycogen	48	30	44
Cat liver glycogen	48	36	53
Waxy maize starch	49	40	50

Muscle phosphorylase action involves a selective shortening of A-chains; the A- and B-chain stubs in a muscle phosphorylase

limit dextrin contain 1 and 6 or 7 glucose residues respectively. It is possible that the exterior chain 'stubs' of a yeast phosphorylase limit dextrin, like those of β-limit dextrins, contain, on the average, 2 or 3 glucose residues.

Thus the available experimental evidence indicates that brewer's yeast phosphorylase differs, in some respects from both potato and muscle phosphorylases; from a preparative point of view, the most significant difference is the sensitivity towards organic solvents and instability in solution.

Section IV.

Isoamylase.

1. Introduction.

The presence of a debranching enzyme in yeast was first reported by Meyer and Bernfeld,[91] who found that yeast "phosphorylase" attacked β-limit dextrins; they did not provide any experimental evidence to show that this reaction was brought about by the action of two distinct enzymes. In 1951, Maruo and Kobayashi[86] reported that autolysates of brewer's yeast contained an enzyme which hydrolysed the interchain linkages of glutinous rice starch producing linear low molecular weight α-1:4-glucosans. It also attacked β-limit dextrin producing a polysaccharide which could be further degraded by β-amylase; it had no action on bacterial dextran (α-1:6-glucosan). This enzyme, previously known as "amylosynthease," was renamed "isoamylase." They also showed that isoamylase (optimum pH 6·0-6·2 and temperature 20°) did not require phosphate ions for its action and concluded that it was similar to R-enzyme, isolated by Hobson, Whelan and Peat[63,110] from potatoes and broad beans, which hydrolyses the interchain linkages in amylopectin.

During studies on brewer's yeast phosphorylase, it was found that protein A_1 (Section III, part 10) degraded waxy maize starch giving 44% conversion into glucose-1-phosphate.

The residual dextrin, on treatment with β-amylase, gave 29% conversion into maltose; this represents 60% total degradation of the original polysaccharide, whereas β-amylase normally degrades only 50% of the molecule. Since the action of phosphorylase and β-amylase is confined to the exterior chains of branched polysaccharides, it was concluded that protein A_1 contained a debranching enzyme. This enzyme catalyses the hydrolysis of a limited number of the α-1:6-linkages in waxy maize starch, thereby removing the barriers to β-amylase action. The factor responsible for this activity will be referred to as isoamylase.

Isoamylase activity has also been observed in some of the yeast proteins described in Section III, part 9, the greatest activity being shown by protein D_1. Acetone fractionation has therefore been used for the preparation of isoamylase.

2. Methods and materials.

(a) Paper chromatography.

Descending chromatograms were carried out at 20° with Whatman No.1 paper and propanol-ethyl acetate-water (6:1:3) as solvent.[20] Development was by spraying with silver nitrate-sodium hydroxide reagent.[132]

(b) Deproteinisation.

Enzymic digests were deproteinized, prior to reducing sugar determinations, according to Nelson[96] using zinc

sulphate solution (5% $ZnSO_4$, $6H_2O$) and barium hydroxide solution (approximately 0·3N). The two reagents were adjusted so that 5 ml. zinc sulphate solution required 4·7 to 4·8 ml. of baryta to produce a definite pink colour to phenolphthalein. 0·5 ml. each of zinc sulphate and barium hydroxide solutions were used for a 3 ml. sample of the enzymic digest, the zinc sulphate being added first as reported by Hers, Beaufays and De Duve.(59)

(c) Determination of reducing sugars.

Reducing sugars were determined by the Somogyi 1952 reagent,(124) prepared to give a glucose range of 0·5 to 1·5 mg., and calibrated against standard glucose and maltose solutions.

(d) β-Amylase.

Barley β-amylase purchased from the Wallerstein Laboratories (New York), (free from α-amylase and maltase) was used for the determination of β-amylolysis limits. The enzyme powder had an activity of 100 units per mg. determined by Hobson, Whelan and Peat's method.(62)

(e) Maltodextrin [Prepared by Dr. D. J. Manners].

Maltodextrin was prepared by the action of malt α-amylase on soluble starch and purified by repeated precipitation with ethanol.

3. Experimental.

(a) Preparation of protein D_1.

Dried brewer's yeast (200 g.) was extracted with 0·1M-sodium bicarbonate solution (1000 ml.) at 35° for 2 hours. The solution was centrifuged (5000 r.p.m. for 15 mins.) at 0°C. and the supernatant (700 ml.) treated with AnalaR acetone (700 ml.) at -7°C. The precipitate was collected on the centrifuge (-4°C) and dissolved in 400 ml. 20% (v/v) ethanol solution in 0·2M-citrate buffer pH 6·0. After removing insoluble material on the centrifuge, the solution (435 ml.) was fractionated with AnalaR acetone at -7°C and the precipitate obtained at 0-24% acetone concentration was collected and washed three times with cold acetone and dried under vacuum. The yield of protein D_1 was 17·6 g.

(b) Experiments with protein D_1.

The following experiments were carried out to characterise some of the enzymes present in protein D_1.

(i) "Debranching" activity.

The following digest was set up:-

10 ml. yeast glycogen (3·9 mg. per ml.),
5 ml. 0·2M-phosphate-citric acid buffer pH 7·0,
10 ml. protein D_1 solution (8 mg. per ml.).

0·5 ml. samples were removed at intervals and treated with 0·25 ml. iodine solution (0·2% iodine in 2% potassium iodide); one drop of 5N hydrochloric acid was added and the volume made

up to 5 ml. The absorption values (A.V.) of the polysaccharide-iodine complex were measured on a Unicam spectrophotometer at 480 mμ in 1 cm. cells against an iodine blank. The following results were obtained.

Time (hours)	0	2	4	24
A.V.	0·270	0·365	0·426	0·610

The experiment was repeated with waxy maize starch, and the β-limit dextrin of potato amylopectin; however these attempts to follow the reaction by iodine-staining were not successful. Hence a unit of activity could not be defined using the method described by Hobson, Whelan and Peat[63] for R-enzyme. Isoamylase activity has therefore been expressed in terms of the increase in β-amylolysis limit of glycogen.

(ii) Phosphorylase activity.

The phosphorylase activity of protein D_1 estimated by Green and Stumpf's method (Section III) was 20 units per g.

(iii) "Branching" activity.

The following digest was set up:-

3·5 ml. amylose (approximately 2 mg. per ml.),
1·5 ml. 0·2M-citrate buffer pH 6·0,
1·0 ml. water,
1·0 ml. protein D_1 solution (30 mg. per ml.).

1 ml. samples were withdrawn at intervals, and treated with 1 ml. iodine solution (0·2% iodine in 2% potassium iodide), 1 drop of 5N hydrochloric acid added and the volume made up to 100 ml. The absorption values (A.V.) of the amylose-iodine

complex were measured on a Spekker photoelectric absorptiometer at 680 mμ in 4 cm. cells against an iodine blank. The following results were obtained.

Time (hours)	0	0·16	1·5	20·0
A.V.	1·210	0·975	0·650	0·440

The reaction digest was reduced to a small volume and examined on a paper chromatogram. Glucose, maltose and small reducing sugars were absent; the fall in iodine-stain of amylose was therefore due to the presence of branching enzyme.

(iv) Maltase activity.

Maltose (5 mg.) was incubated with protein D_1 (10 mg.) in citrate buffer pH 6·0 (1 ml.) for 4 hours. The reaction digest was examined on a paper chromatogram and found to contain glucose and a trace of maltose. Thus maltase is present in protein D_1.

(c) Purification of isoamylase.

Protein D_1 (5 g.) was dissolved in 20% ethanol solution (200 ml.) at -4°C. Anhydrous sodium sulphate (2 g.) was added and the solution stirred slowly with cooling to -7°C. Maize starch (112 g.) was added to the solution and stirring continued for 2 hours. The solution was centrifuged (4500 r.p.m. for 15 mins.) at -4°, and the supernatant (90 ml.) treated with 180 ml. cold AnalaR acetone at -10°C. The precipitate was centrifuged at -4°, washed twice with cold acetone, and dried under vacuum. The yield was 2·09 g.

(d) Properties of isoamylase.

Isoamylase, thus prepared, was a white amorphous powder, soluble in water and contained 8% nitrogen. It was free from phosphorylase, amylases and branching enzyme which had been adsorbed into the maize starch, but contained a trace of maltase. The enzyme preparation had no action on glucose in the presence of acetate buffer. The properties of isoamylase have been determined using this purified preparation.

(e) Maltase activity.

The following digest was set up:-

2 ml. maltose (21·24 mg. per ml.),
5 ml. 0·2M-acetate buffer pH 5·89,
16 ml. water,
2 ml. isoamylase solution (40 mg. per ml.).

3 ml. samples were withdrawn at intervals and heated in a boiling water bath for 3 mins. After deproteinization, the glucose was estimated with the Somogyi 1952 reagent. The following results were obtained.

Time (hours)	24	48
% Conversion into glucose	94	97

The enzymic digest was examined on the paper chromatogram; glucose was the only sugar present.

(f) Effect of heat on isoamylase.

2·5 ml. aliquot portions of isoamylase solution (20 mg. per ml., in acetate buffer pH 5·89) were heated at 35°, 45° and 55° for 1 hour and the debranching activity and maltase activity

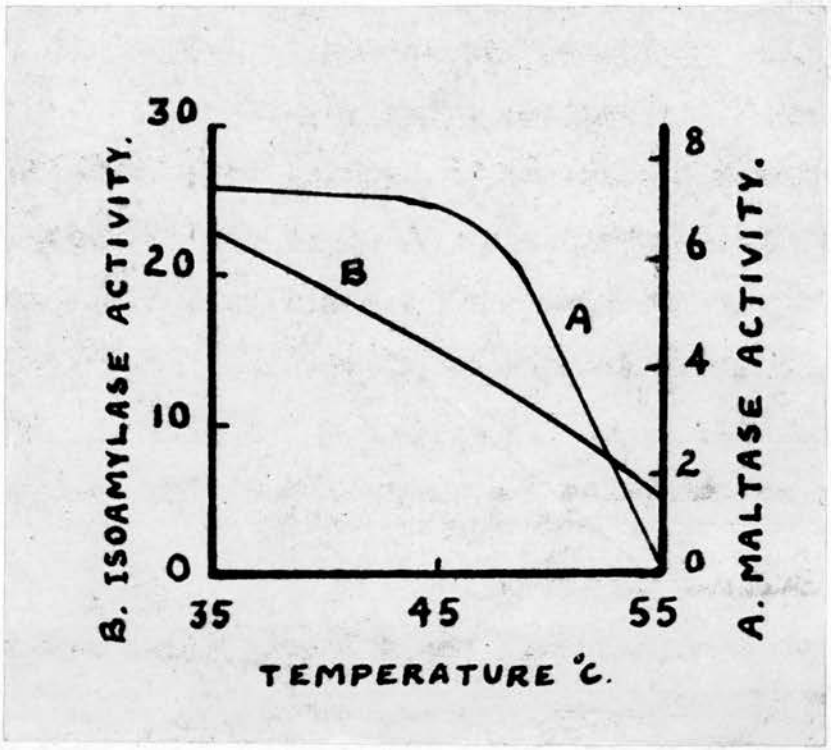


Figure 9. Effect of temperature on isoamylase and maltase.

were tested as follows.

(i) Debranching activity.

Digests consisting of the following were set up.

