Enzymic Degradation of Glycogens.

by

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SECTION I.

INTRODUCTION

1. The occurence of glycogen in nature.

Glycogen, a polysaccharide derived from D-glucose, is present in most animal cells, and was first isolated by Claude Bernard in 1857, from the liver of a dog (1). In higher animals it is found in largest amount in the liver, although there are smaller quantities in skeletal and cardiac muscle and other tissues. Glycogen also occurs in invertebrates. It has been isolated from several species of mollusc (2-4), from the nematode, Ascaris lumbricoides (5), and from two species of aphid (6). The presence of glycogen, or a polysaccharide very closely resembling it, has been reported in yeasts (7,8), fungi (9), protozoa (10,11), and bacteria (12,13). It is not normally found in plants, although a polysaccharide which appears to have similar properties to animal glycogen has been extracted from maize seeds (14-18).

Glycogen is water soluble and is distributed throughout the cell cytoplasm. In this it differs from starch, its counterpart in the plant kingdom, which occurs in discreet granules within the cell.

Whether glycogen, in its native state, is in combination with proteins, is uncertain. From some tissues only part of the glycogen can be extracted with boiling water, or cold trichloracetic acid solution; to obtain the remainder, boiling alkali must be used. Willstätter and Rohdewald have distinguished between the easily

extracted glycogen, or "lyo" glycogen, and the more firmly bound, or "desmo" glycogen; they found that, in well fed animals, a high proportion of the liver glycogen was in the "lyo" form. By contrast, in livers of low glycogen content, the greater part of the polysaccharide was in the "desmo" form (19). More recently, Rozenfeld has reported that the amount of "difficulty extractable" glycogen in frog livers decreases in winter and increases in spring (20). Muscle glycogen, however, is almost entirely in the "desmo" form, and attempts to prepare glycogen from mammalian muscles by aqueous extraction have met with only limited success (21).

Although it is generally agreed that "lyo" glycogen exists, in situ, in an uncombined state, various suggestions have been made to explain the difficulty in extracting "desmo" glycogen from the tissues. Willstätter and Rohdewald have postulated that "desmo" glycogen is held by proteins in a "symplex", i.e. glycogen and protein molecules are held together by secondary valencies (19). Alternatively, the difficulty extractable glycogen may be mechanically entrapped by proteins (22). Meyer has suggested that the solubility of glycogen is related to the degree of polymerisation, and considers that "desmo" glycogen is merely the high molecular weight material which is insoluble in water and soluble in alkali (23). However, alkali-extracted glycogen is water soluble; this would not be expected if Meyer's suggestion were entirely correct.

It seems certain that any combination between glycogen and protein in the native state involves only secondary forces,

and it must be pointed out that the distinction between "lyo" and "desmo" glycogen is very flexible, and depends greatly on the methods of extraction used. It has been shown, however, that glycogen-protein interactions may occur *in vitro*, but again, there is no indication that primary valencies are involved (24-26).

2. The metabolic function of glycogen (27).

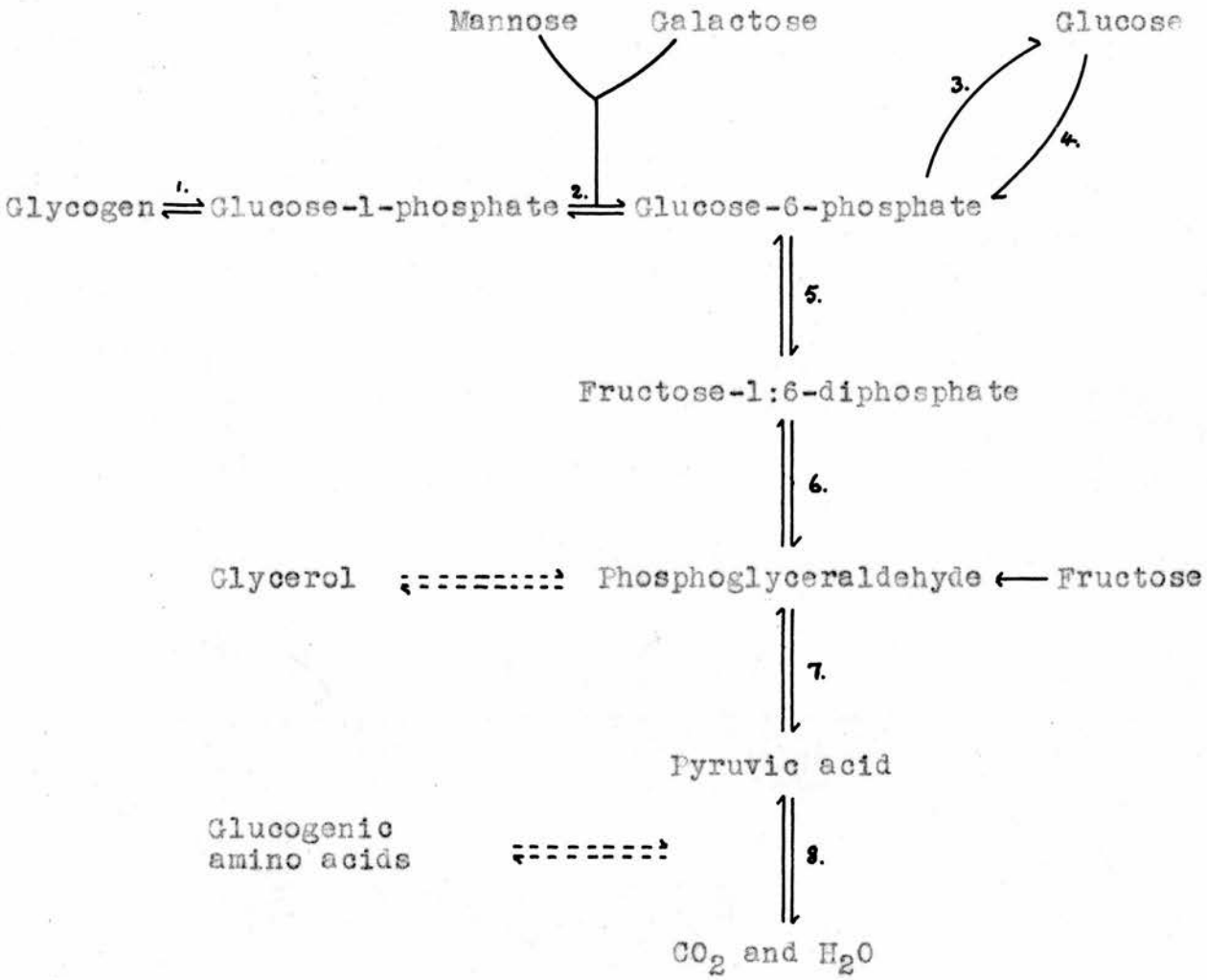
Glycogen is the form in which carbohydrate material is stored in the animal body. It is a substance of vital metabolic importance, since the organism obtains a high proportion of its energy by breakdown of carbohydrates. The routes by which it is metabolised are summarised in Fig. I.

Key to Figure I:

1. Phosphorylase.
2. Phosphoglucumutase.
3. Phosphatase.
4. Hexokinase.
5. Oxoisomerase and Phosphohexokinase.
6. Zymohexase.
7. Enzymes of glycolysis cycle.
8. Enzymes of citric acid cycle.

Figure I.

Metabolism of Glycogen.



The breakdown of glycogen by reactions 1, 2 and 3 is glycogenolysis, and by reactions 1, 2, 4, 5, 6 and 7 is glycolysis. The formation of glycogen from carbohydrate materials (glucose, mannose, galactose and fructose) is glycogenesis, and from non-carbohydrate materials (e.g. glycerol and glucogenic amino acids) is glyconeogenesis.

The principal monosaccharide derived from food is glucose, although small amounts of fructose, galactose, and mannose also occur. These are carried in the blood to the liver where, by enzymic action they are phosphorylated; the phosphorylated intermediates are then converted into glucose-1-phosphate from which glycogen is synthesised by the enzyme phosphorylase and a branching enzyme.

Muscle glycogen is built up mainly at the expense of liver glycogen. The latter is first broken down to glucose-1-phosphate, and then converted via glucose-6-phosphate to free glucose, by a process called glycogenolysis. Glucose passes into the blood stream; it is absorbed by the muscles, and phosphorylated to give glucose-6-phosphate, which is transformed first into glucose-1-phosphate, and thence into glycogen.

Glycogen may also be derived from non-carbohydrate sources, the process being known as glyconeogenesis. Glycerol, arising by saponification of fats, may be phosphorylated and dehydrogenated to give phosphoglyceraldehyde, which, in turn, may be converted to glycogen by a series of enzyme catalysed reactions involving intermediates such as fructose-1:6-diphosphate and glucose-1-phosphate. Certain amino acids (classified as glucogenic amino acids) are interconvertible with glycogen. By simple reactions they give rise to such substances as oxaloacetic, lactic and pyruvic acids. The two former acids are readily transformed into pyruvic acid, and thence into phosphoglyceraldehyde, glucose-1-phosphate, and glycogen.

The complete breakdown of glycogen with concurrent formation of adenosine triphosphate is the ultimate source of much of the energy of the organism. Glycogen is first broken down, via several intermediates, to pyruvic acid, a process known as glycolysis, by reactions which are the reverse of those occuring during glyconeogenesis. Glycolysis, which does not require oxygen and can therefore proceed under anaerobic conditions, causes the release of a certain amount of energy. Under aerobic conditions, pyruvic acid is then completely broken down to carbon dioxide and water, with the release of further energy, by the enzymes of the "citric acid cycle".

3. General Properties.

The term "glycogen" refers not to a single substance of constant chemical structure, but to a group of closely related polysaccharides, which have the following properties:

1. On hydrolysis by weak mineral acids at 90 to 100°, D-glucose is obtained in almost quantitative yield.

2. The pure substance contains carbon, hydrogen and oxygen, empirical formula $C_6H_{10}O_5$. It is nevertheless difficult to prepare glycogen samples which are completely free from nitrogen and phosphorus, and some workers have suggested that glycogen contains a small amount of organically bound phosphorus, possibly in the form of phosphate ester groups (28,4). However it is more probable that both these elements are constituents of contaminating substances, since by careful purification, glycogens

which are virtually free from nitrogen and phosphorus have been obtained (29,30,23).

3. Aqueous solutions are opalescent, although differences in degree of opalescence have been reported (31,32).

4. Aqueous solutions have a high specific dextrorotation, which in the majority of cases is between 190 and 200°.

5. Glycogen is normally stained reddish-brown by iodine, although variations in shade have been noted, especially when iodine is added in small amounts. Some workers have reported that the colour obtained with fish liver glycogen is yellow-brown (30); others describe it as red-brown (33). The glycogen from Mytilus edulis stains yellow-brown (2,21), that from Helix pomatia light brown (3), and that from Avian tubercle baccilli, light yellow (12). Young, in 1934, found that the iodine colorations of muscle glycogen varied from one specimen to another; those of rabbit muscle glycogen ranged from reddish-brown to violet (21).

6. Glycogen has practically no effect on Fehlings solution; its reducing power has been reported to be approximately 0.1% of that of an equal weight of glucose (30).

7. Most samples have molecular weights of the order of 10^6, although examination by ultracentrifugation has indicated that specimens are extremely polymolecular (34).

8. Glycogens have a low viscosity, the limiting viscosity number being less than that of amylopectins of similar molecular weight (35).

4. The structure of glycogen as determined by chemical methods.

A. Characterisation of the basic structural unit.

Early workers noted that D-glucose was produced by acid hydrolysis of glycogen, and that, by the action of certain "ferments", maltose was obtained. In 1921, Karrer and Nageli reported that treatment of glycogen with acetyl bromide gave rise to acetobromomaltose (36). By methylation of glycogen, Haworth, Hirst and Webb obtained a product which analysed as trimethyl glycogen, and which on hydrolysis, gave 2:3:6-tri-O-methyl-D-glucose in 76% yield (37). It thus appeared that glycogen was a polymer of maltose, consisting of chains of 1:4-linked α-glucose units.

In 1931, Haworth and Percival pointed out that the above work did not provide unambiguous proof for this structure of glycogen (38). Theoretically, 2:3:6-tri-O-methyl-D-glucose can be obtained from 1:4-linked glucopyranose units, from 1:5-linked glucofuranose units, or from a structure in which the glucose molecules are in the straight chain form. Maltose, among the products of enzymic degradation, could, in theory, have arisen by reversion from glucose. They even held that the isolation of acetobromomaltose was of little value, as long as hydroxyl groups in the glycogen molecule which was degraded by acetyl bromide, remained unprotected, and therefore available for secondary reactions.

In order to overcome this objection, Haworth and Percival degraded trimethyl glycogen with acetyl bromide, after

showing that molecular rearrangements did not occur during methylation of glycogen. From the reaction they were able to isolate a monoacetyl hexamethyl biose, which, on oxidation and further methylation, yielded the methyl ester of an octamethyl aldobionic acid. On hydrolysis of this ester 2:3:4:6-tetra-O-methyl-D-glucose, and 2:3:5:6-tetra-O-methyl-γ-gluconolactone were obtained, thus proving that in the glycogen molecule α-glucopyranose units are linked to one another through positions 1 and 4.

B. Determination of the length of the unit chain.

At this time, the glycogen molecule was believed to consist of a single chain of α-1:4-linked glucose residues. The next problem was the determination of the length of the chain, and this was first carried out by Haworth and Percival in 1932 (39). They expected that, on hydrolysis of methylated glycogen, 2:3:4:6-tetra-O-methyl-D-glucose would be produced from the non-reducing terminal group, and 2:3:6-tri-O-methyl-D-glucose from the remainder of the molecule. After separation of the products of hydrolysis of methylated rabbit liver glycogen by fractional distillation, they estimated that 9% of the molecule gave rise to 2:3:4:6-tetra-O-methyl-D-glucose. This figure is equivalent to an average chain length of 12 glucose residues.

