A THESIS

Submitted for the degree of

DOCTOR OF PHILOSOPHY

by

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August, 1970.

TO MY MOTHER.

PREFACE

I gratefully acknowledge the help and encouragement by Dr. C.T. Greenwood throughout the period of this research. Thanks are also due to Dr. W. Banks for his critical discussions in certain aspects of this work. Some of the work described in Section 3 has been published in conjunction with Dr. C.T. Greenwood and Dr. W. Banks and the reprint is inserted in the end of this Thesis.

I also wish to thank Dr. R.A. Wall and K.T. Salmon for their help in amino-acid analysis reported in Section 4.

I am also thankful to Professor C. Kemball for providing the laboratory facilities and the Office of Naval Research and Corn Industries Research Foundation for the financial support in the later stages of this work.

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INTRODUCTION

Despite the fact that a great amount of work has been carried out on starch degrading enzymes, they have not yet been adequately characterised. A survey of literature in Section 1 reveals the disagreements over their molecular size, nature of the iso-enzymes and the mode of action of these enzymes. The work presented in this thesis examines some of the controversial points in detail and describes some new approaches to the problems.

In Section 2, the experimental techniques employed are described. Some modifications in the previous techniques were necessary for the experiments carried out. These are outlined and discussed.

Action patterns of alpha-amylases towards amylose have been reported by many workers. In Section 3 this problem has been examined in detail by the techniques applied by the previous authors and a new approach has been made to demonstrate the action pattern of alpha-amylases.

Section 4 describes the studies on the fractionation and amino-acid analyses of the porcine pancreatic alpha-amylase.

Section 5 deals with the preparation and properties of synthetic, long-chain amylopectins, long-chain glycogens and linear amylose oligomers. These samples were prepared by the synthetic action of potato phosphorylase. The action pattern of phosphorylase is also discussed. In the last Section, a preliminary investigation of a method

for determination of degree of polymerisation for linear materials and chain-length for branched materials is described.

SECTION 1

STARCH DEGRADING AND SYNTHESISING ENZYMES

(A GENERAL INTRODUCTION)

1

STARCH DEGRADING AND SYNTHESISING ENZYMES
(GENERAL INTRODUCTION)

Starch degrading and synthesising enzymes are widely distributed in nature. They are found in plants, animals and bacteria. Their action can be represented by the following general equation.

More work appears to have been carried out on starch degrading enzymes as compared to the synthesising enzymes. The following are the most well known enzymes in this field.

1. <u>Alpha</u> -Amylases	(E.C. 3.2.1.1)
2. <u>Beta</u> -Amylases	(E.C. 3.2.1.2)
3. Gluco-Amylases	(E.C. 3.2.1.3)
4. Iso-Amylases	(E.C. 3.2.1.9)
5. Phosphorylases	(E.C. 2.4.1.1)
6. Amylo-1-6-Glucosidases	(E.C. 3.2.1.33)
7. Starch Synthetase	-

1. ALPHA AMYLASES

These enzymes hydrolyse the α -1-4-bonds in starch type substrates and the products formed have α -D-configuration. α -1-6-bonds of amylopectins and glycogen are resistant to alpha amylolytic attack. Alpha-amylases were obtained in crystalline form by Fischer and Bernfeld (1948) from hog pancreas, human pancreas and in 1950 from human saliva. Later on many other workers prepared crystalline alpha-amylases from rat pancreas, Aspergillus oryzae, Bacillus subtilis, Bacillus coagulans, Bacillus stearothermophilus

and malted barley. Highly purified enzymes have also been prepared from malted sorghum, soya beans, malted rye, malted wheat, pigeon pancreas and shore crab. Isolation of enzymes from these sources is achieved by either ammonium sulphate or acetone fractionation, precipitation with rivanol and absorption on starch derivatives. Purification processes include column chromatography, electrophoresis and selective inhibition of contaminating enzymes. Loyter and Schramm (1962) have described a highly effective procedure of precipitating pure alpha-amylase as glycogen complexes.

Very little is known about their structure and the conformation of their dipeptide chains in the solution. The amino acid composition of alpha-amylases from different sources has been reported by a number of workers but the amino acid sequence of any of these enzymes is not known yet.

All alpha-amylases are essentially metallo proteins. Calcium is a vital part of their activity. Reversible inactivation can be effected by chelating agents which remove calcium from the protein. Stein et al (1960) have reported that activity can be restored by the addition of calcium ions. The importance of calcium was further demonstrated by the fact, reported by above authors, that alpha-amylases from which calcium is removed is easily attacked by proteolytic enzymes, while the native enzyme is highly resistant to such attack. The fact that the metal ions render the enzyme resistant to proteolytic

attack indicates that it contributes significantly to the tertiary structure of the protein.

An interesting feature was reported by Isemura et al (1969) that alpha-amylase was not denatured by 0.1 M EDTA and 8M Urea separately, but their simultaneous action denatured the enzyme. This suggests the binding of calcium in alpha-amylases to be a complicated, rather than a simple phenomenon. It appears that some of the calcium is bound loosely. This can be taken off by 0.1 MEDTA, after which alpha amylase loses its configuration and tightly bound calcium sites become vulnerable to the action of urea.

The above authors have also shown by studying the physiological properties of Bacillus subtilus alpha-amylase, that this is a globular protein which has about 20% α -helix content. The enzyme is denatured by 8M urea containing EDTA. In this case secondary and tertiary structures are destroyed. The fact that denatured enzyme can be reactivated shows the importance of the calcium for the stability and the active conformation of the unfolded polypeptide.

Certain groups in proteins show a great affinity for metal ions, but it is not clearly established exactly which are involved. However, one protein group has been well characterised as regard to its metal affinity; the SH-group has a great affinity for mercury and silver compounds. Alpha amylases from different sources show different binding capacities for calcium. The binding strength is in the order, fungal > bacterial > mammalian > plant enzymes. It is not yet known which group is responsible for the

extra binding strength. The role of the calcium in the formation of the enzyme-substrate complex or in the catalytic activity of the enzyme is not yet clear. Mammalian enzymes are activated by monovalent anions like chloride.

All alpha-amylases appear to be of similar molecular size, apart from those of Bacillus stearothermophilus and the shore crab which have rather low molecular weight. The presence of sub units have been reported in alpha-amylases. Muus and Vanchak (1964) have suggested that the molecular weight of human salivary alpha-amylase determined from amino acid analysis is around 18000. As the molecular weight determined by sedimentation is 69000, this enzyme could be a trimer of a small molecular weight enzyme. Noerby (1964) and Muus and Vanchak (1964) have reported the presence of iso-enzymes in human salivary alpha-amylase. Frydenberg and Nielsen (1965) have shown iso-enzymes to be present in barley enzyme. Rowe et al (1968) suggested multiple forms of porcine pancreatic alpha-amylase from their results of gelelectrophoresis and DEAE cellulose column chromatography. Loyter and Schramm (1966) suggested that porcine pancreatic alpha-amylase has two active sites. All these observations indicate that these enzymes are either dimers and trimers of smaller molecular weight enzymes or there are iso enzymes present in the system.

The Bacillus subtilus alpha-amylase is different from other amylases as it forms a dimer with Zn (Stein and Fischer, 1960). Moreover it can be regained by removing the Zn with

metal chelating agents. Alpha-amylase from Aspergillus oryzae is also different from the others because it is a glycoprotein. Akabori et al (1956) showed that it contains a small proportion of carbohydrate.

Very little is known about the active site of the alpha-amylase. Studies on the effect of pH on hydrolysis with plant alpha-amylases (Greenwood et al 1968), with bacterial amylases (Ono et al, 1964) and with porcine pancreatic alpha-amylases (Wakim et al 1969) showed that carboxylate anion and imidazolium cation are the catalytic residues. The importance of histidine is also shown by the action of diazo-benzene sulphonic acid and iodine on plant amylases (Greenwood et al 1965).

Caldwell et al (1945) have ruled out sulphydryl groups for the activity of the alpha-amylases, but Schramm (1964) claimed that masked sulphydryl groups are necessary for hog-pancreatic alpha-amylase. It is possible that these groups are not the part of active site but are necessary for the retention of active enzyme conformation. The role of tyrosine is still a subject of controversy. Isemura et al (1969) have however, suggested that the phenolic-hydroxyl groups of tyrosine, play a stabilizing part in the enzyme molecule, while Weill and Caldwell (1945) thought that these groups are not involved in the system. The role of free amino acids was emphasised by Caldwell et al (1954). When taka-amylase and hog pancreatic alpha-amylase were treated with acetic anhydride, the enzymes were inactivated, it was concluded that the free amino acids are necessary

for alpha-amylase activity.

Alpha-amylases are generally stable between pH 5.5-8.0. Some are stable over an even wider range. Fischer and Bernfield (1948) have however, reported that hog pancreatic alpha-amylase is unstable below pH 6.5. Rowe et al (1969) have reported in contrast that porcine pancreatic alpha-amylase is stable over a range of pH 4.0-10.5. Mammalian enzymes need chloride ions for their activity but they are not inactivated in its absence. Cereal amylases are easily inactivated at pH 3.6 (Greenwood et al, 1968).

The optimum temperature for all alpha-amylases is within the range of 30° - 40°C. They rapidly lose activity above 50°C. This inactivation is irreversible. Alpha-amylases are also inhibited by heavy metals like mercury, silver, copper and lead. (Carlo and Redfern, 1947; Greenwood et al (1965); Urata 1957, Muus et al 1956). The mechanism of inhibition is not yet known. Mercury and silver effect sulphydryl groups as mentioned earlier, but the Bacillus subtilis enzyme does not contain any sulphydryl groups so the mechanism of inhibition is not by mercaptide formation.

The Reaction of Mechanism of Alpha-amylases.

Meyer and Lerner (1959) reported that in the reaction the substrate is first oriented on the surface of the enzyme. An oxonium ion is formed after the protonation of the bridge oxygen and the glucosidic bond is split on the C-1 carbon side leaving an intermediate carbonium ion.

Steric hinderance on the potential reducing residue is responsible for the ultimate configuration of the reducing groups. There are two other possible reaction mechanisms for amylases which are called single displacement in which the C_1-O-C_4 bond is broken while it forms C_1-OH bond simultaneously, and double displacement mechanism in which the C_1-O-C_4 bond breaks and an enzyme-substrate complex is formed. The enzyme-substrate bond is then hydrolysed to give products. Koshland (1954), by analogy with model organic reactions, suggested that alpha-amylases which act by retention of configuration will act by double displacement mechanism. Rowe et al (1969) however, do not agree with Koshland and suggest that alpha-amylases act with single displacement mechanism.

The mechanism can be represented as in Fig. (1.1.)

FIG. 1.1

Mechanism of Amylase action (Meyer & Larner 1959)

1. BETA-AMYLASE

Beta-amylase has been found only in plants. Unlike alpha-amylases this enzyme when isolated from different sources appear to act similarly. It is widely distributed in cereals like wheat, rye, sorghum, oats, barley and in soya beans and sweet potatoes. Balls et al (1948) successfully crystallised beta-amylase from sweet potatoes. Their method is commonly applied to prepare this enzyme from other sources. Crystalline beta-amylase is available commercially. Thachuk and Tripples (1966) reported preparation of highly purified enzyme from wheat, but they failed to crystallise it.

Amino acid analyses of beta-amylases from different sources show a great difference in their composition. The three dimensional structure or amino acid sequence of the enzyme has yet to be determined. Amino acid residues per 10^5 gm. of protein are shown in the Table 1.1. It is clear that the composition of soya bean enzyme differs from that of the enzymes from other sources. Composition of all the enzymes indicates that the soya bean enzyme probably contained some impurities. The number of amino acid residue are roughly comparable to the residues from alpha-amylases. The Table 1.1. shows that five amino acids differ greatly i.e. lysine, serine, leucine and methionine are high, whilst threonine is less than that of alpha-amylases. However, these deviations are not much different from those obtained by comparing residues of alpha-amylases amongst themselves. The only significant variation is in the methionine content,

but the significance of this difference is not yet known.

Beta-amylase does not need any prosthetic group, or any metal ion for its activity. The enzyme is stable at acidic pH and this factor is mainly exploited to purify beta-amylase from alpha-amylase. The optimum pH for this enzyme is between 5 - 6. Beta-amylases are less heat stable than alpha-amylases and can be easily inactivated.

TABLE 1.1.

<u>AMINO ACID RESIDUES OF BETA-AMYLASE</u>				
<u>CALCULATED FOR 100,000 gm. of protein</u>				
Amino Acids	a. Sweet Potato	b. Soya Bean	c. Malted Sorghum	d. Wheat
LYS	51	52	46	32
HIS	13	19	17	31
ARG	31	33	36	46
ASP	95	134	109	93
THR	23	37	43	26
SER	28	53	25	33
GLU	67	113	94	94
PRO	47	65	47	52
GLY	63	87	65	75
ALA	61	68	80	67
VAL	50	61	56	62
MET	27	15	17	19
ILEU	35	50	37	32
LEU	56	99	68	73
TYR	34	49	45	38
Ph.AL	34	36	34	37
TRY	20	20	11	24

Calculated from results of:-

- a. Thoma et al (1965)
- b. Gertler et al (1965)
- c. Botes et al (1967)
- d. Thachuk et al (1966)

Bernfield et al (1965) have reported that beta-amylase is not stable in dilute solutions. Walker and Whelan (1960) and England et al (1951), however, have reported that glutathione and serum albumin can be added as stabilizers. Banks and Greenwood (1969) have shown that under normal conditions of storage and use, no stabilizing agent is required for crystalline sweet potato beta-amylase.

Beta-amylase attacks α -1-4 - glucosidic bonds from non-reducing ends of long chain polysaccharides producing β -D-maltose. α -1-6-bonds are resistant to β -amylolytic attack. The action of this enzyme differs from the alpha-amylases because it causes the inversion of configuration of the newly formed reducing groups. It is believed that the reaction mechanism of beta-amylases is similar to that of alpha-amylases. Both enzymes attack C₁-O-C₄ bond in the α -D(1-4) linked D-glucans between C₁ carbon and oxygen of the bridge. FIG. 1.1. shows a schematic representation of the alpha and beta enzyme action.

Beta-amylase possesses the same type of active sites as alpha-amylases. Zharebtsov (1968), by studying the kinetic curves of pH versus activity, inactivation of enzyme by photo-oxidation and heats of inactivation, has postulated that an electrophilic - nucleophilic pair consisting of carboxyl and imidazol groups is responsible for enzyme activity. The role of sulphydryl groups in alpha-amylases is still ambiguous but for beta-amylases they appear to be necessary for enzyme activity. Rowe et al (1962) have reported that there are 12 sulphydryl groups per molecule

of the enzyme. Fukumato et al (1955), Fischer et al (1951), Gertler et al (1965) and England et al (1951) have provided evidence of the importance of the sulphhydryl groups in beta-amylase activity. Thoma et al (1965) have suggested that although sulphhydryl groups are important for beta-amylase activity, they are not essential, implying that sulphhydryl groups are not present directly at the active site. Modification of one of these groups changes the velocity of enzyme action without altering the fundamental catalytic mechanism.

The action pattern of beta-amylases has been a subject of controversy. Cleveland et al (1951), Hopkins et al (1954) and Cowie et al (1957^a) provided evidence for single chain action for beta-amylase. One disadvantage of the substrate used by the above authors was that native amylose possesses an exponential distribution. It is impossible to differentiate between a single chain and a multichain action because $\overline{DP}_n$ will remain constant for both types of degradation of an exponential distribution. Husemann and Pfannenmuller (1961) have used synthetic amylose as a substrate. This amylose has a Poissonian distribution. It was concluded that beta-amylase acts by a multichain attack pattern.

3. GLUCO-AMYLASE

Glucosyl-amylases degrade starch and glycogen completely into glucose. Terminology to-date for these enzymes is rather diverse. They have been called amyloglucosidases, gamma-amylases, glucamylase and taka-amylase b. This enzyme is found in many species of fungi, yeast and bacteria.

It has also been found in certain tissues.

The amino acid composition, the three dimensional structure and the reaction mechanism is not yet known. Pazur and Okada (1966) have, however, reported that purified enzyme from Aspergillus niger contains two components both having a molecular weight of 100,000. This enzyme is a glycoprotein and contains about 13% carbohydrate.

Gluco amylase hydrolyses both α -1-4 and α -1-6-bonds, without difficulty. The action of the enzyme seems to be dependent on the source from which it is isolated. Barker and Fleetwood (1957) and Phillip and Caldwell (1951) showed that amyloglucosidase has no action on isomaltose. This was also confirmed by Fukumato and Tsujisaka (1958) who showed that crystalline gluco amylase from Aspergillus niger and Rhizopus delemar completely degrade maltose, panose, amylose and β -limit dextrins but isomaltose was resistant to gluco amylase attack. Pazur and Ondo (1955) have, however, reported that purified gluco-amylase from Aspergillus niger can hydrolyse isomaltose and panose. Similar results were reported by Okazaki (1956) for the enzyme isolated from Aspergillus oryzae. It is possible that the later workers obtained the results from an enzyme sample which contained some contaminating enzyme. If both the results are to be believed then enzymes from different sources act differently on different substrates. The specificity of this enzyme is not yet known.

4. ISO-AMYLASES (Amylopectin-6-glucan hydrolases).

Isoamylases specifically attack α -1-6 glucosidic linkages. These enzymes are found in different sources and to-date nomenclature is variable. Isoamylase from yeast was called isoamylase because it attacked iso-maltose linkages in starch. The enzyme isolated from plants was called R-enzyme, and the bacterial enzyme was called Pullulanase because it degraded pullulan. Recently Kobayashi (1969) has applied the term isoamylases to all these enzymes. The presence of yeast isoamylase was first reported by Meyer and Bernfield (1940). It was characterised for the first time by Mauro and Kobayashi (1949) and later on confirmed by Gunja et al (1961).

Hobson et al (1951) had previously reported the presence of an enzyme in potato and broad beans with similar action. This enzyme was called R-enzyme. The enzyme from bacteria was reported by Bender and Wallenfells (1961). This is the only source from which enzyme was obtained in crystalline form.

The structure and properties of enzyme from yeast and plants have not yet been studied. Bacterial enzyme is reported to be heat stable (Abdullah et al, 1966). Frantz et al (1966) have shown that on gel chromatography the bacterial enzyme can be fractionated into two forms of molecular size 150,000 and 50,000. Furthermore the first form is slowly converted to second form on storage.

Isoamylases hydrolyse α -1-6-glucosidic bonds of amylopectin and glycogen, except plant isoamylase which does not attack glycogen. This shows the substrate specificity of enzymes

from different sources. The concurrent action of beta-amylase and isoamylase degrades a branched substrate completely to maltose. Similarly end product is glucose when used along with glucoamylase. French and Abdullah (1966) have reported that at high concentrations bacterial isoamylase shows reaction in the opposite direction e.g. tetrasaccharide and hexasaccharide could be formed from maltose and maltotriose respectively. Iso maltose and pullulan are resistant towards yeast and plant isoamylases while bacterial enzyme attacks pullulan very easily.

5. PHOSPHORYLASES

Phosphorylases are also widely distributed in nature. These are found in plants and are present in mammalian and amphibian skeletal muscles, heart and liver. Phosphorylase activity was first discovered by Cori et al (1939) in liver tissue. Soon afterwards Hanes (1940) observed similar activity in plant tissues. Animal phosphorylases occur in two forms, phosphorylase a and b. Phosphorylase a is the active form, while b shows very little activity. The inactive b form can be converted into the active a form by phosphorylation with adenosine monophosphate (AMP). According to Wang et al (1968) this process is stimulated by the presence of polyamines. Keller and Cori (1953) have reported that active form a can be converted into the inactive form b by removing the prosthetic group enzymically. The molecular weight of the a form is 242,000, while the b form is 135,000. ~~Krebs~~ and Fischer (1955) have reported that phosphorylase extracted from rabbit muscle is largely in the b form and can be readily converted

into the a form by the addition of divalent metal ion and adenosine triphosphate (ATP).

In this reaction phosphate is transferred from the nucleotide to phosphorylase and a dimerisation of the enzyme occurs so that the molecular weight is doubled.

Madsen and Cori (1957), on the basis that phosphorylase a has four binding sites for AMP while phosphophorylase b has only two, suggested that phosphorylase a is made up of 4 subunits while b consists of only two units. This is in agreement with the above equation. Both of these forms dissociate into monomer subunits when treated with excess of p-chloromercuribenzoate. The molecular weight of the monomer subunits was reported to be around 92500. (Serry et al 1967, De Vincenzi and Hedrick 1967, Ulman et al 1968).

Nolan et al (1964) provided evidence that the subunits may be chemically identical. They isolated a unique dipeptide from phosphorylase and elucidated the structure of P³² labelled phosphorine tetradecapeptide from phosphorylase a. However, there is a possibility of non identity of subunits as is suggested by heterogeneity of molecular weights of the subunits in 7.2M guanidine hydrochloride (Serry et al, 1967).

Madsen et al (1967) have suggested from half cystine sequence in phosphorylase that the subunits are identical but the possibility remains that the differences are not in the vicinity of half cystine. Valentine et al (1968)

have suggested from the rhombic shape of the phosphorylase tetramer that the subunits are of different types.

Phosphorylases contain pyridoxal phosphate (PLP) as a prosthetic group. (Baranowski et al 1957, Fischer et al 1958). PLP is a very common factor in a variety of amino acid metabolising enzymes, and is directly involved in catalysis by these enzymes. A significantly different situation is found in phosphorylase, where PLP is not directly involved in catalytic process. Cori and Cori (1945) found that rabbit muscle contains one mole of PLP per mole of enzyme monomer. This was later confirmed by Fischer et al (1958). Lee (1960) has, however, reported that potato phosphorylase contains two moles of PLP per mole of enzyme. The molecular weight of the plant phosphorylase is greater than that of muscle phosphorylase so the amount of pyridoxal is comparable.

TABLE 1.2.

AMINO ACID RESIDUES IN PHOSPHORYLASES

From different sources

CALCULATED FOR 92,500 gms. OF PROTEIN

Amino Acids	a. Lobster	b. Rat	c. Rabbit	c. Human	d. Frog	e. Potato
LYS	49	43	43	47	42	55
HIS	23	19	19	23	20	19
ARG	51	60	59	63	53	35
ASP	92	95	91	94	90	73
THR	40	31	32	32	34	42
SER	37	28	27	24	39	38
GLU	91	91	94	88	84	90
PRO	31	36	39	33	43	29
GLY	48	45	46	46	44	45
AL	57	59	60	62	55	56
VAL	55	53	57	56	57	44
MET	20	22	20	21	20	11
ILEU	61	48	45	46	43	44
LEU	72	74	75	75	70	54
TYR	34	34	34	33	34	26
Ph.AL	31	35	36	39	35	32
TRY	12	13	12	11	12	14

The above values have been calculated

- a. from results of Assaf et al (1969)
- b. from results of Sevilla and Fischer (1969)
- c. from results of Appleman et al (1963)
- d. from results of Metzger et al (1968)
- e. from results of Kamagowa et al (1968)

The presence of AMP is necessary for the activity of muscle phosphorylase b (Cori and Cori 1945) and it activates phosphorylase a (Yunis et al 1962). The role of AMP is not known; it has no effect on potato phosphorylase. In fact AMP can protect phosphorylases from heat denaturation and in some cases may even reverse the action (Grillo, 1959). A similar protective effect is shown by EDTA and K (Akalsuka and Nelson, 1969).

A SCHEMATIC REPRESENTATION OF SITES ON PHOSPHORYLASE
(a) AFTER FUKUI AND KAMAGOWA (1969)

The exact function of PLP is still unknown. Illingworth and Cori (1957) showed that removal of this group causes a total loss of activity which is reversible by incubation with PLP. Kent et al (1958) proposed that, the structure for the linking of PLP in muscle phosphorylase in neutral solution is a substituted aldamine derivative. By treating with acid, base or urea, it changes to Schiff's base form and eventually splits off the enzyme. It can be represented as follows:-

PLP does not take part in the catalytic activity of the enzyme, but it is possible that it is necessary for the active conformation. Appleman et al (1966) showed that although the AMP binding site and the sites involved in the conversion are not noticeably effected by resolution, removal of PLP resulted in a less organised structure as shown by a total loss of activity, a considerable decrease in stability and increased vulnerability to changes in quaternary structure. Kamagowa (1969) has shown that binding of PLP in the potato enzyme is not quite the same as that of the muscle enzyme. This confirms the findings of Lee (1960) that PLP is very tightly bound to potato phosphorylase and requires more vigorous conditions for its removal. According to Kamagowa et al (1968) bound PLP in potato phosphorylase does not respond to colour tests until it is fully reduced. This indicates

that three hydroxyl groups of PLP in the native enzyme should be linked to enzyme in some manner including hydrogen bounding.

Amino acid compositions of enzymes from different sources are shown in Table 1.2.. Although the complete amino acid sequence, or three dimensional structure is not known, the sequence of an important residue peptide has been reported by different workers, for rabbit muscle, human muscle and rat muscle phosphorylase a:

i. Rabbit muscle (Nolan et al 1964)

Ser-Asp-Glu(NH₂)-Glu-Lys-~~Arg~~-Lys-Glu(NH₂)-Ileu-Ser-(P)-Val-Arg-Gly-Leu.

ii. Human muscle (Hughes et al 1962)

Lys-Glu(NH₂)-Ileu-Ser-(P)-Val-Arg.

iii. Rat muscle (Sevilla and Fischer 1969)

Ser-Asp-Glu-Asp-Lys-Arg-Lys-Glu-Ileu-Ser-(P)-Val-Arg-Gly-Leu.

As shown in the Table 1.2. there is a little difference between mammalian and amphibian enzymes, but the potato enzyme is widely different in its composition and so is the enzyme from lobster.

Potato phosphorylase is stable at 30°C. The optimum pH is between 6-6.9 and it is rapidly deactivated below pH 4.9. Muscle phosphorylase requires AMP for its activity whilst potato enzyme is active in its absence. A schematic representation of the sites of PLP and AMP on the potato and muscle enzyme b is given in FIG. 1.2., based on the studies of Appleman et al (1966) and Kamagowa et al (1968).