2 ml. yeast glycogen (4·12 mg. per ml.),

3 ml. 0·2M-acetate buffer pH 5·89,

1 ml. isoamylase solution.

The digest was heated in boiling water bath for 3 mins. after 24 hours incubation at 20°. 2 ml. β-amylase solution (200 units) was added and the β-amylolysis limit determined. The following results were obtained.

Temperature °C.	35	45	55
Isoamylase activity (as increase in β-amylolysis limit)	23	15	6

(ii) Maltase activity.

Digests consisting of the following were set up:-

4 ml. maltose (2·1 mg. per ml.),

2 ml. 0·2M-acetate buffer pH 5·89,

1 ml. water,

1 ml. isoamylase solution.

3 ml. samples were withdrawn, deproteinized and the reducing power, as maltose, determined after 48 hours incubation. The following results were obtained.

Temperature °C.	35	45	55
Maltase activity, (as apparent increase in maltose)	7·3	7·1	0·1

Thus maltase is completely deactivated when heated to 55° at pH 5·89; at the same time, a considerable amount of the debranching activity is also destroyed (Figure 9).

(g) Stability of isoamylase.

The following digest was set up:-

10 ml. yeast glycogen (4 mg. per ml.),

5 ml. 0.2M-acetate buffer pH 5·89,

3 ml. water,

2 ml. isoamylase solution (40 mg. per ml.).

(i) 5 ml. sample was removed after 72 hours incubation at 20°, and the isoamylase destroyed by heating in a boiling water bath for 3 mins. The solution was cooled and 3 ml. β-amylase solution (600 units) added, and the β-amylolysis limit determined.

(ii) To another 5 ml. sample at 72 hours, 2 ml. fresh yeast glycogen solution (4 mg. per ml.) was added and this was incubated at 20° for a further 24 hours. The isoamylase was deactivated and the β-amylolysis limit determined in a similar manner. The following results were obtained.

Time of incubation with isoamylase (hours)		Mg. maltose produced	Increase in β-amylolysis limit (%)
(i)	72	6·02	27
(ii)	24	10·33	7

Thus isoamylase is still active even after 72 hours incubation with glycogen, the β-amylolysis limit of which is 44%.

(h) Time of incubation.

A digest containing B.D.H. glycogen (β-amylolysis limit

45%) was set up as follows:-

10 ml. B.D.H. glycogen (4.04 mg. per ml.),

5 ml. 0·2M-acetate buffer pH 5·89,

2 ml. water,

8 ml. isoamylase solution (10 mg. per ml.).

5 ml. samples were removed after 24, 48 and 72 hours and isoamylase deactivated by heating in a boiling water bath for 3 mins. The solutions were cooled and treated with 3 ml. β-amylase solution (600 units) to determine the β-amylolysis limit of the resulting polysaccharide. The following results were obtained.

Time of incubation with isoamylase (hrs.)	24	48	72
Increase in β-amylolysis limit (%)	20	20	20

Thus isoamylase action is complete within 24 hours.

(i) Action of isoamylase on isomaltose, maltotriose, von Gierke glycogen, α-limit dextrin, β-limit dextrin, and yeast glycogen.

15 mg. substrate was incubated with isoamylase solution (20 mg. per ml. of acetate buffer pH 5·89; 1 ml.) for 24 hours and the enzymic digest then examined on a paper chromatogram. The following results were obtained.

Substrate	Reducing sugars found
Isomaltose	Glucose and isomaltose
Maltotriose	Glucose and maltose
Von Gierke glycogen	Glucose
α-Limit dextrin (starch)	Glucose and maltose
β-Limit dextrin (yeast glycogen	Glucose and maltose
Yeast glycogen	Nil

4. Action of isoamylase on polysaccharides.

(a) Amylose

The following digest was set up:-

2·5 ml. amylose (potato) solution (4 mg. per ml.),

1·0 ml. 0·2M-acetate buffer pH 5·89,

1.5 ml. isoamylase solution (10 mg. per ml.).

A control digest without isoamylase was set up. Both digests were heated in a boiling water bath for 3 mins. after 24 hours incubation (20° C). The β-amylolysis limits were then determined by adding β-amylase solution (3 ml.; 300 units). The following results were obtained.

	β-amylolysis limit (%)
Amylose (control digest)	91
Amylose treated with isoamylase	96

The result indicates that the isoamylase is free from branching enzyme; furthermore, some of the anomalous linkages in amylose may be 1:6 linkages, since the β-amylolysis limit is increased after incubation with isoamylase.

(b) Branched α-1:4-glucosans.

The action of isoamylase on the β-amylolysis limit of a number of branched α-1:4-glucosans was investigated as follows:-

polysaccharide, 0·2M-acetate buffer pH 5·89, water, and isoamylase solution (in water) were incubated at 20° for 24 hours. The digests were then heated at 100° for 3 mins. to

inactivate the enzymes, and after cooling, 3 ml. β-amylase solution (300-600 units) was added. After 24 hours incubation at 35°, the percentage conversion into maltose was determined.

The detailed compositions of the digests are tabulated below.

Digest	Substrate	Weight (mg.)	Buffer (ml.)	Water (ml)	Isoamylase solution (mg); ml.	Total volume
1	Maltodextrin	10·0	1·0	2·5	21; 1·5	5·0
2	β-Limit dextrin	41·3	5·0	12·0	80; 8·0	25·0
3	Waxy maize starch	24·5	4·0	7·0	40; 1·0	12·0
4	Waxy sorghum starch	24·2	4·0	6·0	50; 2·0	12·0
5	Protozoal amylopectin	24·0	4·0	6·0	50; 2·0	12·0
6	Von Gierke glycogen	25·2	4·0	7·0	40; 1·0	12·0
7	B.D.H. glycogen	40·4	5·0	12·0	80; 8·0	25·0
8	Rabbit liver glycogen A	25·3	4·0	8·0	40; 1·0	13·0
9	Rabbit liver glycogen B	24·4	4·0	6·0	50; 2·0	12·0
10	*Helix pomatia* glycogen	25·1	4·0	8·0	40; 1·0	13·0
11	*Trichomonas foetus* glycogen	25·0	4·0	7·0	40; 1·0	12·0
12	Brewer's yeast glycogen	39·8	5·0	12·0	80; 8·0	25·0

The following results were obtained.

Digest No	Substrate	β-Amylolysis limit (%)	Ref.	β-Amylolysis limit after treatment with isoamylase (%)	Increase in β-amylolysis limit (%)
1	Maltodextrin	67	*	94	27
2	β-Limit dextrin	0	*	32	32
3	Waxy maize starch	50	*	66	16
4	Waxy sorghum starch	52	75	76	24
5	Protozoal amylopectin	63	75,*	80	17
6	Von Gierke glycogen	14	82	18	4
7	B.D.H. glycogen	45	*	65	20
8	Rabbit liver glycogen A	53	75	76	23
9	Rabbit liver glycogen B	49	75,*	59	10
10	Helix pomatia glycogen	37	12	60	23
11	Trichomonas foetus glycogen	60	85	80	20
12	Brewer's yeast glycogen	44	p.28	68	24

* Determined in a control experiment, without isoamylase.

In digest 2 the β-amylolysis limits were 19 and 20% after incubation with isoamylase for 5 mins. and 2 hours respectively.

In digest 12 the β-amylolysis limits were 48 and 58% after incubation with isoamylase for 5 mins. and 2 hours respectively.

(c) Action of isoamylase on Von Gierke glycogen.[82]

A preliminary experiment (p. 91) had shown that glucose

was released by the action of isoamylase on the Von Gierke glycogen. This experiment was repeated quantitatively in the following way.

Von Gierke glycogen (65·3 mg.) was incubated with isoamylase solution (2 ml.; 80 mg.), 0·2M-acetate buffer pH 5·89 (3 ml.) and water (5 ml.) at 20°. The liberated glucose was estimated with the Somogyi 1952 reagent; the percentage conversion into glucose after 90 hours incubation was 7·3.

Assuming that the Von Gierke glycogen has an average chain length of 6 glucose residues[82] and contains equal numbers of A- and B-chains, the theoretical amount of glucose which would be released if all the single A-chain linkages were hydrolysed is 8·3%.

(d) <u>Simultaneous action of isoamylase and β-amylase on brewer's yeast glycogen</u>.

The following digest was set up:-

15 ml. brewer's yeast glycogen solution (40·2 mg.),
5 ml. 0.2M-acetate buffer pH 5·89,
2 ml. isoamylase solution (80 mg.),
3 ml. β-amylase solution (375 units).

Since the isoamylase preparation contained traces of maltase, the reaction was followed by estimating the amount of glucose produced. 3 ml. samples were removed at intervals, deproteinized and the glucose content estimated with the Somogyi 1952 reagent. The following results were obtained.

Time of incubation (hours)	24	48	72	120
% Conversion into glucose	66	81	86	92

(e) Stepwise action of isoamylase and β-amylase on brewer's yeast glycogen.

Brewer's yeast glycogen solution (7 ml.; 25·0 mg.) was incubated with isoamylase solution (1 ml.; 40 mg.) and 0·2M-acetate buffer pH 5·89 (5 ml.) at 20° for 24 hours. The enzyme was deactivated by heat and β-amylase (3 ml.; 600 units) was added and the β-amylolysis limit determined after 24 hours by analysis of a 4 ml. portion of the digest. The remaining solution was again treated with isoamylase solution (1 ml.; 40 mg.) after destroying the β-amylase by heating, and incubated at 20° for 24 hours. The isoamylase was then deactivated, and a 3 ml. sample removed for determination of the initial reducing power of the digest. 3 ml. β-amylase (600 units) was added to the solution and the β-amylolysis of the resulting polysaccharide determined. The following results were obtained.

	Maltose content of digest (mg.)	% Amylolysis
First treatment with isoamylase	17·6	67
Second treatment with isoamylase (initial)	18·4	
(after addition of β-amylase)	20·6	14

5. Discussion.

Debranching enzymes bring about the hydrolysis of α-1:6-interchain linkages in branched α-1:4-glucosans; this results in an increase of the β-amylolysis limit and the iodine staining power of the polysaccharide.

The available evidence, from β-amylolysis studies (and in one experiment, from iodine staining power measurements) shows that isoamylase catalyses the hydrolysis of non-terminal α-1:6-linkages in amylopectin, glycogen and their degradation products. Isoamylase has no action on the α-1:4-linkages in glycogen. When yeast glycogen is incubated with protein D_1, during 24 hours a 47% increase in the absorption value of the iodine-glycogen complex at 680 mμ was observed; however attempts to follow the isoamylase action on β-limit dextrin, and amylopectin by iodine-staining were not successful. Determination of a unit of activity by an iodine staining method, similar to that used for R-enzyme activity determinations was not therefore possible.

Protein D_1 contains phosphorylase, branching enzyme, and maltase but is free of amylases; by starch adsorption the phosphorylase and branching enzyme can be completely removed, although the final preparation still contains traces of maltase. Attempts to selectively inactivate the maltase by heating to 53° at pH 5·89 were not successful, since although the maltase was destroyed, a considerable amount of the isoamylase activity was also lost. The presence of

this maltase does not, however, interfere with the degradation studies of polysaccharides since the maltase itself has no action on polysaccharides, and the β-amylase is added after deactivation of the isoamylase preparation. In the special cases of a (i) stepwise, and (ii) simultaneous degradation of a polysaccharide by isoamylase and β-amylase, the effect of the maltase can be eliminated by estimation of the, (i) initial reducing power before the addition of β-amylase and (ii) reducing sugar as glucose.