Since the experiments of Haworth and Percival, methylation end group assays have been carried out on many glycogens. It became apparent that there were two "types" of rabbit liver glycogen; the majority of specimens examined had an average chain length of 12 glucose residues (40), but for certain

specimens a value of 18 was reported. 18-Unit glycogens were first obtained by Bell in 1936, from two separate batches of rabbits which had been fasted, and then fed on galactose (32). Bacon, Baldwin and Bell, in 1944, also isolated an 18-unit glycogen from rabbits which, after fasting, were given a sucrose diet (41). They concluded that the normal chain length of rabbit liver glycogen is 12 units, but that glycogen of higher average chain length was produced when fasted rabbits were fed on galactose or sucrose. When glucose or fructose was fed to fasted rabbits 12-unit glycogen was obtained. 18-Unit rabbit liver glycogen was also reported by Haworth, Hirst and Isherwood in 1937 (42).

End-group assays carried out on glycogens from other organisms have shown that the majority of glycogens have an average chain length of 11 to 13 glucose residues. A selection of results is shown in Tables I and II, and in Table VII (Page 48). The periodate method of end group assay will be discussed in Section II. It will be noted, however, that chain length values may vary considerably, and it is now considered that the term "glycogen" covers a series of gluco-polysaccharides, most of which have an average chain length of approximately 12 glucose residues; a range of about 7 to 18 is however possible.

TABLE I

End group assay of glycogens by methylation studies. Separation of hydrolysis products by non-chromatographic methods.

Date	Source of glycogen	Chain length	Method	Ref.
1932	Rabbit liver	12	A.	39
1935	Rabbit liver	12	B.	40
1935	Fish liver (Gadidae)	12	B.	40
1936	Rabbit liver (galactose ingested)	18	B.	32
1936	Mytilus edulis	18	B.	2
1937	Horse muscle	11-12	A.	43
1937	Rabbit liver	18	A.	42
1938	Dog liver	12	B.	44
1939	Hake liver	12	A.	33
1939	Haddock liver	12	A.	33
1939	Dogfish liver	12	A.	33
1939	Dogfish muscle	12	A.	33
1940	Helix pomatia	11-12	A.	3
1941	Mytilus edulis	11	A.	45
1942	Ascaris lumbricoides	13-14	A.	5
1944	Rabbit liver (glucose injected)	12	A.	41
1944	Rabbit liver (fructose injected)	11-12	A.	41
1944	Rabbit liver (fructose ingested)	11-12	A.	41
1944	Rabbit liver (sucrose ingested)	18-19	A.	41

TABLE I (contd.)

Date	Source of glycogen	Chain length	Method	Ref.
1947	Rabbit liver	18-19	C.	46

A. Separation of methyl glycosides by fractional distillation

B. Separation of methylated sugars by chloroform extraction.

C. Separation of methyl glycosides by partition between light petroleum and water.

TABLE II

End group assay of glycogens by methylation studies. Separation of hydrolysis products by chromatographic methods.

Date	Source of glycogen	Chain length	Method	Ref.
1944	Horse muscle	12	A.	47
1944	Ascaris lumbricoides	15-16	A.	47
1948	Rabbit muscle	11	A.	48
1948	Rabbit liver	12	A.	48
1949	Rabbit liver	12	B.	49
1951	Rabbit liver	11-12	B.	50

A. Separation of methylated sugars on silica gel.

B. Separation of methylated sugars by filter paper chromatography.

C. The branched structure of glycogen.

Although the glycogen molecule was thought to consist of a single chain of approximately 12 glucose residues, it soon became obvious that some of the properties of glycogen could not be explained by this structure.

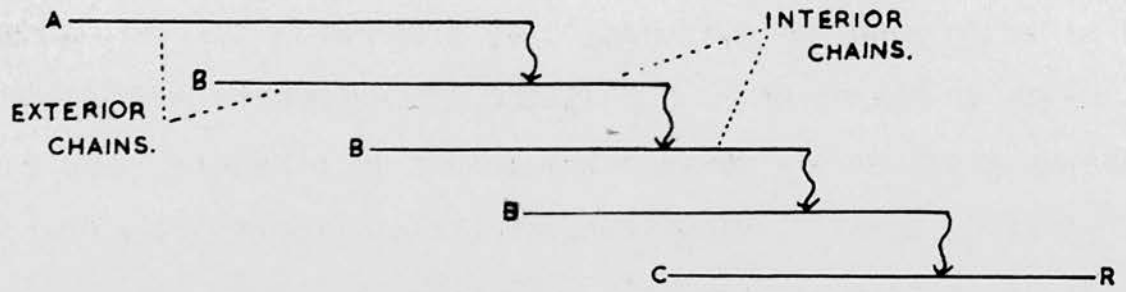
Haworth and Percival had first noted in 1932, that the reducing power of glycogen is very much less than would be expected from a polysaccharide composed of single chains of 12 glucose residues (39). They suggested that modification of the reducing group had occured, either *in vivo*, or during alkali-extraction of glycogen from the tissues.

In 1935, Bell detected the presence of di-*O*-methyl-D-glucose among the hydrolysis products of methylated samples of rabbit and fish liver glycogens (40). He suggested that it arose through incomplete methylation of the glycogen molecule, and concluded that in every dodecasaccharide unit, approximately two hydroxyl groups escaped methyation.

Osmotic pressure studies carried out by Oakley and Young in 1936 revealed that the particle weight of glycogen is of the order of 2×10^6 (51). Approximately the same result was found for methylated glycogen. The physicochemical evidence showed that these results represent the true size of a glycogen macromolecule, and not a physical aggregation of sub-units each containing only 12 glucose residues.

In 1937, Haworth, Hirst and Isherwood suggested that the glycogen molecule was branched (42). To account for the appearance of di-*O*-methyl-D-glucose among the hydrolysis products

FIGURE II. Structures of glycogen.

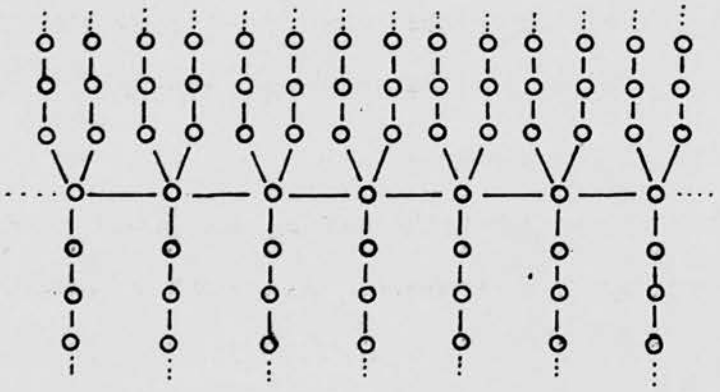


—— Linear chain of α-1:4-linked glucose residues.

ʃ α-1:6-glucosidic linkage.

R Reducing group.

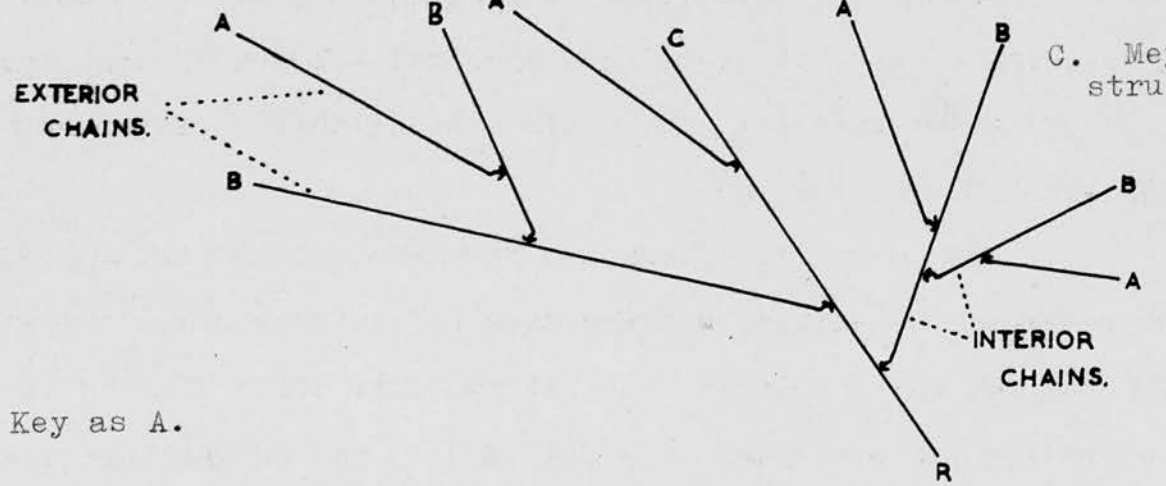
A. Haworth structure.



B. Staudinger structure.

O Glucose residue.

— 1:2-, 1:3-, 1:4- or 1:6-glucosidic linkage.



C. Meyer structure.

Key as A.

of methylated glycogen, for the high molecular weight and the low reducing power of glycogen, they postulated that the molecule consisted of chains of α-1:4-linked glucopyranose units, the reducing group of one chain being linked to a hydroxyl group of an adjoining chain. Di-O-methyl-D-glucose would arise from the branch point if this hydroxyl group were at either the 2,3, or 6 position of a non-terminal glucose residue, and experimentally, some 2:3-di-O-methyl-D-glucose was obtained (42). The authors expressed their structure by the laminated formula (Fig.II.A), although at this time they were unable to specify the nature of the inter-chain bond, or the average position of the branch point residues in the linear chains. It will be seen that, though the molecule may be very large, only one unmodified reducing group is present; the low reducing power of glycogen is therefore explained.

Haworth, Hirst and Smith later suggested that the inter-chain linkages were primary glucosidic bonds (33). A study of methylated starch had shown that inter-chain bonds of this type were present in starch, and it was assumed that glycogen contained similar linkages (52, 53).

In 1937, Staudinger and Husemann carried out osmotic pressure and viscosity measurements on glycogen and its acetyl derivative, and concluded that the molecule had a high molecular weight and was spherical in shape (54). To account for this the authors said that the molecule must be branched; their structure is represented diagrammatically in Fig.II.B.

The main chain is composed of 30-40 glucose residues; the side chains which are attached to the glucose residues of the main chain at positions 2, 3 and 6, are 12 or 18 glucose units in length. Staudinger and Husemann maintained that, with a molecule of this structure, halving or doubling the length of the main chain would not alter the spherical properties.

However, Bell, in 1937, pointed out that the Staudinger and Husemann structure did not account for all the experimental facts (43). On methylation and hydrolysis, such a compound would not give rise to di-O-methyl-D-glucose, but would yield a small quantity of unmethylated glucose, a substance which had not been found in any of the experimental work. Subsequent workers have discounted the Staudinger and Husemann structure.

For several years, most workers in the field of glycogen chemistry considered that the Haworth singly-branched laminated formula adequately fitted experimental facts. However, in 1941, Meyer and Fuld suggested that the glycogen molecule had a multiply-branched structure (45). They treated a sample of mussel glycogen with β-amylase, a hydrolytic enzyme which degrades the outer chains of branched α-1:4-glucosans, but can neither hydrolyse the branch point linkages, nor by-pass them (See Section III). β-Amylolysis ceased when approximately 50% of the glycogen molecule had been removed, and the β-limit dextrin (β-dextrin) was isolated. Methylation end group assays were carried out on the original glycogen and on the β-dextrin; the former had a chain length of 11, and the latter of 5.5 glucose

residues, thus indicating that the same number of end groups were present in the β-dextrin as in the original glycogen molecule. This fact was interpreted as proving that glycogen had the multiply-branched structure shown diagrammatically in Fig. II C.

Before comparing the Meyer structure with that of Haworth, it is necessary to define the various types of "unit chains". These definitions were first formulated by Peat and his colleagues (55). A-chains (side chains) are attached to the rest of the molecule by one linkage only: that involving the reducing group. B-chains (main chains) are those to which other chains are attached. In each molecule there is one C-chain; it is terminated by the reducing group. In addition, A-chains and those parts of B-and C-chains between the branch points and the non-reducing terminal groups, are designated "exterior chains". "Interior chains" are those parts of B-and C-chains which lie between two branch points.

It will be seen that the singly-branched laminated structure has one A-chain and many B-chains per molecule. On the other hand, in the multiply-branched "tree" structure, there are approximately equal numbers of A-and B-chains.

It may be pointed out that the experimental evidence on which Meyer based his multiply-branched structure was equally in accord with the laminated structure. As previously mentioned, he showed that the same number of end groups were present in the β -dextrin as in the original molecule; this was said to be evidence of multiple-branching. However, since the exterior

chain stubs of both A-and B-chains are two to three glucose units in length (See Section III), both the β-dextrin of the laminated structure, and that of the multiply-branched structure would contain the same number of end groups as the original molecule.

Chemically, A-and B-chains are indistinguishable, and it is therefore impossible, by purely chemical methods, to differentiate between the Haworth and the Meyer structures. However, this difficulty has been overcome by the use of enzymes of known action pattern; these enzymic studies have indicated that the glycogen molecule is multiply-branched, and are considered in Section VI.