~~Hayashi~~ ^{OKAZAKI} et al (1968) have, however, studied the involvement of AMP in detail. By using 38 structural analogues of AMP they determined the functional groups of AMP. The results indicated that the amino group at position 6 and the nitrogen atom at position one were effective in the binding. The hydroxyl group at position 2 of the ribose moiety contributed both towards binding and activation. A monophosphate group is absolutely necessary for the activation and should be located at position 5 of the ribose moiety.

No conclusive evidence has yet been given for the active sites of phosphorylase. Although one particular serine residue is phosphorylated during the activation of muscle and liver phosphorylases, yet its direct participation in catalysis is doubtful because Lee (1960) has reported that there are no serine phosphate groups in potato phosphorylase. Fischer et al (1958) have already shown that during catalysis there is no exchange between free inorganic phosphate and phosphate bound to rabbit muscle enzyme.

Holló et al (1967) have shown by the effect of variation of pH and photo-oxidation, acetylation experiments that α -amino and imidazolium groups are necessary for the activity.

Haung and Madsen (1966) by using cyanate as a modifier demonstrated the importance of lysine residues in muscle phosphorylase activity. Philips and Graves (1968) confirmed the involvement of lysine residues by inactivating the enzyme by dinitrophenylation of muscle phosphorylase b with 2,4 dinitrofluorobenzene. In this inactivation, lysine and cystine residues were modified. These findings were further

confirmed by Kamagowa et al (1968). They used glyoxal as an inhibitor. The subsequent amino acid analyses showed modifications in the lysine and arginine residues.

The role of sulphydryl groups is not clear in the catalytic activity. Modifications of the sulphydryl groups with p-mercuribenzoate inactivates the enzyme. Madsen and Cori (1957) found that the enzyme dissociates into subunits and the inactivation is reversible with the addition of cystine. Inhibition by p-mercuribenzoate was further demonstrated by Damjanovich et al (1966), Lee (1960) and Gold (1968).

Phosphorylase plays an important role in metabolism of glycogen and starch. Its action can be expressed as:-

where n is the degree of polymerisation

The forward reaction occurs when D-glucosyl phosphate is in excess, whilst the reaction in opposite direction takes place when inorganic phosphate is present in excess. When phosphorylase was first discovered in liver tissue, it was assumed that this enzyme is responsible for glycogen synthesis and degradation. This assumption was further extended to starch when the enzyme was found in plants as well. However, there are very good reasons to believe that in animal tissues this enzyme functions only in glycogen breakdown rather than its synthesis. Mommaerts et al (1959) showed that phosphorylase is absolutely essential in glycogen breakdown. These conclusions were based on studies of abnormal conditions in human liver and muscles where there was a deficiency of phosphorylase.

In plants, biochemists believe, that starch synthesis is

carried out by transglucosylases rather than phosphorylases. Since transglucosylases need a definite primer to start synthesis, it is argued that phosphorylase is important in primer synthesis. This argument is based on the findings of Illingworth et al (1961) that amylose synthesis with phosphorylase can proceed without any primer. Watkins et al (1965) and Abdullah et al (1965) however, differ from these results. Kamagowa et al (1968) have also shown that no de novo synthesis of amylose can occur in absence of added primer. So it can be said that phosphorylase does not play any part in the synthesis of primer for starch synthesis. It is also important because phosphorylase needs itself at least maltotriose to start synthesis. Smith and Whelan (1963) have suggested that five unmodified α -1-4 linked D-glucose units are necessary for efficient synthesis. It is possible that like animal enzyme, plant phosphorylases mainly function in the degradation of starch. The synthetic action pattern of potato-phosphorylase is discussed in Section 5.

6. Amylo-1-6-glucosidase.

This enzyme is found in animal skeletal muscles and degrades α -1-6 glucosidic linkages. The interesting feature about this enzyme is that it attacks only alpha, beta and phosphorylase limit dextrins of glycogen and amylopectin. It has no effect on undegraded substrates. The action of this enzyme needs to be studied in more detail to clarify the above behaviour. Nothing is yet known about its structure and properties.

7. Starch Synthetase

Synthesis of starch in plants is a complicated process which is not yet fully understood. It was first believed that starch is synthesised by phosphorylases, but Leloir and Cardini (1957) reported glucan synthesis by a nucleotide pathway by a glucosyl transferrase-enzyme in animals.

Leloir et al (1961) reported similar enzyme in beans. It has now been reported in potatoes, maize (Frydman et al 1963) sweet corn (Cardini et al 1964) and rice (Murata et al 1964). There are two types of starch synthetase in plant tissues. One is bound to starch granules and the other is soluble. Due to very small quantities present in plants starch synthetase has not yet been obtained in purified form to evaluate its properties. The enzyme is supposed to react in the following manner.

The reaction results in transferring an α -D-glucopyranosyl group from uridine 5 - (α -D-glucopyranosyl ^{Phos} pyrophosphate) to the O-4 of the D-glucosyl residue of the acceptor which may be starch or any maltodextrin. The reaction takes place with the retention of configuration of the resulting products.

UDPG is synthesised in plants by pyrophosphorylase. Starch synthetase needs a primer to start the reaction and this primer is supposed to be provided by ADPG - which acts as a donor.

Since the discovery of this enzyme it is believed that starch is synthesised by glucosyltransferrases rather than phosphorylases. Starch synthesis is probably a result of more than one kind of enzyme reaction and needs more evidence to reach unambiguous conclusions.

SECTION 2.

GENERAL EXPERIMENTAL TECHNIQUES

1. PREPARATION OF SUBSTRATES(a) Isolation of Starch.

Starch was extracted from potatoes (Pentland Crown) by the method of Banks et al (1959).

(b) Fractionation of starch.

Starch slurry was added to boiling water (final conc. 0.5%) under a nitrogen atmosphere. Heating was continued along with stirring for one hour. The solution was then allowed to cool to 60°C. and powdered thymol (1 gm./litre) added. The solution was stirred for one hour and allowed to stand overnight. Amylose-thymol complex precipitates out and was removed in a Sharples super centrifuge. The complex was recrystallised thrice from hot aqueous butan-1-ol solution. The supernatant from Sharples centrifuge was shaken with ether in a separating funnel to remove thymol. The remaining ether from separated amylopectin was removed by bubbling nitrogen through it. The solution was then freeze dried to obtain solid amylopectin.

(c) Characterisation of Amylose.

Amylose was characterized by measuring the limiting viscosity number $[\eta]$ in 0.15 M potassium hydroxide solution. The weight average molecular weight was calculated from the equation

$$[\eta] = KM^a$$

Where $K = 8.36 \times 10^{-3}$ and $a = 0.77$

The purity of amylose fractions was determined by incubating the amylose with beta amylase and Z-enzyme (Banks and Greenwood 1967). The concentration of Z-enzyme was so low that it did

not cause any noticeable increase in the beta-limit of amylopectin during the same period of incubation (24 hours). A 100% beta-limit showed that amylose was linear and there was no branched material present.

(d) Preparation of Amylopectin beta-limit dextrin.

Amylopectin was incubated with beta-amylase for 48 hours. It was found that after this time the reducing power of the digest did not increase. The enzyme was destroyed by heating the digest in a boiling water-bath for ten minutes, and the solution concentrated on a rotary evaporator, to give the final concentration of 5 - 6 mg./ml. The solution was stored under toluene.

2. DETERMINATION OF ALPHA-AMYLASE ACTIVITY.

Alpha-amylase activity was measured by the method described by Briggs (1961) as modified by Greenwood et al (1965). The unit of activity was described as the amount of enzyme decreasing the iodine stain of a standard amylopectin beta-limit dextrin digest by one third in 100 minutes. Thus the amount of enzyme which decreased the iodine stain of the digest by one third in one minute must be equal to 100 units. Since it was not practicable to remove a sample when the iodine stain of the digest has exactly decreased by one third, a standard graph was prepared from the readings of the digest in which the absorption of iodine polysaccharide decreases from 3.0 - 2.0 in 100 minutes.

Method:- The digests were prepared as follows.

Amylopectin beta-limit dextrin (1-2 ml, 6 mg./ml.)
acetate buffer (1 ml, 0.2M pH 5.5), enzyme solution.

The total volume was made up to 8 ml. and the digest was incubated at 37°C. 2 ml. samples were taken out at different intervals and added to iodine solution (1 ml, 0.2M iodine in 2% KI) and 5N HCl (0.2 ml.) in a 50 ml. graduated flask. The volume was made up to the mark and the absorption of the solution measured at 540 m μ on an Eel Colorimeter using a yellow green filter, no. 625.

The absorption obtained was multiplied by 3/absorption at zero time to give absorption value. From the standard graph the relative time was obtained and divided by actual time. This gives the activity of the enzyme in iodine dextrin colour units. Specific activity of the samples was calculated by dividing the activity with concentration of the enzyme.

3. DETERMINATION OF PROTEIN CONCENTRATION.

Protein concentration was measured by obtaining the optical density of the solutions at 280 m μ in an SP 800 spectrophotometer. The optical density of a 0.1% solution in a 1 cm cell was taken as one.

4. MEASUREMENT OF PHOSPHORYLASE ACTIVITY.

Phosphorylase activity was measured by the method described by Lee (1960). A standard assay mixture containing 0.76% amylopectin, 0.01 MG -1-P and 0.1 M citrate buffer pH 6.3 was prepared. One ml of this standard assay mixture was incubated with suitable amount of enzyme. After five minutes the reaction was stopped by the addition of trichloroacetic acid (5%, 0.5 ml) and the pH of the digest was adjusted to about 4 by the addition of 2 ml of 0.1 M sodium acetate solution. The amount of free inorganic phosphate was estimated

as described below.

This method measures the synthetic activity of the phosphorylase. The enzyme transfers the glucose unit from G-1-P to the amylopectin and the free phosphate is liberated. Under the above conditions, the reaction is first order with respect to G-1-P, so the units are described as $K \times 1000$, where K is the first order velocity constant ($\log_{10} \text{ minutes}^{-1}$), specific activity is expressed as units per mg of protein.

5. MEASUREMENT OF FREE INORGANIC PHOSPHATE.

Free inorganic phosphate was measured by the method of Allen (1940). This method was chosen because of its simplicity and quickness.

A simple method was required to estimate free inorganic phosphate in presence of G-1-P. Most of the methods described in the literature have conditions which degrade G-1-P and the estimation of inorganic phosphate becomes erroneous. Bailey and Whelan (1961) have recommended a modified Allen's method, but it was found that estimation of phosphate was still erroneous. Table 2.1 shows the variations in the amount of reagents tried to find out the correct conditions under which G-1-P is not degraded and the experimental values coincide with the theoretical values.

28A

FIG. 2.1

TABLE 2.1

Variations in amounts of reagents
for estimation of inorganic Phosphate by Allen's method.

No.	REAGENTS (ml)			INORGANIC PHOSPHATE µgm	
	Perchloric Acid	Amidol Reagent	AMM Molybdate	EXP	Theoretical
1	2	2	1	96	50
2	2	1	1	80	50
3	1	2	1	61	50
4	1	1	1	49	50

Method:- Aliquots of solution containing not more than 200 µgm of inorganic phosphate were taken in 25 ml measuring flasks and diluted to about 15 ml. One ml of perchloric acid was added and mixed gently, then one ml of amidol reagent and one ml of ammonium molybdate were added and volume made up to the mark. After standing for 15 minutes, the absorption was measured on a Helger Spectrophotometer using a red filter, with reference to a blank prepared in a similar way. The amount of phosphate was obtained from a standard curve. The standard curve was prepared every time the reagents were changed. A typical curve is shown in the FIG. (2.1)

Reagents: Perchloric acid (60%), Amidol reagent (Amidol 2 gm, sodium bicarbonate 40 gm in 200 ml water and stored at 4°C.), Ammonium molybdate (8.3%).

6. ESTIMATION OF GLUCOSE. (Glucose oxidase method)

Kesten (1956) developed a method for the estimation of glucose by using two enzymes, glucose oxidase and

peroxidase simultaneously. Glucose oxidase catalyses the oxidation of glucose to gluconic acid and hydrogen peroxide. This hydrogen peroxide which is formed in presence of peroxidase and a chromogenic hydrogen donor such as dianisidine forms a coloured substance which is stoichiometrically related to amount of original glucose.

Dahlquist (1961) however, found that commercial preparations of glucose oxidase contain maltase and iso-maltase as impurities which limits the method to pure glucose only. He found that if 0.5M tris buffer is used in the reagent, it selectively inhibits these glycosidases. No interference was observed with the production of colour with free glucose by glucose oxidase reagent containing O-dianisidine as a chromogen. This development was very important because under the conditions described the method can be applied for estimating glucose in presence of maltose and other oligo saccharides.

Although the method was highly successful and accurate, the fact that the reagent used was not stable, made it laborious and time consuming. After standing the reagent develops a colour which makes the estimation of glucose inaccurate. Washko and Rice (1961) found that 40% glycerol makes the reagent stable. Fleming and Pegler (1963) used varying amounts of O-dianisidine hydrochloride to obtain a specific concentration which was most sensitive under the conditions described by Washko and Rice (1961). Catley (1967) further developed this method by using varying quantities of ingredients for reagent to enhance the sensitivity

of the method. In this work the method was slightly modified to improve sensitivity.

Experimental:- Tris glycerol buffer was prepared as described by Banks and Greenwood (1968). 2-amino - 2 hydroxy methyl propane-1-3-diol (15.25 gm) was dissolved in 5N hydrochloric acid (21.5 ml) and the pH adjusted to 7.0 if necessary. In 100 ml of this buffer were dissolved glucose oxidase (40 mg), peroxidase (3 mg) and o-dianisidine hydrochloride (15 mg). The enzyme chromogen mixture was stored at 4°C in a brown bottle.

For the assay, 1 ml of the sample was thoroughly mixed with 1.5 ml of enzyme chromogen solution and incubated for two hours at 35°C. The digests were cooled in ice and acidified with 12 M sulphuric acid (1 ml) and absorbance (A) was measured in a spectrophotometer (Unicam Sp 600) at a wavelength of 5400 Å. The calibration relation for D-glucose in pure water was $D\text{-glucose } (\mu\text{gm}) = 38.4 \times A$ whilst in the presence of 40% glycerol it was $D\text{-glucose } (\mu\text{gm}) = 43.6 \times A$.

7. POTENTIOMETRIC IODINE TITRATION:

Potentiometric iodine titration is the best method for determining the purity of amylose and amylopectin samples. The method was first introduced by Bates et al (1943) who measured the potential difference between a colomel electrode and a bright platinum electrode in the starch solution. From the potential difference the amount of free iodine in the starch solution can be measured. The above method was modified by Gilbert and Marriott (1948) by using a

differential technique to avoid the necessity of reagent blank titrations. This method was later adopted by Anderson and Greenwood (1955) and has been used in the following work with a modification namely a digital volt meter was used in place of an electrometer.

Experimental:- The electrolyte solution contained M/10 potassium iodide (210 ml), M/15 phosphate buffer (15 ml. pH 5.85) and diluted to two litres with distilled water. Each half cell contained 830 ml of this electrolyte. The sample solution (10 ml) and blank were added to their respective cells and the cells immersed in a constant temperature water bath. Small amounts of iodine-iodide solution were added to the half cell containing the polysaccharide by means of an "Agla" micrometer syringe. After equilibrium was reached the potential was balanced. This was achieved by adding iodine to the blank cell until null potential was obtained. The difference between the volumes of iodine added to the two cells gave the amount of iodine bound by the sample. The iodine bound (mg/100 mg of polysaccharide) was plotted as a function of total free iodine in the solution and the iodine affinity obtained by extrapolation of the experimental results to zero free iodine concentration.

8. Determination of $\overline{DP}_n$ of linear polysaccharides.

$\overline{DP}_n$ was determined by the method of Banks and Greenwood (1968). This method is based on the principle that crystalline Beta-amylase will quantitatively degrade any linear α -D-(1-4) glucan to maltose and maltotriose. The maltotriose is being produced from the molecules having an odd number of D-glucose residues. However, if high concentrations of Beta-amylase are used the maltotriose will also be degraded into maltose and glucose. Hence if the polysaccharide sample is considered to contain equal number of chains having an odd and even number of D-glucose residues, the statistical probability of obtaining D-glucose on complete beta-amylolysis is 0.5. Therefore, $\overline{DP}_n$ can be calculated from the amount of glucose produced.

Method.

For determination of D-glucose produced digests were set up as follows.

Polysaccharide solution (1.5 ml 8 mg/ml), glycerol (1.6 ml) 0.1M acetate buffer (PH 4.8, 0.5 ml) and Beta-amylase 4000 units (0.4 ml). After incubation for 48 hours at 37°C the glucose produced (G_B) was estimated by glucose oxidase method as described earlier.

Production of D-glucose (G_t) by total hydrolysis was carried out by incubating the sample with amyloglucosidase at 37°C for 2 hours, glucose produced was estimated by glucose oxidase method.

The number average degree of polymerisation calculated by $\overline{DP}_n = G_t/2G_B$, where G_t is the concentration of D-glucose obtained on complete hydrolysis of the sample and G_B is the D-glucose produced on Beta-amylolysis.

9. ULTRACENTRIFUGATION:

(a) Experimental. Ultracentrifugation provides a simple method for determination of molecular weight and molecular weight distribution in polymers. Molecular weights can be obtained by (i) Sedimentation equilibrium in which the tendency of the molecules to diffuse is balanced by the centrifugal force applied and (ii) Sedimentation velocity measurement in which the molecular weight can be calculated by the movement of the solute.

The apparatus used in this work was a Beckman Spinco ultracentrifuge model E. The samples were contained in a cell in which a sector shaped counter piece was clamped between two quartz discs. The pieces were all firmly enclosed in a cylindrical housing. The cell was assembled and the solution was injected into it with the help of a syringe. The cell was then fitted into the hole of the rotor together with the cell counter-poise in the other hole. These holes have two lines which act as reference marks. The rotor containing both cells was connected to the drive, the vacuum chamber closed and vacuum pump started. All the runs were carried out under high vacuum for minimum thermal disturbance. A constant temperature (as required) was maintained by means of rotor temperature indicator and control unit.

(b) Calculation of Sedimentation co-efficient

Photographs of the sedimenting boundary were taken at different intervals and the distance from the reference line to the mean height of the peak was measured by means of a two-way travelling microscope. This was corrected

for lens magnification M , and then converted to distance from centre of rotation (X). $\log X$ was plotted against time and sedimentation co-efficient (S) calculated from Svedberg equation.

$$S = \frac{2303}{W_2} \times \frac{d \log X}{dt.} \quad (1)$$

The angular velocity W being measured during the run. Individual sedimentation co-efficients can be calculated from successive pictures using the equation.

$$S = \frac{2(X_{t_2} - X_{t_1})}{(X_{t_2} + X_{t_1}) W^2(t_2 - t_1)} \quad (2)$$

where X_{t_1} and X_{t_2} are the distances of maximum ordinate of the sedimentation boundary from the centre of rotation at times t_1 and t_2 respectively. If the photographs are taken at equal time intervals following formula can be used.

$$S_{AV} = 1/10 (2S_1 + 3S_2 + 3S_3 + 2S_4) \quad (3)$$

Kegeles and Gutter (1953) have reported that S_{AV} calculated by this method is equal to the value obtained by determining the slope of $\log X$ versus t plot.

(c) Dependence of Sedimentation co-efficient on concentration

The concentration has a great effect on the sedimentation behaviour of the macro-molecules. For most polymers the sedimentation co-efficient is dependent on the concentration. The degree of dependence is related to the physical properties of the macromolecules. Large molecules with long straight chains like amylose and branched open molecules like amylopectin

exhibit highly concentration dependent sedimentation, whilst rigid and compact molecules like glycogen show very little concentration dependence. To obtain a relative sedimentation co-efficient for different molecules, the values are measured at different concentrations and extrapolated to zero concentration. This eliminates the effect of concentration on sedimentation co-efficients. According to Gralen (1944) extrapolation can be achieved by

$$S = \frac{S_0}{1+Kc} \quad (4)$$

where S is the sedimentation at concentration C and S₀ is sedimentation co-efficient at infinite dilution and K is a constant. Graphs are usually plotted as 1/S versus concentration.

10. VISCOSITY

Polymers have the ability to increase the viscosity of the medium in which they are dissolved even at very low concentrations. The viscosity of a polymer solution depends on the concentration, molecular size and shape of the molecules. Due to simplicity of the technique, viscosity measurements have been widely used in investigations of solution properties of polymers. Viscosity measured in a capillary viscometer are calculated from

$$\eta = Kdt - \frac{Bd}{t}$$

Where K and B are constants, t is the flow time of a fixed volume and d is the density of the solution. B is a kinetic energy factor and has been shown to be negligible for viscometer used, hence

$$\begin{aligned} \eta &= Kdt && \text{for solution} \\ \text{and } \eta_0 &= Kd_0t_0 && \text{for solvent.} \end{aligned}$$

The relative viscosity will be

$$\eta_r = \frac{Kdt}{Kd_0t_0}$$

and specific viscosity

$$\eta_{sp} = \frac{dt - d_0t_0}{d_0t_0}$$

for very dilute solutions $d \approx d_0$

$$\text{therefore } \eta_{sp} = \frac{t - t_0}{t_0}$$

Specific viscosity (η_{sp}) is dependent on concentration. The ratio η_{sp}/C is called the viscosity number. It is necessary to obtain the value of viscosity number at infinite dilution to eliminate concentration effect. This value is known as ~~inter~~intrinsic viscosity and is written as $[\eta]$

Viscosity and molecular properties

The limiting viscosity number $[\eta]$ is related to the molecular weight of the polymer by

$$[\eta] = K^1 M^a$$

where K^1 and a are the constants for different polymer systems.

This equation is a slightly modified form of that proposed by Staudenger (1930). The values of K^1 and a are obtained by separating the polymer into fractions of narrow molecular weight distribution, and molecular weight of the fractions determined by another technique usually by light scattering which also gives weight average molecular weight. The constants are evaluated from the double logarithmic graph of the ~~inter~~intrinsic viscosity and molecular weight.

Most of the theories put forward for the solution properties of the polymer solution show that the intrinsic viscosity can be interpreted on the basis of randomly coiled chain of the molecule.

$$[\eta] = KM^{\frac{1}{2}} \alpha^3$$

$$\text{where } K = \phi (r_0^2/M)^{3/2}$$

$(r_0^2)^{\frac{1}{2}}$ is the linear dimension of the randomly coiling chain and ϕ is a constant. K in the above equation is independent of solvent and molecular weight but α is dependent on molecular weight.

However, it is obvious that intrinsic viscosity can be related to the molecular weight of linear molecules only. For branched molecules $[\eta]$ may be constant over a wider range and the above equation will not be applicable.

Experimental. The viscometer used in this work was a modified Ubbelohde type. Solvent and solutions were both filtered through C.G 4 Sintered glass filter. A definite amount of solvent (>12 ml) was placed in the viscometer, and the viscometer allowed to stand in a constant temperature water bath at 25°C , until the solution attained the temperature equilibrium. The flow time was measured and aliquotes of equal volume of polysaccharide solution were added separately and the flow times measured for each concentration. Another technique was applied, whenever necessary, according to the amount of polysaccharide available. In this technique, polysaccharide solution is put in the viscometer, aliquotes of solvent added and flow times measured for all additions.

Care must be taken while adding solvent or solution aliquotes, that they are directly pipetted in the bulb of the viscometer without any contact with the sides of the tube. Concentration of the polysaccharide solutions was determined after acid hydrolysis by estimating glucose by alkaline ferricyanide - ceric sulphate method as described below.

11. DETERMINATION OF REDUCING VALUE AND POLYSACCHARIDE CONCENTRATION

Polysaccharide concentration and the reducing values were determined by the alkaline ferricyanide-ceric sulphate method of Lampitt et al (1955). This method is accurate for estimating maltose in presence of iodine staining polysaccharide, and in this method there is no need for repeated calibration because the calibration factor is directly proportional to the normality of ceric sulphate.

Method. Three 1 ml samples containing polysaccharide (< 4 mg) were placed in boiling tubes, neutralised if necessary and 1 ml of 3N sulphuric acid was added. The tubes were stoppered and placed in a boiling water bath for two hours, removed and allowed to cool. The contents were neutralised with 1M potassium hydroxide, using bromocresol green as indicator and potassium ferricyanide (2.5 ml) added and diluted to 10 ml with distilled water (potassium ferricyanide 0.05 M + sodium carbonate 0.2 M). The tubes were placed back in the boiling water bath for fifteen minutes, taken out and cooled immediately. H_2SO_4 (5 ml. 5N)

and 2 drops of xylene cyanol F.F indicator was added before titrating against 0.01N ceric sulphate. The colour changes from sage green to whisky yellow at the end point.

Maltose was estimated directly without going through acid hydrolysis stage.

12. CHROMATOGRAPHY.

(a) Paper Chromatography: The one dimensional multiple ascent technique was employed in the following work. Qualitative and quantitative separations were carried out on Whatman No. 3 chromatographic paper using n-propanol:water (70 : 30 V/V) as solvent (French et al 1965). Samples were spotted by the method of Trevelyan et al (1950) and identified by comparison with the distance travelled by standard oligosaccharides.

Preparation of standard solution of sugars.

Amylose solution (5 ml, 3 mg /ml) was incubated with a dilute solution of B. Subtillus alpha amylase. At the achroic stage the enzyme activity was destroyed by heating in boiling water. The solution was filtered and 1 mg of glucose added as only a very small amount of glucose is produced by the action of B. Subtillus alpha amylase on amylose. The solution was stored at 4°C.

Elution of sugars from paper chromatograms.

The floor of a large tray was covered with a moistened filter paper. A petridish containing the eluent is placed on the top of a wooden block or long inverted beaker, in the middle of the tray. Appropriate sections of the sugars were cut and approximately 3 - 4 mm of the end of the paper

was placed between the ends of a pair of microscopic slides. For speedy elution additional filter paper strip was placed between the slides so that one end of the strip touched the chromatogram and the other end was dipping in the eluent. The slides were placed in the petri dish, so that the end of the chromatogram was hanging in an empty beaker. The petri dish and beakers were covered with a large beaker to maintain humid atmosphere above the petri dish. Control experiments showed that all the sugars were eluted quantitatively within 1 - 2 hours.