Isoamylase also catalyses the hydrolysis of terminal α-1:6-glucosidic linkages since it hydrolyses isomaltose, and liberates glucose from a sample of Von Gierke glycogen, which contains a number of single glucose residues attached by 1:6-linkages to the rest of the molecule.(82)

Isoamylase action on glycogens and amylopectins is incomplete since the β-amylolysis limits of the isoamylase treated polysaccharides do not exceed 80%; this is not due to the denaturation of the enzyme in the digest. It is shown that isoamylase, after 72 hours incubation with glycogen, is still capable of hydrolysing the α-1:6-linkages of freshly added glycogen. It seems probable that isoamylase action is mainly confined to the hydrolysis of the 1:6-linkages of A-chains.

The action of isoamylase on various polysaccharides, together with their chain lengths and β-amylolysis limits, are summarised in Table 5.

Table 5.

Effect on the β-amylolysis limits of polysaccharides treated with isoamylase.

Polysaccharides	Chain length*	β-Amylolysis limit (%)	β-Amylolysis limit after isoamylase treatment (%)	Increase in β-amylolysis limit (%)
Amylose (potato)	-	91	96	5
Maltodextrin	-	67	94	27
β-Limit dextrin (waxy maize starch)	11	0	32	32
Waxy maize starch	22	50	66	16
Waxy sorghum starch	25	52	76	24
Protozoal amylopectin	22-23	63	80	17
Von Gierke glycogen	6	14	18	4
B.D.H. glycogen	-	45	65	20
Rabbit liver glycogen A	18	53	76	23
Rabbit liver glycogen B	12	49	59	10
Helix pomatia glycogen	7	37	60	23
Trichomonas foetus glycogen	15	60	80	20
Brewer's yeast glycogen	13	44	68	24

* Determined by potassium periodate oxidation; References (75,82,83).

Debranching enzymes previously reported include amylo-1:6-

glucosidase, limit dextrinase, oligo-1:6-glucosidase, and R-enzyme. The activity of these enzymes, together with isoamylase, towards various substrates is summarised in Table 6.

Table 6.

The specificity of debranching enzymes.

Substrates	Size of A-chain (glucose residues)	Debranching enzymes				
		Amylo-1:6-glucosidase	Limit dextrinase	Oligo-1:6-glucosidase	R-Enzyme	Isoamylase
Muscle phosphorylase limit dextrin	1	+	?	-	+	+
α-Limit dextrin	2-3	-	+	+	+	+
β-Limit dextrin	2-3	-	+	-	+	+
Isomaltose	1	±	?	+	-	+
Amylopectin	10-15	-	-	-	+	+
Glycogen	6-9	-	-	-	-	+
References		33	64	72	63,110	

(+) Indicates that the enzyme is active towards the substrate whereas (-) indicates that the enzyme has no action on the substrate.

Thus amylo-1:6-glucosidase, limit dextrinase and oligo-1:6-glucosidase hydrolyse terminal α-1:6-linkages. The R-enzyme and isoamylase appear to be the only debranching

enzymes which can hydrolyse non-terminal α-1:6-glucosidic linkages. The difference between R-enzyme and isoamylase is that R-enzyme cannot hydrolyse isomaltose and glycogen, whereas isoamylase is active towards both these two substrates. Isoamylase has therefore a wider specificity than any of the debranching enzymes previously reported.

Isoamylase has been used in this Department, for the detection of α-1:6-glucosidic linkages in polysaccharides. For example, the nature of the interchain linkages in Floridean starch, a glucosan containing α-1:4-linkages, isolated from certain algae, have recently been characterised.(38) When treated with isoamylase, the β-amylolysis limit of Floridean starch increased from 42 to 65%, and it was therefore concluded that the interchain linkages are of the α-1:6-type. Later, Dr. Warburton(133) isolated isomaltose from a partial acid hydrolysis of Floridean starch, thus confirming the view that α-1:6-linkages are present in the polysaccharide. In a similar experiment, Mr. A. R. Archibald (unpublished results) has shown that the interchain linkages in the amylopectin of the protozoa Chilomonas paramecium are α-1:6; the β-amylolysis limit of this polysaccharide increased from 58 to 72% on treatment with isoamylase.

Recent experimental evidence indicates that certain samples of amylose contain a small proportion of linkages other than α-1:4-glucosidic linkages since they are not completely hydrolysed by β-amylase. The action of isoamylase

on one sample of amylose suggests that a proportion of the anomalous linkages may be α-1:6-linkages since the β-amylolysis limit of the amylose is increased after incubation with isoamylase. In this case, the fractionation procedure for the separation of the amylose and amylopectin components of starch must have been slightly impaired. Isoamylase may therefore find use for the detection of traces of amylopectin in amylose samples.

Section V

Studies on the Branching Enzyme of Yeast.

1. Introduction.

Cori and Cori[31] showed that extracts of liver, heart and brain tissues contained an enzyme which acted on amylose producing a branched polysaccharide with the same iodine staining power and solubility as glycogen. The existence of a related enzyme, named Q-enzyme, in plant tissues was demonstrated by Haworth, Peat, Bourne and Macey.[17,19,58] Q-Enzyme acts on amylose producing a branched polysaccharide similar in structure to amylopectin. It performs a double function, namely the formation of α-1:6-linkages which follows the scission of an equal number of α-1:4-linkages. Potato Q-enzyme has been extensively studied by Peat and his co-workers[5,6,7,8.18] and recently has been isolated in crystalline form by Baum, Gilbert and Patrick.[9,40]

During studies on yeast phosphorylase, it was found that the iodine staining power of amylose, when treated with protein A_1, decreased from 1·04 to 0·23 within 1 hour (page 69). It was also shown that the β-amylolysis limit of the amylose treated with protein A_1 was 54%, that of the original polysaccharide being 79%. A branching enzyme must therefore be present in protein A_1, and since the presence of a branching enzyme in yeast has not previously been reported,

the properties of this enzyme have been investigated.

Among the yeast proteins obtained during phosphorylase purification, the fractions precipitated with ethanol showed the greatest branching activity and the least phosphorylase activity. Hence, ethanol fractionation has been used for the preparation of the branching enzyme of yeast.

2. Methods and materials.

(a) Iodine staining.

Iodine solution (1 ml.; containing 2 mg. of iodine and 20 mg. potassium iodide per ml.) was introduced into a graduated flask (100 ml.) and water added to ca.80 ml. A drop of 5N hydrochloric acid was added. A sample (1 ml.) of a digest containing polysaccharide was added to the above solution, and the volume made up to 100 ml. The absorption value (.A.V.) of the polysaccharide-iodine complex was measured on a Spekker photoelectric absorptiometer in 4 cm. cells, an Ilford filter No.608 (680 mμ) being used, against an iodine blank.

(b) Paper chromatography and determination of reducing sugars.

Reducing sugars were determined by use of the Somogyi 1952 reagent[124] and descending chromatograms were carried out as described in Section IV, page 83.

(c) β-Dextrins.

β-Dextrins of Light's glycogen and waxy maize starch were

prepared by the prolonged action of barley β-amylase (Wallerstein Laboratories, New York) on the polysaccharide at pH 4·6. The limit dextrin was recovered and the treatment with β-amylase repeated.

(d) Amylose.

Amylose was prepared according to Baum and Gilbert.[10] Fresh potato starch (10 g.) was dispersed in water (1 l.) and the pH of the solution brought to 3 with hydrochloric acid. The suspension was boiled for 30 mins., cooled rapidly to 60°, stirred with excess n-butanol and allowed to stand overnight. The precipitate was washed three times with butanol-saturated water, and redissolved in hot water to give a 0·2% solution. It was reprecipitated with butanol, and the process repeated. Finally the amylose was washed several times with ethanol and dried under vacuum over phosphorus pentoxide at 100° C. The yield of amylose (.B.V. = 1·15) was 2·1 g.

Another sample of amylose (B.V. = 1·33), prepared by Dr. D. J. Manners, was also used in the investigations.

3. Experimental.

(a) Preparation of branching enzyme.

Dried brewer's yeast (100 g.) was extracted with 0·1M-sodium bicarbonate solution (500 ml.) at 35° for 2 hours. The solution was centrifuged at 0° (5000 r.p.m. for 15 mins.) and the supernatant (350 ml.) collected. The solution was

treated with saturated ammonium sulphate (0°C) to 0·5 saturation; the precipitate was collected on the centrifuge and dissolved in 0·1M-citrate buffer pH 6·8 (100 ml.). 50% (v/v) ethanol-citrate solution containing 0·01M-citrate buffer pH 6·8 was added at -4° to 25% alcohol concentration. The precipitate was removed on the centrifuge, and the alcohol concentration of the supernatant solution increased to 30%. The precipitate was collected, and triturated with 0·1M-citrate buffer pH 6·8 (40 ml.) for 30 mins. Insoluble material was removed by centrifugation, and the supernatant solution treated with saturated ammonium sulphate at 0° to 0·5 saturation. The precipitate was collected on the centrifuge, dissolved in 0·1M-citrate buffer pH 6·8 (20 ml.) and freeze dried. The yield was 2·63 g. Found protein nitrogen, 2·3%.

(b) Branching enzyme activity determination.

The branching enzyme activity was determined by the method of Gilbert and Patrick.[40] The following digest containing varying concentrations of the branching enzyme was set up:-

3·5 ml. enzyme solution (10 mg. per ml.) and water,
1.0 ml. 0·2M-citrate buffer pH 6·8,
0·5 ml. 2% soluble starch (AnalaR).

The reaction was followed by determination of the absorption value (A.V.) of starch-iodine complex. The following results were obtained, after 10 and 40 mins.

