UNIVERSITY OF EDINBURGH

PHYSICOCHEMICAL STUDIES ON SOLUTIONS OF SOME PLANT POLYSACCHARIDES: THE ACTION-PATTERN OF SOME STARCH-DEGRADING ENZYMES.

by

Alexander W. MacGregor, B.Sc.

THESIS
submitted for the degree of
DOCTOR OF PHILOSOPHY

August, 1964.

ABSTRACT OF THESIS

Name of Candidate Alexander W. MacGregor, B.Sc.

Address

Degree Doctor of Philosophy *Date* August 1964

Title of Thesis Physicochemical studies on solutions of some plant polysaccharides: the Action-Pattern of some starch-Degrading Enzymes.

SUMMARY

After a brief account of the properties of the starch components, the action patterns of the more important starch-metabolising enzymes, and the importance of these enzymes in determining the structures of amylose and amylopectin have been discussed.

The physical and enzymic techniques used in this study have been outlined. A procedure for the extraction of starch from potatoes has been described and the conventional method of fractionating potato starch by dispersion into boiling water has been discussed.

Starch, pretreated with liquid ammonia, dimethyl sulphoxide or alkali has been dispersed in water at room temperature and successfully fractionated using a "critical concentration" of butanol. The amylose samples obtained from such fractionations were incompletely degraded by β-amylase. These results indicate that the structural-anomaly present in large amylose molecules is unlikely to be an artefact which has been introduced during the fractionation of starch at high temperatures. Fractionation of potato starch by the method of Killion and Foster (1960) yielded samples of amylose and amylopectin whose properties have been shown to be similar to those of the products from a conventional fractionation.

Unheated, aqueous solutions of pretreated potato starch have been examined in the ultracentrifuge and have been shown to contain two components. This result suggests that the "Unitarian Concept" of starch is incorrect.

Investigations on the preparation of samples of linear amylose have been carried out; aqueous leaching of starch granules at 60 - 65°C., followed by a dimethyl sulphoxide/ethanol fractionation of the leached material, was found to be the most efficient method for preparing such samples.

A detailed study on the extraction, purification and properties of soya bean Z-enzyme has been made. The action pattern of this enzyme has been investigated and found to be similar to the action patterns of other plant β-amylases.

β-amylases from broad beans, barley and malted barley have also been examined. Various procedures for determining β-amylase activities have been discussed and the method finally adopted in this work has been described. Using viscometric techniques, kinetic studies on the action

Use other side if necessary.

of these enzymes on linear-amylose substrates have indicated that the α-amylases hydrolysed the substrate in a completely random fashion. However, chromatographic examination of the products of the action of these enzymes on oligosaccharides showed that there are six linkages in such substrates which are relatively difficult to hydrolyse - these are the first five linkages from the non-reducing end and the linkage at the reducing end of the molecule. The extremely slow rate of attack of these plant α-amylases on small oligosaccharides compared to that of salivary α-amylase has been shown by a study on maltotetraose; the salivary enzyme hydrolysed this substrate some 100 x faster than did the plant enzymes.

The relative rates of attack of the α-amylases on various starch-type substrates were found to be: amylose > amylopectin > glycogen.

Investigations of the extraction and purification of R-enzyme from broad beans have shown that the removal of α-amylase impurity from such preparations is extremely difficult. However, methods for removing this impurity have been discussed.

The action pattern of crystalline sweet potato β-amylase on fractions of linear amylose has been examined by measuring the molecular size of the dextrin-product. When rigorously subfractionated amylose samples were used, the reaction mechanism has been found to be almost completely multi-chain at pH 5.0. The results of comparable experiments using fractions with a wider molecular-weight distribution indicated that the reaction was apparently single-chain. This behaviour has been predicted by French (1961).

A commercial preparation of potato phosphatase has been purified and shown to be free from α-amylase. The action of this enzyme on glucose 6-phosphate, amylopectin, amylopectin α,β-limit dextrins has been studied. Although the glucose 6-phosphate and α-limit dextrin were rapidly attacked, only very small amounts of phosphate were removed from the other two substrates. The implications of these results have been discussed.

TO MY
PARENTS

C O N T E N T S

P R E F A C E

I wish to thank Dr. C. T. Greenwood for his unfailing help and encouragement during the period of this work.

I wish to thank Professors Sir Edmund L. Hirst and T. L. Cottrell for the provision of laboratory facilities.

I also wish to thank the Department of Scientific and Industrial Research and Corn Industries Research Foundation Inc. for maintenance grants.

The University Chemistry Department,
The King's Buildings,
Edinburgh 9.

GENERAL INTRODUCTION

Although the main structural features of the starch components - amylose and amylopectin - are now generally accepted, the fine-structures of these polysaccharides are still in dispute. Chemical techniques are not sufficiently sensitive to detect possible slight modifications in the general structures of these large molecules and so enzymic and physical techniques have to be employed. The enzymes used in such studies must be pure, and most of the work in this thesis describes attempts to isolate and characterise several starch-modifying enzymes.

A brief account of the properties of the starch components and of the most widely used starch-metabolising enzymes is given in Section I, while the next section surveys the experimental techniques used in this work.

The heterogeneity of amylose is discussed in Section 3. Solutions of amylose samples which had not been heated above room temperature were investigated to determine whether the barrier, to β-amylolysis is an artefact introduced during fractionation of the starch granules, or whether this barrier is inherent in amylose molecules. This section also includes an investigation and discussion of the "Unitarian Concept" of starch.

With the exception of malted-barley α-amylase, plant α-amylases have not previously been extensively studied. In Section 4, the extraction, purification and properties of some plant α-amylases are discussed. The action-pattern of soya-bean Z-enzyme is compared to those of the α-amylases from

broad bean, barley and malted-barley. The properties of these plant α-amylases have been contrasted with those of α-amylases from various sources.

Section 5 describes attempts to prepare R-enzyme with special reference to the removal of trace amounts of α-amylase impurity from such preparations.

Experiments to determine the action-pattern of sweet potato β-amylase were carried out and the implications of the results from these experiments are discussed in Section 6.

An investigation on potato phosphatase was carried out and the results are described in Section 7. A commercial preparation of the enzyme was used and contaminating α-amylase had to be removed before the action of the enzyme on starch-type substrates could be studied.

SECTION 1.

STARCH AND ITS COMPONENTS

Starch and its Components

Starch is the most abundantly distributed reserve polysaccharide in plants; the resting cereal seed and the potato tuber contain large amounts of starch which can be rapidly assimilated on germination or sprouting. Early methylation studies (Haworth, 1928; Haworth and Percival,1931) showed that starch was a polymer of D-glucose units and was composed of chains of finite length. Kinetic studies on the acid hydrolysis of starch (Freudenberg, 1930, 1936) indicated that the chain-forming linkage in starch was α-glucosidic.

In 1905, Maquenne postulated that starch consisted of two substances, amylose and amylopectin, which had the same chemical properties but had different degrees of physical aggregation. Later, Staudinger (1937) suggested that starch was a polymeric-homologous series, i.e. starch was a mixture of molecules of varying size but which were built to the same chemical pattern. This is the "unitarian" theory of starch structure and it still has some supporters (Bauer and Pacsu, 1953). However, starch is now generally considered to consist of a mixture of amylose and amylopectin.

The Fractionation of starch. Meyer and his co-workers (1940) used the technique of aqueous leaching to obtain fractionation of starch granules. This method involved the extraction of swollen but intact starch granules with successive portions of water at 70°C. until no further soluble material was removed. Separation was achieved by either filtering or centrifuging the resulting suspensions. The soluble extract

represented the amylose component and the residue of swollen granules contained the amylopectin. High temperatures and the use of chemicals were avoided in this procedure but complete separation of the two components was not achieved. Although the amylose samples obtained were apparently pure the amylopectins were contaminated with amylose.

Schoch (1942) devised a more efficient method of fractionation in which he obtained complete dispersion of the starch granule by autoclaving at high temperature and pressure. The amylose component was separated as an insoluble complex by the addition of a polar organic substance to the starch solution and the amylopectin was obtained from the supernatant liquid. Pure amylose was obtained by repeated recrystallisation but the amylopectin fraction could not be purified further. Some of the principal properties of the two components are shown in Table 1.01.

Amylose

The Fine Structure of Amylose. Investigations of the fine structure of this component have been mainly concerned with the problem of heterogeneity.

The methylation procedure of Haworth (1928) was the first technique to be used in this study. Meyer (1940) proposed that amylose possessed an unbranched structure when he found that the degree of polymerisation of maize amylose obtained from methylation studies corresponded to that derived from osmotic pressure measurements on the acetylated derivative. This

Table 1.01.

A comparison of the properties of amylose and amylopectin.

Property	Amylose	Amylopectin
Molecular configuration	Essentially linear	Branched molecule
Molecular weight	ca. 10^6	ca. 10^8
Stability in aqueous solution	Unstable, tends to retrograde	Stable
Iodine colouration	Intense blue	Purple-brown
λmax. of absorption spectra (mμ)	680	530-540
β - amylolysis limit	ca. 70-80% conversion into maltose	ca. 55% conversion into maltose
Action of β-amylase + Z-enzyme	Complete hydrolysis	High molecular weight dextrins
Complex formation	Readily forms complexes with polar substances.	Very limited complex formation with polar substances.
X-ray diffraction	Crystalline	Amorphous or weakly crystalline patterns
Average chain length (glucose residues)	100 - 6,000	20 - 25

view was disputed by Hess (1940) who maintained that amylose was not entirely linear. Meyer, Wertheim and Bernfeld (1941) and Hassid and McCready (1943), however, presented evidence confirming that potato amylose was not branched.

In 1945, Hirst and his co-workers applied the technique of periodate oxidation to end-group analysis. Using this procedure, Potter and Hassid (1948) determined the molecular weights of some amylose samples and compared them with values obtained from osmotic pressure measurements on acetylated samples of the amyloses. They concluded that potato amylose was unbranched, although results for other amyloses (tapioca, wheat, maize, sago) indicated a small degree of branching. These results for potato amylose were substantially confirmed by Meyer and Rathgeb (1948). Potato amylose was subfractionated by successive partial precipitations from aqueous solution with octyl alcohol (Lansky et al. 1949). These subfractions were examined by Potter and Hassid (1951) who again concluded that potato amylose was unbranched.

It is doubtful, however, if much significance can be attached to these early chemical investigations. The amylose samples used in the experiments were probably contaminated with small amounts of amylopectin and the experimental techniques were not sufficiently precise to indicate the presence of a very small number of structural modifications in the amylose molecules.

A more rigorous approach to this problem has been the use of starch-metabolising enzymes. The greatest care, however, must be taken to ensure that the enzymes employed are pure. For instance, additional early evidence for the linearity of amylose

chains was based on the finding that amylose was completely converted into maltose by β-amylase. It was later shown (Peat et al., 1949) that crystalline sweet potato β-amylase converted only ca. 80% of the available amylose into maltose. When an impurity, Z-enzyme, was also present, the hydrolysis approached 100% conversion. These results afforded conclusive proof that amylose was not completely linear. Later workers (Cowie and Greenwood, 1957b; Arbuckle and Greenwood,1958; Banks et al, 1959a) showed that there were two types of amylose molecules present in starch granules: (i) some which contained a randomly situated barrier to β-amylolysis; (ii) others which were completely linear.

The anomalous linkages or units in amylose have been investigated by many workers, but no widespread agreement exists as to the nature of the structural anomaly. As already shown, early workers presented chemical evidence for the existence of a small amount of branching in the amylose molecule. Peat et al. (1952a) thought that the barriers to β-amylolysis were β-1: 3 glucosidic linkages joining single glucose residues to the main chain. This was refuted by Neufeld and Hassid (1955), who showed that the enzymic system used by Peat et al. (1952a) was grossly impure. Barriers to β-amylase action may be inadvertently introduced during fractionation (Banks et al. 1959b), and they may take the form of modified glucose units containing aldehydic or carboxylic groups on C_6 . It appears unlikely that ester-phosphate groups (which are known to be present in potato starch) constitute the barrier (Banks and Greenwood 1961).

Recent physico-chemical studies on the hydrodynamic behaviour of some amylose samples suggest that the anomaly might be due to a small amount of branching in some amylose molecules (Greenwood, 1960). Kjølberg and Manners (1963) have presented enzymic evidence confirming this result.

Amylose in aqueous solution. The instability of aqueous starch solutions is due to the amylose component which rapidly precipitates on standing at room temperature. Dried amylose is only sparingly soluble in hot water and separates again in an insoluble form (retrogradation) when the solution is stored. On the other hand amylose can be readily dispersed in dilute alkali but neutralisation of the alkali again leads in a short time to precipitation. According to Meyer (1942), amylose in aqueous solution is in the form of a long linear chain with a high content of hydrophilic groups. Associative forces cause these chains to aggregate parallelwise until colloidal dimensions are exceeded and precipitation occurs. Retrogradation is exceedingly complex and the rate of precipitation from solution is dependent upon a number of factors including pH, concentration of the amylose solution and the size of the amylose molecules (Foster and Sterman, 1956; Whistler and Johnson, 1948; Loewus and Briggs, 1957). The last consideration is particularly important in studies on the enzymic synthesis and degradation of amylose. Lansky et al. (1949) have shown that the retrogradation time of a series of amylose subfractions is inversely proportional to their chain-length until a certain critical value is reached, below which the molecules are too small to form visible aggregates. These results explain the appearance

of precipitates during the α -amylolysis of concentrated solutions of amylose (Walker and Whelan, 1960).

Since dried amylose samples cannot be maintained in neutral solution (retrogradation), or in acid solution (degradation), alkali is normally used as the solvent and this, too, raises the possibility of degradation. Wolff et al. (1950) showed that at 0°C. the viscosities of amylose samples dispersed in alkali (1M.) did not perceptibly decrease over a period of 48 hours. At higher temperatures, however, oxygen must be excluded from such solutions. Bottle et al. (1953) showed that in the absence of oxygen potato amylose was stable in aqueous solution and in potassium hydroxide (0.2M.) even at 100°C. In the presence of oxygen the viscosities of the samples in aqueous solution decreased slowly but the samples in alkali were very rapidly degraded.

Baum and Gilbert (1954) have suggested that amylose contains oxygen sensitive bonds which are hydrolysed at high temperatures and in the presence of oxygen.

It is obviously very difficult to obtain satisfactory solution of dried amylose samples, but aqueous solutions may be readily prepared from the amylose-butanol complexes. Excess butanol can be removed by centrifugation and the hard-packed complex is readily soluble in cold water. These solutions are stable at room temperature for many hours. Because of the basic instability of amylose in the presence of oxygen, all fractionations and recrystallisations should be carried out in an inert atmosphere.

Amylose Complexes. One of the most important properties of amylose solutions is their ability to form complexes with iodine and with polar organic compounds such as thymol and butanol. It has been shown by optical and X-ray investigations (Rundle and Edwards, 1943; Rundle and French, 1943) that the solid complexes which amylose chains form with iodine and butanol are coiled in the form of a helix, each turn of which contains six glucose residues. Rundle et al. (1944) and, more recently, Rao and Foster (1963) have respectively shown that both the amylose-iodine-iodide complex and the amylose-butanol complex assume the helical form in solution. Katzbeck and Kerr (1950) postulated that complex-formation is not an adsorption phenomenon, but that the complexing agents penetrate inside the helices formed by the long amylose chains. The formation of such chains is supposed to stabilise the helix.

Whistler (1950) reported that the minimum chain-length of amylose molecules required for the formation of insoluble complexes varied according to the nature of the complexing agent. The minimum size of maltodextrin chain which would form a visible complex with butanol was twenty glucose units in length. On the other hand, Thoma and French (1960, 1961) furnished evidence to indicate that iodine will react with units as small as maltose and maltotriose - these complexes, of course, are not visually detectable.

It has been suggested (Mould, 1954) that stable, resonating and extended iodine-iodide chains are formed within the helical structure of amylose molecules. These polyiodine

complexes yield the characteristic blue colour of starch-iodine interactions. As the length of the amylose chain decreases, however, the iodine-iodide resonating structure breaks up into discrete complex groups and the gradually increasing polarisation of the iodine molecules by iodide ions has the effect of shifting the absorption maxima to shorter wave-lengths. Thoma and French (1960) have shown that as the amylose chain decreases in size so the amount of triiodide ion present in the complexes increases. When the glucose chain is less than eight units long, the triiodide ion predominates in the complex but, since this species does not give a visible colour, short chain maltodextrins do not give visible colours with iodine.

Amylopectin.

The Fine structure of amylopectin. Early methylation studies indicated that the average length of unit chain of amylopectin was 20 - 25 glucose residues. This finding was confirmed by Brown et al. (1948), using the periodate oxidation technique. Amylopectin has also been found to have virtually no reducing power and a high molecular weight. The only explanation for these facts is that the amylopectin molecule is highly branched.

The presence of α-1: 6 inter-chain linkages in amylopectin was indicated by Montgomery et al. (1947) when they isolated the branched disaccharide, isomaltose, from enzymic hydrolysates of waxy maize starch. Further proof of their presence was provided by Thomson and Wolfrom (1951) who showed that the partial acid hydrolysis of amylopectin yielded the branched trisaccharide, panose. The technique of periodate oxidation has also afforded convincing proof that the greater proportion of branch-linkages in amylopectin are of the 1: 6 - type. The isolation of small quantities of glucose from the acid hydrolysates of periodate-oxidised amylopectin samples (Abdel-Akher et al, 1952; Hamilton and Smith, 1956) indicated the presence of 1: 2 or 1: 3 linkages in amylopectin. Manners and Mercer (1963), however, were unable to confirm these findings.

From the above results it is clear that amylopectin consists of a number of short chains of α -1: 4-linked anhydroglucose units joined to each other by α -1: 6 - linkages. Three types of structure have been proposed for amylopectin to show how the unit-chains are joined to one another. They are

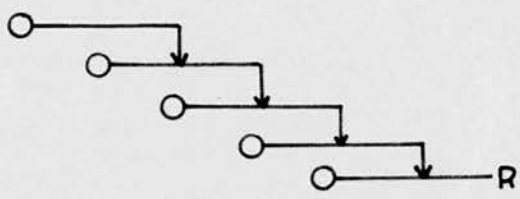
shown in Fig. 1.01. The "laminated" structure of Haworth et al. (1937) and the "herring-bone" structure of Staudinger and Husemann (1937) were originally proposed to account for the great difference between the molecular D.P. (degree of polymerisation) and the unit-chain D.P. of amylopectin.

Myrbäck and Sillén (1949) pointed out that the three structures postulated for amylopectin contained different arrangements of the same basic chains. Peat et al. (1952b) suggested that these chains should be termed A-, B-, and C- chains. The A- chain is linked to the rest of the molecule through its reducing group by an α-1: 6 linkage; the B- chain, in addition to being linked as an A- chain, is also substituted, through the C_6 hydroxyl group, in one or more of its constituent glucose units; the C- chain is joined to the molecule only by substitution through the C_6 hydroxyl group and it carries the sole reducing group in the amylopectin molecule. From Fig.1.01 it is obvious that in a) there is only one A- chain; in b) there are no B- chains; in c) there are approximately equal numbers of A- and B- chains. Since the structures each contain one C- chain they differ only in their proportion of A- and B- chains.

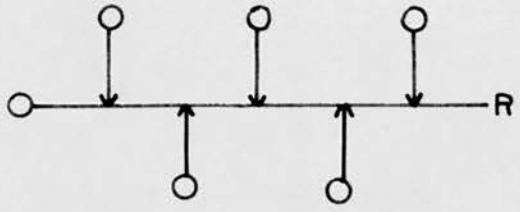
Enzymic evidence has shown that the Meyer structure is the most probable representation of amylopectin. Peat et al. (1952b) distinguished between the three structures by examining the maltosaccharides liberated when the β - limit dextrin of waxy maize starch was submitted to the debranching action of R -enzyme. (See later). The yield of maltose and maltotriose

FIG. 1.01

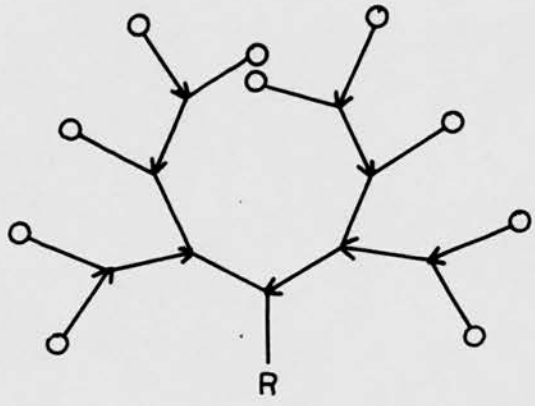
(a) Haworth



(b) Staudinger



(c) Meyer



R Reducing end group

O Non reducing end group

↓ α-1:6 link

from the outer A- chain stubs was 12.8%. A "tree-type" molecule containing equal numbers of A- and B- chains would have yielded 10.4%, whereas "laminated" or "herring-bone" structures would have yielded less than 0.1% or 20.8% of maltosaccharides, respectively.

Larner et al.(1952) treated wheat and maize amylopectins with phosphorylase in the presence of inorganic phosphate. The residual dextrin contained A- chains which had been reduced to single glucose residues, and B- chains which had been reduced to five glucose units (Cori and Larner, 1951). Treatment of the dextrin with amylo -1: 6 - glucosidase removed the exposed A- chain stubs, liberating glucose. After inactivation of the debranching enzyme, the residual polysaccharide was again incubated with phosphorylase. Treatment of this second dextrin with amylo -1: 6 - glucosidase again yielded glucose, but the amount was less than that obtained by the first treatment. These experiments eliminated both the Haworth structure, which would have yielded a much smaller and constant amount of glucose on each successive hydrolysis with amylo -1: 6 - glucosidase, and the Staudinger structure which, after the first treatment with Amylo -1: 6 - glucosidase, would have been completely degraded by phosphorylase.

The isolation of an oligosaccharide, containing two branch points, from the products of salivary α- amylase action on amylopectin (Roberts and Whelan, 1960) provided additional evidence that Meyer's multiply-branched amylopectin structure is probably correct.

Carbohydrase Enzymes

Almost all synthetic and degradative reactions of polysaccharides which are catalysed by enzymes can be expressed in terms of a reaction between a donor and an acceptor substrate as shown in the following equation.

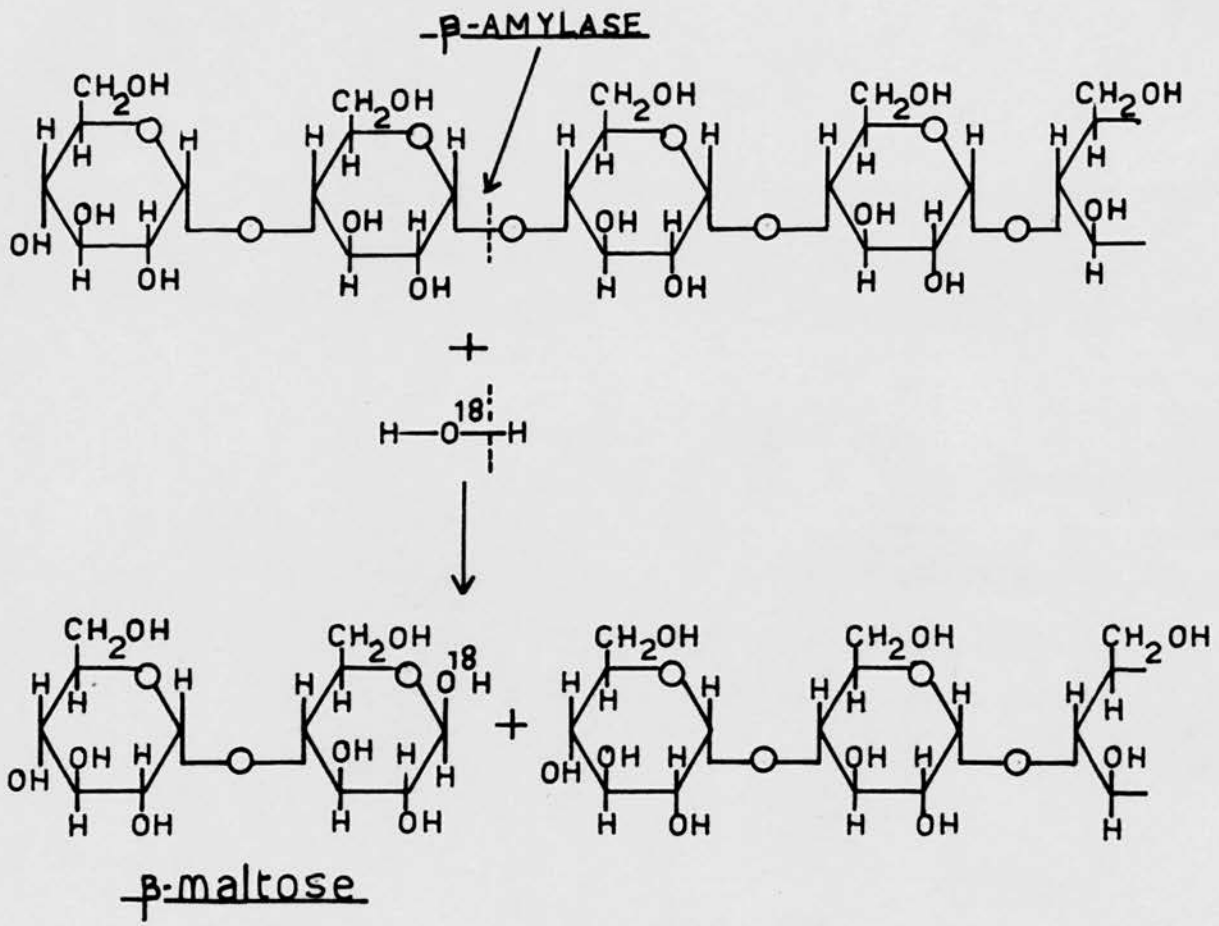
$$\underset{\text{Donor substrate}}{D - OR} + H - OA \rightleftharpoons D - OA + \underset{\text{Acceptor substrate}}{H - OR}$$

In the reaction, the group OR, in the donor substrate, is removed and replaced by OA from the acceptor substrate. For example, the action of β - amylase on glycogen, Fig. 1.02, illustrates this type of reaction (Mayer and Larner, 1959). The glycogen molecule is the donor substrate (D - OR) and the acceptor substrate (H - OA) is water. The enzyme removes an anhydromaltose unit from the glycogen and transfers it to an hydroxyl group of the water to form maltose. In splitting the donor substrate the enzyme could hydrolyse either the C - O bond or the O - H bond. It has been shown for a number of enzymes e.g. muscle phosphorylase, sucrose phosphorylase (Cohn, 1949), almond emulsin (Bunton et al. 1954), β - amylase, pig pancreas ∠ -amylase, B. subtilis ∠ -amylase (Mayer and Larner, 1959) that the transfer involves the glycosyl (D) and not the glucosidic (DO) grouping. This has been done by using acceptor substrates of the type $H - O^{18}H$ and demonstrating that in the products the heavy oxygen isotope is contained in D-OA and not in H-OR. Fig. 1.02.

Starch metabolising enzymes have yielded much valuable information concerning the fine structures of amylose

FIG. 1.02

β-amylolysis of α-1:4 glucans.



and amylopectin. These enzymes may be divided into two main classes - synthesising enzymes ~~and degrading enzymes~~ and degrading enzymes (Whelan, 1958).

Starch Synthesising Enzymes

D-enzyme. (Peat, Whelan and Rees 1953, 1956; Peat, Whelan and Kroll, 1956). This enzyme is a transglycosylase, transferring two or more glucose units from a maltodextrin substrate to a suitable acceptor forming only α- 1: 4 - glucosidic bonds. The maltodextrin chains, themselves, act as acceptors in this reaction. If the glucose produced in the reaction is continuously removed then the enzyme is capable of forming linear chains of amylose from maltodextrin substrates.

Phosphorylase. (Whelan, 1955). The enzyme successively transfers α -glucosyl units from α-D-glucopyranose 1-phosphate (G 1-P) to primer chains containing at least three α-D-glucose units. The single glucose residues are attached by means of α- 1: 4 linkages to the non-reducing end of the primer chains. Phosphate ions are liberated in the reaction. If phosphorylase is incubated with amylose and inorganic phosphate then the reverse reaction takes place; Gl - P is formed by the transfer of α-glucosyl units from amylose to the phosphate groups. This reaction is similar to β-amylolysis in that the hydrolysis of a linear substrate by both β-amylase and phosphorylase is halted by a structural anomaly in the chain.

Q-enzyme. (Barker et al. 1950; Gilbert and Patrick, 1952; Baum and Gilbert, 1953). This enzyme is a transglycosylase. It

severs the α-1: 4 bond in an amylose-type chain and transfers the portion carrying the newly exposed reducing group to another chain with the formation of an α-1: 6 linkage between the reducing end of the severed portion and a primary hydroxyl group in the acceptor molecule. For the reaction to take place at a significant velocity, amylose chains containing at least 40 α-D-glucose residues are required but they do not require to be completely linear. The end-product of the reaction is a branched polysaccharide which is very similar to amylopectin.

Starch Degrading Enzymes.

Exoamylases. These enzymes, β-amylases, hydrolyse alternate linkages in amylose and in the external chains of amylopectin and glycogen with the production of β-maltose. This necessitates a Walden inversion during the hydrolytic process. The enzymic hydrolysis commences at the non-reducing end of the substrate molecule and is inhibited by any structural anomaly in the chain.

Endoamylases. These enzymes, α-amylases, catalyse the random hydrolysis of α-1:4 linkages. With linear substrates the main products of the reaction are maltose and maltotriose. Since the α-amylases cannot hydrolyse the α-1:6 linkages of amylopectin and glycogen, reaction with these substrates leads to the formation of high molecular weight branched dextrins.

Debranching Enzymes. R-enzyme (Hobson et al. 1951; Peat et al. 1954; MacWilliam, 1958) severs the α-1:6 linkage in amylopectin, but it has an almost insignificant action on

glycogen, probably because of the greater compactness of the glycogen molecule.

Amylo -1:6-glucosidase. (Goerdeler, 1958) removes terminal α-glucose units joined by α-1:6 linkages to primary hydroxyl groups in the muscle-phosphorylase limit dextrins of amylopectin and glycogen.

Isoamylase. (Gunja et al, 1961). This enzyme is similar to R-enzyme in that it hydrolyses the outer α-1:6 inter-chain linkages in amylopectin.

SECTION 2.

GENERAL EXPERIMENTAL TECHNIQUES

2a. ULTRACENTRIFUGATION

If the density of a material suspended or dissolved in a liquid differs from that of the liquid, then a force field applied to the system will separate the solute and the solvent. The particles in a suspension will tend to settle out under the influence of the earth's gravitational field, but particles of colloidal dimensions require force fields many times greater than gravity in order to overcome diffusion and promote a measurable sedimentation rate. Centrifugation at high speeds is the most efficient method of achieving such force fields. Speeds as high as 60,000 r.p.m. may be required and they are achieved in an ultra-centrifuge. The technical difficulties involved in the construction of this instrument were overcome by Svedberg (1940).

In this work the ultracentrifuge has been used to study rates of sedimentation and polymer homogeneity.

Sedimentation velocity. When a sufficiently large force-field is applied to a particle in solution it will sediment in the direction of the applied force. The rate of movement of the particle will depend on its size, shape and density, the density and viscosity of the medium and the magnitude of the gravitational field, assuming that no convection or electric currents interfere with its motion.

After initial acceleration, the rate of sedimentation will be constant because the centrifugal force is balanced by a frictional force. At a distance x, from the axis of rotation, the centrifugal force is

$$\omega^2 x (1 - \nabla\rho) m$$

w is the angular velocity; m is the mass of the sedimenting molecule; $\bar{v}$ is the partial specific volume; ρ is the density of the solution. The frictional force is the product of the velocity ($\frac{dx}{dt}$) and a frictional coefficient (f). Thus

$$w^2 x (1-\bar{v}\rho) m = f\frac{dx}{dt} \qquad 2.01$$

$$\text{i.e.} \quad \frac{dx}{dt}\cdot\frac{1}{w^2 x} = \frac{m(1-\bar{v}\rho)}{f} = S. \qquad 2.02$$

The sedimentation coefficient is the velocity of sedimentation per unit force field.

Experimental. A Spinco Model E ultracentrifuge capable of speeds of up to 60,000 r.p.m., was used in this work. The cell containing the solution to be examined consisted of a centrepiece with a sector-shaped cavity clamped between quartz discs, and held in a cylindrical housing. Two types of cell were employed, having cavities 12 mm. and 30 mm in length, respectively. The latter was used with dilute solutions ($<0.1\%$), since the increased optical path length gave better definition. The cell was radially aligned in the rotor by means of scribe marks. It is important that the cell be properly aligned otherwise convectional disturbances affect sedimentation. The rotor chamber was evacuated (pressure $<$ 1μ mercury) prior to acceleration. During the run the temperature was maintained at $20 \pm 0.02^{\circ}C$ by means of a Rotor Temperature Indicator and Control Unit. The schlieren optical system gave a direct measure of the variation in refractive index gradient throughout the cell. Photographs of the sedimentation boundaries were taken at known time intervals. The distance of the maximum ordinate of the peak, on the schlieren pattern, from a reference line was measured with a travelling

microscope. After correction for a magnification factor, this distance was related to the absolute distance (x), of the sedimenting boundary, from the axis of rotation. The sedimentation coefficient was then evaluated from a plot of log x versus time, when

$$S = 2.303 \frac{d \log x}{dt} \Big/ w^2 \qquad 2.03$$

Dependence of sedimentation coefficient on concentration.

Sedimentation studies ought to be carried out on infinitely dilute solutions so that each molecule may sediment freely. At high concentrations the probability of molecular entanglement is greatly increased so that the molecules lose their individual character and there is no free sedimentation. Although nearly all polymers exhibit concentration-dependent sedimentation, the degree of dependence varies according to the physical properties of the macromolecules. Rigid, spherical molecules such as glycogen show very little dependence upon concentration, but rod-like molecules such as amylose are markedly concentration dependent.

Since it is not practicable to measure sedimentation coefficients in very dilute solutions they are measured over a range of concentrations and the values obtained are extrapolated to zero concentration. Gralén (1944) has shown that for many systems the extrapolation may be achieved by

$$S = \frac{So}{1 + k.c} \qquad 2.04$$

where S is the sedimentation coefficient at concentration c, So is the corresponding value at infinite dilution and k is a constant

characterising the concentration dependence. This function is usually plotted as $\frac{1}{s}$ versus c, yielding a straight line. This is a purely empirical relation.

Polymer Homogeneity. The technique of ultracentrifugation may also be successfully applied to the problem of homogeneity in solutions of macromolecules. If the polymeric species under examination has a broad distribution of molecular weights then a broad schlieren pattern is obtained on ultracentrifugation because the smaller molecules tend to trail behind those of average size and the larger ones tend to move ahead. More than one peak may appear showing that the polymeric species is heterogeneous.

This is particularly pertinent to the field of protein chemistry where it is often desirable to know the purity of protein samples after attempted purification by fractionating techniques. Care must be taken in interpreting the results obtained from such an investigation. Although the appearance of two or more peaks signifies that the sample is heterogeneous the converse is not necessarily true; one peak may, in reality be two components of closely overlapping molecular weight distributions. The protein should be re-examined in a radically different solvent and if it still appears to be homogeneous then another method of assessing homogeneity, such as electrophoresis, should be used.

2b. LIGHT SCATTERING

The basis of light-scattering measurements is quite simple. A beam of monochromatic light is allowed to pass through a solution of the polymer and the intensity of the light scattered in various directions is measured by a photoelectric cell and related to the intensity of the primary beam. Since light scattering is essentially an optical diffraction phenomena in which the particles of the medium act as secondary sources of radiation of the same frequency, the method only gives information about the molecular size, weight and shape of the polymer and not about its chemical constitution. Light Scattering is the most satisfactory method of obtaining the weight average molecular weight of a polymeric system.

Theory for small molecules. Rayleigh (1871) investigated the scattering of light by small molecules. As long as a scattering molecule is small, compared to the wave-length of light, it may be treated as a point source.

If light of a given intensity I_o and of wave-length λ_o (in vacuo) falls on a small molecule of finite polarisability α, an alternating dipole of the same frequency is induced in the particle which thus becomes a source of radiation. If the incident light is unpolarised, the intensity I_θ of light scattered at an angle θ from the incident beam, at a distance from the molecule is

$$I_\theta = \frac{8\pi^4}{r^2 \lambda_o^4} \alpha^2 (1+\cos^2\theta) I_o \qquad 2.05$$

This is the intensity of light scattered by a single molecule.

In a dilute gas where the molecules are completely independent of each other, the total scattered intensity is merely the sum of the contributions of the individual molecules. A dilute solution of isotropic spheres can be treated in the same way.

Hence $I_\theta = \frac{8\pi^4}{r^2 \lambda_o^4} \alpha^2 \nu (1+\cos^2\theta) I_o$ 2.06

Here ν = no. of particles /c.c. = NC/M

N = Avogadro's Number,

c = concentration,

M = molecular weight.

Equation 2.06 can be simplified by defining the Rayleigh Ratio, R_θ, as $R_\theta = \frac{I_\theta r^2}{I_o}$

Hence equation 2.06 becomes

$$R_\theta = \frac{8\pi^4}{\lambda_o^4} \cdot \frac{Nc}{M} \cdot \alpha^2 (1+\cos^2\theta) \qquad 2.07$$

Further

$$\alpha = \frac{n_o M \frac{dn}{dc}}{2\pi N}$$ where n_o is the refractive index of the solvent.

Therefore

$$R_\theta = K.c.M(1+\cos^2\theta) \qquad 2.08$$

where $K = \frac{2\pi^2 n_o^2 \left(\frac{dn}{dc}\right)^2}{N \lambda_o^4}$

This relation has been derived for a dilute solution in which the scattered intensity is that derived from solute particles alone, i.e. the excess scattering of solution over solvent.

In concentrated solutions the molecules cannot be treated as individual entities because intermolecular interference takes place. This leads to a decrease in the intensity

of the scattered light. Since no light at all would be scattered from a completely homogeneous medium the scattering from a concentrated solution may be calculated in terms of its departure from complete homogeneity. The result of this calculation is $\frac{KC}{R_\theta}(1 + \cos^2\theta) = \frac{1}{\overline{M}_w} + 2A_2c$ + higher terms in c. 2.09

A_2 is the second virial coefficient and is a measure of intermolecular attraction, and $\overline{M}_w$ is the weight average molecular weight.

$$\overline{M}_w = \frac{\sum n_i M_i^2}{\sum n_i M_i}$$

n is the number of species i with molecular weight M

Theory for large molecules. When a dimension of the solute molecule exceeds $\frac{1}{20}\lambda_o$, differences in path length cause interference in the light scattered from various parts of the molecule, leading to a reduction in scattered intensity. This decrease is not symmetrical, a greater phase difference existing in the backward than in the forward direction. The departure from symmetrical scattering increases with molecular size and is dependent on the shape of the molecule. The Particle Scattering Factor, P_θ, is defined as the ratio of the observed intensity, to that in the absence of interference. The particle scattering factor has been calculated for the various models by which polymer molecules may be represented (Debye, 1947; Zimm, Stein and Doty, 1945), and it can be shown that

$$\frac{Kc}{R_\theta} = \frac{1}{\overline{M}_w P_\theta} + 2A_2c + \cdots \qquad 2.10$$

Equation 2.10 is usually used to derive molecular weights by either (1) the dissymmetry method, or (2) the extrapolation method.

The dissymmetry method. For all models, P_θ decreases smoothly from unity at $\theta = 0$ and so the ratio of intensities at two angles defines the dimensions of a particular model. Light scattering determinations normally involve the measurement of $R_{90}o$, and, if the scattering is not symmetrical, the ratio of the intensities at two other angles. The angles usually selected are 45° and 135°. The ratio I_{45}/I_{135} is known as the dissymmetry ratio and is denoted by Z. From the value of Z, P_{90} is obtained by using the tables constructed by Doty and Steiner (1950). This method is very simple, but it requires a knowledge of the shape of the polymer molecule in solution. Its use is thus limited to molecules for which other physical measurements have indicated their molecular shape.

The extrapolation method. P_0o for all models is unity, and, if the value of R_0o can be established, the molecular weight can be determined. It is not possible to do this directly, but from measurements made at sufficiently low angles, a reliable extrapolation to 0° can be made. The procedure of Zimm (1948) was used. $\frac{Kc}{R_\theta}$ is plotted as a function of $\sin^2\frac{\theta}{2} + k'c$. k' is an arbitrary constant chosen so that the concentration term is comparable in magnitude with $\sin^2\frac{\theta}{2}$. By this method the data is spread out and a grid-like graph is obtained.

By extrapolating the lines of constant angle to zero concentration and the lines of constant concentration to zero angle and extending them both to the $\frac{Kc}{R}$ axis, a common intercept is obtained. From equation 2.10 this intercept $(\frac{Kc}{R})_{\substack{\theta=0 \\ c=0}}$ is equal to the reciprocal of the weight average molecular weight.

Description of the photometer. The instrument used in this work was the commercial model supplied by Phoenix Precision Instrument Co., Philadelphia, built to the design of Brice, Halwer and Speiser (1950).

Because the scattered light is usually of low intensity, it is necessary to have a powerful light source. This is provided by a mercury vapour lamp. A filter is incorporated in the instrument to enable monochromatic light of a desired wavelength to be chosen. A suitable light intensity is obtained by using four neutral filters, either singly or in conjunction with one another. The cell containing the solution is placed on the cell table about which the photomultiplier can be rotated. On the opposite end of the photomultiplier arm an opal glass diffuser, the working standard, is mounted. This is always in the path of the incident light at 0° and serves to reduce the light intensity. The sensitivity of the photomultiplier is adjusted by means of a potentiometer network. The output from the photomultiplier is then measured by a galvanometer.

2c. POTENTIOMETRIC IODINE TITRATION

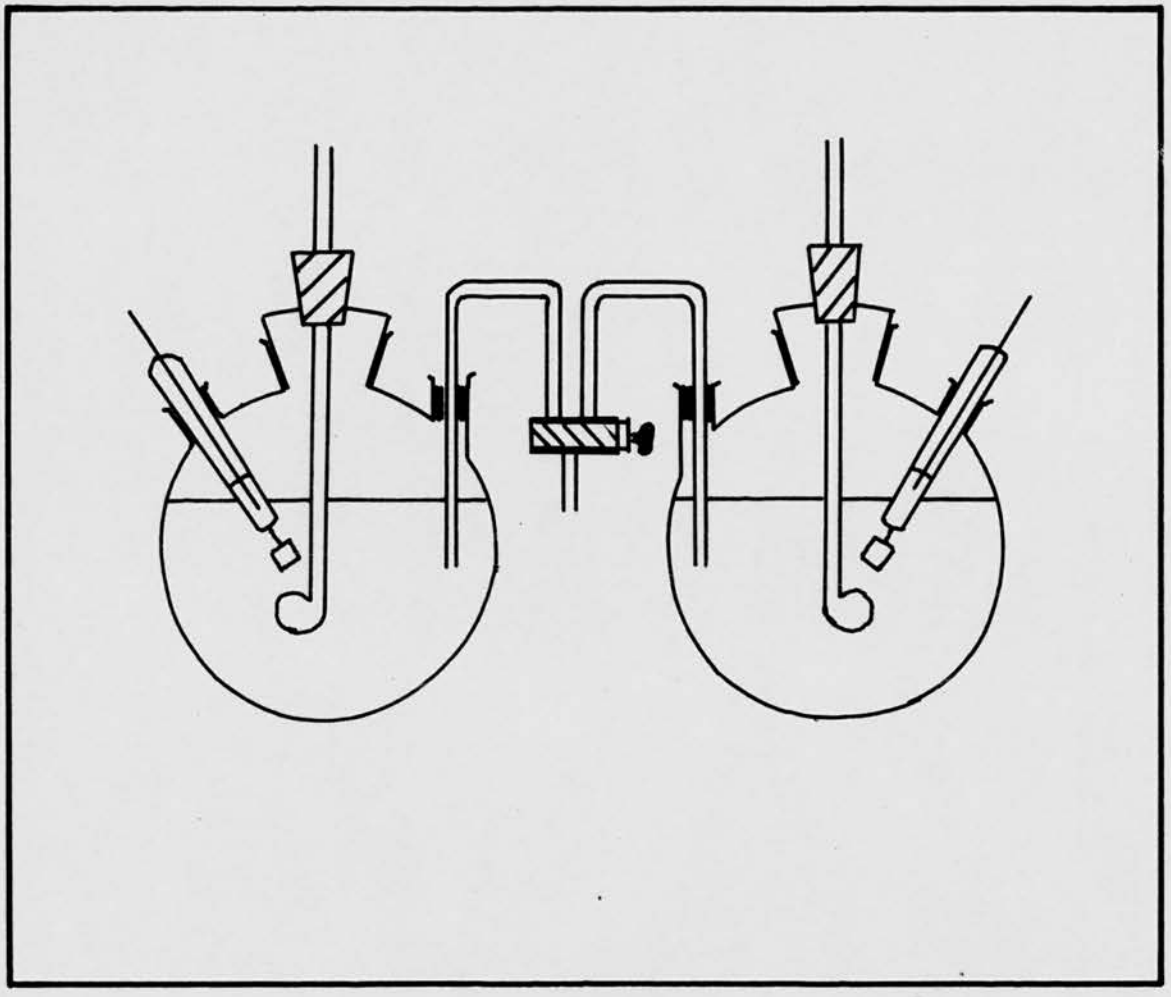
The two components of starch show greatly differing affinities for iodine - amylose stains blue and amylopectin stains red. Bates et al. (1943) introduced a potentiometric iodine titration method which enabled a quantitative estimation of this difference to be made. They measured the potential between a bright platinum electrode in the starch-iodine-iodide solution and a standard calomel electrode and so were able to calculate the equilibrium concentration of free iodine in the mixture.

Anderson and Greenwood (1955) modified a more satisfactory differential technique which had been proposed by Gilbert and Marriott (1948). In this technique, the starch-iodine-iodide solution and the blank-iodine-iodide solution were arranged as opposing half-cells connected by a salt bridge as in Fig.2.01. The equilibrium free-iodine concentration in the starch solution was then found directly. The electrodes were made of platinum foil fused to platinum wires which were each sealed through glass tubing containing mercury. The solutions in the half-cells were stirred continuously.

Preparation of sample solutions. Fatty acids and complexing agents were removed from the sample, by extraction with boiling methanol, prior to titration because they prevent the formation of the iodine complex. This leads to anomalous results.

Samples of amylose (3 - 5mg.) starch (10 - 15mg.) or amylopectin (25 - 30mg) were placed in a weighing stick and dried in a vacuum oven at 70°c. for twelve hours. The samples

FIG. 2.01



Cell Arrangement

Iodine Titration Apparatus

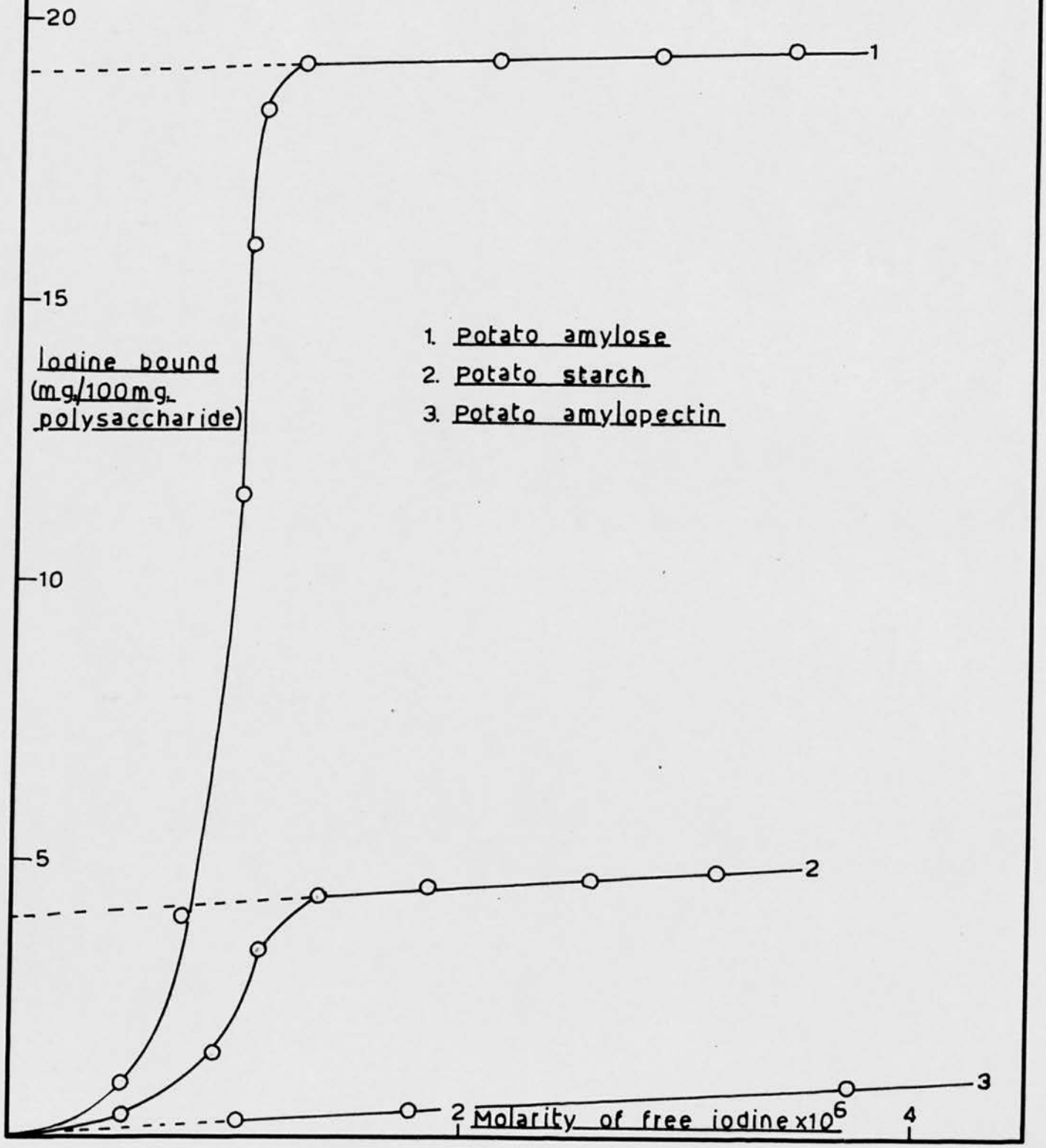
were then carefully weighed out and dissolved in potassium hydroxide (10 ml; 0.2M.). The solution was brought to PH5,85 by the addition of a predetermined amount of phosphoric acid (0.4M.). A blank containing the same volumes of potassium hydroxide and phosphoric acid was also prepared.

Experimental The electrolyte solution contained potassium iodide (210 ml.; $\frac{M}{10}$), phosphate buffer (15 ml.; $\frac{M}{15}$; PH 5.85) and distilled water (1,775 ml.). Equal aliquots (800 ml.) of this solution were added to each half-cell. The solution containing the sample and the blank solution were added to their respective half-cells and the flasks were rinsed with aliquots (25 ml.) of distilled water to make the final volumes in the cells about 840 ml. The salt bridge containing electrolyte was fitted, the stirrers started and the circuit checked to make sure that there was no significant off-balance potential.

Small increments of iodine-potassium iodide solution (0.1 ml.; 0.01M.), were added to the cell containing the solution by means of an "Agla" syringe. About five minutes were then allowed for equilibrium to be reached before the potential produced by the free iodine was balanced by adding the same iodine-iodide solution to the "blank" cell until the galvanometer deflection caused by the potential difference was zero. The difference between the volumes of iodine added to the half-cells gave the amount of iodine bound by the sample. A titration curve was thus constructed by plotting the amount of iodine bound (mg./100 mg. of polysaccharide)against the total free iodine in the solution Fig. 2.02. The iodine affinity of the polysaccharide was obtained by extrapolating the linear portion

FIG. 2.02

Iodine titration curves.



of the graph to zero free iodine concentration.

Under these conditions the large difference between the iodine uptake of amylose and that of amylopectin is readily apparent. Pure potato amylose was found to bind 19.5% of its own weight of iodine and pure potato amylopectin less than 0.2%. Using this value for amylose the amylose-amylopectin content of a particular starch product may be determined from its iodine affinity.

2.d. Estimation of the phosphorus content of starch and its components

Many starches contain phosphorus (present as the ester-phosphate group on C_6 of a glucose residue) although the amount present varies considerably depending on the botanical source of the starch and on the maturity of the starch-containing organ. Although Schoch (1942) has shown that the phosphorus is associated mainly with the amylopectin fraction, Peat et al. (1952a) have reported that potato amylose contains one atom of esterified phosphorus in 2,000 anhydro-glucose units.

Analytical Method

Reagent. Ammonium molybdate-sulphuric acid solution

Ammonium molybdate (10 g.) was dissolved in distilled water (100 ml.). To this solution was added a solution of sulphuric acid (sp. grav. 1.84; 10 ml.) and distilled water (100 ml.).

Procedure

The carbohydrate material must be totally destroyed to enable the liberated phosphate groups to be estimated. The

method of Gieseking et al. (1935) and Smith (1953) was used. The starch-type samples were dried under vacuum at 65°C - 70°C before the estimations were carried out.

Samples of polysaccharide (starch or amylopectin - 100 mg; amylose 500 mg - 1g.) were mixed with concentrated nitric acid (0.25 ml.) in a 25 ml. conical flask. The mixture was heated on a hot plate and, after the initial reaction had subsided, it was boiled until almost dry. The mixture was removed from the hot plate and 0.5 ml. of a mixture of equal parts of nitric acid (8N.) and perchloric acid (70%) was added. The solution was gently heated until it became colourless, indicating that all the organic material had been destroyed, and was then evaporated to dryness. The residual salts were dissolved in hydrochloric acid (0.5 ml; 2N.) and this solution was used for the determination of phosphate by the method of Fogg and Wilkinson (1958).

The acid solution obtained after the destruction of the organic matter was diluted to ca. 40 ml., ammonium molybdate-sulphuric acid solution (4.0 ml.) and ascorbic acid (100 mg.) were added and the solution was boiled for one minute when a blue colour was produced. The solution was cooled, diluted to 50 ml in a graduated flask and its absorbance at 675mμ was measured on a spectrophotometer. A blank control determination in which the polysaccharide was omitted was carried out to determine the phosphate content of the reagents.

Standard solutions of KH_2PO_4 were prepared containing accurately known amounts of phosphorus. A standard graph of

ultra-violet absorbance against phosphorus content was prepared. This enabled the phosphorus content of the polysaccharide to be determined.

2.e. Estimation of Protein Nitrogen

There is a small amount of protein associated with most pure starches, but it has not yet been determined whether it is an integral part of the granule or whether it is an impurity.

The semi-micro Kjeldahl method of Chibnall et al. (1943) was used to determine the α- amino N - content of starch granules. The weighed solid sample was boiled with concentrated sulphuric acid (2 ml. "Analar", N - free grade) containing a catalyst (0.2g.; a mixture of anhydrous sodium sulphate, copper sulphate pentahydrate and sodium selenate in the ratio 80; 20: 1 w/w) until the solution became clear. It was transferred to a Pregle micro-distillation apparatus and potassium hydroxide (10N.; 10 ml.) was run in. The liberated ammonia was reacted with standard hydrochloric acid (ca. N/70) and the amount of protein present was determined by multiplying the weight of nitrogen by 6.25.

2.f. Viscosity

One of the characteristic properties of a polymer is its ability to increase the viscosity of the medium in which it is dissolved. Generally, solutions of large polymers are more viscous than solutions of smaller polymers for the same amount

of dissolved polymer.

To study the behaviour of individual polymer molecules interaction effects must be avoided by measuring the viscosity at a number of concentrations and by extrapolating some functions of it to zero concentration.

The ratio of the viscosity η of a solution to the viscosity η_o of the pure solvent is called the relative viscosity η_r.

$$\eta_r = \eta/\eta_o \qquad 2.11$$

The relative increase of the viscosity is called the specific viscosity η_{sp}.

$$\eta_{sp} = \frac{\eta - \eta_o}{\eta_o} = \eta_r - 1 \qquad 2.12$$

The specific viscosity divided by the concentration is the viscosity number η_c.

$$\eta_c = \eta_{sp}/c \qquad 2.13$$

The value of the viscosity number at infinite dilution is the limiting viscosity number or intrinsic viscosity $[\eta]$

$$[\eta] = \lim_{c \to 0} \eta_c = \lim_{c \to 0} \eta_{sp}/c \qquad 2.14$$

The concentrations of the polymer solutions are given in gm./ml. and the intrinsic viscosity has the dimension of the reciprocal of concentration.

The intrinsic viscosity of a polymer in a particular solvent is dependent not only on the size of the polymer molecules but also on their shape in that solvent. If, for example, the intrinsic viscosity of a polymer containing negatively charged groups was measured in saline and also in water the two results

would generally be different. In water the charges on the molecule would tend to make it rod-like and inflexible whereas in saline, where the charges would be neutralised, the molecule would become more flexible and so have a lower intrinsic viscosity.

The solvent system may also change the shape of uncharged polymer molecules. This was investigated by Alfrey et al. (1942). If a poor solvent is employed, i.e. one which is energetically unfavourable to the process of dissolution, then solvent-polymer contact will be at a minimum. The polymer segments will attract one another in solution and polymer-polymer contact will be enhanced. The polymer will then tend to curl up on itself.

If, however, a good solvent is employed, i.e. one which is energetically favourable to the dissolution of the polymer, the solvent-polymer contact will be at a maximum and the polymer chain will be surrounded by a solvated sleeve which will keep polymer-polymer interaction at a minimum. Uncurled configurations will be favoured and the molecule will become more rod-like.

The Relationship between viscosity and M.W.

Berl (1910) and Biltz (1910) were the first to show that a general relationship existed between the viscosity of a polymer solution and the molecular weight of the polymer. The first mathematical formulation of this relationship was given by Staudinger (1932) in the expression

$$\eta_{sp}/c = K.M \qquad 2.15$$

where K is a constant and M is the molecular weight of the polymer.

At infinite dilution $\eta_{sp}/c = [\eta]$ and the equation becomes

$$[\eta] = K.M \qquad 2.16$$

It has been found by many workers that this simple relationship is not strictly true and the following equation was proposed by Kuhn (1934) and Mark (1938).

$$[\eta] = K' \bar{M}_v^{\beta} \qquad 2.17$$

Houwink (1940) confirmed the validity of the equation for a number of polymers having values of β between 0.5 and 0.8.

The constants K' and β may be found by carefully fractionating a polymer into samples of differing molecular weight and determining for each fraction both $[\eta]$ and the molecular weight; the latter by a separate absolute method giving a weight-average value. A plot of log $[\eta]$ against log M then gives a straight line of slope β and intercept K' on the log $[\eta]$ axis. The constants must be calculated for each solute-solvent system.

The above equations are mainly empirical and a theoretical treatment has been applied to this problem by Kirkwood and Riseman (1948), Flory and Fox (1949, 1950, 1951).

They proposed that the intrinsic viscosity is proportional to the effective hydrodynamic volume of the molecule in solution divided by its molecular weight. If the polymer in solution has the configuration of a randomly coiled chain then its effective volume can be shown to be proportional to the cube of a linear dimension of the chain. A convenient parameter for this linear dimension is the root-mean-square distance $(\bar{r}^2)^{1/2}$ between the ends of the polymer chain. Hence $(\bar{r}^2)^{3/2}$

is a measure of the volume of the polymer molecule in solution. This expression may be split into two factors -

a) $(\bar{r}_0^2)^{3/2}$ which is the unperturbed value of $(\bar{r}^2)^{3/2}$ i.e. where there are no interactions between the chain segments and their environment $(\bar{r}_0^2)^{3/2} \equiv (\bar{r}^2)^{3/2}$

b) α^3 a volume expanding factor by which the linear dimensions of the molecular chains are increased because of segment-segment and segment-solvent interactions.

$$\therefore (\bar{r}^2)^{3/2} = (\bar{r}_0^2)^{3/2} \cdot \alpha^3$$

$$[\eta] \propto \frac{(\bar{r}_0^2)^{3/2} \cdot \alpha^3}{M} \qquad 2.18$$

$$[\eta] = K'' M^{1/2} \alpha^3$$

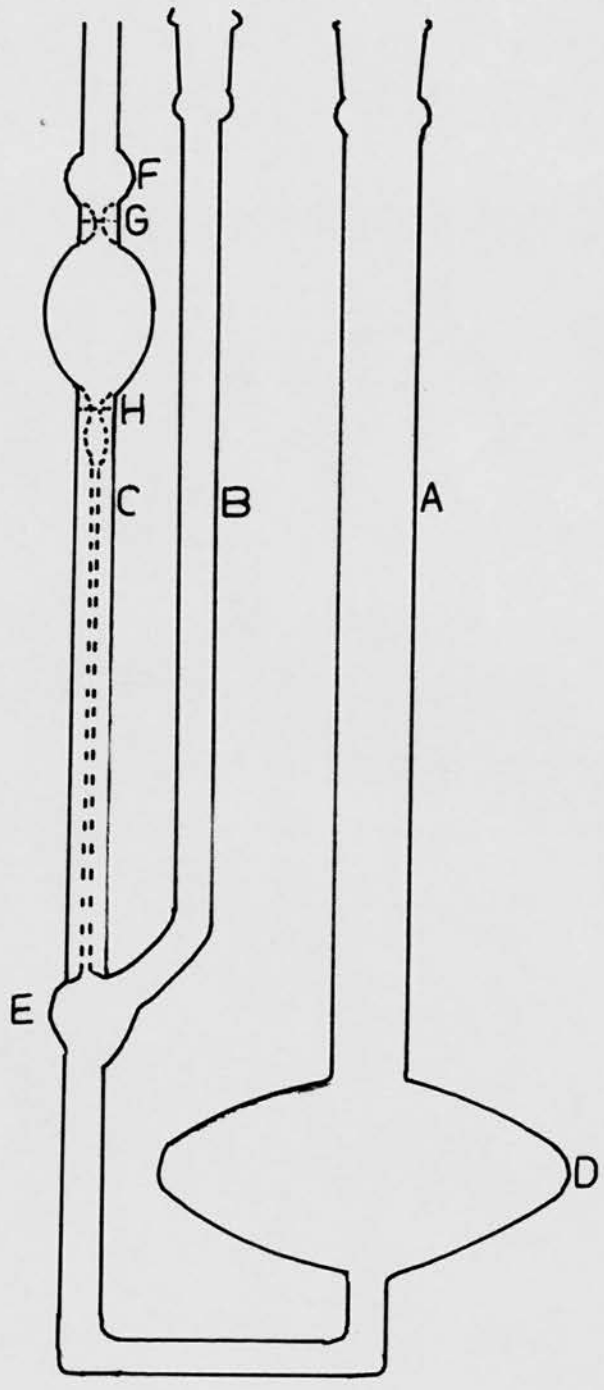
where $K'' = \Phi (\bar{r}_0^2/M)^{3/2}$ 2.19

Φ is a universal constant and has a value of $2.1 \pm 0.3 \times 10^{21}$ $(\bar{r}^2)$ has to be calculated from light scattering data. This relation only applies to linear molecules but there is not a corresponding one for branched molecules.