(b) Column Chromatography.

In the following work "Amberlite C.G. 120" and "Amberlite technicon A" were used for amino acid analyses which are described under a separate heading. For protein fractionation DEAE 52 cellulose and for molecular size determination bio-gel P-60 was used. DEAE 52 cellulose was washed thoroughly with NaOH (0.5N) and then with distilled water to remove the NaOH. It was then washed with 0.5N hydrochloric acid and again with distilled water and finally with eluent buffer until the washings were of the pH of the eluent buffer. The washed resin was equilibrated with eluent buffer for 24 hours, deaerated and packed in the column. The samples were loaded on the top of the column and eluted with buffer. The eluent was constantly monitored at 254 mμ using an L.K.B. "Uvicord" flow photometer and fraction collector, with a photo cell drop counter. The "Uvicord" flow photometer was connected to a servoscribe potentiometric recorder with a logarithmic amplifier to

boost the signal recording of the recorder. The absorption of the eluent and each change of the tube on the fraction collector were recorded. The peaks on the trace can be equated with corresponding tubes and hence with the elution volume of the protein concerned.

13. AMINO ACID ANALYSES.

The techniques of amino acid analyses has varied from straightforward colorimetric analyses, which is limited to a range of only few amino acids, to paper chromatography which although wider in scope lacks resolution and reproducibility and requires extreme delicacy on the part of the operator. Gas phase chromatography has the disadvantage of multiplicity of columns and inapplicability to samples containing proteins or polypeptides. Biological methods are tedious and unwieldy. Column chromatography using columns of 8% cross linked polystyrene sulphonic acid provides accurate fractionation by cationic exchange. The affinity of each amino acid for the resin in the presence of a buffer of known pH and ionic strength results in a characteristic delay in its migration down the column, achieving a degree of separation dependent solely on the length of the column. The necessity of the collection of large number of fractions for colorimetric analysis is avoided by automatically mixing the elute from the column with a ninhydrin solution. The colour is developed by passing through a heated coil and measured by photo electric colorimeters which record the results on a moving chart.

The apparatus used in the present work was the "Technicon Amino Acid Analyser". The method used was developed by

Speckman et al (1958) and later on modified by Patterson and Benson (1965). Amberlite C.G. 120 (type III) fractionated by the method of Hamilton (1958) was normally used in the columns. Long columns (60 x 0.636 cm) were used for acid and neutral amino acids, whilst short columns (16 x 0.45 cm) were used for fractionation of basic amino acids.

Protein samples were hydrolysed with acid, purified and dissolved in buffer solution. Small amounts of these solutions were put on the top of the columns, eluted with buffer and chromatograms obtained from the automatic amino acid analyser. The amount of amino acids was calculated from the peak areas for respective amino acids.

SECTION 3.

ACTION PATTERN OF ALPHA - AMYLASES

INTRODUCTION

Alpha-amylases from different sources differ in their action towards different substrates. They act with different affinities towards chain ends. There is also a difference in affinity towards long chain and short chain molecules. Plant and bacterial alpha-amylases are somewhat similar in their action pattern but mammalian alpha-amylases differ widely from these two groups.

Han²s and Cattle (1938) proposed that malt alpha-amylase has a very much lower affinity for small dextrans than the mammalian enzyme. Similar conclusions were reached by Meyer and Gonon (1951) and they suggested that all alpha-amylases are unable to attack the linkages at the end of substrate molecule and the rest of the bonds are broken randomly. Bernfield (1951) found that the kinetics of starch hydrolysis varied according to the source of the amylase used. He concluded that these variations are due to different affinities of these enzymes towards different substrates. Svanborg and Meyerback (1953) showed that salivary alpha-amylase attacks starch and maltohexaose almost at the same rate, while the malted barley enzyme hydrolysed the polysaccharide at a rate six times faster than for the dextran.

Kung et al (1953) studied the action of alpha-amylases from a different angle. These authors published a series of curves for the decrease in iodine staining power related to the reducing power of the digest. The curves, i.e. the action patterns, were similar but not identical. They

emphasised that the action of alpha-amylases from different sources follow their own pattern during the hydrolysis of a given substrate. It was also shown that bacterial alpha-amylases break down large molecules more rapidly than do mammalian alpha-amylases.

From their studies on the action of malt and Bacillus subtillus alpha-amylases on amylose, Bird and Hopkins (1954) concluded that appreciable quantities of fission products of chain length 2 and upwards are produced during the early stages of hydrolysis. They also concluded that malt and bacterial alpha-amylases do not hydrolyse the first five bonds from the non reducing end, whilst salivary amylase found only the first two bonds to be resistant. At the reducing end salivary and malt alpha-amylases hydrolyse the second linkage whilst the bacterial enzyme attacks the third linkage. The rest of the linkage were equally vulnerable to hydrolysis by enzymes from any source.

Whelan,^{Bailey} and Robert (1953) presented analytical values for ratios of maltose and maltotriose produced from amylose and malto-dextrins by salivary alpha-amylase. These ratios were found to be exactly in agreement with the values calculated on the basis of the Meyer and Bernfield theory (1948) which states that in an amylose type chain alpha-amylase can hydrolyse any linkage except the two terminal ones, and all the susceptible bonds are hydrolysed at equal rate.

Using radioactive malto-oligosaccharides, Pazur and Budovich (1956) showed that salivary alpha-amylase effects

a preferential hydrolysis of linkages. Maltose and maltotriose were the major products from maltopentaose which indicates that attack is either on linkage number two or three. Maltose and maltotetraose were the major products from maltohexaose along with maltotriose. The preferential attack appears to be on bond number two because of the production of radio active maltose. This confirms the theory of preferential attack by alpha-amylases.

Similar observations about pancreatic and bacterial alpha-amylases were reported by Ulman (1958). He studied the products of potato starch by the above enzymes.

Alpha-amylase from Aspergillus oryzae produced maltotriose and maltose as main products; small amounts of maltopentaose, maltohexaose and maltoheptaose were also produced. Pancreatic alpha-amylase released maltose as the main product with maltotriose present in appreciable amounts. These observations confirm the action pattern proposed by Bird and Hopkins (1954).

Walker and Whelan (1960) studied the action of salivary alpha-amylase and found that with a low concentration of the enzyme the end products were maltose and maltotriose but when the concentration was increased 25-fold the products were glucose and maltose only. They also showed that the action of salivary alpha-amylase on maltotriose was much slower than on maltotetraose. This again suggests the preferential attack which is in agreement with the conclusions of previous authors.

Whelan (1960) reviewed the action pattern of five

alpha-amylases and concluded that there are two stages in the hydrolysis of amylose, amylopectin and glycogen namely (1) random splitting of internal linkages and (2) the selective splitting of short chains.

Robyt and French (1963) studied the action of Bacillus subtilis alpha-amylase on different substrates and they found that the main products of hydrolysis of amylose and amylopectin were maltotriose and maltohexaose. The reaction on the maltodextrins was dependent on the size of the molecules. They explained these results on the basis of a dual specificity of the B. subtilis alpha-amylase for the formation of maltotriose and maltohexaose.

Pazur and Okada (1966) showed by the use of auto-radiograms that during the hydrolysis of high molecular weight oligosaccharides by human salivary alpha-amylase, there is a preferential hydrolysis of interior bonds and a slow hydrolysis of terminal bonds. This results in the production of different amount of sugars. This confirms the findings of Bird and Hopkins (1954) and Walker and Whelan (1960).

Up to this stage the studies were made by analysing the digests for the amount of small sugars produced. Greenwood et al (1965) using a viscometeric technique and analysing the data according to the method of Vink (1963) showed that the graph of DP^{-1} against time of hydrolysis is linear, implying that hydrolysis is random. The curve for the acid hydrolysis, which is essentially a random process, was also found to be linear. They suggested

that in the initial stages of hydrolysis the process is random; and in the later stages when the number of resistant terminal bonds becomes significant, the process becomes non-random.

Okada (1967) using labelled olig^oasaccharides as substrates found that malto-oligosaccharides up to maltohexaose were relatively more resistant to bacterial alpha-amylase than long chain molecules. Okada et al (1968) again studied reducing products from maltodextrins in the initial stages of hydrolysis by bacterial alpha-amylases. The products were separated and identified by paper and column chromatography. They concluded that initial attack on maltodextrins occurred much more rapidly on the third or more inner bonds from the non-reducing end.

Robyt and French (1967) obtained blue value as a function of reducing power of the digest for human salivary, Aspergillus oryzae, porcine pancreatic alpha-amylase and 1M sulphuric acid. All these curves were different from one another. These differences were identified as degree of multiple attack by these enzymes. Porcine pancreatic alpha-amylase had a greater degree of multiple attack at pH 4.6 and 6.9 but at pH 10.5 the action changes from a multiple to a random process. This change in action pattern was explained to be due to an unfavourable ionization at the adverse pH. Their alternative explanation - that the change in action pattern at high pH might be due to gross change in the conformation of protein leaving only a small fraction active - seems

unlikely, because reducing value is measured as a function of blue value, so the process should be independent of the rate of reaction and the concentration of both enzyme and substrate.

The dependence of the action pattern of alpha-amylases on the source of the enzyme has again been emphasised by Greenwood and Milne (1968). By a quantitative study of the products for different alpha-amylases at the achroic limit, these authors showed that the experimental results were in agreement with a theoretical model in which certain bonds near the chain ends were considered more resistant to amylolytic attack than those in the interior of the molecule. This confirms the earlier findings of Meyer and Gonon (1951), Svanborg and Meyerback (1953), Kung et al (1953), Bird and Hopkins (1954), Pazur and Okada (1966) and Okada et al (1968).

The above authors also showed that at the achroic limit the products for plant and bacterial alpha-amylases were similar, i.e. maltotriose and maltohexaose in large proportions, whilst in the case of mammalian alpha-amylases the main products were maltose and maltotriose. This can be explained on the suggestion of Hans^f and Cattle (1938) that enzymes from different sources have different affinities for large and small molecules. The mammalian enzymes hydrolyse maltohexaose more easily than do the bacterial and plant alpha-amylases.

From the above survey of literature it is clear that the action pattern of alpha-amylases from different sources

is different. These differences are not fully understood. Basically it is agreed that the final products of alpha-amylases depend on the substrate. Most workers have suggested that hydrolysis in the initial stages is random. This would appear to be specifically confirmed by viscometric technique applied by Greenwood et al (1965).

However, viscosity technique possesses the disadvantage, that the results would be unaffected by the presence of low concentrations of small molecules such as maltose and maltotriose. This has been pointed out by French et al (1967). These authors have suggested that a non-random attack by alpha-amylases near chain ends may be superimposed on the random attack to give a multiple process. They obtained a degree of multiple attack by comparing the reducing value of the digests as a function of blue value during hydrolysis. This technique can be criticised because the blue value is an arbitrary measure of degradation, and it cannot be readily related to changes in the molecular weight in the system.

Ideally the action pattern should be followed by measuring the changes in number-average molecular weight of the substrate during hydrolysis. In the following work a new approach has been made to establish the action pattern of alpha-amylases isolated from different sources. During hydrolysis the changes in number-average degree of polymerisation ($\overline{DP}_n$) and weight average degree of polymerisation ($\overline{DP}_w$) have been measured simultaneously by enzymic and viscometric techniques respectively. The

enzymic determination of $\overline{DP}_n$ requires high concentrations of the substrate. Banks and Greenwood (1968) have reported that the presence of the 40% glycerol in the digests stabilizes the solutions of high concentrations of amylose. Initially 40% glycerol was incorporated in the digests and this led to a study of effect of glycerol and other polyhydroxy compounds on the action pattern of alpha-amylases.

EXPERIMENTAL AND RESULTS

Substrate.

Linear potato amylose was used as the substrate. The amylose was degraded enzymically to a degree of polymerisation of 1800. For quantitative studies undegraded amylose having a weight average degree of polymerisation of ~ 6000 was used.

Enzymes.

Crystalline porcine pancreatic alpha-amylase and B. subtilus amylase were obtained from the Sigma Chemical Company. The specific activities of the enzymes were 4.4×10^4 and 1.8×10^4 respectively. Human salivary alpha-amylase was prepared by the method of Fischer and Stein (1961). Malted rye alpha-amylase was prepared by the method described by Milne (1967) having a specific activity of 1.2×10^4 units. All these enzymes were found to be free from maltase activity.

(a) i. Reducing Power as a function of blue value.

Following digests were set up

1. Amylose solution (30 ml, 3 mg/ml) containing acetate buffer (0.02M pH 4.8) and porcine pancreatic alpha-amylase (0.2 ml).
2. Amylose solution (30 ml, 3 mg/ml) containing acetate buffer (0.02M pH 4.8) and B. subtilus alpha-amylase (0.2 ml).
3. Amylose solution (30 ml, 3 mg/ml) containing glycine-sodium hydroxide buffer (0.02M pH 10.5) and porcine pancreatic alpha-amylase (0.2 ml).

FIG. 3.1

Reducing Power VS Blue Value

4. Amylose solution (30 ml, 3 mg/ml) containing acetate buffer (0.02M, pH 4.8) and 40% glycerol and porcine pancreatic alpha-amylase (0.2 ml).

All the digests were incubated at 40°C, the samples were taken out at different time intervals and their reducing power measured as the percentage of apparent maltose by the alkaline-ferricyanide method as described in Section 2. Blue value of the digest was also measured at the time of withdrawal of samples. Blue values (B.V) are quoted as percentages of the blue value of the starting digest.

$$B.V = \frac{A_t}{A_0} \times 100$$

where A_t and A_0 are the absorbances of the digests when stained with iodine at time t and time zero respectively. The absorbances were measured in the following manner - 0.05 ml of the digest was added to one ml of the iodine solution (0.2% iodine in 2% potassium iodide) and 0.2 ml of 4N HCl in a 50 ml graduated flask. The volume was made up to the mark with distilled water and absorbance measured at EEL colorimeter using a red filter. A blank was also prepared omitting the polysaccharide only.

The results are shown in FIG. 3.1.

- (ii) The effect of Polyhydroxy compounds on the reducing power as a function of Blue value during hydrolysis

Following digests were prepared.

1. Amylose solution (30 ml, 3 mg/ml) containing 1M sulphuric acid.

FIG 3.2

EFFECT OF POLYHYDROXY COMPOUNDS AT PH 4.8.

- 1 Original P.P. without any additives.
 2 In presence of 0.2 M Erythritol.
 3 " " 0.22 M Erythritol.
 4 " " 0.225 M Erythritol.
 5 " " 0.30 M Erythritol.
 6 " " 40 % glycerol.
 7 " " 1 M H_2SO_4
 8 " " 0.30 M Methyl- α -glucoside.

2. Amylose solution (30 ml, 3 mg/ml) containing acetate buffer (0.02M, pH 4.8) and 0.3M α -methyl glucoside and porcine pancreatic alpha-amylase (0.2 ml).
3. Several digests were set up with amylose solution (30 ml, 3 mg/ml) containing acetate buffer (0.02M, pH 4.8) and porcine pancreatic alpha-amylase (0.2 ml) and various concentrations of erythritol i.e. 0.1M, 0.2 M, 0.225 M, 0.25 M, 0.3 M, 0.4 and 0.5 M.

All the digest except No. 1 were incubated at 40°C. The samples were withdrawn at different intervals and their reducing power and Blue value measured as described earlier. Digest 1 was incubated at 60°C and samples taken out were neutralised before measuring their reducing power. Results are shown in FIG. 3.2.

(iii) Possible interference of polyhydroxy compounds in the measurement of Reducing power.

Glucose and maltose solutions (1 mg/ml) were prepared and their reducing power measured in the presence of 40% glycerol, 0.3M erythritol and 0.3M α -methyl glucoside. Results are shown in Table 3.1., with those for a control solution containing only sugar and water.

(b) Quantitative estimation of Malto-dextrins produced during hydrolysis by different alpha-amylases.

- (i) Action of porcine pancreatic and human salivary alpha-amylases.

Two digests were set up with porcine pancreatic alpha-amylase (0.2 ml) and amylose solution

(30 ml, 3 mg/ml) at two different pH 4.8 and 10.5.

Another digest was set up with human salivary alpha-amylase (0.2 ml) and amylose (30 ml, 3 mg/ml) at pH 4.8.

The digests were incubated at 40°C. Samples were withdrawn at different time intervals and their blue value measured. The enzyme activity from the rest of the samples was destroyed by heating for ten minutes in a boiling water bath. Amylose was precipitated by the addition of an equal volume of ethanol. The supernatant was concentrated on a rotary evaporator and streaked on the chromatographic paper. The sugars were separated and eluted as described in Section 2. The quantitative amount of these sugars was estimated by glucose oxidase method as previously described (Section 2). Sugars higher than glucose were degraded to glucose before estimation. The results are shown in Tables 3.2., 3.3. and 3.4.

(ii) Action of Bacillus Subtillus alpha-amylase.

Amylose solution (3 mg/ml 20 ml) was incubated with Bacillus subtillus alpha-amylase at three different values of pH, i.e. 4.8, 7.0 and 8.5. At pH 4.8 another digest was set up in which the alpha-amylase was 10 times more concentrated than usual. Samples were taken out at achroic and two hours after achroic stage and analysed as described for porcine pancreatic and human salivary alpha-amylases. The results are shown in Tables 3.5., 3.6., 3.7.

SS A

FIG 3.3

$\overline{DP}_w^{-1}$ VS TIME

1. B.S. ALPHA AMYLASE
2. H.S. " "
3. M.R. " "
4. P.P. " "

TABLE 3.1.

EFFECT OF GLYCEROL, ERYTHRITOL AND α -METHYL
GLUCOSIDE ON REDUCING POWER OF MALTOSE AND GLUCOSE.

Sample	Control	Concentration (mg/ml) in presence of		
		40% Glycerol	0.3M Erythritol	0.3M Methyl Glucoside
Glucose	0.98	0.96	0.98	0.85
Maltose	0.96	0.95	0.98	0.87

TABLE 3.2.

ACTION OF PORCINE PANCREATIC α -AMYLASE AT pH
10.5 YIELD OF MALTODEXTRINS PRODUCED FROM AMYLOSE,
AS A FUNCTION OF DECREASE IN OPTICAL DENSITY (O.D.)

Sample	Decrease in O.D. %	Mols of Maltodextrins ^a /ml.					
		G ₁	G ₂	G ₃	G ₄	G ₅	G ₆
1	28	4	15	16	8	9	6
2	47	4	47	30	15	14	11
3	68	6	75	48	33	25	21

^aG₁ = Glucose; G₂ Maltose etc.

TABLE 3.3

ACTION OF HUMAN SALIVARY ALPHA-AMYLASE ON AMYLOSE
ATPH 4.8. YIELD OF MALTO DEXTRINS PRODUCED AS A
FUNCTION OF DECREASE IN OPTICAL DENSITY (O.D.)

Sample	% Decrease in O.D.	Moles of Malto Dextrins ^a /ml.					
		G ₁	G ₂	G ₃	G ₄	G ₅	G ₆
1	26	3	32	30	24	10	15
2	45	3	62	68	39	16	30
3	64	6	146	196	130	52	70

TABLE 3.4

ACTION OF PORCINE PANCREATIC ALPHA AMYLASE ON
AMYLOSE AT pH 4.8. YIELD OF MALTODEXTRINS PRODUCED
AS A FUNCTION OF DECREASE IN OPTICAL DENSITY.

Sample	Decrease in O.D. %	Mols of Malto Dextrins ^a /ml					
		G ₁	G ₂	G ₃	G ₄	G ₅	G ₆
1	27	2	290	127	72	38	29
2	50	4	620	268	142	86	70
3	71	6	910	300	222	135	66

^aG₁ = Glucose; G₂ Maltose etc.

TABLE 3.5.

ACTION OF BACILLUS SUBTILLUS ALPHA-AMYLASE ON
 AMYLOSE AT pH 4.8.
 YIELD OF MALTO DEXTRINS AT DIFFERENT STAGES OF
 DIGESTION.

Sample	Digestion Stage	% by weight of maltodextrins						
		G ₁ ^a	G ₂	G ₃	G ₄	G ₅	G ₆	>G ₆
1	Achroic	0.2	6	19	6	5	18	46
2	Post Achroic	0.8	11	26	8	10	29	16
1A	Achroic	0.5	5	20	8.5	6	31	29
2A	Post Achroic	1.5	8	23	8.5	9	29	21

a, G₁ = Glucose G₂ = Maltose etc.

A, Conc. of enzyme ten times in digest 1.

TABLE 3.6

ACTION OF BACILLUS SUBTILLUS ALPHA-AMYLASE ON
 AMYLOSE AT pH 7.00.
 YIELD OF MALTO DEXTRINS AT DIFFERENT STAGES
 OF DIGESTION.

Sample	Digestion Stage	% By weight of Malto Dextrins ^a						
		G ₁	G ₂	G ₃	G ₄	G ₅	G ₆	>G ₆
1	Achroic	0.15	2	6	2	1	8	81
2	Post Achroic	0.3	3	9	2	2	10	74

TABLE 3.7

ACTION OF BACILLUS SUBTILLUS ALPHA-AMYLASE ON
 AMYLOSE AT pH 8.5
 YIELD OF MALTO DEXTRINS AT DIFFERENT STAGES OF
 DIGESTION

Sample	Digestion Stage	% By weight of Malto Dextrins ^a						
		G ₁	G ₂	G ₃	G ₄	G ₅	G ₆	>G ₆
1	Achroic	0.1	1.8	3	1.5	1.3	4	88
2	Post Achroic	0.2	2.4	7	2	1.5	7	80

a, G₁ = Glucose, G₂ = Maltose.

(c) Comparison of $\overline{DP}_w$ and $\overline{DP}_n$ during alpha-amylolysis.

The digests were prepared as follows.

Amylose solution (40 ml, 8 mg/ml) containing 0.02 M acetate buffer (4 ml pH 4.8) and 40% glycerol was incubated at 40°C separately with alpha-amylases from porcine pancreas, human saliva, Bacillus subtilus and malted rye. Samples were taken out at different time intervals and the activity of the enzyme destroyed by heating in a boiling water bath for 10 minutes. Each sample was divided into two equal portions. One portion was precipitated with butanol and the complex dissolved in 0.15 M KOH for the determination of $\overline{DP}_w$. The other portion was analysed for $\overline{DP}_n$ as described by Banks and Greenwood (1968) and detailed in Section 2. Results are shown in Tables 3.8, 3.9, 3.10, 3.11 3.12 and 3.13.

TABLE 3.8

ACTION OF BACILLUS SUBTILLUS ALPHA-AMYLASE ON
 AMYLOSE IN PRESENCE OF GLYCEROL.
 COMPARISON OF CHANGES IN $\overline{DP}_w$ AND $\overline{DP}_n$ DURING
 HYDROLYSIS.

ExPT No.	$\overline{DP}_w$	$\overline{DP}_n$	$\overline{DP}_w/\overline{DP}_n$
1	1975	810	2.44
2	1783	636	2.78
3	1450	589	2.46
4	1212	481	2.51
5	1042	424	2.46

TABLE 3.9

ACTION OF HUMAN SALIVARY ALPHA-AMYLASE ON
 AMYLOSE IN PRESENCE OF GLYCEROL.
 COMPARISON OF CHANGES IN $\overline{DP}_w$ AND $\overline{DP}_n$ DURING
 HYDROLYSIS.

ExPT No.	$\overline{DP}_w$	$\overline{DP}_n$	$\overline{DP}_w/\overline{DP}_n$
1	1810	891	2.03
2	1160	650	1.80
3	1160	584	1.98
4	1042	492	2.11
5	939	477	1.97

TABLE 3.10

ACTION OF MALTED RYE ALPHA-AMYLASE ON AMYLOSE IN
 PRESENCE OF GLYCEROL.
 COMPARISON OF CHANGES IN $\overline{DP}_w$ AND $\overline{DP}_n$ DURING
 HYDROLYSIS.

ExPT No.	$\overline{DP}_w$	$\overline{DP}_n$	$\overline{DP}_w/\overline{DP}_n$
1	1718	474	2.22
2	950	367	2.55
3	512	227	2.25
4	385	191	2.15
5	350	161	2.17

TABLE 3.11

ACTION OF PORCINE PANCREATIC ALPHA-AMYLASE ON
 AMYLOSE IN PRESENCE OF 40% GLYCEROL.
 COMPARISON OF CHANGES IN $\overline{DP}_w$ AND $\overline{DP}_n$ DURING
 HYDROLYSIS.

ExPT No.	$\overline{DP}_w$	$\overline{DP}_n$	$\overline{DP}_w/\overline{DP}_n$
1	1718	774	2.22
2	1042	400	2.60
3	660	295	2.20
4	501	238	2.10
5	527	218	2.41

TABLE 3.12

ACTION OF BACILLUS SUBTILLUS ALPHA-AMYLASE ON
 AMYLOSE IN ABSENCE OF GLYCEROL.
 COMPARISON OF CHANGES IN $\overline{DP}_w$ AND $\overline{DP}_n$ DURING
 HYDROLYSIS.

ExPT No.	$\overline{DP}_w$	$\overline{DP}_n$	$\overline{DP}_w/\overline{DP}_n$
1	1816	810	2.33
2	1543	685	2.25
3	1280	594	2.15
4	1107	505	2.19
5	939	452	2.07

TABLE 3.13

ACTION OF PORCINE PANCREATIC ALPHA-AMYLASE ON
 AMYLOSE IN ABSENCE OF GLYCEROL.
 COMPARISON OF CHANGES IN $\overline{DP}_w$ AND $\overline{DP}_n$ DURING
 HYDROLYSIS.

ExPT No.	$\overline{DP}_w$	$\overline{DP}_n$	$\overline{DP}_w/\overline{DP}_n$
1	2108	847	2.47
2	1763	564	3.12
3	1543	515	3.00
4	1429	408	3.50
5	1225	300	4.08

DISCUSSION

Alpha-amylases come in contact with the substrate randomly, how they act further has been a subject of controversy. Three concepts originally developed for Beta-amylases have been extended to alpha-amylases by Robyt and French (1967).