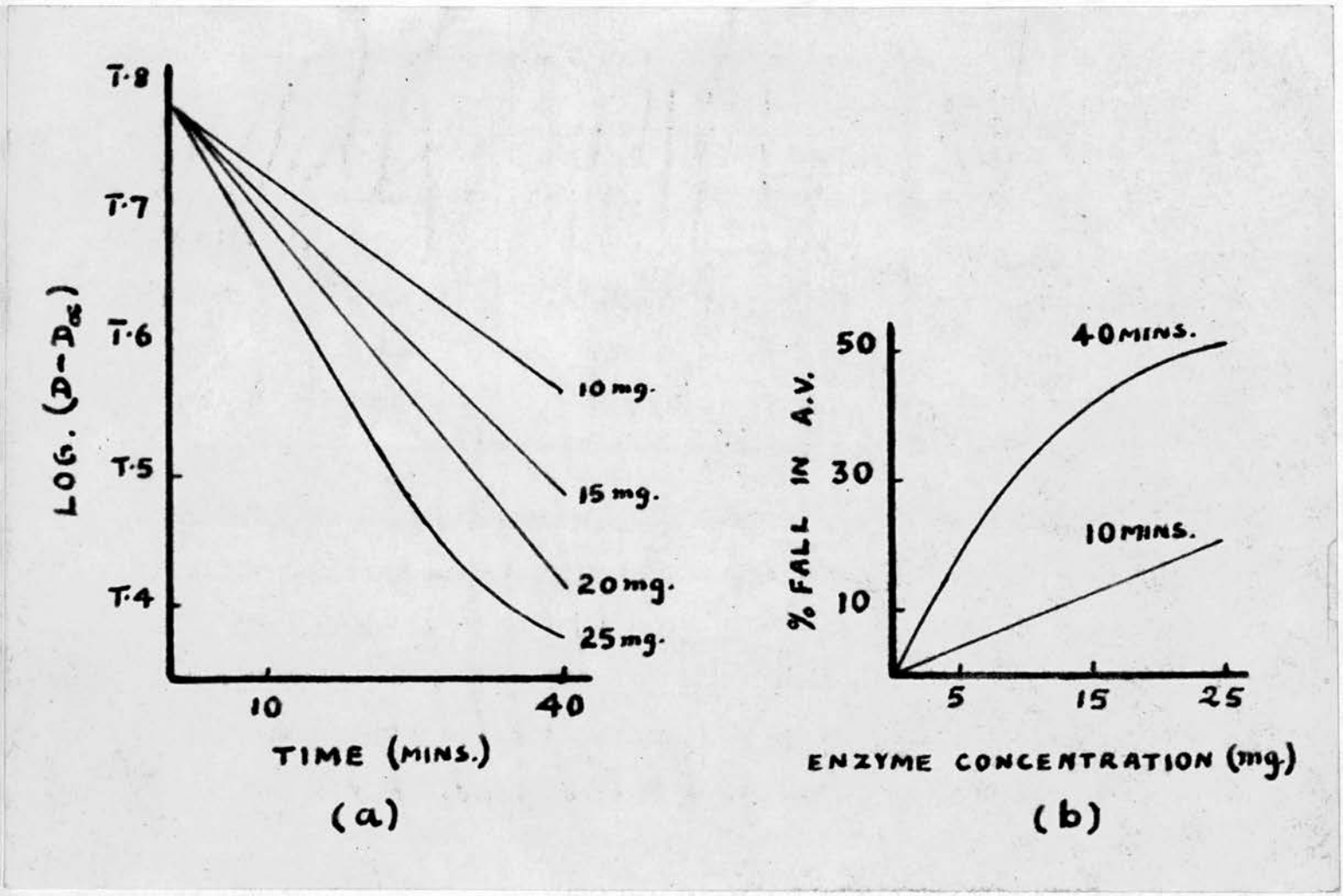


Figure 10. Activity of the branching enzyme.

(a) Activity as a function of time.

(b) Relationship between activity and enzyme concentration.

incubation at 20°.

Initial A.V., 0·75; D_∞ (A.V. at T_∞), 0·132; log $(D-D_\infty)$, $\bar{1}$·80

Concentration of branching enzyme (mg.)	10 mins.		40 mins.	
	% Fall in A.V.	log $(D-D_\infty)$	% Fall in A.V.	log $(D-D_\infty)$
10	8·7	$\bar{1}$.743	33·3	$\bar{1}$.566
15	13·3	$\bar{1}$.714	41·1	$\bar{1}$.491
20	16·7	$\bar{1}$.693	46·8	$\bar{1}$.426
25	20·0	$\bar{1}$.670	50·0	$\bar{1}$.386

Figure 10(b) shows that the percentage fall in A.V. (10 mins. samples) is proportional to the enzyme concentration. Hence, the unit of activity is defined as the amount of enzyme which causes a fall in the absorption value of the starch-iodine complex by 0·1 of the Spekker absorptiometer reading within 10 mins. under the above conditions.

(c) Branching activity.

The following digest was set up:-

2·5 ml. amylose (1 mg. per ml.),

1·0 ml. 0·2M-citrate buffer pH 6.8,

1·5 ml. branching enzyme solution (45 mg.; 3 units).

The reaction was followed by iodine staining and the following results were obtained.

Time (hours)	0·0	0·5	1·5	20·0
A.V.	0·435	0·210	0·150	0·035

The digest was examined on a paper chromatogram and it was found that glucose, maltose and small reducing sugars were absent.

(d) Test for contaminating enzymes.

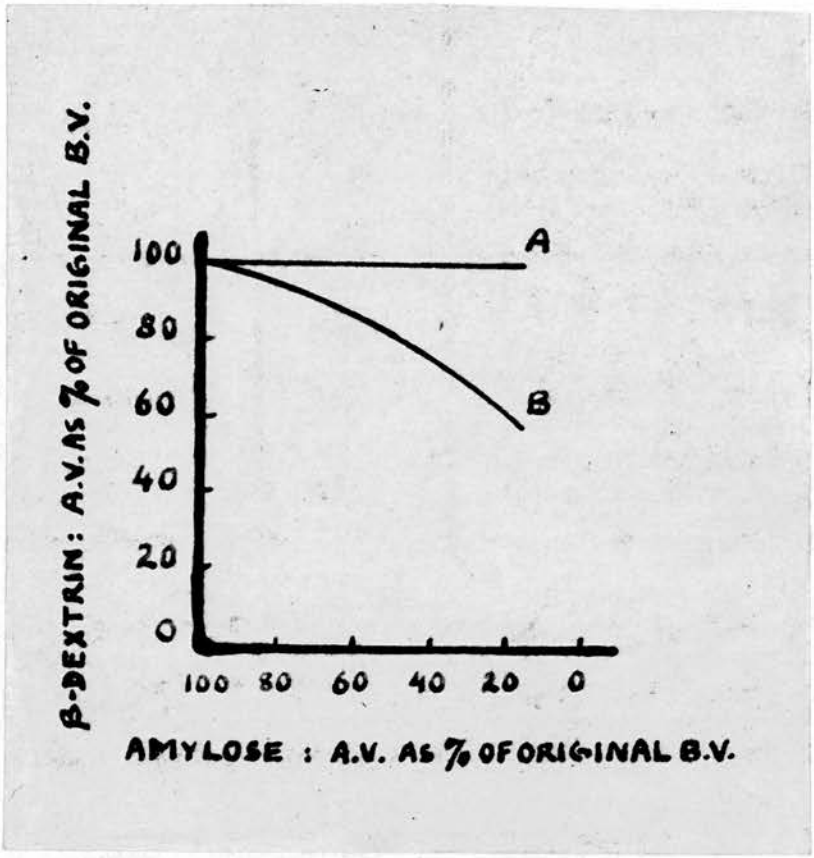
(i) Maltose, maltotriose and isomaltose (10 mg.) were incubated with the branching enzyme preparation (20 mg.) in 0·2M-citrate buffer pH 6·8 (0·5 ml.). The digests were examined on a paper chromatogram after 24 hours incubation at room temperature. The following results were obtained.

Substrates	Reducing sugars found
Maltose	Maltose and glucose
Maltotriose	Maltotriose, maltose and glucose
Isomaltose	Isomaltose and glucose

The enzyme preparation is therefore contaminated with traces of maltase, maltotriase and isoamylase; the presence of these enzymes will not interfere with activity determinations of the branching enzyme preparation.

(ii) α-Amylase.

The α-amylase test was carried out as described by Hobson, Whelan and Peat.[62] Three digests were set up and the iodine staining power determined as follows.



Key: A, Glycogen β-dextrin.

B, Waxy maize starch β-dextrin.

Figure 11. Test for α-amylase.

(After Hobson, Whelan and Peat[62]).

Digest	Amylose	Amylopectin β-dextrin	Glycogen β-dextrin
Weight of substrate (mg.)	5	10	10
Water (ml.)	5	5	5
0·2M-citrate buffer pH 6·8	2	2	2
Branching enzyme solution (60 mg.; 4 units)	3	3	3
Aliquots removed for iodine stain (ml.)	1·0	1·0	1·0
Iodine solution (ml.)	1·0	0·5	0·5
Volume of polysaccharide-iodine solution analysed (ml.)	100	50	50

The absorption values were measured on a Spekker photo-electric absorptiometer in 4 cm. cells at 680 mμ against an iodine blank. The following results were obtained.

Time (mins.)	Percentage of original absorption value		
	Amylose	Amylopectin β-dextrin	Glycogen β-dextrin
0	100	100	100
20	49	81	100
60	36	74	100
1080	16	58	100

α-Amylase is absent from the enzyme preparation since the iodine staining power of glycogen β-dextrin remained

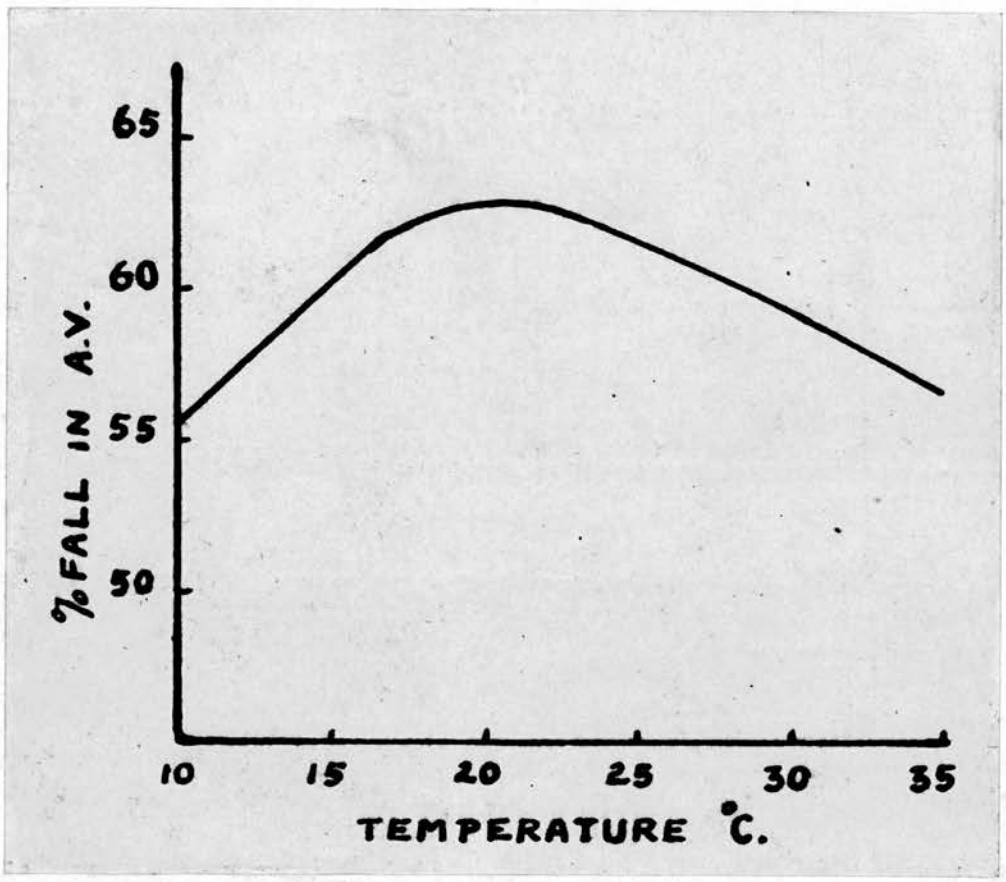


Figure 12. Optimum temperature of yeast branching enzyme.

(Time of incubation 30 mins.)