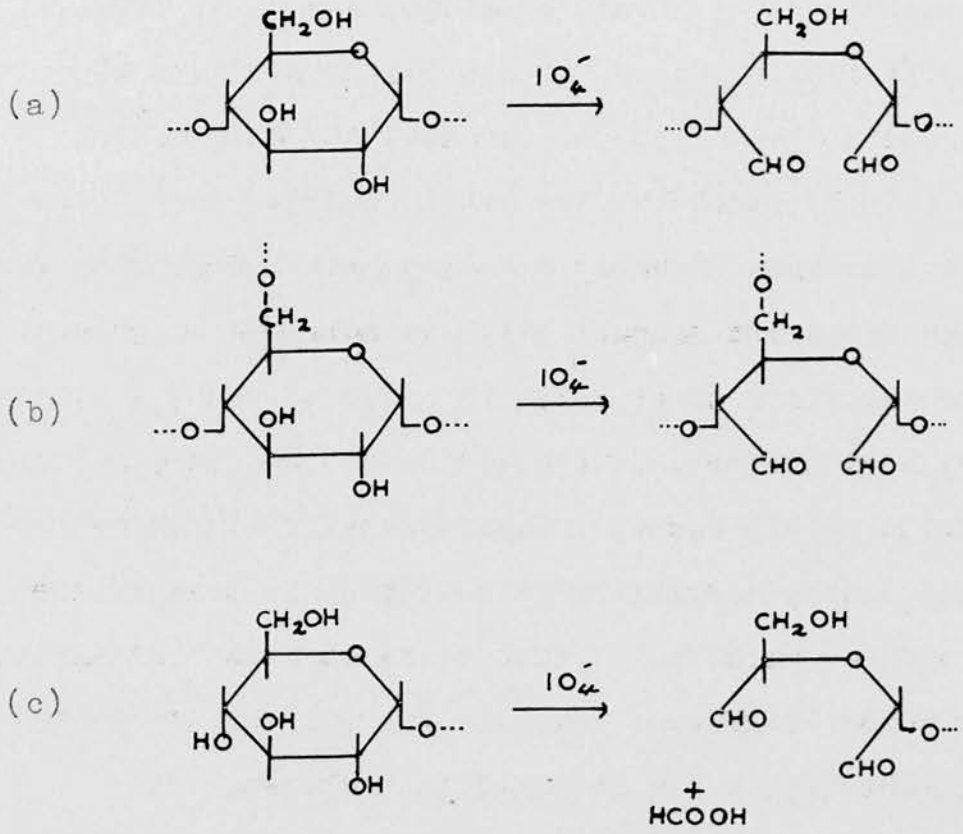
D. The nature of the inter-chain linkage.

The evidence that, in the glycogen molecule, the chains of α-1:4-linked glucose residues are inter-linked by primary glucosidic bonds has already been cited (Page 14). At first it was not known whether this linkage involved the hydroxyl group at position 2, 3, or 6, of the glucose residue at the branch point, although, on examination of the di-O-methyl-D-glucose fraction from the hydrolysate of methylated rabbit liver glycogen, Haworth, Hirst and Isherwood found that ca 30% was 2:3-di-O-methyl-D-glucose, indicating that a proportion of the inter-chain linkages were 1:6 (42).

After this, several workers examined the di-O-methyl-D-glucose fractions isolated from the hydrolysates of methylated glycogen, but no conclusive evidence regarding the nature of the

FIGURE III.

Periodate oxidation of α-1:4-glucosans.



inter-chain linkage was obtained. For example, Bell, in 1948, suggested that a number of the branch points involved a 1:3-glucosidic linkage (48); Hirst, Hough and Jones, in 1949, by chromatographic separation of the hydrolysis products of methylated rabbit liver glycogen obtained two di-O-methyl glucoses in roughly equimolecular yield: 2:3-di-O-methyl-D-glucose, and a second, probably 3:6-di-O-methyl-D-glucose (49).

The large but compact glycogen molecule is extremely difficult to methylate completely; in addition, during hydrolysis of the methylated product, demethylation of the tri-O-methyl-D-glucose fraction may occur. Experimentally, twice as much di- as tetra-O-methyl-D-glucose is obtained, instead of the expected equimolar amounts. Hence it is obvious that analysis of the di-O-methyl-D-glucose fraction can only be of limited value in characterising the inter-chain linkages.

Halsall, Hirst, Jones and Roudier suggested that periodate oxidation could be used for the identification of inter-chain linkages (56). The normal reaction of the periodate ion is the oxidative scission of α-glycol groups (See also Section II): when there are three hydroxyl groups on contiguous carbon atoms, as occurs in non-reducing terminal residues, the carbon atom in the centre is eliminated as formic acid (Fig. III) In glycogen, all glucose residues except those linked through C_2 or C_3, will be oxidised by periodate, and on hydrolysis of the oxo-glycogen, will yield a dialdehyde. If branching were

at either C_2 or C_3, the branch point residue would not be attacked, and, on hydrolysis of the oxo-glycogen, free glucose should be obtained.

Several groups of workers have examined the acid hydrolysates of periodate-oxidised glycogens. Gibbons and Boissonas used paper chromatography, and obtained a very small quantity of glucose; they estimated that 97 to 98% of the inter-chain linkages were 1:6 (57). Abdel-Akher, Hamilton, Montgomery and Smith first reduced the aldehyde groups of oxo-glycogen and hydrolysed the resulting polyalcohol (58). They obtained 1% of glucose, and postulated that a number of 1:3 linkages were present. Bell and Manners, in 1954, suggested that the small quantities of glucose obtained by previous workers, could be due to incomplete periodate oxidation of the glycogen (59). They oxidised four samples of glycogen with saturated potassium periodate at 15° to 20° for 40 to 50 days, hydrolysed the products, and examined the hydrolysates for glucose. In three cases no glucose was detected; in the fourth, the amount of glucose obtained corresponded to less than 1% of the inter-chain linkages being situated at C_2 or C_3.

Carlqvist also showed that on periodate oxidation of glycogen or β-dextrin, the uptake of periodate was slightly more than one mole per anhydro glucose residue (60). This indicated that the majority of inter-chain linkages were 1:6. If branching were at C_2 or C_3, then an uptake of exactly one mole

of periodate per anhydro glucose unit would be expected.

Wolfrom and colleagues further proved the existence of 1:6 linkages in glycogen (61). From a partial acid hydrolysate of rabbit liver glycogen they obtained isomaltose (6-O-α-D-glucopyranosyl-D-glucose) which was isolated as the crystalline octaacetate. This also indicated the configuration of the 1:6 linkage to be α .

5. A comparison between glycogen and starch.

Glycogen is closely related to starch, a polysaccharide found in many photosynthetic plants; both play the same functional role in their respective organisms, acting as reserves of carbohydrate. Starch is a glucosan and contains two components, amylose and amylopectin, in both of which the majority of glucose residues are united by α-1:4 linkages.

Most starches contain approximately 20% of amylose, but in certain waxy starches, e.g. maize and sorghum, the amylose content is only 1 to 2%; for most purposes these waxy starches may be regarded as pure amylopectins. By contrast, the amylose content of wrinkled pea starch is 66% (62). Amylose is only partially soluble in water but dissolves in alkali, the neutralised solution staining blue on addition of iodine. The molecules are long linear chains, each composed of up to 3,000 α-1:4 linked glucose residues. Recent work has shown that a very small degree of branching may occur in some samples of amylose (63).

Most amylopectins dissolve slowly in boiling water and are readily soluble in alkali. A red-purple colour is obtained with iodine. Like glycogens, amylopectins are branched macromolecules, but end group assay has shown that the chain lengths range from 20 to 30 glucose units. Both the Haworth "laminated" structure, and the Meyer "tree" structure, have been suggested for amylopectin. However, as in the case of glycogen, the Meyer formula appears more correct, and recent work has pointed to a method of calculating the ratio of A-chains to B-chains (64).

6. The aim of the present work.

Previous studies have indicated that glycogens are branched α-1:4-glucosans, but that all samples are not identical in properties or structure; this variation occurs even with specimens obtained from different individuals of the same species. For example, rabbit liver glycogen may have average chain lengths of 12 to 18 glucose residues, whilst invertebrate glycogens may vary in average chain length from 7 to 18 glucose residues.

In the present work a more detailed study of the molecular structure of a number of glycogens obtained from various vertebrate and invertebrate organisms has been undertaken. It has been pointed out that chemical methods have a limited application in studying the molecular structure of glycogens. However, by the use of enzymes of known action pattern, controlled break-down of the glycogen molecules can be effected. Since the extent of degradation of the substrate is related to its molecular structure,

enzymic degradation studies provide a method of structural analysis. For example, from the extent of degradation of glycogens by β-amylase the relative positions of the branch points in the unit chains have been calculated.

Glycogens have multiply-branched structures (65,66,67) and most workers have assumed that all specimens contain equal numbers of A- and B-chains. In view of the variation in degree of branching, it seems more likely that the ratio of A-chains to B-chains (the degree of multiple branching) will also vary. A method for the approximate determination of this ratio has accordingly been devised, and the degrees of multiple branching of several glycogens have been compared.

The enzymic degradation of glycogens by α-amylase and potato phosphorylase has also been studied. The former causes random scission of α-1:4-glucosidic linkages in both exterior and interior chains of the glycogen molecule; the relation between the degree of branching and the extent of α-amylolysis has been investigated.

The action pattern of potato phosphorylase towards branched polyglucosans is less well defined; glycogens of known chain length, exterior chain length and $\overline{AB}$ (ratio of A-chains to B-chains) were therefore submitted to the action of potato phosphorylase in an attempt to gain a more precise knowledge of the action pattern of the enzyme.

In a number of cases, similar experiments were carried out with the related α-1:4-glucosan, amylopectin.

The work which has been undertaken may be summarised briefly as follows:

Section II. The use of chemical methods, including end group assay by periodate oxidation, in studying the structure of glycogens.

Section III. Degradation of glycogens by β-amylase, and calculation of the relative lengths of exterior and interior chains.

Section IV. Degradation of glycogens by α-amylase.

Section V. An attempt to determine the action pattern of potato phosphorylase by phosphorolysis of glycogens and amylopectins.

Section VI. Degradation of glycogens by muscle phosphorylase, and calculation of the degree of multiple branching from this data and that from Section III.

SECTION II.

THE STUDY OF GLYCOGENS BY CHEMICAL METHODS.

In the course of this work, several specimens of glycogen were extracted from animal tissues. Before attempting to study their molecular structure by enzymic techniques, certain preliminary observations were made by chemical methods. It was thus shown that the glycogens were pure and that glucose was the only sugar obtained on acid hydrolysis. The absorption spectra of the iodine complexes, and the specific rotations, were measured, and end group assays carried out by potassium periodate oxidation.

A. Isolation of glycogens.

Discussion of methods.

Previous discussion (Page 1-3) has shown that the ease of extraction of glycogen from animal tissues is dependent on the extent of binding of the native glycogen by proteins. Methods of extraction may therefore be divided into two classes by which (a) "easily extractable" glycogen, (b) "difficulty extractable" glycogen, is removed from the tissue.

(a) "Easily extractable" glycogen is that fraction of the total glycogen which is readily soluble in water; it is therefore obtained by boiling the minced tissue in water for a short time. The resulting solution of glycogen is contaminated with proteins, which

are most commonly removed by precipitation, in the cold, with either trichloracetic acid (30), or picric acid (4). The glycogen is then precipitated by the addition of one to two volumes of alcohol.

A variation of this procedure consists in extracting the tissue with cold dilute (ca 10%) trichloracetic acid (68). Glycogen is recovered from the trichloracetic acid extract by alcohol precipitation.

(b) "Difficultly extractable" glycogen is more firmly bound to the tissue proteins. Methods of extracting it depend on complete solubilisation of the tissue, which is normally effected by boiling with 30% aqueous potassium hydroxide for several hours, a method first suggested by Bernard (69), and later used by Pflüger (22). Crude glycogen is then obtained by addition of one to two volumes of alcohol to the alkaline solution.

It has been suggested that exposure of glycogen to hot, strong alkali causes degradation of the molecule (23). In order to avoid any possibility of this, Meyer extracted the tissue with 33% aqueous chloral hydrate for one hour at 80° and pH 6 - 7, and thus obtained the "difficultly extractable" glycogen.

Various workers, however, consider that alkali treatment does not degrade the glycogen molecule. Bell and Young reported that alkali-extracted glycogen had a higher ash content than aqueous-extracted glycogen, but that the polysaccharide was not chemically affected by treatment with alkali (30). In a later investigation, Bell and colleagues carried out molecular weight determinations on two samples of rabbit liver glycogen obtained by alkali-extraction and aqueous-extraction respectively (34).

Both samples had the same molecular weight (4×10^6), although the alkali-extracted glycogen exhibited a higher degree of polymolecularity than the aqueous-extracted glycogen. They concluded, however, that "alkaline extraction of the glycogen from its parent tissue does not effect very serious degradation".

Glycogen prepared by any of the above methods is normally contaminated by a small quantity of inorganic material. Bell and Young found that the ash content of crude glycogen preparations could be substantially reduced by reprecipitating the polysaccharide several times from 80% acetic acid, a technique which had first been used by Claude Bernard in 1877 (30,69).

Another method of freeing glycogen from contaminating proteins was first introduced by Sevag (70), and subsequently used by Meyer (23). It consists of shaking the glycogen solution with chloroform and isoamyl alcohol for several hours. The proteins form a gel with the organic solvents, and can be separated from the aqueous solution of glycogen by centrifugation. Repetition of this procedure several times and subsequent alcohol precipitation of the glycogen, gives a product almost free from ash.

Experimental.

During the course of this work six samples of glycogen have been prepared. The author wishes to record her thanks to Dr. D. J. Bell, who kindly supplied cock liver tissue, and to Dr. G. R. Tristram, who made available tissues of skate liver, Mytilus edulis, Cardium, and Arenicola.

On removal from the organism, the livers were immediately plunged into boiling water to stop glycogenolysis. The tissues of the invertebrate animals were similarly treated. In most cases this water was drained off; weights of wet tissue which were received are reported in Table III.

The skate liver and lug worm glycogens were subsequently extracted by the Pflüger technique (Method A); the remaining samples were obtained by aqueous-extraction (Method B). In all cases the crude glycogens were purified by acetic acid precipitation (30). Details of these methods are as follows:

Method A.