- (i) Single chain attack: In this type of attack enzyme comes in contact with the substrate to form an enzyme-substrate complex, then it acts in a "zipper fashion" towards one end of the chain and does not form a complex with another substrate chain until it completely degrades the first chain it has come across.

The arrow mark indicates the an α -1-4 glucosidic bond hydrolysed.

- (ii) Multichain attack: The enzyme acts randomly in this type of attack and cuts only one bond per effective encounter, then it diffuses away to form a new complex with another substrate molecule.

- (iii) Multiple attack: This type of hydrolysis is in between the two extremes of single chain and multichain attacks. The enzyme comes in contact

with the substrate randomly, forms a complex and cuts one bond, before diffusing away it cuts a few more bonds, i.e. one bond is cut randomly and subsequently one or more bonds are subjected to non-random hydrolysis.

A schematic representation is given below:

Where E represents enzyme, S_n is the substrate molecule with n units, which comes in contact with enzyme and forms a complex, x is > 3 but smaller than n where as y is > 1 and < 4 . P is the product either maltose or maltotriose which is resistant to further attack.

Single chain reaction follows (i), (ii), (iii) and (iv). Multichain reaction proceeds through (i), (v) (vi) followed by (vii), recombining the enzyme with the chain of same molecule or another.

Originally these concepts were applied to exo-enzymes,
for endo-enzymes single chain attack has been rejected

because there will be no decrease in viscosity of the substrate until large number of molecules are degraded completely, which means that $\overline{DP}_w$ will not decrease rapidly and blue value will change slowly. This is not true for the endoenzymes where viscosity and blue value both decrease rapidly without much increase in reducing value. For multiple and multichain attacks conflicting reports are found in the literature. The conclusions reached are by different approaches towards the problem. French et al (1967) have used reducing power - blue value curves for differentiating between the action patterns while Greenwood et al (1965) have applied viscosity technique and quantitative estimation of maltodextrins produced.

ABSORBANCE-REDUCING POWER CURVES.

Curves in FIG. 3.1 show that porcine pancreatic alpha-amylase acts differently from that^{of} Bacillus subtilis enzyme. But at adverse pH 10.5 and in presence of 40% glycerol its action becomes similar to that of bacterial enzyme. Robyt and French (1967) have shown that at pH 10.5 porcine pancreatic alpha-amylase acts randomly because its curve is similar to acid hydrolysis which is essentially a random process. So it can be said that curve No. 2, 3 and 4 in FIG. 3.1 are characteristic of random process while curve No. 1 represents multiple attack. At pH 10.5 and in presence of 40% glycerol, during hydrolysis there is a rapid decrease in absorbance with a low increase in reducing power for porcine pancreatic alpha-amylase while at pH 4.8 there is vast increase in reducing power as compared to

decrease in absorbance. This is in agreement with the results of Robyt and French (1967).

It is rather difficult to explain that why the effect of 40% glycerol on the action pattern of alpha-amylase is similar to that at adverse pH 10.5. Glycerol is a viscous material and in presence of large amount of glycerol such as 40%, the rate of substrate diffusing away from the binding site of the enzyme will be much slower than in its absence, thus increasing the possibility of multiple attack. The explanation put forward by Robyt and French (1967) that at adverse pH there might be a change in the conformation of the enzyme which leaves only a small fraction enzymically active, is not possible because the process is independent of enzyme and substrate concentration.

Isemura et al (1969) have reported that all the hydroxyl groups at the reducing end and the two hydroxyl groups at the non-reducing end of the substrate play an important part in the action pattern of taka amylase on substituted maltose. Any maltose derivative which had an orthomethyl substituent on the reducing glucose unit was resistant to alpha-amylolytic attack. Similarly phenyl-2-O-methyl-alpha-maltoside was found resistant to alpha-amylolytic attack. These observations indicate that hydroxyl groups on the chain ends play an important part in forming the enzyme substrate complex, and when they are substituted the enzyme simply cannot bind the substrate and hydrolysis does not take place. It seems that if large amount of glycerol is present in the digest its hydroxyl groups

occupy the positions on the enzyme surface which are necessary to bind the substrate at the chain ends. Thus the enzyme fails to produce small sugars which rapidly increase the reducing power of the digest with slow decrease in absorbance.

Whitakar et al (1962) had previously found that sugar alcohols like D-sorbitol, D-Mannitol and Inositol have an inhibitory effect on the activity of alpha-amylase. In fact sugar alcohols were more inhibitory than the parent sugars. The replacement of an aldehyde group by a primary alcoholic group greatly increased the inhibition of alpha-amylase action. All these findings confirm the above conclusion that hydroxyl groups when introduced in the digest inhibit alpha-amylase activity. The presence of alcoholic group in place of reducing aldehyde group of either sugars or long chain molecules increases the degree of inhibition.

Inhibitory effect of 50% glycerol has also been reported by Sarivastawa (1959). The degree of inhibition of alpha-amylases, reported by the above author, was 21%. FIG. 3.1 shows that degree of inhibition is around 35% which slightly varies during the course of hydrolysis. This discrepancy could be due to the measurement of degree of inhibition at different stages. Secondly, measurement of activity by the increase in reducing power is more reliable. Wigglesworth (1927) has also reported similar inhibition.

Effect of glycerol on salivary alpha-amylase activity has also been discussed by Lotti (1958). The decrease in

activity is proportional to the concentration of glycerol. With the increase of glycerol in the digest the activity decreases. By studying the changes in activation energy of the reaction in presence of glycerol and sodium chloride, he concludes that glycerol does not modify the enzyme. This also confirms the conclusion that hydroxyl groups of glycerol occupy the position on the enzyme surface which are necessary for binding the substrate near the chain ends and thus change the multiple attack to a random process. There is no change effected in the enzyme structure. This explanation can be extended to the action of the enzyme at pH 10.5. As the pH⁷ the digest increases the basic hydroxyl groups increase and occupy the necessary positions on the enzyme. Robyt and French (1967) explained their results on the possibility of a change of conformation of the enzyme at high pH. This seems unlikely because Rowe et al (1968) have shown that porcine pancreatic alpha-amylase is stable at pH 10.5. Secondly similar effect is produced by glycerol and according to Lotti (1958), glycerol does not modify the enzyme in any way.

From the above discussion it can be suggested that there are two binding sites on the surface of porcine pancreatic alpha-amylase. The simultaneous action of both of these results in the production of small sugars which increase the reducing power of the digest and the process seems to be multiple. One of these binding sites fails to act in presence of glycerol and other alcohols reported by Whitikar (1962) and the process becomes random. It seems that this

second binding sites has a great affinity for hydroxyl groups. It is also indicated that hydroxyl groups of low molecular weight substances can approach this binding site more easily than longer chain molecules. To explore this second possibility the action pattern of porcine pancreatic alpha-amylase was studied in the presence of erythritol and methyl- α -glucoside.

FIG. 3.2 shows the effect of erythritol and methyl- α -glucoside on the reducing power of the digest as a function of blue value during hydrolysis. It was found that the concentration of erythritol in the digest up to 0.2M has no effect on the action of the enzyme. However, the presence of 0.225 M erythritol decreases the reducing power as a function of blue value so that the process becomes random. Further increase in the concentration of the erythritol in the digest does not shift the curve any more. The effect of erythritol is rather a peculiar one in that its presence in the digest inhibits alpha-amylase at a definite concentration. Under the conditions of the experiment the inhibition should be proportional to the concentration. The effect of glycerol has been reported by Lotti (1958) to be proportional to its concentration. Whitikar et al (1962) have also reported that alcohols inhibit alpha-amylase activity in decreasing order of ethanol, isopropanol, propylene glycol, glycerol, ethylene glycol and methanol. Inhibition was directly proportional to the molar concentration of the alcohols. The method used by these authors was similar to the one used in the present

work. FIG 3.2 also shows the effect of 0.3 M Methyl- α -D glucoside and the curve of reducing power as a function of blue value during acid hydrolysis. From all these results it can be concluded that porcine pancreatic alpha-amylase has a multiple attack pattern which is reverted to random attack in the presence of 40% glycerol 0.225 erythritol and 0.3 M methyl- α -glucoside and at pH 10.5. It was found however, that presence of polyhydroxy compounds does not effect the estimation of reducing power of small sugars produced. (Table 3.1). Methyl- α -glucoside gives slightly low results and this could be the reason for the profound shift in the reducing power - blue value curve in FIG. 3.2.

The different behaviour of porcine pancreatic alpha-amylase than other enzymes can be explained on the basis of its composition. Weill and Caldwell (1945) found that this enzyme was not inhibited by sulphydryl group reagents, therefore, it was assumed that sulphydryl groups are not present, or if they are present, they do not play any important role in the activity of the enzyme. Schramm (1964) has claimed that porcine pancreatic alpha-amylase contains masked sulphydryl groups which are important but not necessary for the activity of the enzyme. These masked groups can be partially exposed with EDTA.

p-Chloromercuribenzoate causes inhibition of alpha-amylases from cereals (Fischer and Haselback 1951) and human saliva (Muus et al 1956). Akabori et al (1956) found no cystine or cysteine in Bacillus subtilus alpha-amylase, so they assumed that there are no sulphydryl

groups in this enzyme. This assumption needs further confirmation because the Bacillus subtilus enzyme is inhibited by p-Chloromercuribenzoate (Carlo and Redfern 1947) indicating that sulphhydryl groups are present.

Recently Suzuki Schuichi et al (1968) have reported that the introduction of sulphhydryl groups into Taka amylase increases the enzyme activity, which show that sulphhydryl groups play an important part in the activity of the enzyme. They also found that when sulphhydryl groups were introduced in the enzyme, the rate of the hydrolysis for long chain substrates was increased ^{over} ~~as~~ that for the corresponding short chain substrate. This means that when sulphhydryl groups are present and free to take part in enzyme action, the enzyme has more affinity for longer chains than shorter chains. This could possibly explain the different behaviour of porcine pancreatic alpha-amylase. It has been shown that cereal (Hans and Cattle, 1938) and bacterial enzymes (Meyer and Gonon, 1951) have more affinity for long chain substrates than short chain substrates. Porcine pancreatic alpha-amylase having masked sulphhydryl groups will have equal affinity for both long and short chain substrates.

Thus the enzyme which attacks the long chains preferentially will proceed with a relatively small increase in reducing power while pancreatic enzyme with equal affinity for long and short chain molecules, will cause a large increase in reducing power at the same stage of hydrolysis.

QUANTITATIVE STUDY OF PRODUCTION OF MALTO-DEXTRINS
FROM AMYLOSE BY ALPHA-AMYLASES.

It seems that porcine pancreatic alpha-amylase is the only one differing in its action from alpha-amylases isolated from other sources. This difference was further demonstrated when malto-dextrins produced at different stages of hydrolysis were separated and estimated quantitatively. Tables 3.4 and 3.2 show the malto-dextrins produced by porcine pancreatic alpha-amylase at pH 4.8 and 10.5, respectively, at different stages of digestion shown by decrease in absorbance. The yield of malto-dextrins by porcine pancreatic alpha-amylase^{at pH 10.5} is comparable to that of salivary alpha-amylase at pH 4.8 (Table 3.3). However, at pH 4.8 porcine pancreatic alpha-amylase produces large quantities of malto-dextrins, particularly maltose and maltotriose. This is quite a distinct behaviour. Bacillus subtilus alpha-amylase does not produce such increased amounts of malto-dextrins even at achroic stage. Tables 3.5, 3.6, 3.7 show the amounts of sugars produced by Bacillus subtilus enzyme at pH 4.8, 7.0 and 8.5 at achroic and after achroic stage. Table 3.5 shows that amount of sugars produced at a definite stage is independent of concentration of the enzyme. The results for Bacillus subtilus enzyme at optimum pH 4.8 are similar to those reported by Greenwood et al (1968). It is obvious that this enzyme produces large amounts of G₃ and G₆ while porcine pancreatic enzyme produces mainly G₂ and G₃. This is in agreement with the findings of Robyt and French

(1963) and Greenwood et al (1968). Results of salivary alpha-amylase are similar to those of porcine pancreatic alpha-amylase at adverse pH but in lesser quantity.

Greenwood and Milne (1968) have calculated the production of malto-dextrins by enzymes from different sources. The results for porcine pancreatic alpha-amylase were comparable to experimental yields when calculations were based on the assumption of multiple attack, whilst for bacterial and cereal alpha-amylases results calculated on the basis of preferential attack on certain bonds were comparable to experimental yields. For porcine pancreatic alpha-amylase the degree of multiple attack was assumed to be 2 to get comparable results with the experimental yields. French and Robyt (1967) have however, calculated the degree of multiple attack to be 7 for the same enzyme by a different method.

Studies on the Changes in $\overline{DP}_w$ and $\overline{DP}_n$ during Hydrolysis

Although it is shown in the above discussion that porcine pancreatic alpha-amylase behaves differently from other amylases, but it is not clear that it acts by a multichain or multiple attack pattern. Ideally to differentiate between these two patterns the process should be followed by changes in degree of polymerisation. Greenwood et al (1965) have followed the changes in degree of polymerisation by viscometric technique. In this method the $\overline{DP}$ at different stages of hydrolysis was determined by viscosity of the digest and plotted against time. This gave a linear relationship. Since acid hydrolysis, which

is essentially a random process, also gave a linear relationship, Therefore, it was believed that alpha amylolytic hydrolysis is also random.

Robyt and French (1967) have rejected this explanation on the basis that small sugars produced by a multiple attack will not affect the viscosity of the digest. Following the changes in the weight average degree of polymerisation will not give satisfactory results. Let us imagine that initial substrate contains 2000 glucose units in one chain and one particular alpha-amylase molecule comes in contact in the centre of the chain and hydrolysis the chain into two equal fragments of 1000 glucose units each. If another type of alpha-amylase hydrolysis five additional glucosidic bonds during the same encounter to produce maltose units, one chain will be left with 990 glucose units. The viscosity of the fragments of 1000 + 1000 units will not be different from those of 1000 + 990 units. So during a multiple attack the presence of low concentrations of small sugars will not effect the viscosity of the system and hence the degree of polymerisation.

It is therefore essential to follow the changes in number average degree of polymerisation. However, simply following the number average degree of polymerisation will not reveal how many bonds are broken per effective encounter, so number average and weight average degree of polymerisation should be followed simultaneously. Tables 3.8, 3.9, 3.10, 3.11, 3.12 and 3.13 show the comparison of changes in both type of degrees of polymerisation.

During a random hydrolysis only one bond is broken per

effective encounter, therefore, $\overline{DP}_n$ and $\overline{DP}_w$ will change proportionally while in a multiple attack the $\overline{DP}_n$ will decrease more rapidly than $\overline{DP}_w$ and the ratio between the two will increase rapidly.

In the present work $\overline{DP}_n$ was measured by the method of Banks and Greenwood (1968) and $\overline{DP}_w$ by the viscometric technique. The experimental techniques limit the $\overline{DP}_w$ for studies to be around 2000. For this reason the amylose was initially degraded by alpha-amylase to bring it in the range. The use of total linear amylose as a substrate means that the exponential distribution will be retained on random attack.

Experiments in presence of 40% Glycerol.

Tables 3.8, 3.9, 3.10, 3.11 show the changes in $\overline{DP}_w$ and $\overline{DP}_n$ during hydrolysis. It is clear that there is no change in the ratio of $\overline{DP}_w$ and $\overline{DP}_n$ under the conditions observed, implying a random attack. The constancy of the ratio shows that there is no multiple attack occurring. This is quite in agreement with the Reducing power - blue value curves showing that all the alpha-amylases have random attack in presence of 40% glycerol.

The effectiveness of the method used for the determination depends upon a non preferential distribution of oligosaccharides of even and odd number of glucose units. Quantitative estimation of soluble sugars produced at early stages were carried out chromatographically. There was no preferential build up of any of the oligosaccharide.

(Tables 3.2, 3.3). Moreover, there were only traces of

glucose produced indicating the absence of any maltose splitting activity.

The values of $\overline{DP}_w$ in Tables 3.8, 3.9, 3.10 and 3.11 were plotted against time, linear curves were obtained indicating occurrence of random attack (FIG. 3.3). This is in agreement with the previous reports of Greenwood et al (1965).

All these results are for the initial stages of hydrolysis in which only a small fraction of total bonds is broken. The number of bonds broken can be calculated by the equation

$$P = \frac{\overline{DP}_0}{\overline{DP}_t} - 1 \quad (\text{Cowie and Greenwood})$$

where $\overline{DP}_0$ and $\overline{DP}_t$ are the initial degree of polymerisation and degree of polymerisation after time t , respectively.

Experiments in absence of Glycerol.

The above experiments were carried out in presence of 40% glycerol. ~~To find out~~ whether presence of glycerol effects the action pattern of alpha-amylases, digests were set up without glycerol. In these experiments a lengthy process had to be adopted for determination of $\overline{DP}_n$. The samples taken out at different intervals were evaporated to complete dryness and then dissolved in DMSO and diluted to the required concentrations with distilled water. As indicated by reducing value - blue value curves, presence of glycerol effects only porcine pancreatic alpha-amylase so the experiments were carried out only with porcine pancreatic and Bacillus subtilis alpha-amylases.

Results in Table 3.12 show that the ratio of $\overline{DP}_w$ and $\overline{DP}_n$ for bacterial enzyme does not change at various stages of hydrolysis, implying that presence of glycerol does not effect the action pattern of this enzyme and the process remains random. For porcine pancreatic enzyme there is a profound change in the ratio of $\overline{DP}_w$ and $\overline{DP}_n$ with the time of digestion. (Table 3.13). This shows that small sugars are produced in the digest and the process is not random. This is in agreement with the findings of Robyt and French (1967).

It is concluded from the above results that the attack in the initial stages of hydrolysis by the alpha-amylases from Baccillus subtilus, human saliva and malted rye is not by a multiple pattern. In contrast the action pattern of porcine pancreatic alpha-amylase is completely different. There is no evidence of multiple attack at pH 4.8, in presence of 40% glycerol and at pH 10.5, but there is a build up of small sugars at optimum pH 4.8 in absence of glycerol indicating a multiple attack.

The reason of this different behaviour of porcine pancreatic alpha-amylase may be due to some differences in its structure or in its amino acid composition. Section 4 deals with the fractionation amino acid composition studies of porcine pancreatic alpha-amylase.

SECTION 4.

FRACTIONATION AND AMINO ACID ANALYSES
OF PORCINE PANCREATIC ALPHA - AMYLASE.

INTRODUCTION:-

The observations made in the previous section show that porcine pancreatic alpha-amylase acts differently than other alpha-amylases. This difference could be due to the presence of different structures arising from a different amino acid composition and sequence. Very little is known about the amino acid sequence of alpha-amylases. Even a difference in amino acid sequence is unlikely because if such a difference exists porcine pancreatic alpha-amylase would be an entirely different enzyme from other alpha-amylases which it is not.

Another possibility is that there is more than one protein present in the enzyme with minor differences which play a part in the action pattern of the enzyme. The amylases studied to-date have been judged for purity by four criteria, their crystalline form, their behaviour on ultracentrifugation, paper electrophoresis and free boundary electrophoresis. But with the development of new techniques of gel-electrophoresis and ion-exchange chromatography, it has become possible to study these enzymes in further detail.

Alpha-amylases which were believed to be homogeneous have been shown to contain more than one fraction. Berk et al (1963) found that there are at least two different forms of alpha-amylases in serum. This discovery was not significant because in serum there could be alpha-amylases from different sources like pancreas and salivary glands. Dorane (1967), however, reported that isoenzymes are present in the alpha-amylase prepared from Drosophyllia Melanogesta. Millan and

Smith (1962) using a calcium phosphate column for chromatography obtained three fractions from human salivary alpha-amylase one of which was unstable.

Muus and Vanchak (1964) confirmed the above observations and suggested that the amino acid composition of the human salivary alpha-amylase fits into a minimum molecular weight of 18000, and as the total molecular weight obtained by sedimentation was 69000 so it is possible that human salivary alpha-amylase is a trimer of small fractions which are very similar.

Sick and Nelson (1964) have demonstrated that mouse pancreas produces multiple forms of alpha-amylase. Heler and Kulka (1965) have also reported two forms of alpha-amylases in the developing chick pancreas. Momotani (1966) obtained three fractions with alpha-amylase activity from embryoless barley grains when following induction with gib^{be}berellic acid. Sachio Ogita (1966) reported that human salivary alpha-amylase formed 4 - 6 bands when subjected to thin layer electrophoresis. Pancreatic alpha-amylase was separated into 4 distinct bands. With the results of immuno-electrophoresis he suggested that individual salivary and pancreatic amylase isoenzymes are antigenically identical and are under the control of a single gene.

Wolf and Taylor (1967) compared paper and polyacrylamide gel-electrophoresis of salivary alpha-amylase for iso-amylases. Paper electrophoresis gave only one band where as on gel up to seven bands were obtained. They also found that properties of isoenzymes were dependent on the method of isolating the

pure enzyme. This is in agreement with the earlier findings of Muus et al.

Rowe et al (1968) found that when porcine pancreatic alpha-amylase was passed through DEAE-cellulose coloumn it is resolved into two different fractions. As the separation was highly dependent on pH of the eluting buffer, they concluded that the only difference between these fractions is their ionic strength, otherwise they are quite similar.

Boetcker and Launde (1969) have also shown multiple forms of salivary alpha-amylase when it is subjected to polyacrylamide electrophoresis. They found three major and one minor band which show alpha-amylolytic activity.

This section deals with the studies on the fractionation of porcine pancreatic alpha-amylase on a DEAE-cellulose coloumn, the properties of the fractions obtained and their composition of amino acids.

EXPERIMENTAL

Crystalline porcine pancreatic alpha-amylase was obtained as a suspension in sodium chloride from Sigma Chemical Company and The Worthington Chemical Corporation. All fractionation studies were carried out at pH 8.5 (Tris-HCl buffer 0.01M) unless otherwise stated. The eluting buffer contained 0.03M calcium chloride. Whatman DEAE 52 ion-exchange cellulose with an ion capacity of 1.0 m. eq/gm was used for fractionation. Bio-gel P-60 was used for molecular size of the protein samples.

(a) Chromatography on ion-exchange cellulose.

The cellulose was washed with 0.5N sodium hydroxide, then with distilled water and finally with 0.5N hydrochloric acid. It was then washed with eluting buffer containing 0.03M calcium chloride, until the washings were at pH 8.5. The cellulose was allowed to equilibrate with eluting buffer overnight and was then packed in a 30x1.5 cm glass column. The column was attached to the L.K.B. "Uvicord" flow-photometer and the fraction collector. The details of the apparatus are given in Section 2.

Enzyme (5 mg) dissolved in eluting buffer was put on the top of the column and eluted with the buffer. The buffer was pushed by a small pump to attain the desired flow rate. Samples of the individual peaks obtained were pooled together for further studies. The elution patterns obtained are shown in FIG. 4.2., 4.3 and 4.4.

(b) Concentration and activity of the samples.

Concentrations of the samples were measured manually on SP 500 spectrophotometer at 280 mμ. Measurement of alpha-amylase

FIG 4.1

FIG. 4. 2

82 C

FIG 4.3

FIG. 4.4

FIG. 4.5

Reducing Power as % of maltose.

activity has been described in Section 2.

(c) Molecular size of the protein samples.

Molecular size of the enzyme and its fractions was obtained by using a P-60 Bio-gel column (30x1.5 cm). The column was connected to the photometer and fraction collector. The whole set-up was similar to that for ion-exchange chromatography. The void volume V_0 , of the column was taken as the elution volume for blue dextran 2000. The elution volume V_e for the serum albumin, ovalbumin and trypsin inhibitor was obtained. The ratio V_e/V_0 was plotted as a function of molecular weight to obtain a standard graph (FIG. 4.1). Elution volume for protein samples was obtained and their molecular weights calculated from the standard graph. Results are shown in Table 4.10.

(d) Reducing power and Blue value of the digests.

Methods for measuring the blue value and reducing power are described in Section 2. The results are shown in FIG. 4.5.

(e) Sedimentation co-efficients.

Sedimentation co-efficients were calculated by the method of Bordman et al (1960) using a Beckman Model E. ultracentrifuge. Whatman No. 1 filter paper was cut into three pieces to fit on the base of the cell and these pieces were placed in the cell. Dilute enzyme was introduced in the cell with the help of a syringe. A control cell without filterpapers was also prepared. The enzyme samples in both the cells were centrifuged at a speed of 50740 r.p.m. for 45 minutes. The enzyme samples were taken out very carefully with the help of a syringe and their activity measured as described earlier. One photograph was taken when speed reduced to 4000 r.p.m. This speed was found to be minimum

at which a photograph of a peak could be taken before disorganisation. Results are shown in Table 4.1.

(f) Amino acid analyses

The method used for amino acid analysis was that of Speckman et al (1958) as modified by Patterson (1965). The apparatus used in this was the "Technicon" automatic amino acid analyser and has been described in detail in Section 2.

Hydrolysis of the samples (2 mg) was carried out using 6N (constant boiling) hydrochloric acid (6 ml) in sealed pyrex tubes, which were cooled in liquid nitrogen and evacuated at the water pump prior to sealing. Hydrolysis was carried out in an oven at 105°C. for 24 hours unless otherwise stated. After the required time for hydrolysis, the tubes were again cooled in liquid nitrogen and opened. The contents were transferred to pear-shaped flasks along with washings and evaporated to dryness on a rotary evaporator at 35°C. Samples were dissolved in distilled water and dried again. The evaporation was repeated twice more to get rid of all the acid. The samples were then dissolved in sodium citrate buffer (pH 2.2, 0.0667M) containing Norleucine (NoL) and L-a-amino-B-guanido-propionic acid hydrochloride (AGPA) as internal standards both at a concentration of 0.1 μ M per 0.5 ml of the buffer.

Samples were then loaded on each column in turn. The peak area for each amino acid was calculated by hand measurement from the visible absorption trace on the recorder chart. Using the standard equivalent values for each acid and accurate

starting weight of the sample the amount of each amino acid in the sample was calculated. Results are shown in Tables 4.2 and 4.3.