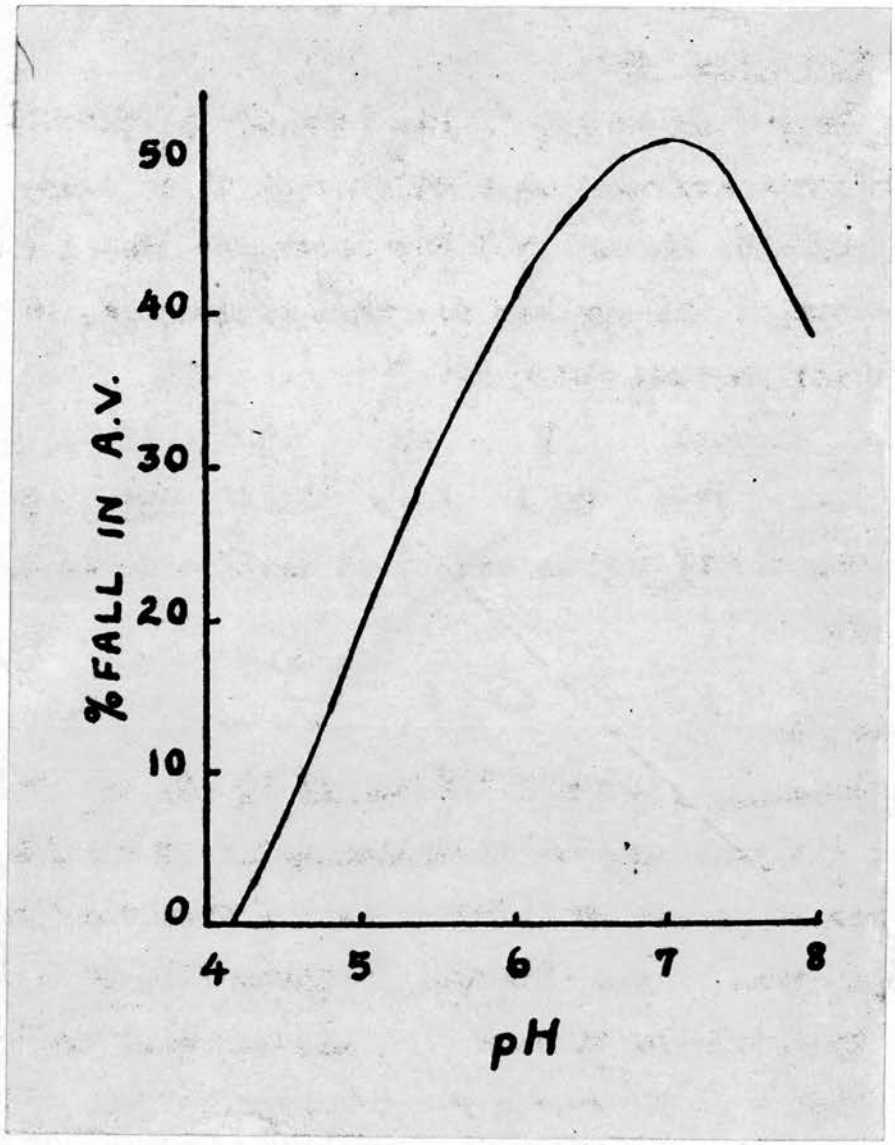


Figure 13. Optimum pH of yeast branching enzyme.

constant. The branching enzyme of yeast acts on waxy maize starch β-dextrin (Figure 11).

(e) Optimum temperature.

Yeast branching enzyme (1 ml. in citrate buffer pH 6·8; 1·33 units) was incubated with amylose (1 ml.; 1 mg. per ml.) at 10, 15, 20, 25, 30 and 35°C for 30 mins. The iodine staining power of the amylose was then determined, when the following results were obtained:-

Temperature °C.	10	15	20	25	30	35
% Fall in A.V.	55·6	60·2	62·9	61·1	59·7	56·6

The rate of the enzyme action is maximal at 20°C. (Figure 12).

(f) Optimum pH.

Yeast branching enzyme (100 mg. in 10 ml. water; 6·66 units) was dialysed against distilled water at room temperature for 12 hours to remove the citrate buffer from the freeze dried preparation. The dialysed solution (1·5 ml.) was incubated with amylose (1 ml.; 2·9 mg. per ml.) and 0·2M-phosphate buffer of pH values ranging from 4·10 to 7·95 (1·5 ml.). The reaction was allowed to proceed at 20° for 15 mins. and the iodine staining power of the amylose then determined.

pH	4·10	5·15	6·10	7·05	7·95
% Fall in A.V.	0·0	22·7	42·9	52·1	39·3

Thus the optimum pH of the yeast branching enzyme is 7·05 (Figure 13).

(g) Activation of yeast branching enzyme.

Barker, Bebbington and Bourne[4] reported that the rate of action of Q-enzyme from Polytomella coeca on amylose was increased by the presence of maltosaccharides (D.P. 2-10) whilst saccharides devoid of α-1:4-glucosidic linkages did not have this effect. The following experiment was carried out to determine the effect of maltosaccharides on the action of yeast branching enzyme.

Amylose (1 ml.; 2·9 mg.) was incubated with branching enzyme (1 ml.; 1 unit), 0·2M-citrate buffer pH 6·8 (1·0 ml.) and activator (1 ml.; 10 mg. in 0·2M-sodium sulphate) at 20°C. The reaction was followed by the determination of the A.V. of amylose-iodine complex. The following results were obtained.

Activators	Percentage of original A.V.		
	10 mins.	20 mins.	40 mins.
None	50	38	29
Maltose	49	38	29
Maltotriose	48	37	29
Cellobiose	49	38	29
Yeast glycogen	53	41	33

Thus the presence of maltosaccharides or cellobiose has no effect on the rate of the branching enzyme of yeast.

(h) Inhibition of yeast branching enzyme.

(i) A digest containing 0·333% ammonium molybdate, which inhibits R-enzyme, was prepared as follows:-

3 ml. amylose (1 mg. per ml.),

1 ml. 0·2M-citrate buffer pH 6·8,

1 ml. 2% ammonium molybdate,

1 ml. enzyme solution (1 unit).

A control digest without ammonium molybdate was also set up and the iodine staining power of the amylose was determined after 10 and 45 mins. incubation at 20°C. The following results were obtained.

Digest	Percentage fall in A.V.	
	10 mins.	45 mins.
Ammonium molybdate (0·333%)	23·4	48·6
Control	27·9	54·5

Thus ammonium molybdate has very little effect on the yeast branching enzyme.

(ii) Mercuric chloride.

The effect of mercuric chloride, which inhibits plant Q-enzymes, on yeast branching enzyme was studied. Amylose (1 ml.; 2 mg.) was incubated with the branching enzyme (0.5 ml. in citrate buffer pH 6·8; 1 unit) and mercuric chloride of varying concentration (0·5 ml.). The reaction was allowed

to proceed for 30 mins. and the iodine staining power of the amylose determined.

Initial A.V. of the amylose-iodine complex 1·01

$HgCl_2$ concentration in digest	0	M/20,000	M/10,000	M/5,000	M/2,000	M/1,000	M/500
A.V.	0·36	0·41	0·47	0·62	1·01	1·01	1·01

Thus brewer's yeast branching enzyme is inhibited by 5×10^{-4}M mercuric chloride.

(i) Action of branching enzyme on amylose, waxy maize starch, and brewer's yeast glycogen.

The following digests containing amylose (40·0 mg.), waxy miaze starch (39·9 mg.) and brewer's yeast glycogen (42·2 mg.) were set up:-

16 ml. polysaccharide solution,
5 ml. 0·2M-citrate buffer pH 6·8,
4 ml. branching enzyme solution (7 units).

The reaction was followed by iodine staining and β-amylolysis limit determination at various intervals. The β-limit determination was carried out by removing 5 ml. samples which were then heated in boiling water bath for 3 mins.; after cooling, 3 ml. β-amylase solution (900 units) was added and the maltose produced after incubation (35°) was estimated by the Somogyi 1952 reagent (with deproteinization). The following results were obtained.

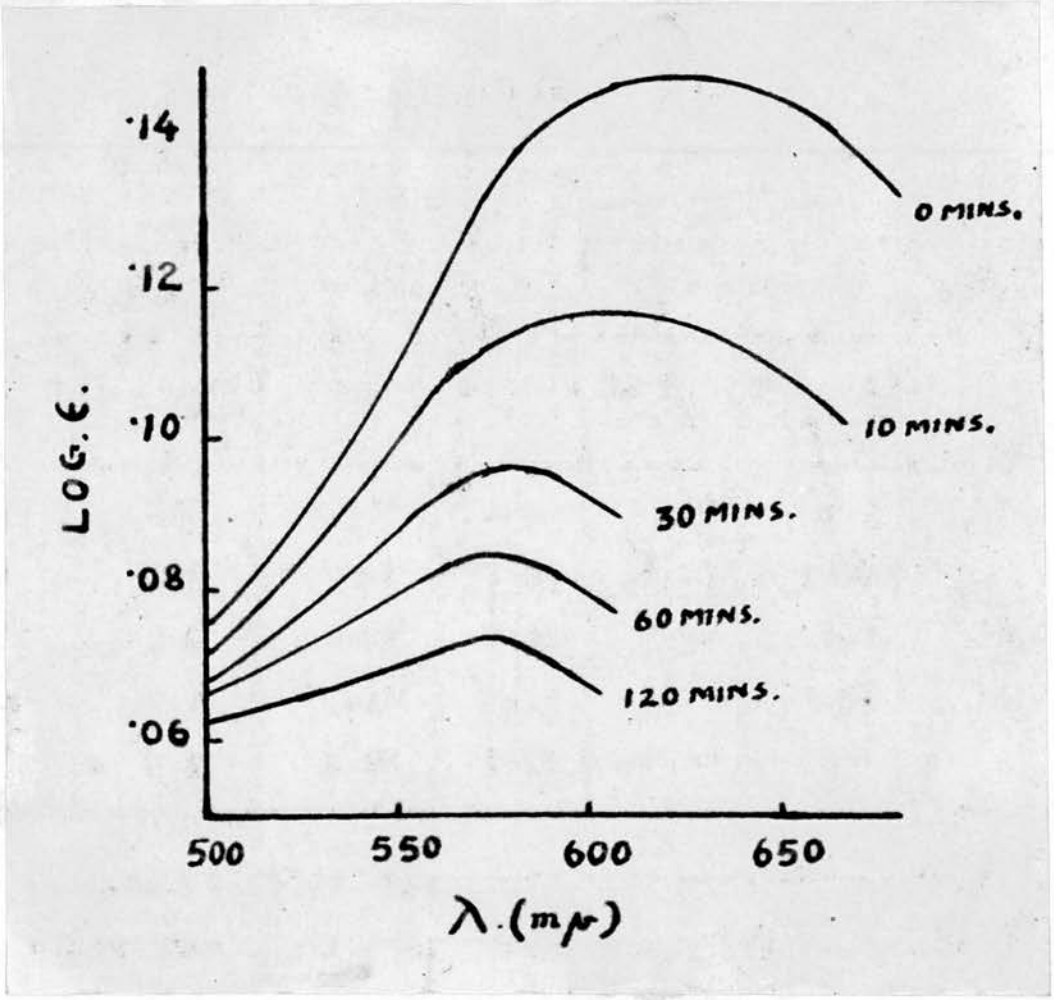


Figure 14. Absorption spectra of the iodine complex of amylose treated with yeast branching enzyme.