The solid tissues were heated with an equal volume of 30% aqueous potassium hydroxide for three hours, during which time the tissue became completely solubilised. After cooling, the fat which had risen to the surface and solidified was removed. On addition of one and a half volumes of alcohol the glycogen precipitated as a black solid. This material was resuspended in 30% aqueous potassium hydroxide and heated for half an hour. The mixture was cooled, an equal volume of water was added, and insoluble material was removed by centrifugation. The glycogen was reprecipitated by the addition of one and a half volumes of alcohol, and when necessary was hardened by trituration with more alcohol.

The crude glycogen was dissolved in water and reprecipitated by adding four volumes of glacial acetic acid. This procedure was repeated three or four times, insoluble material

being removed when necessary by filtering through keiselguhr. The pure glycogen was then freed from traces of acetic acid by reprecipitating six times with 66% ethanol. With the last two or three alcohol precipitations it was necessary to add a trace of electrolyte in the form of ammonium acetate, to facilitate flocculation of the glycogen. The final precipitate was washed once with 90% alcohol, twice with absolute alcohol, twice with ether, and dried over phosphorus pentoxide *in vacuo*. Yields obtained at this stage, without further drying, are given in Table III.

Method B.

The tissues were minced, an equal volume of water added, and the mixture boiled for five to ten minutes. The liquid extract was filtered through muslin and the residue, after grinding with sand, was re-extracted with an equal volume of water. The combined extracts, the pH of which was 7 - 8, were acidified with acetic acid, then saturated with solid picric acid to precipitate the proteins. After filtration through a layer of celite and centrifugation, the glycogen was precipitated by the addition of 1.1 to 1.4 volumes of alcohol.

The crude glycogen was further purified by acetic acid precipitation as detailed in Method A.

Other glycogen samples.

Glycogens from *Mytilus edulis* IX and X were prepared by Dr. G. R. Tristram. The remainder of the glycogen samples used in this work were kindly supplied by Dr. D. J. Manners.

TABLE III.

Extraction of glycogens.

Source of glycogen	Wt.of wet tissue. (g.)	Method of extraction	Wt.of glycogen obtained. (g.)	Apparent glycogen content of tissue (%)
Cock liver	324	B	0.83	0.3
Skate liver	471	A	6.94	1.5
Mytilus edulis VII	218	B	3.47	1.6
Mytilus edulis VIII	274	B	2.38	0.9
Cardium	173	B	1.91	1.1
Arenicola	-	A	0.67	-

Roman numerals refer to different samples of glycogen from the same biological source.

B. Properties of glycogens.

1) Identification of the consituent sugar as glucose by paper chromatography.

Glycogen, in 0.1% concentration, was hydrolysed in 1.5 N sulphuric acid for 2 hours at 100°. The solution was neutralised (barium carbonate), concentrated *in vacuo*, and analysed by paper chromatography.

Descending chromatograms were carried out at room temperature with Whatman No. 1 paper. They were irrigated with benzene-pyridine-butanol-water (1:3:5:3) and developed by means of a silver nitrate - sodium hydroxide reagent (71).

Eight glycogens (from cock liver, skate liver, *Mytilus edulis* VII, *Mytilus edulis* VIII, *Mytilus edulis* IX, *Mytilus edulis* X, *Cardium* and *Arenicola*) were submitted to this technique; in all cases glucose was the only sugar detected.

2) Determination of purity by estimation of the glucose content.

The purity of glycogen samples can be determined by subjecting them to acid hydrolysis and estimating the glucose which is formed.

It has been found, however, that glucose is itself slowly destroyed when it is heated in acid solution. Hydrolysis of starch by acids was studied by Pirt and Whelan who found that sulphuric acid caused less destruction of glucose than did hydrochloric acid, and that loss of glucose was at a minimum when starch, in 0.1%

concentration, was hydrolysed in 1.5 N sulphuric acid for 2 hours at 100° (72). Their conditions have been used for hydrolysing several samples of glycogen including those whose isolation is reported in Section II, A.

Experimental.

a) Analysis of glucose.

Glucose was estimated using the Shaffer-Somogyi reagent 60, as modified by Hanes and Cattle, with a heating time of 15 minutes (73, 74). The reagent was sensitive to weights of glucose between 0.2 and 2.5 mg. in a total volume of 5 ml. It was calibrated against pure glucose; a straight line graph was obtained when weights of glucose were plotted against titre (ml. 0.01 N sodium thiosulphate).

b) Hydrolysis of glycogen.

Glycogen was dried *in vacuo* over phosphorus pentoxide for 4 hours at 100°. *Ca* 20 mg., accurately weighed, was dissolved in water (10 ml.) To an aliquot (4 ml.) of this solution, 3 N sulphuric acid (4 ml.) was added, and the acid solution heated in a boiling water bath for 2 hours. After cooling, it was made faintly alkaline with 5 N sodium hydroxide, using phenolphthalein as indicator; the pH was then brought to the acid side of neutrality by adding 2 N sulphuric acid (1 or 2 drops). The volume was made up to 25 ml. and the glucose content of aliquots

(2 x 3 ml.) of this solution was determined. From this figure, and the original weight of the glycogen sample, the percentage purity of the glycogen could be calculated. The results are reported in Table IV.

In a control experiment, an aliquot (4 ml.) of glucose solution (ca 2 mg./ml.) was heated with acid under conditions identical to those used for the hydrolysis of glycogens. A second aliquot was treated with acid, but was not heated. Estimation of the glucose content of both these solutions indicated that virtually no loss of glucose occured when a 0.1% solution was treated with 1.5 N sulphuric acid for 2 hours at 100°.

Discussion.

In seven cases out of the eight which are detailed in Table IV, the weight of glucose obtained by acid hydrolysis of glycogen is equivalent to 94% or more of the dry weight of the polysaccharide. In view of the fact that the experimental error in this technique may be of the order of 2%. these seven glycogens are considered to be virtually pure. If larger quantities of material had been available, the percentage content of nitrogen, phosphorus and total ash would have been determined. However, since these experiments would require ca 0.5 g. of glycogen, and as the results obtained by acid hydrolysis were satisfactory, it was thought desirable to preserve the material for more important experiments.

Mytilus edulis X glycogen has an apparent anhydro-glucose content of 89%. Insufficient material was available for a determination of ash content, but by analogy with the results obtained for the other seven glycogens of the series it was assumed that the ash content of *Mytilus edulis* X glycogen must be of the order of 7%. In subsequent experiments calculations are based on the assumption that *Mytilus edulis* X glycogen has an anhydro-glucose content of 93%.

TABLE IV.

Anhydro-glucose content of glycogens.

Source of glycogen.	Wt. of glycogen analysed. (mg.)	Wt. of glucose produced. (mg.)	Anhydro-glucose Content. %
Cock liver	20.8	23.1	~~100~~ 97 IDF
Skate liver	21.0	22.7	97
Mytilus edulis VII	19.9	21.0	95
Mytilus edulis VIII	21.5	23.1	98
Mytilus edulis IX	18.8	20.4	98
Mytilus edulis X	20.0	19.8	89
Cardium	20.4	21.2	94
Arenicola	21.3	22.7	96

3) Measurement of the specific rotation.

Solutions of glycogen (dried *in vacuo* over phosphorus pentoxide for 4 hours at 100°) (concentration *ca* 0.2%) were prepared, and rotations were measured in a 2 dm. polarimeter tube. As certain glycogen solutions of this concentration were too turbid to allow accurate polarimeter readings to be obtained, the concentration was decreased. The experimental details and results are summarised in Table V.

TABLE V.

Specific rotations of glycogens.

Source of glycogen.	Concentration. (%)	Observed rotation. (°)	Specific rotation. (°)
Rabbit liver V	0.224	0.88	196
Cock liver	0.0315	0.12	191
Skate liver	0.214	0.84	196
Mytilus edulis VII	0.202	0.81	200
Mytilus edulis VIII	0.211	0.82	194
Mytilus edulis IX	0.202	0.79	196
Mytilus edulis X	0.186	0.74	199
Cardium	0.102	0.41	201
Arenicola	0.195	0.78	200

Discussion.

Since the observed rotations may be in error by 0.01°, the calculated specific rotations of most of the glycogens are accurate only to within ± 3°. In the case of cock liver and Cardium glycogens 0.2% solutions were too opalescent for polarimetric observation and had to be diluted further. The probable error in these specific rotations is therefore proportionately greater.

The specific rotations of the nine glycogens vary from 191° to 201° and are therefore similar to those reported in the literature for other glycogens. The specific rotation of a polysaccharide is not closely related to other chemical and physical properties, and the values are interpreted as indicating the presence of a high proportion of α-glucosidic linkages.

A determination of the molecular weight of cock liver glycogen would be of interest since the glycogen is much less soluble in water than normal, and the resulting solution is very turbid; it is suggested that this glycogen may have an exceptionally high molecular weight.

4) Iodine staining.

The colours obtained when glycogen solutions are stained with iodine have been reported to range from pale yellow, through brown, to violet (See Page 7). These variations in colour are reflected in differences in the wavelength of maximum absorption, and in the optical density of the absorption spectra of the

glycogen - iodine complexes.

A study of the iodine absorption spectra of several glycogens (chiefly from invertebrates) was made, in an attempt to correlate them with known structural features of the polysaccharides.

Experimental.

The method of Peat, Whelan, Hobson and Thomas was used in measuring the iodine absorption spectra of glycogens (75). Glycogen solution (2.5 mg.) to which 6 N hydrochloric acid (1 drop) was added, was stained with iodine - potassium iodide solution (0.2% iodine, 2% potassium iodide; 2.5 ml.) in a total volume of 25 ml. Values of the optical density (log I/I_o) over the wavelength range 390 to 500 mμ were determined, with a Unicam spectrophotometer (Model S.P.500) and 1 cm. cells. Iodine - potassium iodide solution (0.02% iodine, 0.2% potassium iodide) was used as a blank.

The iodine absorption spectra (420 to 700 mμ) of two amylopectins were measured in a similar way.

The wavelengths of maximum absorption ($\lambda_{max.}$) and the corresponding maximum values of the optical density of nine glycogens and two amylopectins are recorded in Table VI. Details of the spectra are shown in Figs. IV and V.

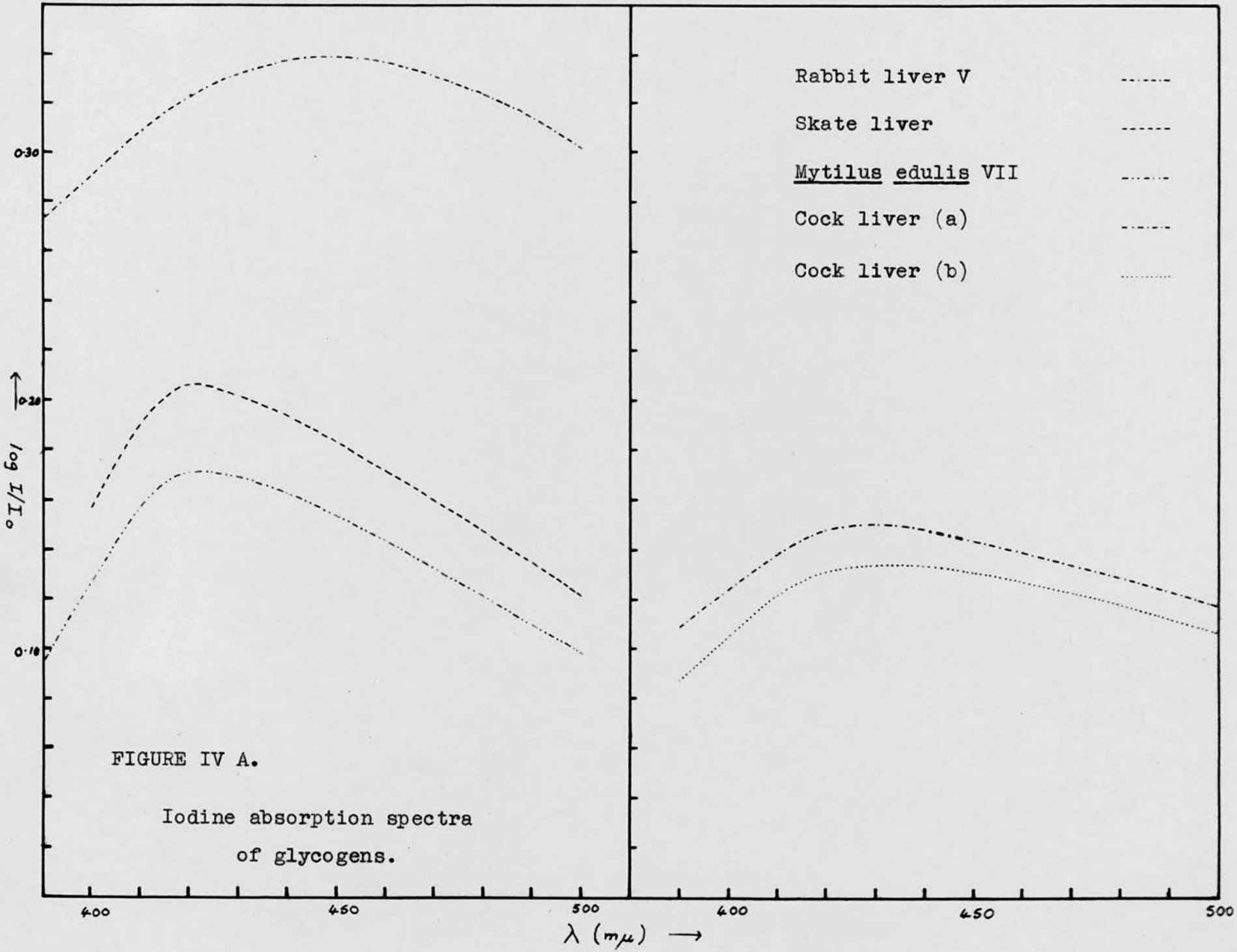


FIGURE IV A.