TABLE: 4.1. SEDIMENTATION CO-EFFICIENTS OF PORCINE PANCREATIC ALPHA-AMYLASE AND ITS FRACTIONS.

Protein	λ	c/c_0	Speed	Time *	$S \times 10^{13}$
ORIGINAL	3.67	0.31	50740	55	4.8
FRACTION 1	3.4	6.28	50740	55	5.4
FRACTION 2	3.2	0.318	50740	55	5.6
FRACTION 3	3.32	0.33	50740	55	5.1
FRACTION 4	3.58	0.33	50740	55	5.3

* Time includes correction for acceleration and deceleration.

TABLE 4.2. AMINO ACID COMPOSITION OF PORCINE PANCREATIC ALPHA-AMYLASE AND ITS FRACTIONS.

<u>Amino Acid</u>	<u>Original</u>	<u>F1</u>	<u>F2</u>	<u>F3</u>	<u>F4</u>
CYSO ₃ H					
ASP	14.6*	14.3	15.5	14.6	14.7
THR	4.3	4.8	4.4	4.3	4.3
SER	5.4	6.0	5.8	5.6	4.7
GLU	9.7	11.5	10.2	9.9	10.1
PRO	4.1	4.8	4.0	4.8	4.4
GLY	6.8	7.0	7.2	7.0	7.3
CYS	2.3	2.3	2.3	2.3	2.3
AL	5.2	5.4	5.4	5.3	5.4
VAL	7.3	8.2	7.4	7.6	7.7
MET	1.7	2.0	2.0	1.8	1.8
ILEU	4.6	5.0	5.2	4.9	4.9
LEU	5.4	5.5	5.9	5.5	5.5
TYR	5.8	3.6	5.6	5.1	5.4
Ph.AL	6.6	5.3	6.5	6.0	5.5
LYS	4.8	5.0	4.6	5.3	5.4
HIS	2.5	2.2	2.0	2.4	2.3
NH ₃	3.3	1.95	2.4	2.3	2.8
ARG	7.5	7.0	5.8	7.2	7.5

* Values are given in gm./100 gm. of protein.

TABLE 4.3. PERCENTAGE OF AMINO ACIDS (μ MOLES) PRESENT IN THE HYDROLYSATE

Amino Acid	Original Enzyme		F1		F2		F3		F4	
	μ moles	%	μ moles	%	μ moles	%	μ moles	%	μ moles	%
NH ₃	2.768	14.6	1.963	12.8	2.416	16.7	2.195	15.1	2.270	17.5
ASP	2.316	12.2	1.817	11.8	1.793	12.4	1.755	12	1.533	11.8
THR	0.768	4	0.691	4.5	0.570	3.9	0.577	3.9	0.444	3.8
SER	1.10	5.8	0.981	6.4	0.851	5.6	0.858	5.9	0.627	4.8
GLU	1.40	7.4	1.330	8.6	1.061	7.3	1.079	7.4	0.953	7.3
PRO	0.762	4	0.710	4.6	0.548	3.9	0.670	4.6	0.527	4.0
GLY	1.915	10.1	1.597	10.4	1.495	10.3	1.490	10.3	1.350	10.4
AL	1.23	6.5	1.089	7.0	0.932	6.5	0.944	6.5	0.852	6.5
VAL	1.32	7.0	1.187	7.7	0.968	6.7	1.04	7.1	0.908	7.0
MET	0.244	1.3	0.233	1.5	0.2.2	1.6	0.194	1.3	0.170	1.3
ILEU	0.758	4	0.657	4.2	0.616	4.3	0.596	4.1	0.521	4.0
LEU	0.868	4.6	0.712	4.6	0.697	4.8	0.672	4.6	0.579	4.5
TYR	0.676	3.6	0.342	2.2	0.476	3.3	0.444	3.1	0.915	3.2
Ph.AL	0.842	4.4	0.549	3.6	0.611	4.2	0.576	4.0	0.968	3.6
LYS	0.697	3.7	0.587	3.8	0.487	3.4	0.580	4.0	0.513	4.0
HIS	0.342	1.8	0.241	1.6	0.200	1.4	0.244	1.6	0.210	1.6
ARG	0.908	4.8	0.682	4.4	0.514	3.7	0.663	4.5	0.596	4.6

TABLE 4.4. MINIMUM AMINO ACID RESIDUES OF PORCINE PANCREATIC ALPHA-AMYLASE AND ITS FRACTIONS

Amino Acid	ORIGINAL		F1		F1a		F2		F3		F3b		F4	
	Residues	Whole No.	RES	W.No	R	W.No	Residues	W.No	R	W.No	R	W.No	R	W.No
ASP	6.7	7	7.5	7	7.2	7	8.9	9	7.1	7	6.9	7	7.3	7
THR	2.5	3	2.8	3	2.6	3	2.8	3	2.3	2	2.6	3	2.3	2
SER	3.7	4	4.0	4	3.6	4	4.1	4	3.5	4	3.6	4	2.9	3
GLU	4.2	4	5.5	6	4.3	4	5.3	5	4.4	4	4.2	4	4.5	4
PRO	2.2	2	2.9	3	2.3	2	2.7	3	2.7	3	2.3	2	2.5	2
GLY	5.6	6	6.6	7	5.7	6	7.4	7	6.1	6	5.6	6	6.4	6
AL	3.6	4	4.5	4	3.4	3	4.6	5	3.8	4	3.3	3	4.0	4
CYS	nd		nd		1.1	1	nd		nd		1.1	1	nd	
VAL	3.8	4	4.9	5	4.2	4	4.8	5	4.2	4	4.2	4	4.3	4
MET	0.7	1	.96	1	.89	1	1	1	0.8	1	.88	1	1	1
ILEU	2.2	2	2.7	3	2.3	2	3.1	3	2.4	2	2.3	2	2.4	2
LEU	2.5	3	2.9	3	2.6	3	3.4	3	2.7	3	2.5	3	2.7	3
TYR	1.9	2	1.5	2	1.9	2	2.4	2	1.8	2	2.1	2	1.9	2
Ph.AL	2.4	2	2.2	2	2.6	3	3.0	3	2.3	2	2.6	3	2.2	2
LYS	2.0	2	2.4	2	2.3	2	2.4	2	2.3	2	2.3	2	2.4	2
HIS	1	1	1	1	1	1	1	1		1	1	1	1	1
ARG	2.6	3	2.8	3	3.0	3	2.6	3	2.7	3	3.1	3	2.8	3
TRYPTO	nd		nd		1.7	2	nd		nd		1.8	2	nd	

a) calculated from Results of Gozzone et al (1970)

b) n.d. not determined.

TABLE 4.5. CALCULATION OF MOLECULAR WEIGHT FROM EXPERIMENTAL VALUES OF AMINO ACIDS
FOR ORIGINAL ENZYME

Amino Acids	gm/100 gm of Protein	mols/10 ⁵	Molar Ratios with Histidineas			Mol Wt.
			1	4	8	
ASP	14.6	231	6.75	27.0	54.0	49191
TYR	4.3	76.8	2.21	8.8	17.6	48706
SER	5.4	110	3.20	12.8	25.6	49777
GLU	9.7	140	4.0	16	32	48494
PRO	4.1	76.2	2.20	8.8	17.6	49356
GLY	6.8	191.5	5.6	22.4	44.8	49411
AL	5.2	123	3.60	14.4	28.8	49292
VAL	7.3	132	3.80	15.2	30.4	48723
MET	1.7	24	0.70	2.8	5.6	49082
ILEU	4.6	76	2.20	8.8	17.6	50121
LEU	5.4	86.8	2.50	10.0	20.0	48518
TYR	5.8	67.6	1.97	7.8	15.7	48999
Ph.AL	6.6	84.2	2.46	9.8	19.6	49000
LYS	4.8	69	2.0	8.0	16.0	49666
HIS	2.5	34.2	1	4	8	49600
ARG	7.5	90.8	2.65	10.6	21.2	49184
Average						49100

TABLE 4.6. CALCULATION OF MOLECULAR WEIGHT FROM EXPERIMENTAL VALUES OF AMINO ACIDS FOR FRACTION 1

Amino Acid	gm/100 gm of protein	Mols/115	Molar Ratios with Histidine as.			Mol. Wt.
			1	4	7	
ASP	14.3	161.7	7.53	30.1	52.7	49014
THR	4.8	69.1	2.86	11.4	20.7	49758
SER	6.0	98.1	4.07	16.28	28.49	49864
GLU	11.5	133.0	5.5	22.0	38.60	49380
PRO	4.8	71.0	2.94	11.7	20.6	49407
GLY	7.0	159.7	6.62	26.4	46.3	49699
AL	5.4	108.9	4.49	17.9	31.4	51801
VAL	8.2	118.7	4.92	19.6	34.4	49193
MET	2.0	23.3	0.96	3.8	6.7	49915
ILEU	5.0	65.7	2.72	10.8	19.0	49997
LEU	5.5	71.2	2.95	11.8	20.6	49257
TYR	3.6	34.2	1.42	5.6	9.9	49943
Ph.AL	5.3	54.9	2.27	9.0	15.9	49643
LYS	5.0	58.7	2.43	9.7	17.0	44785
HIS	2.2	24.1	1	4	7	49318
ARG	7.0	68.2	2.83	11.3	19.8	49239
Average						49702

TABLE 4.7. CALCULATION OF MOLECULAR WEIGHT FROM EXPERIMENTAL VALUES OF AMINO ACIDS FOR FRACTION 2

Amino Acids	gm./100 gm.	Mols/10 ⁵	Molar Ratio with Histidine as			Molecular Weight
			1	4	7	
ASP	15.5	179.3	8.96	35.8	62.7	53217
THR	4.4	57	2.85	11.4	19.9	53995
SER	5.5	81.5	4.07	16.2	28.4	54456
GLU	10.2	106.1	5.30	21.2	37.1	53518
PRO	4.0	54.8	2.74	10.9	19.1	55142
GLY	7.2	149.5	7.47	29.8	52.2	54515
AL	5.4	93.2	4.66	18.64	32.6	53762
VAL	7.4	96.8	4.84	19.3	33.8	53567
MET	2.0	21.2	1.06	4.24	7.4	55274
ILEU	5.2	61.6	3.08	12.3	21.5	54314
LEU	5.9	69.7	3.48	13.9	24.3	54160
TYR	5.6	47.6	2.38	9.5	16.6	53847
Ph.AL	6.5	61.1	3.05	12.2	21.3	54285
LYS	4.6	48.7	2.43	9.7	17.0	54097
HIS	2.0	20.0	1	4	7	54253
ARG	5.8	51.4	2.57	10.2	17.9	53970
Average						54453

TABLE 4.8. CALCULATION OF MOLECULAR WEIGHT FROM EXPERIMENTAL VALUES OF AMINO ACIDS FOR FRACTION 3.

Amino Acids	Experimental Value gm./100gm.	Molar Ratios with Histidine as				Mol. Wt.
		Mols/10 ⁵	1	4	7	
ASP	14.6	175.5	7.19	28.7	50.3	45848
THR	4.3	57.7	2.36	9.4	16.5	45718
SER	5.6	85.8	3.51	14.0	24.5	46068
GLU	9.9	107.9	4.42	17.6	30.9	45941
PRO	4.8	67	2.74	10.9	19.1	45952
GLY	7.0	149	6.1	24.4	42.7	45750
AL	5.3	94.4	3.86	15.4	27.0	45373
VAL	7.6	104	4.26	17.0	29.8	45907
MET	1.8	19.4	07.95	3.1	5.5	45776
ILEU	4.9	59.6	2.44	9.7	17.0	45662
LEU	5.5	67.2	2.75	11.0	19.2	45850
TYR	5.1	44.4	1.81	7.2	12.6	45966
Ph.AL	6.0	57.6	2.36	9.4	16.5	45430
LYS	5.3	58.0	2.37	9.5	16.6	45700
HIS	2.4	24.4	1	4	7	45208
ARG	7.2	66.3	2.71	10.8	18.9	45844
Average						45749

TABLE 4.9. CALCULATION OF MOLECULAR WEIGHT FROM EXPERIMENTAL VALUES OF AMINO ACIDS FOR FRACTION 4

Amino Acids	ExPT Value gm/100 gm of Protein	Mols/10 ⁵ gm	Molar Ratios with Histidine as			Mol Wt.
			1	4	7	
ASP	19.7	153.3	7.3	29.2	51	46233
THR	4.3	49.9	2.35	9.4	16.4	95570
SER	4.7	62.7	2.98	11.9	20.9	46691
GLU	10.1	95.3	4.53	18.1	31.7	46234
PRO	4.4	52.7	2.50	10.0	17.5	45912
GLY	7.3	135.0	6.42	25.6	45.0	46232
AL	5.4	85.2	4.05	16.2	28.35	46807
VAL	7.7	96.8	4.32	17.2	30.2	45989
MET	1.8	17.0	0.81	3.2	5.6	46907
ILEU	4.9	52.1	2.48	9.92	17.3	46429
LEU	5.5	57.9	2.75	11.0	19.3	45969
TYR	5.4	41.5	1.97	7.8	13.8	46367
Ph.AL	5.5	46.8	2.22	8.8	15.6	46800
LYS	5.4	51.3	2.44	9.7	17.1	46233
HIS	2.3	21.0	1	4	7	47173
ARG	7.5	59.6	2.83	11.3	19.8	46090
Average						46352

TABLE 4.10. COMPARISON OF MOLECULAR WEIGHTS OF PORCINE PANCREATIC
ALPHA-AMYLASE AND ITS FRACTIONS
OBTAINED BY DIFFERENT METHODS.

-3
M x 10

SAMPLE	a	b	c	D	Average
ORIGINAL	49.1	48.4	47.6	47.6	48.1
FRACTION 1	49.7	47.9	53.5	52.4	50.9
FRACTION 2	54.4	50.6	55.5	55.3	53.1
FRACTION 3	45.7	47.6	50.5	48.4	48.0
FRACTION 4	46.3	47.9	52.5	46.6	48.3

Calculated by Method of Brand et al	Bio-gel Coloumn	Sedimentation from Table 4.1	Calculated from Minimum Amino Acid Residues Table 4,4.
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DISCUSSIONFractionation of Porcine pancreatic alpha-amylase.

Porcine pancreatic alpha-amylase obtained from Sigma Chemical Company, was found to be homogeneous by ultracentrifugation. This enzyme was put on a DEAE-cellulose coloumn and eluted with buffer pH 8.5 (Tris-HCl, 0.1M) containing $10M^{-4}$ calcium chloride. The resultant elution pattern is shown in FIG. 4.2. It was found that none of the four fractions obtained show alpha-amylolytic activity. In contrast Rowe et al (1968) obtained only two fractions under similar conditions, both of them were found to be active. However, the method followed by these authors was to fractionate the enzyme on the colpumn, and then combine 4 ml samples before measuring the absorbance. It is clear from the elution pattern in FIG. 4.2 that if 4 ml samples were combined together and absorbance measured manually to obtain the elution pattern, peaks No. 1, 2 and 3, 4 would have mixed together to show only two peaks.

Moreover, the buffering capacity of the phosphate buffer at pH 8.5 used for elution by Rowe et al is questionable. This resolution is highly dependent on the pH of the elution buffer, so it is possible that using a more stable Tris-HCl buffer at pH 8.5, a better resolution can be obtained. Cozzone et al (1970) have recently subjected porcine pancreatic alpha-amylase to ion exchange colpumn chromatography under the conditions described by Rowe et al (1968). They have found that the first peak can be resolved into two definite peaks under slightly modified conditions although

they failed to get similar resolution by disc-electrophoresis. The second peak obtained by them was a broad one and it is possible that it contains more than one protein. This is in accordance with the present work that there are 4 different peaks instead of two as shown by Rowe et al (1968).

The amount of protein recovered was 80% in the terms of four definite areas, but no activity was recovered. However, it was found that activity in the fractions cannot be obtained until the amount of calcium chloride was increased to 0.003 M in the buffer. In further experiments therefore, buffer containing 0.003 M calcium chloride was used. It is suggested that activity is lost due to complexing of calcium ions during elution of the enzyme from the DEAE cellulose column. Calcium ions form co-ordinate complexes with side groups of amino acids to link peptide chains together. When calcium is taken out the enzyme loses its configuration which is necessary for its activity. Importance of calcium ions has already been discussed in Section 1. It was found that addition of calcium to the inactive fractions reactivates them.

The above studies were carried out on the enzyme obtained from Sigma Chemical Company. Porcine pancreatic alpha-amylase obtained from Worthington Chemical Company was also subjected to ion exchange chromatography. The resultant elution pattern is shown in the FIG. 4.4. The significance of the differences is discussed later.

Characterisation of the Fractions.

(a) Chromatography on Bio-gel column.

All the four fractions obtained were rechromatographed

on the DEAE cellulose coloumn and were found to emerge as a single peak. (FIG. 4.3). These fractions and the original enzymes were eluted from a standard Bio-gel coloumn and molecular weights calculated from the standard graph. (Table 4.10). It is clear that there is no difference in the molecular weights of the samples indicating the basic similarity of their structure.

(b) Sedimentation behaviour.

Fractions obtained from the coloumn were very dilute and it was not possible to determine their molecular size by conventional studies in the ultra-centrifuge. Bordman et al (1960) have tried another method for sedimentation studies on dilute solutions of enzymes. This method is based on measuring the loss of activity during the course of ultra-centrifugation. The cells of ultra-centrifuge contained a certain thickness of filter papers which traps the sedimented enzyme. The principle involved is that during a certain spinning time, the molecules of the enzyme will sediment according to their size and they will be proportionally trapped by the piece of filter paper. The supernatant enzyme will loose the activity accordingly. The sedimentation co-efficient is related to the loss of activity in the following way

$$S = \frac{-1.75 \log(1 - \Delta C / C_0) \lambda}{\bar{V}^2 t}$$

where ΔC and C_0 are the loss of activity and original activity respectively, $\bar{V}$ is the average speed and t is the time. λ is calculated from $r_p^2 / (r_p^2 - r_m^2)$ r_p^2 is the

distance of centre of rotation from surface of the paper and r_m distance of centre of rotation from the meniscus.

It was found, however, that the above relation was not satisfactory and the results were always greater for the original enzyme which was used as control. A correction to include the time for acceleration and deceleration was necessary and gave satisfactory results when following equation was used.

$$S = \frac{-1.75 \log(1 - \Delta^c / c_0 \lambda)}{\bar{V}^2 (t + x)}$$

where x is the acceleration and deceleration time correction.

The correction was obtained on Beckman model E ultra-centrifuge by counting the revolutions from the reference counter from starting time to the time of reaching the maximum speed, for the time of maximum speed, and from the time of deceleration to the end of spinning. The revolutions at the maximum speed for a definite time gave the revolutions per minute at maximum speed. Therefore, correction for acceleration and deceleration time was calculated from the revolutions in acceleration and deceleration time.

Special care had to be taken while taking the solutions out of the cells, so as not to mix the sedimented enzyme again which will lead to gross errors. A syringe was used to take the solutions out and the tip of the needle was never allowed to touch the filter papers. It was found that 3x1 thickness of Whatman No. 1 filter paper was sufficient to change the activity within range for measurement under the

conditions described in Section 2.

Table (4.1) shows the sedimentation co-efficients of the original enzyme and the four fractions obtained. The sedimentation co-efficients are comparable apart from the fraction 2. which is higher than others indicating its higher molecular weight than the others. The above method is very useful in determining the molecular size of the proteins in dilute solutions as it is independent of concentrations.

(c) Action on Amylose.

Reducing value as a function of Blue value.

FIG. 4.5 shows the increase in reducing power of the digests as a function of blue value for the original porcine pancreatic alpha-amylase and the four fractions obtained from ion-exchange chromatography. It is clear that there is no difference in the action of 4 fractions and the original enzyme at pH 4.8, in presence and in absence of glycerol. (40%). Reducing values increase at the same rate with the decrease in blue value for all the four fractions and it is comparable to that of original enzyme. When 40% glycerol was present in the digests the reaction became random as is shown by slow increase in reducing value of the digest. Rowe et al (1968) have also shown that the two fractions were very similar in their properties and structure in agreement with the present work.

AMINO ACID ANALYSES.

Table (4.2) shows the amino acid composition of the original enzyme and the four fractions obtained. The

hydrolysis was carried out for 24, 48 and 72 hours for the original enzyme. Results shown in the Table are the corrected values for destruction of serine, threonine, and glutamic acid. It is obvious that there is no appreciable difference in the composition of the original enzyme and the four fractions. These results are quite different from the values reported by Caldwell et al (1954) for porcine pancreatic alpha-amylase but are similar to those reported by recent workers (Straub et al 1969, ~~Marchis~~ ^{Cozzone} ~~Murson~~ et al 1970). The major differences from the values reported by Caldwell et al (1954) are in arginine which is rather a higher value although it is in agreement with Cozzone et al (1970).

Table 4.3 shows the percentages of amino acids present in the hydrolysates for the enzyme samples. In addition to tyrosine other hydroxylated amino acids are highly represented (Ser + Thr 9.5%). Alpha-amylase seems to contain large amount of acidic residues (19%) which explains the acidic isoelectric point of the enzyme.

Minimum amount of amino acid residues for the original enzyme and the fractions and the minimum residues calculated from the results of Cozzone et al (1970) for the two fractions they obtained, are shown in Table 4.4 The residues are represented in the nearest whole numbers and are obtained by dividing the amounts (μ mols from Table 4.3) of all the amino acids by that of histidine which is taken as one. The results again show that the original enzyme and the fractions are virtually the same. No structural

difference is indicated from the amino acid composition of the samples. The results are in agreement with those of Cozzone et al (1970), (Table 4.4 coloumn F₁^a and F₃^b). The only difference is that the fraction 1 and 2 have one residue of glutamic acid, glycine and valine each, more than the other fractions. Fraction two also has a rather higher amount of aspartic acid.

The explanation has been put forward by Rowe et al (1968) that a charge difference is responsible for the resolution of enzyme on the DEAE Cellulose coloumn. If this was so, the fractions obtained should be in increasing order of acid contents. Fractions 1 and 2 have higher acid contents than the original enzyme which may explain their separation but fraction 3 and 4 have the same amount of acid contents as the original enzyme. It seems that charge difference is not due to the amount of acids. Cozzone et al (1970) have also reported equal values of acids for fractions obtained by them. So it is possible that charge difference is not in the acid but basic contents of the enzymes.

This work was carried out with the expectation of finding some differences in the structure and properties of porcine pancreatic alpha-amylase, which would explain its different behaviour than the other alpha-amylases. However, no difference was found in the properties and amino acid compositions of the enzyme and its fractions. Tables 4.5, 4.6, 4.7, 4.8, and 4.9, show the molecular weights of the original enzyme and the fractions calculated

from the experimental values by the method of Brand et al (1945) with following equation.

$$M_{wt} = M_{a.a} \times R \times 100/\text{gm in 100 gm.}$$

$M_{a.a}$ is the molecular weight of the amino acid and R is the molar ratio of the amino acid. For the molecular weights calculated by this method, there should be 8 histidine residues in the original enzyme while all the fractions have 7 residues. The residues have been presented by the nearest integer whole number, so the difference is possible if the residues in the original enzyme are the average of the residues of all the fractions.

A comparison of molecular weights of the enzyme and its fractions, obtained by different experimental methods and calculated from amino acid analyses is shown in table 4.10. Coloumn D of the table 4.10 shows the molecular weights calculated by adding up the weights of amino acid residues supposing histidine as 7 residues. Cozzone et al (1970) have suggested the molecular weight of the fractions to be 53000 which is quite in agreement with the present work.

Molecular weights of the original enzyme and the fractions appear to be similar apart from fraction 2. This fact is rather surprising, because no difference in the properties of fraction 2 and other samples is observed. Fraction 2 has been shown to have higher amounts of certain amino acids such as aspartic acid, glutamic acid, valine and alanine. These amino acids probably play no important part in the action pattern of porcine pancreatic alpha-amylase.

As the fractions of the enzyme are of the same molecular weight as the original enzyme, they cannot be sub-unit of the alpha-amylase and might be isoenzymes. The reason of their separation on an ion-exchange coloumn may lie in their tertiary structure. Probably some of the amino acids have more NH_2^+ groups on the outer side of the peptide chains. It is possible that during the course of isolation these groups are preferentially destroyed and all the fractions possess different charge without any basic difference in their structure and properties. This idea was further strengthened, when porcine pancreatic alpha-amylase obtained from a different firm, Worthington Chemical Corporation, was subjected to ion-exchange chromatography under similar condition. This sample emerged as a single peak with only small irregularities in the positions where all the previous fractions appeared (FIG. 4.4).

Rowe et al (1968), however, suggested that multiple forms were not artifacts arising from the purification processes. Marchis Mureen et al (1967) have reported that the enzyme prepared by the precipitation as its glycogen complex has an acylated amino-terminal, while the one prepared by Fischer and Stein procedure has a glycine amino terminal. This shows the differences in the enzymes prepared by different methods. These two enzymes, prepared by different techniques, however, showed similar banding patterns on gel-electrophoresis and similar elution pattern on ion-exchange chromatography.

Comparison of FIG. 4.2 and 4.4 shows the elution

pattern of alpha-amylases obtained from different Companies is different. These Companies probably use different methods for isolation and purification of the enzyme. It is suggested that the method used by Worthington Chemical Corporation is probably better as the enzyme behaves as a homogeneous protein even on ion-exchange chromatography. The method used by Sigma Chemical Company causes some of the basic groups attached to the outer chains of alpha-amylase to be destroyed so the molecules obtain a charge difference. This charge difference is responsible for their resolution on ion-exchange column, but all the fractions remain basically the same enzyme. It is thus unlikely that the phenomenon is due to presence of iso-enzymes in the porcine pancreatic alpha-amylase.

The explanation put forward by Rowe et al (1968) that these multiple forms could be under different genetic control does not explain our results. If the multiple forms are under different genetic control there must be some difference in their composition and behaviour. However, so far, no differences have been found in their specific activity, thermal and pH stability, dependence of $V_{\max}$ and K_m on pH and their overall stereochemical control of hydrolysis as shown by Rowe et al (1968). The present work shows no difference in their action on amylose, in presence and absence of glycerol and in their composition of amino acids. No difference could be found in the ratio of polar and apolar amino acids calculated by

the scheme proposed by Hatch (1965). All these results indicate that we could possibly be dealing with one enzyme in which some of the substituted outer groups or ions are destroyed in the purification procedure giving rise to 2 - 4 fractions with varying charge.