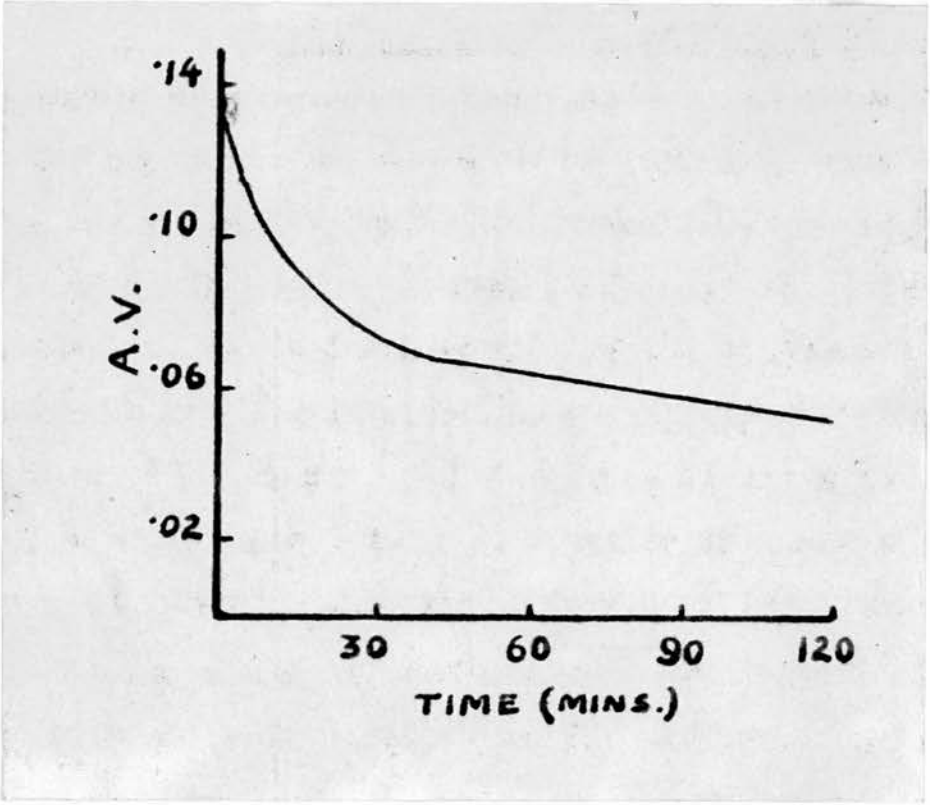


Figure 15. Fall in absorption value of amylose-iodine complex at 680 mμ. after incubation with yeast branching enzyme.

Time of incubation with branching enzyme (hrs.)	Amylose		Waxy maize starch		Yeast glycogen	
	Fall in A.V. %	β-limit %	Fall in A.V. %	β-limit %	Fall in A.V. %	β-limit %
0	0·0	84·1	0·0	50·0	0·0	44·0
1	66.3	50·4	40·6	53·2	0·0	45·5
2	74.3	49·3	46·8	54·4	0·0	47·3
4	80.5	48·6	53·1	56·3	0·0	47·8
24	90·9	54·1	68·7	68·4	0·0	48·5

The results show that the yeast branching enzyme preparation, unlike plant Q-enzyme, attacks waxy maize starch; this finding is not altered by the demonstration of a trace of isoamylase activity in the preparation.

(j) Action of branching enzyme on amylose.

Amylose (5 ml.; 5 mg.) was incubated with branching enzyme (3 ml.; 4 units) in 0·2M-citrate buffer pH 6·8 (2 ml.). 1 ml. samples were withdrawn at intervals and iodine solution (1 ml.) added and the volume made up to 100 ml. The absorption spectrum of the polysaccharide-iodine complex was studied using a Unicam spectrophotometer. The results obtained are shown in Figure (14). Figure (15) shows the decrease in the absorption value at 680 mμ. It was found that the wave length of the maximum absorption falls during incubation (Figure 16). The colour of the solution obtained at 120 mins. incubation was very weak, hence, to obtain the

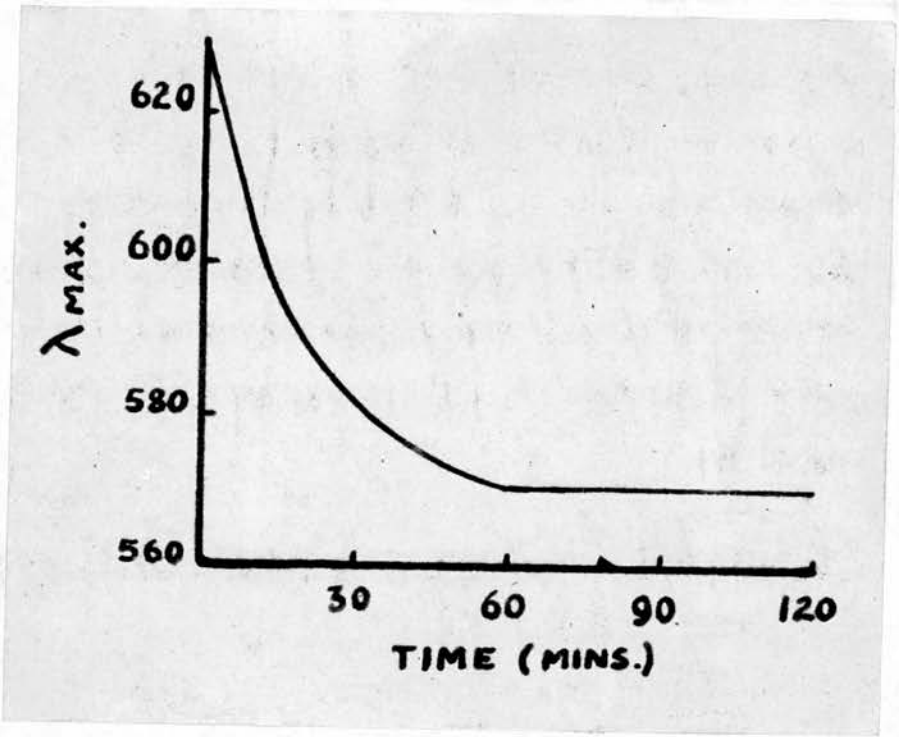


Figure 16. Fall in λ max. of amylose-iodine complex, after incubation with yeast branching enzyme.

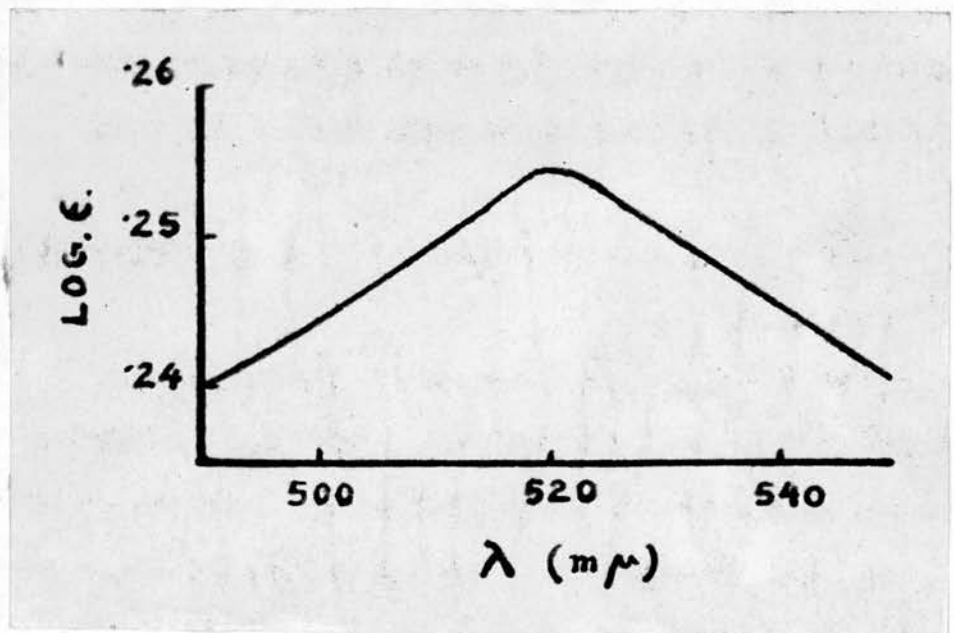


Figure 17. Absorption spectrum of polysaccharide-iodine complex after 20 hours incubation of amylose with yeast branching enzyme.

wave length of maximum absorption after 1200 mins., 1 ml. of the sample was treated with 0·2 ml. of iodine solution and the volume made up to 10 ml. A brown solution which has the maximum wave length at 520 mμ was obtained (Figure 17).

1·5 ml. of the remaining digest was deproteinized and the reducing power determined (Somogyi reagent). The digest had no reducing power.

(k) Action of β-amylase, α-amylase and isoamylase on amylose treated with branching enzyme.

The following digest was set up:-

8 ml. amylose (3·96 mg. per ml.),

4 ml. 0·2M-citrate buffer pH 6·8,

4 ml. branching enzyme solution (80 mg.; 5·3 units).

The digest was divided into three portions (5 ml.) and the reaction allowed to proceed for 4·5 hours at 20°C. The branching enzyme was destroyed by heating in boiling water bath for 3 mins. and the solutions were cooled to room temperature.

to (i) water (1 ml.) and β-amylase (2 ml.; 800 units) was added,

(ii) water (1 ml.) and α-amylase (2 ml.) added,

(iii) isoamylase solution (1 ml.; 25 mg.) added and incubated at 20°C for 18 hours; the isoamylase was then deactivated and β-amylase (2 ml.; 800 units) added.

The apparent percentage conversion into maltose was

determined, and the following results were obtained.

Digest	Degradation of amylose, treated with branching enzyme, with:-	% Amylolysis
(i)	β-Amylase	45
(ii)	α-Amylase	92
(iii)	Isoamylase and β-amylase	65

The results show that the branching enzyme acts on amylose producing a branched polysaccharide having a β-amylolysis limit of 45%. The extent of degradation by α-amylase indicates that the majority of the linkages in the branched polysaccharide are α-1:4. Treatment with isoamylase followed by β-amylase proves that the branch points formed by the branching enzyme are α-1:6-linkages since the β-amylolysis limit increased from 45 to 65%.

4. Discussion.

Fractionation of yeast juice with ethanol-citrate buffer was repeated several times, and it was found that although all the fractions showed branching activity, the greatest activity was obtained at 25-30% alcohol concentration. Hence the protein obtained at this fraction was used in the preliminary studies of this enzyme.

The protein preparation contains traces of maltase, maltotriase and isoamylase since it slowly hydrolyses maltose,

maltotriose and isomaltose respectively. The presence of maltase and maltotriase does not interfere with studies of the branching enzyme since they have no action on amylose or soluble starch. The enzyme preparation also contained traces of isoamylase; this was observed only after prolonged incubation. Thus a 4·5% increase in the β-amylolysis limit of glycogen occurred during 24 hour's incubation, whilst with a normal isoamylase preparation, a 15-20% increase is observed after only 2 hours (see p. 94). The enzyme preparation is free from α-amylase, β-amylase and glucamylase; the absence of α-amylase was shown by the test described by Hobson, Whelan and Peat,[62] whilst β-amylase and glucamylase were absent since (a) no small sugar could be detected (paper chromatography) in the reaction digest of polysaccharide with enzyme, and (b) no increase in the reducing power of the reaction digest occurred.

Like the Q-enzyme of potatoes,[5] the transformation of amylose to a branched polysaccharide by the yeast branching enzyme is optimum at pH 7·05 and 20°C. Unlike the Q-enzyme of *Polytomella coeca*,[4] the yeast branching enzyme does not require activators for its action on amylose. In contrast to yeast phosphorylase the yeast branching enzyme is inhibited by mercuric chloride (5×10^{-4}M); ammonium molybdate (0·33%) has no significant action on the enzyme.