Iodine absorption spectra of glycogens.

FIGURE IV B.

Iodine absorption spectra of glycogens.

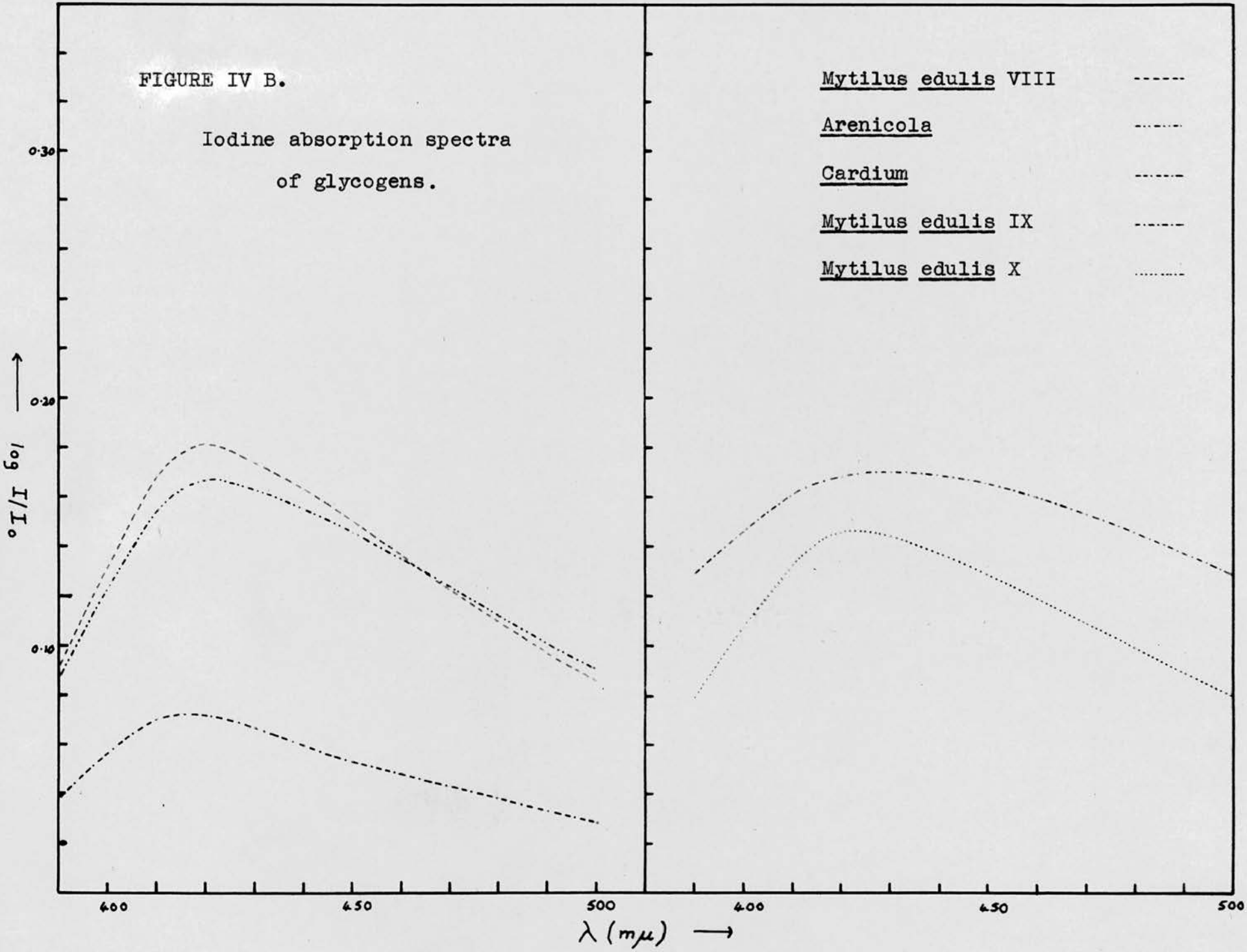


FIGURE V. Iodine absorption spectra of amylopectins.

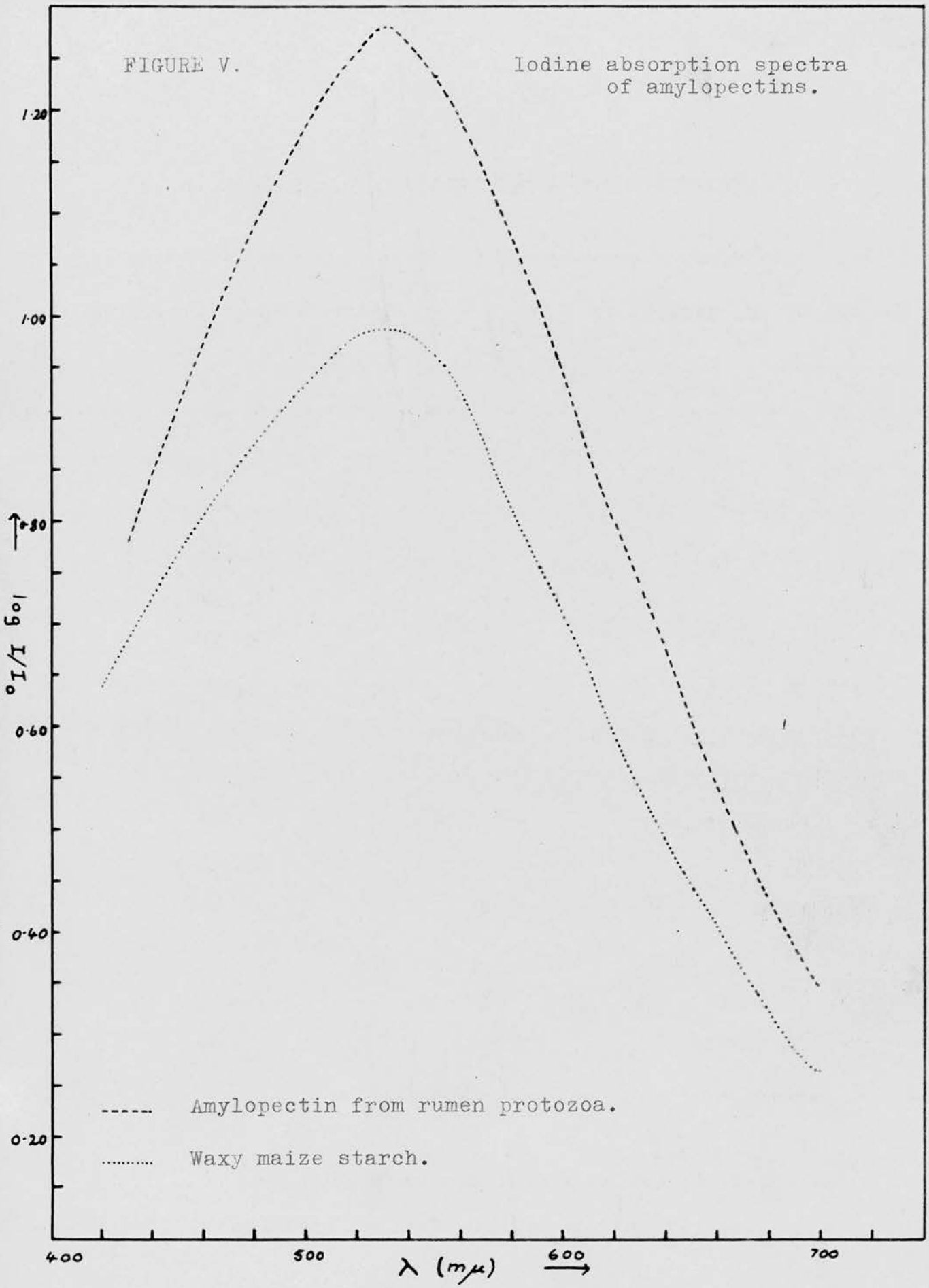


TABLE VI.

Data from iodine absorption spectra of polysaccharides.

Source of polysaccharide.	$\lambda_{max.}$ (mμ)	Maximum optical density.
Glycogens		
Rabbit liver	450	0.339
Cock liver a)	430	0.149
b)	440	0.134
Skate liver	420	0.207
Mytilus edulis VII	420	0.171
Mytilus edulis VIII	420	0.181
Mytilus edulis IX	430	0.170
Mytilus edulis X	420	0.146
Cardium	415-420	0.072
Arenicola	420	0.167
Amylopectins		
Waxy maize	530	0.982
Rumen protozoa	530	1.28

a) Uncorrected for the effect of light scattering.

b) Corrected for the effect of light scattering.

Discussion.

It was possible that light scattering by the glycogen solutions might affect the shape and $\lambda_{max.}$ of the absorption spectra of the iodine complexes. The light absorption (390 to 500 mμ) of glycogen solutions (0.01%) was therefore measured, water serving as a blank. For the majority of glycogens the effect of light scattering was negligible; with the very opalescent cock liver glycogen, however, it was appreciable, and in Fig. IV, two iodine absorption curves are shown for cock liver glycogen: (a) uncorrected, and (b) corrected for the effect of light scattering.

The difference between the iodine absorption spectra of glycogens and those of amylopectins is very marked. In this study, the $\lambda_{max.}$ values of glycogens varied from 420 to 450 mμ, and the optical density was never greater than 0.34 (rabbit liver V glycogen). By contrast, for both amylopectins the $\lambda_{max.}$ value was 530 mμ, while an optical density of 1.28 was attained (amylopectin from rumen protozoa). Thus, the complexes formed between iodine and amylopectins not only absorb at a higher wavelength than do the corresponding complexes of glycogens, but they absorb much more strongly. It was thought that this difference might be due to the longer average exterior chain length of amylopectins, but recent work by Mr. I. D. Fleming in this laboratory has shown that the iodine complex of a β-dextrin of waxy maize starch, has the same $\lambda_{max.}$ as the iodine complex of the parent amylopectin. The interior chains of the β-dextrin

molecule must therefore be involved in complex formation with iodine; this is not unexpected since the average interior chain length is of the order of 8 glucose residues. Herein may lie the difference between the interaction of amylopectins and glycogens with iodine, since binding or absorption of iodine by the shorter interior chains of glycogens would be unlikely to occur.

The iodine absorption spectra of individual glycogens differ considerably; the wavelength of maximum absorption and the optical density of the iodine complexes appear to be dependent, not upon the chain length or the exterior chain length of the glycogen, but upon its source. Previous workers have reported that the colour of the iodine complex formed with some invertebrate glycogens (e.g. from Mytilus edulis and Helix pomatia) and with fish liver glycogens, is yellowish, whereas a red-brown colour is obtained with mammalian glycogens. Similar results were noted here: lower values for both $\lambda_{max.}$ and optical density were found with invertebrate glycogens (Mytilus edulis, Cardium and Arenicola), cock liver glycogen and skate liver glycogen than with rabbit liver glycogen. These differences cannot be correlated with known structural features (chain length, β-amylolysis limit) of the glycogens. Thus, on comparing the data of Fig. IV with that given in Table XI (Page 77), it is seen that rabbit liver V glycogen differs little from Mytilus edulis X glycogen in either chain length or exterior chain length, although the lack of similarity in their iodine absorption spectra is striking.

Moreover, Mytilus edulis X glycogen has a higher chain length and exterior chain length (Table XI) than Mytilus edulis IX glycogen, yet the iodine complex of the former has a slightly lower $\lambda_{max.}$ and corresponding optical density.

Cockle glycogen showed much lower optical density values than the other invertebrate glycogens. This is possibly because a small proportion only, of the exterior chains (average length 3 to 4 glucose units; see Table XI) are long enough to take part in complex formation.

It is noticeable that the shape of the iodine absorption curves varies, and that, in general, sharper peaks are found on those curves where maximum absorption occurs at lower wavelengths. Further work, however, is required before any significance can be attached to this observation.

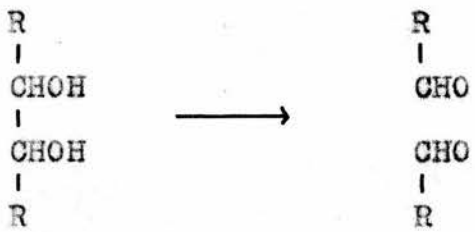
To summarise, it may be stated that the wavelength of maximum absorption of the iodine complexes of invertebrate glycogens is lower than that of mammalian glycogens and that fish liver and bird liver glycogens resemble invertebrate glycogens. (Observations made by Mr. I. G. Jones in this laboratory show that other mammalian glycogens have similar spectra to that reported for rabbit liver V glycogen). The iodine absorption spectra of glycogens cannot be related to their known structural features, although it is noted that amylopectins, which have longer chain lengths, exhibit maximum absorption at higher wavelengths; in general the optical density increases with the $\lambda_{max.}$

C. End group assay by periodate oxidation.

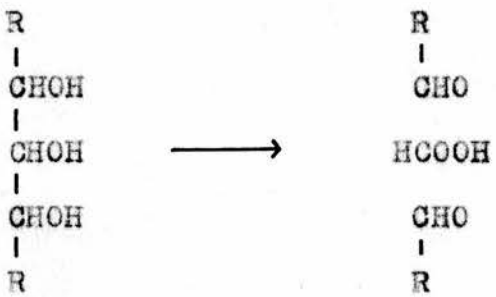
Introduction.

1. Oxidative reactions of periodate.

Compounds containing an α-glycol grouping are oxidised by periodic acid and its salts with the formation of a di-aldehydic structure.



When three vicinal hydroxyl groups are present, two moles of periodate are consumed, the central group being released as formic acid.



Moreover, when a hydroxyl group is adjacent to a primary alcohol group, formaldehyde is produced.