Apparatus and Procedure

All viscosity measurements were carried out in viscometers of the modified Ubbelohde type Fig.2.03 (Ubbelohde 1937; Davis and Elliot 1949). The viscometers were immersed in a bath thermostated at $25 \pm 0.01°C$. Both solvent and solution were filtered through a G4 sintered filter before being inserted into the viscometer. One of the advantages of this type of viscometer is that it is not necessary to know exactly the volume of the liquid present in the viscometer. This permitted the use of modified pipettes containing long stems

FIG. 2.03



Modified Ubbelohde Viscometer

which were able to pass through tube A and reach bulb D. Thus solution and solvent were added directly to bulb D.

Viscosities were measured by either of the two methods outlined below depending on the amount of solute available.

A. Two pipette loads of filtered solvent were added to bulb D and allowed to come to temperature equilibrium. Tube B was closed with a glass stopper and pressure was applied through tube A from a nitrogen cylinder until the liquid level had reached the middle of bulb F. . The pressure was then released, the stopper removed and the time taken by the liquid level to pass the two marks G and H was measured on a stop watch to within 0.1 sec. One pipette load of filtered solution was added to bulb D and thoroughly mixed with the solvent already there by a gentle stream of nitrogen entering at tube B. The flow time of the solution was recorded. This procedure was repeated three times.

B. Two pipette loads of the filtered solution were initially added to bulb D and aliquots of pure solvent were added. The flow times for the various dilutions were determined. The solvent flow time, of course, was determined on a separate run.

In both cases the concentration of the initial polymer solution after filtration was determined by the alkaline ferricyanide method.

Calculation of Results.

In a capillary viscometer of the Ubbelohde type, the viscosity is given by

$$\eta = K\rho t - \frac{B\rho}{t}, \qquad 2.20$$

where K and B are constants; t is the flow time of a fixed volume; ρ is the density of the liquid.

B is the kinetic energy factor and is due to the liquid leaving the capillary with a finite velocity. By the use of standard liquids the value of B was shown to be negligible for the viscometers used in this work.

Hence

$$\eta = K\rho t \quad \text{for the solution}$$

and $$\eta_o = K\rho_o t_o \quad " \quad " \quad \text{solvent}$$

The specific viscosity is, therefore, given by

$$\eta_{sp} = \frac{\rho t - \rho_o t_o}{\rho_o t_o}, \qquad 2.21$$

and for dilute polymer solutions where it may be assumed that $\rho = \rho_o$,

$$\eta_{sp} = \frac{t - t_o}{t_o} \qquad 2.22$$

Having thus obtained experimental values of η_{sp} for several concentrations a graph of $\frac{\eta_{sp}}{c}$ against C was drawn. By extrapolation of the plot to infinite dilution the intrinsic viscosity $[\eta]$ was obtained.

$$\bar{M}_w = \left[\frac{\sum n_i M_i^{1+\beta}}{\sum n_i M_i}\right]^{1/\beta}$$

n is the number of species i with molecular weight M.

2g. Extraction of potato starch

(Banks, Greenwood, Thomson 1959).

The potatoes were thickly peeled and quickly covered with mercurie chloride (10^{-2}M.) to prevent enzymic modification of the starch. They were then chipped, minced and extracted w with mercuric chloride (10^{-2}M.) in an Atomix blendor for three minutes. The slurry was passed through muslin and the filtrate was quickly centrifuged. The residue from the muslin filtration was re-extracted with mercuric chloride (10^{-2}M.) for a further three minutes, passed through muslin and centrifuged. The residues from the sedimentation procedure were suspended in sodium chloride (10^{-2}M.) and purified by sedimentation.

Any protein which might still be present in the starch was denatured by suspending the granules in saline and shaking them overnight with toluene ($\frac{1}{10}$ volume) (Greenwood, Robertson 1954). This was repeated with fresh aliquots of toluene and saline until the toluene layer remained clear. The starch was stored in saline suspension under a layer of toluene at 2°C. Before use the starch was thoroughly washed with water.

2h. Determination of the Gelatinisation Temperature of Starches.

(Schoch and Maywald 1956).

Starch granules are insoluble in cold water but they swell up on warming. Initially, this swelling is reversible. If the starch suspension is gradually heated, microscopic observation shows that the granules continue to swell slowly until, at a certain temperature (55 - 60°C. in the case of potato

starch), they expand in volume very rapidly and lose their sharp outline. This very rapid swelling is irreversible and does not take place at the same temperature for all the granules but extends over a range of 5 - 10 degrees. Immediately prior to the rapid swelling phase the granules lose their polarisation crosses when viewed through an Ahrens analyzer. The gelatinisation temperature is the temperature at which the starch granules lose their birefringent properties. As indicated, the true gelatinisation temperature of a starch is a range of temperatures which is characteristic for that particular starch. In practice, the gelatinisation temperature is very often taken to be the temperature at which half the granules have lost their birefringence.

Experimental. To determine the gelatinisation range, a small quantity of starch was slurried in water to give a suspension (0.1 to 0.2%). One drop of this slurry was placed on a glass slide and surrounded by a ring of highly viscous oil. A cover glass was placed over the slurry and oil in such a way that there were no air bubbles under it. The oil prevented air channels from penetrating under the cover glass and disturbing the field. The glass slide was placed on a "Kofler" electrically heated microscope stage and the temperature raised fairly rapidly to about 45°C. Thereafter it was raised at about 2°C. per minute. The field was watched continuously under normal light until the first few granules had irreversibly swollen and loss of birefringence in these granules was confirmed by introducing the Ahrens analyzer. This was the initial temperature in the gelatinisation range. Heating was

continued until all but the last few granules had lost their polarisation crosses and this temperature was the final temperature in the gelatinisation range.

2.i. Conventional Fractionation of Potato Starch.

Introduction Any detailed investigation into the fine structure of amylose and amylopectin necessitates, that both should be isolated from the parent starch in a manner causing minimum degradation and yet achieving maximum purity of the components. Fractionation techniques, involving selective retrogradation (Maquenne and Roux, 1905), electrophoresis (Samec et al, 1941), or chromatography using an alumina column (Fischer and Settele, 1953; Ulmann and Wendt, 1954), have been used in attempts to isolate the components in a pure, undegraded form. Although separation may be achieved by these procedures, the most successful method yet devised is that of selective precipitation (Schoch, 1942). In this method, an aqueous dispersion of starch is prepared and the amylose is precipitated as an insoluble complex by the addition of a polar organic substance. Whereas it is relatively simple to obtain pure amylose (by repeated recrystallisation), the removal of residual amylose from the amylopectin fraction is more difficult. Methods involving selective adsorption of the amylose on cotton wool (Tanret, 1914) and complex formation with stearic acid (Meyer and Gibbons, 1950) were shown to be misconceived (Gilbert, Greenwood and Hybart, 1954). It is essential, therefore, that the first precipitation should give amylopectin of maximal purity. Greenwood and Robertson (1954) showed that this could

be achieved by using thymol as the initial precipitant.

Experimental. Distilled water was boiled for one hour under nitrogen to remove oxygen. Sufficient starch, in a slurry of cold, deaerated water, was added to the boiling water to give a 0.5% final solution. The suspension was stirred and boiled under nitrogen for 20 - 30 minutes when it was allowed to cool to 60°C. and powdered thymol (1g./litre) was added. The solution was thoroughly mixed and left at room temperature for 72 hours. The amylose complex was removed on a Sharples super-centrifuge and recrystallised three times as the butanol complex. After removing the amylose, the supernatant liquid was shaken with ether to remove excess thymol. The ether was removed on a rotary evaporator at a temperature $< 40^{\circ}C$. The solution was finally freeze-dried to give the amylopectin fraction.

2.j. Estimation of Reducing Sugars and Polysaccharide Concentration

It is often necessary, in starch chemistry, to determine the reducing power of solutions. e.g. in the determination of β- amylolysis limits. This involves the estimation of maltose in the presence of iodine-staining residual polysaccharide. The copper reagent of Somogyi (1945) thus cannot be used, because the iodine used in the titration reacts with the residual polysaccharide making the titration procedure difficult. In the method of Lampitt et al. (1955), there is no interaction between the polysaccharide and the reagents.

The concentration, of any starch solution, may also be conveniently determined by hydrolysing the polysaccharide to its component sugars and then estimating the reducing groups formed.

This is readily carried out by hydrolysing an aliquot of the starch solution with sulphuric acid (3N) for two hours in a boiling water-bath and then estimating the resultant glucose by the technique of Lampitt et al. (1955).

The method has to be calibrated using starch and maltose.

Experimental. "Analar" soluble starch was heated in a vacuum drying line at 65 - 70°C. for twelve hours. A series of standard starch solutions was then prepared in dilute alkali. They contained from ca. 0.5 mg. starch/ml. - 3 mg. starch/ml.

Three aliquots (1ml.) of each solution were placed in Quickfit boiling tubes and sulphuric acid (1ml; 3N) was added to each. The tubes were placed in a boiling water-bath, stoppered, and left for two hours. They were removed and quickly cooled to room temperature when their reducing power was estimated in the following way. Bromocresol green indicator (0.05 ml.) was added to each and the solutions were carefully neutralised with potassium hydroxide (1N.). To each solution were added sodium carbonate solution (2.5 ml; 0.2M.) and potassium ferricyanide solution (2.5 ml; 0.05M.). They were carefully shaken, the insides of the tubes were washed down with distilled water to make the total volume in each tube about 10 ml., and they were then returned to the boiling water-bath for fifteen minutes. The tubes were removed and cooled. Sulphuric acid (5 ml; 5N) and Xylene cyanol FF indicator (4 drops) were added to each tube before it was titrated with standard ceric sulphate solution (ca. 0.0 IN). At the end-point of the titration the colour of the solution changed from sage-green to whisky yellow.

The results are shown in Fig. 2.04..

Standard maltose solutions were prepared in water and aliquots were put through the reducing part of the above procedure. The results are shown in Fig. 2.04..

The ceric sulphate solution was prepared in sulphuric acid (2N.) and it was standardised with standard ferrous ammonium sulphate solution. The calibration factors for maltose and starch, are directly proportional to the normality of the ceric sulphate solution and they are calculated in terms of ceric sulphate (0.01N.).

From Fig. 2.04.

8 ml. 0.01063 N. ceric sulphate ≡ 2.51 mg.starch

∴ 1 ml. 0.01N. ceric sulphate ≡ 0.295 mg.starch

and

7 ml. 0.01063N. ceric sulphate ≡ 3.2 mg. maltose

∴ 1 ml. 0.01N. ceric sulphate ≡ 0.430 mg.maltose

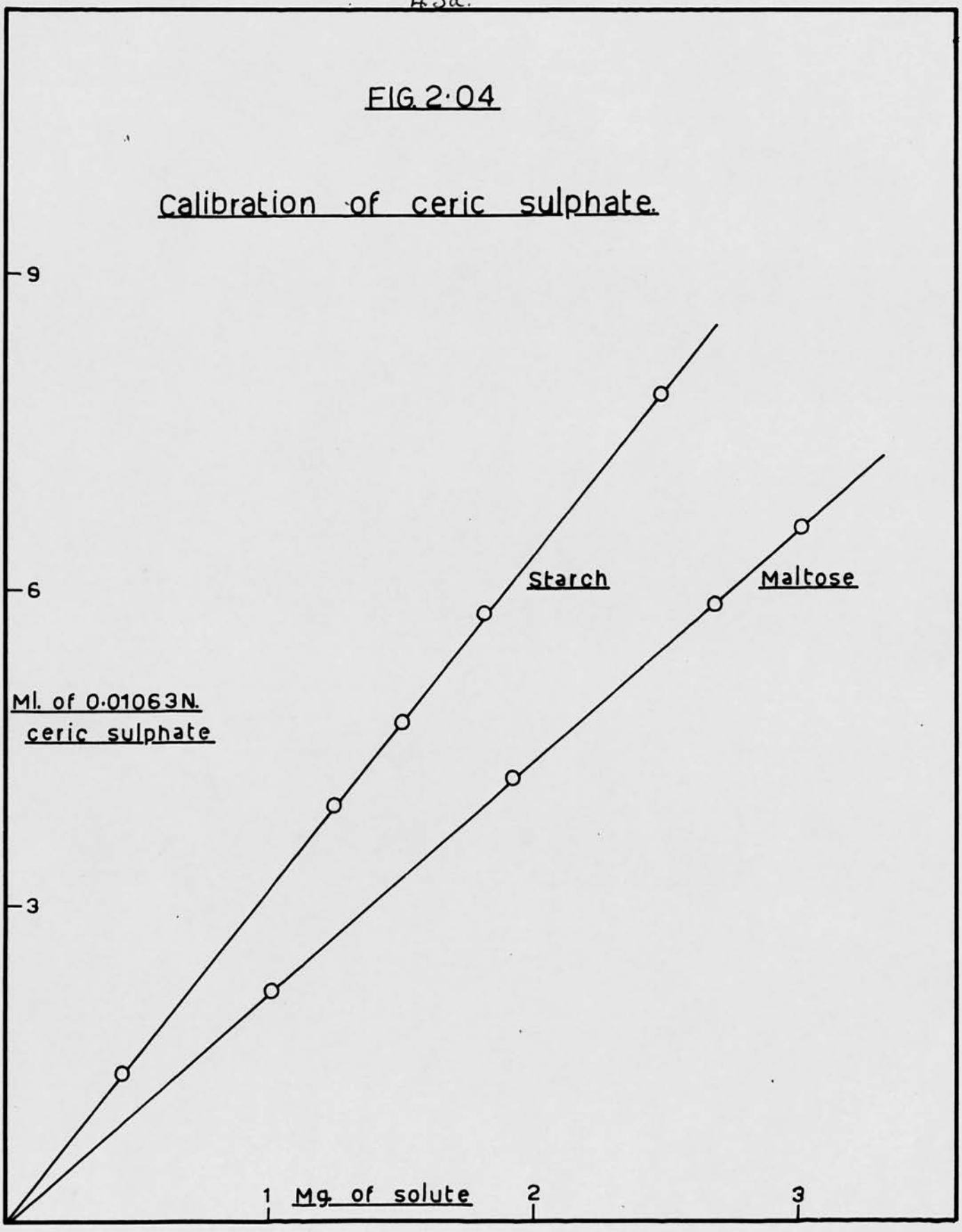
2.k. Estimation of β - amylolysis Limits.

Starch and its components are degraded by β - amylase by the step-wise removal of maltose units from the non-reducing ends of the molecules. The extent of degradation depends upon the number of structural modifications in the polysaccharide molecules - the more abundant they are the smaller is the extent of β - amylase hydrolysis. An important characteristic of starches and their components is, therefore, their percentage hydrolysis into maltose by the action of β - amylase.

Experimental. Digests were prepared containing the poly-

FIG. 2·04

Calibration of ceric sulphate.



saccharide solution (20 ml; 20 mg. polysaccharide) and acetate buffer (4 ml; 0.2M; PH4.8). Aliquots (1 ml. x 3) were removed for concentration determinations. β- amylase solution (200 units) was added and the digests were incubated at 35°C. for twenty four hours when aliquots (3 ml. x 3) were removed for reducing power determinations. Appropriate blanks were also prepared. The percentage conversion of the polysaccharide into maltose was then calculated.

SECTION 3.

PREPARATION OF SUBSTRATES

3.a. Studies on the Fractionation and Subfractionation of Amylose.

Introduction In the present work, α-amylases from various plant sources and crystalline β - amylase from sweet potatoes were investigated in an attempt to elucidate their action patterns. Since these enzymes hydrolyse α- 1: 4 - glycosidic linkages, completely linear amylose (i.e. amylose which is completely hydrolysed by β - amylase) was required as a substrate.

Banks et al. (1959a) showed that completely linear amylose may be obtained by aqueous leaching (at 60 - 65°C.) ~~of~~ potato starch granules which had been pretreated with 80% boiling methanol. By this method about 40% of the total amylose present in potato starch is obtained.

Everett and Foster (1959a) indicated that amylose could be dissolved in dimethyl sulphoxide and fractionated by the stepwise addition of ethanol at 4°C. From viscosity and light scattering results they showed that the fractions had different molecular weights. Later, Banks (1960) investigated this fractionation technique and found that not only did the amylose fractions have different molecular weights but that they also had different β - amylolysis limits. The high molecular weight fractions had low β - limits and the low molecular weight fractions were completely hydrolysed by β - amylase.

Foster and Killion (1960) found that dimethyl sulphoxide was a good solvent for starch granules at room temperature. The addition of butanol to the resulting solution led to quantitative co-precipitation of the amylose and

amylopectin; this mixture was then readily soluble in water at room temperature. The amylose component was separated in the usual way by forming the butanol complex after a brief heating of the aqueous solution at 70°C. The amylose sample, obtained in this way, was almost completely degraded by crystalline β - amylase indicating that it contained very few structural anomalies. It was concluded from light scattering and viscosity results that the amylose was not degraded since its molecular size agreed favourably with that of amylose samples obtained from conventional fractionations.

The results from the latter procedure imply that native amylose is completely linear and that the barriers to β - amylase action are introduced during the conventional preparation of amylose samples. These methods, therefore, were investigated with two aims in view; (1) to find a way of preparing large amounts of linear amylose and (2) to show whether or not native amylose contains structural anomalies.

A difficult problem in starch chemistry is the nature of the starch granule. The extent of the association, if any, of the two components in the "native" state is not readily determined. It has been suggested that starch is a giant homogeneous molecule and that its apparent separation into components is the result of degradative or hydrolytic action. This is the unitarian theory of the starch granule. Pacsu (1947) suggested a structure for starch involving a small number of non-cyclic hemiacetal linkages. Dumazert (1950) postulated a structure involving 1, 5 - acetal cross-linkages between open chains of 1, 4 - linked D - glucose units whilst

Blom and Schwarz (1952) proposed a structure containing chains of alternating D - glucopyranose and D - glucofuranose units. Chemical evidence against these theories can be readily presented (Greenwood, 1956). A phosphate cross-link between the components was suggested by Sutra (1950) and this idea was elaborated by Bauer and Pacsu (1953). These cross-linkages were thought to be easily hydrolysed and so were readily broken during normal fractionation procedures.

Starch granules can be dissolved directly in cold water after their pretreatment with liquid ammonia, alkali or dimethylsulphoxide. An ultracentrifugal investigation was undertaken to determine how many components there were in such solutions in which the granules had received no heat treatment.

Experimental

Isolation of potato-starch. The starches were isolated by the procedure described in the general experimental section.

Pretreatment of starches. If not used directly as prepared, potato starch was pretreated in one of the following ways.

a) Methanol pretreatment (Schoch, 1942). The starch granules were heated under reflux with boiling 80% aqueous methanol for two hours. The methanol was filtered off and the process was repeated for a further two hours.

b) Liquid ammonia pretreatment. (Hodge, Montgomery and Hilbert, 1948)

Liquid ammonia (50 ml.) was added to starch (10 - 15g.), which had been moistened with ethanol. After fifteen minutes a large excess of ethanol (200 - 300 ml.) was added and the

suspension was left overnight to allow the ammonia to evaporate. The starch was filtered off and thoroughly washed with alcohol to completely remove the remaining ammonia.

c) Dimethyl sulphoxide pretreatment. (Killion and Foster, 1960). Starch was added to dimethyl sulphoxide to make a 0.5% suspension which was thoroughly sparged with nitrogen. The suspension was gently shaken at room temperature for three days when dissolution was complete. In later experiments, water (10%) was added to the suspension and solution was achieved more quickly (Schoch, 1962). The starch was precipitated by the addition of excess butanol and the presence of a pinch of salt was found to aid the coagulation of the precipitate. The gummy material was centrifuged, thoroughly washed with butanol, hardened and dried with ethanol.

Fractionation Procedures.

a) The conventional fractionation of potato starch in aqueous dispersions at 100°C., by the addition of a polar organic compound, has been described in the general experimental section.

b) Aqueous leaching at X°C. Distilled water was deaerated and allowed to come to X°C. Starch was added in a cold deaerated water slurry to make a 0.5% suspension which was maintained at X°C., under nitrogen and gentle shaking, for ten minutes. It was then quickly cooled in ice and centrifuged. The clear supernatant was passed through a grade 3 sintered filter and saturated with butanol. The amylose was recrystallised twice as the amylose-butanol complex.

All the amylose samples from dimethylsulphoxide

pretreated starches were purified by four recrystallisations from aqueous dispersions at the appropriate temperature.

Amyloses were stored either in the form of the amylose - butanol complex or in the solid form, after dehydration of the butanol-complex.

Purity of Starch.

The purity of starches and amylopectins was determined from measurements of the iodine-binding power of the samples, using a semi-micro differential potentiometric apparatus. Under the experimental conditions, the iodine affinity of potato amylose was 19.5% by weight (Cowie and Greenwood, 1957). Apparent amylose contents were calculated from [(Iodine affinity of sample) /19.5] x 100.

Enzymic degradation was used to determine the purity of the amylose samples. The percentage conversion of the samples into maltose under the concurrent action of β - amylase and Z - enzyme was measured. This estimation is discussed in detail later.

The accuracy of these measurements is $\pm$ 2%.

Subfractionation of Amylose.

Dimethylsulphoxide/ethanol precipitation. (Everett & Foster 1959) Dried amylose was dissolved in dimethylsulphoxide to give a 0.5% solution. 20% ethanol (v/v) was added and the mixture was cooled to 4°C. Small aliquots of ethanol were slowly added, with continuous stirring, until precipitation occurred. After removal of the precipitate on the centrifuge, the stepwise addition of ethanol was continued until no more polysaccharide remained in solution (ca. 60% ethanol). Precipitates were

washed with ethanol and dried in vacuo at 60°C. The recovery of amylose during this procedure was ca. 90% - the losses being entirely mechanical.

The solid amylose fractions obtained in the above manner were sometimes not readily soluble and they were more easily handled if their butanol - complexes were formed. This was achieved by one of two methods:

(i) The alcohol - washed precipitate (without further drying) was dispersed directly into boiling water (under nitrogen). Excess butanol was added and the amylose - butanol complex was formed on cooling. This was then redispersed and recrystallised with butanol to ensure complete removal of dimethylsulphoxide.

(ii) The dried solid sample was moistened with ethanol, and then dispersed in boiling water with vigorous stirring, under nitrogen, for ten minutes. Butanol was then added to the solution and the required amylose-butanol complex was obtained on cooling.

Preparation of acetyl-derivatives of amylose. (Higginbotham and Morrison, 1948.)

Some potato amylose sub-fractions were acetylated to form the tri-acetate. The amylose-butanol complex was dehydrated using dry butanol and suspended in dry pyridine (15 ml./g. amylose) with vigorous stirring. The suspension was then centrifuged and the precipitate washed twice with further portions of pyridine to displace the butanol. The precipitate was suspended in a mixture of pyridine and acetic anhydride (10 ml. of each/g. of amylose) and left at room

temperature for three days. The clear solution was poured into a mixture of ice and water and the product was washed with distilled water before being dried at low temperature.

Preparation of amylose solutions.

Solid amyloses were dissolved in the appropriate molarity of KOH at 2°C. by standing overnight at this temperature in a nitrogen atmosphere. The solutions were used directly, or, if examination in aqueous KCl was required, they were carefully neutralised with HCl to pH 7 using a pH-meter.

Butanol-amylose complexes were centrifuged and dissolved directly in either water or alkali.

In all cases, concentrations were determined by hydrolysis and estimation of the resultant glucose using the alkaline ferricyanide method (Lampitt, Fuller and Coton, 1955).

Characterisation of amylose samples.

a) Viscosity. Viscosity measurements were carried out using a modified Ubbelohde viscometer as described in the general experimental section. Measurements were made in potassium hydroxide (0.15M) at 25°C. unless otherwise stated.

b) Lightscattering. The instrument used was the model supplied by Phoenix Precision Instrument Co., Philadelphia which was described in the general experimental section.

Clarification of solvent and solutions. Nitroethane was fractionally distilled in an all glass apparatus directly into the lightscattering cell. Solutions were filtered through a grade 4 glass sinter directly into the lightscattering cell.

The results were evaluated by the method of Zimm (1948).

c) Sedimentation. A Spinco Model E Ultracentrifuge was used. Sedimentation coefficients were evaluated as described by Banks and Greenwood, 1963. When dilution-series were obtained, results were expressed in the form

$$S^{-1} = S_o^{-1} + K_S C$$

where S is the sedimentation coefficient at concentration C, S_o is the corresponding value at infinite dilution, and K_s is a constant.

Results and Discussion

Fractionation of dimethylsulphoxide-pretreated starch granules

In all fractionation procedures where potato starch granules are heated to temperatures greater than 65°C. the resulting amylose fractions are incompletely degraded by β-amylase. When methanol pretreated starch granules are leached at 60 - 65°C. amylose samples are obtained which have relatively small values of viscosity but they are completely hydrolysed by β-amylase (Banks et al., 1959a). The possibility has to be considered, therefore, that treating potato starch at temperatures greater than 65°C. modifies the amylose structure.

Killion and Foster (1960) claimed that they had quantitatively fractionated starch at 70°C. and had obtained an amylose sample which was completely hydrolysed by β-amylase. The experiment was repeated and the properties of the resultant amylose were compared to those of a sample obtained from a conventional dispersion without pretreatment. The results were as follows:

Treatment	Dispersion temperature	[η]	[β]	[β+Z]
None	100°C.	525	84	100
Dimethylsulphoxide	70°C.	500	84	101

Amylose samples with large values of [η] were obtained but the β-amylolysis limits were the same in both cases.

The method was examined, therefore, in more detail for two potato starches which, on conventional fractionation, had yielded amyloses with different values of [β] i.e. 80 and 85, respectively. Amyloses were isolated at various dispersion temperatures as shown in Fig. 3.01. It is to be noted that although Killion and Foster (1960) had reported that precipitation of a butanol complex was not possible unless the dispersion had been heated (in their experiments to 70°C.), the use of the "critical concentration of precipitant," introduced by Muetgeert (1961) enabled a complex to be formed at any temperature (see below). The properties of the various amyloses obtained from the two different samples of potato starch are shown in Table 3.01. It can be seen that all the amyloses have essentially the same β-amylolysis limits and limiting viscosity numbers.

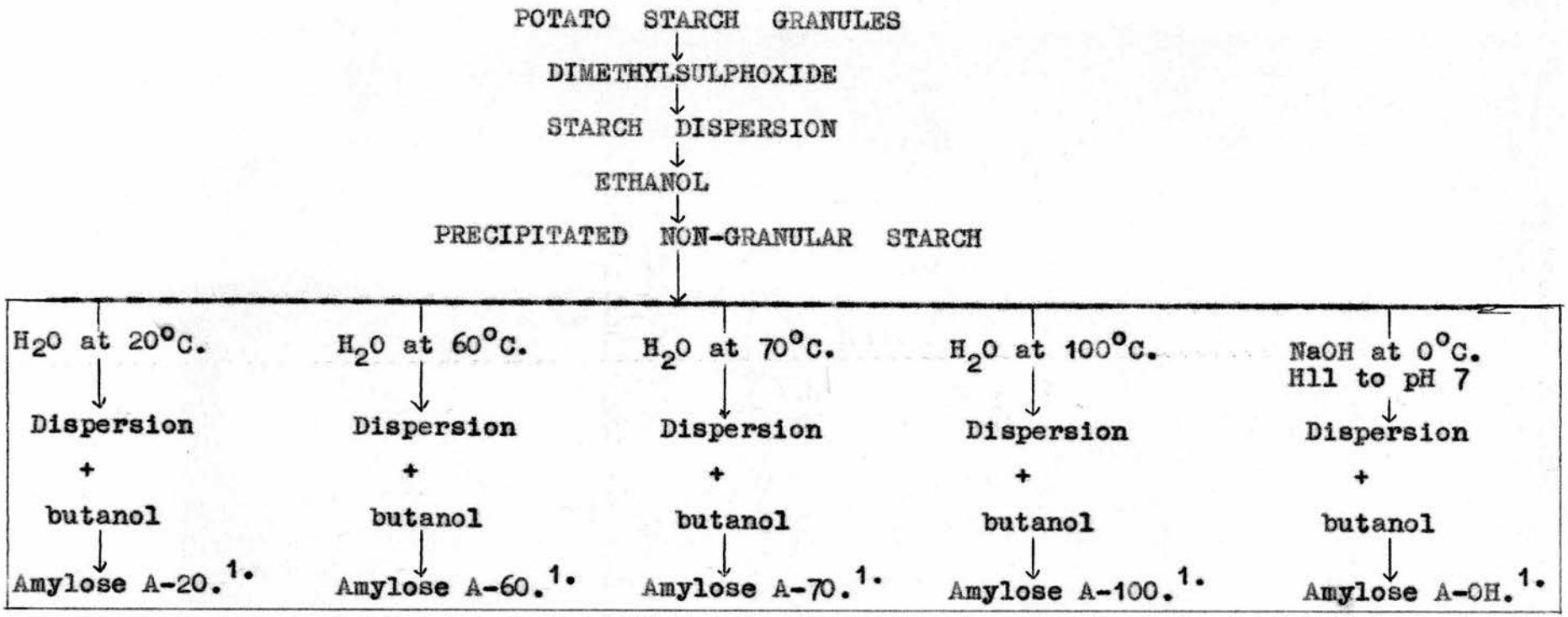


Fig. 3.01.

1. The corresponding amylopectins were obtained from the supernatant liquors after removal of the Amylose-complexes.

Table 3.01

Properties of amyloses obtained from potato starch granules treated with dimethylsulphoxide.

Fraction[1]	$[\beta]$	$[\beta+Z]$	$[\eta]$	% of amylose in[2] amylopectin
var. Redskin				
A-20	79	99	540	0.8
A-60	82	101	560	1.0
A-70	84	100	550	1.0
A-OH	82	99	540	0.8
A^3	80	100	550	0.8
var. Pentlard Crown				
A-20	85	99	620	0.8
A-60	85	99	690	1.0
A-70	85	102	640	0.3
A-100	86	101	630	0.5
A^3	88	100	630	0.8

1. The amyloses were obtained as in Fig. 3.01
2. From measurements of iodine affinity
3. Amylose from conventional dispersion at 100°C. in water; no pretreatment.

There is no evidence that the samples from the pretreated starches were of larger molecular size or contained a smaller number of structural anomalies. This method does not yield linear amylose and in view of the increased experimentation required for the dimethylsulphoxide pretreatment it offers no advantages over the conventional fractionation method for the preparation of amylose.

The "critical concentration" effect.

Bauer and Pacsu (1953) found that the addition of excess butanol to an alkaline dispersion, or a neutralised alkaline dispersion, of potato starch at about room temperature did not cause precipitation of the amylose complex; heating of the mixture to a temperature of at least 60°C. was necesaary before this occurred. Banks et al. (1959a) found a similar result with potato starch which had been pretreated with liquid ammonia.

This anomalous effect has now been explained by Muetgeert (1961) who found that when a precipitant was added to a starch dispersion at room temperature, saturation of the solution with the precipitant did not cause separation of the amylose complex, but for each precipitant there existed a critical concentration at which this occurred. This "critical concentration" is not constant for all starch systems and may depend on the characteristics and relative amounts of the components of a particular starch. The phenomenon has been confirmed and the following results show the effect of adding various concentrations of butanol to a 0.2% dispersion of liquid-ammonia pretreated potato starch in water at 20°C.

% Concentration of butanol (v/v)	2.45	3.60	4.75	5.90	7.00	Saturated
Amount of precipitate* after 48 hrs at 20°C.	-	+++	-	-	-	-

* - = negligible; +++ = large

Hence, using a concentration of butanol of 3.6% (v/v), quantitative isolation of amylose was achieved from a starch dispersion which had not been heated above 20°C. This critical concentration of butanol still works efficiently for dispersions at higher temperatures including 100°C.

The Subfractionation of amylose.

In the past, the successful subfractionation of native amylose into species of differing molecular weight has not proved to be easy. Lansky, Kooi and Schoch (1949) obtained some fractionation by the addition of n-octyl alcohol to an aqueous dispersion of the amylose, whilst Hollo and Szejtli (1958) utilised the preferential formation of an iodine-complex by larger amylose molecules. It has been shown, however, that degradation occurs when these procedures are used. Goodison and Higginbotham (1950) made use of the fact that the solubility of a butanol complex is dependent both on the molecular weight of the amylose and the temperature of the amylose solution. They achieved fractionation by slowly cooling an amylose solution saturated with butanol and removing the precipitate at a given temperature by centrifugation at that temperature. This method appeared to be successful but it was rather laborious. The method of Everett and Foster (1959),

however, was found to be relatively simple and remarkably successful.

Potato amylose was prepared by the conventional fractionation of starch dispersions at 100°C. using thymol followed by butanol as the precipitants. The amylose was dissolved in dimethylsulphoxide and subfractionated by the stepwise addition of ethanol at 4°C. Although precipitation occurred over a relatively narrow concentration-range of ethanol, subfractionation was fairly good. The results are shown in Table 3.02.

The efficiency of molecular weight fractionation is shown by the wide range in values of the limiting viscosity number, $[\eta]$. It is significant that the subfractions also varied in the extent to which they were degraded by β-amylase. Table 3.02 shows that the percentage conversion into maltose, $[\beta]$, for different subfractions varied from a lower limit of about 60 up to values for complete conversion of 100. These results substantiate the claim of Banks et al. (1959) that native amylose is heterogeneous - some molecules consisting of a linear chain of unmodified glucose-units, whilst others must contain some type of structural anomaly. In addition, the values of $[\eta]$ indicate that this anomaly is associated primarily with the high molecular-weight fractions, and the low molecular-weight samples are, essentially, linear. The incomplete enzymic degradation of the fractions is unlikely to be due to contaminating branched material because their conversion into maltose under the concurrent action of β-amylase and Z-enzyme, $(\beta+2)$, was complete.

Table 3.02.

Properties of subfractions of potato (var. Pentland Crown) amylose obtained by precipitation from dimethylsulphoxide solution.

Fraction[1,2]	$[\eta]$	$[\beta]$	$[\beta+Z]$	S_s[3].	S_f[3].
P1a.	1200	58	101	12.9	27
P1b.	700	66	101	-	-
P2.	800	65	100	11.0	24
P3.	525	72	99	-	-
P4.	480	76	99	-	-
P5.	410	82	101	-	-
P6.	300	95	99	8.6	21
P7.	160	100	100	5.0	-
P8.	90	100	100	4.0	-

1. a, b, represent refractionation products.
2. Fraction weights were not determined as the samples were immediately converted into their butanol-complexes.
3. The sedimentation coefficients were measured in potassium chloride (0.16M.).

The ultracentrifugation of amylose and its subfractions. (in collaboration with Mr. A. R. Procter).

The behaviour of amylose and its subfractions on ultracentrifugation in 0.16M. potassium chloride and 0.16M. potassium hydroxide was studied. Although the samples appeared to be homogeneous in the alkali some of the fractions were shown to be heterogeneous in salt solution. It has to be emphasised that this heterogeneity is not apparent when a total amylose is ultracentrifuged in saline solution because the molecular weight distribution is too broad.

Table 3.02 shows the apparent sedimentation coefficients for the amylose subfractions dissolved in 0.16M potassium chloride at a concentration of ca. 0.2g./100ml. It can be seen that each sample which is incompletely degraded by β-amylase, shows the presence of a minor fast-moving component with a sedimentation coefficient, S_f , somewhat greater than that for the main slower-moving component S_s.

Ultracentrifugal examination of a heterogeneous subfraction after β-amylolysis showed the absence of the major slow-moving component, but a peak was obtained which had a sedimentation coefficient intermediate between S_f and S_s. It would appear, therefore, that the structural anomaly responsible for incomplete β-amylolysis of amylose is present in the minor fast-moving component, which must correspond to the high molecular-weight material.

It is apparent from Table 302 that only two subfractions of amylose were obtained which could be completely degraded by β-amylase. These two subfractions contained only a small

proportion of the total amylose fractionated and so the yield of linear amylose was rather small. It would appear from the ultracentrifugal studies that the linear amylose is distributed among the other fractions and so the fractionation was not completely efficient.

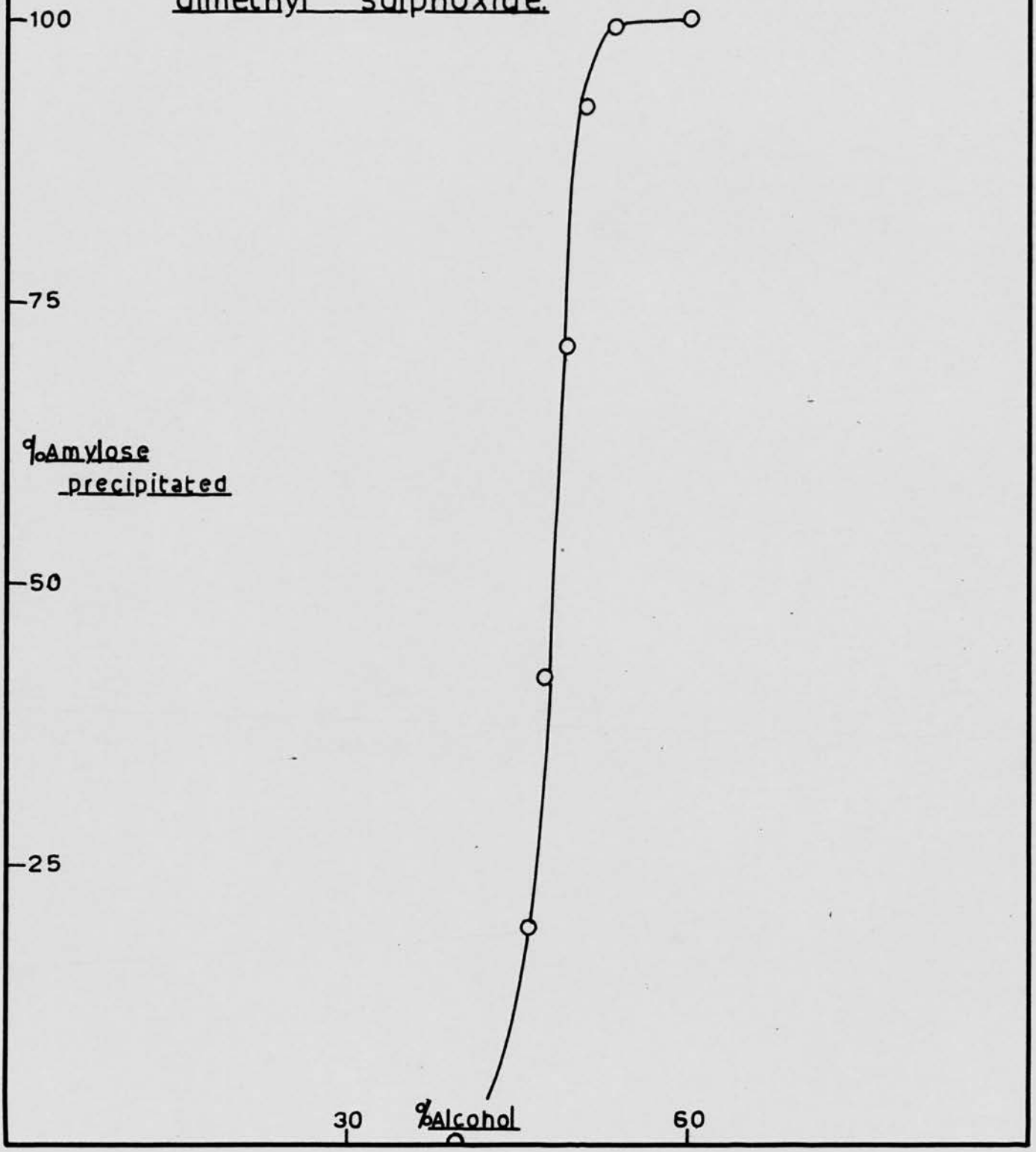
Subfractionation of amylose samples obtained from aqueous leaching of methanol-pretreated starch granules.

As large-scale leaching experiments were found to be neither convenient nor efficient, a large number of small-scale (one litre) extractions were carried out. Suspensions (0.5%) of methanol-pretreated potato starch were leached at 61°C. for ten minutes in a nitrogen atmosphere. The purified amylose-butanol complexes which were obtained from the above procedure were dehydrated with dry butanol and dried at 60 - 65°C. in vacuo. This would appear to be the most efficient method, so far studied, for obtaining reasonably large quantities of linear amylose. About 40% of the total amylose present in potato starch was extracted by the aqueous leaching technique and shown to be practically homogeneous. The dry amylose was dispersed in dimethylsulphoxide (0.5% concentration) and left to stand, at room temperature, for three days before being fractionated with ethanol. The fractionation curve is shown in Fig. 3.02.

Each fraction was washed free from dimethylsulphoxide, dried at 60 - 65°C. in vacuo and weighed. The fractions were then dispersed carefully in boiling deaerated water under nitrogen and their butanol complexes were formed by allowing the solutions to cool in an excess of butanol. The amylose-butanol

FIG. 3.02

Fractionation of amylose from dimethyl sulphoxide.



complexes were characterised by the following procedures:

i) viscosity measurements in 0.15M.KOH and 0.33M.KCl

ii) the extents of degradation by β-amylase, [β] and the concurrent action of β-amylase and enzyme.

The results are shown in Table 3.03.

It is evident from the spread of the limiting viscosity values of the fractions that effective fractionation of the amylose sample has been achieved. Since the β-limit of the "parent" amylose was high it is not surprising that fractions having low β-limits were not obtained. The viscosities of the fractions in alkali are obviously higher than the viscosities in saline. This illustrates the well-known phenomenon that salt solutions are poor solvents for amylose (Everett and Foster, 1959).

The efficiency of the fractionation is clearly shown by comparing the properties of the "parent" amylose with those of the summative values of the fractions.

	$[\eta]_{KOH}$	$[\eta]_{KCl}$	β-limit
Experimental Value	260	120	96
Calculated Value	270	121	98

There is reasonably good agreement between the calculated and the experimental values, showing that fractionation has been achieved without degradation occurring.

<u>The effect of starch-pretreatments on the properties of the amylose component</u>.

Some starches - of which those from cereals are the most common - are extremely difficult to disperse directly into

Table 3.03

Properties of amylose subfractions obtained by dimethylsulphoxide-ethanol subfractionation of aqueous leached potato amylose.

Fraction	% Alcohol	% Amylose pptd.[1.]	$[\eta]_{KOH}$	$[\eta]_{KCl}$	β-limit	β+Z-limits
1	45.8	19.4	470	195	90	100
2+3	47.0	22.3	365	158	98	100
4	48.1	23.5	240	110	98	102
5	49.0	6.6	175	90	100	99
6	49.4	5.6	165	80	101	99
7	51.0	15.1	130	60	98	101
8	54.1	7.5	75	40	99	100

1. The recovery of amylose during the fractionation was ca. 90%; the losses being entirely mechanical. The weight of each fraction was, therefore, corrected for this loss before the fraction-percentages were calculated.

aqueous solution. Pretreatment of the granule is often necessary for effective dispersion but care must be taken to ensure that the properties of the starch components are not modified. Because potato starch can be dispersed quite readily in boiling water, it provides a useful standard by which the effect of various pretreatments may be examined. Samples of potato starch were, therefore, pretreated with alkali, liquid ammonia and dimethylsulphoxide. They were all dispersed in boiling water under nitrogen and the amylose components were separated in the usual manner using thymol, followed by butanol, as the precipitants. The results are shown in Table 3.04. Although the β-amylolysis limit is independent of the pretreatment, the molecular size of the amylose from a liquid ammonia pretreated starch tends to be slightly higher than the others. Earlier results also show this trend (Banks et al, 1959). Table 3.04 also shows that amylose may be satisfactorily stored in the form of the butanol complex for a long period without degradation occurring. This would appear to be the best method for keeping amylose, since the solubility of dehydrated amylose certainly decreases with length of storage.

The apparent molecular size of amylose.

Viscosity measurements are the most convenient way of determining the relative sizes of amylose samples. Values of the limiting viscosity number, $[\eta]$, vary with plant maturity and also with the source of the amylose (Greenwood and Thomson, 1962). Random selections of potato tubers examined in these laboratories, however, have consistently yielded amyloses

Table 3.04.

Properties of amyloses obtained by precipitation from aqueous dispersions of potato starch, var. Pentland Crown (at 100°C.) after various pretreatments of the granules.

Method of pretreatment of the granules	$[\beta]$	$[\beta+Z]$	$[\eta]$	% of amylose in the corresponding amylopectin
None	88	100	630	0.4
Liquid ammonia	86	100	730	0.4
KoH/HCl at 0°C.	86	101	625	0.5
Liquid ammonia[1]	87	100	720	0.4
None[1]	88	100	610	0.4

1. Re-examination of the samples after storage of the butanol-complexes at room temperature for six months.

having values of $[\eta]$ of 400 - 500. Amylose samples having even higher values of $[\eta]$ have been obtained from a new variety of potato "Pentland Crown".

High values of $[\eta]$ have been criticised by Muetgeert (1961). He suggested that they may be caused by the presence of molecular aggregates of amylose and amylopectin which had not been completely dispersed during the fractionation process. This postulate seems very unlikely since, in the present work, all amylose samples have been recrystallised at least three times from dilute solution and values of $[\eta]$ as high as 1200 have been obtained (Table 3.02). Since these samples are completely degraded by a mixture of β-amylase and Z-enzyme they cannot contain a significant amount of amylopectin. Further, since amylopectin molecules are more compact than those of amylose, amylopectin impurity would tend to lower the viscosities of amylose solutions.

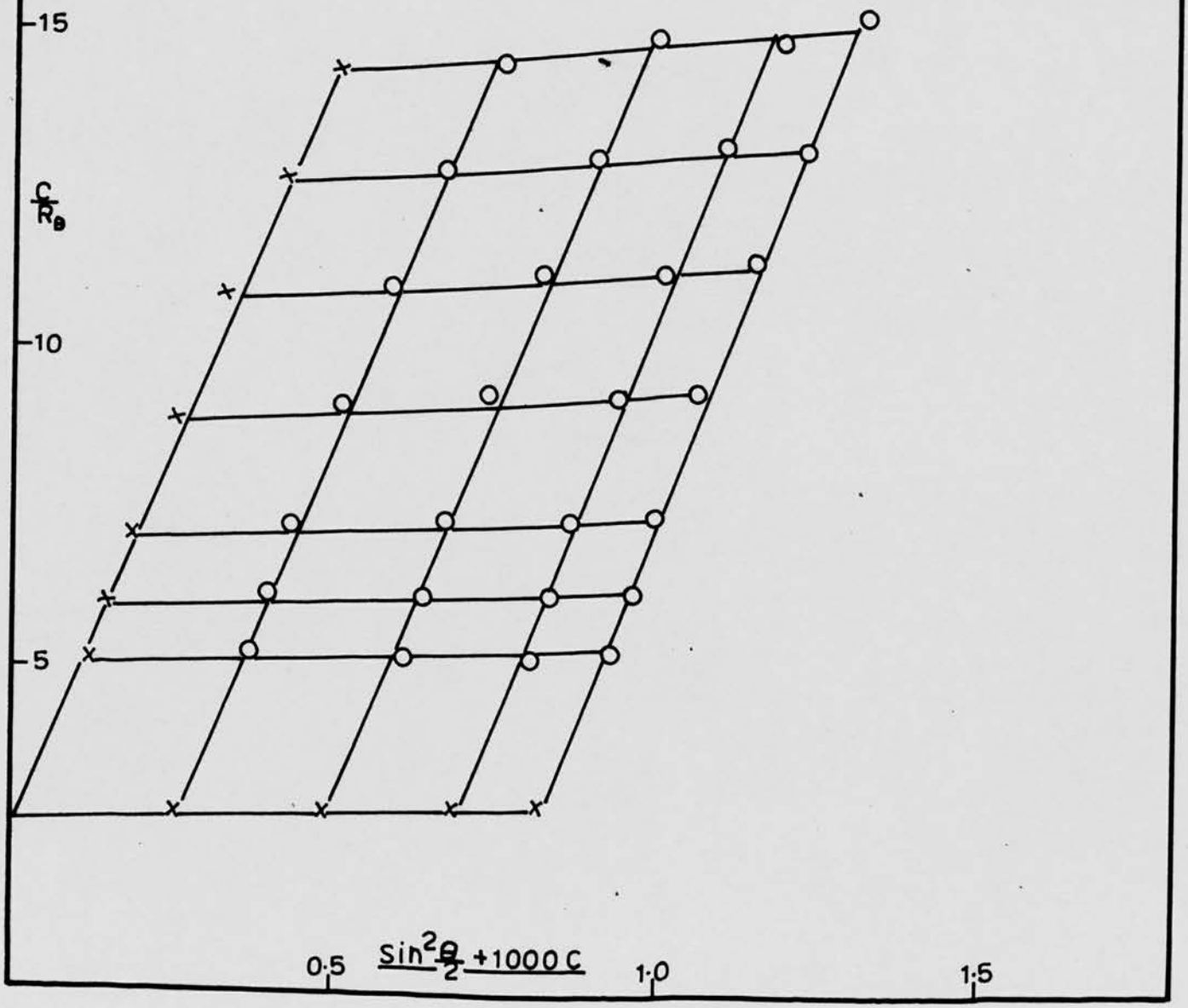
It is rather more difficult to show that the high values of $[\eta]$ are not due to molecular aggregates of amylose. For this purpose some potato amylose subfractions were converted to the triacetate form and lightscattering measurements were carried out. The solvent used was nitroethane and the results were evaluated by the method of Zimm (1948). The molecular weights of the fractions with the corresponding values of $[\eta]$ in potassium hydroxide (0.2M.) are shown below.

Fraction	$[\eta]_{KOH}$	$10^{-6}\bar{M}_w$
1	500	20.6
2	315	12.8
3	230	3.3
4	75	1.4

FIG. 3.03

M.Wt. of amylose acetate fraction 3 = 6.9×10^6

$\therefore$ M.Wt. of original amylose = 3.3×10^6



The Zimm plot for fraction 3 is shown in Fig.3.03. It is apparent that these amylose samples do have large molecular weights.

Examination of pretreated starches.

The granular structure of potato-starch was disrupted by the use of dimethylsulphoxide and liquid ammonia. The iodine-binding power of these pretreated starch samples was shown to be the same as that of the original starch (see below). Solutions of the starches were prepared in water at room temperature, and the extent to which they were hydrolysed, by β -amylase and by the concurrent action of β -amylase and Z-enzyme, was determined The untreated starch was dissolved in alkali at 2°C. and the solution was neutralised to PH4.8 before the addition of buffer and the appropriate enzyme system.

The results are shown below.

Starch pretreatment	Iodine Affinity	β -limit	β+Z -limit
None	4.25	64	70
Dimethylsulphoxide	4.25	64	69
Liquid ammonia	4.20	65	71

Although these results indicate that no profound change in the structure of the starch samples has taken place, as a result of the pretreatments, these techniques would not be sensitive enough to shew minor changes in the components.

As already indicated, Table 3.01, a normal fractionation of amylose can be made from these cold water dispersions if the critical concentration of precipitant is used. Amylose,

with similar properties, was obtained from a neutralised alkaline dispersion of dimethylsulphoxide treated starch.

The Ultracentrifugation of starch dispersions.

Few workers have examined starch dispersions in the ultracentrifuge. Senti et al. (1960) investigated the behaviour of aqueous dispersions of maize starch, and Erlander and French (1958) studied waxy maize starch in aqueous lithium bromide. Killion and Foster (1960) examined dispersions of potato starch in dimethylsulphoxide.

Potato starch, which had been pretreated with either dimethylsulphoxide or liquid ammonia, was dispersed directly into dimethylsulphoxide, potassium hydroxide (0.2M.) and water with gentle agitation at room temperature. The resultant dispersions were then examined in the ultracentrifuge. In all cases, the presence of two components was observed. Apparent sedimentation coefficients - S_f, for the faster-moving, and S_s, for the slower-moving component - are given below for 0.4% dispersions.

Pretreatment	Solvent	$10^{13}\ \underline{S}_f$	$10^{13}\ \underline{S}_s$
Dimethylsulphoxide	Dimethylsulphoxide	9.3	2.4
Dimethylsulphoxide	0.2M. potassium hydroxide	73	8.2
Liquid ammonia	Water	80	20

These values of $\underline{S}_f$ and $\underline{S}_s$ are similar to those which have been found for the separate amylopectin and amylose

components, respectively (Cowie and Greenwood, 1957).

Because of its large molecular weight the amylopectin component sediments very rapidly and care must be taken to ensure that there is no insoluble material present in these solutions. There was, however, little evidence of rapidly sedimenting material during acceleration of the rotor to full speed. These experiments in alkali confirm the results of Banks, Greenwood and Thomson (1959) who presented evidence that complete solution of starch granules was achieved in sodium hydroxide (0.5M.). It is not surprising, therefore, that the fractionation of potato starch by treating it with alkali (Baum and Gilbert, 1956) is not very satisfactory.

Although the ultracentrifugation of starch dispersions is complicated, the results for these pretreated starches show unambiguously that there are two separate components present when neither the starch granule nor its dispersion has been heated above 20°C.

3.b. Preparation of Substrates other than amylose.

As already indicated, linear amylose is a useful substrate in a study of the action patterns of carbohydrase enzymes. However, for a comprehensive investigation of these enzymes further substrates were required.

Preparation of amylopectin. It was prepared by the conventional dispersion method outlined in the section on general techniques.

Preparation of glycogen. (Sahyun and Alsberg, 1930). The liver from a freshly killed rabbit was treated with trichloracetic acid (10%) at 0-2°C. in a high-speed atomix blendor for 2 - 3 minutes. The resulting suspension was filtered through muslin and the glycogen was immediately precipitated with ice-cold ethanol (1½ volumes). After a few minutes the precipitate was obtained by centrifugation at 0-2°C. It was dissolved in cold water and re-precipitated with ethanol. This procedure was repeated until the addition of a small amount of an electrolyte (usually sodium chloride) was required to induce flocculation of the precipitate. The glycogen was washed with ethanol and finally dried with ether.

Preparation of β-amylase limit dextrins. The polysaccharide (100 mg.) was dissolved in water (90 ml.) and acetate buffer (10 ml; 0.2M; pH 4.8) was added. Sufficient β-amylase was added to ensure that the enzyme action was complete within twelve hours. The digest was incubated at 35°C. for 24 hours when it was heated in a boiling water-bath for ten minutes and filtered. The polysaccharide was precipitated with an excess of ethanol, dissolved in water and subjected to further enzyme

action. After a further period of 24 hours the dextrin was isolated by precipitation with excess ethanol, dialysed against running water for 48 hours, and freeze-dried.

Preparation of Maltodextrins. They were prepared by hydrolysing solutions of amylose with crude salivary α-amylase. Dilute solutions were employed to prevent precipitation of the amylose. When the digests gave no colour with iodine they were heated on a boiling water-bath for five minutes, filtered and evaporated to small volume on a rotary evaporator. The syrup was streaked on to 3mm. chromatographic paper and eluted with n-propanol: ethyl acetate: water (14: 2: 7) (Commerford et al., 1963). Strips were cut from each side of the chromatogram and developed by the method of Trevelyan et al. (1950). The oligosaccharides were identified by running a set of standards on the same chromatogram - this portion of the chromatogram was also cut out and developed. The remaining part of the chromatogram was then cut into sections - each section corresponding to an oligosaccharide - which were separately eluted with water. The resulting sugar solutions were concentrated before being re-run in the same system. This procedure was continued until the maltodextrins were chromatographically pure.

SECTION 4.

STUDIES ON PLANT α-AMYLASES

4.a. **Z-ENZYME**

INTRODUCTION

Early workers in the field of starch chemistry (Hanes, 1936; Ford and Peat, 1941; Haworth, Kitchen and Peat, 1943) used crude extracts of ungerminated grain of wheat or barley as a source of β-amylase. However, such preparations were often contaminated with α-amylase. It was later found to be more satisfactory to use soya bean as the source of the crude enzyme since this cereal did not appear to contain a detectable amount of α-amylase (Bourne et al, 1945). In 1949 Peat, Pirt and Whelan showed that, whereas this crude soya bean extract completely hydrolysed amylose, crystalline sweet potato β-amylase hydrolysed only 70% of the amylose into maltose. They concluded, therefore, that there was present in soya beans another factor - which they named Z-enzyme - which rendered amylose completely accessible to degradation by β-amylase.

Peat et al. (1950) compared the actions of Z-enzyme and dilute salivary α-amylase on amylose and amylopectin. The α-amylase caused a large fall in the amylopectin-iodine absorption value with a corresponding increase in reducing power, but the Z-enzyme had no apparent effect. A mixture of β-amylase and Z-enzyme acting on amylose gave a higher conversion into maltose than did a corresponding β-amylase and salivary α-amylase mixture. Later, Peat et al, (1952) partially purified Z-enzyme and showed that it was not a phosphatase; that it hydrolysed laminarin, cellobiose, gentiobiose and crown gall polysaccharide, all of which are β-linked glucose polymers. Amylose

was completely hydrolysed to maltose when it was incubated with a mixture of emulsin (a crude mixture of enzymes containing β-glucosidase) and β-amylase. From these results the authors concluded that amylose contained side-chains, consisting of single glucose residues joined to the main chain through β-glucosidic linkages. These glucose residues constituted the barrier to β-amylase and they were preferentially removed by Z-enzyme.

When Hopkins and Bird (1953) examined the products from the initial attack of a mixture of Z-enzyme and β-amylase on amylose β-limit dextrin they could not detect any glucose. Hence, they postulated that Z-enzyme was merely a weak α-amylase. They also showed that active β-glucosidases in emulsin did not liberate glucose from amylose β-limit-dextrin so that Z-enzyme was distinct from β-glucosidase. In their comments on this communication, Peat and Whelan (1953) emphasised that they could distinguish Z-enzyme from α-amylase by pretreating an amylose with Z-enzyme or weak salivary α-amylase before β-amylase action. The β-amylolysis limits were 98% and 89% for Z-treated and α-treated amyloses, respectively. The Z-enzyme appeared, therefore, to have exerted a true debranching action and allowed complete β-amylolysis, whereas the α-amylase had fragmented the amylose molecules in random fashion to leave intact the barriers to β-amylase. These authors modified their concept of amylose to explain the results of Hopkins and Bird (1953) and suggested that the amylose side-chains could contain more than one glucose residue.

The theory that Z-enzyme had the specificity of a

β-glucosidase was finally shown to be incorrect by the following experiments: (1) Neufeld and Hassid (1955) showed that the Z-enzyme activity in emulsin could be destroyed without removing the β-glucosidase activity; (2) Baba and Kojima (1955) found that a mixture of purified apricot β-glucosidase and crystalline β-amylase hydrolysed sweet potato amylose to 73% conversion into maltose. This was the conversion achieved by pure β-amylase, acting on the amylose; (3) Baba (1959) further showed that the emulsin used by Peat et al. (1952) could be fractionated, by heating in the presence of calcium, to yield an enzyme which possessed only amylolytic activity.

Banks et al. (1960) showed conclusively that Z-enzyme exerted a random hydrolytic attack on amylose by following the rapid fall in viscosity of amylose - Z-enzyme digests. Since this enzyme also attacked linear amylose (i.e. amylose which is completely degraded to maltose by pure β-amylase) the postulate that Z-enzyme specifically removed the barrier to β-amylase was proved to be incorrect. These authors further showed that the enzyme attacked amylopectin and its β-limit dextrin, but, under their experimental conditions they found no evidence to show that the enzyme also attacked glycogen and its β-limit dextrin. Their results also indicated that the enzyme was inactive below PH3.6.