Determination of branching enzyme activity by Gilbert and Patrick's method[40] shows that enzyme action is directly

proportional to its concentration within 10 mins. Hence, a simple method for activity determination could be devised in which a unit of activity is defined as the amount of enzyme which causes the fall in absorption value of the starch-iodine complex by 0·1 Spekker absorptiometer reading within 10 mins. under the standard conditions.

The absorption spectrum of the amylose-iodine complex after treatment with branching enzyme clearly indicates that a branched polysaccharide is formed. The iodine staining power of amylose falls rapidly, with time, at 680 mμ and the wave length of maximum absorption falls from 630 to 520 mμ. The β-amylolysis limit of the branched polysaccharide so obtained is 45%, and since the polysaccharide is rapidly and extensively degraded by α-amylase (92% apparent conversion to maltose), the majority of the linkages are α-1:4. The branch points formed by the enzyme are α-1:6-linkages since treatment of the polysaccharide with isoamylase increased the β-amylolysis limit from 45 to 65%. The polysaccharide formed is therefore similar to glycogen with respect to degradation by β-amylase and isoamylase; for example, the β-amylolysis of B.D.H. glycogen is increased from 45 to 65% on treatment with isoamylase (p. 94.).

The yeast branching enzyme differs from the related plant Q-enzyme in that the latter does not act on amylopectin. Normal plant cells do not contain glycogen type polysaccharides and their absence may be due to the inability of plant

Q-enzymes to attack interior chains of less than 6-8 glucose residues (as in amylopectin), thus preventing further branching of amylopectin with the production of a more highly branched glycogen-type molecule. By contrast, yeast branching enzyme can cause branching of interior chains containing 6-8 glucose residues, although enzyme action ceases when the interior chains are shorter than 3-4 glucose residues. However, glycogen type polysaccharides have been reported in certain varieties of sweet corn; unfortunately data on the specificity of the Q-enzyme from these plants is not yet available.

Summary.

The phosphorylase, isoamylase and branching enzyme of brewer's yeast (Saccharomyces cerevisiae) which catalyse the synthesis and degradation of α-1:4-glucosans have been investigated.

Various methods of extraction of phosphorylase from brewer's yeast are described, and a protein preparation, having a phosphorylase activity similar to that of plant phosphorylase preparations has been obtained by ammonium sulphate fractionation. The enzyme preparation, free from amylases, was contaminated with phosphatase, phosphoglucomutase, branching and debranching enzymes. The inhibition of these impurities by specific inhibitors is described. Organic solvents are not suitable for the purification of phosphorylase; adsorption studies using alumina C_γ and calcium phosphate indicate that phosphorylase cannot easily be recovered from these adsorbents.

Yeast phosphorylase is unstable in solution, and the rate of inactivation is especially rapid on dialysis. It does not require activators for degradative or synthetic activity; the latter activity is primed more effectively by glycogen than any other maltosaccharide. The optimum pH and temperature of yeast phosphorylase are 6·6 and 35° respectively.

The action pattern of yeast phosphorylase is generally similar to that of other phosphorylases, but some criteria

which differentiate brewer's yeast phosphorylase from muscle and potato phosphorylases have been discovered.

Brewer's yeast isoamylase, which hydrolyses both terminal and non-terminal α-1:6-glucosidic linkages, has been studied. A method for the purification of isoamylase is described. It is shown that isoamylase has a wider specificity than any of the debranching enzymes previously reported. Isoamylase has been used to detect α-1:6-linkages in branched α-1:4-glucosans.

The branching enzyme of brewer's yeast, responsible for the transformation of linear α-1:4-glucosans to branched polysaccharides, has been studied. This enzyme acts on amylose, producing a branched polysaccharide having a structure resembling that of glycogen. The enzyme preparation is contaminated with traces of maltase and isoamylase, but is free from other hydrolytic enzymes. Enzyme action is inhibited by mercuric chloride (5×10^{-4}M); it is optimum at pH 7·0 and 20°, and it is not activated by maltosaccharides. The branch points synthesised by the branching enzyme are shown to be α-1:6-glucosidic linkages.

The specificity of the above enzymes was determined by studying their action on starch-type polysaccharides, including brewer's yeast 'glycogen,' the molecular structure of which has been determined. This reserve polysaccharide is rapidly degraded by α-amylase and has a molecular weight of ca. 2×10^6; it has an average chain length of 13 glucose

residues, a β-amylolysis limit of 44% and a muscle phosphoryolysis limit of 30%. The molecular structure therefore resembles that of a typical animal glycogen. Small but significant differences between the molecular structures of baker's and brewer's yeast glycogens have been revealed.

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α-1 : 4-*Glucosans. Part III.* The Molecular Structure of Brewer's Yeast Glycogen.*

By D. J. Manners and Khin Maung.

[Reprint Order No. 5858.]

Brewer's yeast (*Saccharomyces cerevisiae*) contains a glucosan which is stained reddish-brown by iodine, is degraded by salivary α-amylase, and has a molecular weight of $\sim 2 \times 10^6$. The polysaccharide has an average chain length of 13, a β-amylolysis limit of 44%, and a phosphorolysis limit of 23%. The molecular structure therefore resembles that of a typical glycogen. The properties of the glycogens from baker's yeast and brewer's yeast are compared.

Although the glycogen from baker's yeast has been studied several times (*e.g.*, Northcote, *Biochem. J.*, 1953, **53**, 348; Jeanloz, *Helv. Chim. Acta*, 1944, **27**, 1501), little attention has been paid to the corresponding polysaccharide from brewer's yeast. A sample of the latter, isolated by Daoud and Ling (*J. Soc. Chem. Ind.*, 1931, **50**, 365T), had $[\alpha]_D$ +179° in water and was stained brownish-red by iodine; Yokoyama (*Beitr. Physiol.*, 1925, **3**, 95) found $[\alpha]_D$ +192° in water. No structural examination appears to have been published. The present communication deals with an investigation of the molecular structure of brewer's yeast glycogen; this is being used as a substrate in studies on the carbohydrate-metabolising enzymes in this organism.

The glycogen was extracted from yeast cell-wall material (obtained by disruption of the whole yeast with hot dilute sodium hydroxide) with 0·5N acetic acid (Bell and Northcote, *J.*, 1950, 1944; Northcote and Horne, *Biochem. J.*, 1952, **51**, 232), and was purified by several precipitations with 80% acetic acid (cf. Bell and Young, *Biochem. J.*, 1934, **28**, 882) and with ethanol. The purified glycogen gave an opalescent solution in water ($[\alpha]_D$ +198°), which was stained reddish-brown by iodine. The iodine binding power of the glycogen was quantitatively determined by Mr. D. M. W. Anderson, using the potentiometric titration method described by Anderson and Greenwood (*Chem. and Ind.*, 1953, 642); it was similar to that of mammalian glycogens. Acid hydrolysis of the glycogen gave glucose (96%) and no other reducing sugar. The glycogen was readily attacked by salivary α-amylase, as shown by the rapid loss of iodine staining power, and the production of glucose, maltose, and α-dextrins; the glucosidic linkages in the glycogen are therefore predominantly of the α-1 : 4 type. Since maltulose was absent from the α-amylolytic digest, fructose is not a constituent of the glycogen (cf. Peat, Roberts, and Whelan, *Biochem. J.*, 1952, **51**, xvii). Oxidation of the glycogen by potassium periodate (cf. Halsall, Hirst, and Jones, *J.*, 1947, 1399; Bell and Manners, *J.*, 1952, 3641) and determination of the maximum amount of formic acid produced indicated a unit-chain length of thirteen glucose residues. In an attempt to detect 1 : 2 or 1 : 3 linkages, periodate-oxidised glycogen was hydrolysed with acid, and the hydrolysate analysed for glucose (cf. Hirst, Jones, and Roudier, *J.*, 1948, 1779; Bell and Manners, *J.*, 1954, 1891). Paper chromatography showed that glucose was absent; the glycogen must therefore contain only α-1 : 4 and 1 : 6 glucosidic linkages. Treatment of the glycogen with β-amylase gave 44% conversion into maltose, indicating that the exterior chains comprise 8 glucose residues; the interior chains, on the average, thus contain 4 glucose residues. Treatment of the glycogen with muscle phosphorylase in the presence of excess of inorganic phosphate

* Part II, *J.*, 1954, 3527.

resulted in a 23% conversion into glucose 1-phosphate; 12-unit and 13-unit glycogens from various animal tissues have phosphorolysis limits of 20—25% (Liddle and Manners, unpublished). Examination of the glycogen in an ultracentrifuge, by Dr. C. T. Greenwood, showed it to be multimolecular, the sedimentation constant (S_{20}) being 52×10^{-13} c.g.s. units [equivalent to a molecular weight of *ca.* 2×10^6, the diffusion constant being assumed to be of the same order as that of other glycogens (cf. Bell, Gutfreund, Cecil, and Ogston, *Biochem. J.*, 1948, **42**, 405)]. An estimate of the shape of the molecule, and the degree of multiple branching in the molecule will be described in a later communication.

The available data indicate that brewer's yeast glycogen consists of *ca.* 10^3 unit-chains, each comprising, on the average, 13 α-1 : 4-linked glucose residues, and randomly interlinked by 1 : 6-glucosidic linkages. The molecular structure thus resembles that of the majority of the glycogens from mammalian, invertebrate, and protozoan tissues examined in our previous studies (Bell and Manners, *loc. cit.*; Manners and Ryley, *Biochem. J.*, 1952, **52**, 480); nevertheless, small but significant differences in degree and position of branching between the baker's yeast and the brewer's yeast glycogen are revealed (see Table).

A comparison of the properties of yeast glycogens with rabbit-liver glycogen.

Source :	Baker's yeast		Brewer's yeast	Rabbit-liver
	A	*B*		*C*
$[\alpha]_D$ in water	+184°	+187°	+198°	+196°
Unit-chain length	11—12	—	13	12—13
β-Amylolysis limit	50	46—48·5	44	43
Exterior chain length *	8	—	8	8
Interior chain length †	2—3	—	4	3—4

A, Data from Northcote (*loc. cit.*). *B*, Data from Jeanloz (*loc. cit.*). *C*, Data from Bell and Manners (*loc. cit.*).

* No. of glucose units removed on β-amylolysis plus 2·5. † Unit-chain length — Exterior chain length — 1.

In our previous papers (Bell and Manners, *loc. cit.*; Manners and Ryley, *loc. cit.*) exterior chain lengths were calculated on the assumption that the exterior " stubs " of β-limit dextrins contained 1·5 glucose residues (cf. Meyer, *Adv. Enzymology*, 1943, **3**, 109); Peat, Whelan, and Thomas (*J.*, 1952, 4546), however, have shown that a proportion of the " stubs " in a β-dextrin from waxy-maize starch contain two or three glucose residues. A mean of these latter figures has been used in the present calculation.

In view of the different conditions of growth and fermenting properties of baker's and brewer's yeast, and hence, enzymatic composition, it is not unexpected that the glycogens show small differences in molecular structure; the unit-chain length and the position of branching in the chains depend upon the " balance " between the activities of phosphorylase and the branching and debranching enzymes, *i.e.*, on the metabolic condition of the organism at the time of isolation of the glycogen.

Experimental

Analytical Methods.—(a) *Determination of reducing sugar.* Reducing sugars were determined by use of the Shaffer–Somogyi reagent 60 (*J. Biol. Chem.*, 1933, **100**, 695) as modified by Hanes and Cattle (*Proc. Roy. Soc.*, 1938, *B*, **125**, 387) or by the Somogyi reagent (*J. Biol. Chem.*, 1945, **160**, 61) which had been calibrated against glucose and maltose.