Malaprade was the first to apply periodic acid oxidation to the study of carbohydrates (76). He oxidised sugar alcohols

of the general structure $CH_2OH.(CHOH)_n.CH_2OH$, and confirmed that they reacted according to the equations detailed in the preceeding paragraph. (n+1) Moles of periodate were consumed with the production of two moles of formaldehyde and n moles of formic acid.

Periodate oxidation techniques have found wide application in structural studies in carbohydrate chemistry (77,78,79). Jackson and Hudson showed that periodic acid would react with carbohydrate molecules which were stabilised in the ring formation: α- and β-methyl glucosides (and other methyl glycosides) were oxidised yielding one mole of a dialdehydic compound together with one mole of formic acid. This work suggested that periodate oxidation could be used as a means of end group assay of 1:4-linked glucosans. Each glucopyranose radical at an intermediate position in the polymer chain has an α-glycol group at carbon atoms 2 and 3, and will consume one mole of periodate with the formation of a dialdehyde (Fig.III a,b). Non-reducing terminal radicals have free hydroxyl groups on carbon atoms 2,3 and 4, and will react with two moles of periodate, one mole of formic acid being liberated (Fig. III c). Measurement of the amount of formic acid released on periodate oxidation of branched glucopolysaccharides such as glycogen, should provide an accurate means of end group assay. Owing to the size of the molecule and the high degree of branching, the reaction of the periodate ion with the single reducing group (which can give three or more moles of formic acid) may be neglected.

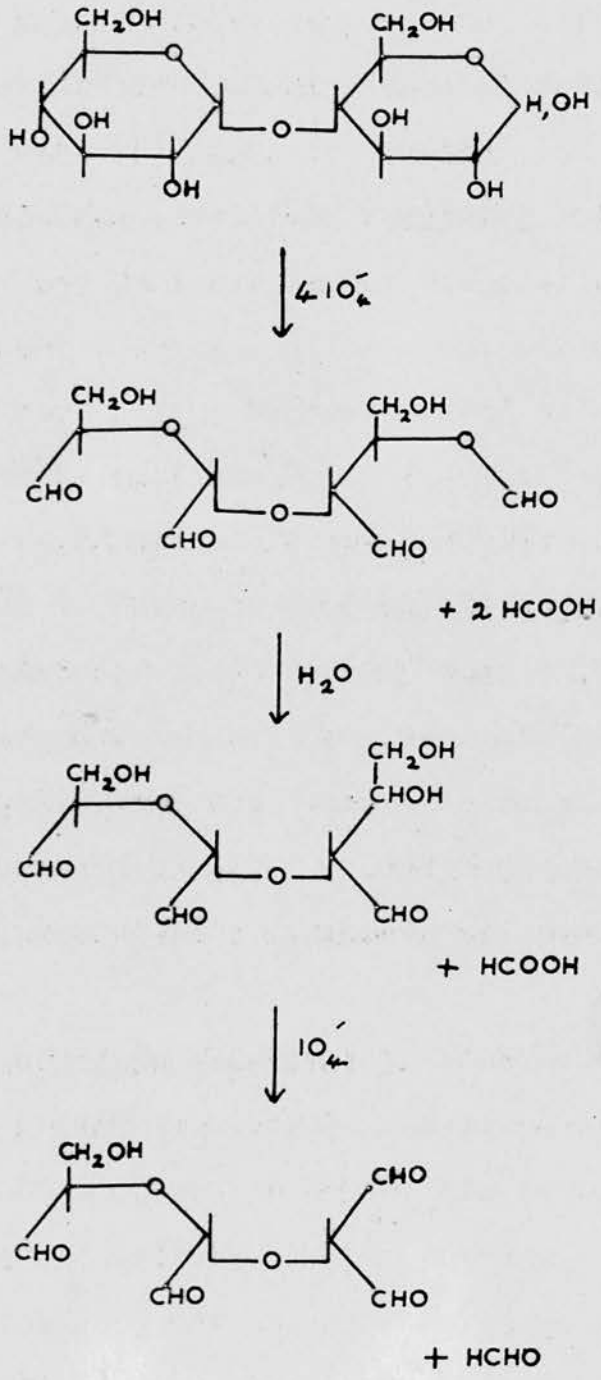
2. Methods of periodate oxidation of glycogens.

Earlier workers found that during oxidation of 1:4-glucosans the periodate uptake was often greater than expected, an effect which was usually accompanied by increased liberation of formic acid and formaldehyde, and which has been termed "over-oxidation" (80). Before quantitative studies on polysaccharides could be attempted, conditions which would avoid over-oxidation had to be devised. With this end in view, Hirst and coworkers to whom the idea of using periodate oxidation as a method of end group assay must be credited, carried out model experiments with methyl pyranosides of various hexoses and pentoses, and with methyl biosides (81). Oxidation of the methyl glycosides of monosaccharides with sodium periodate gave the expected results. However, when this technique was applied to the methyl glycosides of reducing disaccharides, over-oxidation occured. Thus it was obvious that sodium periodate oxidation could not be applied to polysaccharides.

Oxidation of the same compounds with potassium periodate was attempted. This salt is less soluble than sodium periodate, and was prepared by adding potassium chloride to a solution of sodium periodate and glycoside. The bulk of the potassium periodate precipitated out in the reaction mixture, ensuring that a small though constant quantity of periodate ion was present. Although the rate of the reaction was inevitably decreased the production of formic acid from β-methyl maltoside ceased after 150 hours at 15°, and the theoretical quantity of formic acid was produced from the methyl glycosides of hexopyranoses,

FIGURE VI.

Periodate oxidation of maltose.



pentopyranoses, and the reducing disaccharides, provided that the final concentration of formic acid in the reaction mixture was kept low (ca 10 mg./ml.). Using these conditions end group assays of various polysaccharides gave results which agreed fairly well with those obtained from methylation studies (46,82).

Bell and Manners, in 1952, reported that on oxidation of glycogens with potassium periodate, production of formic acid did not cease within 150 hours, and that the values of unit chain length based on the 150 hour figure, were incorrect (83). They suggested that the large branched polysaccharide molecule was oxidised less rapidly than were small molecules such as β-methyl maltoside. The reaction was therefore allowed to proceed beyond 150 hours and it was found that after 250 - 300 hours, no further increase in formic acid concentration occured; oxidation was then considered to be complete, and the maximum yield of formic acid was used in calculation of the unit chain length. These observations are supported by those of Anderson, Greenwood and Hirst who examined the potassium periodate oxidation of starches (84).

Other methods of periodate oxidation of glucopolysaccharides have been suggested. Potter and Hassid, in 1948, criticised the potassium periodate method on the grounds that it was too slow (85). They approached the problem of the end group assay of polysaccharides by first studying the periodate oxidation of maltose. Their aim was to find conditions under which maltose would yield three moles of formic acid (see Fig. VI), and then to apply

these conditions to the end group assay of polysaccharides. Potter and Hassid found that, by carrying out the oxidation of maltose with sodium periodate in sodium chloride solution at 2°, formic acid production was rapid, until, after 25 hours, the theoretical value of three moles was reached. However, the reaction did not stop completely; continued slow production of formic acid occured, due to over-oxidation of the reducing end group. Nevertheless, in experiments in which amylose and amylopectin were oxidised, the amount of formic acid produced in 25 hours was used in calculation of the percentage end group.

Schlamowitz applied the Potter and Hassid sodium periodate method of end group assay to glycogens, and reported chain length values which were consistently higher than those obtained by other workers (See Table VII) (86). It is suggested that although the oxidation of maltose might be complete in 25 hours, oxidation of glycogen would proceed more slowly, and only part of the maximum theoretical yield of formic acid would be released in that time. The error involved in basing calculations on the 25 hour figure would be greater than that arising from over-oxidation of the single reducing group. Moreover, recent experiments by Archibald and Manners have shown that sodium periodate oxidation of maltose by the method of Potter and Hassid is slower than claimed by these authors; less than 2.5 moles of formic acid are released in 25 hours (87).

Meyer and Bathgeb studied the periodate oxidation of both simple sugars and glucopolysaccharides (88). They found that sodium periodate was a satisfactory oxidant, provided that

the temperature of the reaction was kept at 0°, and the pH between 4.2 and 5.8. Under these conditions the authors found that the oxidation of branched glucopolysaccharides such as amylopectin was complete in 150 - 200 hours.

Results of end group assays on a number of glycogens were published by Abdel-Akher and Smith in 1951 (89). They claimed that, with a low concentration of sodium periodate and a low temperature, over-oxidation was avoided; a maximum yield of formic acid was obtained in 80 - 90 hours at 5 - 6°, although the liberation of formic acid took 160 - 180 hours when the oxidation was carried out at 0°.

A selection of results obtained by previous workers who have carried out end group assays on glycogens are reported in Table VII.

3. Over-oxidation.

The term over-oxidation covers oxidative reactions which occur subsequent to the straightforward attack on vicinal hydroxyl groups. For example, on periodate oxidation of reducing disaccharides, such as maltose, the primary product is an ester of formic acid. The ester linkage is readily hydrolysed, giving a structure which is again susceptible to periodate oxidation (See Fig.VI.). The compound resulting from this oxidative step has a grouping of the type $-\overset{O}{\overset{\|}{C}}-\overset{H}{\overset{|}{C}}-\overset{O}{\overset{\|}{C}}-$, where the hydrogen atom is activated by the presence of the neighbouring carbonyl groups. Several workers have postulated that the active hydrogen atom is oxidised to a hydroxyl group, thus opening the way for a further

series of normal periodate oxidations (90,91,92,81). The mechanism of subsequent reactions is uncertain (c.f.93); the final products are formic acid, formaldehyde, and carbon dioxide.

Formic acid and formaldehyde also undergo slow oxidation to carbon dioxide and water. Sarkar, in 1951, studied the problem of oxidative loss of formic acid, and showed that, in control mixtures of formic acid and potassium periodate, oxidation of formic acid occured when it was present in fairly high concentrations (94). At concentrations of N/500 or less, no loss of formic acid could be demonstrated. Subsequently, Head and colleagues have studied the effect of light on periodate oxidation (95,96,97). They conclude that normal Malapradian reactions are not noticeably accelerated by exposure to light, but that the rate of over-oxidation, including the further oxidation of formic acid, is greatly increased.

It is therefore apparent that over-oxidation can be controlled by carrying out the raction in the dark, and by keeping both the concentration of periodate in the reaction mixture (81,89), and the concentration of formic acid formed during the reaction, low. Under these conditions, over-oxidation of the small percentage of reducing groups present in the large, highly branched glycogen molecule, is negligible. Furthermore, loss of formic acid does not occur.

TABLE VII.

End group assay of glycogens by periodate oxidation.

Source of glycogen.	Method of oxidation.[a]	Chain length.	Ref.
Rabbit liver	A	12	89
Rabbit liver	A	17	86
Rabbit liver	A	21	86
Ox liver	A,B	13,12	89
Horse muscle [b]	B	14[c]	46
Horse muscle [b]	B	11[d]	83
Ascaris lumbricoides	B	12	46
Helix pomatia	B	7	83
Mytilus edulis	B	16	83
Moniezi expansi	A,B	12,12	89

a) A, sodium periodate; B, potassium periodate oxidation.

b) Both analyses were carried out on the same specimen of horse muscle glycogen.

c) Calculated from 150 hour titre.

d) Calculated from maximum titre.

4. Estimation of formic acid.

Malaprade, in 1934, made the first attempt to estimate formic acid produced by periodic acid oxidation of carbohydrates (98). He titrated the acidic mixture to the phenolphthalein or thymolphthalein end point, and applied corrections for the periodic acid, and iodic acid which were present.

A similar, but more complex method was suggested by Khouvine and Arragon in 1941 (99). They removed periodic acid and iodic acid from the reaction mixture by precipitation with barium hydroxide. The filtrate was acidified, and steam distilled, and the steam volatile formic acid estimated by titration to the phenolphthalein end point.

Abdel-Akher and Smith, in 1951, claimed that an iodometric method for estimation of formic acid was most efficient (89). After destruction of the excess periodate in the reaction mixture by the addition of ethylene glycol, they added potassium iodide in excess, and measured the amount of iodine liberated according to the equation:

$$IO_3^- + 5I^- + 6H^+ \rightarrow 3H_2O + 3I_2$$

However, most workers, using sodium or potassium periodate for oxidation, have found that destruction of excess periodate with ethylene glycol, followed by straightforward titration of formic acid with barium hydroxide or sodium hydroxide is the most suitable method. Hirst and co-workers titrated to a methyl red endpoint and their example has been followed by several others (46,83,100), although Meyer and Rathgeb claimed that the pK of this indicator

was too low for titration of a weak carboxylic acid, and preferred to titrate to pH 5.8, using bromocresol purple as indicator (88).

In 1952, Kerr and Cleveland plotted the curve obtained by potentiometric titration of formic acid with barium hydroxide (101). They showed that the mid-point was at pH 7.1 and stated that an accurate estimate of formic acid should be obtained by titrating to pH 7.1. However, Morrison, Kuyper and Orten pointed out that the potentiometric curve rose steeply from pH 5.5 to pH 8.0, and claimed that pure formic acid could be accurately determined by titrating to any pH within these limits (102). They noted, however, that at an alkaline pH, in the presence of periodate-oxidised glycogen, other acidic substances were produced, and that above pH 7, the experimental potentiometric curve no longer followed the theoretical curve for pure formic acid.

Anderson, Greenwood and Hirst, in 1955, gave a more detailed account of this effect. Potentiometric titration curves of periodate oxidation mixtures deviated from the theoretical formic acid curve above pH 6.25 (84). The authors considered that this was due to acidic substances formed by alkaline hydrolysis of the acetal linkages, and by Cannizzaro type reactions in which -CHO groups were converted to -COOH. They affirmed that titration of formic acid was complete at pH 6.25.

Experimental.

End group assays have been carried out on a number of glycogens by the method of Halsall, Hirst and Jones, as modified

by Bell and Manners (83,85).