Cunningham et al. (1960) confirmed these findings and later, (1962), suggested that the enzyme, if present in a sufficiently high concentration, might also attack glycogen and glycogen β-limit dextrin. They also showed that the α-amylolytic activity was decreased when calcium was removed

from the enzyme preparations by chelating agents such as ethylenediaminetetraacetate (E.D.T.A.).

Since most of the published work on Z-enzyme has been carried out with crude, weakly active preparations, an investigation was undertaken to attempt to prepare pure Z-enzyme and to examine its properties in more detail.

EXPERIMENTAL

Initial attempts to prepare Z-enzyme from soya bean. A crude sample of soya bean Z-enzyme, containing β-amylase impurity was obtained by a modification of the procedure of Bourne et al. (1945). Dry, defatted soya bean flour (200 g.) was extracted with aqueous ethanol (800 ml; 20%) at room temperature for 12 hours. The resulting suspension was centrifuged, passed through muslin and the alcohol concentration was raised to 30%. After 15 minutes stirring the precipitate was removed on the centrifuge and discarded. Further precipitates, 2 and 3, which were obtained at 50 and 70% of alcohol, respectively, were separated by centrifugation and dried with ethanol. The β-amylase and Z-enzyme activities of these fractions were determined as described later and the results are shown in Table 4.01.

Table 4.01

Protein sample	Z-enzyme specific activity	β-amylase specific activity
Original solution	5.3	10
Fraction 2	1.7	17
Fraction 3	8.2	57

The Z-enzyme activities are expressed in I.D.C.* units /mg. of

protein; the β-amylase activities are expressed in mg. of maltose produced/mg. of protein (see p. 83). * I.D.C. = iodine-dextrin colour.

The effect of mercuric chloride on the enzyme fraction 3 was investigated. Digests were prepared in the following manner:

1. Enzyme fraction 3(5mg.) + acetate buffer (2ml.; 0.2M.; pH 3.6) + mercuric chloride (8 ml.; 1.9×10^{-6}M.)
2. Enzyme fraction 3(5mg.) + acetate buffer (2ml.; 0.2M.; pH 4.6) + mercuric chloride (8 ml.; 1.9×10^{-6}M.)

These were incubated at room temperature for 2 hours before being added to amylose solutions containing the appropriate buffer and concentration of mercuric chloride. These solutions were incubated at 35°C. for 24 hours when the amyloses were precipitated by the addition of excess butanol. The viscosities of the amylose samples were determined in potassium hydroxide (0.2M.) at 25°C. The results are shown in Table 4.02

Table 4.02

	Original amylose	Amylose from digest 1	Amylose from digest 2
Viscosity	540	550	205

These results indicate that mercuric chloride (1.5×10^{-6}M.) does inhibit the β-amylase activity without completely inhibiting the Z-enzyme activity. This inhibition technique is not very satisfactory, however, since its effect on the Z-enzyme activity is difficult to determine.

In an attempt to obtain a fraction of Z-enzyme free from β-amylase, fraction 3 was subfractionated with solid ammonium sulphate in the following manner.

A portion of fraction 3(400mg.) was suspended in water (35ml.) and carefully stirred for 20 minutes at room temperature before being centrifuged (20,000 r.p.m.; 15 minutes), yielding a clear brown solution which was cooled to 2°C. Solid ammonium sulphate was slowly added with stirring and fractions were removed on a refrigerated centrifuge at 20, 30 and 60% saturation with ammonium sulphate to give fractions 3A, 3B and 3C, respectively. These precipitates were stored as suspensions in saturated ammonium sulphate solution at 2°C. Aliquots (1ml.) of the suspensions were centrifuged and the resulting solids were suspended in water (5ml.) to give clear solutions which were used to determine the Z-enzyme and β-amylase activities of the fractions. The results are shown in Table 4.03.

Table 4.03

	Fraction 3A	Fraction 3B	Fraction 3C
β-amylase specific activity	67	75	180
Z-enzyme specific activity	8.2	11.3	23.1

The effect of heat on these enzymes was investigated in an attempt to separate their activities. A portion of fraction 3(550mg.) was suspended in calcium acetate solution (100ml.; 0.2%) for 30 minutes and carefully stirred. The suspension was centrifuged (20,000 r.p.m.; 15 minutes) to

yield clear solution 3D which was heated at 70°C. for 15 minutes and the resulting cloudy suspension was quickly cooled to room temperature. It was centrifuged to yield clear solution 3E which was further cooled to 2°C. and saturated with ammonium sulphate. After 24 hours a portion (5ml.) of the suspension was centrifuged and the hard-packed precipitate dissolved in water (2ml.) to give solution 3F. Portions of these solutions (3D, 3E, 3F) were tested for Z-enzyme and β-amylase activities. The results are shown in Table 4.04.

Table 4.04

	Original	3D	3E	3F
Z-enzyme specific activity	8.2	8.0	40.2	40
β-amylase specific activity	57	55	0	0

Final Preparation of Z-enzyme from soya beans. Dry, defatted finely ground soya bean flour (300g.) was shaken with calcium chloride solution (0.2%; 1500ml.) for 4 hours. The resulting suspension was centrifuged and filtered to yield a clear solution (35mg. protein/ml.) which was cooled to 2°C. Sufficient acetone at -15°C. was added to make its concentration in the solution 10% (v/v). The temperature of the solution was then lowered to -5°C. and protein fractions, precipitated at acetone concentrations of 0-20, 20-30, 30-35, 35-40, 40-45, 45-50, 50-55 and 55-60% (v/v), were collected, partially air-dried to remove excess acetone and suspended in cold water. The suspensions were centrifuged to yield clear solutions. During fractionation the chilled acetone was added slowly with continuous stirr-

ing to the protein solution and each precipitate was left for 15 minutes before being separated by centrifugation. The Z-enzyme and β-amylase activities of the fractions were determined and are shown in Table 4.05.

Table 4.05

Acetone concentration % (v/v)	0	20	30	35	40	45	50	55	60
Fraction number	S0	S1	S2	S3	S4	S5	S6	S7	S8
Z-enzyme specific activity[+]	6.4	5.0	5.3	7.8	15.4	20.0	35.0	36.0	4.6
β-amylase specific activity[x]	7.6	6.7	7.0	8.5	13.0	12.0	12.0	9.0	5.0

[+]expressed in I.D.C. units/mg. of protein; [x]expressed in mg. of maltose produced/mg. of protein (see p. 83.)

To portions of fraction S7 (10 ml.) were added calcium acetate (20 mg.) and acetate buffer (1ml.; pH4.8; 0.2M.). The solutions were heated (70°C.; 20 minutes), cooled, centrifuged and combined. Appropriate dilutions of the clear solution were tested for protein content, Z-enzyme and β-amylase activities. The results, Table 4.06, indicated that the specific Z-enzyme activity had been enhanced but the β-amylase activity had been reduced to a negligible value.

Table 4.06

	Specific Activities	
	Z-enzyme	β-amylase
Fraction S7 before heating	36	9
Fraction S7 after heating	51	0

The heated enzyme fraction S7 was subfractionated with chilled acetone at -5°C. between the limits 0-35, 35-42, 42-47, 47-52 and 52-57%. The enzyme activities are shown in Table 4.07.

Table 4.07

Acetone concentration %(v/v)	0	35	42	47	52	57
Fraction number	S7	$S7_1$	$S7_2$	$S7_3$	$S7_4$	$S7_5$
Z-enzyme specific activity	51	6.8	33.0	90.0	450	91.2

Subfraction $S7_4$ was further purified, via glycogen-complex formation (Loyter and Schramm, 1962), in the following way:- The enzyme solution (5.3ml.) was cooled to 3°C. and chilled ethanol (3.5ml.) was slowly added with stirring. After 15 minutes the precipitate was centrifuged off and dissolved in cold water-solution (1). To the clear solution, obtained on centrifugation, were added phosphate buffer (0.25ml.; pH8.0; 0.2M.), glycogen solution (0.1ml.; 2%) and cold ethanol (0.23ml.). The suspension was stirred for 10 minutes and then

centrifuged, yielding a small precipitate and a clear solution (3). The precipitate was suspended in phosphate buffer (0.02M.; pH6.7) - solution (2). The three solutions were assayed for activity and protein content - the results are shown in Table 4.08.

Table 4.08

Solution number	1	2	3
Fraction number	$S7_4 1$	$S7_4 2$	$S7_4 3$
Z-enzyme specific activity	69	790	12

The enzyme fraction $S7_4 2$ was maintained at 35°C. for six hours (to aid digestion of the contaminating glycogen), cooled to 2°C. and precipitated with excess, chilled acetone. The precipitate was dissolved in cold distilled water and stored, under toluene, at 2°C. It was found to retain its activity, at this low temperature, for a few weeks.

Z-enzyme activity. A modification of the procedure devised by Briggs (1961) for the determination of α-amylase activities was employed. The decrease in the capacity of amylopectin β-limit dextrin solutions to yield red colours with iodine after digestion with Z-enzyme, was used as a measure of Z-enzyme activity. (see p. 95). The times taken for the iodine absorption values (A.V.), of various α-amylase/amylopectin β-limit dextrin digests, to fall by a fixed amount were noted. The reciprocals of these times were proportional to the α-amy-

lase concentrations in the digests. This enabled a standard graph to be constructed from which all Z-enzyme activities were calculated.

Digests were prepared containing amylopectin β-limit dextrin and various concentrations of salivary α-amylase (see later). Aliquots were removed from these digests at appropriate intervals and stained with iodine. The A.V. of the β-limit dextrin control was set equal to 3.00 "corrected colorimeter units" and the dextrin-iodine colours of the digests were correspondingly adjusted by multiplying them by the factor 3.00/ observed β-limit dextrin-iodine control colour. The "time reference point" is the time taken for the colour of the dextrin-iodine complex to reach a corrected value of 2.00. A "relative time" scale was established in which the α-amylolysis commenced at zero time and 100 denoted the "time reference point". The observed times were then converted into "relative time" intervals by multiplying them by 100/actual time taken to reach the "time reference point". The standard graph was then constructed by plotting the corrected dextrin-iodine A.Vs. on a logarithmic scale and the corresponding "relative times" on a linear scale Fig. 4.01.

Unless otherwise stated α-amylase and Z-enzyme digests were prepared in the following way. They contained acetate buffer (1ml.; 0.2M.; pH5.5), amylopectin β-limit dextrin (2ml.; ca.5-7mg./ml.) and a suitable amount of enzyme in a total volume of 8ml. Before the addition of the enzyme samples the digests were preincubated at 35°C. and they were

maintained at this temperature throughout the digestion period. Aliquots (2ml.) were removed at intervals and stained with iodine. The dextrin-iodine A.Vs. were converted to "corrected colorimeter units" and the corresponding "relative times" were obtained from the standard graph. Enzymic activities were calculated directly from ("relative time"/time of taking the sample) and were expressed in I.D.C. units (iodine-dextrin colour). The specific activity of an enzyme sample was obtained by dividing the enzyme activity by the corresponding protein concentration (mg. of protein/ml.) in the digest. The average value of three separate determinations per digest was found to be satisfactory.

Example of the calculation of Z-enzyme activity. Two typical activity digests were prepared and incubated at 35°C. To one was added an appropriate amount of Z-enzyme solution and aliquots (2ml.) were removed at intervals for iodine staining measurements. An equal volume of water was added to the other digest (control digest) and its iodine stain was also determined. The results are shown in Table 4.09.

Table 4.09

Sample No	Time of taking sample	Colorimeter reading	Corrected colorimeter reading	Relative time (from graph)	α-amylase activity
Control digest	-	3.20	3.00	-	-
1	15 minutes	2.60	2.44	50	3.34
2	30 minutes	2.10	1.97	101	3.37
3	60 minutes	1.36	1.27	202	3.37
				Average =	3.36

Protein concentration in the digest = 0.02mg./ml.

∴ Z-enzyme specific activity = 3.36/0.02 = 168 I.D.C. units/ mg. of protein.

The A.V. of the control digest was set equal to 3.00 and the values for the samples from the enzymic digest were correspondingly adjusted to yield the "corrected colorimeter readings". Using these and the standard graph the appropriate "relative times" were determined. The enzymic activity for each sample was obtained directly from "relative time"/ time of taking sample. The specific activity of the enzyme was obtained by dividing the activity by the concentration of enzyme in the digest and is expressed in I.D.C. units/mg. of protein.

β-amylase activity. Digests contained starch solution (0.6%; 25ml.), acetate buffer (4ml; 0.2M.; pH3.6) and sufficient enzyme in an aliquot (1ml.) to produce 2 - 3mg./ml. of maltose in the digest under the experimental conditions. The digests were incubated at 35°C. for 30 minutes when aliquots (1ml.) were removed and their maltose content determined by the alkaline ferricyanide method. The β-amylase activities were expressed as mg. of maltose produced/mg. of protein/ml. in the digest.

Concentration of polysaccharide solutions. The concentration and reducing power of polysaccharide solutions were determined by the routine procedures outlined in the general experimental section

Concentration of protein solutions. The routine determination of the protein content of clear solutions was carried out on an SP500 ultraviolet and visible spectrophotometer at 280 mμ. Micro Kjeldahl protein estimations were also carried out on some

of the enzyme solutions and a quantitative relation was obtained between these results and those from the absorption values at 280 m μ . It was thus possible to convert an absorption value at 280 m μ into a quantitative estimation of the amount of protein in a particular solution.

Iodine reagent. A solution containing potassium iodide (2%) and iodine (0.2%) was prepared in water.

Determination of amylopectin β -limit dextrin/iodine absorption values. Aliquots (2ml.) were removed from dextrin-enzyme digests and added to the standard iodine solution (1ml.) containing hydrochloric acid (0.2ml.; 5N.) in a total volume of 50ml. The colour intensity was measured on an "EEL" photo-electric colorimeter using yellow-green filter No.625 which has its maximum absorption at 540 m μ . The instrument was initially set to zero with a blank consisting of water, iodine and hydrochloric acid.

Soluble starch solution. Soluble starch (Analar, B.D.H.; 0.6g) was suspended in distilled water (10-15ml.) and added, in the form of a thin slurry, to boiling distilled water (50ml.). The suspension was boiled for 5 minutes, cooled to room temperature, filtered and made up to 100ml. These solutions were always freshly prepared before use.

Enzyme substrates. These were prepared as outlined in the general experimental section.

Paper chromatography. Solutions of pure sugars were applied directly to Whatman No.1 chromatographic paper. Portions withdrawn from enzymic digests, however, were treated in the

following way before being subjected to chromatography. They were boiled for 5 minutes to stop the enzymic reaction and to coagulate the protein, cooled and centrifuged. The clear solutions were shaken with Bio-Deminrolit mixed-bed resin in the carbonate form (Woolf, 1953) to remove any inorganic ions present, and were then concentrated to small volume, in a rotary evaporator, before being applied to the chromatograms. The solvents and developing agents used are described in the general experimental section.

Viscosity. Viscosity measurements were carried out in modified Ubbelohde viscometers as described in the general experimental section.

R E S U L T S

Purity of enzyme fraction $S7_4 2$. Aliquots of the enzyme solution (0.1ml.) were added to samples (1mg.; 0.1ml.) of maltose, maltotriose, cellobiose and laminarin. These micro-digests were incubated at 35°C. for 48 hours when portions were removed for chromatographic examination. No glucose was detected in the maltose, cellobiose or laminarin digests indicating that this Z-enzyme fraction is free from maltase, cellobiase and laminarinase. After prolonged digestion (72 hours), small amounts of glucose and maltose were detected in the maltotriose digest indicating that, either a trace of β-amylase was present in the enzyme fraction or these sugars were being produced by the very slow action of Z-enzyme itself on maltotriose. The absence of higher sugars in the maltotriose digest indicated that the enzyme fraction was not contaminated with D-enzyme.

Removal of β-amylase activity from Z-enzyme preparations. Two portions of soya bean Z-enzyme fraction S5 at pH6 were heated in a water-bath at 70°C. - one contained calcium acetate (2mg./ml.) and the other contained no added calcium. Aliquots were removed from these solutions at appropriate intervals, quickly cooled and centrifuged to yield clear solutions. The protein contents, Z-enzyme and β-amylase activities of these solutions were determined. The enzymic activities were calculated as a percentage of the activities present in the original, unheated enzyme solution. The results are shown in Fig. 4.02. Curves (1) and (2) indicate the effect of prolonged heating at 70°C. in

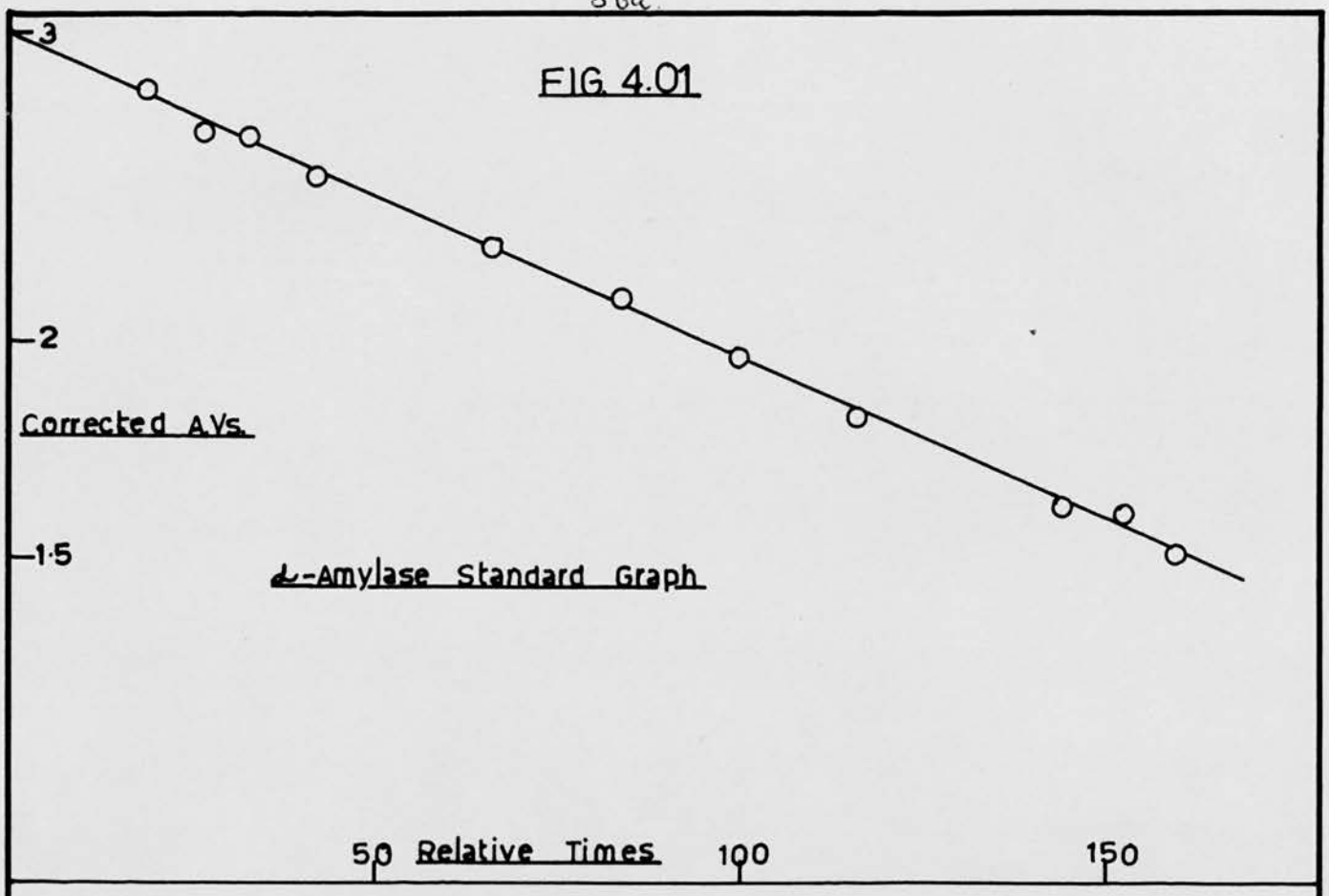


FIG. 4.01

α-Amylase Standard Graph

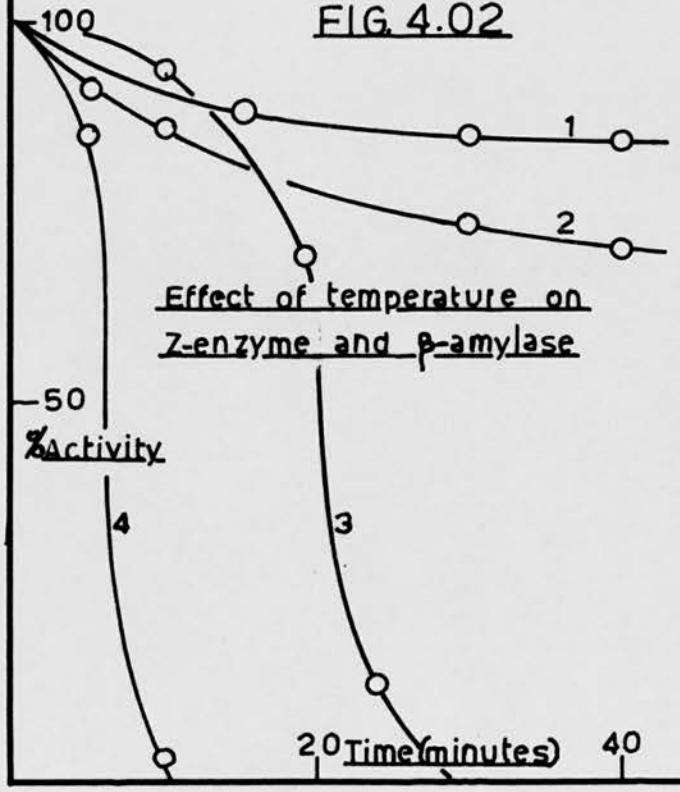


FIG. 4.02

Effect of temperature on Z-enzyme and β-amylase

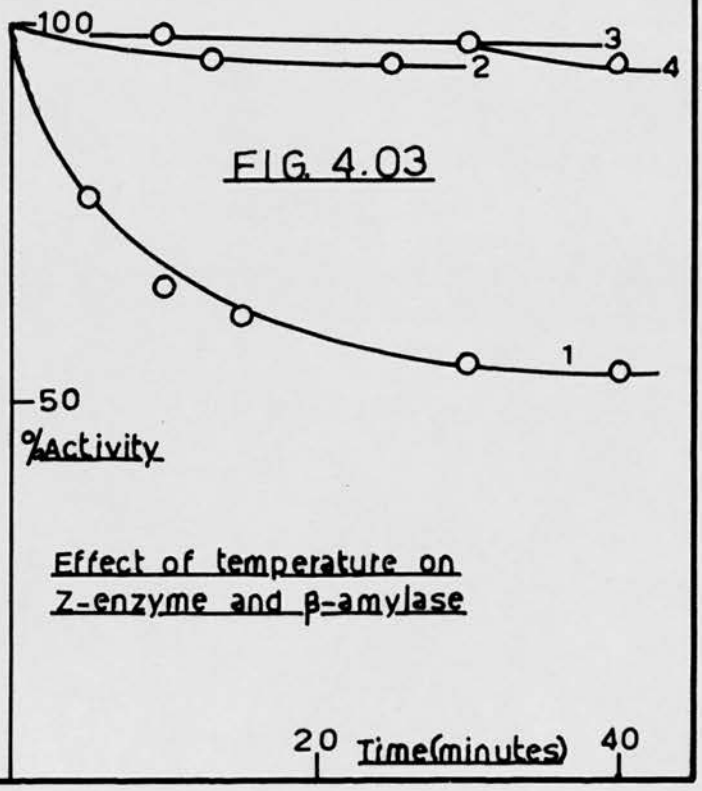


FIG. 4.03

Effect of temperature on Z-enzyme and β-amylase

the presence and absence of calcium, respectively, on the activity of soya bean Z-enzyme. Curves (3) and (4) show the corresponding β-amylase activities. Similar experiments were carried out at 63°C. and pH4.8 (Fig. 4.03).

Variation of Z-enzyme activity with temperature. Digests were prepared containing amylopectin β-limit dextrin solution (2ml.), acetate buffer (1ml.; 0.2M.; pH5.5) and water (4.8ml.). These were preincubated at various temperatures until temperature equilibrium had been established when portions of enzyme solution (0.2ml. fraction $S7_4 2$) were added to each. The enzyme activities were determined in the usual manner. The results are shown in Fig. 4.04.

Effect of temperature on the stability of Z-enzyme. Digests were prepared containing acetate buffer (1ml.; 0.2M.; pH5.5) and water (4.8ml.). They were equilibrated at their respective temperatures and aliquots of enzyme solution (0.2ml. fraction $S7_4 2$) were then added to each. After 1 hour the digests were allowed to come to equilibrium at 35°C. and samples of amylopectin β-limit dextrin solution (2ml.) were added to each. The enzymic activities of the digests were determined in the usual manner and the results were calculated as a percentage of a standard enzyme activity which was carried out at 35°C. without preincubation of the enzyme at this temperature. The results are shown in Fig. 4.04.

Variation of Z-enzyme activity with pH. Digests were prepared

containing amylopectin β-limit dextrin solution (2ml.), water (4.8ml.) and portions (1ml.) of McIlvaine's standard buffer solutions at various pH values. These were preincubated at 35°C. before the enzyme was added (0.2ml. fraction $S7_4 2$). The enzymic activities were determined in the usual way and were expressed as a percentage of the maximum activity obtained. The results are shown in Fig. 4.05.

Activity of Z-enzyme at pH 3.6. Four digests were prepared in the following manner.

A. Acetate buffer (2ml.; 0.2M.; pH5.5) + enzyme (0.2ml. fraction $S7_4 2$) + amylose solution (20ml.; 3mg./ml.).

B. Acetate buffer (2ml.; 0.2M.; pH5.5) + amylose solution (20ml.; 3mg./ml.).

C. Acetate buffer (2ml.; 0.2M.; pH3.6) + enzyme (0.2ml. fraction $S7_4 2$) + amylose solution (20ml.; 3mg./ml.).

D. Acetate buffer (2ml.; 0.2M.; pH3.6) + enzyme (0.2ml. fraction $S7_4 2$. This digest was incubated for 2 hours at room temperature before the amylose solution (20ml.; 3mg./ml.) was added.

These digests were incubated at 35°C. for 20 hours when they were heated on a boiling water-bath for 5 minutes, cooled, filtered and precipitated with excess butanol. There was no precipitate in digest A but the viscosities of the amylose samples obtained from digests B, C and D were determined in potassium hydroxide (0.2M.) at 25°C. The results are shown in Table 4.10.

FIG. 4.04

Z-enzyme activity/temperature

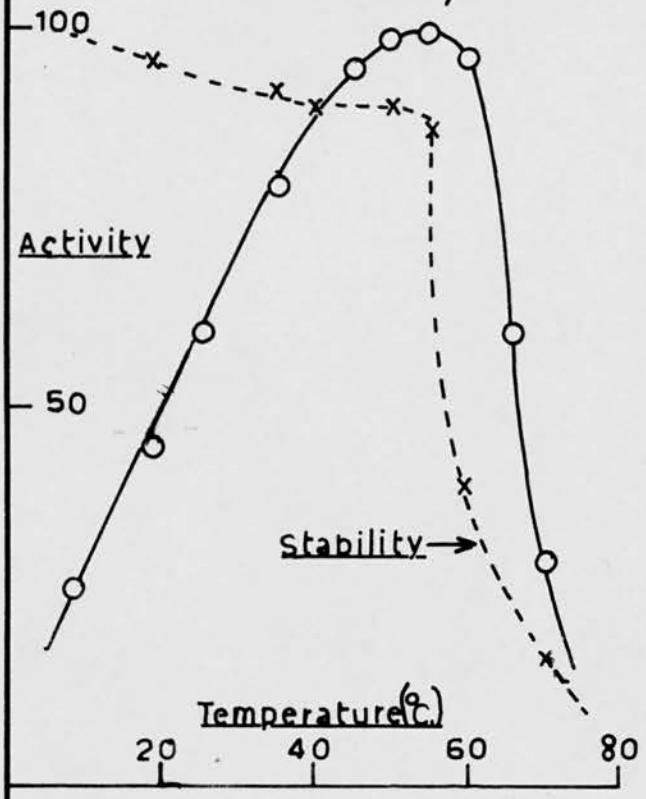


FIG. 4.05

Z-enzyme activity/pH

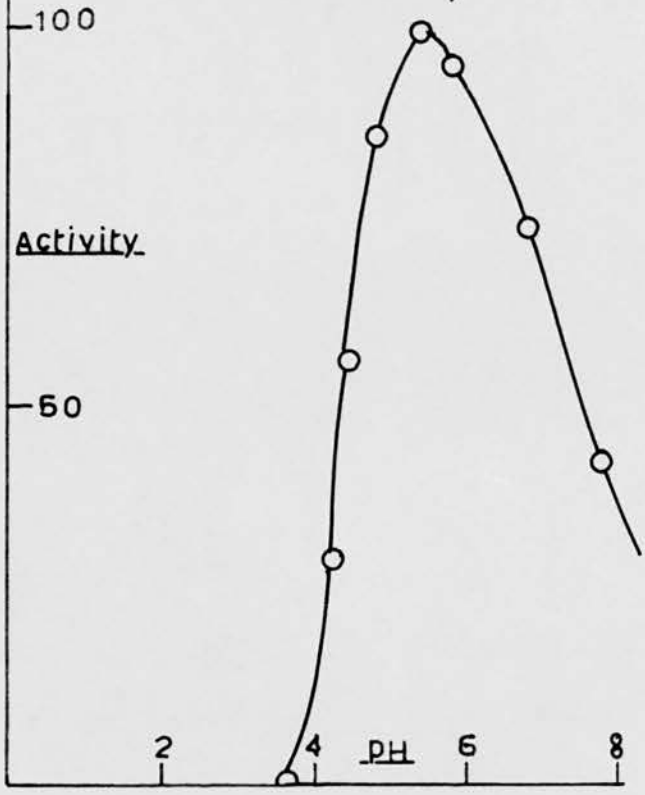


FIG. 4.06

Action of Z-enzyme on amylose

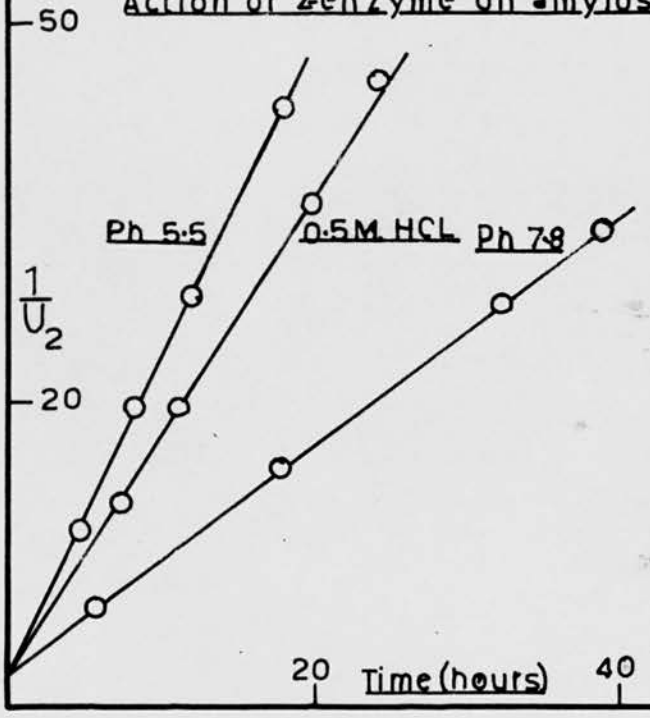


FIG. 4.07

Action of Z-enzyme on amylose

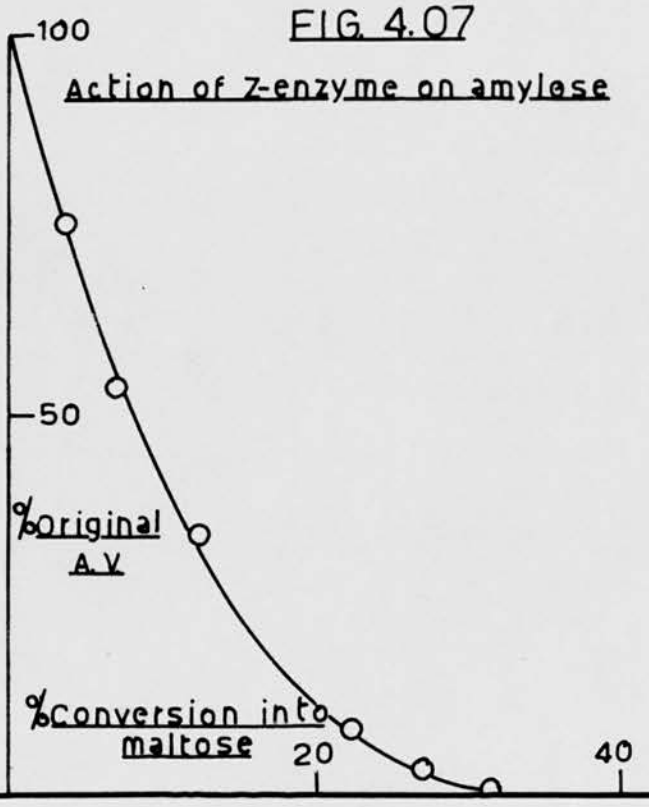


Table 4.10

Digest	A	B	C	D
Viscosity ($[\eta]$)	0	510	400	496

Effect of various reagents on Z-enzyme activity.

1. Effect of Ions. Digests were prepared containing acetate buffer (1ml.; 0.2M.; pH5.5), enzyme solution (0.2ml. fraction $S7_4 2$), water (0.5ml.) and the requisite amount of ionic species in water (5.3ml.). These were preincubated at 35°C. for 2 hours when the precipitates, which had appeared in the mercuric chloride digests, were removed by centrifugation. Aliquots of amylopectin β -limit dextrin solution (1ml.) were then added to each digest. The Z-enzyme activity in the various digests was determined in the usual manner. The results are shown in Table 4.11.

Table 4.11

	Ionic Concentration			
	10^{-3}M	10^{-4}M	10^{-5}M	10^{-6}M
Ionic species		% Inhibition		
Mercuric chloride	100	100	42	15
Potassium cyanide	0	0	0	0
Ammonium molybdate	20	0	0	0
Calcium chloride	0	0	0	0

2. Effect of other reagents. Similar experiments were carried out with ascorbic acid and tryptophane. The results are shown in Table 4.12.

Table 4.12

	10^{-3}M	10^{-4}M	10^{-5}M	10^{-6}M
		% Inhibition		
Tryptophane	0	0	0	0
Ascorbic acid	100	90	70	42

3. Effect of Z-enzyme samples, pretreated with mercuric and calcium chloride, on the viscosity of amylose solutions.

Digests were prepared in the following manner.

A. Acetate buffer (1ml.; 0.2M.; pH5.5), enzyme (0.2ml.fraction $S7_4 2$), water (0.5ml.) and mercuric chloride (6.3ml.; 1.3 10^{-3}M.)

B. Similar to above but containing mercuric chloride (6.3ml.; 1.3 10^{-4}M.).

C. Similar to above but containing calcium chloride (6.3ml.; 1.3 10^{-3}M.).

D. Similar to above but containing water (6.3ml.) instead of ions.

These digests were incubated at room temperature for 2 hours when those which had become cloudy were clarified by centrifugation. They were then added to equal volumes of the same amylose solution and incubated at 35°C. After 1 hour excess butanol was added to digests C and D, but A and B were left for 24 hours before receiving the same treatment. The viscosities of the amylose samples were determined in potassium

hydroxide (0.2M.) at 25°C. The results are shown in Table 4.13

Table 4.13

Digest	A	B	C	D
Viscosity ($[\eta]$)	495	500	80	75
The viscosity of the original amylose sample was 510				

4. Effect of ethylenediaminetetraacetate (E.D.T.A.) and trypsin. Digests were prepared in the following way.

Two digests were prepared, labelled A_1 and A_2, containing E.D.T.A. (0.1ml.; 10^{-1}M.) and trypsin (0.1ml.; 0.4mg.); two digests were prepared, labelled B_1 and B_2, containing E.D.T.A. (0.1ml.; 10^{-1}M.); two digests were prepared, labelled C_1 and C_2, containing trypsin (0.1ml.; 0.4mg.); one digest was prepared, labelled D, containing water

Water (10ml.), McIlvaine's standard buffer solution (1ml.; pH6.8) and enzyme (0.1ml. fraction $S7_42$) were added to each digest. They were left at room temperature for 12 hours when aliquots of calcium chloride solution (1ml.; 1M.) were added to digests (1). There was an immediate precipitation which was removed by centrifugation. Equal volumes of the same amylose solution were added to the digests which were incubated at 35°C. for 24 hours when excess butanol was added to each. Precipitates immediately formed in digests A_1, A_2 and B_2 but, even after 48 hours, there were only small precipitates present in digests C_2 and B_1. No precipitates were obtained in digests C_1 and D. The viscosities of the

amylose samples were determined in potassium hydroxide (0.2M.) at 25°C. The results are shown in Table 4.14.

Table 4.14

Digest	A_1	A_2	B_1	B_2	C_1	C_2	D
Viscosity ($[\eta]$)	75	455	30	280	-	40	-
The viscosity of the original amylose sample was 510.							

Action of Z-enzyme on amylose

1. Kinetics of the initial reaction. The enzymic and acid hydrolyses were carried out at 25°C. in a modified Ubbelohde viscometer.

1a. Action of Z-enzyme on amylose at pH5.5. The flow time of the solvent (0.01M. acetate buffer pH5.5) was measured and then an appropriate amylose solution was placed in the viscometer and allowed to come to temperature equilibrium. Its flow time was then obtained and enzyme solution (0.002ml. fraction $S7_4 2$) was added. The flow time of the amylose solution was taken at regular intervals over a period of 24 hours.

1b. Action of Z-enzyme on amylose at pH 7.8. The above procedure was repeated using, as solvent, McIlvaine's standard buffer solution (0.01M.; pH7.8).

1c. Action of acid on amylose. The above procedure was repeated using hydrochloric acid (0.5M.) as the solvent. Enzyme, of course, was not added in this instance.

In the above hydrolyses, $\eta_{sp.}$ was calculated from each flow time as indicated in the general experimental section.

A function of $\frac{1}{\eta_{sp}}$ was plotted against time (see discussion) for each system and the results are shown in Fig. 4.06.

2. Products of the final reaction. Portions of Z-enzyme solution (0.75ml. fraction $S7_4 2$) were added to two identical amylose solutions A and B (80ml.; 1.3mg. amylose/ml.) which were incubated at 35°C. At appropriate intervals aliquots (2ml. and 0.5ml.) were removed from digest A for reducing power and iodine staining measurements, respectively. The results are shown in Fig.4.07. When digest A approached its achroic limit an aliquot (10ml.) was removed from digest B. Further aliquots were removed from digest B at appropriate intervals as indicated in Table 4.15. (p. 94). They were quickly heated in a boiling water-bath for 5 minutes, evaporated to dryness in a rotary evaporator and suspended in water (0.25ml.). The solutions were centrifuged and portions of the clear supernatants were applied to Whatman No.1 chromatographic paper. The oligosaccharides were separated on the paper strip by the multiple descent technique using ethyl acetate: pyridine: water (10:4:3) as solvent. Each time the solvent front reached the bottom of the paper the chromatogram was removed from the chromatographic tank and dried before being replaced. With an average time of 6 hours/descent it was found, by using the appropriate standard substances, that 6 - 7 descents gave satisfactory resolution of the higher maltodextrins G_7, G_8 and G_9. (Since these oligosaccharides are linear, glucose, maltose, maltotriose ..... etc. will be referred to as G_1,

G_2, G_3....etc. in this thesis.) The chromatograms were treated as described in the general experimental section and the results are shown in Table 4.15.

Table 4.15

Time of extraction of aliquot from the digest.					
Maltodextrin	33 hours	70 hours	96 hours	120 hours	140 hours
G_1	+	+ +	+ + +	+ + + +	+ + + + +
G_2	+	+ +	+ + +	+ + + +	+ + + + +
G_3	+	+ +	+ + +	+ + + +	+ + + + +
G_4	+	+ +	+ + +	+ + + +	+ + +
G_5	+	+ +	+ + +	+ +	+
G_6	+	+ +	+ + +	+ +	+
G_7	+	+ +	+ + +	+ +	+
G_8	+	+ +	+ + +	+	0
G_9	+	+ +	+ +	+	0
Iodine stain of concentrated digest	Blue	Blue	Red	Achroic	Achroic

In Table 4.15 the depth of colour developed by the oligosaccharide spots is indicated by the number of " + " units. These numbers only afford a satisfactory basis for comparing various amounts of the same sugar; even then, when a certain depth of colour is exceeded, it is difficult to detect any further increase. It was quite possible, however, to form rough estimates of the relative quantities of a particular sugar,

e.g. G_5, present in the digest at various times. There has been no attempt to estimate the relative amounts of different sugars present on the chromatograms since, the longer the saccharide chain, the less is the depth of colour produced.

Action of Z-enzyme on T.C.A. glycogen β-limit dextrin. The dextrin (30mg.) was dissolved in water (22ml.) and acetate buffer (2ml.; 0.2M.; pH5.5). The solution was divided into two portions; to (1) was added β-amylase (400 units) and to (2) were added β-amylase (400 units) and Z-enzyme (0.2ml. fraction $S7_4 2$). The digests were incubated at 35°C. and aliquots were removed at intervals for reducing power determinations (Table 4.16).

Table 4.16

	Apparent conversion into maltose (%)		
Digest	24 hours	48 hours	72 hours
1	0	0	0
2	2	2.5	3

Action of Z-enzyme on amylopectin and amylopectin β-limit dextrin. Digests were prepared containing amylopectin or amylopectin β-limit dextrin solution (5ml. ca. 5mg./ml.), acetate buffer (2ml; 0.2M; pH5.5), water (12ml.) and enzyme solution (0.1ml. fraction $S7_4 2$). They were preincubated at 35°C. before the enzyme was added. Aliquots (3ml.) were removed at intervals and stained with iodine. The results are shown in Fig. 4.08.

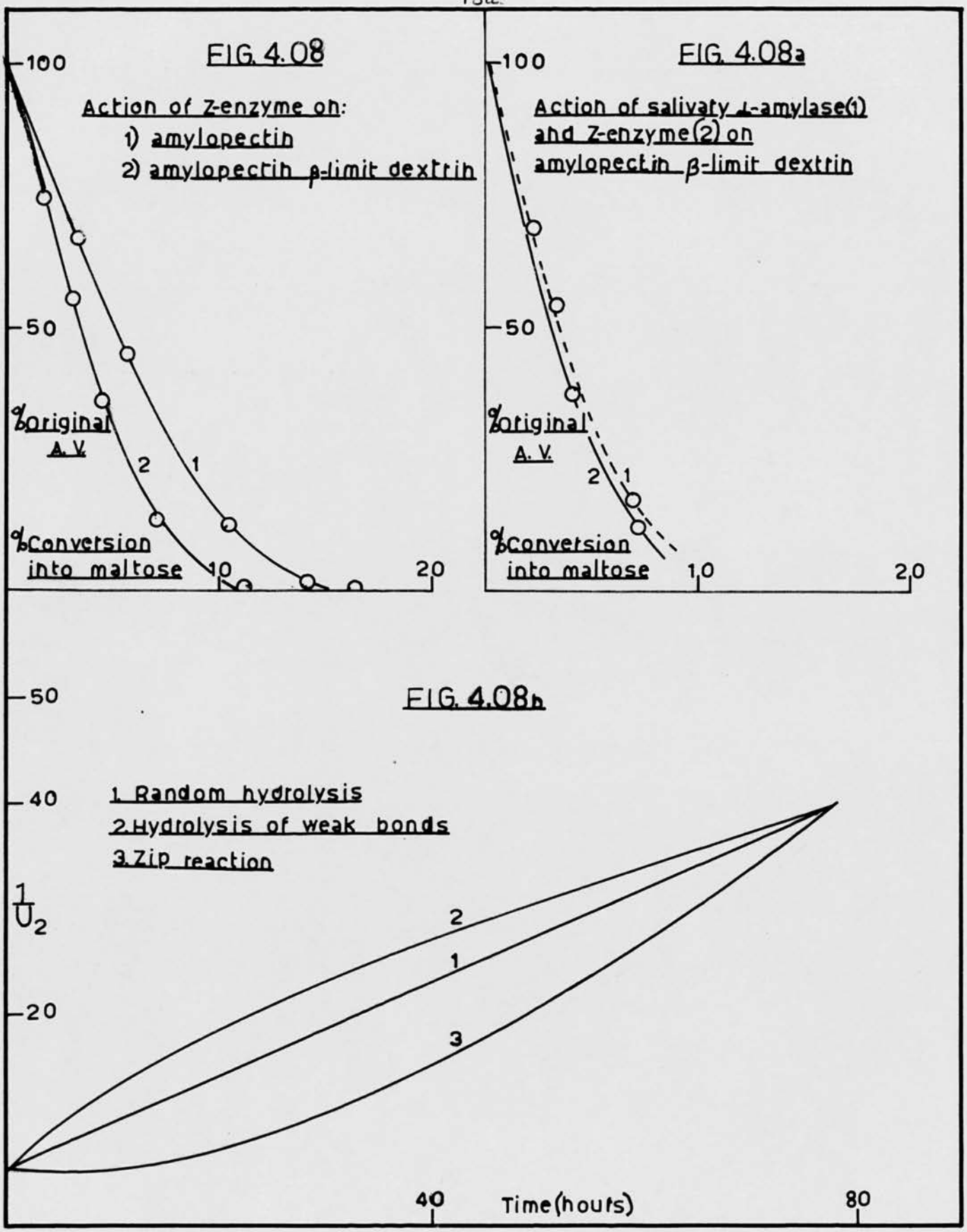


FIG. 4.08
100
50
Action of Z-enzyme on:
1) amylopectin
2) amylopectin β-limit dextrin
% original A.V.
% conversion into maltose
10
20
2
1
FIG. 4.08a
100
50
Action of salivary α-amylase(1) and Z-enzyme(2) on amylopectin β-limit dextrin
% original A.V.
% conversion into maltose
1,0
2,0
2
1
FIG. 4.08b
50
40
20
1. Random hydrolysis
2. Hydrolysis of weak bonds
3. Zip reaction
1/U2
2
1
3
40
Time (hours)
80

Relative initial rates of attack of Z-enzyme on various substrates. A sample (ca. 60mg.) of each substrate listed in Table 4.17 was dissolved in acetate buffer (40 ml.; 0.02M; pH5.5). The exact concentration of each solution was determined by hydrolysing an aliquot and estimating the resulting glucose by the alkaline ferricyanide method outlined in the general experimental section. Portions of Z-enzyme solution (0.002ml. fraction $S7_4 2$) were added to each digest and the rates of hydrolyses were determined by measuring the change in the molecular weights of the substrates. These measurements were carried out in collaboration with Mr. R. Geddes using the Brice Phoenix lightscattering instrument described earlier. The results are shown in Table 4.17.

Table 4.17

Substrate:	Amylose	Amylose β-limit dextrin	Amylopectin	Amylopectin β-limit dextrin	Glycogen	Glycogen β-limit dextrin
Relative Rate:	330	77	18	13.6	5	1

DISCUSSION

Preparation and purification of soya bean Z-enzyme. Peat et al. (1952a) reported the isolation of Z-enzyme from soya beans, but later workers (Banks et al., 1960) were unable to repeat their preparation. Since pure Z-enzyme could not readily be obtained, these latter authors employed the technique of selective inhibition to prepare samples of the enzyme. They showed that mercuric chloride (1.5×10^{-6}M.) effectively inhibited β-amylase activity without apparently affecting Z-enzyme activity in mixtures of the two. This procedure was investigated further using a crude enzyme fraction obtained from soya bean flour by alcohol precipitation (Table 4.01, p. 74) The results in Table 4.02 (p. 75) indicate that mercuric chloride (1.5×10^{-6}M.) does indeed inhibit β-amylase, leaving the Z-enzyme activity apparently intact. It is rather difficult, however, to determine whether or not the mercuric chloride modifies the Z-enzyme activity to any extent and so, studying the properties of a sample of the enzyme prepared in this way is not at all satisfactory. An attempt was made to separate the two activities by an ammonium sulphate fractionation, but, although a fraction was obtained containing enhanced Z-enzyme activity, the β-amylase content was also increased (Table 4.03, p. 76).

It has been reported (Myrbäck, 1948; Preece, 1949) that β-amylase impurity may be removed from barley malt α-amylase preparations by heating a solution of the enzyme mixture at 70°C. in the presence of calcium ions. The

similarity between α-amylase and Z-enzyme suggests that these two enzymes might be expected to react in the same manner to high temperatures. When a soya bean fraction was heated at 70°C. in the presence of calcium acetate the results (Table 4.04, p. 77) indicated that this treatment completely destroyed the β-amylase activity while the specific activity of the Z-enzyme was increased through the removal of inert protein.

These preliminary experiments show that Z-enzyme may be purified to a certain extent by careful fractional precipitation and - of greater importance - that the inclusion of a simple heat-treatment in the procedure completely removes β-amylase impurity.

The initial extractions of soya bean flour were carried out in water, but since these were difficult to clarify, later extractions utilised calcium chloride solutions (0.2%) from which clear solutions of crude protein were readily obtained. The salt and water extracts contained similar activities of Z-enzyme.

The final method adopted for the preparation of Z-enzyme included two acetone fractionations, which must be carried out at very low temperatures otherwise the enzymic activity may be drastically reduced. When the enzyme fractions obtained from these acetone fractionations were completely dehydrated they became rather insoluble in cold water and lost much of their activity. This procedure was replaced by one involving the removal of excess acetone from the freshly precipitated fractions with a current of air - the still moist

protein fractions then dissolved fairly readily in either cold water or cold dilute buffer.

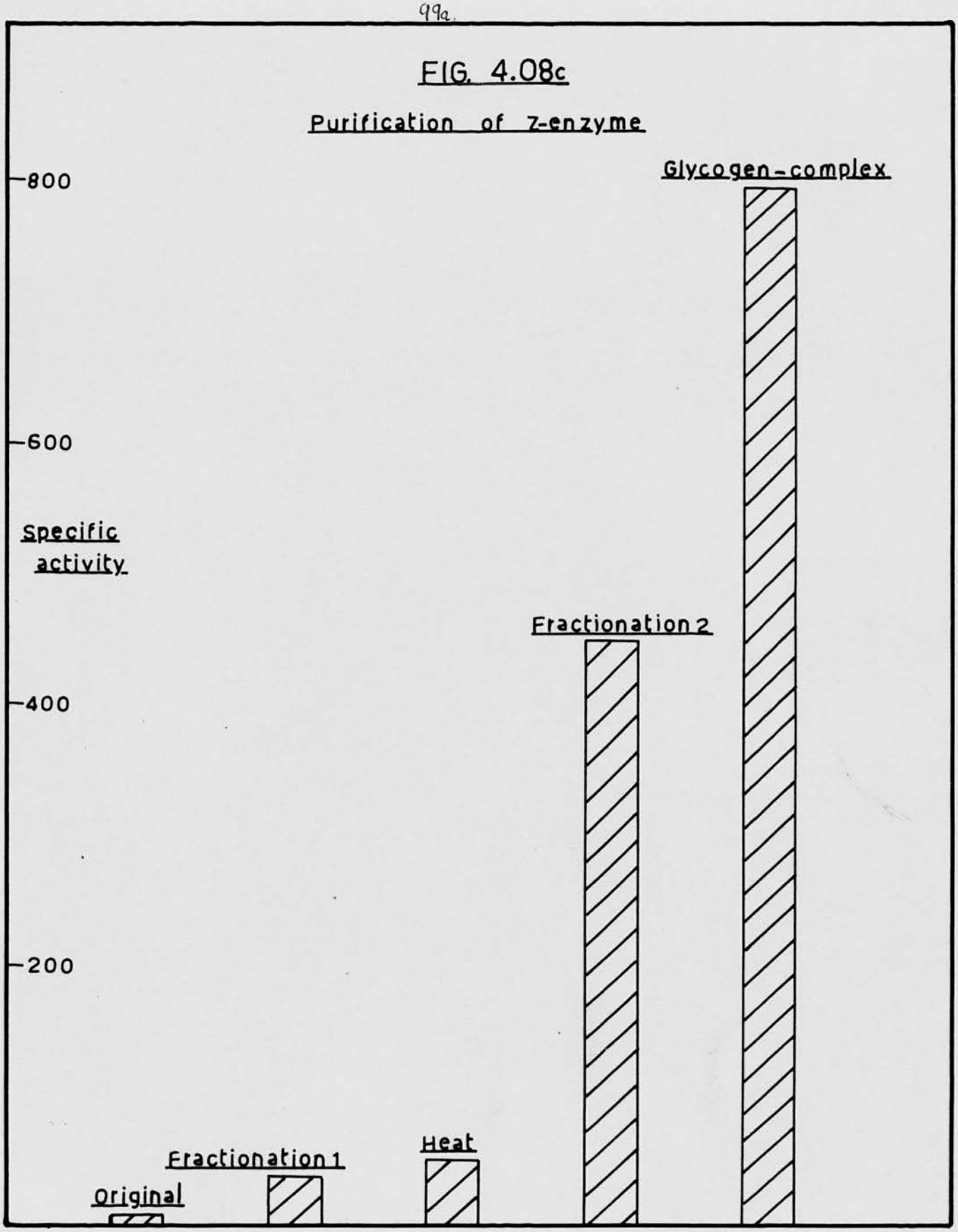
Recently it has been shown that α-amylase preparations will form complexes with glycogen and this method has been suggested as a general technique for preparing α-amylases with very high specific activity (Loyter and Schramm, 1962; Levitzki, Heller and Schramm, 1964). The method was, therefore, adapted for use in the preparation of Z-enzyme. The efficiency of the glycogen/Z-enzyme complex formation procedure in yielding a highly purified enzyme fraction is illustrated in Table 4.08 (p. 80).

The increase in the specific activity of the Z-enzyme with each stage in the purification procedure is shown in Fig. 4.08c. The enzyme has been purified by a factor of ca. 120 and appears to be free from the contaminating enzymes with which the preparation of Peat et al. (1952) was associated.

It is often very difficult to repeat enzymic preparations reported in the literature because full experimental conditions are not given; procedures involving fractional precipitation techniques are only reproducible when certain parameters present in the systems are carefully controlled (cf. Dixon and Webb, 1961). To obtain consistent results from the fractionation scheme reported here the following points should be noted: (1) the protein concentrations of the original soya bean flour/salt solution extracts must be the same; (2) the extracting medium must be of constant ionic strength; (3) during the heating stage to remove β-amylase activity, equal

FIG. 4.08c

Purification of Z-enzyme



volumes of the protein solutions should be treated i.e. consistent results will not be obtained if, on one occasion a small volume of solution (ca. 200ml.) is heated under the appropriate conditions, and, on another, a larger volume (ca. 500ml.) is heated under the same conditions. It was found convenient to heat the protein solutions in standard volumes (10 - 15ml.).

Removal of β -amylase from Z-enzyme preparations. The results shown in Fig. 4.02 (p. 86a) indicate that, although the activities of a crude preparation of Z-enzyme is remarkably stable at pH6 and 70°C. (curve 2), the activity of the enzyme is further increased by the addition of calcium acetate to the protein solution (curve 1). The presence of excess calcium, under the same conditions of temperature and pH, also affects the stability of β -amylase since in its presence the enzyme is almost completely denatured after 10 minutes while in its absence, the fall in activity is not so rapid and is still detectable after 40 minutes. These results agree with those of Myrbäck (1948) and Preece (1949), who showed that β -amylase impurity may be removed from barley malt α-amylase preparations by heating the enzyme solutions at 70°C. in the presence of excess calcium. The heat labilities of crude preparations of malt α-amylase and soya bean Z-enzyme would, therefore, appear to be similar.

Peat et al. (1952c) investigated the effect of heat on crude mixtures of soya bean Z-enzyme and β-amylase. They found that in the absence of calcium and at an acid pH(4.8) a

temperature of 63°C. completely destroyed the Z-enzyme activity after 15 minutes while the β-amylase activity was reduced to 70% of its original value. These findings are not inconsistent with the results shown in Fig. 4.03 which illustrate the effect of heating crude Z-enzyme and β-amylase mixtures at 63°C. and pH4.8 in the presence and absence of calcium. β-amylase appears to be quite stable under these conditions but there is a significant change in the activity of the Z-enzyme component of the mixture; although it is stable in the presence of calcium (curve 2), its activity is rapidly reduced in the absence of this ion (curve 1). Since the enzyme is more stable at 70°C. than at 63°C. in the absence of calcium, the extent, of denaturation must depend on the pH of the system. This result is in agreement with Peat et al. (1952) who found that β-amylase and Z-enzyme were equally thermostable at pH5.5.

The Z-enzyme activity is not completely destroyed under the conditions of the experiment in Fig. 4.03 (curve 1), in contrast to the report of Peat et al. This discrepancy might be due to a difference in the concentration of the enzyme in the samples used for the heating experiments; in the present work reasonably concentrated solutions (10mg./ml.) of crude Z-enzyme were employed, whereas the samples used by Peat et al. appeared to contain weakly active enzyme. It is known that the stability of an enzyme in solution may be dependent upon its concentration (Walker and Whelan, 1960) - the more concentrated the solution the more stable is the enzyme - and so the Z-enzyme samples investigated by Peat et al. would be more readily

denatured. This argument also explains the 30% fall in activity of β-amylase, maintained at 63°C., reported by the latter authors.

The Z-enzyme samples from which the β-amylase impurity had been removed by heat were retested for β-amylase after 4 days but none was found. The heat treatment had, therefore, irreversibly denatured the β-amylase.

Determination of enzymic activities. Because the action patterns of Z-enzyme and α-amylase are similar (i.e. they both exert a random hydrolysis on α-1:4 linkages) general methods for the assay of α-amylase activity were investigated to obtain one for measuring the activities of Z-enzyme preparations. There are many methods available but most of them are unsuitable because they do not distinguish between the activities of α- and β-amylases. For example, the method of Fischer and Stein (1954) necessitates the estimation of liberated maltose, from starch digests, by the enzyme system. A very small amount of β-amylase impurity would thus invalidate these results. The method of Hultin (1949), which involves measuring the fall in viscosity of enzyme-polysaccharide digests after various times of incubation, will also be suspect in the presence of β-amylase. Many methods utilise the property of α-amylases to decrease the colour of starch-iodine digests (Smith and Roe, 1957) - these also are affected by β-amylase. All of these methods are suitable for determining the activities of pure α-amylases and so could be used for most of the measurements presented in this

work. However, a method of estimation was sought which could be utilised to determine the activities of the initial fractions, obtained in the preparation of Z-enzyme, which are grossly contaminated with β -amylase. The method of Briggs (1961) appeared to be the most satisfactory one for this purpose. It utilises amylopectin β -limit dextrin as the substrate and, since β -amylase cannot attack this molecule, the initial attack, indicated by a fall in the iodine staining power of the substrate, must be due to α -amylolytic scission of the molecule. The substrate is not ideal since, in the presence of excess β -amylase, α-amylolytic scission will be rapidly followed by β-amylase attack which will also tend to reduce the iodine colour. However, because the dextrin molecule is fairly compact and the lengths of chain available to the β -amylase after an attack by α -amylase are small there will not be many β -amylase scissions/ α -amylase attack. A further possible substrate is the β -limit dextrin of amylose, but this is a more open molecule and the ratio of β -amylase scissions/ α -amylase attack would be much higher with this polysaccharide as the substrate. Thus,although amylopectin β -limit dextrin is not entirely satisfactory it is probably the best substrate available for determining α -amylase activities in the presence of β -amylase.

The action of salivary α -amylase on amylopectin β -limit dextrin was used in constructing the standard graph from which subsequent Z-enzyme activities were calculated. It has been found that the hydrolyses of samples of

dextrin by salivary α-amylase and Z-enzyme are similar in the initial stages and only close to the achroic limit do the action patterns vary (Fig. 4.08a). This is caused by the difference in affinities of the enzymes for the small maltodextrins present in the digests at the achroic limit, but in the construction of the graph, measurements were not made close to the achroic limit.

The β-amylase samples examined in this investigation were all contaminated with Z-enzyme and so their activities were determined at pH3.6. These conditions inhibit Z-enzyme activity but do not greatly decrease the activity of β-amylase.

Effect of temperature and pH on Z-enzyme. The results shown in Figs. 4.04 and 4.05 (p. 88a) indicate that the temperature of maximum activity is ca. 55°C. and the optimum pH is 5.4. The stability experiments show that the enzyme loses only about 10% of its original activity after 1 hour at 50°C., but then there is a very rapid decrease between 55 and 60°C. Since the activity of the enzyme increases with temperature to a maximum at 55°C. (Fig 4.04), high temperatures appear to have two opposing effects on the enzyme activity: (1) the higher the temperature the greater is the rate of reaction; (2) the higher the temperature the faster is the enzyme denatured. Effect (1) predominates up to a temperature of 55°C. but, at higher temperatures, the enzyme is very rapidly denatured. Since the enzyme sample used in these experiments is highly purified, the concentration of protein in the digests is much less than that used in the heating

experiments Fig. 4.02 (p. 86a). There are, therefore, only small amounts of "protecting" protein in these stability digests and so the enzymic activity is more readily destroyed.