As there is ^{no} difference found in the structure and properties of the fractions obtained it is difficult to specify the reason for the different behaviour of the porcine pancreatic alpha-amylase than the other alpha-amylases. The reason might be due to a difference in amino acid sequence which is highly improbable. Again this could be due to differences in secondary and tertiary structure of this alpha-amylase in which the peptide chains are so conformed that either they bring two binding sites close enough to produce small sugars from a long chain substrate, or the conformation is well suited to hold the substrate even after the first cleavage.

SECTION 5

PREPARATION AND PROPERTIES OF SYNTHETIC
LONG-CHAIN AMYLOPECTINS, GLYCOGENS AND
LINEAR AMYLOSE OLIGOMERS.

INTRODUCTION

Synthesis of blue iodine-staining polysaccharides was achieved as long ago as 1940, immediately after the discovery of phosphorylase (Cori and Cori, 1939) but these synthetic polysaccharides were not characterised until Swanson (1948) studied a series of synthetic oligomers in detail. She found that a purple iodine stain was observed at a DP of 6, then there was a transition of colour from purple to red and finally to blue, with the increase in DP. Chains as long as 50 - 150 glucose units gave absorption spectra closely resembling those of amylose.

Bailey and Whelan (1961) further investigated the iodine staining properties of synthetic linear polysaccharides. They prepared a series of amylose oligomers of $\overline{DP}_n$ varying from 6 to 580 and reported that these oligomers become iodine-staining at a $\overline{DP}_n$ of 18. The iodine stain increases linearly with increase in $\overline{DP}_n$ up to 72 and thereafter the relation becomes non-linear. The blue value of the naturally occurring amylose was not reached until the chain exceeds 400 units. It appears, therefore, that $\overline{DP}_n$ of the polysaccharides can be related to their iodine staining ability. Recently Pfannemuller et al (1969) have also related the iodine binding capacity to the $\overline{DP}_n$ of the polysaccharides.

Phosphorylase is unable to synthesise long-chain polymers without added primer (Fukui and Kamagowa, 1969) Whelan and Bailey (1954) have shown that to start the

synthesising reaction of phosphorylase, at least 4 glucose units - unsubstituted and joined linearly - are needed. They found that maltotriose was an inefficient primer for phosphorylase, whilst maltotetraose and maltopentaose were very efficient primers. These findings were confirmed by Pfannemuler and Burchard (1969) who found that for preparation of blue-staining polysaccharides, maltotetraose and maltohexaose were good primers. The reaction with maltotriose was 400 times slower. The priming capability of larger molecules i.e. amylose, amylopectin and glycogen has also been reported by Swanson (1948). The products synthesized by using amylopectin and glycogen as primers, by phosphorylase, have not been studied to date.

The properties of synthetic amylose oligomers have been reported by many workers. Husemann (1960) found that synthesis of amylose with pure phosphorylase proceeds with increase in degree of polymerisation, without a terminating point. Husemann and Pfannemuller (1961) have shown that enzymically-prepared amylose behaves differently than natural amylose. Synthetic amylose was much more easily soluble than natural amylose. The reaction of beta-amylase was also different on both the samples. However, it was found that after long storage, synthetic amylose acquires the properties of natural amylose. These differences can be explained on the basis of there being a different helical content in natural amylose. It is widely recognised now that helix is an important structural characteristic of amylose. A freshly

synthesised amylose is not expected to coil immediately; it will acquire the characteristics of natural amylose only after long storage.

The studies on synthetic amyloses by the above authors, and those of Swanson (1948), and Bailey and Whelan (1954) have been helpful to understand the properties and structure of natural amylose. But many properties of amylopectin and glycogen are still not clear. The structure of amylopectin and glycogen might be thought to be very similar, because both of them appear to have tree-like structure proposed by Meyer and Bernfield (1940). The only major difference between the two, is in their degree of branching, which is 4 - 5% in amylopectin and 7 - 10% in glycogen. However amylopectin gives a violet stain with iodine and has a β -amylolysis limit of around 55% conversion into maltose, whilst glycogen gives a red stain with iodine and has a β -limit of about 40%. Even greater differences are found in their solution behaviour. The limiting viscosity number of glycogen in water is about 10 ml/g, but the corresponding value for amylopectin is of greater magnitude. It is to be noticed that weight average molecular weight of both polysaccharides is about 10^8 .

In order to elucidate many points in the structure of these polysaccharides, and the action pattern of phosphorylase, synthetic amylose oligomers, long chain amylopectins and glycogens were prepared by using maltohexaose, and maltoheptaose, waxy-maize amylopectin and ~~horse~~ Oyster glycogens as primers, respectively. Their iodine binding capacities and solution behaviours are detailed.

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and was checked for the presence of any glucose by glucose oxidase method (Section 2). No glucose was found whatsoever and so the G-1-P was used without further purification.

2. Isolation and purification of phosphorylase

a. Juice extraction

Potatoes (1 kg) were peeled and steeped in dithionite solution (0.5%) for twenty minutes and washed thoroughly with cold distilled water. Citrate buffer (100 ml, 0.5 M, pH 7.0) was added and the potatoes were pulped in a hand mincer. The juice was separated from the pulp by filtering through muslin and allowed to stand for 4 hours to allow inert protein and non protein material to settle out. The supernatant liquor was centrifuged and the precipitate (mainly starch) was rejected. The clear juice was heated to 56°C in a constant temperature water bath for 15 minutes after attaining temperature equilibrium and immediately cooled in an ice bath. The volume of the juice was made up to 500 ml if necessary.

b. Ammonium Sulphate fractionation.

Ammonium sulphate fractionation was carried out as described by Holló et al (1964). All the fractionation was carried out at 4°C. Ammonium sulphate was added to the juice slowly, with stirring, to bring the specific gravity (S.g) of the solution to 1.085. The solution was stirred for 15 minutes,

EXPERIMENTAL AND RESULTS

1. Substrate

a. Glycogen

Oyster glycogen was obtained from B.D.H. Limited.

b. Preparation of Maltohexaose (G₆) and Maltoheptaose (G₇)

Amylose was incubated with Bacillus Subtillus alpha-amylase, and G₆ and G₇ were isolated from the digest by paper chromatography. (Whatman, 4.mm paper). The sugars were rechromatographed to obtain pure samples and after elution of the sugars the solutions were freeze dried..

c. Waxy Maize Amylopectin

Amylopectin was isolated from waxy maize starch. Solid starch (15 gm) was added to boiling water (100 ml) and stirred vigorously for 30 minutes. The viscous solution was diluted to 3 litres with distilled water and boiling along with stirring continued for a further one hour. After cooling to 60°C, thymol (1 gm/l) was added and stirring continued. The solution was then passed through Sharples supercentrifuge. A small amount of polysaccharide thymol complex was removed. The supernatant was shaken repeatedly with chloroform to remove any thymol present, concentrated to 500 ml on a rotary evaporator and freeze dried.

d. Glucose-1-phosphate (G-1-P)

The dipotassium salt was obtained from B.D.H. Limited,

allowed to stand for 30 minutes and centrifuged in a refrigerated centrifuge (2500 xg, 20 minutes).

The ~~centrifuge~~ ^{Precipitate} was discarded and the specific gravity of the solution was brought up to 1.152 by adding more ammonium sulphate as described above. The solution was then centrifuged and the supernatant liquor discarded. The precipitate was dissolved in distilled water (250 ml) to yield fraction 1.

This solution was further fractionated between S.g 1.095 and 1.145. The precipitate obtained was dissolved in 150 ml distilled water to yield fraction 2. The fractionation was repeated between S.g 1.10 and 1.14 to obtain fraction 3. Fraction 4 was

obtained by fractionating fraction 3 between S.G. 1.10 and 1.135 and was dissolved in citrate buffer (10 ml 0.1M pH 6.3).

Results obtained for the activity and protein concentration are shown in Table 5.1. The percentage yield and the concentration of the solution with respect to specific activity are given in Table 5.2.

c. Measurement of phosphorylase activity.

Measurement of phosphorylase activity has been detailed in Section 2.

d. Tests for purity of phosphorylase.

(i) Presence of Alpha-amylase

Amylose solution (20 ml, 2 mg/ml) containing citrate buffer (2 ml 0.1M pH 6.3) was placed in a viscometer and allowed to obtain temperature

TABLE 5.1

PHOSPHORYLASE ACTIVITY AND PROTEIN CONCENTRATION OF
THE SOLUTIONS DURING AMMONIUM SULPHATE FRACTIONATION.

Samples	Volume	Enzyme Activity K x 1000	Total Protein (mg)
Juice	500	53000	21000
Fraction 1	250	21570	2060
Fraction 2	150	13800	760
Fraction 3	50	8750	350
Fraction 4	10	3510	115
Dialysate	13	4563	104

TABLE 5.2

CHANGES IN SPECIFIC ACTIVITY, CONCENTRATION AND YIELD
OF PHOSPHORYLASE DURING AMMONIUM SULPHATE FRACTIONATION.

Samples	Specific Activity (Activity/mg of protein)	Concentration	Yield (%)
Juice	2.5	-	100
Fraction 1	10.8	4.3	40.5
Fraction 2	18	7.0	26
Fraction 3	25	10.0	16.5
Fraction 4	30.5	12.6	6.7
Dialysate	43.7	17.5	8.5

equilibrium in a constant temperature water bath at 25.5°C. Phosphorylase solution 0.5 ml was added and flow time measured immediately and after 15 hours.

(ii) Presence of Debranching enzyme

A digest containing amylopectin (3 mg/ml, 5 ml) containing citrate buffer (0.5 ml, 0.1M, pH 6.3) and phosphorylase (0.2 ml) was incubated at 37°C. Aliquot portions were taken out as a function of time and their iodine staining ability measured.

3. Preparation of long chain Amylopectins.

The following digests were prepared.

- (i) Amylopectin (70 mg), G-1-P (400 mg) in 10 ml solution containing citrate buffer (2 ml, 0.1M, pH 6.3) and phosphorylase solution (0.2 ml)
- (ii) Amylopectin (50 mg), G-1-P (400 mg) in 10 ml solution containing citrate buffer (1 ml 0.1M, pH 6.3) and phosphorylase solution (0.2 ml).
- (iii) Amylopectin (25 mg), G-1-P (400 mg) in 10 ml solution containing citrate buffer (1 ml, 0.1M, pH 6.3) and phosphorylase solution (0.2 ml).
- (iv) Amylopectin (10 mg), G-1-P (400 mg) in 10 ml solution containing citrate buffer (1 ml 0.1M pH 6.3) and phosphorylase solution (0.2 ml).

All these digests were put in dialysis tubing and incubated at 37°C for 5 hours while being dialysed against citrate buffer (0.1M pH 6.3). The digests were then transferred to flasks and

enzyme activity destroyed by heating for 10 minutes in a boiling water bath. Amylopectin was then precipitated with three volumes of ethanol. The precipitate was repeatedly shaken with 50% aqueous^u methanol (200 ml aliquotes) in order to remove unused G-1-P. It was found that the amount of G-1-P used in the digests (400 mg) could be dissolved in this way. The amylopectin precipitate was washed with absolute alcohol and dried overnight in vacuo at 65°C.

4. Preparation of Glycogens with long external chains

The following digests were prepared.

- (i) Glycogen (50 mg), G-1-P (500 mg) citrate buffer (1 ml, 0.1M, pH 6.3) in a total volume of 10 ml, and incubated with phosphorylase (0.2 ml) at 37°C for 2.5 hours.
- (ii) Glycogen (40 mg), G-1-P (500 mg), citrate buffer (1 ml, 0.1M, pH 6.3) in a total volume of 10 ml and incubated with phosphorylase (0.2 ml) at 37°C for 5 hours.
- (iii) Glycogen (25 mg), G-1-P (500 mg) citrate buffer (1 ml, 0.1M pH 6.3) in a total volume of 10 ml and incubated with phosphorylase (0.2 ml) at 37°C for 5 hours.

The enzyme activity was destroyed by heating for 10 minutes in a boiling water bath.

Synthetic glycogen samples were isolated and purified as described for amylopectins.

5. Preparation of amylose oligomers.

A master equimolar solution of G_6 and G_7 ($1.08 \times 10^{-3}M$) dissolved in 0.1M citrate buffer (pH 6.3) was prepared and stored at $4^\circ C$ under toluene. Digests were prepared in the following way:-

$G-1-P$ (600 mg) was dissolved in an aliquote of primer mixture solution and diluted to 10 ml with citrate buffer (0.1M, pH 6.3), including phosphorylase solution (0.2 ml) and incubated at $37^\circ C$ for varying time periods. The details of amounts of master solution and incubation time are given in Table 5.3.

TABLE 5.3

Conditions for preparation of amylose oligomers

Sample	1	2	3	4	5	6	7	8	9
Master Sol: ution (ml)	10	7.5	7.5	5	2.5	2.5	2.5	2	1
Time of incubation (hr)	5	3.5	5	5	5	10	18	18	18

6. Dissolution of synthetic samples.

The most suitable solvent for synthetic samples was found to be dimethyl sulphoxide (DMSO). Long chain amylopectin, glycogen and amylose oligomers were accordingly dissolved in DMSO to give approximately 1%, 4% and 1.5% solutions (W/V) respectively.

(c) Measurement of $\overline{DP}_n$ of the synthetic amylose oligomers

$\overline{DP}_n$ was measured as described by Banks and Greenwood (1968) with the modification of the method of estimating glucose, described in Section 2. This method depends on the presence of equal numbers of odd and even-numbered glucose chains. This equality was ensured by using equimolar mixture of G_6 and G_7 primer. Results are shown in Tables 5.8 and 5.11.

(d) Sedimentation behaviour of long chain Glycogens.

Sedimentation studies were carried out on the Beckman Spinco model E ultracentrifuge as described in Section 2. The temperature indicator and control unit was used to maintain the temperature of the rotor at 24°C . All the runs were made at 20410 r.p.m.

For the parent glycogen and glycogens $G_{(1)}$ and $G_{(2)}$ measurements were carried out in a DMSO-water mixture (1:9 V/V). It was found, however, that sample $G_{(3)}$ precipitates during the course of sedimentation, therefore, pure DMSO was used for studies on $G_{(3)}$. A control run using original glycogen $G_{(0)}$ was also carried out in DMSO. The concentration range for dilutions was $(0.1-0.5)10^{-2}$ gm/ml. Results are shown in FIG. 5.1.

(e) Measurement of iodine binding capacity.

The potentiometric titrations were carried out as described in general experimental techniques. Results are shown in Tables 5.4, 5.5, 5.7, 5.10 and 5.12. Iodine bound and iodine free are plotted in FIG. 5.4, 5.5, 5.6, 5.7 and 5.8.

FIG 5.1

SEDIMENTATION BEHAVIOUR OF LONG CHAIN GLYCOGENS

(f) Measurement of limiting viscosity number.

The limiting viscosity number was measured in DMSO as described in Section 2.

(g) Concentration of synthetic products.

Synthetic products were diluted to a concentration of 20 - 50 $\mu\text{g}/\text{ml}$ and hydrolysed with amyloglucosidase. The resulting glucose was estimated by glucose oxidase method.

7. Characterisation of synthetic products.

- (a) Measurement of the wave length of maximum absorption ($\lambda_{\max}$) of the polysaccharide iodine complex.

Polysaccharide solution in DMSO (0.05 ml, approx 0.5 mg) and standard KI solution (0.2 ml, 5 gm, KI + 0.5 gm I_2 in 250 ml) were diluted to 10 ml in a graduated flask. $\lambda_{\max}$ was measured on a Unicam SP 800 spectrophotometer using a 10 mm cell, with reference to a blank prepared in a similar way omitting the polysaccharide only. Results are shown in Tables 5.4, 5.5 and 5.8.

- (b) Measurement of the β -Amylolysis Limit.

The β -amylolysis limit for the synthetic products was obtained by the concurrent action of beta-amylase and pullulanase as described by Banks and Greenwood (1967).

Digests were prepared as follows:-

- (i) Polysaccharide solution, (0.8 ml) acetate buffer (2.5 ml, 0.1M pH 4.9) beta-amylase solution (0.15 ml, 8 units) and diluted to 25 ml with distilled water.
- (ii) 8 ml of digest (i) was incubated with crude beta-amylase and pullulanase.

The digests were incubated at 37°C overnight. The amount of maltose produced was calculated by estimating the reducing power as described in Section 2. Taking the amount of maltose produced by concurrent action of beta-amylase and pullulanase as 100%, the amount produced by beta-amylase only gives the beta amylolysis limit. Results are shown in 5.4, 5.5, and 5.7.

FIG 5.2

FIG 5.3

IODINE TITRATION CURVES FOR LONG CHAIN GLYCOGENS

FIG. 5.4

IODINE TITRATION CURVES FOR LONG CHAIN GLYCOGENS

Molarity of total free iodine ($\times 10^6$)

FIG. 5.5

IODINE TITRATION CURVES FOR LONG CHAIN AMYLOPECTINS

119d

FIG 5.6

FIG. 5.7

119f

FIG 5.8

LINEAR AMYLOSE OLIGOMERS

IODINE BOUND VS IODINE FREE AT HIGH TEMPERATURE

FIG 5.9

LINEAR AMYLOSE OLIGOMERS

FIG. 5.10

LONG CHAIN AMYLOPECTINS

FIG 5.11

LONG CHAIN AMYLOPECTINS

FIG. 5.12

FIG 5.13

LINEAR AMYLOSE OLIGOMERS

TABLE 5.4

PROPERTIES OF WAXY-MAIZE AMYLOPECTIN, AND OF
SYNTHETIC LONG-CHAIN AMYLOPECTINS.

Sample	Waxy- maize amylo- pectin Ap ⁰	<u>Synthetic amylopectin</u>			
		Ap1	Ap2	Ap3	Ap4
$\lambda_{\max}(\text{m}\mu)$	529	544	556	575	587
$[\beta]^1$	58	71.5	75	85	93
$[\beta + \text{pu}]^1$	100	101	102	101	102
$\overline{\text{CL}}_n^1$	19.7	29.2	36.0	52	115
External Chain-length	13.7	23.4	29.9	46.5	109.5
Internal Chain-length	6.0	5.8	6.1	5.5	5.5
I.B.C. at 20°C ²	0.10	(0.3)	(7.1)	(10.0)	14.3
I.B.C. at 1.5°C ²	0.15	(7.3)	(9.6)	16.1	17.1
$\lambda_{\max}$ of β -limit dextrin	525	525	525	525	525

¹ See Text.

² I.B.C. = Iodine binding capacity expressed as mg of bound per 100 mg of polysaccharide. Values in parentheses ambiguous - see discussion.

TABLE 5.5

PROPERTIES OF GLYCOGEN, AND OF SYNTHETIC GLYCOGENS
HAVING LONG EXTERNAL-CHAINS.

Sample	G(0)	G(1)	G(2)	G(3)
λ_{max} of Polysaccharide/ iodine complex	—*)	584	610	630
β -amylolysis limit ¹	40	49	56	82.5
Iodine binding capacity at 1.5°C ²	—	2.71	5.82	15.3
Iodine binding capacity at 20°C ²	—	2.28	5.24	13.3
$[\eta]$ in DMSO ml/g	11	13	30	80

¹ Percentage conversion into maltose by crystalline β -amylase.

² mg. of iodine bound per 100 mg. of polysaccharide.

*) No stain under conditions used.

TABLE 5.6

VALUES OF CHAIN-LENGTH AND EXTERNAL CHAIN-LENGTH
FOR THE SYNTHETIC GLYCOGEN SAMPLES, CALCULATED FROM
THE DATA IN TABLE 1.

Sample	Chain-length	External Chain-Length
G(0)	12.5	7.5
G(1)	14.7	9.7
G(2)	17.1	12.1
G(3)	42.8	37.8

TABLE 5.7

IODINE AFFINITIES OF AMYLOSE, GLYCOGEN AND LONG-CHAIN
SYNTHETIC GLYCOGEN, AND PARAMETERS CALCULATED FROM
THESE MEASUREMENTS.

Sample	<u>Iodine binding</u> <u>capacity</u>		<u>% amylose</u>		<u>β-amylolysis</u> <u>limit</u>	
	1.5°C	20.4°C	1.5°C	20.4°C	Calc.	Exptl.
Amylose	22.0	19.5	100	100	-	100
G(0)	-	-	-	-	-	40
G(1)	2.71	2.28	12.3	11.7	47	49
G(2)	5.82	5.24	26.5	26.8	56	56
G(3)	15.3	13.3	69.5	68.0	81	82.5

TABLE 5.8

VALUES OF NUMBER-AVERAGE DEGREE OF POLYMERISATION $\overline{DP}_n$
AND WAVE LENGTH OF MAXIMUM ABSORPTION OF IODINE
COMPLEX ($\lambda_{\max}$) FOR SYNTHETIC AMYLOSE OLIGOMERS.

Sample	1	2	3	4	5	6	7	8	9
$\lambda_{\max}$	496	524	530	546	574	588	595	606	610
$\overline{DP}_n$	22.2	28.9	31.3	36.4	50.7	71	93	105	134

TABLE 5.9

COMPARISON OF THE CONSTANTS A AND B OBTAINED BY
VARIOUS WORKERS

Constant	$(1/A) \times 10^3$	$(B/A) \times 10^4$	$B \times 10^5$
Bailey and Whelan (1961)	1.56	88	1.37
Pfannemuller <u>et al</u> (1969)	1.637	92.5	1.51
Present work	1.558	102.5	1.60

TABLE 5.10

VALUES OF IODINE-BINDING CAPACITIES FOR AMYLOSE
OLIGOMER AT VARIOUS TEMPERATURES.

Sample	Iodine-binding capacity (mg I ₂ /100 mg amylose)			
	1.4°C	7,7°C	14.2°C	20.4°C
1	7.8	n.d. ^a	n.d.	— ^b
2	9.4	n.d.	n.d.	—
3	12.9	n.d.	n.d.	—
4	19.2	n.d.	n.d.	3.6
5	19.6	18.8	14.8	11.1
6	20.7	n.d.	n.d.	n.d.
7	21.0	20.2	18.9	16.2
8	21.4	20.5	19.5	16.4
9	21.8	20.9	20.1	17.3
Amylose	22.2	21.7	20.7	19.5

^a n.d. = not determined

^b — = not possible to extrapolate experimental values to positive iodine affinities.

TABLE 5.11

THE RATIOS, $F(I)$, OF IODINE-BINDING CAPACITY AT 20.4°C TO THOSE AT 1.4°C AS A FUNCTION OF $\overline{DP}_n$.

Sample	$\overline{DP}_n$	$F(I)$
4	36.4	0.187
5	50.7	0.566
7	93	0.772
9	1,500	0.880

TABLE 5.12

VALUES OF FREE IODINE CONCENTRATION AT HALF-SATURATION, $[I_f]_v$, AS A FUNCTION OF TEMPERATURE AND $\overline{DP}_n$ AND THE DERIVED ENTHALPIES.

Sample	$\overline{DP}_n$	$[I_f]_v \times 10^6$				$-\Delta H$ Kcal/ mole
		1.4°C	7.7°C	14.2°C	20.4°C	
5	50.7	2.44	3.70	5.86	9.08	11.4
7	93	0.90	1.61	2.61	4.40	13.4
8	105	0.70	1.35	2.36	3.75	14.4
9	134	0.50	0.91	1.73	2.60	14.8
Amylose 1,500		0.25	0.50	0.92	1.50	15.7

DISCUSSIONPreparation and purification of phosphorylase.

The method used for isolation of phosphorylase was that described by Holló et al (1964). The crude preparation of phosphorylase was found to be free from any contaminating carbohydrases and could be used without further purification. Alpha-amylase was absent from the phosphorylase as it was found that there is no change in the flow-time of an amylose solution over a period of 15 hours. Absence of alpha-amylase is further confirmed by the fact that experimental values of chain length determined for long chain amylopectin are in agreement with the theoretical values (FIG. 5.10). If any trace of alpha-amylase had been present, the experimental values could not have fitted the theoretical curve.

Holló et al (1964) have, however, reported a small amount of alpha-amylase in potato phosphorylase prepared by this method. It was found that there is some variability in the amount of alpha-amylase surviving the heat treatment. Huseman et al (1958) have reported that enzyme, isolated from potato juice subjected to heat treatment for 15 minutes at 54°C, contained a small amount of carbohydrase activity, which was shown by the change in viscosity of the amylose solution. In these experiments no hydrolase activity whatsoever was found if the juice was heated at 56°C for 15 minutes, although the phosphorylase activity also decreased by 20%. For the studies described in this Section, a pure phosphorylase preparation was

needed which should be free from carbohydrase activities and the specific activity was of secondary importance. Accordingly, the juice was heated at 56°C for 15 minutes before the ammonium sulphate fractionation.

No debranching activity was observed in the phosphorylase preparation as shown by no change in the iodine staining ability of amylopectin when incubated with phosphorylase. Branching activity was also absent as the amylose oligomers prepared by phosphorylase were found to be linear by their complete conversion into maltose by beta-amylase.

The purification of phosphorylase during ammonium sulphate fractionation is shown in Table 5.1. Specific activity and yield of phosphorylase are shown in Table 5.2. The specific activity of the final phosphorylase solution is of the same order as obtained by Hollo et al (1964), but the yield of phosphorylase is considerably less. As already pointed out, this might be due to heat treatment at 56°C instead of the temperature 54°C used by the above authors.

STUDIES ON LONG-CHAIN AMYLOPECTIN.

Table 5.4 shows the various measurements made on the original waxy maize amylopectin and the four long-chain samples prepared from it. It is clear that $\lambda_{\max}$ increases with the increase in average chain-length.