Reagents.

Reagents of AnalaR grade were used where possible.

Ethylene glycol: Ethylene glycol was distilled over solid potassium hydroxide to eliminate all traces of acid.

Sodium hydroxide: Sodium hydroxide (approximately 0.01 N) was prepared. It was rendered carbonate-free by passing through a column of "Deacidite F", in the OH form (103). This is a strongly basic anionic exchange resin on which divalent carbonate ions are preferentially adsorbed.

Periodate oxidation of glycogens and analysis of formic acid produced

1) Experimental procedure.

Glycogen (200 to 300 mg.) (dried in vacuo over phosphorus pentoxide for 4 hours at 100°) was dissolved in 5% potassium chloride (90 ml.). An aliquot (10 ml.) of this solution was removed to serve as a blank. To the bulk of the glycogen solution 0.2 M sodium metaperiodate solution (30 ml.) was added, causing potassium periodate to precipitate out of solution. The reaction mixture was placed in a brown glass bottle (to avoid sunlight) and gently shaken by mechanical means.

At suitable time intervals, aliquots (10 ml.) of the solution were removed, care being taken to include none of the solid potassium periodate (which settled readily on standing). Ethylene glycol (1 ml.) was added, and the solution allowed to stand for 20 to 30 minutes, to ensure complete destruction of excess periodate. The formic acid was then titrated potentiometrically to pH 5.7 with carbonate-free sodium hydroxide

(0.01 N), contained in a semi-micro burette. The titration was followed by means of a glass electrode, and a Pye mains-operated pH meter. In order to ensure that no interference from carbonate ions occured, nitrogen was bubbled through the solution for 10 minutes prior to commencement of the titration, and throughout the duration of the titration.

A blank titration was carried out in order to correct for traces of acidity caused by substances other than formic acid. To an aliquot (10 ml.) of glycogen solution removed before addition of sodium metaperiodate to the bulk of the reaction mixture, ethylene glycol (1 ml.) and then 0.2 M sodium metaperiodate (30 x 10/80 = 3.75 ml.) were added. The solution was titrated to pH 5.7, with carbonate-free sodium hydroxide (0.01 N). The titre obtained was the blank value equivalent to 13.75 ml. of the reaction mixture. It was therefore multiplied by 10/13.75 = 0.73 to obtain the figure equivalent to 10 ml. of the reaction mixture. This blank value, which was normally between 0.00 ml. and 0.03 ml. was subtracted from the observed formic acid titre.

Aliquots were removed from the reaction mixture after ca 260 hours, and at 48 or 72 hour intervals thereafter, until no further increase in titre was observed. The maximum titre, corrected for the blank, corresponded to the amount of formic acid released when periodate oxidation of glycogen was complete, and was therefore used in calculating the unit chain length of the glycogen. Normally the maximum titre was attained between 260 and 300 hours after the commencement of the reaction.

2. A typical experiment: end group assay of Mytilus edulis VII glycogen.

Glycogen (263.5 mg.) was dissolved in potassium chloride solution (5% ; 90 ml.). An aliquot (10 ml.) was removed for the blank; sodium metaperiodate solution (0.2 M; 30 ml.) was then added. The reaction mixture therefore contained 234.22 mg. of glycogen in 110 ml. i.e. the concentration of glycogen in the reaction mixture was 21.29 mg./10 ml.

Titrations were carried out with 0.01177 N sodium hydroxide solution.

Results.	Blank.		Titre.
			0.01 ml.
	Reaction.	Time.	Titre. (corrected for blank)
		264 hr.	0.84 ml.
		336 hr.	0.86 ml.

The increase in titre between 264 hours and 336 hours was negligible. It was therefore concluded that periodate oxidation of the glycogen was complete, and these titration figures were used to calculate the average chain length of the glycogen.

3. Calculation of results.

One molecule of formic acid is released from each unit chain in the glycogen molecule. Assuming that each chain comprises an average number of n glucose residues, 46 mg. (the mg. equivalent weight) of formic acid will be obtained from [179 + (n-2)162 + 145] mg. of glycogen, i.e. from (162n) mg. of glycogen.

Experimentally, the weight of formic acid which is released from a known weight of glycogen is obtained from the sodium hydroxide titre, 1 ml. of 0.01 N sodium hydroxide being equivalent to 0.46 mg. of formic acid. e.g. the weight of formic acid released by 21.29 mg. of Mytilus edulis VII glycogen is

$$\frac{0.46 \times 0.86 \times 0.01177}{0.01} \text{ mg.}$$

By proportion, therefore, $n = \frac{21.29 \times 46 \times 0.01}{0.46 \times 0.86 \times 0.01177 \times 162}$

$= 13.0$ glucose residues.

i.e. Mytilus edulis VII glycogen has an average chain length of 13 glucose residues.

Using the titre of 0.84 ml. (0.01177 N sodium hydroxide) obtained after 264 hours, the chain length value is 13.3 glucose residues: the difference between 13.3 and 13.0 is negligible and within the limits of experimental error.

Results.

End group assays were carried out on a number of glycogens, and the results obtained are summarised in Table VIII.

Discussion.

In none of the experiments reported in Table VIII, did the concentration of formic acid in the reaction mixture reach the value of 0.002 N above which Barkar considered that oxidative loss of formic acid occured (94). The highest concentration attained was 0.0016 N (Cardium glycogen), but in most experiments

TABLE VIII

End group assay of glycogens by potassium periodate oxidation.

Source of glycogen	Chain length (glucose radicals)
Rabbit liver V	14
Rabbit liver XI	16
Rabbit liver XII	17
Rabbit liver XIII	15
Rabbit muscle II	11
Human muscle II	11
Cock liver	13
Skate liver	13
Mytilus edulis VI	13
Mytilus edulis VII	13
Mytilus edulis VIII	13
Mytilus edulis IX	10
Mytilus edulis X	14
Cardium	8
Arenicola	11

the figure was appreciably lower. Moreover, access of sun light to the reaction mixture was prevented. It was therefore con-

sidered that conditions were such that over-oxidation of formic acid would not take place.

Duplicate experiments carried out on several glycogens showed that the results were accurate to within ± one glucose unit. Periodate oxidation as a method of end group assay is therefore fully as reliable as the methylation technique, and in addition to being more convenient, requires much smaller quantities of material.

Titrations were carried out to pH 5.7. As this point lay on the vertical portion of the titration curve for pure formic acid and sodium hydroxide (102), complete neutralisation of all the formic acid present was ensured. Anderson, Greenwood and Hirst suggested that pH 6.25 should be regarded as the end point of the titration (84); however, chain length values obtained by titrating to pH 6.25 did not differ significantly from those obtained by titrating to pH 5.7. The titre differences were 0.01 - 0.03 ml. of 0.01 N sodium hydroxide, equivalent to chain length differences of ca 0.3 glucose residues.

The values of the average chain lengths of 15 specimens of glycogen are reported in Table VIII. They vary from 8 to 17 glucose residues, although the majority are within the range of 10 to 14 glucose residues; the same order of distribution of results has been found previously (See Table VII; page 48).

The glycogen of Cardium is particularly interesting in that it has a very short chain length. Its structure will be discussed in greater detail in Section III.

The four specimens of rabbit liver glycogen examined during the course of this work all have chain lengths higher than the more usual value of 12. The occurence of 18-unit rabbit liver glycogens has, however, been occasionally reported (32,41,42). It may therefore be considered that, although a chain length value of *ca* 12 for rabbit liver glycogen is most commonly found, values between 12 and 18 are by no means abnormal.

Several specimens of glycogen from *Mytilus edulis* have been examined, and again variations on the average chain length have been found. This study of both rabbit liver and *Mytilus edulis* glycogens emphasises the fact that normal glycogens from a particular species may vary in chain length from one specimen to another, and thus, that the structure of glycogen is not constant even within a species.

SECTION III

β-AMYLOLYSIS OF GLYCOGENS.

Introduction.

β-Amylase occurs in germinated and ungerminated cereal grains such as barley, rye and wheat, and in other higher plants e.g. sweet potatoes and soya beans; it has not been found in animal tissues.

This enzyme causes hydrolytic degradation of starch-type polysaccharides and related oligosaccharides. Starting from the non-reducing end of each chain, it catalyses the hydrolysis of alternate α-1:4-linkages with the production of maltose. This maltose initially has a β-configuration, indicating that the enzymic reaction involves a Walden inversion. The enzyme can neither hydrolyse nor by-pass α-1:6-glucosidic linkages, or other anomalous linkages which may be present in the substrate.

β-Amylase is an extremely efficient catalyst, with a turnover number, in terms of glucosidic linkages hydrolysed per minute at 30° and pH 4.8 of *ca* 250,000 (104). Degradation of the substrate may occur by either of two mechanisms, involving a) single-chain action, in which one chain of α-1:4-linked glucose residues is completely degraded before a second chain is attacked or b) multi-chain action, in which there is random attack on all chains. Experimentally it is found that during β-amylolysis of amylose, the mechanism is somewhere between

these two extremes, but that the proportion of multi-chain action increases as the temperature is raised (105, 106).

β-Amylase is inhibited by mercury, copper and silver ions, and by various other substances including oxidising agents. The latter act by transforming -SH groups which are essential for enzymic activity, into -S-S- bonds (104).

Certain samples of amylose have been shown to contain linkages which are not attacked by β-amylase. Early studies of the β-amylolysis of amylose indicated that, provided retrogradation was avoided, the molecule was completely degraded to maltose (107-109). More recently, β-amylase preparations of a high degree of purity, often crystalline, have been used, and it has been found that β-amylolysis of amylose ceases when about 70% of the molecule has been degraded (63). Further work has shown that amorphous preparations of β-amylase are contaminated with a system of β-glucosidases which is removed by subsequent purification (110). This may indicate that a small percentage of β-glucosidic linkages is present in the amylose molecule; whether these are anomalous bonds in the single chain of α-1:4-linked glucose residues, or points of chain ramification, has not been conclusively proved, although on the basis of work by Kerr and Cleveland, the latter appears more likely (101). These authors studied the rates of β-amylolysis of various amyloses; their results indicate that in both potato and tapioca amylose, a small degree of branching is present. There has been no experimental confirmation of the suggestion made by Peat,

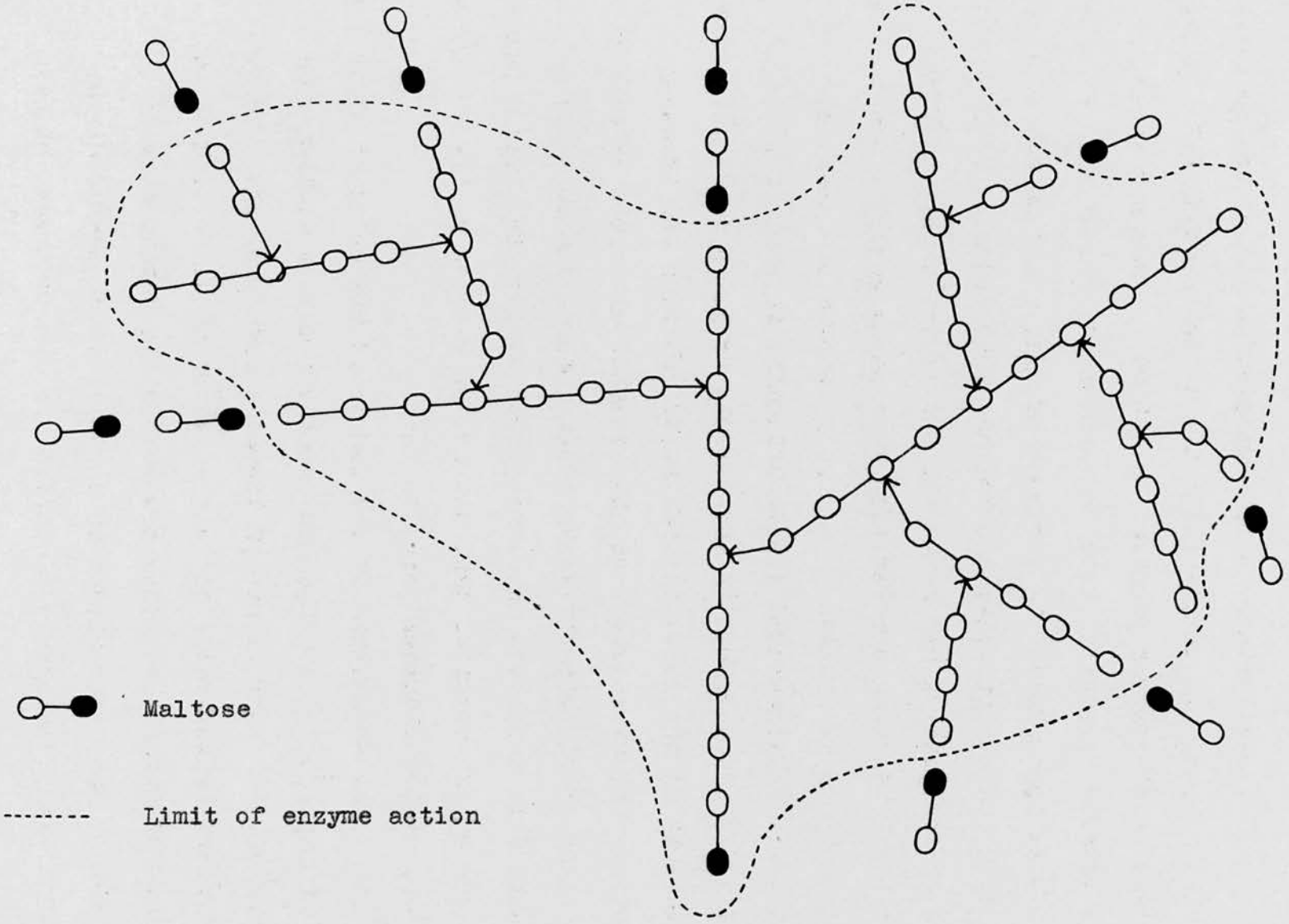


FIGURE VII.