Activity of Z-enzyme at pH3.6. The difference between the viscosities of amylose samples B and D in Table 4.10 (p. 89) is within experimental error and does not indicate hydrolysis of amylose sample D. There is, however, a significant decrease in the viscosity of amylose sample C, showing that the substrate has been hydrolysed before complete inhibition of the enzyme had been achieved. Preincubation at pH3.6 for at least 2 hours is required for the complete inhibition of soya bean Z-enzyme. Crude preparations of β-amylase containing Z-enzyme impurity may, therefore, be used to determine the β-amylolysis limits of starch-type polysaccharides if the enzyme sample is pre-incubated at pH3.6 before use.

Effect of various reagents on Z-enzyme. Mercuric chloride (10^{-4}M.) and ascorbic acid (10^{-3}M) are very efficient inhibitors of Z-enzyme activity (Tables 4.11, 4.12 and 4.13 pp. 89-91). It is interesting to note that even more dilute mercuric chloride solutions (10^{-6}M.) inhibit 15% of the enzymic activity and so the Z-enzyme sample used by Banks et al. (1960), in mercuric chloride (1.5×10^{-6}M.) must have been inhibited to an even greater extent. Calcium ions do not appear to activate the enzyme but their presence is necessary to enable the enzyme to exert its activity (Table 4.14, p. 92).

E.D.T.A. is a metal chelating agent which removes calcium ions from solution, whereas trypsin is a protease and exerts a hydrolytic action on various proteins. Tests for amylolytic inhibition using viscosity measurements are more sensitive than those procedures involving iodine-stain. The fall (45%) in viscosity of amylose sample B_2 (Table 4.14) indicates that although the enzyme is greatly inhibited, limited hydrolysis has taken place in the E.D.T.A./Z-enzyme/amylose digest. This inhibition is largely reversible because on adding excess calcium ions to digest B_1 the extent of amylose hydrolysis is greatly increased. Trypsin, by itself, appears to have a very small effect on the enzymic activity as shown by the large decrease in viscosity of amylose sample C_2. It should be noted that these very small values of viscosity are approximate because they are too small to be accurately determined by the viscosimetric technique. Since the viscosity of amylose sample A_2 has only fallen by 10% of its original value a mixture of E.D.T.A. and trypsin is a more efficient inhibitor than E.D.T.A. by itself. The inhibition of the enzyme achieved under the combined action of E.D.T.A. and trypsin is not completely reversible since, on the addition of excess calcium ions to the system, amylose sample A_1 is not hydrolysed to the same extent as sample B_1. This might be caused by the trypsin irreversibly destroying some of the enzyme molecules from which E.D.T.A. has removed the calcium ions.

Action of soya bean Z-enzyme on amylose.

a) Kinetics of the initial attack. Banks et al. (1960) investigated the action of soya bean Z-enzyme on amylose samples by examining the viscosities of the residual polysaccharides in amylose/Z-enzyme digests. In all cases where the digests were maintained at pH values conducive to enzymic attack, a large reduction in the viscosities of the substrates was obtained. The enzyme also caused a large decrease in the viscosity of linear amylose and from these results it was concluded that a random hydrolytic action was taking place.

Perhaps the most satisfactory method of investigating the kinetics of polymer degradation is to follow the decrease in the average molecular weight of the polymer molecules. During these reactions the number of molecules in the system increases and so such processes should be followed by techniques designed to yield number-average molecular weights. These methods are insensitive to small initial changes in the polymeric species, however, but weight-average methods provide a sensitive measure of these. Viscosity measurements, therefore, may be used to study these reactions since the average molecular weight obtained from this technique approximates to a weight average value. This procedure is very convenient because the degradation process under investigation can be carried out in a viscometer and the measurements are readily and rapidly performed enabling fast reactions to be followed. Because of the high sensitivity of the viscosimetric measurements very slow processes can also be studied.

It has been pointed out by Bryce and Greenwood (1957) that the decrease in viscosity ($[\eta]$), with time, of a polymer undergoing degradation is not itself a true measure of the degradation rate but for either a zero - or first order reaction the rate is proportional to $[\eta]^{-1}$. Since it is more convenient to follow these reactions by the changes in η_{sp} with time, the relation established by Vink (1963) was employed:

$$\frac{1}{U_2} = \left[K'c\left(\frac{1}{\eta_{sp}} + k\right)\right]^{\frac{1}{a}} \qquad 4.01$$

where U_2 is the weight average degree of polymerisation.

C is the concentration of the polymer (g./ml.)

a is the exponent in the viscosity equation $\eta = K.M.^a$ and, under the present experimental conditions $= 0.5$ (Banks and Greenwood, 1963).

K^1 is a constant.

k is Huggin's constant and for this system $= 0.65$ (Everett and Foster, 1959b).

The validity of this relation depends on the following conditions being fulfilled: (1) the viscosity-average molecular weight closely approximates to the weight-average molecular weight; (2) the initial weight distribution of the substrate does not contain two or more peaks e.g. a high molecular weight sample containing an appreciably lower molecular weight portion; (3) the rapid formation of a random distribution of substrate molecules in the reaction system.

If $^1/_{U_2}$ is plotted against t(length of duration of the reaction) the shape of the resulting graph is an indication of the type of degradation process taking place. (See Fig. 4.08b). A straight line (curve 1) obtained over a period of extensive degradation provides strong evidence for the presence of random degradation. The converse is not necessarily true, however, since the rate constant k, may vary during the reaction thus changing the degradation kinetics. If the slope of the line is high at the beginning of the reaction and then decreases to a constant value (curve 2) this indicates the presence of randomly situated weak links in the polymer chain. These links are preferentially consumed at the beginning of the reaction at a higher rate of degradation than the normal ones and when they have been completely destroyed the rate of the reaction decreases to a constant value characteristic of the degradation of these normal bonds. A "zip reaction" - i.e. a polymer degradation reaction in which small units are successively split off from the chain ends - is characterised by a slow initial degradation rate which gradually increases as the reaction proceeds. (curve 3).

A typical example of a random degradation process is the hydrolysis of linear amylose with acid; this reaction was investigated and the results are shown in Fig. 4.06 (p. 88a) The straight line which was obtained indicates that the system is fulfilling the conditions, outlined above, for the establishment of equation 4.01. The hydrolysis of amylose samples by soya bean Z-enzyme at two different values of pH also yielded

straight lines Fig. 4.06. These results indicate that the enzyme exerts a random hydrolytic action on the substrate molecules. It must be emphasised, however, that although a large change (ca. 15-fold) has taken place in the degree of polymerisation of the amylose molecules, these results represent only the initial stage in the degradation process. This may be readily seen from the following calculation. The number of bonds broken/molecule during a random degradation reaction may be obtained from the relation

$$\text{No. of bonds broken} = \frac{D.P._o}{D.P._t} - 1 \quad \text{(Cowie and Greenwood, 1957)}, \qquad 4.02$$

where $D.P._o$ is the number-average degree of polymerisation at the beginning of the reaction and $D.P._t$ is the number-average degree of polymerisation after time t.

Since the amylose sample used in this investigation was a linear fraction its number-average molecular weight would be similar to its weight-average molecular weight. Therefore

$$\frac{D.P._o}{D.P._t} \equiv \frac{U_2^o}{U_2 t} \qquad 4.03$$

Therefore, the number of bonds broken/molecule during the hydrolyses experiments =

$$\frac{U_2^o}{U_2 t} - 1. \qquad 4.04$$

In the reaction of Z-enzyme on amylose at pH5.5 the number of bonds broken/molecule = 15.6. The viscosity of the amylose sample in potassium hydroxide (o.2M.) was 400. Using the relation D.P. = 7.4 $[\eta]$. (Cowie and Greenwood, 1957a) the D.P. of this amylose sample is 2,960. The percentage of bonds broken during the hydrolysis is ca. 0.5.

b) End products of the reaction. It is apparent (Fig. 4.07, p. 88a) that there are two distinct stages in the hydrolysis of amylose by Z-enzyme. First, there is a rapid decrease in the size of the amylose molecules as shown by the fall in the colour of the iodine-stain. This is accompanied by an increase in the reducing power of the amylose solution. It was the initial stage in this reaction which was studied viscosimetrically. The second part of the reaction begins when the achroic limit of the amylose solution is reached and is characterised by a slow increase in the reducing power of the solution. The discontinuity in the reaction occurs at ca. 30% apparent conversion into maltose (Fig. 4.07) and the disappearance of the amylose-iodine stain indicates that there are only small maltodextrin molecules present at this stage in the reaction.

Bird and Hopkins (1954) have shown that the achroic point of an amylose/barley malt α -amylase digest depends on the amylose concentration. The achroic point occurs at a higher apparent conversion into maltose in a solution containing a high amylose concentration than it does for a lower

concentration of amylose. In a concentrated digest there will be a large accumulation of maltodextrins coexisting with partially degraded substrate molecules and so the probability of dextrin - enzyme reaction will be high. These maltodextrins will then be hydrolysed to smaller units such as glucose, maltose, maltotriose ..... which will greatly increase the reducing power of the solution without lowering the iodine colour in proportion. This will lead to the production of small saccharides before the achroic limit is reached. In a dilute digest, however, the enzyme/substrate ratio is increased and so a larger proportion of the substrate molecules are degraded to chains of 8 to 10 units in length before these chains are themselves, attacked to a significant extent. The achroic limit is not therefore, constant but depends on the amylose/enzyme ratio.

The results in Table 4.15 (p. 94) illustrate the action of Z-enzyme on the maltodextrins present in amylose/Z-enzyme digests at the apparent achroic point. Chromatograms indicate that maltodextrins larger than G_6 predominate at this stage in the reaction, although small quantities of G_1 and G_2 and trace amounts of G_3, G_4 and G_5 are also present. As the hydrolysis proceeds all the saccharides detected on the chromatograms (G_1 - G_9) increase in amount and then the higher ones begin to decrease until only trace amounts of G_5, G_6 and G_7 remain. G_4 is hydrolysed extremely slowly and even after many days it still persists in the digests. It is not possible to determine whether or not G_3 is hydrolysed

under these conditions. The small amounts of saccharides G_1 - G_5 present in the digests at the achroic stage were probably formed by the action of Z-enzyme on the amylose molecules and not by the enzymic hydrolysis of large oligosaccharides. The initial formation of these saccharides, therefore, is due to primary fission of the amylose molecules.

It is at first surprising that the yields of large maltodextrins G_8 and G_9 continue to increase after the achroic point has been reached. This indicates that there are still long chain substrate molecules present in the digest which should stain with iodine. Normally, aliquots are removed from enzymic digests, diluted 50 or 100 times and then stained with iodine. The achroic point is taken to be the stage at which these dilute solutions no longer give a colouration with iodine. Obviously, then, the achroic point will vary with the dilution employed and the value will be meaningless unless the experimental details by which it is obtained are given. Portions of the concentrated digests which were applied to the chromatograms were tested with iodine and the results are shown in Table 4.15. They indicate that there is still blue-staining material in the digests at the "achroic limit". Not until many hours later is the "true" achroic point reached and the chromatographic evidence suggests that at this stage in the hydrolysis there are no maltodextrins higher than G_9 present in the digests.

Effect of Z-enzyme on various substrates. Banks et al. (1960)

showed by light scattering measurements that Z-enzyme hydrolyses amylopectin and amylopectin β-limit dextrin. In this present work very active preparations of Z-enzyme have been used and so enzymic attack on these substrates can readily be shown by iodine-staining measurements (Fig. 4.8, p. 95a). Both the amylopectin and its dextrin are hydrolysed to achroic dextrins, and so the validity of measuring the purity of amylose samples by the combined action of β-amylase and Z-enzyme may be questioned (Greenwood and Thomson, 1962). However, it must be remembered that the concentration of Z-enzyme impurity in most preparations of β-amylase is so low that it does not produce changes in amylopectin which are measurable by changes in iodine stain.

There has been some controversy over the question of whether or not Z-enzyme attacks glycogen β-limit dextrin. From light scattering studies, Banks et al. (1960) suggested that there was no attack, while Cunningham et al. (1962), using much larger amounts of enzyme, obtained an increase in the reducing power of glycogen β-limit dextrin solutions after prolonged incubation. The light scattering technique, which follows the change in the molecular weight of the substrate, is perhaps not the most sensitive method of indicating attack by Z-enzyme on the β-limit dextrin of glycogen. This molecule is very compact and so it will be extremely difficult for the enzyme to penetrate it to any extent. The internal bonds in the molecule will thus be inaccessible and only the bonds on the outside will be available for attack by the enzyme. Hydrolysis

of these external linkages will not greatly change the molecular weight of the substrate. However, the addition of β-amylase to such a system might be expected to produce a detectable amount of maltose from the short, newly exposed chains in the dextrin molecules. The results in Table 4.16 (p. 95) indicate that maltose is produced under these conditions. With a suitable concentration of enzyme (10 x concentration used by Banks et al, 1960), light scattering results (Table 4.17, p. 96) also indicate that glycogen β-limit dextrin is attacked by Z-enzyme.

4b. BARLEY α-AMYLASE

INTRODUCTION

Crude preparations of barley α-amylase contain an impurity which renders the amylose component of starch completely susceptible to β-amylase attack (compare Section 4a). Because such preparations hydrolysed laminarin (Manners, 1952) and their action on amylose was similar to that ascribed to soya bean Z-enzyme (Peat et al, 1952a), the amylose-attacking enzyme which they contained was first thought to be a β-glucosidase (Manners, 1952). It has been shown, however, that soya bean Z-enzyme is an α-amylase (Banks et al, 1960) and that preparations of barley flour also contain α-amylolytic activity (Cunningham, et al., 1960). These latter authors examined the action of crude preparations of barley α-amylase on various starch-type substrates and concluded that the enzyme readily attacks amylose, amylopectin and their β-limit dextrins; glycogen and its limit dextrin were hydrolysed to a small extent when very large concentrations of enzyme were used. They did not purify the enzyme in order to show whether barley contained a very small amount of highly active enzyme or a larger amount of weakly-active enzyme.

The present study was undertaken in an attempt to prepare pure barley α-amylase and to compare its properties with those of the α-amylase prepared from a malted sample of the same barley.

EXPERIMENTAL

Initial attempts to prepare barley α-amylase

(1) Finely ground, ether-defatted barley flour (400g.) was extracted with aqueous alcohol (1400 ml.; 20% alcohol) for 3 hours at room temperature. The suspension was centrifuged to yield a clear brown solution (900 ml.) which was cooled to 2°C. and fractionated with chilled alcohol (1920 ml.). The precipitate was removed by centrifugation, suspended in calcium acetate solution (200 ml.; 0.2% solution) and heated on a water-bath (70°C.; 15 minutes) yielding a cloudy suspension which was cooled and centrifuged to give a clear solution (185ml.). Chilled alcohol was added to 60% concentration (278 ml.) and the resulting precipitate was removed and dried with alcohol followed by ether. This preparation was rather insoluble in water and dilute buffer and had a low activity.

(2) Finely ground, ether defatted barley flour (400g.) was extracted with water (1500 ml.) for 6 hours at room temperature- a few drops of octanol were added to prevent excess frothing of the suspension. On centrifugation a clear brown solution (1100ml.) was obtained which was cooled to 2°C. and fractionated with chilled acetone (-15°C.) at a low temperature (-10°C.) between the limits 0-20, 20-30, 30-40, 40-50, 50-60 and 60-70% with respect to acetone. The precipitates were immediately suspended in cold water (150ml.), stirred, centrifuged and the resulting clear solutions were saturated with solid ammonium sulphate and stored at 2°C. The original flour extract was labelled solution (1) and the fractions were labelled suspensions

2 to 7 respectively.

α-amylase activities. Aliquots (5ml.) of the suspensions were centrifuged and the precipitates were dissolved in water (5ml.). Samples (1ml.) of these diluted solutions and of solution (1) were added to digests containing amylopectin β-limit dextrin solution (3ml.), acetate buffer (3ml; pH5.5; 0.2M.) and water (16ml.). Aliquots (3ml.) were extracted at intervals and stained with iodine as already described. The specific activities of the fractions are shown in Table 4.18.

Table 4.18

Fraction Number	1	2	3	4	5	6	7
α-amylase specific activity	0.53	1.13	1.74	3.14	2.75	0.27	0.18
β-amylase specific activity	5.6	8.3	14.6	14.8	36.5	27.6	10.4

β-amylase activities. These were carried out as described in Section 4a and the results are shown in Table 4.18.

Fraction (4) was centrifuged, the precipitate suspended in water (80ml.), and re-centrifuged to yield a clear brown solution (75ml.). Aliquots (1ml.) were removed for α-amylase, β-amylase and protein concentration determinations. Calcium acetate (150 mg.) was added to the remaining solution and it was heated at 70°C. for 15 minutes before being cooled and centrifuged. Samples (1ml.) were withdrawn for activity determinat-

ions and the results are shown in Table 4.19.

Table 4.19

	α-amylase specific activity		β-amylase specific activity	
	Before heating	After heating	Before heating	After heating
Fraction 4	3.14	0	14.8	0

(3) An extraction and fractionation were carried out as described in (2), but the solid protein fractions were air-dried to remove excess acetone and suspended in water. They were stored in aqueous solution at 2°C. The specific activities of these fractions are shown in Table 4.20.

Table 4.20

Fraction Number	1	2	3	4	5	6	7
Specific α-amylase activity	0.43	0.27	1.1	3.9	0.72	0	0
Specific β-amylase activity	2.44	2.70	7.3	8.1	27.6	10.2	0.7

Calcium acetate (60 mg.) was added to a portion (30ml.) of fraction (4) and a sample (5ml.) was removed for activity determinations. The remaining solution was heated at 70°C. and samples (5ml.) were removed after intervals of 5, 10, 15, 30 and

40 minutes. They were quickly cooled and centrifuged to yield clear solutions 3 to 7. The activities and specific activities of the solutions are shown in Table 4.21.

Table 4.21

Solution Number	Original	2	3	4	5	6	7
α-amylase activity	1.22	1.18	0.90	0.74	0.73	0.73	0.73
α-amylase specific activity	3.9	5.9	8.7	7.5	7.5	7.3	7.3
β-amylase specific activity	8.1	7.9	0.3	0	0	0	0

A further sample of fraction 4 (145ml.) was heated (70°C.; 15 minutes) in the presence of calcium acetate (290mg.) cooled, centrifuged and the clear supernatant was saturated with solid ammonium sulphate and stored at 2°C. This was barley α-amylase sample B A having a specific activity of 7.5.

Final method adopted for the preparation of barley α-amylase. Dry, defatted flour (250g.) was shaken for 2 hours with calcium acetate solution (800 ml.; 0.2%) containing n-octanol (0.2 ml.). The suspension was centrifuged and the clear brown supernatant (containing 11.3 mg. protein/ml.) was heated (70°C.; 15 minutes) in aliquots (10-15 ml.). The resulting cloudy suspension was quickly cooled and centrifuged

to yield a clear brown solution (430 ml.; 9.76 mg. protein/ml.). Enzymic activities were measured on the extract before and after heating; the results are shown in Table 4.22.

Table 4.22

α-amylase activity		α-amylase specific activity		β-amylase activity		β-amylase specific activity	
Before heating	After heating	Before heating	After heating	Before heating	After heating	Before heating	After heating
0.64	0.64	0.46	0.53	3.5	0	2.44	0

The aqueous extract was cooled to 2°C. and fractionated with chilled acetone between the limits 0 - 30, 30 - 35, 35 - 40, 40 - 45, 45 - 50, 50 - 55 and 55 - 60%. When the acetone concentration was 20% the temperature of the solution was lowered to - 5°C. and was maintained at this low value throughout the fractionation.

The enzymic activities of the fractions are shown in Table 4.23.

Table 4.23

Fraction Number	Original	B1	B2	B3	B4	B5	B6	B7
α-amylase specific activity	0.53	1.93	5.8	2.3	2.1	1.4	0	0

Fraction B2 was subfractionated with acetone between the limits 0 - 27, 27 - 37, 37 - 42, 42 - 47, 47 - 52, 52 - 63%. The activities of the fractions are shown in Table 4.24.

Table 4.24

Fraction Number	B2	$B2_1$	$B2_2$	$B2_3$	$B2_4$	$B2_5$	$B2_6$
β-amylase specific activity	5.8	1.3	33.2	20.1	9.2	2.1	0

Fractions B1 (75 ml.) and $B2_2$ (10 ml.) were combined and further subfractionated with acetone between the limits 0 - 22, 22 - 27, 27 - 32, 32 - 37 and 37 - 70%. The activities of the fractions are shown in Table 4.25.

Table 4.25

Fraction Number	$(B1+B2_2)$	$(B1+B2_2)1$	$(B1+B2_2)2$	$(B1+B2_2)3$	$(B1+B2_2)4$	$(B1+B2_2)5$
β-amylase specific activity	4.8	0.41	0.47	0.62	51.3	9.8

Further purification of the enzyme via glycogen - complex formation was not achieved, probably, because of the very small quantity of enzyme used. The purifications obtained in the various stages of the procedure are shown in Fig. 4.09a.

FIG. 4.09a

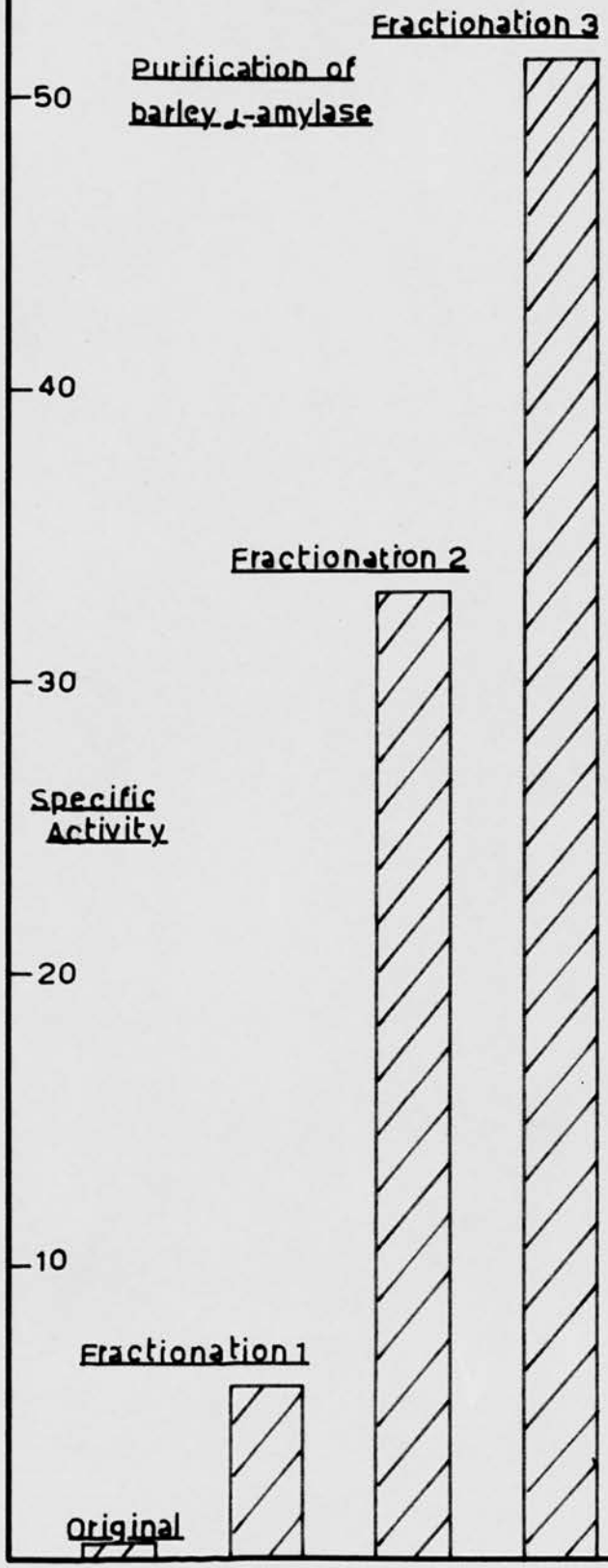


FIG. 4.09b

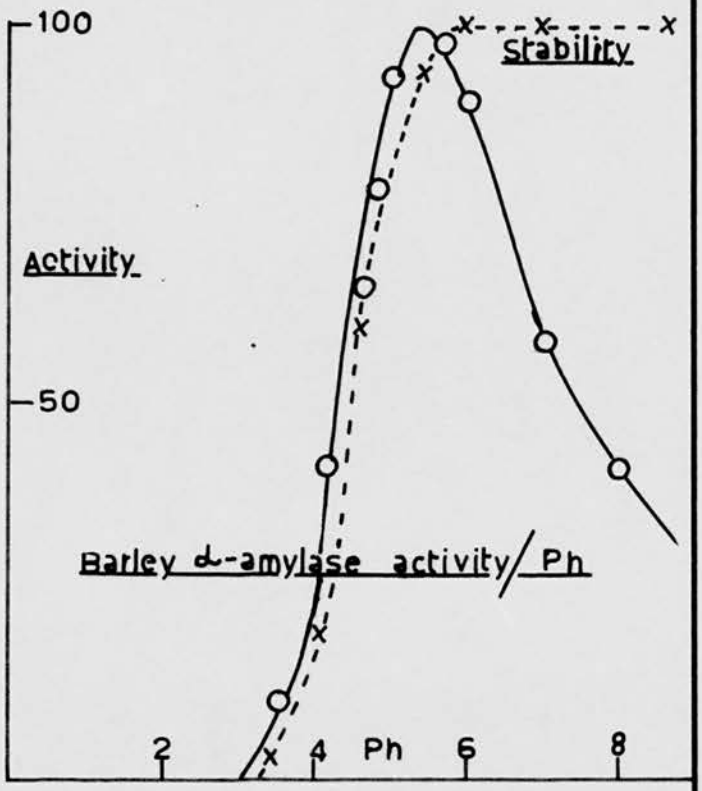
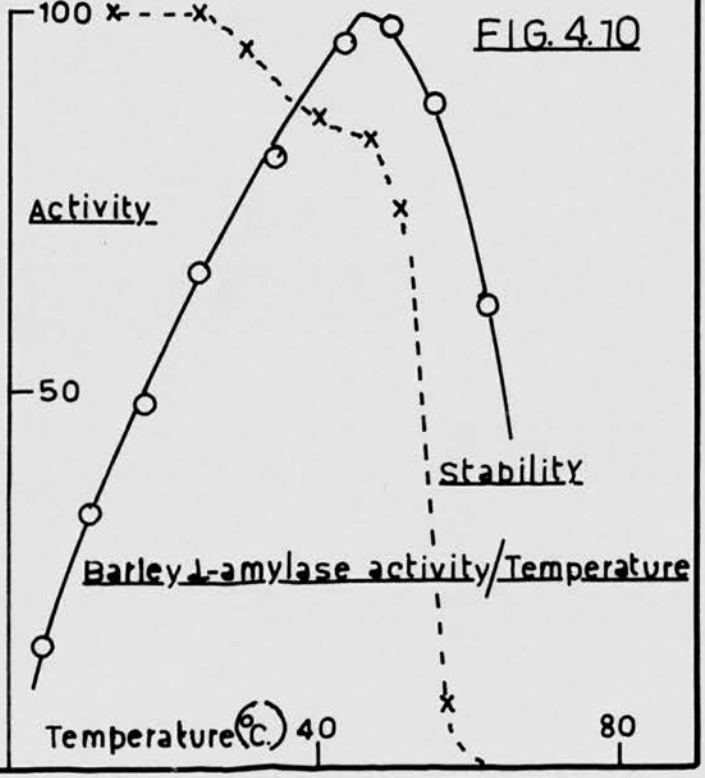


FIG. 4.10



RESULTS

Effect of pH on barley α-amylase. (a) Activity of barley α-amylase/pH. Digests were prepared containing enzyme solution (0.1 ml. fraction ($B1+B2_2$)4), acetate buffer (1ml.; 0.2M.; appropriate pH), amylopectin β-limit dextrin solution (2 ml.) and water (4.9 ml.). They were incubated at 35°C. and the α-amylase activities were determined. The results are shown in Fig. 4.09.

(b) Stability of barley α-amylase/pH. Digests were prepared containing enzyme solution (0.3 ml. fraction ($B1+B2_2$)4), water (4 ml.) and buffer (2ml.; 0.2M. appropriate pH). Acetate buffer was used to obtain pH values between 3 and 8, whilst borate buffer was used between pH 8 and 12. These digests were left at room temperature for 1 hour when their pH values were adjusted to 5.5 with either dilute ammonia or dilute acetic acid. Aliquots (6ml.) of amylopectin β-limit dextrin solution were then added to each and the α-amylase activities of the various digests were determined. A standard digest (pH 5.5), similar to those above, was prepared and its α-amylase activity was determined without prior incubation of the enzyme. The activities of the enzymic digests were calculated as a percentage of the activity of the standard digest. The results are shown in Fig. 4.09.

Effect of temperature on barley α-amylase. (a) Activity of barley α-amylase/temperature. Digests were prepared containing water (4.7 ml.), acetate buffer (0.2M.; pH 5.5; 1ml.) and amylopectin β-limit dextrin solution (2 ml.). They were

equilibrated at various temperatures between 9 and 71°C. when enzyme solution (0.3 ml. fraction ($B1 + B2_2$) 4) was added to each and the respective α-amylase activities were determined. The results are shown in Fig. 4.10.

(b) Stability of barley α-amylase/temperature. Digests containing water (4.7 ml.) and acetate buffer (1 ml.; 0.2M.; pH 5.5) were equilibrated at various temperatures between 9 and 71°C. Aliquots of enzyme solution (0.3 ml. fraction ($B1 + B2_2$) 4) were added to each digest and they were incubated at the various temperatures for 1 hour. The digests were then quickly brought to equilibrium at 35°C; aliquots (2 ml.) of amylopectin β-limit dextrin were added to each prior to determining the α-amylase activities. A "standard activity" was carried out using an enzyme sample which had been stored at 2°C., and the activities of the enzymic digests were calculated as a percentage of the activity of the standard digest. The results are shown in Fig. 4.10.

(c) Activation Energy of barley α-amylase. The results showing the variation of α-amylase activity with temperature were utilised in determining the activation energy of the enzyme as shown in Fig. 4.11.

Effect of E.D.T.A. and trypsin on barley α-amylase. Digests were prepared as follows:

1. Acetate buffer (pH 5.5; 0.2 M.; 2 ml.) + E.D.T.A. (2 ml.; 10^{-1}M.) + enzyme solution (1 ml. fraction B A) + water (13 ml.).

FIG. 4.11

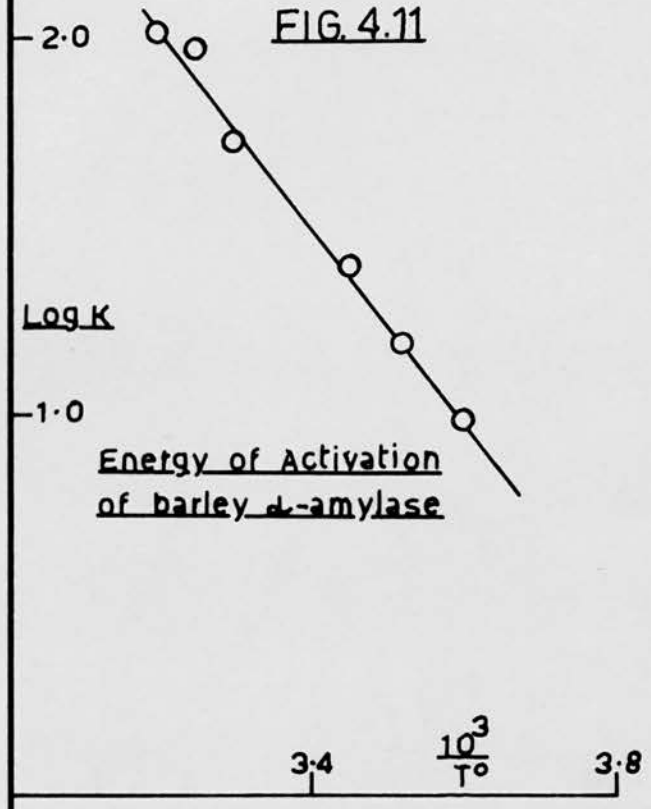


FIG. 4.12

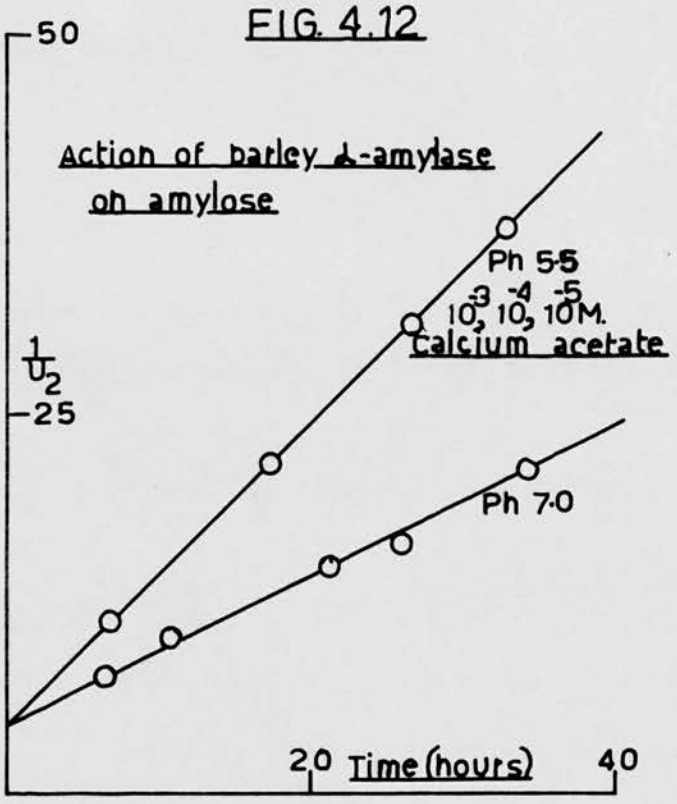


FIG. 4.13

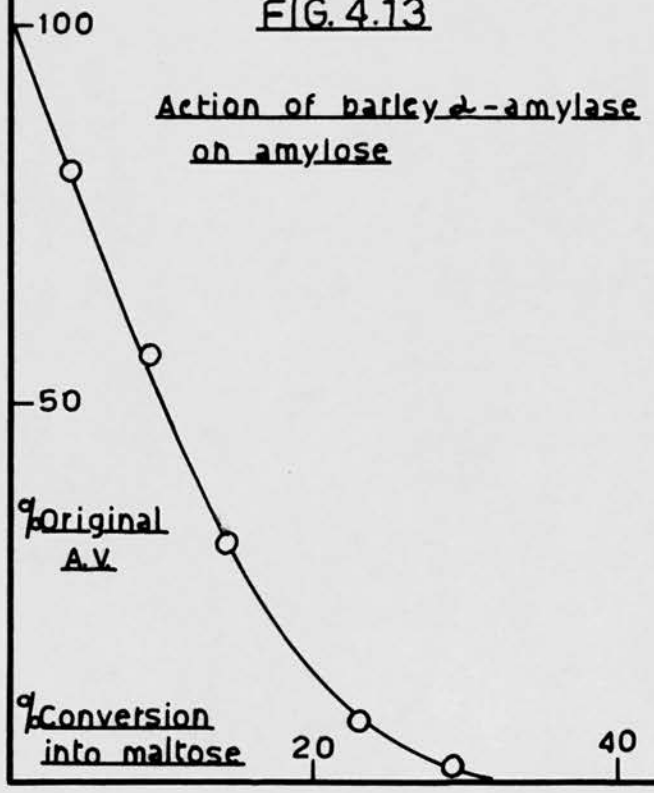
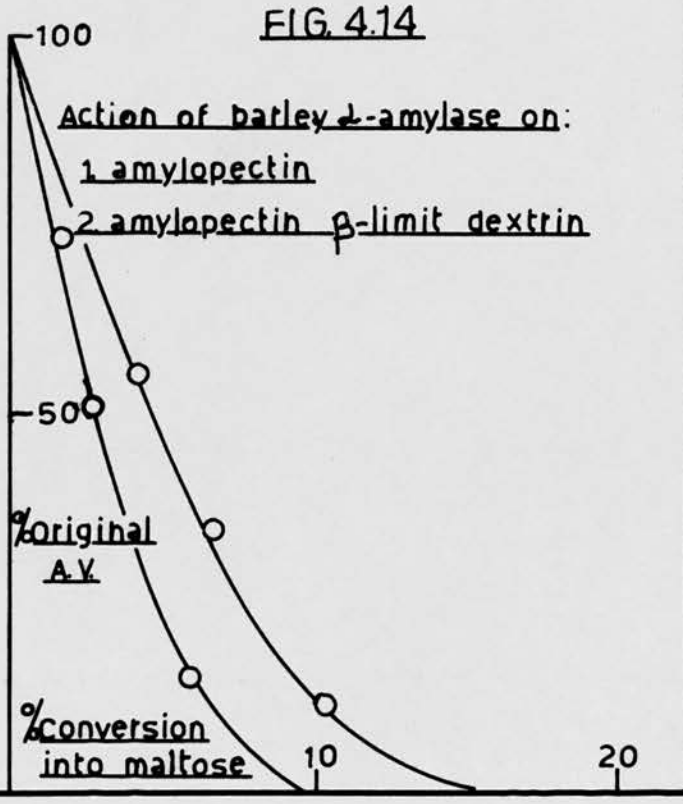


FIG. 4.14



2. Acetate buffer (pH 5.5; 0.2M.; 2 ml.) + E.D.T.A. (2 ml.; 10^{-1}M.) + trypsin (0.2 ml.; 6 mg./ml.) + enzyme solution (1 ml. fraction B A) + water (13 ml.).
3. Acetate buffer (pH 5.5; 0.2M.: 2 ml.) + trypsin (0.2 ml.; 6 mg./ml.) + enzyme solution (1 ml. fraction B.A) + water (15 ml.).
4. Acetate buffer (pH 5.5; 0.2M.; 2ml.) + enzyme solution (1 ml. fraction B A) + water (15 ml.).

These were incubated at 35°C. for 15 minutes after which amylopectin β-limit dextrin (5 ml.) was added to each and the α-amylase activities were determined. The above experiments were repeated, but the time of preincubation was increased from 15 minutes to 12 hours. The results are shown in Table 4.26.

Table 4.26

	% Inhibition	
Digest	15 minutes preincubation	12 hours preincubation
1	51	90
2	74	100
3	0	0
4	0	0

Effect of ions on barley α-amylase. Digests were prepared containing acetate buffer (2 ml.; 0.2M.; pH 5.5),

enzyme solution (1 ml. fraction B A), and the requisite amount of salt solution and water to make the total volume of the digests 10 ml. The results are shown in Table 4.27.

Table 4.27

	% Inhibition			
Salt	10^{-3}M	10^{-4}M	10^{-5}M	10^{-6}M
$HgCl_2$	100	100	90	30
K C N	0	0	0	0
Na Cl	0	0	0	0
$CaCl_2$	0	0	0	0
Ammonium Molybdate	20	2	0	0

Purity of barley α-amylase fraction (B1 + $B2_2$)4. Digests were prepared containing enzyme solution (0.1 ml. fraction (B1 + $B2_2$)4) and samples (ca. 1 mg.) of laminarin, maltose and maltotriose. They were incubated at 35°C. and aliquots were removed at intervals for examination by paper chromatography. No glucose was detected in the laminarin and maltose digests but trace amounts of glucose and maltose were found in the maltotriose digest after prolonged digestion (96 hours).

A digest was prepared containing enzyme solution (0.3 ml. fraction (B1 + $B2_2$)4), glucose 6-phosphate (1 ml.; 5mg./ml.), acetate buffer (1ml.; 0.2M.; pH 4.6) and water (3.7 ml.). It was incubated at 35°C. and aliquots (1 ml.)

were removed at intervals for the determination of free phosphate by the method of Allen (1940). After 96 hours there was no detectable phosphatase activity.

Action of barley α-amylase on amylose. (a) Kinetics of the initial reaction. The experiments were similar to those carried out with soya bean Z-enzyme described in Section 4a. Amylose solution (19 ml.; 3mg./ml.), dilute enzyme solution [0.1 ml. of fraction (B1 $B2_2$)4 was diluted to 0.3 ml. with water and 0.1 ml aliquots of this dilute solution were used] and acetate buffer (1 ml.; 0.2M.; pH 5.5 or 7.5) were added to a viscometer at 25°C. The flow times of the solution were determined at various times. Similar experiments were carried out in calcium acetate solutions (10^{-3}, 10^{-4} and 10^{-5}M.) of constant ionic strength (μ = 0.118). The results are shown in Fig. 412.

(b) Products of the reaction. A digest was prepared containing amylose solution (60 ml.; 2mg./ml.) and enzyme solution (1 ml. fraction (B1 + $B2_2$)4). It was incubated at 35°C. and aliquots (1.25 ml.) were removed at intervals for reducing power determinations (1 ml.) and iodine staining measurements (0.25 ml.). Larger aliquots (5 ml.) were removed after 24, 48, 72 and 120 hours, evaporated to dryness on a rotary evaporator, suspended in water (0.75 ml.) and applied to paper chromatograms. The results of these experiments are shown in Fig. 4.13 and Table 4.28.

Small aliquots (0.25 ml.) of the concentrated solutions which were applied to the chromatograms were tested with

iodine solution (0.05 ml. of 0.2% iodine in 2% potassium iodide). The results are shown in Table 4.28.

Table 4.28

	Relative amounts of saccharides			
Time of aliquot extraction	24 hours	48 hours	72 hours	120 hours
G_1	+	+ +	+ + +	+ + + +
G_2	+	+ +	+ + +	+ + + +
G_3	+	+	+ +	+ + +
G_4	+	+	+ +	+ + +
G_5	+	+	+ +	+ + +
G_6	+	+	+ +	+ + +
G_7	+	+ +	+ + +	+ +
G_8	+	+ +	+ + +	+ +
Higher oligosaccharides	+ + + + +	+ + + +	+ +	+
Iodine stain of the concentrated digests	Blue	Blue	Red	Red

Action of barley α-amylase on amylopectin and amylopectin β-limit dextrin. Digests were prepared containing enzyme solution (1 ml. fraction B A), amylopectin or

amylopectin β-limit dextrin solution (5 ml.; 3.5 mg./ml.), acetate buffer (0.2M.; pH5.5; 2 ml.) and water (12 ml.). Aliquots were removed at intervals for iodine staining and reducing power determinations. The results are shown in Fig. 4.14.

Action of barley α-amylase on amylose, glycogen and glycogen β-limit dextrin. (In collaboration with Mr. R. Geddes). The degradation of these substrates with barley α-amylase fraction ($B1 + B2_2$)4 was followed by lightscattering measurements and the relative rates of attack are given in Table 4.29.

Table 4.29

Substrate	Amylose	Glycogen	Glycogen β-limit dextrin
Rate of attack	850	9.8	1

DISCUSSION

Preparation of barley α-amylase. The initial enzyme preparation (p. 117) was unsatisfactory because the dried protein fraction was very insoluble in water. Ammonium sulphate precipitated protein fractions are usually readily soluble in cold water and so, in preparation (2) (p. 117) the enzyme fractions were stored as suspensions in saturated ammonium sulphate solutions. During the heating stage, however, both the α- and β-amylase activities were destroyed in fraction 4 and this was thought to be due to a lowering of the pH of the heated solution caused by the presence of a small amount of ammonium sulphate. In preparation (3) (p. 119), therefore, the acetone-precipitated fractions were air-dried to remove excess acetone and stored in aqueous solution at 2°C. at which temperature they remained stable over a period of 4 weeks.

The results in Table 4.21 (p. 120) illustrate the effect of a short heat treatment on the α- and β-activities of fraction 4 (preparation 3) in the presence of calcium ions. After 15 minutes, the β-amylase activity is completely destroyed whereas the α-amylase activity is decreased by ca. 40% and its specific activity is increased by ca. 90%. These results are similar to those obtained with soya bean Z-enzyme, and the increase in the specific activity of the α-amylase is due to the removal of a large amount of inert protein by coagulation during the heating stage. The 40% loss in activity during the heating stage is not very satisfactory, but this fall might be reduced if the heated protein solution contained a higher concentration

of protein. In the final preparation procedure, therefore, the heat-treatment was carried out on the initial aqueous extraction which contained a relatively high protein concentration.

The results in Table 4.22 (p. 121) show that, once again, the β-amylase activity has been destroyed but, in this experiment, the α-amylase activity has not been reduced.

The overall purification factor is ca. 110 but the yield of enzyme is very small and this is probably the reason for the inability of the relatively pure fraction $(B1 + B2_2)4$ to form an insoluble glycogen complex. It is known that if there is insufficient enzyme present then the complex does not form satisfactorily (Levitzki et al, 1964).

Effect of pH and temperature on barley α-amylase. The pH of maximum activity is 5.4 (Fig. 4.09), and it should be noted that the activity falls rapidly at pH levels where the enzyme is stable for 1 hour. The enzyme shows surprising stability at high values of pH, but its activity is rapidly decreased when it is maintained at low levels of pH (< pH 4.6). This denaturation is irreversible since a sample, which has been treated with buffer at pH 4, does not recover its activity on being stored at 2°C. in the presence of buffer at pH 5.5 over a period of 3 - 4 days.

The temperature of maximum activity is ca. 47°C. and the enzyme is fairly stable at this temperature, losing only 17% of its activity after 1 hour. (Fig. 4.10).

The energy of activation of barley α-amylase is 12 K cals. (Fig. 4.11) and this is extremely low in comparison with

values for non-enzymic processes. The acid-catalysed hydrolysis of similar compounds yields a value of 30 K. cals (French, 1957). One explanation of the lowering of the activation energy is that the enzyme more readily attacks a strained form of the substrate, and so only a small additional amount of activation energy is required to raise the enzyme-substrate complex to the fully activated state when reaction will be rapid.

Effect of reagents on barley α-amylase. The results in Table 4.26 (p. 125) illustrate the effect of E.D.T.A. and trypsin, acting separately and together, on the activity of barley α-amylase. Inhibition is complete when E.D.T.A. and trypsin act together on the enzyme. Trypsin, by itself, has no effect on the enzyme and E.D.T.A. requires a long digestion time before it completely deactivates the α-amylase. In the presence of calcium ions, trypsin is unable to attack the α-amylase but, when these ions are removed from the enzyme by E.D.T.A., the configuration of the enzyme must be altered and it is rendered susceptible to attack by trypsin.

Mercuric chloride (10^{-3}M. and 10^{-4}M.) completely inhibits barley α-amylase activity but ammonium molybdate (10^{-3}M.) has only a slight effect (Table 4.27; p. 126).

Purity of enzyme fraction (B1+ $B2_2$)4. The results in Table 4.22 (p. 121) show that there is no β-amylase present in any of the enzyme fractions obtained during the final α-amylase preparation. Chromatographic evidence suggests that maltase and laminarinase are absent from fraction (B1+ $B2_2$)4 and the inability of a sample of this fraction to produce free phosphate from

a solution of glucose 6-phosphate indicates the absence of phosphatase activity.

Action of barley α-amylase on amylose. The straight lines shown in Fig. 4.12 indicate the random nature of the enzymic hydrolysis of amylose. In the presence of various amounts of calcium acetate (10^{-3}M.; 10^{-4}M.; 10^{-5}M.), the action pattern does not appear to vary and the calcium ions do not inhibit or activate the α-amylase activity.

Because of the low α-amylase activity in barley-flour extracts, final reaction products for the action of the enzyme on amylose were not obtained, but the general features are readily apparent from Fig. 4.13 and Table 4.28 (p. 128). The initial detectable products of the reaction are the small oligosaccharides G_1 and G_2, small amounts of G_7 and G_8, and larger amounts of higher oligosaccharides. As the hydrolysis proceeded all the maltodextrins (G_1 - G_8) increased in amount but the larger dextrins (> G_8) steadily disappeared. Not even after prolonged digestion, however, did the digest become achroic indicating the very slow hydrolysis of maltodextrins less than 8 to 10 glucose units in length.

Action of barley α-amylase on amylopectin and amylopectin β-limit dextrin. Both of these substrates are hydrolysed by the enzyme (Fig. 4.14). This degradation is accompanied by loss in iodine staining capacity and increase in reducing power. The dextrin reached its achroic limit at a smaller percentage conversion into maltose than did the amylopectin. This result is to be expected from the molecular

structures of the two substrates.

Relative rates of attack on amylose, glycogen and its β-limit dextrin. The results (Table 4.29 p. 129) indicate that the enzyme hydrolyses amylose some 100 x faster than glycogen. Attack on the glycogen limit dextrin is slight, being some 10 x less than that on the glycogen itself. The enzyme is thus not readily able to penetrate into the compact limit dextrin molecule.

4.c. MALTED BARLEY α-AMYLASE

INTRODUCTION

The most widely studied plant α-amylase is that obtained from malted barley. Many investigations of the action pattern of this enzyme have been made but much of the early work is invalid because very little attempt was made to obtain the enzyme in a pure form. Although this enzyme has been crystallised (Schwimmer and Balls, 1949) amorphous enzyme preparations may be used in action-pattern studies if care is taken to eliminate other carbohydrase enzymes.

Perhaps the simplest method of obtaining information about the action pattern of malt α-amylase is to study the production of oligosaccharides from amylose/α-amylase digests, but such investigations have given rise to many conflicting results. From studies on the degradation of amylose by malt α-amylase, Meyer and his associates (Meyer and Bernfeld, 1941; Meyer and Gonon 1951a, b) concluded that all the bonds in amylose, except the terminal ones, are equally susceptible to attack by the enzyme. This indicates that the hydrolysis is almost completely random. The glucose produced during the reaction was thought to arise solely from the slow hydrolysis of maltotriose and so the end-products of the reaction would be glucose and maltose. Bernfeld and Studer - Pécha (1947) showed that malt α-amylase reacted much more slowly with maltodextrins (D.P. ca. 8) than it did with starch. They attributed this lowering of the reaction rate, not to a difference in enzyme-

substrate affinity, but to retrogradation of the substrate. Other workers, however, (Hanes and Cattle, 1938; Myrbäck, 1948; Schwimmer, 1950) proposed that the difference in the rate of hydrolysis was due to a difference in enzyme-substrate affinity. This view was further confirmed when Myrbäck (1950) showed that at the stage where the reaction decreased nearly all of the amylose substrate was recovered as small dextrins having average chain-lengths of 6 glucose units. Retrogradation of these oligosaccharides is highly unlikely.

The presence of glucose in the early stages of amylose/malt α-amylase hydrolysis has been shown by Myrbäck (1953), and so the postulate of Meyer and Bernfeld, that glucose is formed only from maltotriose, is unlikely. Myrbäck further showed that the hydrolysis of a small starch dextrin (Average D.P. ca. 6) gave rise to glucose as a primary product. These results indicate that at least one of the terminal linkages in amylose-type molecules is susceptible to attack by malt α-amylase. Further, when the starch dextrin, mentioned above, was converted to the hexaonic acid and digested with malt α-amylase, glucose was not formed. Since the dextrins differed only in the nature of their reducing groups (the acidic grouping is formed at the reducing end of the G_6 molecule), it must be concluded that malt α-amylase splits off glucose from the reducing end of a substrate molecule. This result was later confirmed by Hopkins and Bird (1954). These authors further suggested that malt α-amylase does not readily attack the first five linkages from the non-reducing end of substrate molecules. The linkage nearest to the reducing end

of the molecule is hydrolysed with difficulty, but the second and third linkages are more susceptible to attack, provided that they are not within the vicinity of the non-reducing group. This hypothesis explains the build-up of oligosaccharides having chain-lengths of 6 - 8 glucose units in amylose/malt α-amylase digests because the enzyme more reddily hydrolyses iodine-staining dextrins (D.P. > 10) than the achroic ones. These results suggest then, that towards the end of the amylase action on amylose the hydrolysis is not random but specific.

The increase in the amylase content of germinating cereals such as barley, wheat and rye has been known for many years (Mayer and Klinga - Mayer, 1940; Kneen, 1944). Because of the small amount of α-amylase activity present in the resting seed, no studies have previously been carried out to determine if the α-amylase activity in the germinated seed is a more highly active form of that in the resting seed or whether the action patterns of the two enzymes are different. In order to make such an investigation a sample of the barley studied in the previous section was carefully malted and the resulting α-amylase activity was extracted and examined. In addition, the action of the malt α-amylase on amylose was investigated in an attempt to elucidate the kinetics of the initial stages in the hydrolysis (i.e. whether the reaction is random or specific) and to find out at what stage in the reaction glucose is produced. The enzymic hydrolysis of oligosaccharides (G_4, G_7 - G_{10}) was also studied to test the hypothesis of Hopkins and Bird.

EXPERIMENTAL

Preparation of barley-malt. The procedure of Whitmore and Sparrow (1957) was used.

Samples (200 g.) of barley seed were placed in each of three large jam jars (2 lb.), covered with water and left at 22°C. for 4 days. Each day the water was removed and replaced by a fresh sample. After the 4th day, the water was not replaced and the barley was maintained at 22°C. for a further 5 days when much sprouting took place. Each day the barley was turned out on filter paper and the rootlets were carefully disentangled. After 9 days the barley was placed in a ventilated oven, heated for 24 hours at 122°F., followed by 24 hours at 140°F. The barley was then sieved to remove the shoots and rootlets, finely ground into flour and thoroughly extracted with ether.

Initial attempt to prepare malt α-amylase. Malt flour (110 g.) was shaken with water (410 ml.) containing *n*-octanol (0.2 ml.) for 6 hours at room temperature. The suspension was clarified, by centrifugation and filtration, cooled to 2°C. and fractionated with chilled acetone between the limits 0 - 30, 30 - 35, 35 - 40, 40 - 45, 45 - 50, 50 - 55, 55 - 60 and 60 - 70%. The initial aqueous extract had a protein concentration of 20 mg. protein/ml. The fractions were dried with a stream of air, dissolved in water and stored at 2°C. Suitable aliquots were removed and tested for enzymic activity; the results are shown in Table 4.30.

Table 4.30

Fraction Number	Original	1	2	3	4	5	6	7	8
α-amylase specific activity	710	2,800	3,400	2,140	940	275	102	0	0
β-amylase specific activity	99	128	250	270	350	375	200	170	20

Fraction 2(40 ml.) was heated (70°C.; 15 minutes) in the presence of calcium acetate (80 mg.) and tested for enzymic activity. Both the α- and β-amylase activities were destroyed. Fraction 3(30 ml.) was reprecipitated with cold acetone (-15°C.; 30 ml.), dissolved in a small volume of water and heated (70°C.; 15 minutes) in the presence of calcium acetate. The β-amylase activity was completely destroyed but some α-amylase activity (40%) remained. The clear solution was dialysed against distilled water for 12 hours at 2°C., tested for activity, and placed on a G - 25 "Sephadex" column which was eluted with water. The column speed was 35 ml./hour and 5 ml. fractions were collected. Fig. 4.15 illustrates the elution diagram obtained. α-amylase activity determinations were carried out on the fractions containing protein, but no activity was detected.

A further sample (30 ml.) of fraction 3 was heat-treated in the presence of calcium ions and dialysed against calcium chloride solution (0.2%) at 2°C. for 12 hours. This enzyme sample was placed on a G - 25 "Sephadex" column which had

FIG. 4.15

Fractionation of malted barley β-amylase on a G-25 Sephadex column (eluant: water)

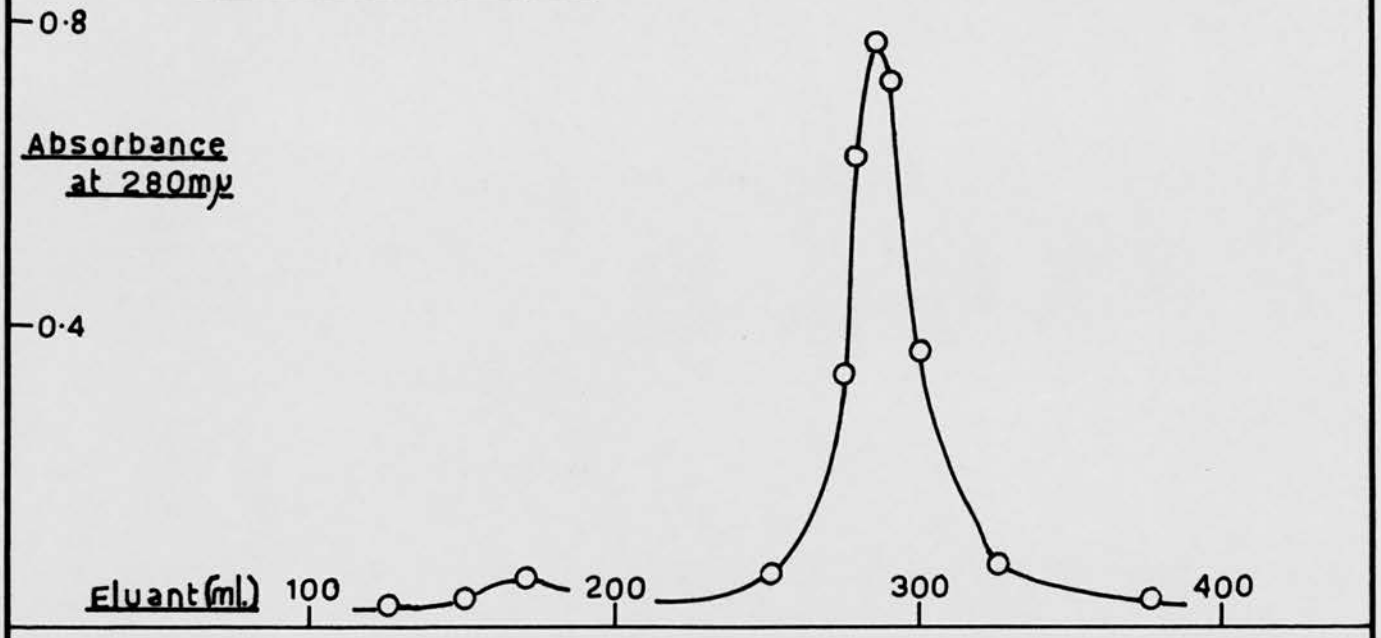
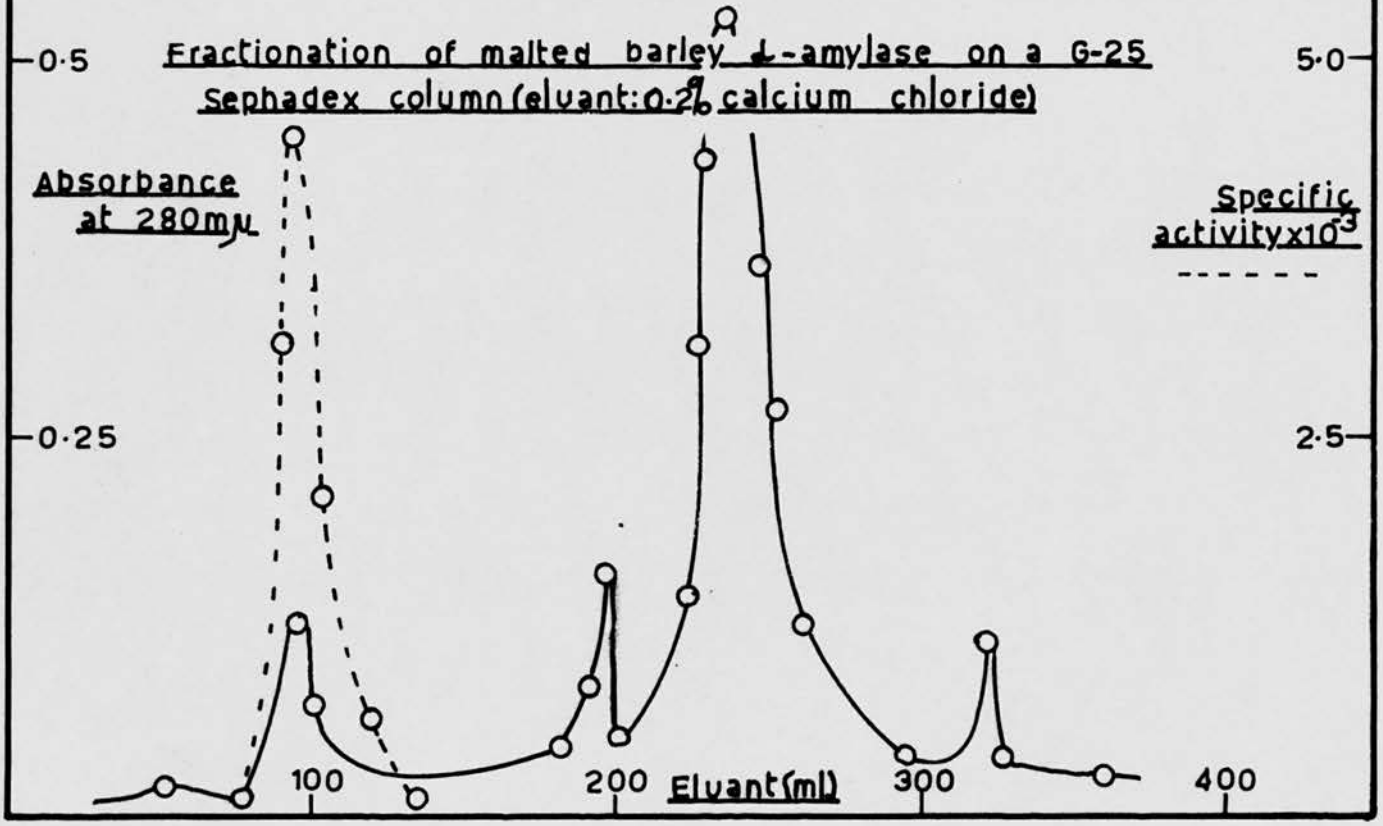


FIG. 4.16

Fractionation of malted barley β-amylase on a G-25 Sephadex column (eluant: 0·2% calcium chloride)



been equilibrated with calcium chloride (0.2%). The column was eluted with calcium chloride solution (0.2%) and 5 ml. fractions were collected. The results are shown in Fig. 4.16. The α-amylase activity was increased by a factor of 2.5.