Pfannemuller et al (1969) have shown that the degree of polymerisation ($\overline{DP}_n$) of a linear amylose oligomer and the

$\lambda_{\max}$ of its iodine complex can be related by the Langmuir isotherm.

$$\lambda_{\max} = \frac{A (\overline{DP}_n)}{B + \overline{DP}_n}$$

or

$$\frac{1}{\lambda_{\max}} = \frac{1}{A} + \frac{B}{A} \frac{1}{\overline{DP}_n} \quad \text{--- (1)}$$

where A is the asymptotic limit at high $\overline{DP}_n$ and B is a constant.

FIG. 5.10 shows the graph of $1/\lambda_{\max}$ against the average chain-length instead of $\overline{DP}_n$. These results are quite in agreement with the above equation. (1) with $1/A = 1.67 \times 10^{-3}$ and $B/A = 44 \times 10^{-4}$. Pfannemuller et al (1969) find for the linear series $1/A = 1.64 \times 10^{-3}$ and $B/A = 92.5 \times 10^{-4}$. The intercepts are quite comparable in both cases, but the slopes are different. This can be explained on the basis that in one case there is purely linear long-chain molecules, and in the other case, there is a complex molecule in which the interior part is highly branched while the external chains are long and linear.

The relation between Chain-length and Beta-amylolysis limit

Beta-amylase does not hydrolyse α -1-6- glucosidic bonds. Therefore the enzymic hydrolysis of amylopectin should stop at branching points. But practically the enzymic hydrolysis of amylopectin and glycogen by beta-amylase is halted when, on average, 2.5 glucose units remain before the α -1-6- branching point. If the external chain-length Y is defined as the number of glucose units beyond the branching limit, it can be related to the total chain-length X by

$$\text{Internal chain-length (C.L.)} = X - Y \quad (2)$$

$$\text{and } Y = BX + 2.5 \quad (3)$$

where B is the fraction converted to maltose by the action of beta-amylase. Substitution of equation (3) into equation (2) gives

$$\text{Internal chain-length} = X(1 - B) - 2.5 \quad (4)$$

In the present work all the long-chain amylopectin samples were prepared from the same waxy-maize amylopectin, therefore, their internal chain-lengths must be comparable. The following relation can be derived from equation (4)

$$X_1 = X_0 (1 - B_0) / (1 - B_1)$$

where 0 refers to the parent amylopectin and 1 to the synthetic sample.

Thus using the experimental values of X_0 and B_0 a theoretical curve can be constructed relating B to X. FIG. 5.11 shows the theoretical curve together with the experimental points. It is obvious that theoretical values are in agreement with experiment values.

Two points about 5.11 should be noted. Firstly

the relation is valid no matter how the anhydroglucose units are added to the amylopectin, i.e. whether the addition is random with respect to chain-ends. This is a necessary consequence of $\overline{CL}_n$ being independent of the mode of addition. (Of course, the weight average chain-length is sensitive to whether the synthesising mechanism is multichain or single chain). Secondly, the absence of alpha-amylase is demonstrated. If there was any alpha-amylase present in the phosphorylase preparation, it would have degraded the synthesised sample and chain-length determined experimentally must have been much lower than the theoretical values.

Table 5.4 also shows the internal chain-length of all the samples and original amylopectin which reveal no change in the internal structure of the parent amylopectin. This is further confirmed as the $\lambda_{\max}$ for the iodine complex of the Beta-amylolysis limit dextrin is the same for the parent waxy-maize amylopectin and all the four synthetic long-chain amylopectins.

Iodine binding capacity of Long-chain Amylopectins

The iodine binding capacities of the parent and synthetic amylopectins were measured at two different temperatures (1.5°C and 20.4°C). The curves of % iodine bound as a function of free iodine concentration are shown in FIG. 5.5 (a, b).

It is obvious that the parent amylopectin binds very little iodine at both the temperatures which was expected from the low value of $\lambda_{\max}$ (529 mμ) of the

iodine complex (Table 5.4).

The iodine binding curves for the samples and parent amylopectin are shown in FIG. 5.5. These experiments were carried out at 1.5°C. At this low temperature, the curve for sample Ap1 is different than others. This sample seems to continue binding the iodine and thus has a much greater slope between $(5 - 10) \times 10^{-6}$ M iodine and cuts the curve for Ap2. To clarify this behaviour of Ap1, a separate experiment was carried out for the measurement of iodine binding at higher concentrations of free iodine. FIG. 5.6 shows the resultant curve. It was found that under the conditions used the amylopectin 1 sample continues to bind iodine over a wider range of concentration of free iodine. Mould (1954) also reported a similar phenomenon during his studies on fractions of amylose obtained by degradation with alpha-amylase. He explained this behaviour on the basis of suggestion of Dvornik et al (1950) that surface absorption of the iodine molecules on the polysaccharide is occurring, and that below a degree of polymerisation of 50, it is difficult to differentiate between binding of iodine and absorption as the two processes appear to merge with each other. This means that absorption is only confined to those molecules which have insufficient chain-length to form long stable helices. This explanation can be applied to Ap1 and to some extent to Ap2. It is obvious from the FIG. 5.5a that only Ap3 and Ap4 give curves in a manner expected from an amylose-like structure.

This surface absorption does not appear to contribute to the $\lambda_{\max}$ of the iodine-polysaccharide complex because if surface absorption of iodine on the samples of short chain length, did effect $\lambda_{\max}$, a linear relation between $\lambda_{\max}$ and $1/\overline{CL}_n$ would not be obtained contrary to the results shown in FIG. 5.1.

FIG. 5.5b shows the iodine binding curves at 20°C. It is clear that at this temperature sample Ap¹ does not behave differently from the parent amylopectin Ap⁰. There is very little difference between curve for Ap¹ and Ap⁰. Again the curves for Ap₂ and Ap₃ behave differently. The curve for Ap₂ is curved over the whole range of free iodine concentration whilst that for Ap₃ has a very high slope. This again could be explained on the basis of surface absorption. Only the curve for Ap₄ reaches the limiting value for iodine-binding capacity.

Ap₄ has a chain-length of 115 units. The studies on amylose oligomers (Table 5.9) show that for a $\overline{DP}_n$ of 105, the iodine binding capacity at 1.5°C is 21.8 mg iodine per 100 mg of polysaccharide and at 20°C the value is 17.3 mg. Using these values the amount of "amylose" present in the sample Ap₄ can be calculated from Table 5.4. At both temperatures the value is about 82%, but the amount obtained by amylolysis is about 93%. This discrepancy might be due to the difference in the distribution function for the external chain-length of the amylopectin sample and for the linear oligomers. The linear oligomers correspond to a "living polymer". In

this all the molecules have equal probability of growth and the resulting polymer has a Poisson distribution. But in case of a branched molecule acting as a primer, it is highly probable that all the non-reducing end-groups do not have the same probability of acting as primers due to the steric requirements of the synthesising enzyme (phosphorylase). Therefore, there will be a wider chain-length distribution. This difference in distribution is probably responsible for the discrepancy in "amylose" content of the amylopectin obtained by different methods.

From the above discussion, it can be concluded that when the external chain-length is less than about 15 glucose units, no iodine is bound at low (1.5°C) and high (20°C) temperatures. As the external chain-length increases to 20 - 25 glucose units, the molecule binds appreciable amounts of iodine at 1.5°C but virtually none at room temperature (20°C). The amount bound at 1.5°C also includes absorption of iodine on the surface of the molecule. When the external chain-length grows to 30 units, iodine is bound by the polysaccharide at both the temperatures (1.5°C and 20°C). The binding may involve some surface absorption as well.

When the external chain-length grows to about 45 glucose units, the molecule binds iodine at low temperature similar to amylose, but at room temperature, the curves are anomalous and surface absorption becomes apparent. Finally, at an average chain-length of about 100 units iodine binding is similar to that of amylose both at high and low temperatures.

STUDIES ON LONG-CHAIN GLYCOGENS.(i) The Chain-length of the Synthetic Glycogen.

The chain-length of the glycogen samples can be related to beta-amylolysis limit by equation (5) as for the long-chain amylopectins. The values of X_0 determined by the Banks and Greenwood method (1970) for parent glycogen was 12.5 glucose units. Table 5.6 shows the values of chain-length and external chain-length for the synthetic glycogen samples. These values are calculated by equation (5) from the beta-amylolysis limit of these samples, as shown in Table 5.5. The values of beta-amylolysis limit are shown as fractional conversion to maltose, and they are converted to % conversion before calculation of chain-length.

The values shown in Table 5.6 reveal the relatively small changes which have taken place as a result of addition of D-glucose units by the synthetic activity of phosphorylase. Samples G_1 and G_2 possess an average chain-length of less than native amylopectin.

(ii) The reaction of Long-chain Glycogens with Iodine.

Table 5.5 shows the reaction of these samples with iodine. It is clear that the reaction of sample G_1 and G_2 with iodine is not as expected for a material intermediate between glycogen and amylopectin, but it is more like an amylose-like polysaccharide. The significance of this behaviour is discussed later.

The values of wavelength of maximum absorption of the synthetic glycogen iodine complexes range from

584 - 630 m μ , whilst the average external chain-length has varied from 10 to 40 glucose units only. In comparison the corresponding values of $\lambda_{\max}$ for the long-chain amylopectins is between 544 - 587 m μ for an increase of 23 - 110 glucose units in the external chain-length (Table 5.4).

This difference can be explained on the basis of the structures of parent amylopectin and glycogen. In the case of amylopectin a comparatively large number of the non-reducing end-groups are accessible to the synthesising activity of phosphorylase, whereas in glycogen, which has a more compact structure, only a few groups have the necessary requirements to act as primers.

Larner et al (1952) have pointed out that if glycogen is assumed to possess an ideal "tree-like structure" proposed by Meyer and Bernfield (1940), then the number of branching points increase rapidly as the successive tiers are added. Thus the density of chain-ends on the surface of the macromolecule increases rapidly. Eventually this would be a limiting factor in the molecular size attained by the polysaccharide. The present work is in agreement with this concept. But it is suggested that the surface of glycogen molecule is not even and some of the chain ends protrude out slightly more than the rest of the chain ends. Only these few chain ends act as primers for the phosphorylase activity and this results in the production of a few long chains on the surface of parent glycogen. In case of amylopectin, the results obtained represent a

much more open structure in which a large proportion of chain ends can act as primers.

The model suggested above, that is, a compact glycogen molecule with a few external chains explain the iodine binding results for synthetic glycogen samples shown in FIG. 5.3 and 5.4.

At low temperature, the iodine binding capacities of both G_2 and G_3 reach limiting values, whilst that for G_1 shows a slight slope even at high concentrations of free iodine. At the higher temperatures none of the graphs of iodine bound as a function of free iodine reaches the limiting value, however, all possess linear portions from which extrapolation can be made to zero free iodine concentration.

Table 5.7 shows the values of iodine affinities for the various glycogen samples at the both high and low temperatures. Table 5.7 also includes the corresponding values for a linear amylose having a number-average degree of polymerisation of about 1500 glucose units. From the values for the linear amylose, the weight % of "amylose" present in glycogen can be calculated. This amylose being completely linear will have a beta-amylolysis limit of 100% conversion into maltose. If w is the weight fraction of linear material, the beta-amylolysis limit $\beta_{calc.}$ may be found for the compound polysaccharide from the relation

$$\begin{aligned} \beta_{calc.} &= w \times 100 + (1 - w) 40 \\ \text{or } \beta_{calc.} &= 60w + 40. \end{aligned} \quad (6)$$

The values calculated for the synthetic long-chain glycogens using equation (6) are given in Table 5.7. It is obvious that the agreement between the two values is excellent.

The possibility that these long chains, or "amylose" can exist separately is eliminated when no glucose was detected after incubation of polysaccharide samples with high concentrations of beta-amylase, showing that the linear chains have no free reducing end groups. Therefore, the long amylose chains are definitely linked to the glycogen and must be the extensions of the outer chains of the glycogen.

SOLUTION BEHAVIOUR OF SYNTHETIC LONG-CHAIN GLYCOGENS

The differences in the degree of branching in glycogen and amylopectin lead to a profoundly different solution behaviour of these polysaccharides. This is demonstrated in FIG. 5.2 by the concentration dependence of the sedimentation coefficients (Banks 1960). The glycogen used for this work was a sub-fraction of a laboratory isolated horse liver glycogen. This was chosen because its sedimentation coefficient, S_0 at infinite dilution was the same as that of a sample of laboratory prepared potato amylopectin. It is clear that for glycogen the dependence of S on concentration C is very small, while for amylopectin there is a profound dependence of S on C , therefore the extrapolation to infinite dilution may be quite erroneous.

The reason for this different behaviour can again

be ascribed to the compact nature of glycogen macromolecule as opposed to the more open structure of amylopectin. It is accepted that with an increase in the degree of branching, the possibilities of molecule to explore more conformations, become limited. Thus a highly branched macromolecule structure may be regarded as rigid. The hydrodynamic model for this type will be sphere or ellipsoid type which offers hydrodynamic resistance to the surrounding medium and is impenetrable to the solvent. Thus it will be incapable of molecular interaction and the sedimentation will not be dependent on concentration. This is true for glycogen (FIG. 5.1).

On the other hand, if there is a completely linear flexible chain, the so-called random coil, the segments of the molecule, are in constant motion because of thermal energy and the number of possible conformations which the macromolecule may adopt is infinite. Even in very poor solvents, the sedimentation of this type molecule is dependent on concentration. Moreover in good solvents the polymer coil expands causing a greater amount of polymer-solvent interaction and therefore, a greater dependence of S on C . In a lightly branched material, the characteristics of a random coil would still be apparent and S would remain dependent on C . This is true for amylopectin. Thus the observed behaviour of amylopectin (FIG. 5.2) may be explained on the basis of macromolecule possessing some degree of flexibility.

The outer chains of glycogen are lengthened by the

addition of glucose units by phosphorylase. This will result in more flexible chains in glycogen. This lengthening of outer chains of glycogen would be expected to change the hydrodynamic behaviour of the macromolecule and the polymer should behave more like amylopectin. As mentioned already, ~~that~~ glycogen possesses a structure which has a greater density of chain ends at the outer surface. If the growth from these chain ends occur without branching, this will result in a progressive decrease of density of chain ends on the surface of the molecule. Obviously these long chains will be more flexible and the molecule will no longer behave as hydrodynamic sphere. From this model it is obvious that if phosphorylase acts by a multichain reaction mechanism and all the chain-ends grow to the same extent, then the hydrodynamic behaviour of the synthetic glycogen will be little different from the parent glycogen, even when the external chain length has doubled. On the other hand, if the enzyme acts by a single chain action pattern and glucose residues are added to only a few chain-ends, these long chains would be capable of considerable coiling and this will influence the hydrodynamic behaviour, even at a very small growth of the original chains.

FIG. 5.1 shows the sedimentation behaviour of the long-external-chain glycogens, and the parent glycogen. FIG. 5.1a shows the results G_0 , G_1 and G_2 in a DMSO water mixture, and FIG. 5.1b shows the graphs of the data for G_0 and G_3 in pure DMSO.

It is apparent that concentration dependence of the sedimentation coefficient is extremely sensitive to change in the external chain length. There is a large difference in sedimentation behaviour between G_0 and G_1 for an external chain length increase from 7.5 to 9.7 residues. This confirms the explanation that these macromolecules have only a few long external chains with the remainder the same length as in the native glycogen.

The model proposed for glycogen is also consistent with the measurements of limiting viscosity numbers of the samples. Table 5.5 shows that the limiting viscosity number increases very rapidly as more glucose units are added to the external chains. This reflects the ability of outer chains to coil which is expected from very long external chains.

STUDIES ON SYNTHETIC AMYLOSE OLIGOMERS.

Relation between $\overline{DP}_n$ and $\lambda_{\max}$

Table 5.8 shows the values of $\overline{DP}_n$ and $\lambda_{\max}$ measured for linear amylose oligomers. The $\lambda_{\max}$ increases with the increase in $\overline{DP}_n$. FIG. 5¹² shows a graph of $\lambda_{\max}$ as a function of $\overline{DP}_n$. This type of graph was also obtained by Swanson (1948) and Bailey and Whelan (1961). The latter authors found that amylose becomes iodine-staining when $\overline{DP}_n$ is 18. The iodine-stain increases linearly with the increase in $\overline{DP}_n$ up to 72 and thereafter the relation becomes non-linear.

Szejtli et al (1967) plotted the iodine bound and iodine free as a function of $\overline{DP}_n$ for a mechano-chemically

degraded, as well as periodate-oxidised amylose. They obtained a curve consisting of two linear sections. The break in the curve occurred between $\overline{DP}_n$ 110 and 130. This value was also confirmed by Pfannemuller et al (1969). However, several comments can be made about the experimental results of these authors. Szejtli et al (1967) used a series of amylose samples obtained by mechano-chemical degradation of natural amylose so these would possess a broad molecular-weight distribution. Furthermore the $\overline{DP}_n$ of the samples determined by the relation of Cowie and Greenwood (1957).

$$\overline{DP}_n = 7.4 [\eta] \quad (7)$$

where $[\eta]$ is the limiting viscosity number of amylose (in ml/g) in IM KOH. This relation was established for $140 < [\eta] < 560$. Szejtli et al have employed it in the range of $7.5 < [\eta] < 190$, hence the potential error could be large. Again $[\eta]$ is a weight-average value and its use to determine number-average value is questionable. Pfannemuller et al (1969) on the other hand have used enzymically synthesised amylose samples for which $\overline{DP}_w/\overline{DP}_n \sim 1.001$, and $\overline{DP}_n$ was determined by the same enzymic technique used in this work.

The investigations in this work were restricted to a narrow range of $\overline{DP}_n$, in which the major changes occur as reported by the above authors. FIG. 5.12 does not reveal any sharp break in the graph of λ_{max} as a function of $\overline{DP}_n$, at either $\overline{DP}_n=72$, or between 110 and 130. On the other hand, a smooth curve may be drawn through the points. FIG. 5.12

also shows the $\lambda_{\max}$ for a linear amylose ($\overline{DP}_n$ 1500). This value can be taken as the asymptotic limit for the curves for the oligomer series.

FIG. 5.13 shows the results graphed according to the equation (1). A linear relation is obtained within experimental error. Moreover, when this relation is extrapolated to zero, the value of $1/A$ obtained is $1.550 \times 10^{-3} \text{ (nm)}^{-1}$ from which A is 642 nm. This is exactly the value obtained experimentally for natural amylose. The relation seems to be quite satisfactory as it predicts the asymptotic value of $\lambda_{\max}$ correctly. The results of Bailey and Whelan (1961) can also be calculated with equation (1). Table 5.9 shows the parameters calculated for the present work and for that of previous authors. The value of $1/A$ is quite in agreement with the value obtained for the data of Bailey and Whelan (1961). There is however, a large difference in the value of parameter B , which is rather difficult to explain. The results of Pfannemuller (1969) are different from the present work. This large difference was first thought to be due to different conditions used for measurement of $\lambda_{\max}$. The above authors have used 0.4 g oligomer/l ($2.47 \times 10^{-3} \text{ M monomer/l}$), 0.1045 g I_2 /l ($0.412 \times 10^{-3} \text{ M/l}$) and 0.1045 gm KI/l ($0.63 \times 10^{-3} \text{ M/l}$) i.e. the molar ratio of iodine/anhydroglucose of 1:6. The conditions employed in this work were similar to those employed by Bailey and Whelan (1961) i.e. 0.015 g amylose/l ($0.093 \times 10^{-3} \text{ M/l}$), 0.041 g I_2 /l ($0.157 \times 10^{-3} \text{ M/l}$) and 0.40 g KI/l ($2.41 \times 10^{-3} \text{ M/l}$)

That is molar ratio of iodine/anhydroglucose is 1.5 : 1. There is a difference of a factor of 9 between the two conditions. However, on measuring $\lambda_{\max}$ of linear amylose by both methods, no difference was detected although it was necessary to reduce the polysaccharide concentration when using the conditions of Pfannemuller et al (1969) in order to avoid precipitation of amylose-iodine complex.

Iodine binding capacities of the Synthetic Amylose Oligomers.

The iodine binding capacities of the amylose oligomer series at different temperatures are given in Table 5.10. These iodine binding capacities are defined as mg iodine bound / 100 mg polysaccharide at zero free iodine concentration. It is clear from the Table 5.10 that the iodine binding capacity increases with the increase in $\overline{DP}_n$ and at a certain $\overline{DP}_n$ the iodine binding capacity increases with decrease in temperature. The effect of temperature becomes much more apparent as the $\overline{DP}_n$ decreases. Table 5.11 shows the changes in the function $F(I)$ with the changes in $\overline{DP}_n$. $F(I)$ is defined as:-

$$F(I) = \frac{(\text{iodine binding capacity at } 20.4^\circ\text{C})}{(\text{iodine binding capacity at } 1.4^\circ\text{C})}$$

It is obvious that the changes in iodine binding capacities become more apparent as the $\overline{DP}_n$ and temperature are varied. This behaviour can be explained on the structure of amylose. At high temperature, the helices of the polysaccharide will expand more, and consequently the iodine binding capacity would decrease. Bailey and Whelan (1961) have reported that amylose becomes iodine staining when $\overline{DP}_n$ is 18.

Table 5.10 shows that at 20.4°C, the lowest $\overline{DP}_n$ to give a positive iodine affinity is 36.4 (sample 4), but at lower temperature even lower $\overline{DP}_n$ show iodine affinity. FIG. 5.8 shows that a lengthy extrapolation has to be made for this sample and the linear portion has an unexpected high slope. This is again a difficult phenomenon to explain. This might be due to some error in iodine affinities.

The enthalpy of the amylose-iodine interaction:

The results graphed in FIG. 5.7 show that binding of iodine is a complex function of three factors, i.e. $\overline{DP}_n$, free iodine concentration and temperature. Szejtli et al (1967) have proposed that at a given temperature, the interaction of amylose and iodine can be represented as

where I_f and I_b are the concentrations of free and bound iodine respectively and Am_f and Am_b are functions of the amylose concentrations expressed in terms of iodine binding sites. Therefore,

$$K = [I_b Am_b] / [I_f] [Am_f] \quad (10)$$

where K is the equilibrium constant. If the point of half saturation is represented by V it follows that

$$[Am_f]_V = [I_b Am_b]_V \quad (11)$$

$$\text{and} \quad K = 1/[I_f]_V \quad (12)$$

The value of $[I_f]_V$ can be obtained directly from Figures 5.7 and 5.8. This value is reciprocal of the equilibrium constant K .

Szejtli et al (1967) have also related the equilibrium constant K to the temperature of measurement by

$$K = A \exp(-\Delta H/RT) \quad (13)$$

When equation (13) is expressed in logarithmic form and the value of K is substituted from equation (12) it can be written as

$$\log_{10} [I_f]_v = -\log_{10} A + \Delta H/2.3RT$$

The values of $[I_f]_v$ as a function of temperature and $\overline{DP}_n$ are shown in Table 5.12. The values of H derived from the graph of $\log [I_f]_v$ as a function of reciprocal temperature (FIG. 5.9) are also shown in Table 5.12.

The value of ΔH for amylose is -15.7 Kcal/mole iodine. This value is in agreement with values reported by Gilbert and Marriot (1948), Kerr (1950), Hollo and Szejtli (1957), and Kuge and Ono (1960). It is also obvious that ΔH decreases with the decrease in $\overline{DP}_n$, which means that at low $\overline{DP}_n$ the reaction between polysaccharide and iodine is less exothermic.

From Table 5.12, it can also be suggested that the asymptotic limit for ΔH will be attained when $\overline{DP}_n$ of the polysaccharide reaches > 200 . Kuge and Ono (1960) have examined the temperature dependence of $[I_f]_v$ for three samples of amylose of different $\overline{DP}_n$. They showed that ΔH was the same for all the three samples. The limiting viscosity number of the samples used by these authors reveal the minimum $\overline{DP}_n$ to be around 500. This confirms the above suggestion that the asymptotic limit will be attained when the $\overline{DP}_n$ reaches around 200.

While measuring the ΔH (Table 5.12) an experimental difficulty was observed. At low temperatures the equilibrium between iodine free and iodine bound is attained within a minute, while at high temperature - especially at low $\overline{DP}_n$ - for sample 5, periods of up to 30 minutes were required of each addition of iodine at 20.4°C. This factor restricts the measurements to a comparatively narrow range of temperature.

The use of $[I_f]_v$ for these calculations is also doubtful. Szejtli et al (1967) have used the total free iodine as in the present work, but Gilbert and Marriot (1948) and Kuge and Ono (1960) have used concentration of molecular iodine. This can be obtained from the equilibrium constant K of the reaction

where $K = [I_2][I^{\bullet}] / [I_3^-] = 1.4 \times 10^{-3}$ at 25°C and the heat of reaction is -4.22 Kcal/mol (Jones and Kaplan, 1928). The use of the concentration of free iodine will considerably alter the values of ΔH . But the experience of these experiments suggests that neither the concentration of molecular iodine nor that of the I_3^- ion controls the equilibrium.

SECTION 6

A NEW METHOD FOR DETERMINATION OF $\overline{DP}_n$.

(PRELIMINARY INVESTIGATIONS.)

THEORETICAL BASIS.

The survey of the literature for the synthetic and degradative action of phosphorylase revealed that maltotetraose was the limit-dextrin for this enzyme in both types of reactions. Phosphorylase needs maltotetraose as the lowest primer for efficient synthesis of longer chain molecules (Whelan and Bailey 1954), and it is unable to degrade a molecule of 4 or less glucose units.

Hestrin (1949) found that the phosphorylase limit dextrin of glycogen and amylopectin can still be degraded by beta-amylase. He suggested that the outer stubs of phosphorylase limit-dextrin are at least three glucose units long. When the structure of phosphorylase limit dextrin was studied in detail by Walker and Whelan (1960), they found that there were four glucose units on either side of the branch point.

The action pattern of this enzyme can be used as the basis of a new enzymic method for the determination of the number-average degree of polymerisation ($\overline{DP}_n$) of linear amylosic materials. It can also be extended to determine the average chain length of the branched materials, if they are completely debranched before estimation. The theoretical basis for this method is as follows:-

Phosphorylase will degrade every amylosic chain to produce one molecule of maltotetraose (G_4) in addition to glucose-1-phosphate. If the amount of G_4 can then be estimated and related to the total polysaccharide

concentration, the $\overline{DP}_n$ can be calculated from:

$$\overline{DP}_n = \frac{[G_t]}{[G_4]}$$

where $[G_t]$ and $[G_4]$ are the molar concentrations of total glucose and maltotetraose respectively.