(b) *Paper chromatography.* Descending chromatograms were carried out at room temperature with Whatman No. 1 paper and benzene–pyridine–butanol–water (1 : 3 : 5 : 3) as solvent. Development was by spraying with aniline oxalate or with a silver nitrate–sodium hydroxide reagent (Trevelyan, Procter, and Harrison, *Nature*, 1950, **166**, 444).

(c) *Iodine stain.* Polysaccharide solution (2 ml.) was added to iodine solution (1 ml.; containing 1 mg. of iodine and 10 mg. of potassium iodide per ml.) and water (2 ml.), and the absorption value of the polysaccharide–iodine complex measured on a Spekker Photoelectric Absorptiometer (1 cm. cells), an Ilford filter No. 603 being used, against an iodine blank.

(d) *Determination of glucose 1-phosphate.* Glucose 1-phosphate was determined by a slight modification of Allen's method (*Biochem. J.*, 1940, **34**, 858).

Preparation of Glycogen.—A dispersion of washed brewer's yeast (1·5 kg.) in 3% sodium hydroxide (1 l.) was heated at 95° for 6 hr. (cf. Northcote and Horne, *loc. cit.*). After the mixture had cooled, the cell-wall material was collected on the centrifuge and treated again with hot 3% sodium hydroxide. (The sodium hydroxide extracts did not contain any appreciable

amount of glycogen.) The cell-wall material was extracted by three successive treatments with 0·5N-acetic acid (each 500 ml.) at 75° for 2 hr. The combined acetic acid extracts were concentrated under reduced pressure to about 500 ml., and ethanol (6 vols.) was added. The crude precipitate of glycogen was purified by three precipitations from 80% acetic acid (Bell and Young, *loc. cit.*) and finally from ethanol. The yield was 17·7 g.

Properties of the Glycogen.—The glycogen had $[\alpha]_D^{18}$ +198° (*c*, 0·25 in H_2O); +175° (*ca.* 0·50 in N-NaOH) (Found: N, 0·05%; P, nil; Ash, 0·10%). An aqueous solution was opalescent, and was stained red-brown with iodine. Hydrolysis by 1·5N-sulphuric acid at 100° for 2 hr. gave glucose and no other sugar (paper chromatography). The glycogen had a glucose content of 96%, determined by quantitative acid hydrolysis (Pirt and Whelan, *J. Sci. Food Agric.*, 1951, **2**, 224).

Salivary α-Amylolysis of the Glycogen.—Salivary α-amylase solution was prepared by dissolving freeze-dried human saliva in distilled water, and removing insoluble material by centrifugation. The amylase solution showed no maltase activity, but was contaminated with maltotriase. During the digestion of waxy-maize starch, maltose, maltotriose, and α-dextrins were the initial products of the reaction, and glucose, maltose, and α-dextrins the end-products (cf. Whelan and Roberts, *Nature*, 1952, **170**, 748; *J.*, 1953, 1298).

An enzymic digest was set up containing glycogen (50·0 mg.), phosphate–citrate buffer (0·16M with respect to phosphate) of pH 7·0 (20 ml.), sodium chloride (25·0 mg.), salivary amylase solution (1 ml.), and water (29 ml.). Aliquot portions (2 ml.) were analysed at intervals (Shaffer–Somogyi reagent 60), after incubation at 35°.

The decrease in iodine-staining power was as follows:

Time of incubation (min.)	0	4	10	20
Absorption value	0·305	0·035	0·020	0·010

The apparent percentage conversion into maltose after 1, 2, and 48 hours' incubation was 62, 68, and 95, respectively.

Paper chromatography showed the presence of glucose ($R_G = 1$), maltose ($R_G = 0·55$), and a series of sugars of higher molecular weight ($R_G < 0·09$) in the digest. Maltulose was absent.

Potassium Periodate Oxidation of the Glycogen.—Glycogen (549·0 mg.) was dissolved in 5% potassium chloride solution (100 ml.); 10 ml. were withdrawn for a control determination. 8% (w/v) sodium metaperiodate (20 ml.) was added to the bulk, from which 10-ml. portions were withdrawn at intervals for determination of formic acid by titration in a carbon dioxide-free atmosphere against 0·01N-sodium hydroxide, methyl-red being used as indicator (cf. Halsall, Hirst, and Jones, *loc. cit.*; Bell and Manners, *loc. cit.*). The following results were obtained.

Time (hr.)	96	168	266	386
Total formic acid produced (mg.)	8·9	10·2	10·6	10·5
Apparent chain length * (glucose residues) ...	15·6	13·6	13·2	13·2

* Calculated from the weight of glycogen oxidised.

A 12-unit or 14-unit glycogen would yield 11·6 or 10·0 mg. of formic acid, respectively.

The remaining solution of periodate-oxidised glycogen was neutralised with ethylene glycol (5 ml.) and dialysed against running tap water for 36 hr., and the non-diffusible material collected by freeze-drying. 50 mg. of this were hydrolysed by 2N-sulphuric acid (2 ml.) at 100° for 3 hr. No glucose could be detected in the hydrolysate by paper chromatography; brewer's yeast glycogen does not therefore contain 1 : 2- or 1 : 3-glucosidic linkages.

β-Amylolysis of the Glycogen.—β-Amylase was prepared from soya beans by Bourne, Macey, and Peat's method (*J.*, 1945, 882). A solution of β-amylase was prepared by dissolving soya bean β-amylase (50 mg.) in 0·2M-acetate buffer (pH 4·6; 20 ml.), and removing insoluble material by centrifugation; the supernatant liquid had an activity of 125 units/ml., estimated by Hobson, Whelan, and Peat's method (*J.*, 1950, 3566). Control experiments with maltose and starch showed it to be free from maltase and α-amylase. Glycogen (48·4 mg.) was incubated with 0·2M-acetate buffer (pH 4·6; 6 ml.), water (21 ml.), and β-amylase solution (3 ml.; 375 units) at 37°. Samples (3 ml.) were withdrawn at intervals and analysed for maltose. The course of degradation was as follows:

Time of incubation (hr.)	1	2	20	44
% Conversion into maltose	30·7	38·4	43·5	44·0

In a duplicate experiment with 50·4 mg. of glycogen, the β-amylolysis limit was 43·8%.

Phosphorolysis of the Glycogen [with A. MARGARET LIDDLE].—Glycogen (52·4 mg.) was incubated at 35° with 0·5M-phosphate buffer (pH 6·8; 2·0 ml.), adenylic acid (1 mg.), muscle

phosphorylase solution (0·20 ml.), and water to a total volume of 10 ml. Crystalline rabbit-muscle phosphorylase was prepared by Green and Cori's method (*J. Biol. Chem.*, 1943, **151**, 21).

Time of incubation (hr.)	5	24	48
% Conversion into glucose 1-phosphate	20·0	23·2	23·2

In a duplicate experiment with 53·4 mg. of glycogen, the phosphorolysis limit was 22·8%.

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OBSERVATIONS ON THE SPECIFICITY OF YEAST *iso*AMYLASE

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In the course of studies on carbohydrate metabolising enzymes in brewer's yeast, we have subjected waxy maize starch to degradation by yeast phosphorylase; 44% conversion to glucose-1-phosphate was observed. The residual dextrin was then treated with β-amylase, resulting in 29% conversion to maltose. This represents 60% total degradation of the original polysaccharide, whereas β-amylase normally removes 50% of the molecule. The phosphorylase was prepared by ammonium sulphate fractionation of an extract of brewer's yeast; it was free of α-amylase, and had a phosphorylase activity similar to that reported[1] for preparations from potatoes and broad beans, namely *ca.* 100 units per g.

Since the action of phosphorylase and β-amylase is confined to the exterior chains of amylopectin and glycogen,[2] we concluded that the phosphorylase preparation contained a debranching enzyme which catalysed the hydrolysis of a proportion of the 1 : 6 inter-chain linkages in waxy maize starch, thereby removing the barriers to β-amylase action.

In 1951, Maruo and Kobayashi[3] reported that autolysates of brewer's yeast contained an enzyme which hydrolysed the inter-chain linkages in glutinous rice starch producing a polysaccharide of lower molecular weight and increased susceptibility to β-amylase. This enzyme, previously known as amylosynthease, was renamed *iso*amylase. It appears probable, therefore, that our phosphorylase preparation contained isoamylase.

By acetone fractionation (at − 10°) of a brewer's yeast extract, we have obtained a protein preparation (hereafter referred to by the provisional name of *iso*amylase) which catalyses the hydrolysis of a proportion of the 1 : 6-linkages in glycogen[4] (from brewer's yeast), β-dextrin (prepared by the action of β-amylase on waxy maize starch) and maltodextrin (a mixture of linear and branched maltosaccharides prepared by the action of malt α-amylase on potato starch). The polysaccharides were incubated with *iso*amylase at pH 7·0 at 20° for various times; after inactivation of the enzyme, the β-amylolysis limit was determined.

Time of incubation with *iso*amylase	β-Amylolysis limit (%)		
	Glycogen	β-Dextrin	Maltodextrin
Nil ..	44	0	67
5 min. ..	49	19	—
2 hr. ..	54	20	—
24 hr. ..	57	32	94

These results demonstrate hydrolytic activity towards non-terminal α-1 : 6-glucosidic linkages. During 24 hr. a 47% increase in the absorption value of the iodine-glycogen complex at 680 mμ was observed. However, attempts to follow enzyme action on β-dextrin by iodine-staining were not successful; this criterion of activity has also been found to be unsatisfactory in similar experiments with the debranching enzyme of higher plants (R-enzyme)[5].

*iso*Amylase action on glycogen is incomplete, and is presumably limited to A-chains (side chains); incubation of BDH glycogen with *iso*amylase for 24, 48, and 72 hours gave a similar increase in β-amylolysis limit, from 45 to 65%. If yeast glycogen and β-dextrin have multiply branched " tier " structures, with 50% of the branch points in the outermost " tier,"[6] then hydrolysis of the outermost branch points (which join A-chains to the rest of the molecule) would increase the β-amylolysis limits to 58 and 32%, respectively.

*iso*Amylase also catalyses the hydrolysis of terminal α-1 : 6-glucosidic linkages, since it hydrolyses *iso*maltose. Furthermore, on incubation with a glycogen of abnormal structure,[7] which contains a number of single glucose residues attached by 1 : 6-linkages to the rest of the molecule, glucose was liberated.

The *iso*amylase preparation was free from α-amylase, phosphorylase and branching enzyme, but contained a trace of maltase.

Debranching enzymes previously reported include R-enzyme[8] (from potatoes and broad beans), and amylo-1 : 6-glucosidase[9] (from rabbit muscle). R-enzyme catalyses the hydrolysis of non-terminal α-1 : 6-glucosidic linkages in amylopectin and its β-dextrin, and in α-dextrins from amylopectin and glycogen; it has no appreciable action on glycogen or on *iso*maltose. By contrast, amylo-1 : 6-glucosidase action is limited to the hydrolysis of *iso*maltose and terminal α-1 : 6-glucosidic linkages in muscle phosphorylase limit dextrins from glycogen and amylopectin. *iso*Amylase has, therefore, a wider specificity than either R-enzyme or amylo-1 : 6-glucosidase.

*iso*Amylase, together with α-1 : 4-glucosidases, is now being used in investigations of the fine structure of amylopectin and glycogen.

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