Final preparation of malt α-amylase. Barley malt flour (250 g.) was shaken with calcium acetate solution (800 ml.; 0.2%) in the presence of n-octanol (0.2 ml.) for 2 hours at room temperature. The suspension was then centrifuged and filtered to yield a clear brown solution (590 ml.; pH 5.7; 16.8 mg. protein/ml.). The effect of pH on the stability of the α- and β-amylases towards heat was examined by preparing the following digests:

1) Aqueous extract (3ml.) + water (0.25ml.)

2) Aqueous extract (3ml.) + acetate buffer (0.25ml.; 0.2M.; pH5.8)

3) " " " + " " (0.25ml.; 0.2M.; pH5.4)

4) " " " + " " (0.25ml.; 0.2M.; pH4.8)

5) " " " + " " (0.25ml.; 0.2M.; pH4.4)

These solutions were heated (70°C.; 15 minutes), quickly cooled and centrifuged to yield clear solutions which were examined for enzymic activity. The results are shown in Table 4.31.

Table 4.31

Digest Number	Aqueous extract	1	2	3	4	5
α-amylase specific activity	415	420	418	418	420	420
β-amylase specific activity	70	0	0	0	0	0

The remaining aqueous extract (560 ml.) was heated (70°C.; 15 minutes) in small aliquots (10 - 15 ml.), quickly cooled and centrifuged to yield a clear solution which was cooled to 2°C. and fractionated with acetone between the limits 0 - 30, 30 - 35, 35 - 40, 40 - 45, 45 - 50, 50 - 55, 55 - 60 and 60 - 70%. When the solution was 20% with respect to acetone, the temperature was lowered to -5°C. and was maintained at this low value throughout the fractionation. These protein fractions were air-dried and dissolved in water to give solutions A1 to A8 which were stored at 2°C. The activities of the fractions were determined and the results are shown in Fig. 4.17. The distribution of the total λ-amylase activity throughout the fractions is shown in Table 4.32.

Since fractions A2 and A3 contained 52% of the original activity they were combined and subfractionated with acetone between the limits 0 - 30, 30 - 33, 33 - 38, 38 - 40, 40 - 43, 43 - 48, 48 - 53, 53 - 58, 58 - 63 and 63 - 70% with respect to acetone. The fractionation was carried out at low temperature (-5°C.) and the fractions were air-dried before being dissolved in water to yield solutions 2A1 to 2A10.

The λ-amylase activities of the fractions were determined and the results are shown in Fig. 4.18. Fraction 2A3 was further purified, via glycogen complex formation, in the following way. An aliquot (15 ml.) of fraction 2A3 was cooled to 2°C. and chilled alcohol (10 ml.) was slowly added with stirring. After a short time (10 minutes) there was no precipitate and glycogen solution (0.4 ml.; 2%) was added. The precipitate was

FIG. 4.17

Fractionation of malted barley α-amylase

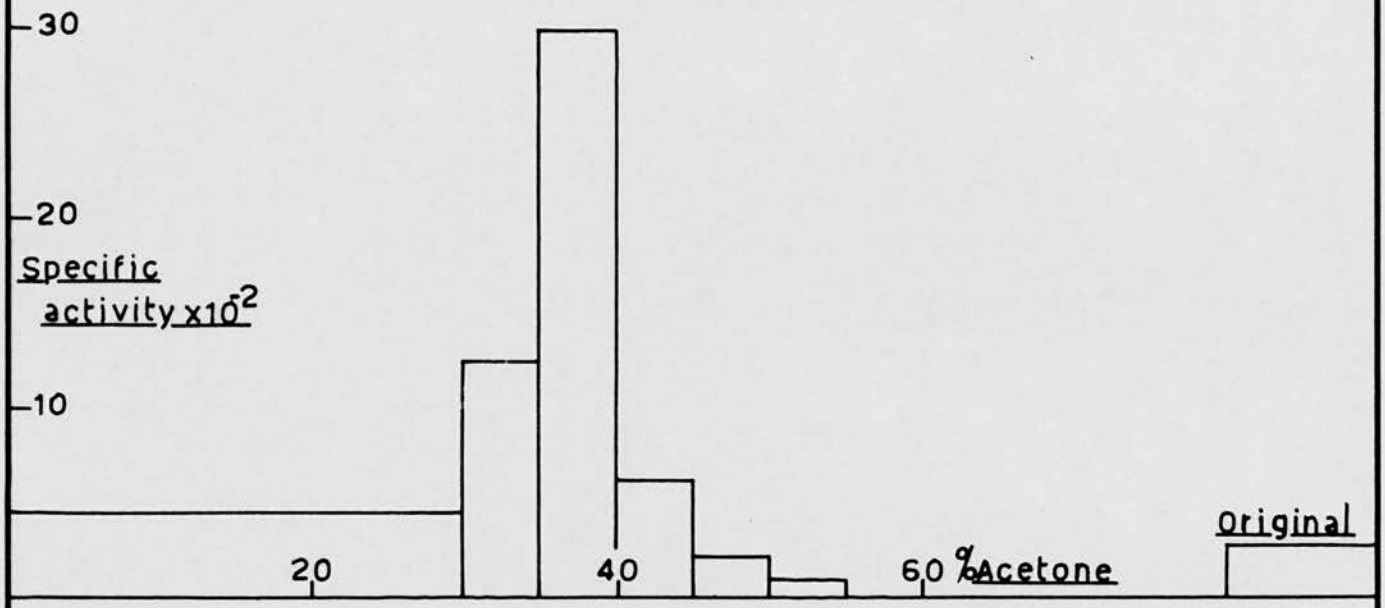
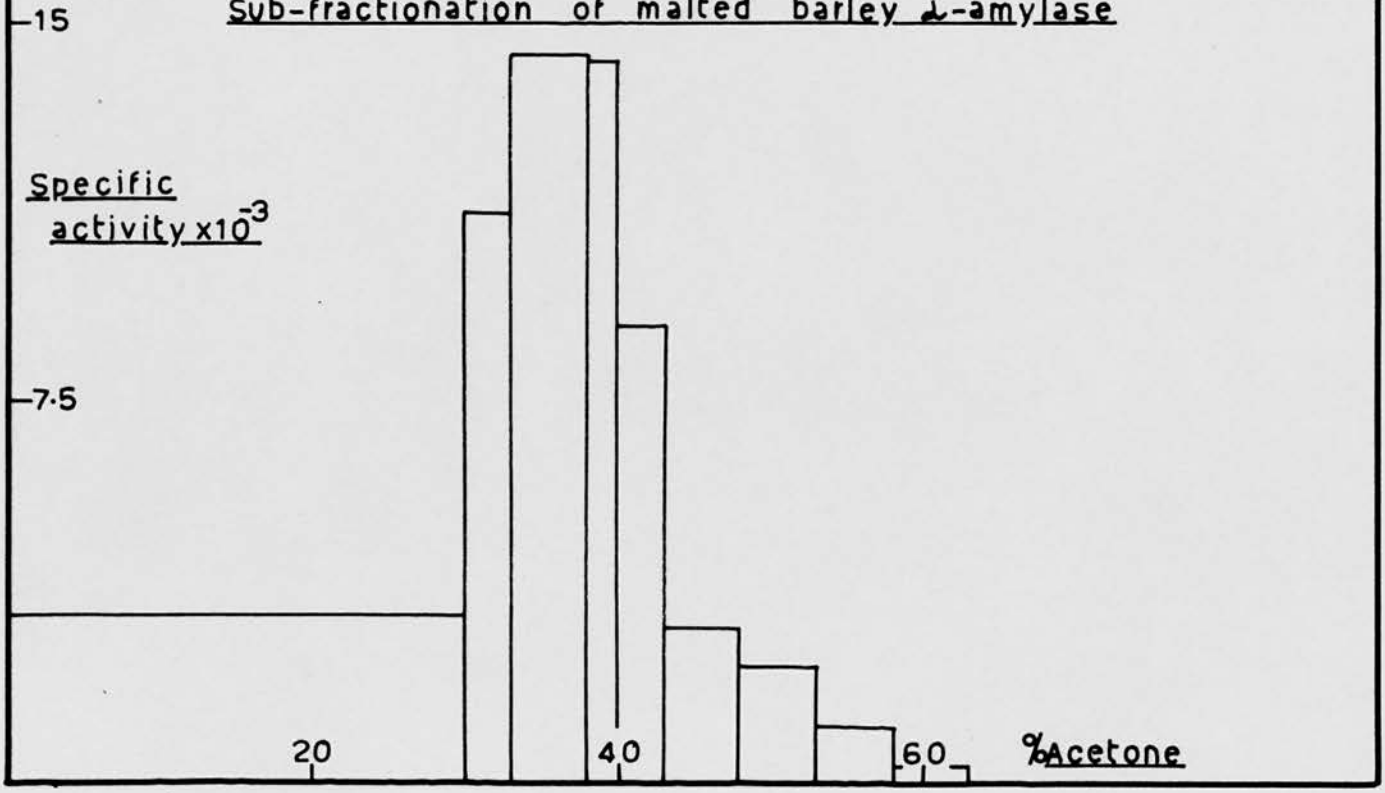


FIG. 4.18

Sub-fractionation of malted barley α-amylase



immediately removed by centrifugation, washed with aqueous alcohol (5 ml.; 40%) and dissolved in distilled water. The solution was left at room temperature for a time (4 - 5 hours) to enable the α-amylase to completely hydrolyse the glycogen, reprecipitated with cold acetone (50%), dialysed against dilute buffer (0.01M. acetate buffer pH5.5) and stored at $2^{\circ}C$. This was enzyme sample G2A3. A portion (0.2ml.) of it was placed on a chromatogram and tested for reducing sugar but none was detected. The specific activity of the sample was found to be 56.7×10^3 which represents an overall purification of 170. The purifications obtained at each stage in the procedure are shown in Fig. 4.19

Table 4.32

Fraction Number	Total Activity	% Original Activity
Aqueous extract after heat treatment	250×10^4	100
A1	156×10^3	6
A2	530×10^3	21
A3	770×10^3	31
A4	180×10^3	7
A5	32×10^3	2
A6	20×10^3	0.8
A7	4×10^3	0.16
A8	2×10^2	0.08

FIG. 4.19

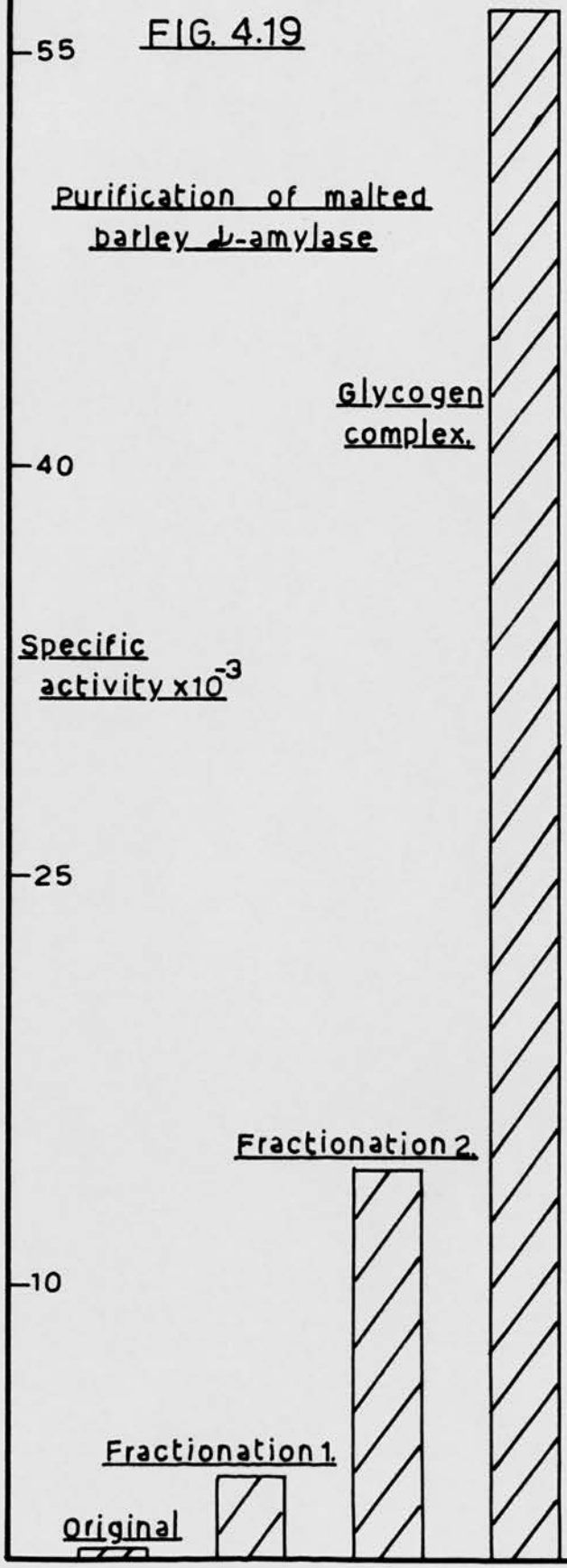


FIG. 4.20

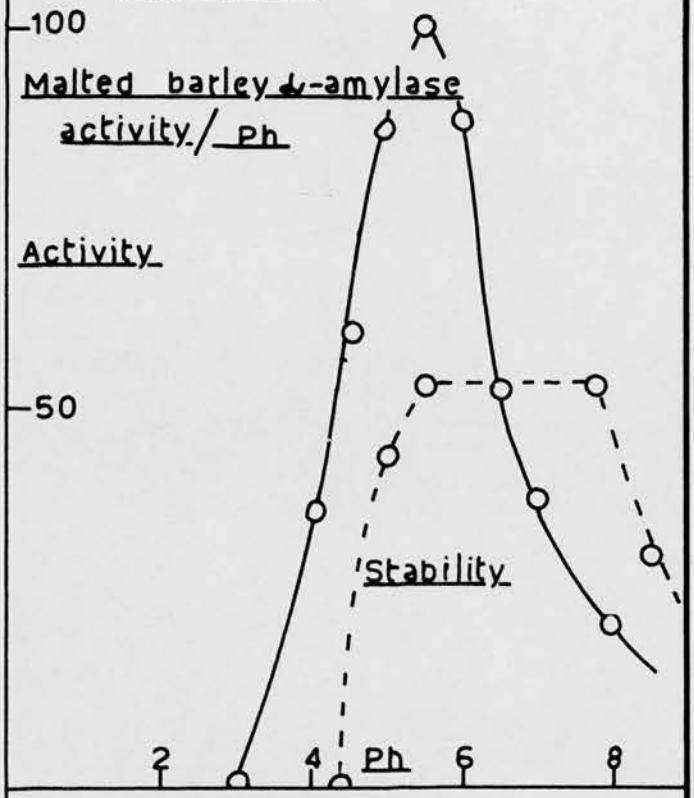
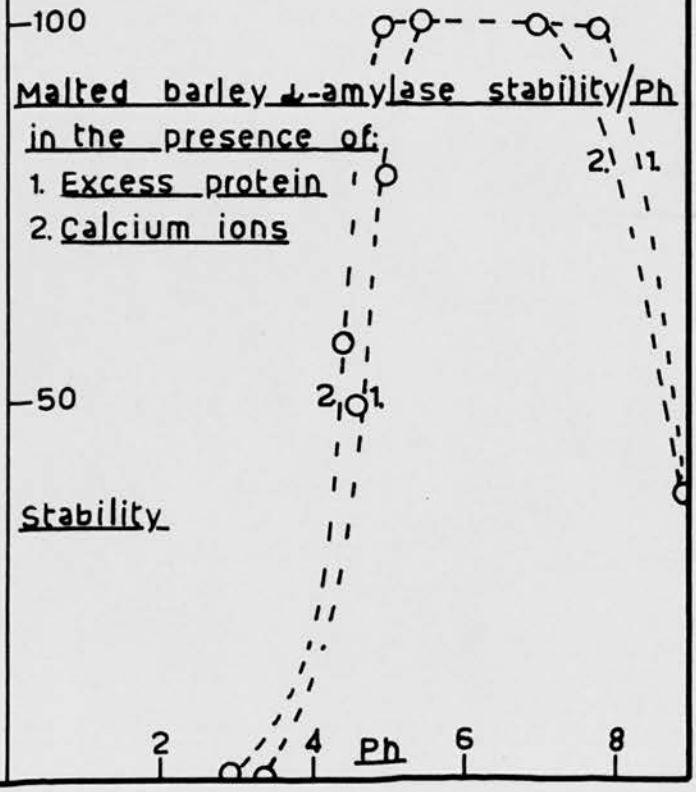


FIG. 4.21



Preparation of "Sephadex" column. Dry G - 25

"Sephadex" powder (80 g.) was stirred into a beaker containing dilute salt solution (0.05M.). After sedimentation and decantation to remove the finest gel particles, the suspension was left for 24 hours to complete its swelling. A chromatographic tube (120 cm. x 2.5 cm.) was partially filled with water, and mounted vertically. A large filter funnel was fitted to the top such that its tip was below the level of water in the tube. The suspension was added to the funnel, slowly stirred, and allowed to sediment to the bottom of the tube. When a layer of 2 to 5 cm. had formed, the lower part of the column was opened to allow through a slow stream of water. When all the gel grains had settled a circular piece of filter paper was placed on the top of the bed. The column was well washed with water, and the initial flow-rate was 70 ml./hour.

RESULTS

Purity of enzyme sample G2A3. Digests were prepared containing enzyme solution ($\frac{1}{40}$ ml.) and aliquots (ca. 1 mg. in 0.1 ml. of water) of laminarin, maltose and cellobiose. These were incubated at 35°C. and aliquots were removed at intervals for examination by paper chromatography. No glucose was detected in any of the digests indicating the absence of laminarinase, maltase and cellobiase in the enzyme preparation.

Effect of pH on malt α-amylase. (a) Activity of malt α-amylase/pH. Digests were prepared containing enzyme solution ($\frac{1}{40}$ ml.), acetate buffer (1 ml.; 0.2M.; appropriate pH) amylopectin β-limit dextrin solution (2 ml.) and water (5 ml.). These were incubated at 35°C. and the α-amylase activities were determined. The results are shown in Fig. 4.20.

(b) Stability of malt α-amylase/pH. Digests were prepared containing enzyme solution ($\frac{1}{40}$ ml.), water (4 ml.) and buffer (2 ml.; 0.2M.; appropriate pH). They were left at room temperature for 1 hour when their pH values were adjusted to 5.5. with either dilute ammonia or acetic acid. Aliquots (6 ml.) of amylopectin β-limit dextrin solution were then added to each and the α-amylase activities of the various digests were determined and expressed as a percentage of the activity of a standard digest (pH 5.5) in which the enzyme was not preincubated. The results are shown in Fig. 4.20.

(c) Stability of malt α-amylase/pH in the presence of calcium ions. The above experiment was repeated but the digests

included calcium acetate (1.4×10^{-3}M.). The results are shown in Fig. 4.21.

(d) Stability of malt α-amylase/pH in the presence of inert protein. The above experiment was repeated but aliquots (1 ml.) of inert protein (fraction 2A10) were added to each digest. The results are shown in Fig. 4.21.

Effect of temperature on malt α-amylase. (a) Activity of malt α-amylase/temperature. Digests were prepared containing water (5 ml.), acetate buffer (1 ml.; 0.2M.; pH 5.5) and amylopectin β-limit dextrin solution (2 ml.). They were equilibrated at various temperatures between 5°C. and 75°C. before enzyme solution ($\frac{1}{40}$ ml. G2A3) was added to each. The α-amylase activities were determined and the results are shown in Fig. 4.22.

(b) Stability of malt α-amylase/temperature. Digests containing water (5 ml.) and acetate buffer (1 ml.; 0.2M.; pH 5.5) were equilibrated at various temperatures between 5°C. and 75°C. Portions of enzyme solution ($\frac{1}{40}$ ml. fraction G2A3) were added to each and, after 1 hour, the digests were quickly brought to equilibrium at 35°C. before portions (2 ml.) of amylopectin β-limit dextrin solution were added to each. The α-amylase activities were determined in the usual manner and expressed as a percentage of the activity of a standard digest (pH 5.5; 35°C.) in which the enzyme was not preincubated. The results are shown in Fig. 4.22.

(c) Stability of malt α-amylase/temperature in the presence of inert protein. The above experiment was repeated but inert

FIG. 4·22

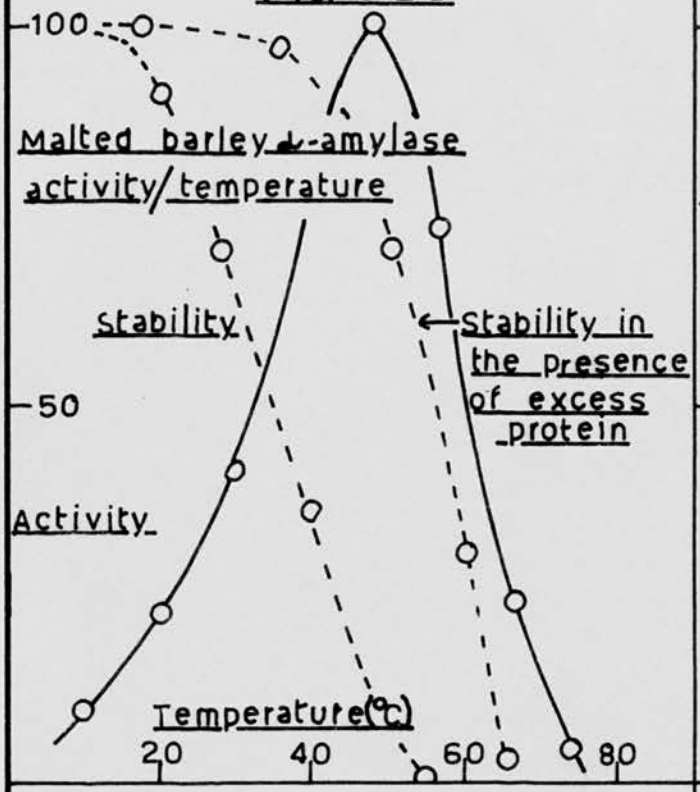


FIG. 4.23

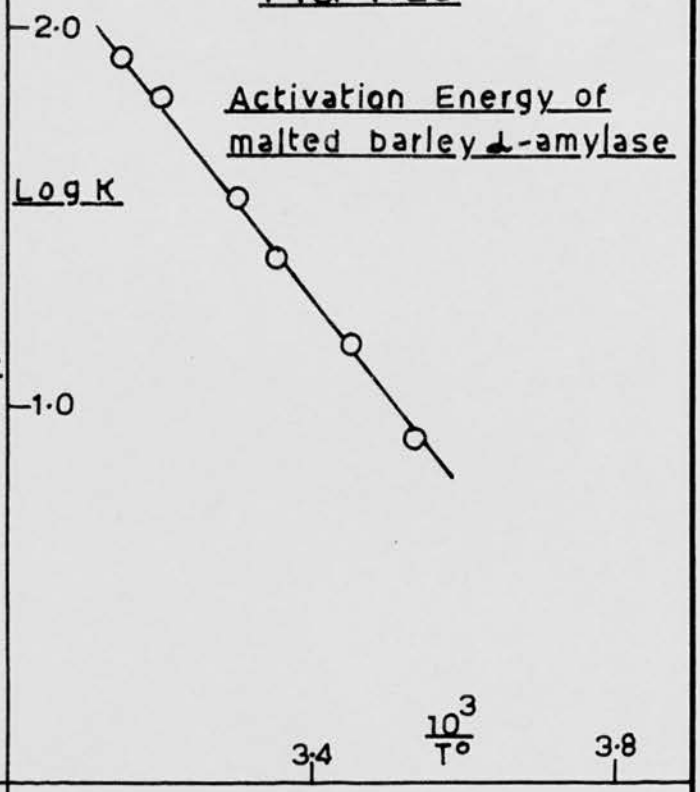


FIG. 4.24

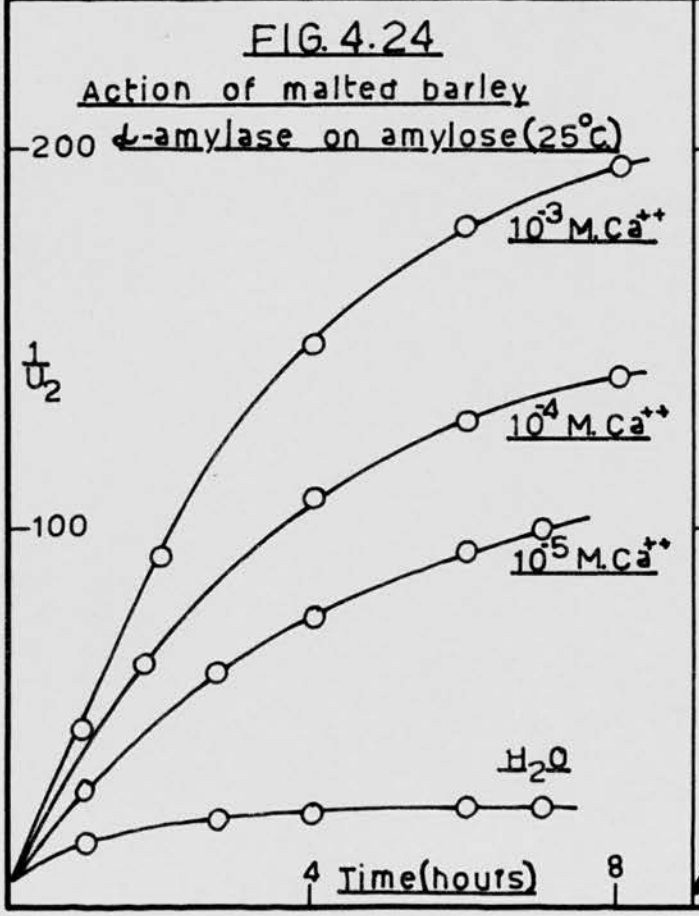
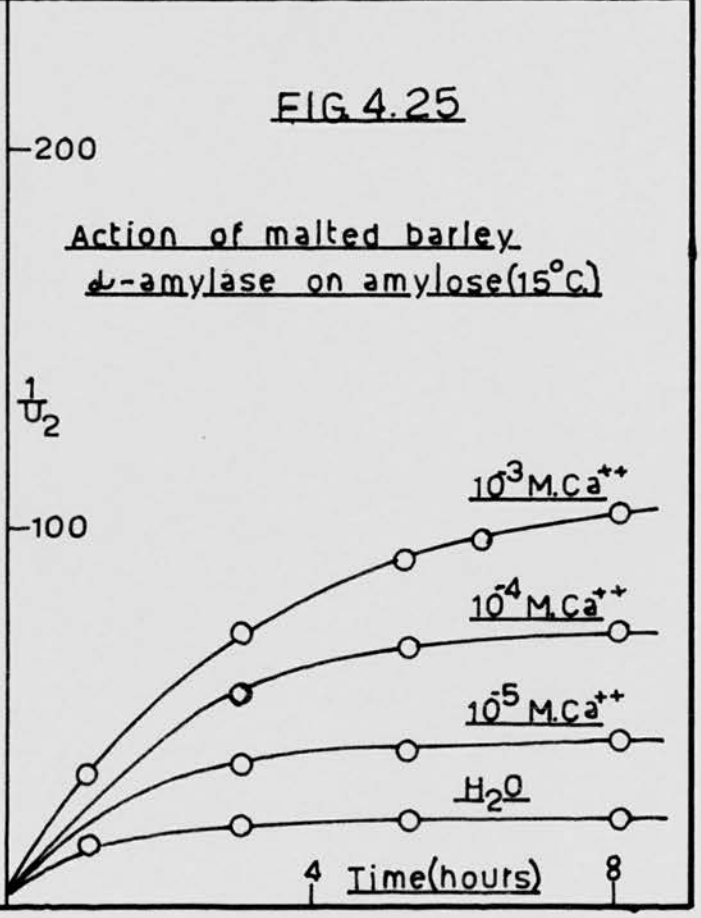


FIG. 4.25



protein (1 ml. of fraction 2A10) was added to each. The results are shown in Fig. 4.22.

(d) Effect of pH and calcium ions on the stability of malt α-amylase at 70°C. The following digests were prepared:

1. Water (7 ml.) + acetate buffer (1 ml.; 0.2M.; pH4.8) + enzyme solution (8 ml. of fraction A4)

2. Water (7 ml.) + acetate buffer (1 ml.; 0.2M.; pH4.8) + enzyme solution (8 ml. of fraction A4) + calcium acetate (200 mg.)

3. Water (7 ml.) + acetate buffer (1 ml.; 0.2M.; pH5.5) + enzyme solution (8 ml. of fraction A4)

4. Water (7 ml.) + acetate buffer (1 ml.; 0.2M.; pH5.5) + enzyme solution (8 ml. of fraction A4) + calcium acetate (200 mg.)

These solutions were heated at 70°C. and aliquots (2 ml.) were removed after 0, 5, 10, 15, 20, 30 and 40 minutes, quickly cooled, centrifuged and examined for α-amylase activity. The results are shown in Table 4.33.

Stability of malt α-amylase at 2°C.

Since the malt α-amylase appeared to be very unstable the activity of fraction 2A3, stored at 2°C., was determined at various intervals. The results are shown in Table 4.34.

Table 4.33

Time of heating	% Original Activity			
	Digest 1	Digest 2	Digest 3	Digest 4
0 minutes	100	100	100	100
5 "	36	80	49	91
10 "	6	80	17	87
15 "	1	68	5	80
20 "	0	55	1.4	76
30 "	0	28	0	68
40 "	0	15	0	63

Table 4.34

	% Original Activity				
	1 week	2 weeks	3 weeks	4 weeks	5 weeks
Fraction 2A3	85	79	75	70	68

Energy of Activation of barley malt α-amylase. Digests, similar to those used in the determination of the optimum temperature for malt α-amylase activity, were prepared and the enzymic activity at various temperatures was determined. The

logarithm of the rate of reaction at each temperature was plotted against $10^3/T^\circ$ (where T° is the absolute temperature) and the results are shown in Fig. 4.23.

Effect of prolonged treatment at pH 3.6 and various temperatures on malt λ-amylase. Three similar digests were prepared containing acetate buffer (3 ml.; 0.2M.; pH 3.6) and enzyme solution (1 ml. of fraction A4). They were incubated at 2, 18 and 35°C. for 12 hours when the pH of each was adjusted to pH 5.5 and aliquots (1 ml.) were removed for activity determinations. The results are shown in Table 4.35.

Table 4.35

	Digest at 2°C	Digest at 18°C	Digest at 35°C
% Original activity	80	8	5

Effect of various substances on malt λ-amylase. Digests were prepared containing acetate buffer (2 ml.; 0.2M.; pH 5.5), enzyme solution ($\frac{1}{40}$ ml. of fraction 2A3), the requisite amount of compound under investigation and water to make a total volume of 10 ml. They were incubated at room temperature for a time (2 hours) and those containing precipitates at the end of this time were clarified by centrifugation. Aliquots (2 ml.) of amylopectin β-limit dextrin were added to each and the λ-amylase activities were determined at room temperature. The results are

shown in Table 4.36.

Table 4.36

	% Inhibition			
	10^{-3}M	10^{-4}M	10^{-5}M	10^{-6}M
$HgCl_2$	100	100	80	43
K C N	0	0	0	0
Na Cl	0	0	0	0
$CaCl_2$	0	0	0	0
Ascorbic acid	93	80	-	-
Histidine	0	0	0	0
Tryptophane	0	0	0	0
Formamide	10% (aq.;v/v)	20%	30%	40%
% Inhibition	50	70	95	100

Effect of E.D.T.A. and trypsin on malt α-amylase. (a) Dilute α-amylase solution.

The following digests were prepared:

1. Dilute enzyme solution (0.1 ml. 2A4) + water (10 ml.) + E.D.T.A. (1 ml.; 10^{-1}M.) + trypsin (1 ml.; 6 mg./ml.) + McIlvaine's buffer (2 ml.; 0.2M.; pH 7.0).

2. Dilute enzyme solution (0.1 ml. 2A4) + water (12 ml.) + McIlvaine's buffer (2 ml.; 0.2M.; pH7.0).

These were incubated at room temperature and aliquots (1 ml.) were removed at intervals for α-amylase determinations. The activity of the enzyme in digest 1 was calculated as a percentage of the activity in digest 2 at each time interval. The results are shown in Table 4.37.

Table 4.37

Time interval (hours)	0	1¼	3	24
α-amylase activity in digest 1.	100	34	0	0
α-amylase activity in digest 2.	100	92	80	25
% Inhibition		58	100	100

(b) Strong α-amylase solution. The following digests were prepared;

1. Enzyme solution (5 ml. fraction 2A4) + water (5 ml.) + E.D.T.A. (1 ml.; 10^{-1}M.) + trypsin (1 ml.; 6 mg./ml.) + McIlvaine's buffer (2 ml.; 0.2M.; pH 7.0).

2. Enzyme solution (5 ml. fraction 2A 4) + water (7 ml.) + McIlvaine's buffer (2 ml.; 0.2M.; pH 7.0).

These were incubated at room temperature and aliquots

(0.3 ml.) were withdrawn at intervals for α-amylase determinations. The results are shown in Table 4.38.

Table 4.38

Time interval (hours)	0	2	24	48	72
α-amylase activity in digest 1	100	94	60	43	32
α-amylase activity in digest 2	100	95	72	60	52
% Inhibition		0	12	17	20

The above experiment with the strong α-amylase solution was repeated using borate buffer at pH 9.5. The results are shown in Table 4.39.

Table 4.39

Time interval (hours)	0	2	24	40	72
α-amylase activity in digest 1	100	88	44	20	0
α-amylase activity in digest 2	100	94	74	61	55
% Inhibition		6	30	41	100

Adsorption of malt α-amylase on starch. (a) Dilute α- amylase

Solution. Two similar digests were prepared containing enzyme solution (0. 1 ml. fraction 2 A 4), water (15 ml.) and potato starch (5 g.). One was maintained at 2°C. and the other at 18°C. They were both carefully shaken and, at intervals, the suspensions were centrifuged and the supernatants tested for α-amylase activity. The results are shown in Table 4.40.

Table 4.40

Time interval (hours)	1	12	24
% Removal of activity from solution at 2°C.	76	80	80
% Removal of activity from solution at 18°C.	79	84	95

(b) Strong α-amylase solution. Two digests were prepared containing enzyme solution (5 ml. of fraction 2A4), acetate buffer (1 ml.; 0.2M.; pH 5.5) and water (4 ml.). Potato starch (3g.) was added to one digest (starch digest) and they were both incubated at 50°C. At various times they were tested for α-amylolytic activity. The results are shown in Table 4.41.

Action of malt α-amylase on amylose. (a) Kinetics of the initial reaction. Digests were prepared containing amylose (31.2 mg.), acetate buffer (1 ml.; 0.2M.; pH 5.5) and calcium acetate solutions (19 ml.) of different concentrations (10^{-3}M.; 10^{-4}M.; 10^{-5}M.; 0). The ionic strength of each

strength of each solution was adjusted to 0.118 by the addition of the requisite amount of sodium chloride. These solutions were equilibrated at 25°C. in viscometers and an aliquot (0.1 ml.) of dilute enzyme solution (0.1 ml. of fraction G2A3 was diluted to 10 ml.) was added to each. The degradations were followed by measuring the flow times of the solutions at various times. The results are shown in Fig. 4.24.

Table 4.41

Time interval (hours)	1	15	38
% Removal of activity from starch digest	40	71	100
% Removal of activity from standard digest (deactivation)	5	30	100
% Adsorption	35	41	-

The above experiments were repeated at 15°C. and the results are shown in Fig. 4.25.

Two amylose solutions A and B were prepared in acetate buffer (20 ml.; 0.01M.; pH 5.5) and they were equilibrated in viscometers at 25°C. Three times as much enzyme was added to A as was added to B and the rates of degradation were followed. The results are shown in Fig. 4.26.

The effect of inert protein on the degradation of amylose was investigated. Two digests, C and D were prepared in the following manner:

c. Amylose (31.2 mg.) + acetate buffer (1 ml.; 0.2M; pH 5.5) + calcium acetate solution (19 ml.; 1.05×10^{-3}M.)

D. Amylose (31.2 mg.) + acetate buffer (1 ml.; 0.2M.; pH 5.5) + inert protein solution (1 ml. of fraction 2 A 10) + water (18 ml.) + requisite amount of sodium chloride to maintain the two solutions at constant ionic strength.

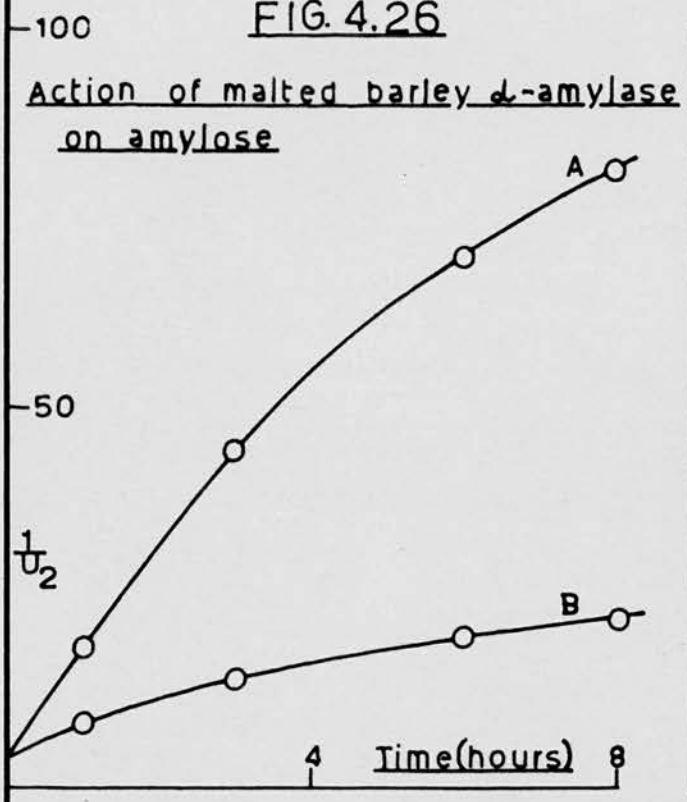
These were equilibrated at 25°C. and an aliquot (0.1ml.) of dilute enzyme solution (0.1 ml. of fraction G 2 A 3 was diluted to 10 ml.) was added to each. The rates of degradation were followed and the results are shown in Fig. 4.27.

(b) Products of the reaction. Two indentical amylose solutions A and B, were prepared containing amylose (40 ml.; 1.52 mg. amylose/ml.) and enzyme solution (0.25 ml. of fraction G 2 A 3). They were incubated at 35°C. and aliquots (1.5 ml.) were removed from digest A at intervals for reducing power (1 ml.) and iodine staining measurements (0.25 ml.). Because of the instability of the malt α-amylase, fresh portions of enzyme were added to the digests after 24 and 96 hours. The results are shown in Fig.4.28.

Aliquots (5 ml.) were removed at intervals, from digest B, heated on a boiling water-bath (5 minutes) to coagulate the protein, clarified by centrifugation and evaporated to dryness. The residue was taken up in a small volume of water (0.75 ml.) and applied to paper chromatograms. The results are shown in Table 4.42.

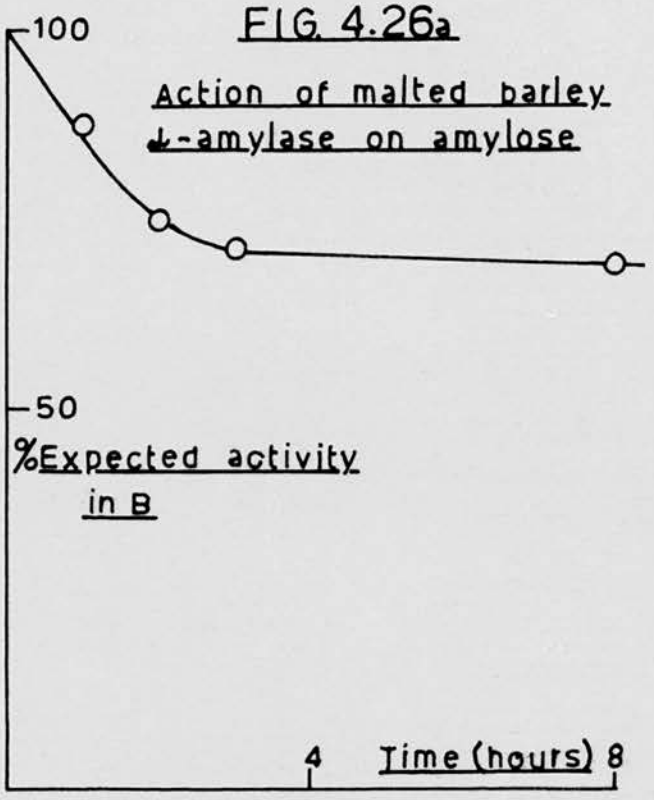
Action of malt α-amylase on oligosaccharides. Microdigests were prepared containing enzyme solution (0.025 ml. fraction G 2 A 3) and samples of oligosaccharides G_7, G_8 G_9 and G_{10} in small volumes of water (o.1 ml.). They were incubated at 35°C.

FIG. 4.26



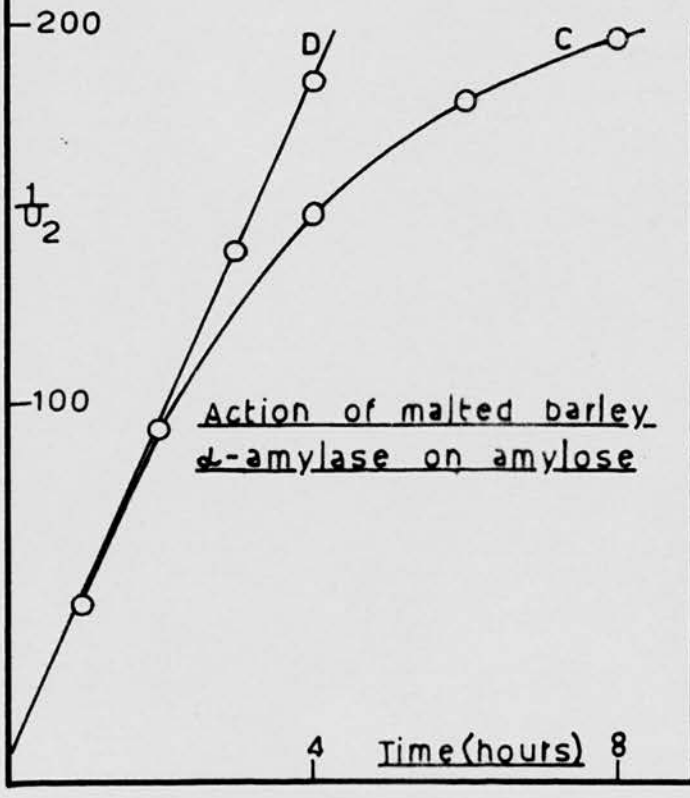
Action of malted barley ⍺-amylase on amylose

FIG. 4.26a



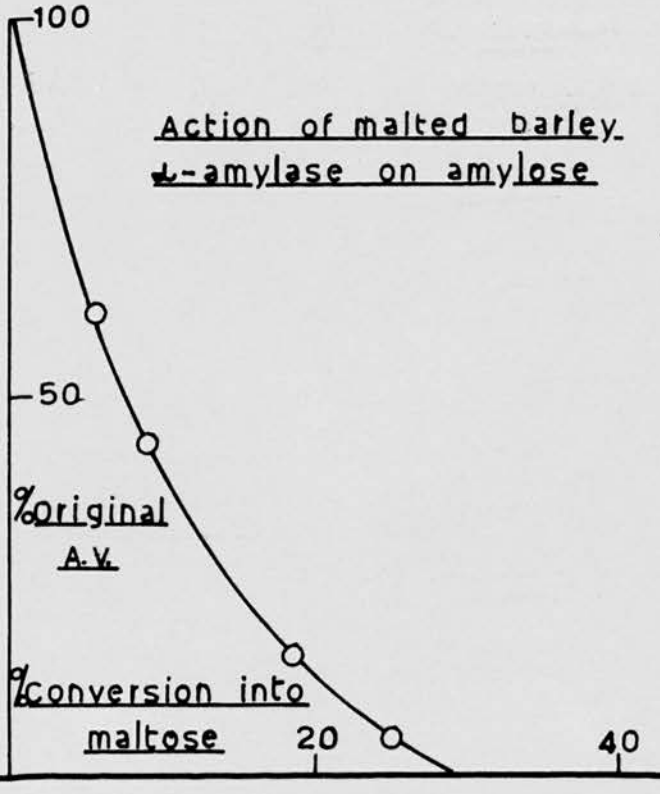
Action of malted barley ⍺-amylase on amylose

FIG. 4.27



Action of malted barley ⍺-amylase on amylose

FIG. 4.28



Action of malted barley ⍺-amylase on amylose

and small aliquots were removed at intervals for chromatographic analysis. The results are shown in the following tables. Standard saccharides G_1 to G_9 were also run on the chromatograms.

Table 4.42.

Relative amounts of oligosaccharides							
Time of aliquot extraction (hours)	0.5	1	2	12	50	96	120
Oligosaccharide							
G_1	0	+	++	+++	+++++	+++++	+++++
G_2	+	++	+++	++++	+++++	+++++	+++++
G_3	+	++	+++	+++	+++	++++	++++
G_4	+	++	+++	++++	+++++	+++++	+++++
G_5	+	++	++	+++	++++	++++	++++
G_6	++	+++	++++	+++++	++++	+++	+++
G_7	++	++++	+++++	++	+	0	0
G_8	+++++	++++	+	0	0	0	0
Higher oligosaccharides	+++++	++	0	0	0	0	0

Action of malt α-amylase on G_{10}

Table 4.43

Relative amounts of oligosaccharides						
Time of aliquot extraction (hours)	0	1	7	17	24	48
Oligosaccharide						
G_1	0	0	+	++	+++	++++
G_2	0	+	++	+++	++++	+++++
G_3	0	+	++	+++	++++	+++++
G_4	0	0	0	+	++	+++
G_5	0	0	0	0	+	++
G_6	0	0	+	++	+++	++++
G_7	0	+	++	+++	++++	++++
G_8	0	0	+	++	+++	++
G_9	0	0	0	+	++	0
G_{10}	+++++	++++	++	0	0	0

Action of malt α-amylase on G_9

Table 4.44

	Relative amounts of oligosaccharides					
Time of aliquot extraction (hours)	0	1	7	17	24	48
Oligosaccharide						
G_1	0	0	+	++	+++	++++
G_2	0	+	++	+++	++++	+++++
G_3	0	0	0	+	+	++
G_4	0	0	0	0	+	++
G_5	0	0	0	0	+	++
G_6	0	+	++	+++	++++	+++++
G_7	0	+++	+++	++	++	+
G_8	0	+	++	+++	++	++
G_9	+++++	++++	++	0	0	0

Action of malt α-amylase on G_8.

Table 4.45

	Relative amounts of oligosaccharides					
Time of aliquot extraction (hours)	0	1	7	17	24	48
Oligosaccharide						
G_1	0	+	++	+++	+++	++++
G_2	0	+	++	+++	++++	+++++
G_3	0	0	0	+	+	++
G_4	0	0	+	+	++	+++
G_5	0	0	+	+	++	+++
G_6	0	+	++	+++	++++	+++++
G_7	0	+	+	++	++	++
G_8	+++++	++++	+++	+++	++	++

Action of malt α-amylase on G_7

Table 4.46

	Relative amounts of oligosaccharides					
Time of aliquot extraction (hours)	0	1	7	17	24	48
Oligosaccharide						
G_1	0	0	+	++	+++	++++
G_2	0	0	0	0	+	++
G_3	0	0	0	0	0	+
G_4	0	0	0	0	+	++
G_5	0	0	0	0	+	++
G_6	0	0	+	++	+++	++++
G_7	+++++	++++	++++	+++	++	+

Comparison of the action of malt α-amylase and salivary α-amylase on G_4. Saliva was collected in distilled water, maintained at room temperature for three hours and centrifuged - the clear supernatant was stored, under toluene, at $2^{\circ}C$. Two identical digests were prepared containing water (5 ml.), acetate buffer (1 ml.; 0.2M.; pH 5.5) and amylopectin β-limit dextrin solut-

ion. They were incubated at 35°C. To one was added an aliquot (0.05 ml.) of the crude saliva, and an aliquot (0.0025 ml.) of malt α-amylase fraction G2A3 was added to the other. Aliquots (2 ml.) were removed from the digests at intervals and stained with iodine. The results are shown in Table 4.47.

Table 4.47

	Iodine stain A.Vs.			
Time of aliquot extraction (minutes)	0	15	30	60
Salivary α-amylase	4.70	3.40	2.60	1.62
Malt α-amylase	4.70	3.30	2.50	1.60

Two microdigests were prepared: (1) contained salivary α-amylase (0.05 ml.) and an aliquot of G_4 (0.05 ml.); (2) contained malt α-amylase fraction G2A3 (0.025 ml.), water (0.025 ml.) and G_4 solution (0.05 ml.). These were incubated at room temperature and aliquots were removed at intervals for examination by paper chromatography. Standards G_1, G_2, G_3 and G_4 were also applied to the chromatograms. The results are shown in Table 4.48.

Action of malt α-amylase on amylose, amylopectin, amylopectin β-limit dextrin, glycogen and glycogen β-limit dextrin. (In collaboration with Mr. R. Geddes). The degradation of these substrates with malt α-amylase fraction G 2A3 was followed by light-scattering measurements and the relative rates of attack are given in Table 4.49.

Table 4.48

Relative amounts of oligosaccharides						
Time of aliquot extraction (hours)		0	1	5	8	20
	Oligosaccharide					
Saliva Digest	(G_1	0	+	+	++	++
	(G_2	0	++++	+++++	+++++	+++++
	(G_3	0	+	++	++	+
	(G_4	+++++	+	0	0	0
Malt Digest	(G_1	0	0	0	0	0
	(G_2	0	0	+	++	+++
	(G_3	0	0	0	0	0
	(G_4	+++++	+++++	++++	+++	+++

Table 4.49

Substrate	Amylose	Amylopectin	Amylopectin β-limit dextrin	Glycogen	Glycogen β-limit dextrin
Rate of Attack	700	22	35.6	10	1

DISCUSSION

Preparation of malt α-amylase. Samples of crystalline malt α-amylase have been prepared (Schwimmer and Balls, 1949; Fischer and Haselbach, 1951), but these procedures were not examined in this work. One of the aims in this study, was to compare the properties and action patterns of the α-amylases from barley and malted barley; these enzymes, therefore, were prepared by the same isolation scheme. Ungerminated barley contains only a small amount of α-amylase (Kneen, 1944) and so a simple procedure for isolating and concentrating the enzyme was required. Acetone fractionation techniques were reasonably effective and - provided that the experimental conditions were adhered to - they were reproducible. The enzymes obtained in this work have been extensively purified - the barley α-amylase by a factor of 100, the malt α-amylase by a factor of 170 - and do not contain detectable amounts of other carbohydrases.

Dilute solutions of malt α-amylase are particularly susceptible to heat inactivation and the removal of β-amylase impurity from α-amylase preparations by heat treatment was best carried out on the aqueous extract in the presence of calcium ions - these ions increased the stability of α-amylase but decreased that of β-amylase (Kneen, Sandstedt and Hollenbeck, 1943).

Effect of pH on malt α-amylase. The pH of maximum activity was 5.5 which is comparable to the range of 4.7 - 5.4 obtained by Fischer and Haselbach (1951). The crystalline enzyme of these authors appeared to be more active at low values

of pH (3 - 6) than the enzyme studied in this work.

The stability of the enzyme at various values of pH is very dependent on the presence of inert protein and calcium ions. Whilst, in 1 hour at 20°C., the enzyme loses 46% of its activity when it is maintained at pH values between 5.5 and 7.8 on either side of this "plateau" the activity falls even more rapidly (Fig. 4.20). However, the addition of either inert protein or calcium ions (Fig 4.21) renders the enzyme much more stable in this pH range, but at lower and higher values of pH the enzyme is still rapidly deactivated. This apparent instability of malt α-amylase is not in agreement with the findings of Fischer and Haselbach (1951), who suggested that crystalline malt α-amylase is highly stable. It has been reported (Hultin, 1950) that the stability of pure malt α-amylase is only $\frac{1}{50}$ to $\frac{1}{100}$ of that of a crude malt extract and so perhaps the stability of relatively pure samples of the enzyme is dependent upon the concentration at which the enzyme is maintained. The inert protein added to the malt α-amylase solutions must stabilise and protect the active centres of the enzyme in a manner similar to the stabilisation of dilute β-amylase solutions by glutathione (Walker and Whelan, 1960).

Effect of temperature on malt α-amylase. The temperature of optimum activity was ca. 48°C. which is in good agreement with the range of 45 - 55°C. reported by Fischer and Haselbach (1951). Fig. 4.22 illustrates the stability of the enzyme towards heat and shows that a temperature of 50°C. almost completely destroys the activity of the enzyme in 1 hour. Once

again, inert protein greatly increases the enzyme stability (Fig. 4.22) and 70% of the activity remains in the solution after 1 hour at 50°C. Calcium ions, too, greatly retard the deactivation of malt λ-amylase under unfavourable temperature conditions (Table 4.33 p.147). They are not able, however, to completely overcome the effect of unfavourable pH and temperature; even in their presence the enzyme loses a large amount of its activity at pH 4.8.

The loss in enzymic activity at 2°C. is initially rapid but then it decreases. (Table 4.34 p.147) No stabilising protein or calcium ions were added to this stock enzyme solution since the properties of the pure enzyme were being studied.

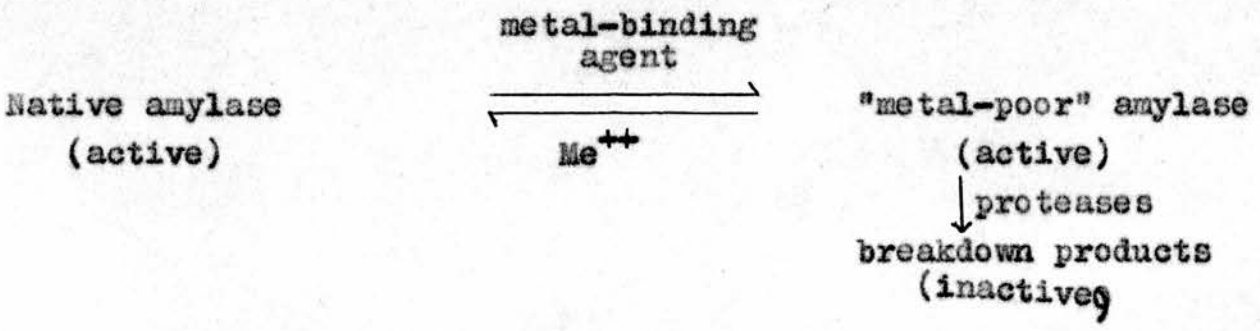
Low temperature lessens the effect of unfavourable pH on the enzyme activity (Table 4.35 p. 148). Even after 12 hours at pH 3.6 the enzyme maintained 80% of its activity whereas, at room temperature, it lost 92% of its activity. These results suggest that the inactivation of the enzyme by unfavourable conditions of pH is a reversible process and so is very unlikely to be caused by a protease which would irreversibly destroy the enzyme.

The energy of activation of the enzyme was found to be 11.7K cals. which is similar to that found for salivary λ-amylase (13.5 K cals.; Meyer et al, (1948).

Effect of various substances on malt λ-amylase. Of the compounds investigated only mercuric chloride (10^{-3}M.; 10^{-4}M.) and formamide (40%) completely inhibited the enzymic activity.

Neither calcium chloride nor sodium chloride activated the enzyme (Table 4.36. p.149).

When calcium ions are removed from malt α-amylase there is a large decrease in the enzymic activity (Table 4.37. p.150). These results are in agreement with those reported by Hultin (1950), Schwimmer and Balls (1949), Fischer and Haselbach (1951). These authors removed the calcium with metal chelating agents or by precipitation as an insoluble complex (Hultin, 1950). In these experiments, the loss in activity was reversible and the addition of calcium ions to the inert enzyme restored its activity completely. The effect of E.D.T.A. alone on the malt α-amylase was not investigated in the present work, but the combined action of E.D.T.A. and trypsin effected irreversible deactivation of the enzyme. This phenomenon has been examined by Stein and Fischer (1958) who investigated the effect of E.D.T.A. and trypsin on α-amylases from human saliva, swine pancreas, Bacillus subtilis and Aspergillus oryzae. They suggested that these enzymes are maintained in a rigid and stable configuration by divalent metal linkages and, as such, they are immune to proteolytic attack. When, however, the α-amylases are no longer combined with their full complement of metal ions they become susceptible to proteolysis. These results are consistent with the following scheme:



Calcium-free malt α-amylase solutions may be stabilised by many divalent ions including barium, cadmium, manganese and zinc (Hultin, 1950), whereas monovalent ions do not restore the enzymic activity.

The results in Table 4.39 (p.151) indicate that the removal of calcium ions by E.D.T.A. is more effective at relatively high values of pH (9.5). Relatively concentrated solutions of malt α-amylase were inactivated very slowly under the combined action of E.D.T.A. and trypsin (Table 4.38. p.151). The enzyme sample used in these experiments was somewhat crude and earlier results (Fischer and Haselbach, 1951) suggest that E.D.T.A. removes calcium from impure samples of enzyme only with difficulty. The contaminating protein appears to exert a stabilising influence on the enzyme.

An apparently efficient and simple method of rendering malt α-amylase inactive is to pass it through a "Sephadex" column in a water medium. The protein passes quickly through the column leaving the inorganic ions behind.

Adsorption of malt α-amylase on starch. The complete adsorption of enzymic activity on starch granules is very difficult to obtain. Results in Table 4.40 (p.152) indicate that 75% of the enzyme is quickly adsorbed (1 hour) but, thereafter the adsorption is slow and only a further 4% is removed in the subsequent 23 hours. These results are in agreement with those of Schwimmer and Balls (1949).

Action of malt α-amylase on amylose. (a) Kinetics of initial reaction. The kinetics of the initial reaction

were rather difficult to obtain because of the marked instability of the enzyme sample (Figs. 4.24 and 4.25). There are two possible explanations for these curved lines: (1) the enzyme rapidly loses its activity under the experimental conditions; (2) these lines genuinely represent the course of the degradation. Two digests were prepared, therefore, containing different amounts of the enzyme solution and equal amounts of amylose. Their rates of degradation were investigated and the results are shown in Fig. 4.26. Digest A contained three times as much enzyme as digest B and so, if these lines represent the amylose - degradation reaction, hydrolysis A should proceed at approximately three times the velocity of B. The results in Fig. 4.26a indicate that this relation does not hold because the difference in the reaction rates increases rapidly at the beginning of the degradations and then remains almost constant - the enzyme in digest B having only ca. 70% of its expected activity. It is reasonable to assume, therefore, that these curves are a function of enzyme deactivation and not of amylose degradation. As already indicated (Fig. 4.22), inert protein may be used to stabilise the enzyme and so an amylose degradation was investigated in the presence of protein. The results are shown in Fig. 4.27. and indicate that calcium ions are not such efficient stabilising agents as protein, although they do have a large effect on the enzyme activity (Fig. 4.24). Calcium ions, however, do not activate the enzyme but they are essential for it to attain its full activity.

In these experiments, the enzyme appears to lose its activity very rapidly and this may attributed to the large dilut-

ions of enzyme employed. Since only a small fraction (0.1 - 0.5%) of the amylose bonds is broken during the initial stages of these degradations, extremely weak enzyme solutions are required. Usually, aliquots of $\frac{1}{1000}$ ml. are added to 20 ml. of amylose solution in the viscometers whereas, in the usual digests for activity determinations, aliquots of $\frac{1}{40}$ ml. are added to a total volume of 8 ml. - there is, therefore, a 60-fold difference in enzymic concentration between the two digests.

The results in Fig. 4.27 indicate that the initial stage in the degradation of linear amylose by stable malt α-amylase proceeds by a random scission of α-1: 4 - glucosidic bonds.