PRELIMINARY EXPERIMENTS:

The amount of G_4 can be estimated enzymically by degrading it to glucose by amyloglucosidase and estimation by the glucose oxidase method. Total concentration (G_t) of the polysaccharide can also be obtained enzymically by the glucose oxidase method after acid hydrolysis of the ester phosphate group.

There are two possible sources of error in this technique. First, amyloglucosidase might degrade G-1-P (produced by phosphorylase) to glucose. Second, the method of estimation of glucose involves two impure enzyme preparations, i.e. glucose oxidase and peroxidase, either of which might contain a contaminant which would hydrolyse G-1-P.

To check the effect of amyloglucosidase on G-1-P the following digest was set up.

G-1-P (1mg/ml) 5 ml, containing acetate buffer, 1 ml, (0.1M, pH 4.8) and amyloglucosidase solution (0.1 ml).

The digest was incubated at 37°C for 22 hours and the amount of glucose estimated enzymically. No glucose was produced, therefore, the first source of error is eliminated and amyloglucosidase can be safely used for the selective degradation of G_4 in the presence of G-1-P.

This experiment also shows that the glucose-oxidase reagent does not cause any interference in the assay. Experiments were also performed to show that glucose could be accurately estimated in presence of G-1-P. It was found that the calibration figure for this system was identical to that for glucose itself.

MEASUREMENT OF $\overline{DP}_n$.

Only the preliminary investigations using maltoheptaose (G₇) are reported here.

Production and Estimation of G₄.

The following digest was set up:-

Maltoheptaose 0.5 ml. (1mg/ml) citrate buffer 0.1 ml (0.1M pH 6.3) and phosphorylase solution (0.2 ml).

The digest was incubated at 37°C for 5 hours. Then the pH of the digest was changed to 4.8 by adding acetate buffer 0.5 ml (0.1M pH 4.8). Amyloglucosidase (0.1 ml) was added and the digest diluted to 5 ml with distilled water. The digest was incubated again at 37°C for 3 hours and the glucose produced estimated by glucose oxidase method.

ESTIMATION OF TOTAL GLUCOSE.

1.00 ml of the above digest was hydrolysed by 1.0 ml of 0.5 N H₂SO₄ in a boiling water bath for 1.5 hours, neutralised and glucose estimated by the glucose oxidase method.

Calculation.

$$\text{Molar concentration of G}_4 \text{ produced} = 12 \times 10^{-5}$$

$$\text{Molar concentration of total glucose} = 87 \times 10^{-5}$$

$$\overline{DP}_n = 7.2$$

Therefore, this method appears suitable for the determination of $\overline{DP}_n$ and should be applicable to amylose samples as well.

This method will have two advantages over the method of Banks and Greenwood (1968). Firstly, every substrate chain will produce 4 molecules of glucose. Therefore, this method would be 8 times more sensitive. Secondly, very small amounts of amylosic samples would be needed for the estimation. Furthermore, this method could be applied to estimate average chain length ($\overline{CL}_n$) of branched materials, if these samples are debranched before degradation with phosphorylase.

SUMMARY

A survey of the literature for starch-degrading enzymes has been made, and their physical properties, structure, reaction mechanism and action pattern have been discussed.

Action patterns of alpha-amylases from different sources have been examined by different methods i.e. by blue value reducing power curves, by a quantitative estimation of maltodextrins produced during alpha - amylolysis, and by a comparison of changes in $\overline{DP}_n$ and $\overline{DP}_w$ during hydrolysis. All alpha-amylases appear to act by a multi-chain attack pattern, except the enzyme from porcine pancreas, which has a multiple attack pattern.

The multiple attack pattern of porcine pancreatic alpha-amylase changes to multi-chain attack at an adverse pH and in presence of polyhydroxy compounds such as glycerol, erythritol and methyl- α -glucoside. The effect of glycerol has also been examined on bacterial alpha-amylase, and it seems to have no effect on the action pattern of this enzyme. Various theories about the action pattern of alpha-amylases are critically discussed in view of the results obtained in the present work.

Some commercial samples of porcine pancreatic alpha-amylase resolve into four fractions on a DEAE-cellulose column. The action of these fractions on amylose has been examined, and their amino-acid

compositions determined and compared with that of parent enzyme. No difference has been found in their behaviour towards amylose and their amino-acid compositions.

Other commercial enzyme preparations do not resolve into different fractions under similar conditions. The possible reasons for this discrepancy are discussed.

Samples of synthetic glycogens and amylopectins with long external chains have been prepared from glycogen and G-1-P and waxy-maize amylopectin and G-1-P, by the action of amylase-free phosphorylase. The iodine staining properties of these materials have been investigated. A theoretical relation between $\lambda_{\max}$ and average chain-length has been proposed. The dependence of sedimentation coefficient on concentration for long chain glycogens has been examined.

Addition of glucose units to the glycogens seems to be made by a single-chain pattern, while for amylopectins the pattern is multi-chain. The significance of this difference is discussed.

A series of linear amylose oligomers have also been synthesised from maltohexaose and maltoheptaose. The interaction of these materials with iodine has been studied. The $\lambda_{\max}$ has been found to be related to $\overline{DP}_n$ by the Langmuir isotherm.

$$1/\lambda_{\max} = 1.558 \times 10^{-3} + 102.5 \times 10^{-4}(1/\overline{DP}_n)$$

Differential ^{Potential}~~photometric~~ iodine titrations show that the iodine binding capacity of linear oligomers is a

function of both $\overline{DP}_n$ and temperature.

A new experimental method for the determination of the number average degree of polymerisation of linear amylosic material is proposed and preliminary investigations made.

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STUDIES ON STARCH-DEGRADING ENZYMES

PART XII*. THE INITIAL STAGES OF THE ACTION ON AMYLOSE OF THE ALPHA-AMYLASES FROM *B. subtilis*, HUMAN SALIVA, MALTED RYE, AND PORCINE PANCREAS

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(Received June 27th, 1969)

ABSTRACT

The action pattern on amylose of the alpha-amylases from *B. subtilis*, human saliva, malted rye, and porcine pancreas has been studied. In the initial stages, the reaction has been followed by measuring the weight-average degree-of-polymerization ($\overline{DP}_w$) by viscometry, and also the number-average value ($\overline{DP}_n$) by enzymic assay. The mode of enzymic hydrolysis was established by following changes in the value of the ratio $\overline{DP}_w/\overline{DP}_n$, and also by chromatographic examination of the products of low molecular weight. We have confirmed that the porcine, pancreatic alpha-amylase is unique amongst those enzymes examined, in showing evidence of multiple attack at certain pH-values, and have shown that the presence of glycerol (40%, v/v) changes the mode of action in the initial stages to an essentially random form. The significance of this finding is discussed.

INTRODUCTION

It is now known that the mode of action of alpha-amylases isolated from various sources is not the same, but the exact details of these differences are not yet completely established¹. Elsewhere²⁻⁵, we have suggested that the initial stages of the alpha-amylolysis of amylose involves the random degradation of the α -D-(1 $\rightarrow$ 4) linkages in the polysaccharide. These conclusions were based on the change in degree-of-polymerization (as measured by the viscosity number) with time of enzymic hydrolysis.

Viscosity experiments possess the inherent disadvantage, however, that they would be unaffected by the presence of low concentrations of such small molecules as maltose or maltotriose, as has been pointed out by Robyt and French⁶. From studies of the change in absorbance of the residual amylose-iodine complex and reducing-power measurements, these authors suggested that the alpha-amylases from human saliva, porcine pancreas, and *Aspergillus oryzae* degraded amylose by varying degrees of multiple attack. Robyt and French⁶ obtained a measure of this degree of multiple attack by comparing the reducing value of the residual polisaccharide-

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fraction with that of the corresponding oligosaccharide-fraction at various stages of digestion. Here, we present another approach to the problem of investigating the initial stages of the alpha-amylolysis of amylose which avoids the disadvantage of the viscosity technique.

If an alpha-amylase initially degrades amylose by multiple attack⁶, the resulting oligosaccharides of low molecular weight will have a profound influence on the number-average degree-of-polymerization, ($\overline{DP}_n$) of the total system⁶, whereas their effect on the weight-average value ($\overline{DP}_w$) will be insignificant. Multiple attack will, in fact, cause the ratio of $\overline{DP}_w/\overline{DP}_n$ to increase with time of enzymic hydrolysis. On the other hand, if linear amylose, which has the most probable, or exponential, distribution⁷, is used as the substrate, random attack will be reflected by constancy in the ratio $\overline{DP}_w/\overline{DP}_n$, the numerical value being *ca.* 2.

In this work, we have investigated changes in the value of this ratio by determining $\overline{DP}_w$ from viscosity measurements⁸, and measuring $\overline{DP}_n$ enzymically⁹. This approach has been used to investigate the early stages of the action on amylose of the alpha-amylases from *B. subtilis*, human saliva, malted rye, and porcine pancreas.

EXPERIMENTAL

Substrate. — Samples of linear amylose were obtained by leaching potato-starch granules¹⁰.

Enzymes. — Crystalline alpha-amylases from porcine pancreas and *B. subtilis* were obtained from the Sigma Chemical Company, London. The specific activities were 4.4×10^4 and 1.8×10^4 iodine-dextrin colour units^{5,11} per mg of protein per ml of digest, respectively. Human salivary alpha-amylase was prepared by the method of Fischer and Stein¹² (specific activity = 2.8×10^4 units), and the alpha-amylase from malted rye was prepared by the method of Greenwood and Milne⁵ (specific activity = 1.2×10^4 units).

Control experiments showed that all of the enzymes were free from maltose-splitting activity.

Digestion conditions. — Digests were set up at pH 4.8 (0.02M acetate buffer), 7 and 8.5 (0.02M phosphate buffer), and 10.5 (0.02M sodium hydroxide-glycine buffer), and incubated at 37° under toluene. For convenience in the measuring of $\overline{DP}_n$, high concentrations of amylose (20 mg per ml) were required; digests were therefore made 40% v/v with respect to glycerol, to stabilize the polysaccharide⁹, unless otherwise stated.

Analytical techniques. — The reducing power of the digests was measured by the alkaline ferricyanide method¹³, and results were expressed as the apparent percentage of maltose.

Absorbance measurements of the amylose-iodine complex¹⁴ were carried out on a Hilger Spekker (filter 608).

Quantitative estimations of the oligosaccharides produced at different stages of hydrolysis in the digests were made after precipitating the residual polysaccharide

with ethanol⁶. Salts were removed by shaking the supernatant liquors with resin (Amberlite IRA-400, saturated with CO₂). The oligosaccharides were then concentrated on a rotary evaporator before quantitative separation by multiple, ascending, paper (3 MM) chromatography with 70% aqueous propyl alcohol as solvent¹⁵ at 20°. Standard sugars were run as controls. After elution, the oligosaccharides were estimated by enzymic degradation with amyloglucosidase¹³, and then the amount of liberated D-glucose was determined by using D-glucose oxidase, as described below, after appropriate dilutions. Control experiments with standard sugars showed that recovery of oligosaccharide was > 95%.

For determinations of $\overline{DP}_w$ and $\overline{DP}_n$, samples of the digest were removed at appropriate intervals, and the enzyme was destroyed by heating for 10 min at 98°. Digests for $\overline{DP}_n$ were then set up as described⁹, whilst for $\overline{DP}_w$ -values, the amylose product was precipitated as the complex with butyl alcohol, before drying and dissolving in 0.15M potassium hydroxide⁸.

Determination of $\overline{DP}_w$ by viscosity measurements. — The technique for measuring the limiting-viscosity number, $[\eta]$, has been detailed¹⁶. Measurements were made in 0.15M potassium hydroxide. Weight-average molecular weights were found from the relation⁸:

$$[\eta] = 8.36 \times 10^{-3} \times \overline{M}_w^{0.77},$$

and hence the corresponding value of $\overline{DP}_w$ was calculated.

Determination of $\overline{DP}_n$ by an enzymic technique. — The enzymic technique used to measure the number-average molecular weight, and hence $\overline{DP}_n$, was that described earlier⁹, except for the assay of D-glucose by D-glucose oxidase*.

Assay for D-glucose. — Tris-glycerol buffer was prepared as previously described⁹. In 100 ml of this buffer were dissolved²¹ D-glucose oxidase (30 mg), peroxidase (3 mg), and *o*-dianisidine hydrochloride (15 mg). The enzyme-chromogen mixture was stored at 4° in a brown bottle.

For the assay, a 1.0-ml sample was thoroughly mixed with 1.5 ml of enzyme-chromogen solution, and incubated for 2 h at 35°. The digests were then cooled in ice-water, and acidified with 12M sulphuric acid (1 ml). The absorbance (*A*) at a wavelength of 5400 Å was then measured in a spectrophotometer (Unicam SP.600).

The resultant calibration relation for D-glucose in pure water was: D-glucose content in $\mu\text{g} = 38.4 \times A$; in the presence of 40% aqueous glycerol, the relation was: D-glucose content in $\mu\text{g} = 43.6 \times A$.

RESULTS AND DISCUSSION

The essential differences between random degradation and multiple attack have been discussed by Robyt and French⁶. These authors stressed the profound effect

*In our original paper⁹, we included a description of the technique given to us by Professor W. J. Whelan, which we had erroneously assumed to have been taken from the literature¹⁷⁻²⁰. We now learn that we gave a method described by Dr. Catley²¹, but not at that time published. We apologise to Dr. Catley for this oversight.

that the presence of small oligomers (arising from multiple attack) would have on the number-average of the system.

The best method of establishing the extent of multiple attack is to measure simultaneously $\overline{DP}_w$ and $\overline{DP}_n$. As a measure of the former, Robyt and French⁶ used the absorbance of the amylose-iodine complex, but the range of degradation over which the absorbance is proportional to the $\overline{DP}_w$ of the amylose is quite limited²². Similarly, the accuracy of reducing-power measurements to determine $\overline{DP}_n$ for a range of molecular sizes is questionable; Robyt and French⁶ had to extrapolate from a control sugar of $\overline{DP}_n = 4$ to values of $\overline{DP}_n \sim 1000$ for amylose. Our present technique is inherently more accurate, provided any small sugars produced by multiple attack are statistically divided between odd and even numbers of residues. This point is dealt with in more detail later.

Experiments in the presence of 40% of glycerol (v/v)

Value of the ratio, $\overline{DP}_w/\overline{DP}_n$. — In our first experiments, the enzymic digests contained 40% by volume of glycerol for convenience in dealing directly with high concentrations of amylose in the $\overline{DP}_n$ -determination. Results obtained under these conditions for the four enzymes are shown in Table I. It can be seen that, in the

TABLE I

ACTION OF VARIOUS ALPHA-AMYLASES ON AMYLOSE IN PRESENCE OF 40% OF GLYCEROL:
VALUE OF $R = \overline{DP}_w/\overline{DP}_n$

<i>B. subtilis</i>				<i>Human saliva</i>			
<i>Sample</i>	$\overline{DP}_w$	$\overline{DP}_n$	R	<i>Sample</i>	$\overline{DP}_w$	$\overline{DP}_n$	R
1	1980	810	2.5	1	1810	890	2.0
2	1780	635	2.8	2	1160	650	1.9
3	1450	590	2.5	3	1160	585	2.0
4	1210	480	2.5	4	1040	490	2.1
5	1040	425	2.5	5	940	480	2.0

<i>Malted rye</i>				<i>Porcine pancreas</i>			
<i>Sample</i>	$\overline{DP}_w$	$\overline{DP}_n$	R	<i>Sample</i>	$\overline{DP}_w$	$\overline{DP}_n$	R
1	1720	775	2.2	1	1720	775	2.2
2	950	367	2.6	2	1040	400	2.6
3	510	230	2.2	3	660	295	2.2
4	385	190	2.0	4	500	240	2.1
5	350	160	2.2	5	530	220	2.4

initial stages of enzymic action, there is no change in the ratio $\overline{DP}_w/\overline{DP}_n$, within experimental error. Multiple attack would cause $\overline{DP}_n$ to change more rapidly than $\overline{DP}_w$, and so the ratio between the two would increase. The constancy of the experi-

mental ratio implies that multiple attack is not occurring for any of the four enzymes under our experimental conditions.

These results are in contrast to those of Robyt and French⁶, who found evidence for a degree of multiple attack for the three enzymes that they examined, particularly so for porcine, pancreatic alpha-amylase.

The effectiveness of our method of determining $\overline{DP}_n$ depends on there being no preferential build-up in the digest of oligosaccharides having an even number of D-glucose residues. The digests were therefore analysed chromatographically for soluble sugars at the early stages of hydrolysis. Traces of these were found (Table II)

TABLE II

ACTION PATTERN OF HUMAN SALIVARY ALPHA-AMYLASE: YIELDS OF MALTODEXTRINS PRODUCED FROM AMYLOSE AS FUNCTION OF DECREASE IN ABSORBANCE AT pH 4.8

Sample	Decrease in absorbance (%)	Nmoles of maltodextrin ^a per ml					
		G ₁	G ₂	G ₃	G ₄	G ₅	G ₆
1	26	3	32	30	24	10	15
2	45	3	62	68	39	16	30
3	64	6	146	195	130	52	70

^aG₁ = D-glucose; G₂ = maltose; etc.

for the action pattern of human salivary alpha-amylase, but there was no evidence for any pronounced build-up of even-numbered sugars. It should be noticed that there were only traces of D-glucose, indicating the absence of any maltase-splitting activity, and also no appreciable build-up of maltose and maltotriose, indicating the absence of multiple attack.

When the results of the viscosity experiments were treated as previously²⁻⁵, the resultant graphs of $\overline{DP}_w^{-1}$ versus time were linear, indicating again the occurrence of a random, hydrolytic scission of α -D-(1→4) bonds in the earlier stages of reaction (see Fig. 1).

Fig. 1. Graph of $\overline{DP}_w^{-1}$ against time (h) for the action on amylose of the alpha-amylase from 1. *B. subtilis*, 2. human saliva, 3. malted rye, 4. porcine pancreas.

Experiments in the absence of 40% of glycerol

These experiments differed from the earlier ones⁶, however, as the digests contained 40% of glycerol. In order to investigate whether the presence of this reagent was influencing the action pattern of the enzyme, digests without this reagent were set up.

Value of the ratio $\overline{DP_w}/\overline{DP_n}$. — Experiments were made with the alpha-amylases from *B. subtilis* and porcine pancreas at pH 4.8. Table III shows that the value of

TABLE III

ACTION OF *B. subtilis* AND PORCINE PANCREAS ALPHA-AMYLASES ON AMYLOSE IN ABSENCE OF 40% OF GLYCEROL: VALUE OF $R = \overline{DP_w}/\overline{DP_n}$

<i>B. subtilis</i>				<i>Porcine pancreas</i>			
Sample	$\overline{DP_w}$	$\overline{DP_n}$	R	Sample	$\overline{DP_w}$	$\overline{DP_n}$	R
1	1820	810	2.2	1	2110	850	2.5
2	1540	685	2.2	2	1760	565	3.1
3	1280	595	2.2	3	1540	575	3.0
4	1110	505	2.2	4	1430	410	3.5
5	940	450	2.1	5	1230	300	4.1

the ratio $\overline{DP_w}/\overline{DP_n}$ was unchanged for the *B. subtilis* enzyme, but that there was a profound alteration in the value of this ratio with time of digestion for the porcine, pancreatic enzyme. This result implies that, under these experimental conditions, the enzyme was producing an excess of small sugars, in agreement with the results of Robyt and French⁶.

Absorbance-reducing-power curves. — Robyt and French⁶ showed that, at the adverse pH of 10.5, porcine, pancreatic alpha-amylase appeared to have a multichain action, and that this was reflected by characteristic changes in absorbance-reducing-power curves. We compare in Fig. 2, therefore, the behaviour of porcine, pancreatic alpha-amylase at pH 4.8 without glycerol (curve 1) with its behaviour in the presence of glycerol (curve 2) and also at pH 10.5 (curve 3). It can be seen that the action pattern of the enzyme at pH 4.8 in the presence of glycerol and at pH 10.5 are the same. From the values of the ratio $\overline{DP_w}/\overline{DP_n}$ under these conditions (Table I) and from Robyt and French's conclusions⁶, we suggest that curves 2 and 3 in Fig. 2 are characteristic for random degradation, i.e., under these conditions, there is a rapid fall in absorbance with a corresponding relatively small increase in reducing power. The fact that the same shape of graph is obtained for the action of *B. subtilis* alpha-amylase at pH 4.8 without glycerol (curve 4), together with the results in Table III, confirms this suggestion.

In contrast, curve 1 (Fig. 2) for a digest of porcine, pancreatic alpha-amylase at pH 4.8 without glycerol shows a much more rapid increase in reducing power for much smaller changes in absorbance, and this type of curve must be characteristic for enzymic action that results in a build-up of small sugars in the digest.

Fig. 2. Graph of the change in absorbance of the amylose-iodine complex as a function of the apparent reducing-power for the action on amylose of 1. porcine, pancreatic alpha-amylase at pH 4.8 without glycerol; 2. porcine, pancreatic alpha-amylase at pH 4.8 in presence of 40% aqueous glycerol; 3. porcine, pancreatic alpha-amylase at pH 10.5; 4. *B. subtilis* alpha amylase at pH 4.8 without glycerol.

It was found that the enzymes from human saliva and malted rye also gave absorbance-reducing-power curves which indicated that random degradation still occurred in the absence of glycerol.

Quantitative estimations of oligosaccharide products

Porcine, pancreatic alpha-amylase appears to be the only enzyme of those examined which has any pronounced degree of multiple attack on amylose. However, at an adverse pH of 10.5, the enzyme reverts to an essentially random attack, in agreement with the results of Robyt and French⁶. Additionally, we have now shown that the presence of 40% of glycerol, at optimal values of pH, is analogous to the use of high pH.

The difference in action pattern of the porcine amylase was further demonstrated when the sugars produced in the early stages of the digestion were determined quantitatively after paper-chromatographic separation. Digests corresponding to the two extremes of action pattern, *i.e.*, pH 4.8 without glycerol, and pH 10.5, were analysed with the results shown in Table IV. It can be seen that at pH 10.5 the amounts of oligosaccharides produced are comparable to those in Table II. However, at pH 4.8 and without glycerol, vastly increased amounts of all sugars (with the exception of D-glucose) are formed. Particularly noticeable are the relatively large amounts of maltose and maltotriose.

Even under optimal conditions and allowing the reaction to proceed to the

achroic point, the other alpha-amylases do not produce such excessive amounts of the small sugars. Table V shows typical results for estimations of sugar composition at pH 5.5, 7.0, and 8.5 using *B. subtilis* alpha-amylase. The same trends as in Table II are apparent.

TABLE IV

ACTION PATTERN OF PORCINE, PANCREATIC ALPHA-AMYLASE: YIELDS OF MALTODEXTRINS PRODUCED FROM AMYLOSE AS A FUNCTION OF pH AND DECREASE IN ABSORBANCE

Sample	pH of digest	Decrease in absorbance (%)	Nmoles of maltodextrin ^a per ml					
			G ₁	G ₂	G ₃	G ₄	G ₅	G ₆
1	4.8	27	2	290	127	72	38	29
2	4.8	50	4	620	268	142	86	70
3	4.8	71	6	910	300	222	135	66
1a	10.5	28	4	15	16	8	9	6
2a	10.5	47	4	47	30	15	14	11
3a	10.5	68	6	75	48	33	25	21

^aG₁ = D-glucose; G₂ = maltose; etc.

TABLE V

ACTION PATTERN OF *B. subtilis* ALPHA-AMYLASE: YIELDS OF MALTODEXTRINS PRODUCED FROM AMYLOSE AT THE ACHROIC LIMIT AS A FUNCTION OF pH

Digest	pH	Digestion stage	Percentage by weight ^a						
			G ₁	G ₂	G ₃	G ₄	G ₅	G ₆	> G ₆
1	4.8	Achroic	0.2	6	19	6	5	18	46
	4.8	Post-achroic	0.8	11	26	8	10	29	16
2	4.8 ^b	Achroic	0.5	5	20	8.5	6	31	30
	4.8 ^b	Post-achroic	1.5	8	23	8.5	9	29	21
3	7.0	Achroic	0.1	2	6	2	1	8	81
	7.0	Post-Achroic	0.3	3	9	2	2	10	74
4	8.5	Achroic	0	1.8	3	1.5	1.3	4	88
	8.5	Post-achroic	0.2	2.4	7	2	1.5	7	80

^aG₁ = D-glucose; G₂ = maltose, etc. ^bConcentration of enzyme 5 × greater than in digest 1.

CONCLUSIONS

The initial stages of the action pattern on amylose of the alpha-amylases from *B. subtilis*, human saliva, and malted rye do not involve multiple attack, as measured by changes in the ratio of $\overline{DP}_w/\overline{DP}_n$. This conclusion holds for digests at pH 4.8 with, and without, the presence of 40% of glycerol.

In contrast, the action of the enzyme from porcine pancreas is different. Although there is no evidence of multiple attack at pH 4.8 in the presence of 40%

of glycerol, there is a preferential build-up of small sugars in its absence. This difference in action pattern is reflected by changes in the absorbance-reducing-power curves, in agreement with Robyt and French⁶. The action pattern at the adverse pH of 10.5 is the same as that at pH 4.8 in the presence of 40% of glycerol. Reasons for this difference in action pattern are difficult to establish. Aqueous glycerol (40%) is a viscous medium in which it might be expected that the rate of breakdown of the enzyme-substrate complex would be slower than in a purely aqueous system: multiple-chain attack would, therefore, be expected to be favoured in this medium. The experimental evidence suggests, in fact, that the converse effect occurs. Unfavourable ionization of the catalytic groups, as suggested by Robyt and French⁶, is unlikely to be occurring in aqueous glycerol (40%). This problem is being investigated further, but we tentatively suggest that the porcine, pancreatic alpha-amylase may be a mixture of two multiple forms²³ having slightly different action-patterns, one of which is apparently suppressed in the presence of aqueous glycerol (40%).

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