(b) Products of the reaction. The achroic limit for the action of malt α-amylase on amylose occurs at ca. 30% apparent conversion into maltose (Fig. 4.28). This figure is in good agreement with the results of Bird and Hopkins (1954) who showed that the achroic point was dependent upon the amylose concentration. With amylose concentrationsof 0.44% and 0.022% they found that the achroic limits occurred at 41.5% and 26.4% apparent conversions into maltose, respectively. In the present work the amylose concentration was ca. 1.5 mg./ml., and so an achroic limit of ca. 30% is to be expected. At the achroic limit no oligosaccharides larger than G_8 should be found, but chromatographic evidence (Table 4.42. p.155) suggests that there is quite a large amount of these higher dextrins present although they cannot be detected by the iodine-staining measurements. That the achroic limit depends on the concentration of substrate solution used has already been pointed out (p.111).

The action of malt α-amylase on amylose consists of two stages: (1) the rapid breakdown of the amylose into short chain dextrins resulting in loss in iodine staining power, large decrease in viscosity and increase in the reducing power of the amylose solution, (2) a slow hydrolysis of the small dextrins resulting in a slow increase in the reducing power of the solution. There has been much controversy about the production of glucose in amylose/α-amylase digests. Myrbäck (1948) suggested that glucose is produced early in the reaction and is split from large as well as small molecules although most of it is very slowly formed from short-chain products during the second part of the reaction. On the other hand, Meyer and Bernfeld (1941), and Meyer and Gonon (1951b) suggested that glucose is only formed from maltotriose and so cannot be produced until some maltotriose is present in the digest. Now a certain amount of maltotriose may be formed in the first part of the reaction by random hydrolysis and so glucose, produced from maltotriose, may be present at the achroic stage of the reaction. The formation of glucose in this way was also supported by Somogyi (1940) who studied the action of α-amylase on starch. He concluded that, at a given stage of hydrolysis, the more dilute the starch solution, the lower was the percentage of glucose produced. Hopkins and Bird (1954) examined the action of malt α-amylase on extremely dilute solutions of amylose (0.025%) and still found traces of glucose at the achroic limit.

The results in Table 4.42 (p.155) suggest there is only a very small amount of glucose present at the achroic limit

indicating that this saccharide is formed as a primary fission product from the amylose molecules. After ½ hour there is a large accumulation of G_6, G_7, G_8 with lesser amounts of G_2 and trace amounts of G_3, G_4 and G_5. As the hydrolysis proceeds, G_8 and larger oligosaccharides disappear while G_7 builds up and then disappears, but G_6 increases in amount and is hydrolysed extremely slowly. All the smaller oligosaccharides increase in amount but the yields of G_3, G_4 and G_5 are small. The enzyme appears to hydrolyse G_6 and smaller maltodextrins only very slowly.

Action of malt α-amylase on oligosaccharides. (1) G_4. (Table 4.48 p.161). The enzyme hydrolyses G_4 extremely slowly and the products of the reaction are shown in Table 4.48. Only maltose is formed and there is no trace of either glucose or maltotriose. Since the hydrolysis of G_4 is so slow the suggestion that glucose is formed only from maltotriose during the hydrolysis of amylose with malt α-amylase (Meyer and Bernfeld, 1941; Meyer and Gonon, 1951) does not seem to be correct.

(2) G_7. (Table 4.46. p. 159). The initial products were G_1 and G_6 which increased in amount before G_2, G_4 and G_5 appeared. Only very small quantities of G_3 and G_4 were produced so that both the G_1 and G_2 could not have been formed from them. Under the experimental conditions the fission products most readily formed were G_1 and G_6 followed by G_2 and G_5.

(3) G_8 (Table 4.45. p.158). The initial products were G_1, G_2, G_6 and G_7 and the first three of those increased in amount during the hydrolysis. The preferred products appeared to be G_2 and G_6 followed by G_1 and G_7. There were only trace

amounts of G_3, G_4 and G_5. The relative amounts of G_6 and G_7 present after 24 and 48 hours are somewhat misleading because of the relative stabilities of G_6 and G_7 towards the enzyme. Since G_7 is hydrolysed faster than G_6, it is not allowed to accumulate whereas the G_6 remains largely undegraded and may increase in amount through secondary fission of G_7.

(4) G_9 (Table 4.44. p.157) The initial products of the reaction were G_2, G_6, G_7 and G_8, but G_2 and G_7 appeared to predominate as the reaction proceeded. Once again, there was an accumulation of G_6, but it did not appear to be formed as a primary fission product from G_9 because there was no accompanying production of G_3. There were relatively small amounts of G_3, G_4 and G_5 produced.

(5) G_{10} (Table 4.43. p.156) The initial reaction products appeared to be G_2, G_3 and G_7. As the reaction proceeded the amounts of these four dextrins increased and G_8 was also produced in quite a large amount. Only a very small quantity of G_5 was formed and the system is too complex to decide whether this dextrin was produced on primary or secondary fission.

The relative rates of attack of salivary α-amylase and malt α-amylase on G_4 are shown in Table 4.48 (p.161). Whereas the enzyme samples showed the same activity on reaction with amylopectin β-limit dextrin solution they differed widely in their rates of attack on G_4. An excess (x 10) of malt enzyme was added but there was still a large difference in the hydrolysis of the dextrin. Whereas the salivary amylase had completely hydrolysed the G_4 in 5 hours the malt enzyme had only

effected a detectable hydrolysis. The main product from the salivary α-amylase digest was maltose but reasonable amounts of G_1 and G_3 were also produced. G_3 would appear to be much more slowly hydrolysed than G_4. These results are in agreement with those of Whelan and Roberts (1953) and Pazur (1953).

Specific action of malt α-amylase. In an attempt to determine whether α-amylolysis took place at the reducing or non-reducing ends of amylose-type molecules, Hopkins and Bird (1954) examined the low molecular weight products obtained near the beginning of the reaction of malt α-amylase on dilute amylopectin solutions. This substrate contains, essentially, only non-reducing end groups but enzymic degradation will greatly increase the number of reducing end groups - the reaction must be stopped and the products examined before this stage is reached. The above authors found that G_6 was the smallest oligosaccharide detected under these conditions. They concluded, therefore, that the first five bonds from the non-reducing end of the molecule are resistant to malt α-amylase attack and are hydrolysed very slowly, thus explaining the slow degradation of G_4. These authors also found that G_6 was preferentially split into G_4 and G_2 with smaller amounts of G_1 and G_5 indicating that the second linkage from the reducing end of the molecule is more easily hydrolysed than the first one. If this is correct then G_7 should be split to form G_5 and G_2 as primary fission products, but this does not happen (Table 4.46. p.159); the enzyme prefers to hydrolyse a less susceptible bond to produce G_6 and G_1. This result is rather difficult to understand, but the results in

Tables 4.43, (p.156) - 4.46. (p.159) all indicate that substantial quantities of G_1 are formed from all the oligosaccharides examined showing that malt α-amylase fairly readily hydrolyses the linkage next to the reducing group in intermediate oligosaccharides (G_7 - G_{10}). These results also indicate, however, that the second and third linkages from the reducing end of G_8, G_9 and G_{10} are preferentially attacked.

Rates of attack of malt α-amylase on various substrates.

The results in Table 4.49 (p. 161) illustrate the action of the enzyme on various starch-type substrates. Amylose is attacked some 30 x faster than amylopectin and 700 x faster than glycogen β-limit dextrin. The lightscattering technique measures only changes in the weight-average molecular weight of the substrates and is, therefore, insensitive to the formation of oligosaccharides during the initial stages in the hydrolysis of branched substrates. This explains why amylopectin is apparently attacked less readily than its β-limit dextrin. The dextrin is a more compact molecule and enzymic attack must take place on its interior linkages and so perceptibly reduce the molecular weight. With amylopectin, however, the enzyme may hydrolyse a large number of the external branches without appreciably changing the molecular weight. These results suggest that the external linkages in amylopectin are hydrolysed before those in the interior of the molecule.

4.d. BROAD BEAN α-AMYLASE

INTRODUCTION

Many carbohydrase enzymes are present in broad bean flour, e.g. phosphorylase and Q-enzyme from this source have been isolated and studied by Hobson et al, (1950) and Wild (1954), whilst the presence of R-enzyme has also been shown [Hobson et al, (1951); Wild, (1954); Peat et al, (1954); MacWilliam, (1958)]. The results from some of these investigations indicated that there was present in broad beans an enzyme having α-amylolytic activity. Hobson et al, (1950) reported that the pH optimum of this enzyme was 6.1 - 6.3 but it has not been further characterised. In this work, the isolation, purification and characterisation of the α-amylase in broad beans are described.

EXPERIMENTAL

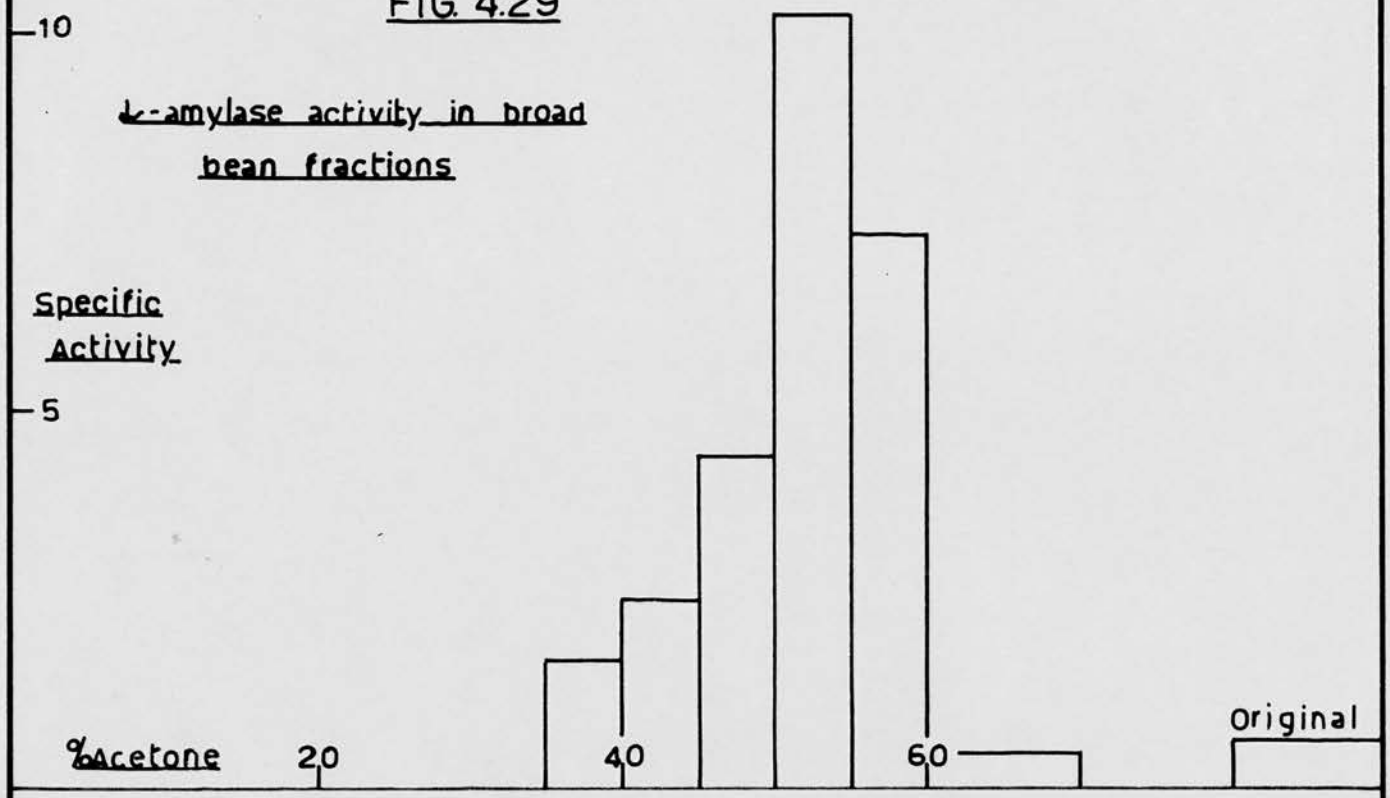
Throughout this work Dobbie's "Champion Long Pod" broad beans were used.

Initial attempts to prepare broad bean α-amylase. Dry, defatted, finely ground broad bean flour (100 g.) was extracted for 5 hours at room temperature with aqueous calcium chloride solution (500 ml.; 0.2%) containing *n*-octanol (0.2 ml.). The suspension was centrifuged and the supernatant dialysed against running tap water for 12 hours. The solution was then filtered to yield a clear solution (B B 1; 360 ml.; 33 mg. protein/ml.)

which was cooled to 2°C. and fractionated with chilled acetone between the limits 0 - 20, 20 - 30, 30 - 35, 35 - 40, 40 - 45, 45 - 50, 50 - 55, 55 - 60 and 60 - 70%. Each fraction was air-dried and suspended in water to yield solutions 2 - 10 which were stored at 2°C. These fractions were examined for the presence of α-amylase (Fig. 4.29). Solutions 5 - 8 contained α- activity, but R-enzyme activity in the initial fractions was suspected. Since ammonium molybdate inhibits this enzyme (Bailey et al., 1951) digests incorporating samples from solutions 3 and 8 were prepared as indicated below.

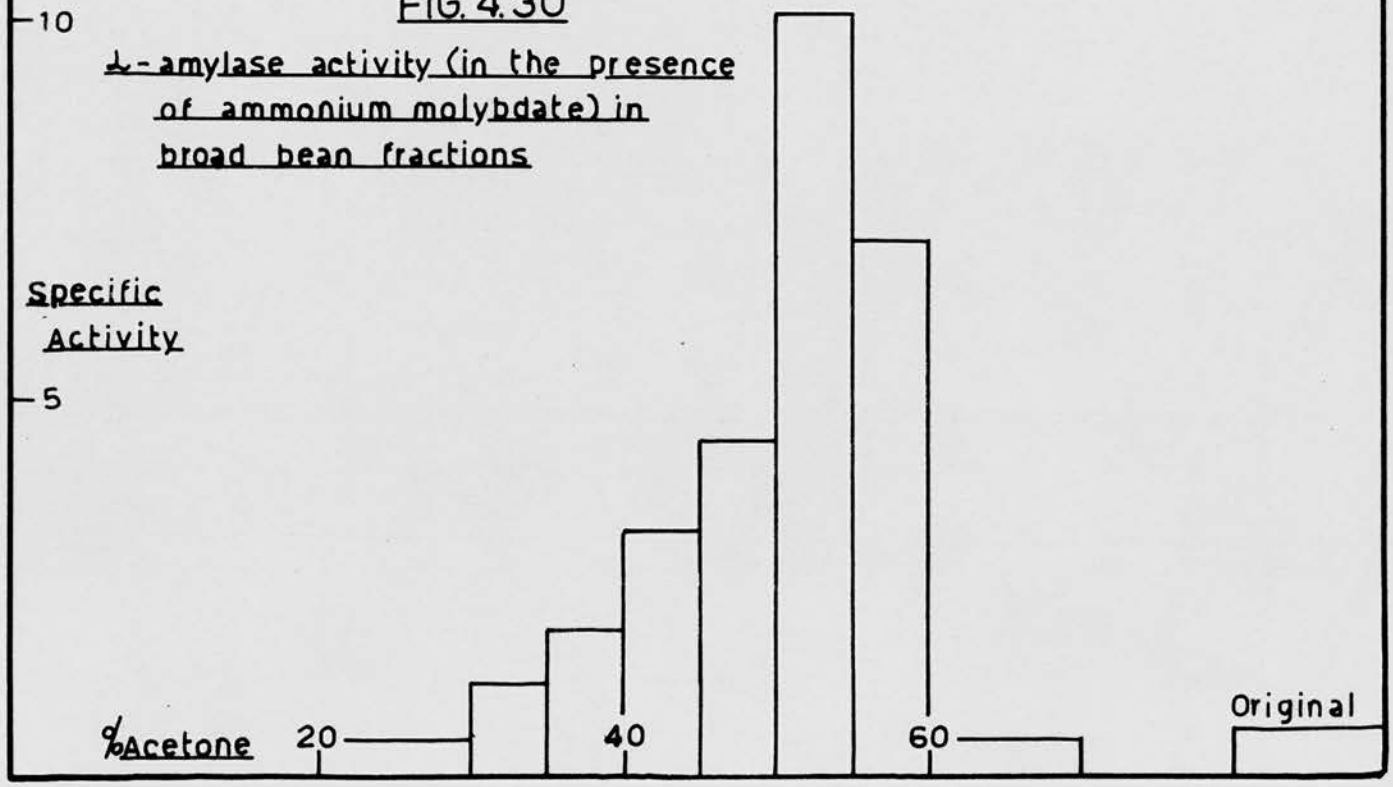
1. Acetate buffer (2 ml.; 0.2M.; pH 5.5) + water (6 ml.) + solution 3 (1 ml.)
2. Acetate buffer (2 ml.; 0.2M.; pH 5.5) + water (5 ml.) + solution 3 (1 ml.) + ammonium molybdate (1 ml.; 10^{-1}M.).
3. Acetate buffer (2 ml.; 0.2M.; pH 5.5) + water (5 ml.) + solution 3 (1 ml.) + ammonium molybdate (1 ml.; 10^{-2}M.).
4. Acetate buffer (2 ml.; 0.2M.; pH 5.5) + water (5 ml.) + solution 3 (1 ml.) + ammonium molybdate (1 ml.; 10^{-3}M.).
5. Acetate buffer (2 ml.; 0.2M.; pH 5.5) + water (6 ml.) + solution 8 (1 ml.)
6. Acetate buffer (2 ml.; 0.2M.; pH 5.5) + water (5 ml.) + solution 8 (1 ml.) + ammonium molybdate (1 ml.; 10^{-1}M.)
7. Acetate buffer (2 ml.; 0.2M.; pH 5.5) + water (5 ml.) + solution 8 (1 ml.) + ammonium molybdate (1 ml.; 10^{-2}M.)
8. Acetate buffer (2 ml.; 0.2M.; pH 5.5) + water (5 ml.) + solution 8 (1 ml.) + ammonium molybdate (1 ml.; 10^{-3}M.)

FIG. 4.29



α-amylase activity in broad bean fractions

FIG. 4.30



α-amylase activity (in the presence of ammonium molybdate) in broad bean fractions

These were incubated at 35°C. for 30 minutes when aliquots (1 ml.) of amylopectin β-limit dextrin solution were added to each. Aliquots (4 ml.) were removed immediately, and after an interval of 30 minutes, for iodine staining measurements. The results are shown in Table 4.50.

Table 4.50

Digest	1	2	3	4	5	6	7	8
A.V. (0 hour)	2.90	2.87	2.90	2.90	2.92	2.91	2.91	2.88
A.V. (½ hour)	4.10	2.88	3.34	3.70	0.51	1.60	1.27	1.07

Therefore, ammonium molybdate (10^{-2}M.) completely inhibits R-enzyme activity and partially inhibits (45%) the β amylase activity. Similar activity determinations were carried out on all the fractions both in the presence and absence of ammonium molybdate (10^{-2}M.). The results are shown in Figs. 4.29 and 4.30.

Presence of phosphatase in the fractions. Digests were prepared containing acetate buffer (2 ml.; 0.2M.; pH 4.6), water (3 ml.), glucose 6 - phosphate (10 mg./ml.; 1 ml.) and enzyme solution (1 ml.). These were incubated at 35°C. for 2 hours when aliquots (1 ml.) were extracted and their phosphate content determined by the method of Allen (1940). The results are shown in Fig. 4.31.

FIG. 4.31

Phosphatase activity in broad bean ⍺-amylase fractions

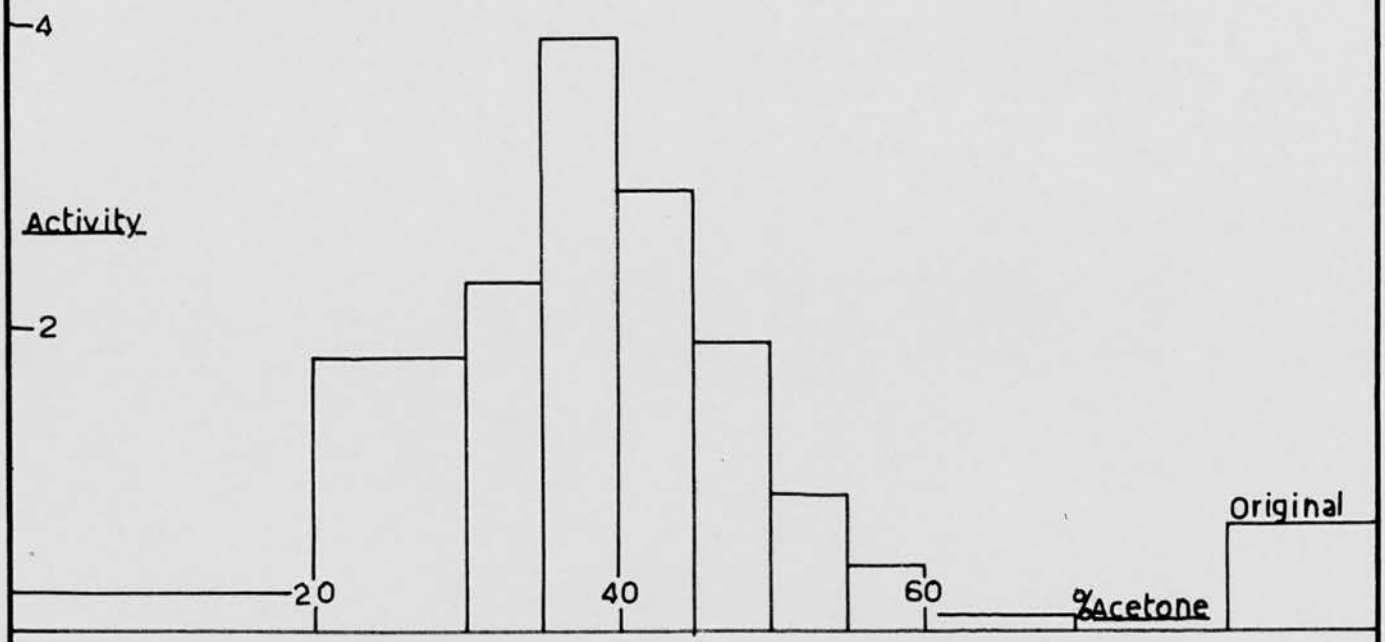
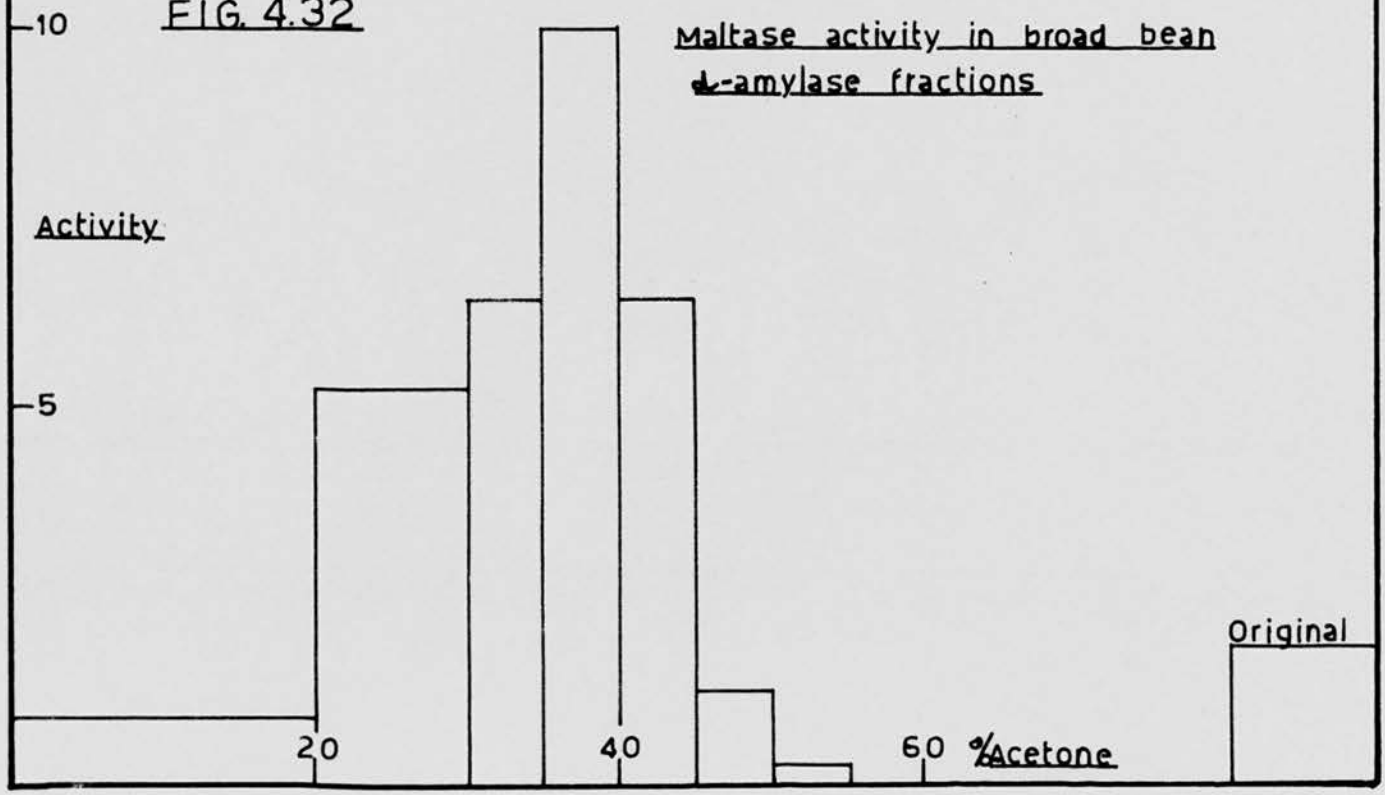


FIG. 4.32

Maltase activity in broad bean ⍺-amylase fractions



Presence of maltase in the fractions. Digests were prepared containing acetate buffer (2 ml.; 0.2M.; pH 5.5), maltose (5 ml.; 3 mg./ ml.) and aliquots (1 ml.) of the enzyme solutions. These were incubated at 35°C. and samples (1 ml.) were extracted at intervals for reducing power determinations. The results are shown in Fig. 4.32.

Attempted purification on starch column. A small starch column (10 cm. x 2.5 cm.) was prepared and equilibrated with aqueous alcohol (20%) at 2°C. Ethanol (0.5 ml.) and calcium acetate (5 mg.) were added to a sample (5 ml.) of fraction B B 8. The solution was cooled to 2°C., centrifuged and added to the column which was then eluted with calcium acetate solution (0.2%). Fractions (5 ml.) were removed and tested for α-amylase activity. The results are shown in Fig. 4.33.

Alcohol sub-fractionation of broad bean α-amylase. Acetate buffer (4 ml.; 0.2M.: pH 5.5) and calcium acetate (100 mg.) were added to a portion of fraction B B 9 which, on centrifugation, yielded a clear solution (1). This solution was heated on a water-bath at 70°C. for 5 minutes, cooled and centrifuged to give solution (2) which was dialysed against distilled water at 2°C. for 6 hours. The cold solution was then fractionated with chilled alcohol between the limits 0 - 20, 20 - 30, 30 - 40, 40 - 50, 50 - 60 and 60 - 70%. Each fraction was air-dried and dissolved in water to give solutions B B 9(3) - B B 9(7). The α -amylase activities of the fractions were determined and they are shown in Table 4.51.

FIG. 4.33

Fractionation of broad bean α-amylase on a starch column.

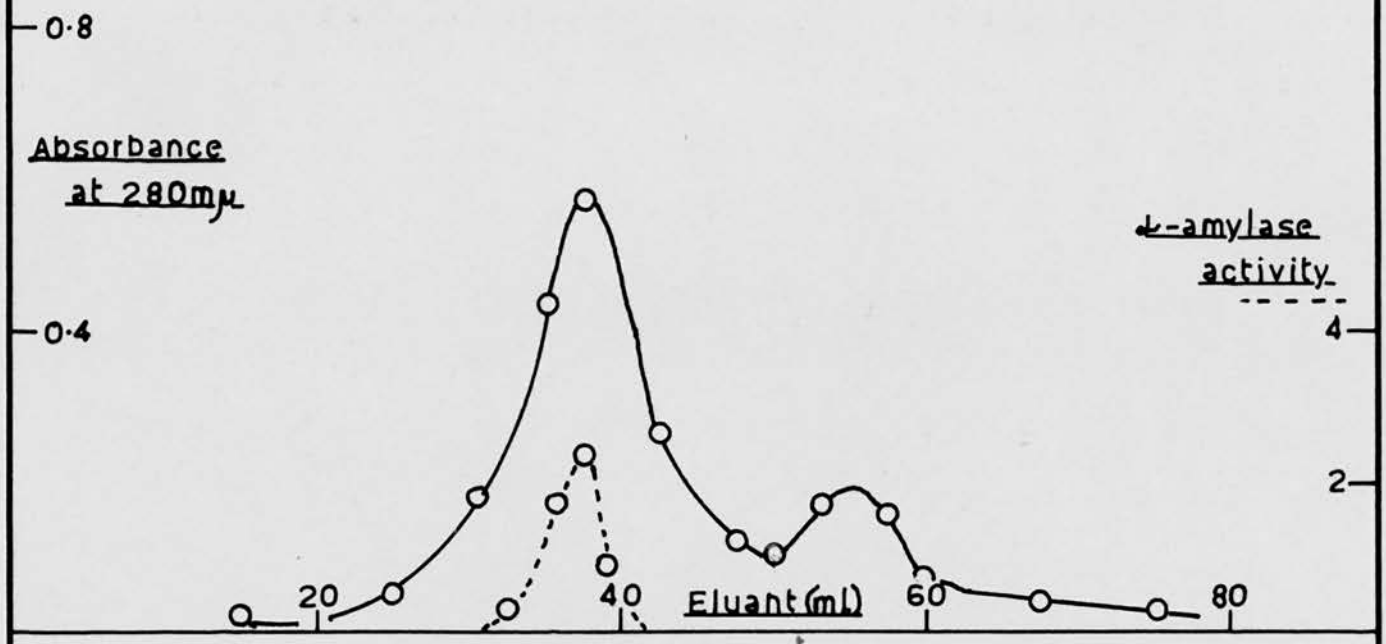


FIG. 4.34

Fractionation of broad bean α-amylase on a G-25 Sephadex column.

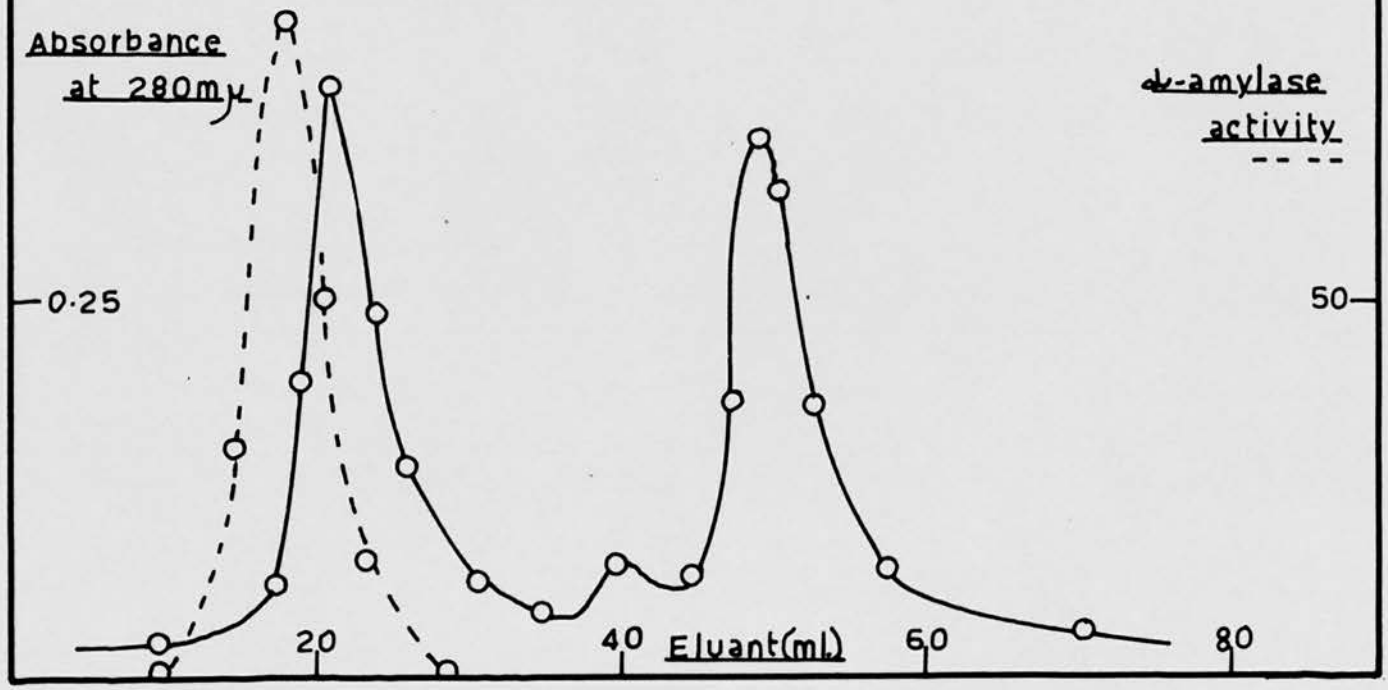


Table 4.51

Fraction Number	B B 9(1)	2	3	4	5	6	7
λ-amylase specific activity	5.7	6.8	12.7	12.0	11.4	12.0	7.4

Fractionation of broad bean λ-amylase on a "Sephadex" column. A sample (5 ml.) of fraction B B 8 was placed on a G - 25 "Sephadex" column, equilibrated with calcium chloride (0.2%), and eluted with calcium chloride solution (0.2%). The λ-amylase activities of the protein - containing fractions were determined and the results are shown in Fig. 4.34.

Final preparation of broad bean λ-amylase. Stage 1. Dry, defatted broad bean flour (100 g.) was shaken at room temperature for 2 hours with calcium chloride solution (500 ml.; 0.2%) containing n-octanol (0.2ml.). The suspension was centrifuged and filtered to yield a clear solution B B A0 (320 ml.; 35 mg. protein/ ml.) which was cooled to 2°C. and fractionated with chilled acetone (-15°C.) between the limits 0 - 20, 20 - 25, 25 - 30, 30 - 35, 35 - 40, 40 - 45, 45 - 50, 50 - 55, 55 - 60 and 60 - 70%. The fractions were air-dried and dissolved in water to give clear solutions 1 - 10 which were stored at 2°C. The λ-amylase activities of the fractions were determined and the results are shown in Table 4.52.

Table 4.52

Fraction Number	BBAO	1	2	3	4	5	6	7	8	9	10
α-amylase specific activity	0.54	0.17	0.56	0.86	1.00	1.33	1.43	2.25	3.30	0.42	0

Stage 2. Since the most active fractions were B B A 7 and B B A 8 these were combined and subfractionated with acetone between the limits 30 - 35, 35 - 40, 40 - 45, 45 - 50, 50 - 55, 55 - 60, and 60 - 70%. These were air-dried, dissolved in water to give clear solutions B B(7 + 8)A - B B (7 + 8) H which were stored at 2°C. The α-amylase activities of these fractions were determined and the results are shown in Table 4.53.

Table 4.53

Fraction Number	BB(7+8)	BB(7+8)A	B	C	D	E	F	G	H
α-amylase specific activity	2.72	0.96	3.30	6.4	10.6	24.0	12.0	3.3	0

Stage 3. Glycogen - complex formation. A portion (9.5 ml.) of fraction B B (7 + 8) E was cooled to 2°C. and chilled ethanol (2°C.; 6.5 ml.) was slowly added with stirring. After 10 minutes, the precipitate was removed by centrifugation at 2°C.

and dissolved in water to give a clear solution B B E 1. Phosphate buffer (0.5 ml.; 0.2M.; pH 8.0), glycogen solution (0.1 ml.; 2%) and ethanol (0.48 ml.) were added to the supernatant obtained from the initial centrifugation. The resulting turbid solution was stirred at 2°C. for 5 minutes before being centrifuged to yield a clear supernatant and a small precipitate which was dissolved in water (solution B B E 2). The clear supernatant was made 60% with respect to alcohol, stirred for ten minutes at 2°C., centrifuged and the precipitate was dissolved in a small volume of water (solution B B E 3). The λ-amylase activities of these solutions were determined and the results are shown in Table 4.54.

Table 4.54

Solution Number	BBE1	BBE2	BBE3
λ-amylase specific activity	22	360	15

Enzyme solution B B E 2 was maintained at room temperature for ca. 6 hours and then dialysed against cold distilled water for 12 hours before being stored at 2°C.

The purification obtained in each stage of the procedure is shown in Fig. 4.35.

Preparation of starch column. Equal weights of "Celite" and laboratory-prepared potato starch were slurried with water,

FIG. 4.35

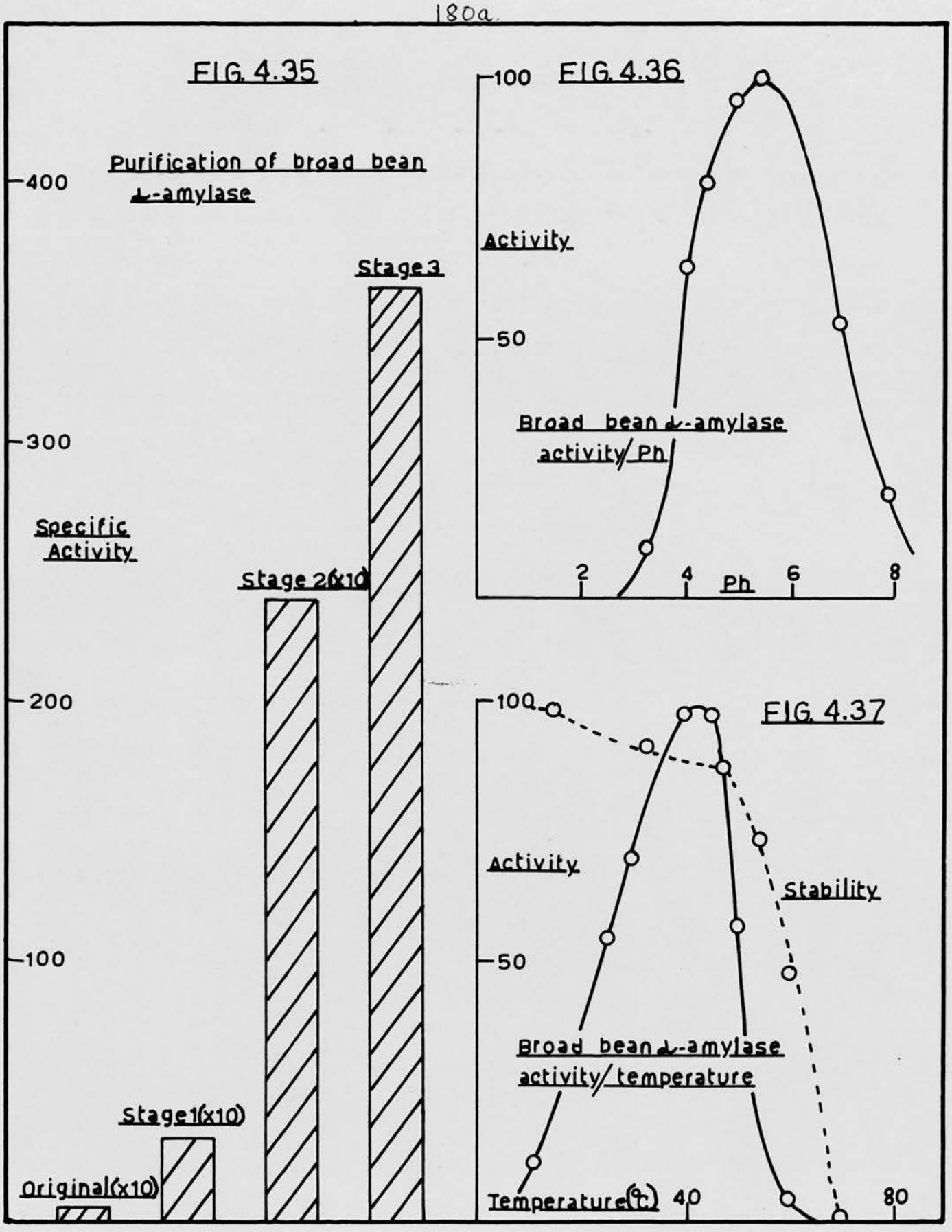


FIG. 4.36

FIG. 4.37

added to a small chromatographic tube (15 cm. x 2.5 cm.) and allowed to sediment. A column height of 10 cm. gave a satisfactory flow-rate. The column was well washed with water before being equilibrated with aqueous alcohol (20%) at low temperature (2°C.).

Preparation of "Sephadex" column. A column similar to that described in the previous section was used.

RESULTS

Purity of enzyme fraction B B E 2. Digests were prepared containing laminarin (1 mg. 0.1 ml.), maltose (1 mg.; 0.1 ml.) and cellobiose (1 mg.; 0.1 ml.). Portions (0.1 ml.) of the enzyme solution were added to each and the digests were incubated at 35°C. Portions were removed at intervals for chromatographic examination. No glucose was detected in any of the digests.

A digest was prepared containing maltotriose (1 mg.; 0.1 ml.) and enzyme solution (0.1 ml.). It was incubated at 35°C. and portions were removed at intervals for chromatographic examination. Glucose and maltose were detected after 10 hours incubation but even after 48 hours sugars larger than maltotriose were not found.

A digest was prepared containing acetate buffer (0.5 ml. 0.2M.; pH 4.6), glucose 6 - phosphate solution (1 ml.; 5 mg./ml.), water (2 ml.) and enzyme solution (0.2 ml.). It was incubated at 35°C. and, after 24 hours, it was tested for the presence of free phosphate groups by the method of Allen (1940). No phosphate was detected.

A digest was prepared containing amylose solution (10 ml.; 1 mg./ml.), acetate buffer (1 ml.; 0.2M.; pH 5.5), disodium hydrogen phosphate solution (1.5 ml.; 23 mg./ml.) and enzyme solution (0.2 ml.). It was incubated at 35°C. and portions (1 ml.) were removed at intervals for free phosphate determinations. After 48 hours there was no reduction in the amount of free phosphate present in the digest.

Effect of pH on broad bean α-amylase. Digests were prepared containing McIlvaine's standard buffer (1 ml.; 0.2M.; pH3 - pH7.8), amylopectin β-limit dextrin solution (2 ml.), water (4.7 ml.) and enzyme solution (0.3 ml. fraction (7 + 8) D). They were incubated at 35°C. and the α-amylase activities were determined. The results are shown in Fig. 4.36. Two further digests, A and B, were prepared containing water (4.7 ml.) and enzyme solution (0.3 ml. fraction (7 + 8) D). A portion (1 ml.) of acetate buffer (0.2M.; pH 5.5) was added to A and a similar amount of pH 3.6 buffer was added to B. They were left at room temperature for 2 hours when equal aliquots (2 ml.) of amylopectin β-limit dextrin solution were added to each. The α-amylase activities of the two digests at room temperature were determined. The results are shown in Table 4.55.

Table 4.55

% Original activity retained by digest A	95
% " " " " " B	0

The following digests were prepared:

1. Acetate buffer (2 ml.; 0.2M.; pH5.5) + enzyme (0.3 ml. fraction (7 + 8)D) + amylose (10 ml.; 2 mg./ml.)
2. Acetate buffer (2 ml.; 0.2M.; pH3.6) + enzyme (0.3 ml. fraction (7 + 8)D) + amylose (10 ml.; 2 mg./ml.)
3. Acetate buffer (2 ml.; 0.2M.; pH3.6) + enzyme (0.3 ml. fraction (7 + 8)D)

Digest 3 was maintained for 2 hours at room temperature before being incubated with the amylose solution (10 ml.; 2 mg./ml.) at 35°C. After 5 hours the amylose present in each digest was precipitated with excess butanol, washed with water saturated with butanol and dissolved in potassium hydroxide (15 ml.; 0.2M.) for a viscosity determination. The results are shown in Table 4.56.

Table 4.56

Digest	1	2	3
Viscosity of amylose sample	95	430	490
Viscosity of original amylose = 490			

Effect of temperature on broad bean α-amylase. (a) Activity of the enzyme/temperature. Digests were prepared containing acetate buffer (1 ml.; 0.2M.; pH 5.5), water (4.7 ml.) and amylopectin β-limit dextrin solution (2 ml.). They were equilibrated at various temperatures between 5 and 60°C. when aliquots of enzyme solution (0.3 ml. of fraction (7 + 8)D) were added to each. The α-amylase activities of the various digests were determined and the results are shown in Fig. 4.37.

(b) Stability of the enzyme/temperature. Digests were prepared containing acetate buffer (1 ml.; 0.2M.; pH 5.5) and water (4.7 ml.). They were equilibrated at various temperatures between 10 and 71°C. when aliquots of enzyme solution (0.3 ml.

fraction (7 + 8)D) were added to each. After 1 hour, the digests were quickly brought to equilibrium at 35°C. and portions (2 ml.) of amylopectin β-limit dextrin solution were added to each. The α-amylase activities were determined and the results are shown in Fig. 4.37.

Effect of E.D.T.A. and trypsin on broad bean α-amylase. The following digests were prepared:

1. Acetate buffer (1 ml.; 0.2M.; pH 5.5) + enzyme solution (0.3 ml. (7 + 8)D) + E.D.T.A. (1 ml.; 10^{-1}M.)
2. Acetate buffer (1 ml.; 0.2M.; pH 5.5) + enzyme solution (0.3 ml. (7 + 8)D) + E.D.T.A. (1 ml.; 10^{-1}M.) + trypsin (1 ml.; 6 mg./ml.)
3. Acetate buffer (1 ml.; 0.2M.; pH 5.5) + enzyme solution (0.3 ml. (7 + 8)D) + trypsin (1 ml.; 6 mg./ml.)
4. Acetate buffer (1 ml.; 0.2M.; pH5.5) + enzyme solution (0.3 ml.(7 + 8)D).

These were incubated at room temperature for 12 hours when equal aliquots (10 ml.) of an amylose solution (2 mg./ml.) were added to each. After 48 hours, excess butanol was added to each digest, but there was no precipitate in 3 and 4. The viscosities of the amylose samples obtained from 1 and 2 were determined in potassium hydroxide (0.2M.). The results are shown in Table 4.57.

Table 4.57

Digest	1	2	3	4
Viscosity	470	495	0	0
Viscosity of the original amylose = 490				

The following digests were prepared:

1. Acetate buffer (2 ml.; 0.2M.; pH5.5) + E.D.T.A. (1 ml.; 10^{-1}M.) + enzyme (0.3ml. fraction (7 + 8)D).
2. Acetate buffer (2 ml.; 0.2M.; pH 5.5) + trypsin (0.2 ml. 6 mg./ml.) + enzyme (0.3 ml. fraction (7 + 8)D).
3. Acetate buffer (2 ml.; 0.2M.; pH 5.5) + E.D.T.A. (1 ml.; 10^{-1}M.) + trypsin (0.2 ml.; 6mg./ml.) + enzyme (0.3 ml. fraction (7 + 8)D).
4. Acetate buffer (2 ml.; 0.2M.; pH 5.5) + enzyme (0.3 ml. fraction (7 + 8)D).

These were incubated at room temperature for 12 hours when each was divided into two equal portions. Calcium acetate (50 mg.) was added to one set of digests and they were left at room temperature for a further 3 hours, when aliquots of water (3 ml.) and amylopectin β-limit dextrin (1 ml.) were added to each. The α-amylase activities were determined and are shown in Table 4.58.

Table 4.58.

% Original Activity				
Digest	1	2	3	4
Before addition of calcium ions	0	90	0	100
After addition of calcium ions	30	88	0	100

Effect of various substances on broad bean α-amylase. Digests

were prepared containing acetate buffer (2 ml.; 0.2M.; ph 5.5), enzyme (0.3 ml. (7 + 8) D), requisite amount of the compound under investigation and water, to make a total volume of 10 ml. They were left at room temperature for 2 hours when digests containing precipitates (especially those containing mercuric chloride) were clarified by centrifugation. Aliquots of amylopectin β-limit dextrin solution (2 ml.) were added and the α-amylase activities of the digests were determined. The results are shown in Table 4.59.

Table 4.59

	% Inhibition			
	10^{-3}M	10^{-4}M	10^{-5}M	10^{-6}M
$HgCl_2$	100	100	85	52
KCN	0	0	0	0
$CaCl_2$	0	0	0	0
NaCl	0	0	0	0
Ammonium molybdate	32	24	16	10
Tryptophane	0	0	0	0

Action of broad bean α-amylase on amylose. (a) Kinetics of the initial reaction. A digest was prepared containing amylose solution (19 ml.; 1.5mg./ml.) and acetate buffer (1 ml.; 0.2M.; pH 5.5). It was incubated in a Ubbelohde viscometer at 25°C. and its flow-time determined. 0.1 ml. of fraction B B E 2 was

diluted to 5 ml. with water and an aliquot (0.1 ml.) of this dilute solution was added to the amylose solution. The degradation of the amylose was followed by measuring the flow-times of the solution at various times. The results are shown in Fig. 4.38.

(b) Products of the reaction. A digest was prepared containing amylose solution (74 ml.; 3 mg./ml.) and enzyme (0.5 ml. fraction B B E 2). It was incubated at 35°C. and aliquots (1.5 ml.) were removed at intervals for iodine staining (0.25 ml.) and reducing power determinations. The results are shown in Fig. 4.38a. Larger aliquots (5 ml.) were also removed at intervals, evaporated to dryness and suspended in a small volume (0.75 ml.) of water. Portions of these were tested with iodine and further portions were applied to chromatograms. The results are shown in Table 4.60. Standard sugars G_1 - G_{10} were also placed on the chromatograms. 5 descents of ca. 8 hours duration were required for complete separation of saccharides G_1 to G_{10}.

Action of broad bean β-amylase on maltotetraose. A digest was prepared containing enzyme solution (0.1 ml. fraction B B E 2) and G_4 (0.1 ml.; 1 mg.). It was incubated at 35°C. and aliquots were removed at intervals for chromatographic examination. The results are shown in Table 4.61.

FIG. 4.38

Action of broad bean β-amylase on amylose

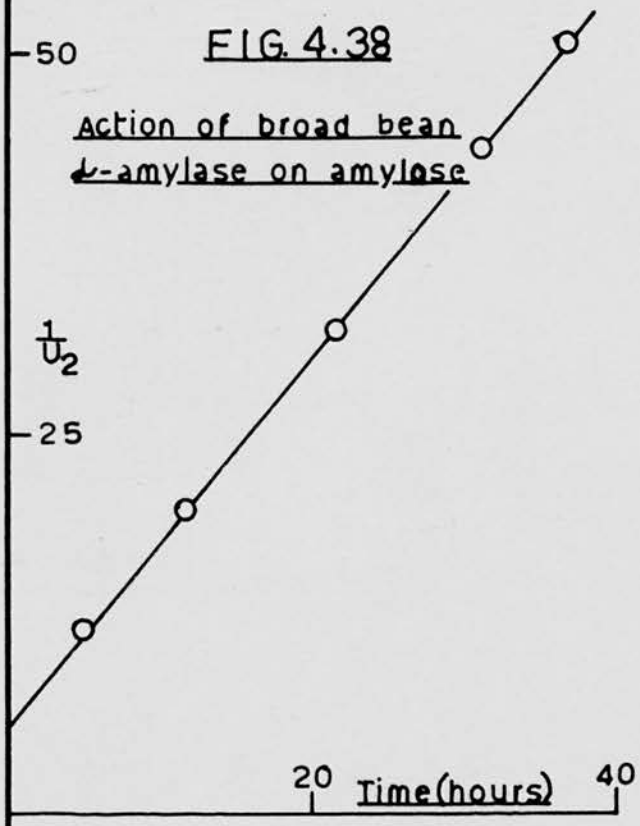


FIG. 4.38a

Action of broad bean β-amylase on amylose

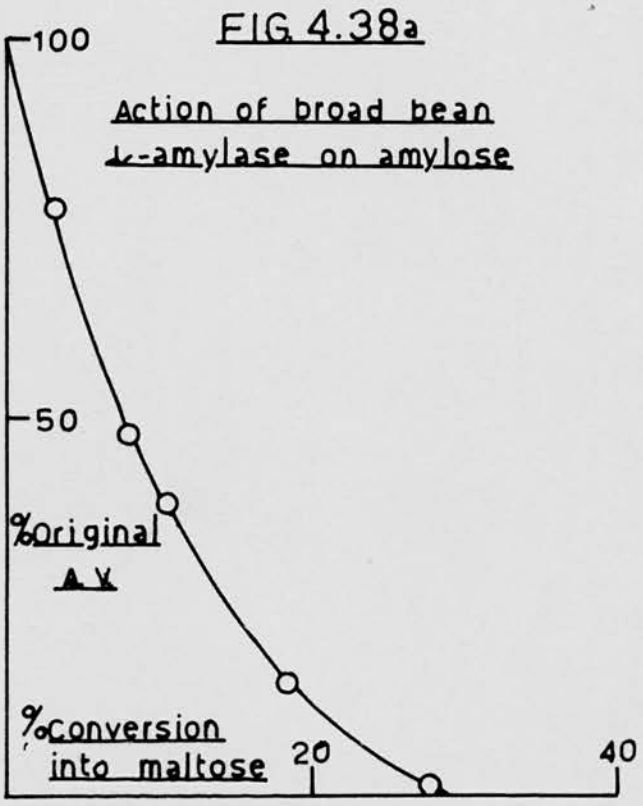


FIG. 4.39

Action of broad bean β-amylase on:
1. amylopectin
2. amylopectin β-limit dextrin

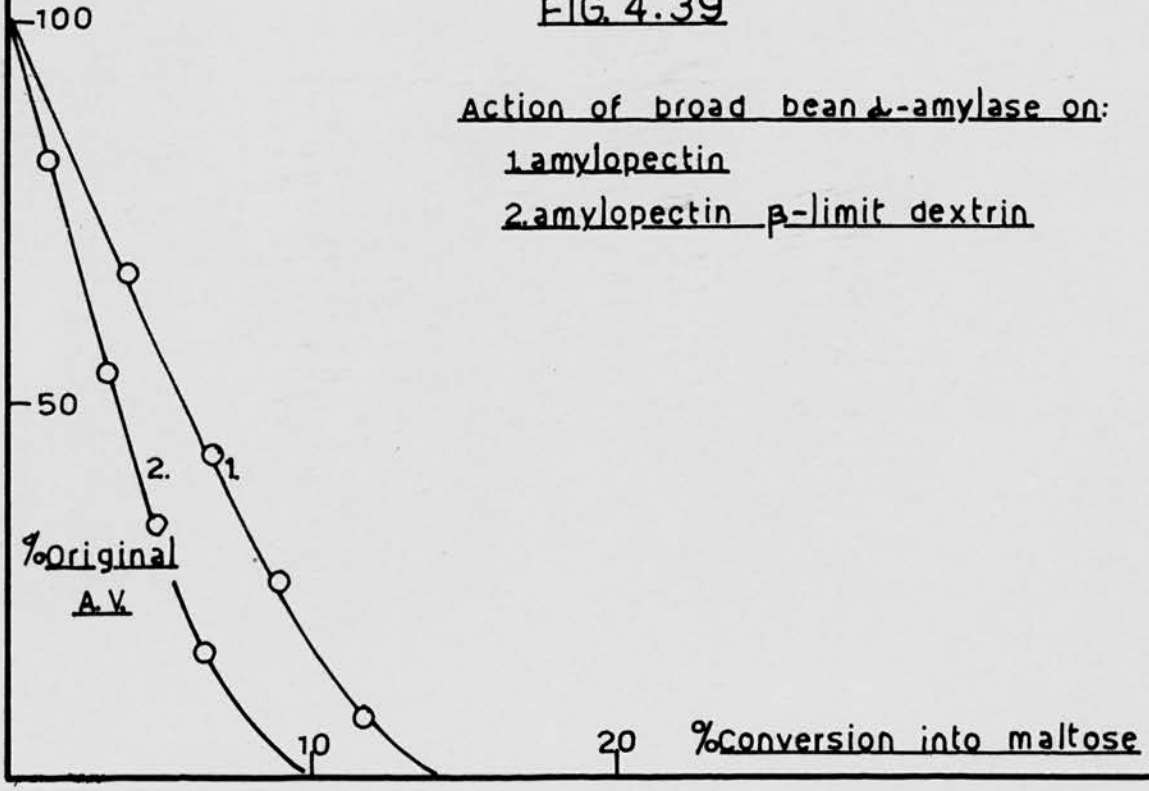


Table 4.60

Time of extraction of aliquot from the digest						
Maltod-extrin	22 hours	32 hours	45 hours	58 hours	104 hours	140 hours
G_1	trace	+	++	+++	++++	+++++
G_2	+	++	+++	+++	++++	+++++
G_3	+	++	+++	+++	++++	+++++
G_4	+	++	++	++	+++	++++
G_5	+	++	++	++	++	++
G_6	+	++	+++	+++	+++++	++
G_7	+++	++++	++++	++++	+++	trace
G_8	+++	+++++	++++	+++	trace	0
Higher than G_8	++++	+++++	++++	+++	trace	0
Iodine stain of concen-trated digest	Purple	Red	Brown	Brown	Faint Brown	Achroic

Table 4.61

Relative amounts of oligosaccharides						
Time of aliquot -removal(hours)	0	5	8	26	48	69
Oligosaccharide						
G_1	0	+	++	+++	+++	+++
G_2	0	+++	++++	+++++	+++++	+++++
G_3	0	+	++	+++	+++	+++
G_4	+++++	+++	++	0	0	0

Action of broad bean α-amylase on amylopectin and amylopectin β-limit dextrin. Digests were prepared containing acetate buffer (2 ml.; 0.2M.; pH 5.5), enzyme solution (0.5 ml. B B E 2) and amylopectin or amylopectin β-limit dextrin (30 ml.; 4.5 mg./ml.). They were incubated at 35°C. and aliquots (2 ml.) were extracted at intervals for iodine staining (1 ml.) and reducing power (1 ml.) determinations. The results are shown in Fig. 4.39.

Action of broad bean α-amylase on glycogen and glycogen β-limit dextrin. Digests were prepared containing glycogen or glycogen β-limit dextrin (10 ml.; 2 mg./ml.), acetate buffer (1 ml.; 0.2M.; pH 5.5) and enzyme solution (0.5 ml. fraction B B E 2). They were incubated at 35°C. and aliquots (1 ml.) were removed at intervals for reducing power determinations. The results are shown in Table 4.62.

Table 4.62

% Apparent Conversion into maltose					
Time of aliquot extraction (hours)	0	5	12	30	48
Glycogen digest	0	4	8	10	11
Glycogen β-limit dextrin digest	0	0	2	2	3

DISCUSSION

Preparation of broad bean α-amylase. The presence of R-enzyme activity is detected by a rise in the iodine-staining power of amylopectin β-limit dextrin solution (Hobson et al, 1951). The results in Fig. 4.29 indicate that R-enzyme is present in fractions B B 2, B B 3 and B B 4 of the initial preparation; it may also be present in some of the other fractions, but its activity would not be detected because of the relatively large amount of α-amylase also present. The determination of R-enzyme activity in the presence of excess α-amylase is a difficult problem, which was not investigated in this section, and so the activities shown in Fig. 4.29 are not very precise, but they do indicate the presence of R-enzyme in those particular fractions. Also, R-enzyme affects the determination of α-amylase activities but this is not such a difficult problem as ammonium molybdate (10^{-2}M.) completely inhibits R-enzyme activity whilst it only partially inhibits (45%) that of α-amylase. The activities shown in Fig. 4.30 have been adjusted to take into account the inhibitory effect of ammonium molybdate.

The results shown in Figs. 4.31 and 4.32 indicate the distribution of maltase and phosphatase in the initial acetone fractionation procedure. They are both relatively weak in the fractions which contain the highest α-amylase activities.

Various workers (Holmberg, 1933 a, b; Hockenhull and Herbert, 1945; Hobson et al, 1950; Schwimmer and Balls, 1949) have shown that α-amylases from different sources may be adsorbed

on to starch granules - the adsorption being more efficient in the presence of alcohol. The results shown in Fig. 4.33 indicate that ca. 80% of the broad bean β-amylase was adsorbed on the starch column but was not eluted under the experimental conditions.

An experiment was carried out to determine the efficiency of alcohol as a fractionating agent for broad bean β-amylase. The results, shown in Table 4.51 (p.178) indicate that efficient fractionation has not been accomplished because all the fractions contain a substantial amount of the total enzymic activity. There is, therefore, nothing to be gained by using alcohol as a precipitant in these fractionations.

A further attempt to subfractionate the enzymic activity was made on a G - 25 "Sephadex" column (Fig. 4.34). Extensive purification (x 10) was obtained, but the activity curve was skew, i.e. the enzymic activity did not correspond to a clear-cut protein fraction. This method of purification, however, deserves further study.

The enzyme was finally purified by the double acetone-fractionation and glycogen-complex techniques which have already satisfactorily fractionated the β-amylases from soya bean and malted barley. The results in Tables 4.52 and 4.53 indicate that the acetone fractionations of the broad bean flour have not been as efficient as were those of the barley and malted barley flour. This loss in efficiency was probably caused by the higher concentrations (x 2) of protein present in the broad bean solutions. Protein concentration plays an important part in the

efficiency of the fractionation obtained in a particular system (Dixon and Webb, 1961).

Purity of enzyme sample B B E 2. Since the enzyme did not produce glucose on digestion with maltose, laminarin or cellobiose, the sample does not contain maltase, laminarinase or cellobiase. The slight hydrolysis of maltotriose detected indicates that the enzyme attacks this oligosaccharide only very slowly and the absence of higher sugars in the digest shows that D-enzyme is not present in the system. Phosphatase is also absent as shown by the inability of the enzyme sample to hydrolyse glucose 6 -phosphate. In the digest containing amylose, phosphate and enzyme, no lowering of the free-phosphate concentration was detected indicating that phosphorylase is not present. This enzyme, in the presence of free phosphate, hydrolyses amylose to produce glucose 6 - phosphate and so lowers the free phosphate concentration of the digest (Barker and Bourne, 1953).

Effect of pH on broad bean α-amylase. The optimum pH for enzymic activity is pH 5.5. (Fig. 4.36). The results in Tables 4.55 and 4.56 (p.183-184) indicate that the enzyme is completely inhibited after incubation (2 hours) at pH 3.6. This inhibitory effect at pH 3.6 is not instantaneous as shown by the fall in viscosity of digest 2 (Table 4.56. p.184) and the small activity shown by the enzyme even at pH 3.0 (Fig. 4.36).

Effect of temperature on broad bean α-amylase. The enzyme shows its maximum activity at 43°C. and it retains 90% of its activity after a 1 hour incubation at this temperature (Fig. 4.37). At 70°C. the activity is quickly destroyed, however.

Effect of E.D.T.A. and trypsin on broad bean β-amylase. The results in Tables 4.57 and 4.58(p.185-186) indicate that the enzyme requires calcium ions for its activity. In the absence of these the enzymic activity is reversibly inhibited and it may be restored on the addition of calcium ions to the enzyme solution. A protease (e.g. trypsin) is not able to attack the enzyme when it contains its full complement of calcium ions, but when these are removed by a complexing agent (e.g. E.D.T.A.) the enzymic activity is irreversibly destroyed.

Effect of various substances on broad bean β-amylase. Of the substances investigated, only mercuric chloride and ammonium molybdate had any effect on the activity of the enzyme (Table 4.59. p. 187). Neither sodium chloride nor calcium chloride activated the enzyme.

Action of broad bean β-amylase on amylose. The linear relation obtained from the viscosity results during the initial stage of the hydrolysis (Fig 4.38) indicates that the amylose was degraded by a completely random mechanism. (cf. p.168)

The final products of the reaction are shown in Table 4.60 (p.189). They suggest that at 28% conversion of the amylose into apparent maltose only very small amounts of oligosaccharides G_1 - G_6 are present in the digest. There are only detectable amounts of G_1, G_4, G_5 and G_6 formed at this stage in the reaction but larger quantities of G_2 and G_3 are present. In general, the amounts of these saccharides increase as the hydrolysis proceeds, but G_6 is itself hydrolysed towards the end of the reaction.

G_4 and G_5 are only slowly formed but, whereas G_4 is present in a relatively large amount after 140 hours, G_5 does not increase appreciably in amount throughout the hydrolysis. The concentrations of G_1, G_2 and G_3 increase steadily as the reaction proceeds. Oligosaccharides having chain-lengths greater than 8 are present in the reaction products even after 104 hours as shown by the chromatogram and the slight brown colour which a sample of the concentrated digest yields with iodine. During the period in which the reaction was investigated the amounts of G_7 and G_8 increase initially, but then rapidly decrease, with G_8 being more quickly hydrolysed than G_7.

Only a small amount of G_1 is present in the digest after 22 hours hydrolysis and it is difficult to determine whether it is a primary fission product or whether it was formed as a secondary fission product from the large oligosaccharides already present in the digest.

However, larger amounts of G_2 and G_3 are initially present, and these may well have been formed by the hydrolysis of relatively long amylose molecules. If the enzyme acts in a purely random manner then small dextrins ought to be present as primary scission products of the reaction. Because the amounts of these small saccharides increase as the reaction proceeds, they must be preferentially formed as secondary products from the hydrolysis of the larger oligosaccharides. The small amounts of G_4 and G_5 produced during the intermediate stage in the reaction (32 - 104 hours) suggest that these are not readily formed from small dextrins (D.P. ca. 10) and so the initial production of these sugars is due to primary fission of amylose molecules.

Action of broad bean α-amylase on G_4. The distribution of the reaction products is shown in Table 4.61 (p.189). G_2 is preferentially formed, but detectable amounts of G_1 and G_3 were also present. Since the amount of G_1 did not increase when the G_4 was completely hydrolysed, maltose did not appear to be present in the system.

Action of broad bean α-amylase on various starch-type substrates. The results in Fig. 4.39 and Table 4.62 (p.190) indicate that the enzyme attacks amylopectin, glycogen and their β-limit dextrins. Glycogen β-limit dextrin is attacked much more slowly (x 3) than glycogen, showing that the exterior chains of the parent molecule are more readily attacked than those in the interior.

4e. GENERAL PROPERTIES OF α-AMYLASES

Since the properties of the Z-enzyme from soya beans were similar to those of α-amylases extracted from broad beans, barley and malted barley, this enzyme must also be an α-amylase. The α-amylase from malted barley had a much higher activity (ca. 10^3) than the other α-amylases but the remaining properties were very similar. They were all shown to require calcium ions for their activity, to be unaffected by the presence of excess chloride ions and to be completely inhibited in the presence of mercuric chloride (10^{-4}M.) In the absence of calcium ions, they were susceptible to protease attack and were irreversibly denatured by the combined action of E.D.T.A. and trypsin. Inactivation caused by E.D.T.A. alone was reversible because the addition of excess calcium ions to E.D.T.A. - deactivated enzyme systems restored the enzymic activity. Malt α-amylase was the most unstable of these enzymes, but this may have been due to the necessarily high dilutions of the enzyme used in the investigations.

The enzymes were all rendered inactive by a two-hour incubation period at pH 3.6, and each enzyme showed its maximum activity at pH 5.5. The temperatures of optimum activity were somewhat more variable, ranging from 43°C. in the case of broad bean α-amylase to 55°C. for soya bean Z-enzyme. This property depends upon the stability of the particular enzyme at relatively high temperatures and may vary with the protein concentration of the enzyme solution.

All of these enzymes hydrolysed amylopectin, glycogen

and their β-limit dextrins and the relative rates of enzymic attack on these substrates showed that amylopectin was hydrolysed much more quickly than glycogen, and not at the same velocity, as indicated by Schwimmer (1950) from studies on the rates of attack of malt α-amylase on similar substrates. This author also stated that amyloses were hydrolysed at twice the velocity of amylopectins and glycogens by malt α-amylase; the results in Table 4.49 (p. 161) show, however, that this enzyme hydrolysed amylose 32× faster than amylopectin and 70× faster than glycogen. Although the two sets of results are widely different, they may both be correct. Schwimmer followed the hydrolyses by reducing power determinations and so, in effect, measured the attack of the malt enzyme on the outer chains of amylopectin and glycogen. The initial velocities of the enzymic attacks on these may not be very different and they may be similar to the initial attack on amylose. Since the external chains of amylopectin and glycogen are much shorter than the chains in amylose, then short-chain reducing saccharides will be formed more rapidly in the digests containing these substrates. At the beginning of the reaction this will help to compensate for the inevitable rapid fall in enzymic attack as the enzyme approaches branch-points in the amylopectin and glycogen molecules. In the present work, however, the degradations have been followed by a light-scattering technique which indicates the extent of hydrolysis of the internal bonds in the substrate molecules. Results of initial velocities of attack obtained in this way would be expected to show a large variation in the degradations of amylose,

amylopectin and glycogen.

Because amylose is an essentially linear molecule, it was used as the substrate for examining the action patterns of the α-amylases from soya beans, broad beans malted barley and barley. In all cases the initial hydrolyses were shown to take place by a random attack on the substrate molecules and the enzymes were found to have a much smaller affinity for short-chain oligosaccharides (D.P. < 7) than for large amylose molecules. The curves relating iodine stain to reducing power during the α-amylolysis of amylose were similar for all the enzymes, indicating that the action patterns of the various enzymes were comparable.

There are two widely used methods for comparing the action patterns of different α-amylases on starch-type substrates. The first of these entails measuring the loss in iodine-stain and the corresponding increase in reducing-power of a solution of the substrate during α-amylolysis. In the second method, the products of the reaction are examined by chromatography.

Bernfeld and Fuld (1948) examined the action patterns of various α-amylases by allowing the enzymes to degrade starch to a limited extent and comparing the increase in reducing power (Saccharogenic activity) with the decrease in iodine stain (dextrinogenic activity). They obtained, therefore, a quotient of saccharogenic power/dextrinogenic power. This quotient was found to be the same for all the α-amylases tested and these authors suggested that the action patterns of the amylases were

identical.

This view was contested by Myrbäck (1950) and results reported by Kung et al. (1953) suggested that the conclusions of Bernfeld and Fuld (1948) were not correct. Kung et al. (1953) investigated the hydrolysis of corn amylose by various α-amylases and examined the relation between, the disappearance of the substrate - as shown by blue value determinations - and the increase in the reducing value of the hydrolysate. The nature of the substrate at the achroic limit, as shown by reducing power, was thus determined for a number of α-amylase/amylose systems. The results indicated that the enzymes differed widely in their reactions with the amylose substrate, e.g. 23% of the glucosidic linkages in the amylose substrate were broken at the achroic limit by swine pancreatic α-amylase, but only 12% by Taka amylase (from mold Aspergillus oryzae). The discrepancy between the results of the Meyer-Bernfeld saccharogenic/dextrinogenic quotient and those reported by Kung et al. has been explained by Whelan (1958). He suggested that since the quotient was determined at a very early stage in the reaction sufficient degradation of the substrate to reveal any differences in reaction pattern would not have taken place.

Hopkins and Bird (1954) have examined the action of crystalline malt α-amylase on amylose solutions of varying concentration and concluded that the percentage reducing power (which is equivalent to 2 x percentage of glucosidic linkages broken) attained at the achroic point was dependent upon the amylose concentration. These results suggest that measurements

of iodine stain and reducing power are not very satisfactory for determining the reaction mechanisms of such enzyme systems.

The technique of separating oligosaccharides, both qualitatively and quantitatively, by chromatography enables the intermediate and final products of an amylase/substrate system to be investigated. The reaction products are simplified if a linear substrate, such as amylose, is used because only linear oligosaccharides will then be formed. In this work, the reaction products from the degradation of linear amylose by soya bean, broad bean, barley and malted barley α-amylases have been examined by paper chromatography. The distribution of oligosaccharides present at the achroic limit was examined for each enzyme, but no significant difference was detected. The most abundant species were oligosaccharides of chain-lengths 7 - 10 glucose units. Smaller amounts of G_2, G_3, G_4, G_5 and G_6 were detected but only a trace of G_1 was present. After the achroic limit, dextrins larger than G_6 slowly disappeared and the amounts of G_1, G_2 and G_3 present rapidly increased. G_4 and G_5 did not appreciably increase in amount until dextrins larger than G_7 had been hydrolysed. Because of the extremely slow hydrolysis of dextrins smaller than G_7, the true end-products of the reactions were not determined. However, hydrolysis of G_4 and G_3 by these enzymes was detected (cf. Parrish (1958) Bines (1956)) and so these plant α-amylases should degrade amylose to glucose and maltose only.

The following reaction scheme explains the presence and relative quantities of the oligosaccharides near the

achroic limit in an amylose/α-amylase digest. Although the discussion pertains to the action pattern of malt α-amylase it may also be applied to the other plant α-amylases investigated in this work.

Many workers (e.g. Myrbäck, 1947; Hanes and Cattle, 1938; Schwimmer, 1950; Meyer and Gonon 1951 b) have shown that malt α-amylase has a much lower affinity for small oligosaccharides (D.P. < ca. G_8) than for long-chain amylose or starch molecules. Hopkins and Bird (1954) indicated that these results may be explained by assuming that the enzyme does not readily attack the first 5 linkages from the non-reducing end and the linkage at the reducing end of a substrate molecule. The action pattern of the enzyme, therefore, cannot be a completely random hydrolysis of the substrate. However, assuming that the other bonds in the substrate molecule are equally susceptible to attack, the accumulation of oligosaccharides having D.Ps. > 6 should be observed in the reaction mixture. Detectable amounts of G_2 - G_6 should also be found because the enzyme ought to attack linkages 2, 3, 4 etc. from the reducing end of the substrate molecule. Since the bond at the reducing end is not so readily hydrolysed, smaller amounts of G_1 are to be expected. As the achroic limit is approached, the number of substrate molecules containing more than ca. 12 - 15 glucose units becomes increasingly small and the enzyme is forced to react with the smaller dextrins to produce G_1, G_2 and G_3. The very small amounts of G_4 and G_5 produced during the hydrolysis may be explained in the following way. During the hydrolysis of G_{12} the following dextrins are most probably formed

as primary fission products $G_6 + G_6$, $G_7 + G_5$, $G_8 + G_4$, $G_9 + G_3$, $G_{10} + G_2$ and smaller amounts of $G_{11} + G_1$. Assuming that G_7 is relatively slowly attacked then the following secondary fission products will be formed:

$G_8 \rightarrow G_6 + G_2$; $G_9 \rightarrow G_6 + G_3$; $G_7 + G_2$; $G_{10} \rightarrow G_8 + G_2$, $G_7 + G_3$, $G_6 + G_4$. The only tertiary fission which will occur is $G_8 \rightarrow G_6 + G_2$,

Small amounts of glucose will also be split off from dextrins > G_6. It can thus be shown that G_{12} produces $3G_7 + 6G_6 + 1G_5 + 2G_4 + 3G_3 + 5G_2$ + glucose. There are, therefore, relatively small amounts of G_4 and G_5 produced. However, dextrins such as G_6 and G_7 will be preferentially split to form $G_4 + G_2$, $G_5 + G_1$ and $G_5 + G_2$, $G_6 + G_1$, $G_4 + G_3$, respectively. This explains the relatively high production of G_4 and G_5 near the end of the α-amylolysis of amylose by these plant α-amylases. The reaction scheme described above is in agreement with the observations and conclusions of Bird and Hopkins (1954). These results may be compared and contrasted with the action patterns and characteristics of α-amylases from various sources.

Pazur and Sandstedt (1954) have examined the action of wheat malt α-amylase on starch. Using paper chromatography they have shown the production of glucose and higher saccharides during the degradation. The enzyme attacked G_4 to produce G_2, G_3 and G_1 and it also attacked G_3 to form G_2 and G_7.

An α-amylase has been extracted from sorghum malt and some of its properties have been examined (Dube and Nordin 1961,

1962). Since this is a further example of a plant amylase, it is of added interest to compare the properties of this enzyme with those of the other plant α-amylases studied in the present work. The sorghum malt α-amylase required calcium ions for stabilisation, was inactivated at pH values below 3.5 and had a pH optimum of 4.6. Only the latter result differs from those obtained in this study, but it is closer to the values for the plant α-amylases than for Bacillus subtilis α-amylase (pH 5.3 - 6.8. Meyer et al., 1947; Meyer, 1952), salivary α-amylase (pH 6.9. Meyer et al., 1948) or Aspergillus oryzae (5.5 - 5.9. Fischer and Montmollin, 1951 a, b). The hydrolysis of starch by sorghum α-amylase showed the same characteristics at the achroic limit as did the hydrolysis of amylose by the plant α-amylases. There was a build-up of dextrins G_6 - G_8 and G_1 - G_3 with smaller amounts of G_4 and G_5. The distribution of oligosaccharides suggests that the sorghum α-amylase does not readily attack those bonds which have already been shown to be relatively immune to plant α-amylase attack.

The action of salivary α-amylase on amylose has given rise to much controversy, but some of the features of the reaction have now been established. Only a small amount of glucose is produced during the reaction but its source is difficult to determine. Initial results (Roberts and Whelan, 1951, 1952; Whelan and Roberts, 1953) suggested that the enzyme did not attack maltotriose but later workers (Pazur, 1953, 1955; Walker and Whelan, 1960) showed conclusively that extremely large concentrations of enzyme effected a slow hydrolysis of maltotriose into

maltose and glucose. The production of a trace of glucose during the hydrolysis of a starch-type substrate is, therefore, mostly due to an extremely slow hydrolysis of maltotriose.

Investigation of the increase in reducing power of an amylose/salivary α-amylase digest indicated that the reaction took place in two stages (Walker and Whelan, 1960). During the first stage, amylose was rapidly hydrolysed to maltose and maltotriose and, in the slow, second stage, maltotriose was further degraded to glucose and maltose. This indicates that salivary α-amylase hydrolyses amylose and all dextrins larger than maltotriose at comparable velocities. Results obtained by Myrbäck (1947) and Walker and Whelan (1960), who showed, respectively, that G_6 and G_4 were hydrolysed at rates comparable to that of amylose, were in agreement with this conclusion. A comparison of the action of salivary α-amylase and malt α-amylase on G_4 carried out in this work (p. 161) showed that the salivary enzyme hydrolysed the substrate many times faster than did the malt enzyme.

The end-products of the action of salivary α-amylase on short-chain oligosaccharides has also been studied (Pazur and Budovich, 1956; Whelan and Roberts, 1953). The results of Pazur and Budovich are probably more precise, since these workers used oligosaccharides labelled at the reducing end with C^{14} in their studies. They showed that G_5 was preferentially split to form G_3 + G_2. G_6 was preferentially split to form G_2 + G_4 but G_3 was also readily formed. In both cases the G_2 formed was radioactive, indicating that the second bond from the reducing end was preferentially hydrolysed in both dextrins. These

results show that the hydrolysis of short-chain oligosaccharides, by the α-amylase, is not random although only two bonds (as against six in reactions with plant α-amylases) are difficult to hydrolyse - those nearest the reducing and non-reducing ends of the substrate molecule. The linkage nearest to the non-reducing end of the substrate was not hydrolysed by either the salivary α-amylase or the plant α-amylases.

Pazur et al. (1950) reported results which, they claim, indicate that salivary α-amylase hydrolyses amylodextrin (D.P. ca. 23) via a "random encounter, multiple attack" mechanism. They followed the degradation of amylodextrin in the presence of salivary α-amylase at pH 7 and pH 10.3 by paper chromatography, iodine staining and reducing power determinations. At pH 7 no significant amount of dextrins larger than G_4 were detected and, from this result, the authors concluded that intermediate oligosaccharides (G_5 - G_{10}) were not formed. However, since such species are hydrolysed very rapidly by the enzyme they may have been produced and degraded before the digest was examined by chromatography (cf. Bird and Hopkins, 1954). At pH 10.3 (an unfavourable pH for the enzymic activity) intermediate dextrins were detected but the reaction under these conditions took place extremely slowly and so these dextrins would be present in the digest for a relatively long period. The results, therefore, may be explained quite simply without recourse to postulating a complex action pattern.

The α-amylase from Bacillus subtilis resembles malt α-amylase in having a very small affinity for short-chain

oligosaccharides. It is also capable of splitting glucose from amylose at a fairly early stage (achroic limit) in the reaction. Investigations on the action pattern of this enzyme have been carried out by Hopkins and Kulka (1942), Bird and Hopkins, (1954) and Robyt and French (1962). The results of Hopkins and Bird suggest that the enzyme is further similar to malt α-amylase in that it does not readily attack the first five linkages from the non-reducing end of a substrate molecule. The bacterial enzyme, however, split the first two linkages from the reducing end less readily than did the malt enzyme. There are, therefore, seven bonds in an amylose molecule relatively immune to Bacillus subtilis α-amylase compared to six in the case of malt α-amylase. This means that the bacterial enzyme will form relatively less G_1 and G_2 but more G_3 on reaction with amylose than will malt α-amylase. Therefore, towards the end of the Bacillus subtilis α-amylase hydrolysis of amylose an accumulation of G_3 and G_6 would be expected. The chromatograms of Robyt and French (1962) indicate that this does happen but these authors interpreted the results in terms of a dual product specificity of the enzyme for the formation of G_3 and G_6. Their results on the action of the α-amylase on amylose and dextrins may be explained as above but interpretation of the results pertaining to the enzymic degradation of branched substrates is more difficult.

A unique property of the α-amylase from Bacillus subtilis is that it contains small amounts of zinc which induce dimerisation in the protein molecules (Stein and Fischer, 1960). One atom of zinc per molecule of dimer is taken up by the enzyme

during dimerisation and released with subsequent dissociation of the dimer in the presence of metal-binding agents.

Proteases irreversibly deactivate Bacillus subtilis in the presence of E.D.T.A. which suggests that the enzyme requires divalent ions for its activity (Stein and Fisher, 1958). The presence of calcium in the enzyme has been shown by these authors and further studies (Stein and Fischer, 1960; Vallee et al., 1959) have indicated that this metal is more firmly bound than zinc by the enzyme. Later workers (Stein et al., 1964; Hsiu et al., 1964) examined the effect of removing the calcium ions from Bacillus subtilis α-amylase on the activity of the enzyme and compared the results with similar ones obtained for salivary α-amylase. Their main conclusions were that the bacterial and salivary α-amylases contained 5 and 2 g. atoms/mole of calcium, respectively; the bacterial enzyme appeared to bind its associated calcium much more firmly than did the salivary enzyme. The enzymes were rendered inactive when calcium was removed but, in the absence of proteases they were stable and quantitatively recovered their activity on the addition of excess calcium ions. α-amylases from different sources appeared to liberate their bound calcium at different rates, decreasing in the order mammalian > bacterial > fungal. The results obtained for malt α-amylase and the other plant α-amylases examined in this work suggest that these enzymes do not bind calcium very firmly and that its rate of release would be similar to that of the calcium associated with salivary α-amylase.

A fungal α-amylase has been extracted from Aspergil-

lus oryzae and its properties have been investigated (Fischer and Montmollin, 1951a, b; Underkofler and Roy, 1951; Hanrahan and Caldwell, 1953 a, b; Bird and Hopkins, 1954). The results of these authors suggest that the action pattern of this enzyme is similar to, although not necessarily identical to the action patterns of pig pancreatic (Meyer and Gonon, 1951 b) and human salivary (Whelan and Roberts, 1953) α-amylases i.e. in each case there is a random hydrolysis of non-terminal linkages. The presence of calcium in the enzyme has been shown (Hanrahan and Caldwell, 1953 b) but it is so firmly bound that attempts to remove it have, so far, proved unsuccessful (Stein et al., 1964). The properties of the enzyme in the absence of bound calcium have not yet, therefore, been studied.

The end-products (glucose and maltose) of the action of α-amylases on amylose are similar (except for Bacillus subtilis α-amylase) but the reaction mechanism by which complete degradation of the substrate is attained certainly varies widely. However, the results of many investigations on α-amylases suggest that these enzymes may be divided into various groups such as plant, mammalian, bacterial and fungal. The enzymes within each group may well have identical action patterns.

SECTION 5.

STUDIES ON R-ENZYME

5. STUDIES ON R-ENZYME

INTRODUCTION

R-enzyme catalyses the scission of α-1; 6 - glycosidic bonds in starch-type polysaccharides. The enzyme was first detected in extracts of broad bean and potato by Hobson et al. (1950), who later made a more thorough investigation of its properties (1951). They examined the action of the enzyme on amylose, amylopectin and amylopectin β-limit dextrin. Their main conclusions were: (1) the enzyme effected an increase in the A.Vs. of amylopectin and its dextrin which was accompanied by a decrease in viscosity and an increase in the substrate reducing power (1% in the case of amylopectin; 11.4% in that of amylopectin β-limit dextrin), (2) the successive actions of R-enzyme and β-amylase converted amylopectin and its limit dextrin into 74% and 65% maltose, respectively, (3) the simultaneous action of R-enzyme and β-amylase converted amylopectin β-limit dextrin into 80% maltose, (4) the enzyme had no detectable action on amylose.

Mould and Synge (1954) have separated the reaction products from the action of R-enzyme on amylopectin and its β-limit dextrin, into two fractions: one was able to diffuse through a "Cellophane" membrane, and the other was retained. The indifusible fractions of the R-treated amylopectin and limit dextrin were, respectively, 84% and 63.5% of the original material. These results, which indicate that amylopectin is less amenable to attack than its limit dextrin, agree with the find-

ings of Hobson et al. (1951).

The action of R-enzyme on the phosphorylase-limit dextrin and β-amylase limit dextrin of starch led to the production of small dextrins containing 3 - 4 and 2 - 3 glucose units, respectively (Wild, 1954). These results show that the enzyme is capable of removing small side-chains, containing 2 - 4 glucose units, joined to the base chain by an α - 1: 6 - link. The enzyme does not hydrolyse isomaltose, (Hobson et al. 1951), nor does it remove single glucose residues joined by an α-1: 6 - link to maltodextrin molecules (Roberts, 1953).

The action of the enzyme on various glycogens has also been studied (Peat et al. 1954; Fleming and Manners, 1958), and the results indicate that only a small hydrolysis takes place. Evidence is given to show that the higher degree of branching, and the subsequent greater compactness, of the glycogen molecule compared to that of amylopectin, may explain the difference in the extents of hydrolysis of these two substrates containing α-1: 6 - interchain linkages (Peat et al. 1954).

MacWilliam (1958) and MacWilliam and Harris (1959) have extracted R-enzyme from broad bean, barley malt and potato. They have fractionated these enzyme preparations on alumina columns and shown that each contains two components which are capable of attacking α - 1: 6 - linkages in amylopectin or its degradation products. One enzyme (so-called "R-enzyme") increases the blue values of amylopectin and its dextrin rendering these substrates more susceptible to attack by β-amylase. It does not, however, have any apparent effect on the small α-limit dextrins obtained

from amylopectin by exhaustive hydrolysis with salivary α-amylase. The other enzyme (limit dextrinase) hydrolyses these α-limit dextrins, but appears to have no action on larger substrates.

The properties of an R-enzyme sample prepared from malt were found to differ in certain respects from those reported by Peat et al. (1952, 1954, 1956) for broad bean and potato R-enzyme. (1) The R-enzymes of potato and broad bean released 12.8% of reducing sugar and a series of higher dextrins, amounting to a further 27.2%, from β-limit dextrin, but malt R-enzyme gave only 1.5% of reducing sugar consisting only of maltose and maltotriose. (2) Whereas the broad bean and potato enzyme, acting in conjunction with β-amylase on starch β-limit dextrin, yielded almost 100% of reducing sugar, malt R-enzyme, acting under the same conditions, yielded only 14% of apparent maltose. These results suggest a profound difference in the action-patterns of the R-enzymes studied. The enzymes from broad bean and potato are similar in that they are both capable of hydrolysing α-1: 6 - linkages joining two relatively large chains of α - 1: 4 linked glucose units. Malt R-enzyme, however, appears to hydrolyse only α- 1: 6 - bonds linking short chains (D.P. ca. 3 glucose units) to a main chain in the substrate.

Many of the above results are inconclusive and contradictory and some of the R-enzyme preparations were almost certainly contaminated with α-amylase impurity. An investigation was, therefore, undertaken in an attempt to prepare pure broad bean R-enzyme and to examine its properties.

EXPERIMENTAL

The source of enzyme used throughout this work was Dobbie's "Champion Long Pod" broad beans.

Attempts to prepare R-enzyme. (1) Dry, defatted broad bean flour (200 g.) was extracted with water (800 ml.) at room temperature for 5 hours when the suspension was centrifuged and a protein fraction obtained by the lead acetate precipitation technique of Barker, Bourne and Peat (1949). The ammonium sulphate precipitate was suspended in water (75 ml.) and clarified by centrifugation (solution 1). A sample (2 ml.) was removed for an activity determination (Table 5.01). The remaining solution was cooled to 2°C., potato starch (42 g.), sodium sulphate (0.75 g.) and ethanol (18 ml.) were added and the suspension was carefully shaken at 2°C. for 30 minutes. A clear solution (2) was obtained on centrifugation which was tested for activity and shaken with a further portion of starch (28 g.). After 30 minutes shaking the suspension was centrifuged to yield a clear solution (3) which was assayed for R-enzyme activity. The R-enzyme activity determinations were carried out in the following way. Aliquots (1 ml.) of each enzyme solution were added to digests containing citrate buffer (2 ml.; 0.2M.; pH 6.5) and amylopectin β-limit dextrin solution (10 ml.; ca. 1.5 mg./ml.) These were incubated at 35°C. and aliquots (1 - 2 ml.) were removed at intervals, heated on a boiling water-bath for 5 minutes to coagulate the protein and centrifuged. Aliquots (1 ml.) of the clear supernatants were stained with iodine (1 ml. of an iodine solution containing 0.2% iodine in 2% potassium iodide +

4 drops of 5 N. HCl) and made up to 100 ml. with water. The absorbances of the solutions, in 4 cm. cells, were determined on a Spekker Photoelectric Absorptiometer using an appropriate blank and Ilford Filter No. 608. The results are shown in Table 5.01.

Table 5.01

	Absorption Values (A.Vs.)				
Time (hours)	0	1	4	14	24
Solution 1	0.179	0.170	0.121	0.05	-
" 2	0.180	0.223	0.250	0.270	0.230
" 3	0.179	0.183	0.180	-	0.179

(2) An aqueous extract of broad bean flour was prepared and cooled to 2°C. A saturated solution of ammonium sulphate was added to a final concentration of 19 g./100 cc. After 12 hours the precipitate was removed by centrifugation and suspended in cold water; the resulting suspension was centrifuged to yield a clear solution (1) which was treated in the following way.

An aliquot (30 ml.) was shaken with potato starch (10g.), sodium sulphate (0.3 g.) and ethanol (6 ml.) for 30 minutes at 2°C. before being centrifuged. Chilled acetone (40 ml.) was added to the clear supernatant and the resulting precipitate was suspended in cold water (solution 2). The starch was extracted

with two portions of citrate buffer (10 ml.; 0.2M.; pH 6.5) to yield solutions 3 and 4. The activities of these solutions are shown in Table 5.02.

Table 5.02

	A.Vs.					
Time (hours)	0	1	3	5	23	48
Solution 1	0.178	0.165	0.150	0.142	0.05	-
" 2	0.178	0.192	0.198	0.208	0.256	0.213
" 3	0.181	0.190	0.220	0.240	0.192	-
" 4	0.180	0.163	0.150	0.142	-	-

Solutions 2 and 3 were treated with further portions of starch and re-tested for enzymic activity. Neither α-amylase nor R-enzyme were present.

The above procedure was repeated with a further portion (30 ml.) of solution (1) but aliquots (10 g.) of "superlose" were used in place of starch. The activities of the fractions obtained are shown in Table 5.03.

Table 5.03

	A.Vs.				
Time (hours)	0	1	3	5	23
Solution 2	0.180	0.187	0.182	0.178	0.121
" 3	0.179	0.206	0.220	0.233	0.203
" 4	0.180	0.193	0.208	0.193	0.162

A portion (30 ml.) of solution (1) was heated at 70°C. for 15 minutes, cooled, centrifuged and tested for enzymic activity. Both the R-enzyme and λ-amylase activities had been destroyed.

(3) An ammonium sulphate precipitate (19 g./100 ml.) of broad bean flour was prepared as in 2, dissolved in water (solution A) and treated in various ways. A portion (30 ml.) was treated with potato starch (10 g.), sodium sulphate (0.3 g.) and ethanol (6 ml.) at 2°C. for 30 minutes. The suspension was centrifuged to yield solution (1). Calcium phosphate gel suspension (10 ml.) was added to solution (1) and the suspension was carefully stirred for 5 minutes before being centrifuged to give clear supernatant (2). The gel was extracted with successive portions of citrate buffer (20 ml.; 0.2M.; pH 6.5), citrate buffer (20 ml.; 0.2M.; pH 7.5), phosphate buffer (20 ml.; 0.2 M.; pH 7.5) and phosphate buffer (20 ml.; 0.2M.; pH 6.5) containing ammonium sulphate (10%), to yield solutions 3, 4,5 and 6. They were tested for R-enzyme activity and the results are shown in Table 5.04.

Table 5.04

	A.Vs.				
Time (hours)	0	1	5	20	40
Solution 1	0.180	0.191	0.203	0.250	0.220
" 2	0.180	0.172	0.167	0.143	0.120
" 3	0.179	0.192	0.207	0.232	0.201
" 4	0.178	0.170	0.158	0.132	0.110
" 5	0.179	0.180	0.178	0.180	0.183
" 6	0.181	0.179	0.180	0.178	0.180

Calcium chloride solution (6 ml.; 0.5%), ethanol (3 ml.) and freshly precipitated starch (500 mg.) were added to a portion (5 ml.) of solution A at 2°C. The suspension was stirred and aliquots (2 ml.) were removed after 0, 2, 10, 15 and 30 minutes (Solutions 1, 2, 3, 4 and 5) for activity determinations which are shown in Table 5.05.

Table 5.05

	A.Vs.		
Time (hours)	0	2½	17½
Solution 1	0.180	0.134	0.042
" 2	0.180	0.149	0.045
" 3	0.180	0.152	0.081
" 4	0.179	0.150	0.090
" 5	0.180	0.152	0.088

The above procedure was repeated using glycogen (500mg.) as an adsorbent in place of starch. The activity results are shown in Table 5.06.

Table 5.06

	A.Vs.				
Time (hours)	0	1	2	8	22
Solution 1	0.180	0.205	0.210	0.191	0.080
" 2	0.180	0.223	0.225	0.208	0.164
" 3	0.180	0.219	0.241	0.238	0.210
" 4	0.180	0.180	0.178	0.170	0.153
" 5	0.180	0.180	0.180	0.175	0.160

A portion (30 ml.) of solution A was cooled to 0°C. and fractionated with acetone between the limits 0 - 20, 20 - 30, 30 - 40, 40 - 50 and 50 - 60% with respect to acetone. The fractions (1 - 5) were dried by washing with acetone on a Buchner and they were stored in a vacuum desiccator. Their enzymic activities were examined in the following way. Portions (20 mg.) of each fraction were suspended in citrate buffer (3 ml.; 0.5 M.; pH 6.5) and left at room temperature for 1 hour. They were centrifuged and portions (2 ml.) of the clear supernatants were added to digests containing amylopectin β-limit dextrin (5 ml.; 1.5 mg./ml.) and water (3 ml.) at 35°C. Aliquots (1.5 ml.) were removed at intervals, heated on a boiling water-bath for 5 minutes, cooled and centrifuged before their iodine-stains were measured. The results are shown in Table 5.07.

Table 5.07

	A.Vs.				
Time (hours)	0	1	2	23	72
Solution 1	0.180	0.120	0.122	0.190	0.152
" 2	0.178	0.191	0.207	0.219	0.201
" 3	0.178	0.193	0.205	0.202	0.192
" 4	0.182	0.164	0.145	0.098	-
" 5	0.179	0.143	0.115	-	-

(4) Dry, defatted broad bean flour (300 g.) was extracted with water (1500 ml.) for 30 minutes at room temperature and

centrifuged. A sample (200 ml.) of the solution was put through the lead acetate precipitation procedure and ammonium sulphate was added to the resulting sodium bicarbonate-protein solution to a concentration of 19 g./100 c.c. After 12 hours the precipitate was removed by centrifugation, dissolved in water and treated with starch and ethanol to yield solution A. The starch treatment was repeated on solution A to yield solution A1. The protein solution remaining after the removal of the initial ammonium sulphate precipitate was further fractionated with ammonium sulphate at 25, 35, 45 and 50g./100 ml. Each fraction was treated twice with starch/ethanol to yield solutions B, B1, C, C1, D, D1, E and E1.

A further sample (300 ml.) of the initial aqueous extract was brought to 19 g./ 100 ml. concentration with respect to ammonium sulphate and left for 24 hours. The precipitate was removed and the supernatant further sub-fractionated at 25, 35 and 50 g./100 ml. The fractions were treated twice with starch and ethanol to yield solutions F, F1, G, G1, H, H1, J and J1. The activities of these solutions are shown in Table 5.08.

A portion of solution F(25 ml.) was cooled to 2°C. and degraded amylose suspension (7 ml.; 5 mg./ml.), precooled to 2°C., was added slowly with stirring. After 15 minutes stirring, the amylose was removed by centrifugation to yield a clear supernatant (F2), which was treated with a further portion of degraded amylose solution to yield solution F3. The amylose precipitates were combined and extracted with citrate buffer (10 ml.; 0.2M.; pH 6.5) at room temperature for 30 minutes when the suspension

was centrifuged to yield solution F4. These solutions were tested for activity and the results are shown in Table 5.09.

Table 5.08

	A.Vs.			
Time (hours)	0	2	5	24
Solution A	0.180	0.197	0.223	0.251
" A1	0.180	0.183	0.190	0.190
" B	0.180	0.167	0.135	0
" B1	0.178	0.180	0.172	0.141
" C	0.180	0.146	0.098	0
" C1	0.179	0.175	0.168	0.135
" D	0.181	0.182	0.174	0.137
" D1	0.182	0.180	0.180	0.169
" E	0.180	0.180	0.180	0.178
" E1	0.180	0.180	0.183	0.180
" F	0.180	0.250	0.228	0.100
" F1	0.180	0.188	0.197	0.192
" G	0.179	0.200	0.169	0
" G1	0.180	0.180	0.175	0.151
" H	0.180	0.127	0.040	0
" H1	0.180	0.165	0.150	0.08
" J	0.180	0.180	0.175	0.175
" J1	0.180	0.182	0.183	0.181

Table 5.09

	A.Vs.			
Time (hours)	0	2	5	24
Solution F	0.180	0.250	0.228	0.100
" F2	0.180	0.231	0.220	0.111
" F3	0.180	0.190	0.208	0.210
" F4	0.178	0.180	0.162	0.05

(5) Dry, defatted broad bean flour (200 g.) was extracted with water (1000 ml.) for 45 minutes at room temperature and centrifuged. The cloudy supernatant was cooled to 2°C. and ammonium sulphate was added to 25 g./100 ml. concentration. After 12 hours the precipitate was removed by centrifugation, suspended in cold water (110 ml.) and centrifuged to yield a clear solution (A). Ethanol (20 ml.; - 5°C.) and potato starch (50 g.) were added and the suspension was carefully shaken for 10 hours. After centrifugation the clear supernatant was maintained at 0°C. and ammonium sulphate was added up to 20 g./100 ml. concentration. The precipitate, which had coagulated, was removed on the centrifuge after 9 hours and dissolved in cold water (solution B). The supernatant from the ammonium sulphate precipitation was solution C. The activities of these solutions are shown in Table 5.10.

Table 5.10

	A.Vs.					
Time (hours)	0	½	1	3	5	24
Solution A	0.180	0.200	0.208	0.218	0.230	0.211
" B	0.182	0.230	0.270	0.300	0.301	0.172
" C	0.182	0.186	0.190	0.191	0.183	0.101

A portion (5 ml.) of solution B was placed on a G - 75 "Sephadex" column and eluted with water. Although fractionation of protein was effected, neither R-enzyme nor λ-amylase activities were detected. Portions (10 ml.) of B and C were freeze-dried. This procedure also destroyed the enzymic activities.

(6) Dry, defatted broad bean flour (200 g.) was extracted for 5 hours at room temperature with water (1,000 ml.) containing *n*.-octanol (0.2 ml.). The suspension was cooled to 2°C. and centrifuged (20,000 r.p.m.; 20 minutes). An acetone fractionation was carried out between the limits 0 - 20, 20 - 30, 30 - 35, 35 - 40, 40 - 50 and 50 - 60% with respect to acetone. The fractions were air-dried and suspended in water to give solutions 1 - 6. Portions of these (containing ca. 10 mg.) were added to digests consisting of citrate buffer (2 ml.; 0.2M.; pH 6.5), amylopectin β-limit dextrin solution (2 ml.; 4 mg./ml.) and water to make the total volume 10 ml. These were incubated at 35°C. and aliquots (2 ml.) were removed at intervals and stained with iodine (1 ml. of normal iodine solution + 0.2 ml. N. HCl in

a total volume of 50 ml.). The absorbances of the solutions were measured against an appropriate blank on an "E E L" Colorimeter using red filter no. 608. The results are shown in Table 5.11.

Table 5.11

	A.Vs.			
Time (hours)	0	0.5	1	5
Fraction (Original)	2.90	1.70	1.21	0.05
1	2.90	3.40	3.42	2.33
2	2.90	4.53	4.31	2.97
3	2.88	3.15	3.01	2.10
4	2.90	2.79	2.70	2.51
5	2.90	2.41	1.50	-
6	2.89	2.10	1.30	-

A portion of fraction 2(10 ml.) was cooled to $2^{\circ}C$. and sub-fractionated with chilled alcohol between the limits 0 - 15, 15 - 25, 25 - 30, 30 - 35, 35 - 45 and 45 - 60%. Activity measurements, similar to those above, were carried out on these fractions (2A - 2F). The results are shown in Table 5.12.

Table 5.12

	A.Vs.			
Time (hours)	0	0.5	1	5
Fraction 2A	2.90	4.70	4.85	4.45
" 2B	2.91	4.12	4.15	3.50
" 2C	2.91	3.73	3.61	3.11
" 2D	2.90	3.13	2.97	2.81
" 2E	2.90	2.78	2.68	2.41
" 2F	2.90	2.53	2.40	2.21

Attempted separation of λ-amylase and R-enzyme on a starch column. A portion (5 ml.) of fraction 2A was placed on a small starch column and eluted with water. Fractions were collected in 5 ml. samples and their protein contents were determined by measuring their absorbances at 280 mμ. The fractions were examined for enzymic activity but neither λ-amylase nor R-enzyme was detected.

Preparation of freshly-precipitated potato starch. The starch was dissolved in a water/chloral hydrate (2: 1) mixture at 80°C. and slowly poured with vigorous stirring into acetone. The resulting precipitate was dried with acetone followed by ether and stored in a vacuum desiccator.

Preparation of degraded amylose (Peat et al, 1959). A sample of an amylose-butanol complex containing about 0.5 g. amylose was dissolved in boiling water (200 ml.). The solution was reduced in volume to 100 ml., citrate buffer (2 ml.; 0.5M.; pH 6.0) was added, and it was allowed to cool under constant stirring to 2°C. A flocculant precipitate was obtained which was stored at 2°C.

Preparation of calcium phosphate gel. The procedure of Levin (1962) was used.

Disodium phosphate (2l.; 0.5M.) and calcium chloride (2l.; 0.5M) were slowly added, at the same rate (ca. 15 ml./minute), to a large beaker (5l.). The mixture was mechanically stirred (ca. 80 r.p.m.). When precipitation was complete, the supernatant was removed and the precipitate washed with water (3 x 1 l.portions) - complete settling was allowed each time. Water (3 l.) and sodium hydroxide (100 ml.; 40%) were added, with stirring, to the precipitate. The suspension was heated to boiling point (45 minutes) and maintained there for 1 hour. The calcium phosphate was then allowed to sediment (ca. 5 minutes), the turbid supernatant was removed and water (3½ l.) was added. The suspension was stirred (5 minutes), allowed to sediment and the supernatant was removed. This procedure was repeated three times. After the 4th washing phosphate buffer (3½ l.; 0.01M.; pH 6.8) was added to the precipitate and stirring and heating were commenced. When the suspension had begun to boil, the heating was stopped, the calcium phosphate allowed to settle (5 minutes) and the supernatant was removed. This procedure

was repeated twice with 0,01M. buffer and twice with 0.001M. buffer. On each occasion boiling was continued for 15 minutes. After the 5th boiling the calcium phosphate gel was stored in phosphate buffer (1½ l.; 0.001M.; pH 6.8) under toluene.

DISCUSSION

A crude preparation of R-enzyme was obtained by following the procedure outlined by Hobson et al. (1951). The removal of contaminating α-amylase was found to be very difficult because the starch/ethanol adsorption procedure did not appear to be completely efficient (Table 5.01 p. 214). The results in Table 5.02 (p. 215) indicate that the starch does adsorb most of the activity but a detectable amount remains in solution. If the starch treatment is repeated then both the R-enzyme and α-amylase activities are removed from solution. "Superlose" (a commercial amylose preparation) was even less efficient than starch (Table 5.03 p. 215). Heating a solution containing the two activities was not effective; both activities were destroyed. This suggests that the thermal stabilities of the two enzymes are comparable.

The effect of calcium phosphate gel on a mixture of α-amylase and R-enzyme was also studied (Table 5.04 p. 216). The results suggest that neither enzyme is preferentially adsorbed by the gel but that they are both partially removed from the solution. Using various buffer systems attempts were made to desorb the activities from the gel but they, too, were unsuccessful.

Since many adsorbents require to be activated before they reach their highest efficiency an attempt was made to prepare a more highly adsorbent sample of starch. This was carried out by carefully precipitating starch with acetone from an aqueous chloral hydrate solution. In this way starch in a highly divided state was obtained. The results in Table 5.05 (p. 217)

show that the experiment was not successful. In fact, the β-amylase is even more active in these solutions than it was in previous similar attempts. This may be caused by the addition of calcium chloride to the enzyme - starch-ethanol suspension. Since the β-amylase is not fully active when there is a deficiency of calcium ions in its solution (p.185), it may not be so readily adsorbed by starch under similar conditions. Excess calcium ions were, therefore, added but, as already indicated, the results in Table 5.05 are not satisfactory.

Glycogen has been shown to adsorb broad bean β-amylase (p.180) and so its effect on a mixture of R-enzyme and β-amylase was examined. Since R-enzyme has only a very slight action on glycogens (Peat et al. 1954; Fleming and Manners, 1958) it would not be expected to attack the glycogen molecules and so would not be adsorbed. The results (Table 5.06 p.217) indicate that the β-amylase is incompletely removed from solution. It should be stressed that there is only a trace of β-amylase present in these solutions and so there may be insufficient enzyme to form an insoluble complex with the glycogen. The results also indicate that R-enzyme is adsorbed by the glycogen.

An acetone fractionation of an ammonium sulphate precipitate (19 g./100 c.c.) was attempted but the results (Table 5.07 p. 218) indicate that all the fractions were contaminated with β-amylase to such an extent that the R-enzyme was barely detectable.

An investigation was undertaken to determine the

efficiency of the lead acetate precipitation technique for preparing R-enzyme and to confirm that ammonium sulphate, present in 19 g./100 ml. concentration, quantitatively precipitated the enzyme. Samples of initial aqueous extract and lead acetate prepared protein were fractionated with ammonium sulphate. The fractions were treated with starch to remove most of the α-amylase and so enable the R-enzyme activity to be detected. The results are shown in Table 5.08 (p. 220) and indicate that a straight forward ammonium sulphate fractionation yields more R-enzyme, with correspondingly more α-amylase, than does the more complex lead acetate procedure. Most of the required enzyme is precipitated at an ammonium sulphate concentration of 19 g./100 ml. and at higher salt concentrations larger amounts of α-amylase are precipitated.

Degraded amylose has been used as an adsorbent in the purification of starch metabolising enzymes (Peat et al, 1959). A sample of fraction F, (Table 5.08 p. 220) which contained a relatively high concentration of R-enzyme, was treated with degraded amylose but the results (Table 5.09 p. 221) were not satisfactory. Not only was the very small amount of α-amylase incompletely removed but R-enzyme was adsorbed, to a certain extent, by the amylose. Extraction of the amylose with citrate buffer yielded a very small amount of R-enzyme which appeared to be free from α-amylase.

A relatively active preparation of R-enzyme was obtained by subfractionating, with ammonium sulphate, a starch treated protein solution (Table 5.10 p. 222). Further purification was attempted on a "Sephadex" column but this was unsuccessful as was

the attempt to freeze-dry a solution of the fraction.

Results obtained during an investigation of broad bean β-amylase suggested that R-enzyme could be prepared by an acetone fractionation of an aqueous extract of broad bean flour. The results are shown in Table 5.11 (p. 223) and indicate that a fraction containing high R-enzyme activity was obtained. Unfortunately, β-amylase was also present. An attempt was made to subfractionate the active fraction with ethanol but this was not very successful (Table 5.12 p. 224). Fractionation was not really achieved because R-enzyme was present in at least two of the fractions and β-amylase in all of them. A surprising result was that alcohol, in 15% concentration, precipitated a large amount of the R-enzyme activity. Therefore, all the starch-ethanol treatments must have removed most of the R-enzyme activity from the previous preparations. The activity of fraction 2A (Table 5.12 p. 224) was extremely high and an attempt was made to remove the β-amylase impurity by passage through a starch column but this was not successful.

Although the work described in this section did not result in the preparation of a sample of R-enzyme which was completely free from β-amylase activity the acetone fractionation procedure (Table 5.11, p. 223) gave fractions which were some 15 times more active than those reported by Hobson et al. (1951). However, complete removal of contaminating β-amylase is obviously difficult and further work is necessary - possibly using methods involving adsorption on amylose.

SECTION 6.

STUDIES ON β-AMYLASE

INTRODUCTION

β -Amylase hydrolyses a chain of α -1: 4 - linked glucose units to form β -maltose as the only reaction product (Kuhn, 1925; Ohlsson, 1930). The enzymic attack ceases when the enzyme approaches a modified α-glucose residue, e.g. amylopectin is incompletely hydrolysed largely because it contains α -1: 6 - linkages, and maltohexaose containing an ester-phosphate grouping is also resistant to attack by β -amylase (Posternak, 1951). Myrbäck and Nycander (1942) found that the enzyme attacked maltohexaose and maltohexaonic acid, liberating three and two molecular proportions of maltose, respectively. These results indicate that the enzyme attacks a substrate molecule from the non-reducing end and splits alternate α -1: 4 - linkages to liberate maltose units. The above conclusions have been confirmed many times and they are not in dispute.

There are two main mechanisms by which β -amylase attack may take place - (1) a single-chain mechanism in which the enzyme completely hydrolyses one substrate molecule at a time, (2) a multi-chain mechanism in which the enzyme hydrolyses the substrate molecules in a random manner. During single-chain action, only maltose and undegraded substrate molecules will be present in the system. Because the turnover number of β -amylase is extremely high (Englard and Singer, 1950; Englard, Sorof and Singer, 1951), the molar ratio of enzyme to substrate must be very small if the β -amylolysis is to proceed at a measurable velocity, and so there will be only negligible amounts of partly degraded amylose molecules in the reaction

mixture at any one time. During multi-chain attack all the amylose molecules will be degraded by approximately the same amount. Enzymic degradation may, however, take place via a mixture of these two action patterns i.e. "multiple" attack. During such degradations the enzyme splits off several molecules of maltose per encounter with the substrate molecule before diffusing away and recombining with the same or with a different substrate molecule.

Investigations of the action pattern of β-amylase may be conveniently considered in two groups: (1) those involving substrate molecules of small size i.e. D.P. < 50, and (2) those involving large amylose molecules as a substrate.

Investigations with small substrate molecules. Results presented by Hopkins and Jelinek (1949) on the action of β-amylase on an amylose dextrin (D.P. ca. 20) suggested the presence of multi-chain attack. These authors studied the iodine staining power of the dextrin at various stages of hydrolysis.

French et al. (1950a, b) investigated the action of β-amylase on maltoheptaose. By chromatographic examination of the products of the reaction at various stages in the hydrolysis, these authors concluded that under optimum conditions of temperature and pH only maltose and maltotriose were formed. If multi-chain attack was taking place then maltopentaose should also have been present. However, these authors further showed that under unfavourable conditions for enzymic reaction (pH 10.0; 70°C.) maltopentaose was, indeed, produced. The reaction mechanism, therefore, appears to depend upon the experimental

conditions.

Further evidence for multi-chain action has been obtained by Hopkins and Jelinek (1954) and Bird and Hopkins (1954b). These authors confirmed that amylose and its short-chain fission products are hydrolysed by β-amylase at the same velocity. The preferential disappearance, therefore, of the short-chain molecules in mixtures of long - and short-chain polysaccharides was attributed to multi-chain attack - all substrate molecules were attacked to the same extent and so the short-chain molecules were more quickly completely hydrolysed. Studies on the hydrolyses by β-amylase, of short amylose chains (D.P. 16 - 30) and maltohexaose indicated that multi-chain action was also operative in these systems.

The action pattern of β-amylase on maltohexaose, maltoheptaose and a synthetic amylose (average chain-length 49 units) has been studied under a variety of experimental conditions (Bailey and Whelan, 1957). The results suggested that with all the substrates, and under all the conditions investigated, the action pattern was always intermediate between single - and multi-chain attack, i.e. a multiple attack mechanism was in operation (French and Wild, 1953). Under favourable conditions for enzymic reaction there was a higher proportion of single-chain attack but, as the conditions became more unfavourable, the proportion of multi- chain attack increased.

Bailey and French (1957) examined the action of β-amylase on synthetic amylose molecules labelled with C^{14} at the non-reducing end and having average chain-lengths of 44 glucose units.

They concluded that for each effective encounter which the enzyme made with an amylose molecule four maltose units were removed. These results further support the concept of multiple attack.

Investigations with large D.P. substrates. Although the mechanism of the action of β-amylase on small substrate molecules (i.e. D.P. < 50) seems reasonably well established to be "Multiple-attack", conflicting evidence has been presented for the action-pattern of the enzyme on large substrate molecules (i.e. amylose molecules whose D.Ps. approximate to those of natural amylose).

Evidence for single-chain action was provided by Swanson (1948) who studied the hydrolysis of maize amylose by β-amylase. This worker showed that during the degradation no short-chain fission products were formed and, although the intensity of the amylose-iodine stain decreased its colour and wavelength of maximum absorption remained unchanged. These results were confirmed by Cleveland and Kerr (1948), Kerr (1949) and Kerr and Cleveland (1951). These latter authors isolated an amylose β-limit dextrin and examined its viscosity, iodine stain and osmotic pressure. They concluded that the dextrin possessed physical and chemical properties similar to those of the original amylose. These conclusions, however, were criticised by Bourne and Whelan (1950) who supported the multi-chain hypothesis for which evidence had apparently been furnished by Hopkins and Jelinek (1949).

More evidence for the single-chain action pattern of

β-amylase was supplied by Cowie et al. (1958) who suggested that the physical properties of various amylose β-limit dextrins were comparable to those of the original amylose. Husemann andPfannemüller (1961) carried out a lightscattering study on natural and synthetic amyloses at various stages of degradation by β-amylase. Their results confirmed the findings of Cowie et al. (1958) for the natural amylose but the synthetic amylose was degraded by a multi-chain mechanism.

Although most of the investigations on the action pattern of β-amylase have been carried out with reasonably pure samples of amylose, very little attempt has been made to determine the molecular weight distribution of these samples. French (1961) has indicated that if the original amylose contains the "most probable" distribution of molecules, then attack by β-amylase via any of the three action patterns will lead to the formation of amylose β-limit dextrins having average molecular weights and size distributions precisely the same as those of the original amylose. This theoretical conclusion invalidates the results of Cowie et al. (1958) and Husemann and Pfannemüller (1961) on samples of natural amylose, as these authors used unfractionated material. In the work reported here an investigation of the action of β-amylase on narrow molecular weight fractions of amylose has been carried out.

EXPERIMENTAL AND RESULTS

Worthington's 2 x crystallised sweet potato β-amylase

was used throughout this work. The enzyme was further purified by passing it through a small G - 25 "Sephadex" gel column (cf. Thoma and Koshland, 1960)

Preparation of β-amylase solutions. A portion (0.15 ml.) of the suspension of β-amylase crystals in ammonium sulphate was centrifuged. The resulting pellet of enzyme was suspended in glutathione solution (10 ml.; 0·5m M.) carefully stirred and centrifuged to yield a clear solution which was covered with toluene and stored at 2°C. The enzymic activity was measured by the method of Hobson et al. (1950a).

In agreement with the results of Walker and Whelan (1960) diluted aqueous solutions of β-amylase were found to be unstable on storage at 2°C. Glutathione, therefore, was used as a stabilising agent in the dilute stock solution of the enzyme. However, when digests were also prepared under conditions which maintained the total concentration of glutathione at 5m M., the estimation of maltose by the alkaline ferricyanide - ceric sulphate method was found to be inaccurate because of the reducing power exerted by the glutathione. Since the enzyme was not inactivated very readily in the presence of substrate no additional glutathione was added to enzymic digests.

Measurements of limiting viscosity numbers in potassium hydroxide (0.15M.) and β-amylolysis limits were carried out as described in the General Experimental section.

Variation of β-amylase activity with temperature. Digests were prepared containing starch solution (10 ml.; 0.6%) and acetate buffer (1 ml.; 0.2M.; pH5.0). They were equilibrated

at various temperatures between 10 and 70°C. Portions (0.025 ml.) of enzyme solution were added to each and samples (2 ml.) were removed after 10 minutes for reducing power determinations.

Variation of β-amylase activity with pH. Digests were prepared containing starch solution (10 ml.; 0.6%) and acetate buffers (1 ml.; 0.2M.) having various pH values between 3.55 and 7.0, inclusive. They were incubated at 35°C. when portions (0.025 ml.) of enzyme solution were added to each. Samples (2ml.) were removed from the digests after 10 minutes for reducing power determinations.

Preparation of amylose samples. "Redskin" potato starch granules were aqueous leached at 60°C. after pretreatment with aqueous methanol. The sample had a β-amylolysis limit of 100% and limiting viscosity number of 260. A portion of this amylose was subfractionated with ethanol from dimethylsulphoxide solution and the properties of these fractions are shown in Table 3.03 (p.62). Mr. A. R. Procter subfractionated a further portion of the leached-amylose with acetone from dimethysulphoxide solution and characterised the subfractions. Their properties are shown below.

Table 6.01

Fraction	[η]	β-limit	Mw $x10^{-6}$	D.P.
2A	435	100	0.91	3,200
3A	295	100	0.60	2,200
3B	167	100	0.31	1,200
4	120	100	0.21	900

A, B represent refractionation products.

Molecular weights were calculated from short-column equilibrium runs on the ultracentrifuge.

To show the absence of α-amylase impurity in the β-amylase sample. A portion of the amylose obtained by aqueous leaching starch granules at 60°C. was incubated at pH 5.0 and 35°C. with β-amylase (0.025 ml.). When a 50% conversion into maltose was obtained, the residual amylose was isolated as the butanol complex. After 24 hours the complex was thoroughly washed with butanol -saturated water to remove the maltose. Viscosity determinations (0.15M. potassium hydroxide; 25°C.) were carried out on the 50% dextrin and on a sample of the original amylose. The two results were identical, indicating that the β-amylase sample was free from α-amylase.

Action of β-amylase on amylose. Samples of amylose subfraction 4 (Table 3.03, p. 62) were incubated with β-amylase at pH 5.5 and 35°C. Amylose-dextrins were isolated as butanol complexes at various stages of conversion into maltose. Their limiting viscosity numbers in potassium hydroxide (0.15 M.) were measured and the results are shown in Table 6.02.

Table 6.02

% Conversion into maltose	0	25	57	60	66	79
Viscosity $[\eta]$	260	225	197	192	194	194
% Fall in $[\eta]$	0	6	18	20	19	19

Samples of the fractions shown in Table 6.01 were incubated with β-amylase at 35°C. and different values of pH. β-limit dextrins were isolated at various stages and their

limiting viscosity numbers in potassium hydroxide (0.15 M.) were determined. The results are shown in Table 6.03.

Table 6.03

Fraction	pH of digest	% Conversion into maltose	$[\eta]$	Calculated $[\eta]$ for multi-chain action. [1]
2A	4.0	73	246	118
	5.0	42	240	250
3A	5.0	40	184	173
3B	7.0	93	60	12
4	5.0	77	120	28
	7.0	67	120	40

1. Calculated assuming uniform molecular weight for each fraction.

DISCUSSION

Effect of temperature and pH on β-amylase activity. The temperature of optimum activity was 43°C. (Fig. 6.01) and the pH of optimum activity was 5 (Fig. 6.02). This latter result is in agreement with that quoted by Balls et al. (1948).

Action - Pattern of β-amylase on amylose. The samples of amylose used in this work had D.Ps. of 900 - 3,200. The results for the original amylose subfraction in Table 6.02 suggest that there is only a relatively small decrease (19%) in the viscosity.

FIG. 6.01

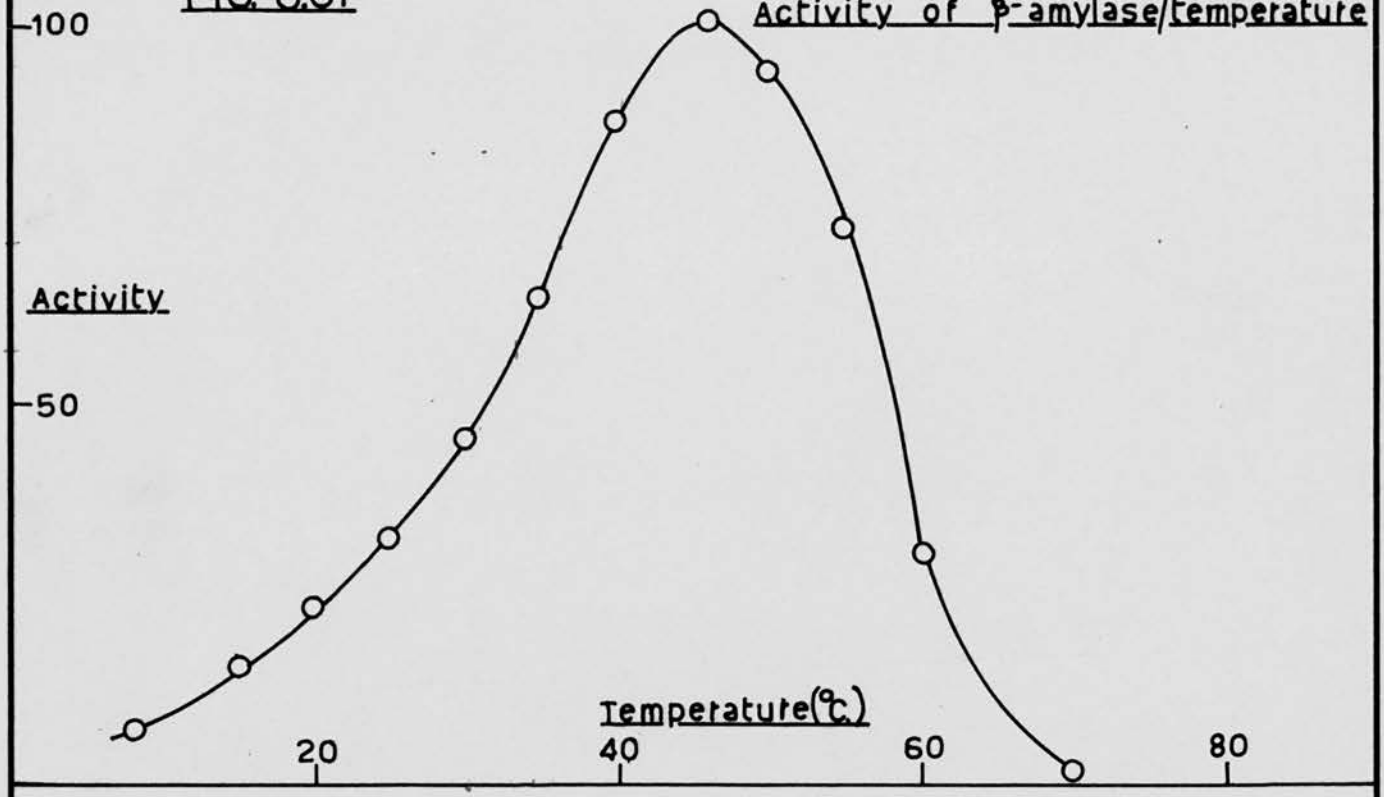
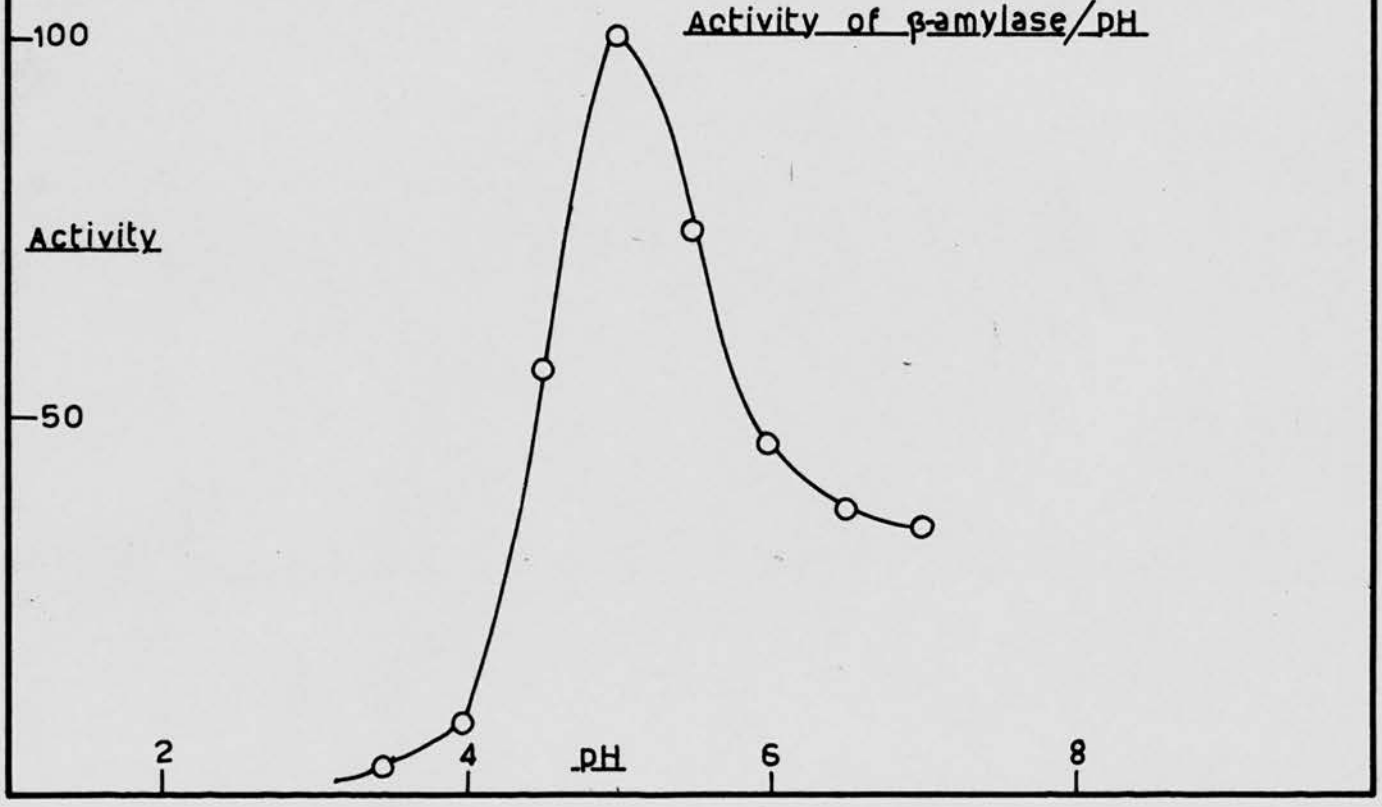


FIG. 6.02



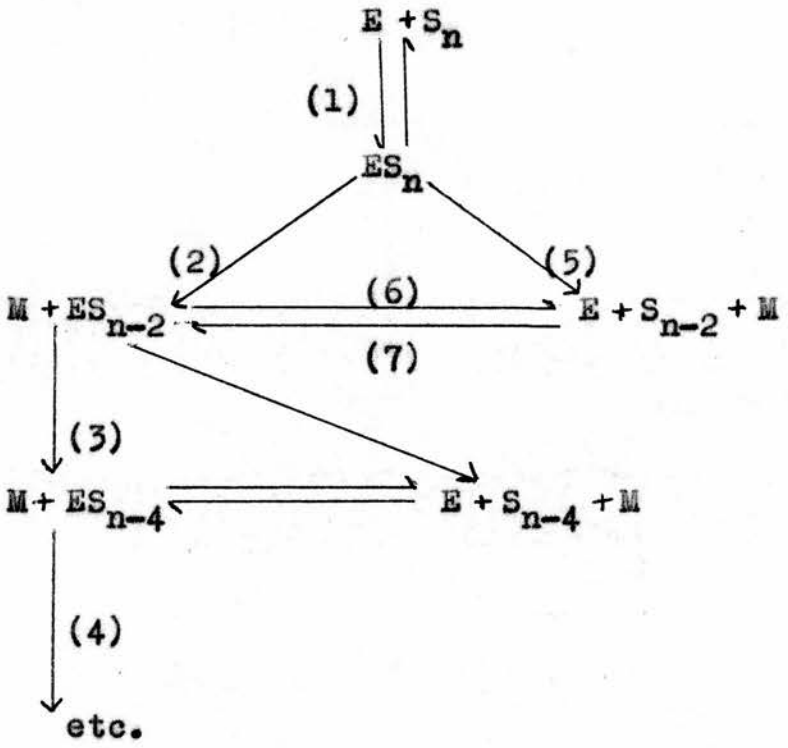
of the amylose after extensive degradation (79%) by β-amylase. This indicates, therefore, that the degradation is largely single-chain.

However, the results for the more rigorously subfractionated samples in Table 6.03 show that large falls in the viscosity were found, thus indicating multi-chain attack. It is pertinent that fractions 2A, 3A and 3B have been subfractionated twice and so were expected to have much sharper distributions than the first fraction investigated (Table 6.02). It is also significant that the viscosity of fraction 4 does not alter; this fraction had not been refractionated and so its molecular weight distribution would be similar to that of the fraction investigated in Table 6.02.

Table 6.03 shows the calculated values for the limiting viscosity numbers for multi-chain action. From a comparison of observed and calculated $[\eta]$ values, it is concluded that for samples of amylose with a relatively narrow molecular weight distribution, the action of β-amylase at pH 5.0 is not inconsistent with multi-chain attack. At unfavourable values of pH, i.e. 4 and 7, there is obviously a certain amount of single-chain action also, as the fall in molecular weight is less than expected for multi-chain attack (see Table 6.03). It is significant that evidence of multi-chain action was only found for resubfractionated samples: for samples with a wide molecular weight distribution the action pattern is apparently single-chain as predicted by French (1961).

These results are in general agreement with the conclusions of Husemann et al.(1964). These authors showed

that β-amylase degrades both natural and synthetic amyloses by a multi-chain mechanism. A schematic representation of such an action pattern is shown below (Bailey and Whelan, 1957).



Single-chain action is the result of the successive reactions (1), (2), (3), (4) etc. Multi-chain action proceeds either by reactions (1) and (5), or (1), (2) and (6), followed, in reaction (7), by recombination of the enzyme with the same or with a different substrate molecule.

SECTION 7.

STUDIES ON PHOSPHATASE

INTRODUCTION

Many starches contain phosphorus, but Samec and his co-workers (1924, 1931) have shown that it is not present in the same form in all starches.

Posternak (1935) showed that most of the phosphorus in cereal starches is present in the form of phosphatides which may be extracted with hot methanol, whereas phosphorus is chemically bound to potato starch. Samec (1914) and Schoch (1942) have indicated that most of the phosphorus in potato starch is associated with the amylopectin component and Posternak (1935, 1951) has shown that this phosphorus is present as an ester-phosphate group on C_6 of a glucose residue. This author further concluded that these groups are not in the viscinity of reducing groups or of branching points in the amylopectin molecule. Posternak (1951) isolated and examined dextrins containing the ester-phosphate groups from α-amylolysates of potato starch and showed that the phosphorus-containing groupings prevented β-amylase attack on these dextrins.

Peat et al. (1952) reported the presence of small amounts of esterified phosphorus in samples of potato amylose. These workers and Banks and Greenwood (1961) investigated the effect of various phosphatases on samples of amylose - such enzymes should remove ester-phosphate groups - but their results suggested that the removal of these phosphorus - containing groups did not alter the β-amylolysis limits of the amylose samples. These results, therefore, are somewhat unexpected after the findings of Posternak (1951). However, neither Peat et al. nor Banks and Greenwood showed that the phosphorus anomaly in their samples of amylose

had, in fact, been removed by the phosphatase. There is evidence that the phosphorus in amylopectin is difficult to remove (Fukui, 1957) and so an investigation was undertaken to obtain pure, highly active phosphatase and to examine its action on amylose and amylopectin.

EXPERIMENTAL AND RESULTS

A sample of potato phosphatase (acid) obtained from Mann Research Laboratories Inc. was used in this work.

Presence of β-amylase in the sample. The following digests were prepared:

1. Acetate buffer (2 ml.; 0.2M.; pH 5.5) + phosphatase (1 ml.; 5 mg./ml.) + water (10 ml.)
2. Acetate buffer (2 ml.; 0.2M.; pH.3.6) + phosphatase (1 ml.; 5 mg./ml.) + water (10 ml.).

These digests were incubated at 35°C. for 2 hours when aliquots (2 ml.) of amylopectin β-limit dextrin solution (4 mg./ml.) were added to each. Samples were removed at intervals for iodine -staining determinations. The results are shown in Table 7.01.

Table 7.01.

A.Vs.

Time (hours)	0	2½	10	20	48
Digest 1	4.41	2.72	0	0	0
Digest 2	4.40	4.20	4.15	4.02	3.71

Effect of mercuric chloride on α-amylase activity. The following digests were prepared:

1. Acetate buffer (2 ml.; 0.2M.; pH 5.5) + phosphatase (5 ml.; 2 mg./ml.) + mercuric chloride (3 ml.; 3.3×10^{-3}M.)
2. Acetate buffer (2 ml.; 0.2M.; pH 5.5) + phosphatase (5 ml.; 2 mg./ml.) + mercuric chloride (3 ml.; 5×10^{-4}M.).
3. Acetate buffer (2 ml.; 0.2M.; pH 5.5) + phosphatase (5 ml.; 2 mg./ml.) + mercuric chloride (3 ml.; 3.3×10^{-4}M.)
4. Acetate buffer (2 ml.; 0.2M.; pH 5.5) + phosphatase (5 ml.; 2 mg./ml.) + water (3 ml.)

These were incubated at room temperature for 24 hours when equal aliquots (10 ml.) of an amylose solution (2 mg./ml.) containing requisite amounts of mercuric chloride were added to each. After 48 hours at room temperature excess butanol was added to each digest and the amylose samples were isolated. No amylose was obtained in digest 4. The viscosities of the amyloses were determined in potassium hydroxide (0.2M.) at 25°C. The results are shown in Table 7.02.

Table 7.02

Digest	1.	2.	3.	4.
Viscosity	615	510	250	0
Viscosity of original amylose sample = 620.				

Effect of mercuric chloride on phosphatase activity. The

following digests were prepared:

1. Acetate buffer (2 ml.; pH 4.6; 0.2M.) + phosphatase (4 ml.; 2 mg./ml.) + water (3 ml.) + mercuric chloride (1 ml.; 10^{-3}M).
2. Acetate buffer (2 ml.; pH 4.6; 0.2M.) +phosphatase (4 ml.; 2mg./ml.) + water (3 ml.) + mercuric chloride (1 ml.; 1.5×10^{-3}M.).
3. Acetate buffer (2 ml.; pH 4.6; 0.2M.) +phosphatase (4ml.; 2 mg./ml.) + water (3 ml.) + mercuric chloride (1 ml.; 10^{-2}M.).
4. Acetate buffer (2 ml.; pH 4.6; 0.2M.) + phosphatase (4 ml.; 2 mg./ml.) + water (4 ml.).

These were incubated at room temperature for 24 hours when aliquots (1 ml.) of glucose 6 - phosphate (20 mg./ml.) were added to each. They were incubated at 35°C. for 24 hours when aliquots (1 ml.) were extracted and their phosphate contents were measured by the method of Allen (1940). The coloured solutions were read against an appropriate blank on a Spekker Absorptiometer using Ilford filter No. 608. The results are shown in Table 7.03.

Table 7.03

Digest	1	2	3	4
A.V.	0.484	0.425	0.123	0.510

Attempted Fractionation of phosphatase. A sample of phosphatase (100 mg.) was dissolved in water (13 ml.) and centrifuged to give a clear solution P1 which was cooled to 2°C. and

fractionated with alcohol between the limits 0 - 33, 33 - 37.5, 37.5 - 44.4, 44.4 - 70% with respect to alcohol. The precipiptates were air-dried and dissolved in water to give solutions P2 - P5. The α-amylase and phosphatase activities of the fractions were determined as already indicated, using amylopectin β-limit dextrin and glucose 6 - phosphate, respectively. The results are shown in Table 7.04.

Table 7.04

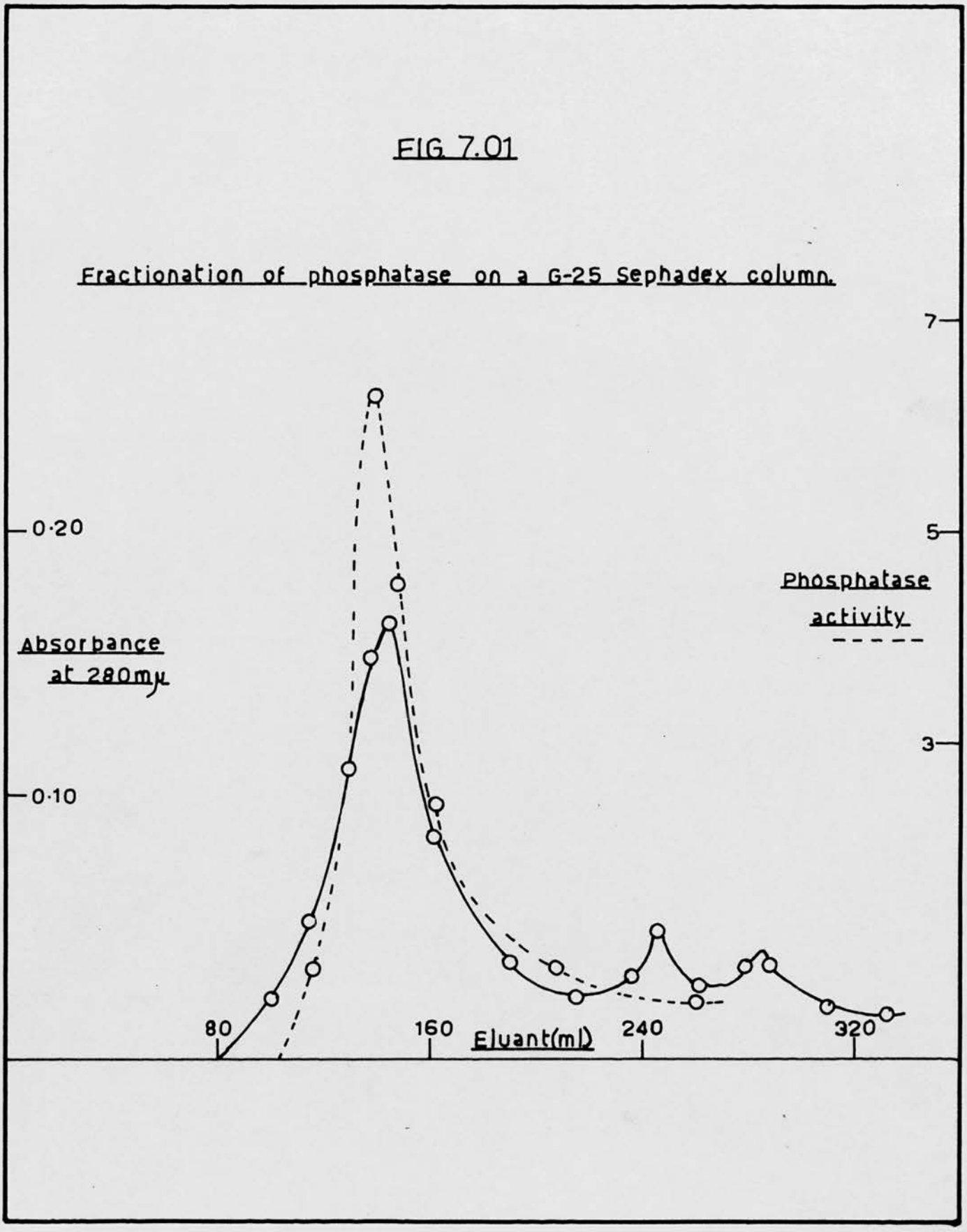
Fraction	P1	P2	P3	P4	P5
Phosphatase activity[x]	0.033	0.38	0.31	0.29	0.10
α-amylase activity	0.051	0.22	0.15	0.12	0.10

[x] calculated as follows: $\frac{\text{A.V.(0 hours)} - \text{A.V.(X hours)}}{\text{A.V.(0hours)} \times X \times \text{protein concentration in the digest.}}$

Purification of phosphatase on a "Sephadex" column. A sample of phosphatase (100 mg.) was suspended in water (3 - 4 ml.) and centrifuged to yield a clear brown solution which was placed on a G - 25 "Sephadex" gel column and eluted with water. Fractions were collected in 7 ml. aliquots and the phosphatase activities of those containing protein were determined by incubating a portion of the fraction with glucose 6 - phosphate. The results are shown in Fig. 7.01. The fractions containing phosphatase activity were tested for α-amylase activity by incubating portions with amylose solutions for 24 hours and then

FIG. 7.01

Fractionation of phosphatase on a G-25 Sephadex column.



determining the viscosities of the isolated amylose samples. However, no α-amylase activity was detected in any of the phosphatase - containing fractions. The most active fractions (25 - 30) were added together and this composite sample of phosphatase was used in the following experiments.

Effect of pH on phosphatase activity. Digests were prepared containing water (3 ml.), glucose 6-phosphate solution (1 ml.; 5 mg./ml.), acetate buffer (1 ml.; appropriate pH) and phosphatase (0.2 ml.). These were incubated at 35°C. and aliquots (1 ml.) were withdrawn after 4 hours for phosphate determinations. The results are shown in Fig. 7.02.

Effect of phosphatase on the β-limits of amylose and amylopectin. Digests were prepared as follows:

1. Amylose solution (10 ml.; 1.2mg./ml.) + acetate buffer (1 ml.; 0.2M.; pH4.6) + β-amylase (200 units).
2. Amylose solution (10 ml.; 1.2mg./ml.) + acetate buffer (1 ml.; 0.2M.; pH4.6) + β-amylase (200 units) + phosphatase (2 ml.).
3. Amylopectin solution (10 ml.; 2mg./ml.) + acetate buffer (1 ml.; 0.2M.; pH4.6) + β-amylase (200 units).
4. Amylopectin solution (10 ml.; 2mg./ml.) + acetate buffer (1 ml.; 0.2M.; pH4.6) + β-amylase (200 units) + phosphatase (2 ml.).

These were incubated at 35°C. for 48 hours when samples were removed for reducing power determinations. The presence of phosphatase did not alter the β-amylolysis limits of the substrates in these digests.

FIG. 7.02

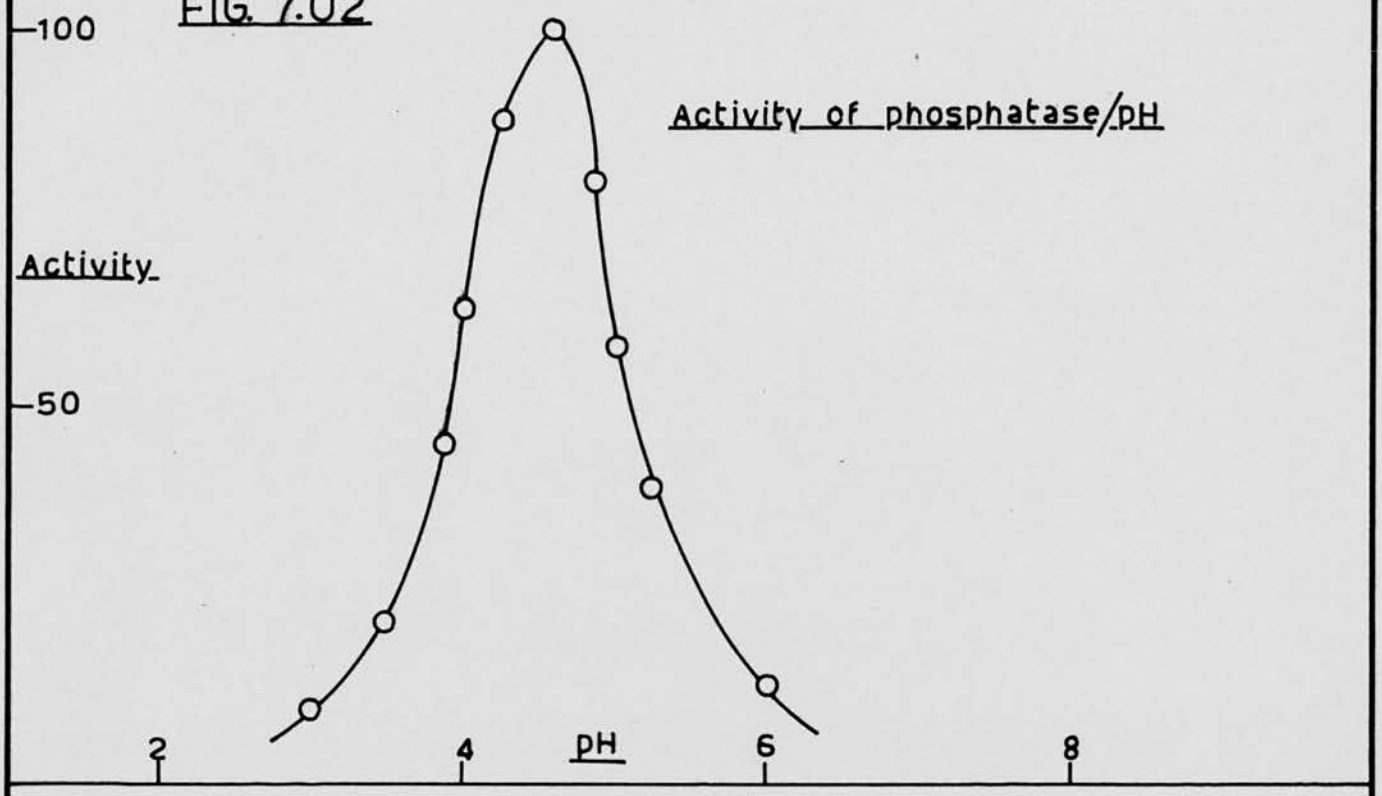
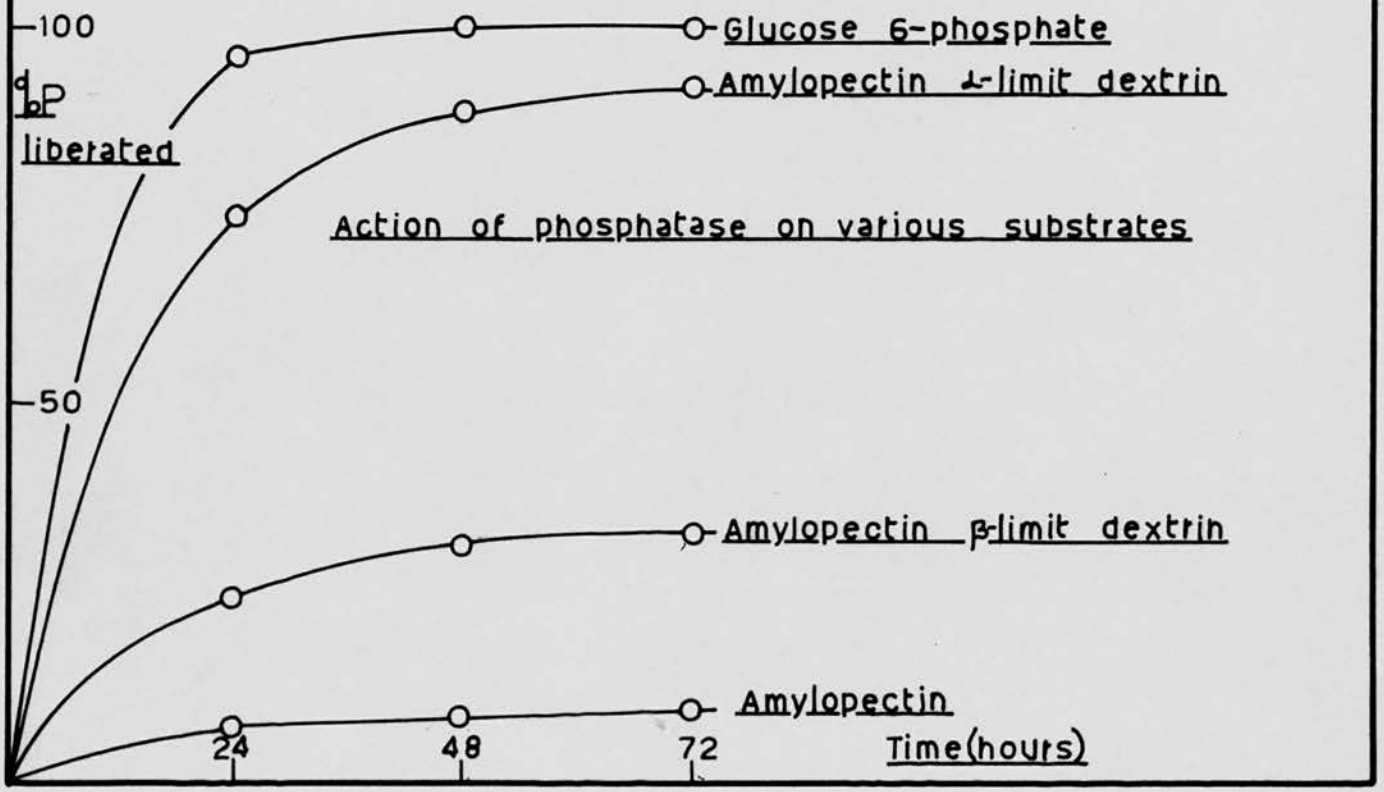


FIG. 7.03



Preparation of amylopectin α-limit dextrin. An amylopectin solution was incubated at pH 6.0 and 35°C. for 48 hours with a portion of purified salivary α-amylase. The digest was then heated on a boiling water-bath for 5 minutes and the resulting small precipitate was removed by centrifugation.

Action of phosphatase on various substrates. The following digests were prepared:

1. Amylopectin solution (9.84 mg./ml.; 105 ml. containing 0.01M. acetate buffer pH 4.6) + phosphatase (4 ml.).
2. Amylopectin β-limit dextrin solution (7.62 mg./ml.; 105 ml. containing 0.01M. acetate buffer pH 4.6) + phosphatase (4 ml.).
3. Amylopectin α-limit dextrin solution (5.56 mg./ml.; 105 ml. containing 0.01M. acetate buffer pH 4.6) + phosphatase (4 ml.).
4. Glucose 6-phosphate solution (1 mg./ml.; 105 ml. containing 0.01M. acetate buffer pH 4.6) + phosphatase (4 ml.).

These were incubated at 35°C. and after 24, 48 and 72 hours portions (digests 1 and 2 - 35 ml.; digests 3 and 4 - 20 ml.) were removed for free-phosphate determinations. These aliquots were passed through a small column (IRA - 400; 1.5 cm. x 7 cm.) in the chloride form when the free-phosphate was completely removed from the substrate solution. On eluting with sodium sulphate (50 ml.; 4%) the phosphate was completely removed from the column and estimated by the method of Allen (1940). Sodium sulphate did not interfere with this estimation and 95 - 100% recovery of the phosphate was obtained from the procedure. The results are shown in Fig. 7.03.

DISCUSSION

Purification of the phosphatase. The commercial preparation contained α-amylase impurity (Table 7.01) and an attempt was made to inhibit this enzyme with mercuric chloride (Table 7.02). Although complete inhibition of the α-amylase was achieved in this way the relatively high concentration of mercuric chloride required had an adverse effect on the phosphatase activity (Table 7.03.).

An attempt to separate the two activities by fractionation with ethanol was not successful (Table 7.04) - both α-amylase and phosphatase activities were detected in all of the fractions.

Fractionation on a "Sephadex" column was, however, successful. Active phosphatase fractions were obtained which were completely free from α-amylase impurity.

Optimum pH for phosphatase activity. This was found to be 4.6 (Fig. 7.02) and so all phosphatase-containing digests were incubated at pH 4.6.

Action of phosphatase on amylose. Since there is such a small amount of phosphate - if any - associated with pure samples of amylose no attempt was made to determine if free -phosphate groups were present in amylose digests after incubation with phosphatase. However, it is clear that even after prolonged incubation (48 hours) phosphatase had no effect on the β-limit of an amylose sample. This result is in agreement with the findings of Banks and Greenwood (1961) and suggests that ester-phosphate is not the

barrier to β-amylolysis present in large amylose molecules.

Action of phosphatase on amylopectin and limit dextrins. The results in Fig. 7.03 show that phosphatase rapidly removes phosphate groups from glucose 6-phosphate and amylopectin α-amylolysates but that amylopectin and its β-limit dextrin are much less readily attacked. This indicates that most of the phosphorus associated with amylopectin must be linked to interior portions of the molecule, and not to the exterior branches. Further evidence for this conclusion was provided when the inability of phosphatase to increase the β-amylolysis limit of amylopectin was illustrated. When the external branches in amylopectin were removed by β-amylase a much larger amount of phosphate was liberated on incubation with phosphatase. The difficulty experienced in removing phosphorus from amylopectin by enzymic action would, therefore, appear to be due to steric effects.

SUMMARY

After a brief account of the properties of the starch components, the action patterns of the more important starch-metabolising enzymes, and the importance of these enzymes in determining the structures of amylose and amylopectin have been discussed.

The physical and enzymic techniques used in this study have been outlined. A procedure for the extraction of starch from potatoes has been described and the conventional method of fractionating potato starch by dispersion into boiling water has been discussed.

Starch, pretreated with liquid ammonia, dimethyl sulphoxide or alkali has been dispersed in water at room temperature and successfully fractionated using a "critical concentration" of butanol. The amylose samples obtained from such fractionations were incompletely degraded by β-amylase. These results indicate that the structural -anomaly present in large amylose molecules is unlikely to be an artefact which has been introduced during the fractionation of starch at high temperatures. Fractionation of potato starch by the method of Killion and Foster (1960) yielded samples of amylose and amylopectin whose properties have been shown to be similar to those of the products from a conventional fractionation.

Unheated, aqueous solutions of pretreated potato starch have been examined in the ultracentrifuge and have been shown to contain two components. This result suggests that the "Unitarian Concept" of starch is incorrect.

Investigations on the preparation of samples of linear amylose have been carried out; aqueous leaching of starch granules at 60 - 65°C., followed by a dimethyl sulphoxide/ethanol fractionation of the leached material, was found to be the most efficient method for preparing such samples.

A detailed study on the extraction, purification and properties of soya bean Z-enzyme has been made. The action pattern of this enzyme has been investigated and found to be similar to the action patterns of other plant α-amylases.

α-Amylases from broad beans, barley and malted barley have also been examined. Various procedures for determining α-amylase activities have been discussed and the method finally adopted in this work has been described. Using viscometric techniques, kinetic studies on the action of these enzymes on linear-amylose substrates have indicated that the α-amylases hydrolysed the substrate in a completely random fashion. However, chromatographic examination of the products of the action of these enzymes on oligosaccharides showed that there are six linkages in such substrates which are relatively difficult to hydrolyse - these are the first five linkages from the non-reducing end and the linkage at the reducing end of the molecule. The extremely slow rate of attack of these plant α-amylases on small oligosaccharides compared to that of salivary α-amylase has been shown by a study on maltotetraose; the salivary enzyme hydrolysed this substrate some 100 x faster than did the plant enzymes.

The relative rates of attack of the α-amylases on various starch-type substrates were found to be: amylose > amylopectin > glycogen.

Investigations of the extraction and purification of R-enzyme from broad beans have shown that the removal of α-amylase impurity from such preparations is extremely difficult. However, methods for removing this impurity have been discussed.

The action patternof crystalline sweet potato β-amylase on fractions of linear amylose has been examined by measuring the molecular size of the dextrin-product. When rigorously sub-fractionated amylose samples were used, the reaction mechanism has been found to be almost completely multi-chain at pH5.0. The results of comparable experiments using fractions with a wider molecular-weight distribution indicated that the reaction was apparently single-chain. This behaviour has been predicted by French (1961).

A commercial preparation of potato phosphatase has been purified and shown to be free from α-amylase. The action of this enzyme on glucose 6-phosphate, amylopectin, amylopectin α-limit and β-limit dextrins has been studied. Although the glucose 6-phosphate and α-limit dextrin were rapidly attacked, only very small amounts of phosphate were removed from the other two substrates. The implications of these results have been discussed.

BIBLIOGRAPHY

Abdel - Ahker, M., J. K. Hamilton, H. Montgomery and F. Smith (1952)	J. Amer. Chem. Soc., 74, 4970.
Alfrey, T., A. Bartovics and H. Mark (1942)	J. Amer. Chem. Soc., 64, 1557.
Allen, R. J. L. (1940)	Biochem. J., 34, 858.
Anderson, D. M. W. and C. T. Greenwood (1955)	J. Chem. Soc., 3016.
Arbuckle, A. W. and C. T. Greenwood (1958)	J. Chem. Soc., 2626.
Baba, A. (1959)	Nippon Nogei Kagaku Kaishi, 33, 596. Via Chem. Abs., 57, 15489c.
Baba, A. and H. Kojima (1955)	Bull. Agr. Chem. Soc. Japan, 19, 167. Via Chem. Abs., 50, 16898h.
Bailey, J. M. and D. French (1957)	J. Biol. Chem., 226, 1.
Bailey, J. M., G. J. Thomas and W. J. Whelan (1951)	Biochem. J., 49, LVI.
Bailey, J. M. and W. J. Whelan (1957)	Biochem. J., 67, 540.
Balls, A. K., R. R. Thompson and M. K. Walden (1946)	J. Biol. Chem., 163, 571.
Balls, A. K., R. R. Thompson and M. K. Walden (1948)	J. Biol. Chem., 173, 9.
Banks, W. (1960)	Ph.D. Thesis, Edinburgh.
Banks, W. and C. T. Greenwood (1961)	Chem. and Ind., 714.
Banks, W. and C. T. Greenwood (1963)	Makromol. Chem., 67, 49.

Banks, W., C. T. Greenwood and I. G. Jones (1960)	J. Chem. Soc., 150.
Banks, W., C. T. Greenwood and J. Thomson (1959a)	Makromol. Chem., 31, 197.
Banks, W., C.T. Greenwood and J. Thomson (1959b)	Chem. and Ind., 928.
Barker, S. A. and E. J. Bourne (1953)	Quart. Rev. Chem. Soc., Lond., 7, 56.
Barker, S. A., E. J. Bourne and S. Peat (1949)	J. Chem. Soc., 1705.
Barker, S. A., E. J. Bourne, I. A. Wilkinson and S. Peat (1950)	J. Chem. Soc., 84.
Bates, F. L., D. French and R. E. Rundle (1943)	J. Amer. Chem. Soc., 65, 142.
Bauer, A. W. and E. Pacsu (1953)	Textile Research J., 23, 853.
Baum, H. and G. A. Gilbert (1953)	Nature, 171, 983.
Baum, H. and G. A. Gilbert (1954)	Chem. and Ind., 489.
Baum, H. and G. A. Gilbert (1956)	J. Colloid Sci., 11, 428.
Berl, E. (1910)	Z. ges. Schiess -u Sprengstoffw, 5, 82.
Bernfeld, P. and M. Fuld (1948)	Helv. Chim. Acta, 31, 1423.
Bernfeld, P. and H. Studer - Pécha (1947)	Helv. Chim. Acta, 30, 1904.

Biltz, W. and A. von Vegesack (1910)	Z. physik. Chem., 73, 481.
Bines, B. J. (1956)	Ph. D. Thesis, University of Wales. Via Whelan, W. J. (1960) Starke, 12, 358.
Bird, R and R. H. Hopkins (1954)	Biochem. J., 56, 86
Bird, R. and R. H. Hopkins (1954a)	Biochem. J., 56, 140.
Blom, J. and B. Schwarz (1952)	Acta Chem. Scand., 6, 697.
Bottle, R. T., G. A. Gilbert, C. T. Greenwood and K. N. Saad (1953)	Chem. and Ind., 541.
Bourne, E. J., A. Macey and S. Peat (1945)	J. Chem. Soc., 882.
Bourne, E. J. and W. J. Whelan (1950)	Nature, 166, 258.
Brice, B. A., M. Halwer and R. Speiser (1950)	J. Opt. Soc. Amer., 40, 768.
Briggs, D. E. (1961)	J. Inst. Brewing 67, 427.
Brown, F., T. G. Halsall, E. L. Hirst and J. K. N. Jones (1948)	J. Chem. Soc., 27.
Bryce, W.A. J. and C. T. Greenwood (1957)	J. Polymer Sci., 25, 481.
Bunton, C. A., T. A. Lewis, D. R. Llewellyn, H. Tristam and C. A. Vernon (1954)	Nature, 174, 560.
Chibnall, A. C., M. W. Rees and E. F. Williams (1943)	Biochem. J., 37, 354.

Cleveland, F. C. and R. W. Kerr (1948) — Cereal Chem., 25, 133.

Cohn, M. (1949) — J. Biol. Chem., 180, 771.

Commerford, J. D., G. T. Van Duzee and B. L. Scallet (1963) — Cereal Chem., 40, 482.

Cori, G. T. and J. Larner (1951) — J. Biol. Chem., 188,17.

Cowie, J. M. G., I. D. Fleming, C. T. Greenwood, D. J. Manners (1958) — J. Chem. Soc., 697.

Cowie, J. M. G. and C. T. Greenwood (1957a) — J. Chem. Soc., 2862.

Cowie, J. M. G. and C. T. Greenwood (1957b) — J. Chem. Soc., 4640.

Cowie, J. M. G. and C. T. Greenwood (1957c) — J. Chem. Soc., 2658.

Cunningham, W. L., D. J. Manners, A. Wright and I. D. Fleming (1960) — J. Chem. Soc., 2602.

Cunningham, W. L., D. J. Manners and A. Wright (1962) — Biochem. J., 85, 408.

Davis, W. E. and J. H. Elliott (1949) — J. Colloid Sci., 4, 313.

Debye, P. (1947) — J. Phys. Chem., 51, 18.

Dixon, M. and E. C. Webb (1961) — Adv. Protein Chem., 16, 197.

Doty, P. and R. F. Steiner (1950) — J. Chem. Phys., 18, 1211.

Dube, S. K. and P. Nordin (1961) — Arch. Biochem. Biophys., 94, 121.

Dube, S. K. and P. Nordin (1962) — Arch. Biochem. Biophys. 99, 105.

Dumazert, C. (1950)	Bull. Soc. Chim. biol., 32, 983.
Englard, S. and T. P. Singer (1950)	J. Biol. Chem., 187, 213.
Englard, S., S. Sorof and T. P. Singer (1951)	J. Biol. Chem., 189, 217.
Erlander, S. R. and D. French (1958)	J. Amer. Chem. Soc., 80, 4413.
Everett, W. W. and J. F. Foster (1959a)	J. Amer. Chem. Soc., 81, 3459.
Everett, W. W. and J. F. Foster (1959b)	J. Amer. Chem. Soc., 81, 3464.
Fischer, E. H. and C. H. Haselbach (1951)	Helv. Chim. Acta, 34, 325.
Fischer, E. H. and R. de Montmollin (1951a)	Helv. Chim. Acta, 34, 1987.
Fischer, E. H. and R. de Montmollin (1951b)	Helv. Chim. Acta, 34, 1994.
Fischer, E. H. and W. Settele (1953)	Helv. Chim. Acta, 36, 811.
Fischer, E. H. and E. A. Stein (1954)	Arch. Sci., 7, 131.
Fleming, I. D. and D. J. Manners (1958)	Chem. Ind., 831.
Flory, P. J. and T. G. Fox (1949)	J. Phys. Chem., 53, 197.
Flory, P. J. and T. G. Fox (1950)	J. Polymer Sci., 5, 745.
Flory, P. J. and T. G. Fox (1951)	J. Amer. Chem. Soc., 73, 1904.

Fogg, D. N. and N. T. Wilkinson (1958) — Analyst, 83, 406.

Ford, L. H. and S. Peat (1941) — J. Chem. Soc., 856.

Foster, J. F. and P. J. Killion (1960) — J. Polymer Sci., 46, 65.

Foster, J. F. and M. D. Sterman (1956) — J. Polymer Sci., 21, 91.

French, D. (1957) — Baker's Digest, 31, 24.

French, D. (1958) — Proc. Intern. Symposium Enzyme Chem., Tokyo, 2, 530.

French, D. (1961) — Nature, 190, 445.

French, D., D. W. Knapp and J. H. Pazur (1950a) — J. Amer. Chem. Soc., 72, 1866.

French, D., M. L. Levine, J. H. Pazur and E. Norberg (1950b) — J. Amer. Chem. Soc., 72, 1746.

French, D. and R. W. Youngquist (1963) — Stärke, 15, 425.

Freudenberg, K. (1936) — Trans. Faraday Soc., 32, 74.

Freudenberg, K., W. Kuhn, W. Dürr, F. Bolz and G. Steinbrunn (1930) — Ber., 63, 1510.

Fukui, T. (1958) — Mem. Inst. Sci. Ind. Res., Osaka Univ., 15, 201.

Gieseking, J. E., H. J. Snider and C. A. Getz (1935) — Ind. Eng. Chem., Anal. Ed., 7, 185.

Gilbert, G. A., C. T. Greenwood and F. J. Hybart (1954)	J. Chem. Soc., 4454.
Gilbert, G. A. and J. V. R. Marriott (1948)	Trans. Faraday Soc., 44, 84.
Gilbert, G. A. and A. D. Patrick (1952)	Biochem. J., 51, 181.
Goerdeler, J. (1958)	In "Encyclopedia of Plant Physiology," Springer-Verlag, Berlin, Vol. VI, p.245.
Goodison, D. and R. S. Higginbotham (1950)	Shirley Inst. Mem., 24, 235.
Gralén, N. (1944)	Dissertation, Uppsala.
Greenwood, C. T. (1956)	Adv. Carbohydrate Chem., 11, 335.
Greenwood, C. T., (1960)	Stärke 12, 169.
Greenwood, C. T. and J. S. M. Robertson (1954)	J. Chem. Soc., 3769.
Greenwood, C. T. and J. Thomson (1962)	J. Chem. Soc., 222.
Gunja, Z. H., D. J. Manners and K. Maung (1961)	Biochem. J., 81, 392.
Hamilton, J. K. and F. Smith (1956)	J. Amer. Chem. Soc., 78, 5910.
Hanes, C. S., (1936)	Biochem. J., 30, 168.
Hanes, C. S. and M. Cattle (1938)	Proc. Roy. Soc. Lond., B 125, 387.

Hanrahan, V. M. and M. L. Caldwell (1953a)	J. Amer. Chem. Soc., 75, 2191.
Hanrahan, V. M. and M. L. Caldwell (1953b)	J. Amer. Chem. Soc., 75, 4030.
Hassid, W. Z. and R. M. McCready (1943)	J. Amer. Chem. Soc., 65, 1157.
Haworth, W. N., E. L. Hirst and F. A. Isherwood (1937)	J. Chem. Soc., 577.
Haworth, W. N., E. L. Hirst and J. I. Webb (1928)	J. Chem. Soc., 2681.
Haworth, W. N., H. Kitchen and S. Peat (1943)	J. Chem. Soc., 619.
Haworth, W. N. and E. G. V. Percival (1931)	J. Chem. Soc., 1342.
Hess, K. and B. Krajnc (1940)	Ber., 73, 976.
Hess, K. and E. Steurer (1940)	Ber., 73, 1076.
Higginbotham, R. S. and G. A. Morrison (1948)	Shirley Inst. Mem., 22, 141.
Hirst, E. L., T. G. Halsall and J. K. N. Jones (1947)	J. Chem. Soc., 1427.
Hobson, P. N., W. J. Whelan and S. Peat (1950a)	J. Chem. Soc., 3566.
Hobson, P. N., W. J. Whelan and S. Peat (1950b)	Biochem. J., 47, XXXIX.
Hobson, P. N., W. J. Whelan and S. Peat (1951)	J. Chem. Soc., 1451.

Hockenhull, J. D. J. and D. Herbert (1945)	Biochem. J., 39, 102.
Hodge, J. E., E. M. Montgomery and G. E. Hilbert (1948)	Cereal Chem., 25, 19.
Holló, V. J. and J. Szejtli (1958)	Kolloid. Zhur. 20, 229.
Holmberg, O. (1933)	Ark. Kemi Mineral. O. Geol., 11B.
Holmberg, O. (1933)	Biochem. Z., 258, 134.
Hopkins, R. H. and R. Bird (1953)	Nature, 172, 492.
Hopkins, R. H. and B. Jelinek (1949)	Nature, 164, 955.
Hopkins, R. H. and B. Jelinek (1954)	Biochem. J., 56, 136.
Hopkins, R. H. and D. Kulka (1942)	J. Inst. Brewing, 48, 170.
Houwink, R. (1940)	J. Prakt. Chem., 157, 15.
Hsiu, J., E. H. Fischer and E. A. Stein (1964)	Biochemistry 3, 61.
Hultin, E. (1949)	Acta Chem. Scand. 3, 697.
Hultin, E., (1950)	Arkiv. Kemi (Stockh.) 2, 135.
Husemann, E., W. Burchard and B. Pfannemüller (1964)	Stärke, 16, 143.
Husemann, E. and B. Pfannemüller (1961)	Makromol. Chem., 43, 156.

Katzbeck, W. J. and R. W. Kerr (1950)	J. Amer. Chem. Soc., 72, 3208.
Kerr, R. W. (1949)	Nature, 164, 757.
Kerr, R. W. and F. C. Cleveland (1951)	J. Amer. Chem. Soc., 73, 2421.
Kirkwood, J. G. and J. Riseman (1948)	J. Chem. Phys., 16, 565.
Kjølberg, O. and D. J. Manners (1963)	Biochem. J., 86, 258.
Kneen, E. (1944)	Cereal Chem., 21, 304.
Kneen, E., R. M. Sandstedt and C. M. Hollenbeck (1943)	Cereal Chem., 20, 399.
Kuhn, W. (1934)	Kolloidzschr., 68, 2.
Kuhn, R. (1925)	Liebigs Ann., 443, 1.
Kung, J. T., V. M. Hanrahan and M. L. Caldwell (1953)	J. Amer. Chem. Soc., 75, 5548.
Lampitt, L. H., C. H. F. Fuller and L. Coton (1955)	J. Sci. Food and Agric., 6, 656.
Lansky, S., M. Kooi and T. J. Schoch (1949)	J. Amer. Chem. Soc., 71, 4066.
Larner, J., B. Illingworth, G. T. Cori and C. F. Cori (1952)	J. Biol. Chem., 199, 641.
Levin, O. (1962)	Methods in Enzymology, 5, 27.
Levitzki, A., J. Heller and M. Schramm (1964)	Biochim. Biophys. Acta, 81, 101.

Loewus, F. A. and D. R. Briggs (1957)	J. Amer. Chem. Soc., 79, 1494.
Loyter, A. and M. Schramm (1962)	Biochim. Biophys. Acta, 65, 200.
MacWilliam, I. C. (1958)	Nature, 181, 1143.
MacWilliam, I.C. and G. Harris (1959)	Arch. Biochem. Biophys., 84, 442.
Manners, D. J. (1952)	Ph. D. Thesis, Cambridge.
Manners, D. J. and G. A. Mercer (1963)	J. Chem. Soc., 4317.
Maquenne, L. and E. Roux (1905)	Compt. rend., 140, 1303.
Mark, H. (1938)	"Der Feste Körper", Leipzig, 103.
Mayer, K. and M. Klinga - Mayer (1940)	Z. physiol. Chem. 267, 115. Via Chem. Abs. (1941) 3290.
Mayer, F. C. and J. Larner (1959)	J. Amer. Chem. Soc., 81, 188.
Meyer, K. H. (1942)	Adv. Colloid Sci., 1, 143.
Meyer, K. H. (1952)	Experientia (Basel), 8, 405.
Meyer, K. H. and P. Bernfeld (1941)	Helv. Chim. Acta, 24, 359E.
Meyer, Wolff (K. H., P. Bernfeld and E. (1940a)	Helv. Chim. Acta, 23, 854.

Meyer, K. H., E. H. Fischer, A. Staub and P. Bernfeld (1948)	Helv. Chim. Acta, 31, 2158.
Meyer, K. H., M. Fuld and P. Bernfeld (1947)	Experientia (Basel), 3, 411.
Meyer, K. H. and G. C. Gibbons (1950)	Helv. Chim. Acta, 33, 210.
Meyer, K. H. and W. F. Gonon (1951a)	Helv. Chim. Acta, 34, 290.
Meyer, K. H. and W. F. Gonon (1951b)	Helv. Chim. Acta, 34, 294.
Meyer, K. H. and P. Rathgeb (1948)	Helv. Chim. Acta, 31, 1545.
Meyer, K. H., M. Wertheim and P. Bernfeld (1940b)	Helv. Chim. Acta, 23, 865.
Meyer, K. H., M. Wertheim and P. Bernfeld (1941)	Helv. Chim. Acta, 24, 378.
Montgomery, E. M., F. B. Weakley and G. E. Hilbert (1949)	J. Amer. Chem. Soc., 71, 168 2.
Mould, D. L. (1954)	Biochem. J., 58, 593.
Mould, D. L. and R. L. M. Synge (1954)	Biochem. J., 58, 571.
Muetgeert, J. (1961)	Adv. Carbohydrate Chem., 16, 299.
Myrbäck, K. (1948)	Adv. Carbohydrate Chem., 3, 251.
Myrbäck, K. (1950)	Ark. Kemi (Stockh.) 2, 417.

Myrbäck, K. and G. Nycander (1942)	Biochem. Z., 311, 234.
Myrbäck, K. and L. G. Sillén (1949)	Acta Chem. Scand., 3, 190.
Myrbäck, K. and K. Svanborg (1953)	Arkiv. Kemi (Sockh.), 6, 113.
Neufeld, E. F. and W. Z. Hassid (1955)	Archiv. Biochem. Biophys., 59, 405.
Ohlsson, E. (1930)	Hoppe-Seylers Z., 189, 17. Via Whelan (1958).
Pacsu, E. (1947)	J. Polymer Sci., 2, 565.
Parrish, F. W. (1958)	Ph.D. Thesis, University of London. Via Whelan, W. J. (1960) Stärke, 12, 358.
Pazur, J. H. (1953)	J. Biol. Chem., 205, 75.
Pazur, J. H. and I. Budovich (1955)	Science, 121, 702.
Pazur, J. H. and T. Budovich (1956)	J. Biol. Chem., 220, 21.
Pazur, J. H., D. French and D. W. Knapp (1950)	Proc. Iowa Acad. Sci., 57, 203.
Pazur, J. H. and R. M. Sandstedt (1954)	Cereal Chem., 31, 416.
Peat, S., S. J. Pirt and W. J. Whelan (1949)	Nature, 164, 499.
Peat, S., S. J. Pirt and W. J. Whelan (1952c)	J. Chem. Soc., 714.
Peat, S., G. J. Thomas and W. J. Whelan (1950)	Biochem. J., 47, xI.

Peat, S., G. J. Thomas and W. J. Whelan (1952a)	J. Chem. Soc., 722.
Peat, S., J. R. Turvey and G. Jones (1959)	J. Chem. Soc., 1540.
Peat, S. and W. J. Whelan (1953)	Nature, 172, 494.
Peat, S., W. J. Whelan, P. N. Hobson and G. J. Thomas (1954)	J. Chem. Soc., 4440.
Peat, S., W. J. Whelan and G. W. F. Kroll (1956)	J. Chem. Soc., 53.
Peat, S., W. J. Whelan and W. R. Rees (1953)	Nature, 172, 158.
Peat, S., W. J. Whelan and W. R. Rees (1956)	J. Chem. Soc., 44.
Peat, S., W. J. Whelan and G. J. Thomas (1952b)	J. Chem. Soc., 4546.
Peat, S., W. J. Whelan and G. J. Thomas (1956)	J. Chem. Soc., 3025.
Posternak, T. (1935)	Helv. Chim. Acta, 18, 1351.
Posternak, T. (1951)	J. Biol. Chem., 188, 317.
Potter, A. L. and W. Z. Hassid (1948)	J. Amer. Chem. Soc., 70, 3488.
Potter, A. L. and W. Z. Hassid (1951)	J. Amer. Chem. Soc., 73, 593.
Preece, I.A. and M. Shadaksharaswamy (1949)	Biochem. J., 44, 270.

Rao, V. S. R. and J. F. Foster (1963)	Biopolymers, 1, 527.
Rayleigh, Lord (1871)	Phil. Mag., 41, 447.
Roberts, P. J. P. (1953)	Ph.D. Thesis, University of Wales.
Roberts, P. J. P. and W. J. Whelan (1951a)	Biochem. J., 49, Ivi.
Roberts, P. J. P. and W. J. Whelan (1952)	Biochem. J., 51, xviii.
Roberts, P. J. P. and W. J. Whelan (1960)	Biochem. J., 76, 246.
Robyt, J. and D. French (1963)	Arch. Biochem. Biophys., 100, 451.
Rundle, R. E. and F. C. Edwards (1943)	J. Amer. Chem. Soc., 65, 2200.
Rundle, R. E., J. F. Foster and R. R. Baldwin (1944)	J. Amer. Chem. Soc., 66, 2116.
Rundle, R. E. and D. French (1943)	J. Amer. Chem. Soc., 65, 558.
Sahyun, M. and C. L. Alsberg (1930)	J. Biol. Chem., 89, 33.
Samec, M. (1914)	Kolloid- Beihefte, 6, 23. Via Chem. Abs.(1915) p.468.
Samec, M. and R. Klemen (1931)	Kolloid - Beihefte, 33, 254. Via. Chem. Abs. (1931) p.5913.

Samec, M., M. Minaeff and N. Ronzin (1924)	Kolloid - Beihefte, 19, 203. Via Chem. Abs. (1924) p. 2975.
Samec, M., C. Nucic and V. Pirkmaier (1941)	Kolloid - Z., 94, 350.
Schoch, T. J. (1942)	J. Amer. Chem. Soc., 64, 2957.
Schoch, T. J. and H. W. Leach (1962)	Cereal Chem., 39, 4.
Schoch, T. J. and E.C. Maywald (1956)	Analyt. Chem., 28, 382.
Schwimmer, S. (1950)	J. Biol. Chem., 186, 181.
Schwimmer, S. and A. K. Balls (1949)	J. Biol. Chem., 179, 1063.
Senti, F. R., G. E. Babcock, R. Tobin and R. J. Dimler (1960)	Cereal Chem., 37, 645.
Smith, G. F. (1953)	Analyt. Chim. Acta, 8, 397.
Smith, B. W. and J. H. Roe (1957)	J. Biol. Chem., 227, 357.
Somogyi, M. (1940)	J. Biol. Chem., 134, 301.
Somogyi, M. (1945)	J. Biol. Chem., 160, 61.
Staudinger, H. (1932)	"Die Hochmolekularen Organischen Verbindungen," Springer, Berlin.

Staudinger, H. and E. Husemann (1937)	Ann., 527, 195.
Stein, E. A. and E. H. Fischer (1958)	J. Biol. Chem., 232, 867.
Stein, E. A. and E. H. Fischer (1960)	Biochim. Biophys. Acta, 39, 287.
Stein, E. A., J. Hsiu and E. H. Fischer (1964)	Biochemistry 3,56.
Summer, R. and D. French (1956)	J. Biol. Chem., 222, 469.
Sutra, M. R. (1950)	Bull. Soc. Chim. France, M, 294.
Svedberg, T. and K. O. Pedersen (1940)	"The Ultracentrifuge", Oxford Univ. Press, London.
Tanret, C. (1915)	Bull. Soc. Chim. France, 17, 83.
Thoma, J. A. and D. French (1960)	J. Amer. Chem. Soc., 82, 4144.
Thoma, J. A. and D. French (1961)	J. Phys. Chem., 65, 1825.
Thoma, J. A. and D. E. Koshland, Jr. (1960a)	J. Biol. Chem., 235, 2511.
Thoma, J. A. and D. E. Koshland, Jr. (1960b)	J. Amer. Chem. Soc., 82, 3329.
Thompson, A. and M. L. Wolfrom (1951)	J. Amer. Chem. Soc., 73, 5849.
Trevelyan, W. E., D. P. Procter and J. S. Harrison (1950)	Nature, 166, 444.

Ubbelohde, L. (1937)	Anal. Chem., 9, 85.
Ulmann, B. and B. Wendt (1954)	Makromol. Chem., 12, 155.
Underkofler, L. A. and D. K. Roy (1951)	Cereal Chem., 28, 18.
Vallee, B. L., E. A. Stein, W. N. Sumerwell and E. H. Fisher (1959)	J. Biol. Chem., 234, 2901.
Vink, H. (1963)	Makromol. Chem., 67, 105.
Walker, G. J. and W. J. Whelan (1960a)	Biochem. J., 76, 257.
Walker, G. J. and W. J. Whelan (1960b)	Biochem. J., 76, 264.
Whelan, W. J. (1955)	Methods in Enzymol. 1, 192.
Whelan, W. J. (1958)	In "Encyclopedia of Plant Physiology", Springer - Verlag, Berlin, Vol VI, p.154.
Whelan, W. J. and P. J. P. Roberts (1953)	J. Chem. Soc., 1298.
Whelan, W. J. and J. G. Roberts (1954)	Biochem. J., 58, 569.
Whistler, R. L., W. Dvonch and H. J. Yearian (1950)	J. Amer. Chem. Soc., 72, 1748.
Whistler, R. L. and C. Johnson (1948)	Cereal Chem., 25, 418.

Whitmore, E. T. and D. H. B. Sparrow (1957) — J. Inst. Brew., 63, 397.

Wild, G. M. (1954) — Iowa State Coll. J. Sci., 28, 419. Via Chem. Abs. 11505c.

Wolff, I. A., L. J. Gundrum and C.E. Rist (1950) — J. Amer. Chem. Soc., 72, 5188.

Woolf, L. I. (1953) — Nature, 171, 841.

Zimm, B. H. (1948) — J. Chem. Phys., 16, 1093.

Zimm, B. H., R. S. Stein and P. Doty (1945) — Polymer Bull., 1, 90.