β-Amylolysis of a branched polyglucosan.

Thomas and Whelan that single glucose residues are attached to the main chain by β-glucosidic linkages (111,112).

β-Amylolysis of both amylopectins and glycogens follows a similar pattern and is unaffected by the presence of β-glucosidases in the enzyme preparation (63,83). Enzymic attack on the exterior chains of both polysaccharides ceases when a branch point is approached; the products of enzyme action are therefore maltose and a high molecular weight limit dextrin in which all the branch points, and hence all the interior chains of the original molecule, are retained, but whose exterior chains are much reduced in length. β-amylolysis of a branched polyglucosan is represented diagrammatically in Fig. VII. The extent of β-amylolysis of amylopectin or glycogen is therefore dependent on the relative lengths of exterior and interior chains. By measurement of the β-amylolysis limit of an amylopectin or glycogen of known average chain length, it is possible to calculate the average position of the branch point on the chain, provided that the action pattern of the enzyme as it approaches a branch point is known.

An indication of the length of the exterior chain stub of an amylopectin or glycogen β-dextrin may be derived from a study of the β-amylolysis of linear α-1:4-glucosans. Maltotetraose, maltohexaose and higher dextrins containing an even number of glucose residues are completely broken down to maltose. On β-amylolysis of maltopentaose, equimolecular quantities of maltose and maltotriose are produced; higher

dextrins having an odd number of glucose residues also yield mixtures of these two sugars. Maltotriose is not normally hydrolysed by β-amylase, although, when the enzyme is present in high concentration, some hydrolysis to maltose and glucose does occur (113). It is probable that this is due to a specific maltotriase which is a contaminant of β-amylase preparations.

When the reducing group of a linear maltodextrin is modified the pattern of degradation remains unchanged: it is found that maltotetraonic acid and maltohexaonic acid yield the expected quantities of maltose and maltobionic acid (114, 115). It therefore appears that the normal minimum substrate requirement for β-amylase is a linear chain containing at least three α-1:4-glucosidic linkages.

This conclusion appears to be substantiated by work of Posternak (116). He attempted to degrade with β-amylase, maltohexaose which was esterified with phosphate on carbon atom 6 of either the fourth or fifth glucose residue from the non-reducing end of the molecule, but found that no attack took place. It may therefore be assumed that not only are three α-1:4-glucosidic bonds necessary for β-amylase action, but that position 6 of the fourth glucose residue must be free.

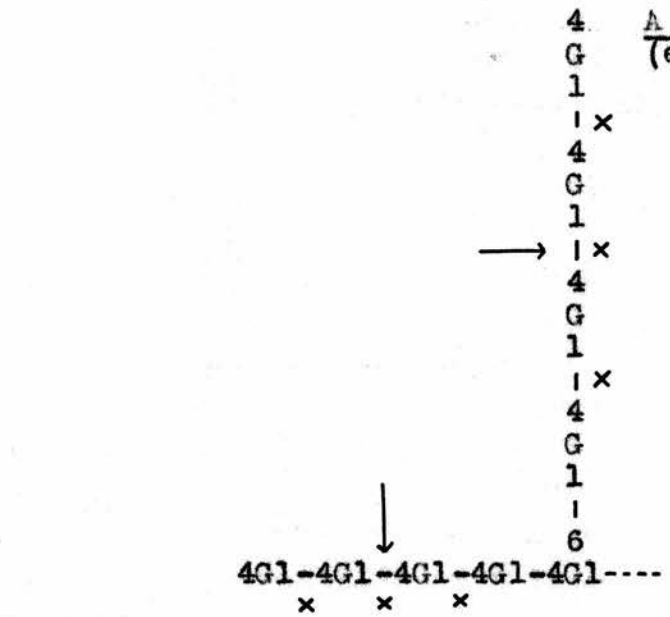
On the basis of this work, Illingworth, Larner and Cori concluded that the exterior chain stubs of glycogen and amylopectin β-dextrins must be either two or three glucose units in length, depending on whether the original chain

contained an even or an odd number of glucose residues (117). They assumed that substitution of carbon atom 6 of the branch point glucose residue by an A-chain, would have the same effect as esterification of the analogous glucose residue in maltohexaose, by phosphate. In Fig.VIII the three α-1:4-glucosidic bonds which must be intact before further β-amylolysis can occur, are marked. A- and B-chains having either even or odd numbers of glucose residues are considered.

It should be pointed out, however, that substitution by a small, but highly polar, phosphate group, is not necessarily analogous to substition by a chain of glucose units. Moreover the argument of Cori and colleagues is invalidated if the phosphate group on phosphohexaose is on the fifth glucose unit from the non-reducing end group, and not on the fourth as has been assumed.

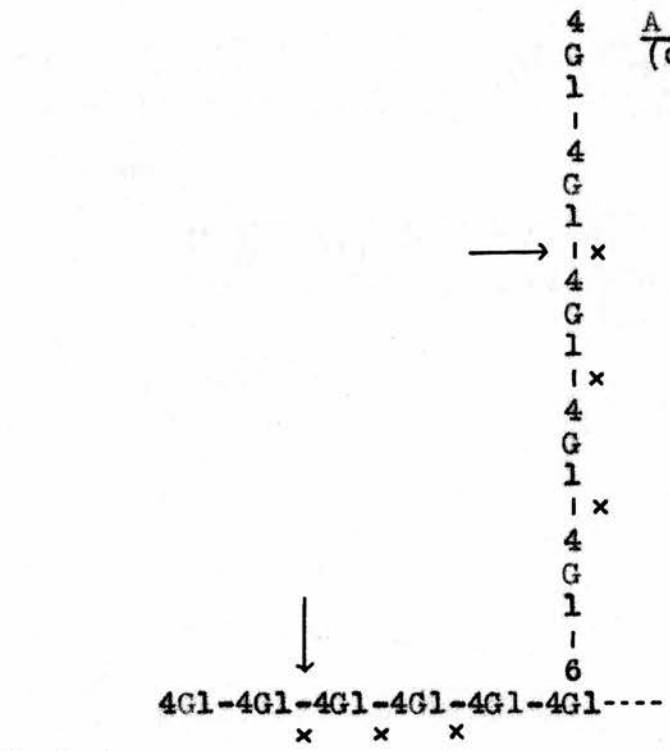
Peat and his colleagues have provided definite evidence that the A-chain stubs of an amylopectin β-dextrin contain either two or three glucose units (55). When β-dextrin of waxy maize starch was treated with R-enzyme, which hydrolyses α-1:6 linkages, maltose and maltotriose were the only low molecular maltosaccharides obtained. Maltose and maltotriose could have arisen only from the A-chain stubs, thus proving that β-amylase must have approached to within two or three glucose units of the branch point. Moreover, the quantity of maltose and maltotriose was such that the number of A-chains was far in excess of one per molecule: this work thus provides good evidence of multiple branching in amylopectin.

FIGURE VIII



A chain
(even number of glucose residues)

B chain
(even number of glucose residues)



A chain
(odd number of glucose residues)

B chain
(odd number of glucose residues)

× These bonds must be intact before further β-amylolysis can occur.

⟶ Attack by β-amylase.

To summarise, it has been proved that the A-chain stub of the β-dextrin from amylopectin (and hence from glycogen) is either two or three glucose units in length, and there is tentative evidence that the B-chain stub has a similar size. Accordingly, it will be assumed that the length of the exterior chain stubs of glycogen β-dextrins is either two or three glucose units; statistically this gives a figure of 2.5 glucose units.

Experimentally β-amylolysis of glycogens is found to reach a limit when about 40 to 50% of the molecule has been degraded (83). The corresponding figures for amylopectins are 50 to 60%. From the average chain length, the number of glucose residues which have been removed per chain by β-amylase, can be calculated. Addition of 2.5 to this figure gives the average length of the exterior chains, and hence the average length of the interior chains can be deduced. Table IX gives a selection of β-amylolysis data which has been obtained by previous workers. In the calculations it is considered that the branch point residue belongs neither to the interior nor to the exterior chain.

In this type of work it is essential that the β-amylase be free from enzymic impurities which might attack α-1:4-glucosidic linkages. β-Glucosidases present in amorphous preparations of β-amylase are without action on glycogen: degradation with amorphous or crystalline preparations of β-amylase gives identical percentage conversions to maltose, showing that glycogen contains no β-glucosidic linkages (118).

TABLE IX

β-Amylolysis of glycogens.

Source of glycogen.	C.L.	β-amylolysis limit.	Ext.C.L.	Int.C.L.	Ref.
Rabbit liver	13	43	8	4 3	7
Rabbit muscle *	12	45	8	3	83
Horse muscle *	11	42	7	3	33
Mussel (Merck)	11	47	7-8	2-3	45
Yeast (Baker's)	12	50	8-9	2-3	7
Yeast (Brewer's)	13	44	8-9	3-4	8
Trichomonas foetus *	15	60	11-12	2-3	11
Corn (Golden Bantam)	10	48	7-8	1-2	18

* These glycogens were degraded with crystalline sweet potato β-amylase.
In the remaining experiments the enzyme was obtained from cereal grains.

α-Amylase and maltase, however, affect the course of β-amylolysis of glycogen. The latter causes the maltose produced by action of β-amylase to be hydrolysed to glucose, thus preventing the exact determination of the β-amylolysis limit. Maltase has no effect on the β-dextrin. On the other hand, α-amylase causes random scission of both exterior and interior chains of glycogens. Attack on the interior chains allows further β-amylolysis to occur, and the apparent β-amylolysis limit is greatly increased. It is obvious that both α-amylase and maltase must be removed before β-amylase can be used in structural work.

In the work to be described, β-amylolysis limits of a large number of glycogens were determined, in order to discover whether there was any variation in the position of the branch point on the unit chain.

Experimental.

1. Source of β-amylase.

Attempts have been made to prepare β-amylase from both rye grains and from soya beans. In the former case the method used was that by which Hanes prepared β-amylase from barley (119). This involved extraction of the finely milled flour with dilute salt solution, and inactivation of the contaminating α-amylase by treatment overnight with acid (pH 3.4) in the cold. After neutralisation, the β-amylase was obtained from solution by alcohol fractionation. This procedure yielded a product which

was still contaminated with α-amylase; it was concluded that the α-amylase in rye flour was not completely inactivated by the overnight acid treatment.

α-Amylase is reported to be absent from soya beans, but maltase is a common contaminant of β-amylase from this source. Soya bean β-amylase was prepared by the method of Bourne, Macey and Peat, in which soya bean flour was extracted with 20% ethanol (120). At this concentration of alcohol, maltase is inactivated but β-amylase is not affected. After overnight extraction, the β-amylase was precipitated from solution by raising the alcohol concentration to 80%. Two enzyme preparations were carried out using this method, but in each case β-amylase was contaminated with a slight trace of maltase, which was not removed by further treatment with 20% ethanol.

A commercial preparation of β-amylase from the Wallerstein laboratories, New York, was then examined, and found to have a high activity, and to be free of α-amylase and maltase. This preparation was used in the β-amylolysis of glycogens.

2. General methods used in β-amylolysis.

a) Preparation of β-amylase solution.

β-Amylase solution was prepared according to Peat, Pirt and Whelan (63). The enzyme was dissolved in water, and an equal volume of 0.2 M acetate buffer, pH 4.6 was added. Only traces of material remained undissolved and were removed

by centrifugation. Enzyme solutions of various concentrations were used.

b) Analysis of maltose.

In the β-amylolysis experiments, reducing sugars were estimated using the Shaffer-Somogyi reagent 60, as modified by Hanes and Cattle, with a heating time of 15 minutes (73,74). The reagent was suitable for the determination of between 0.4 and 4.0 mg. of maltose, in a total volume of 5 ml. A calibration graph was prepared; weights of maltose were plotted against titre (ml. of 0.01 N sodium thiosulphate).

c) Precautions against mould contamination.

To prevent the growth of moulds, toluene was used as an antiseptic in all digests.

d) Control of pH.

All experiments were carried out at pH 4.6; at this pH, cereal α-amylases are inactive (121).

3. Examination of β-amylase obtained from Wallerstein laboratories, New York.

a) Activity of β-amylase.

One unit of activity of β-amylase is defined as the weight of enzyme which liberates 1 mg. of maltose when the following digest is incubated at 35° for 30 minutes (122).

Digest (set up in duplicate).

25 ml. AR soluble starch solution (0.6%)
3 ml. acetate buffer (0.2 M; pH 4.6) } preheated to 35°.
2 ml. β-amylase solution (0.063 mg./ml.)

After 30 minutes an aliquot (3 ml.) was removed from each digest, and the reducing power determined.

	Titre (ml.)	Maltose equivalent (mg.)
Digest A.	2.63	1.49
Digest B.	2.63	1.49

In the entire digest (30 ml.) 14.9 mg. of maltose was liberated by 0.126 mg. of enzyme. The weight of maltose which would be liberated by 1 mg. of enzyme was therefore 118 mg.; i.e. 1 mg. of enzyme powder contained 118 units of β-amylase.

b) Reducing power of β-amylase.

A digest was set up containing only acetate buffer and enzyme, in order to determine the reducing power of the enzyme.

Digest. 10 ml. distilled water
5 ml. acetate buffer (0.2 M; pH 4.6)
1 ml. β-amylase solution (8 mg./ml.; ca 950 units)

The digest was incubated at 35° and aliquots (3 ml.) were removed at intervals for reducing power determination.

Time (hr.)	Titre (ml.)
0	0.05
24	0.08
72	0.05

It was concluded that the enzyme had negligible reducing power.

c) Maltase activity of β-amylase.

To determine whether or not the enzyme was contaminated with maltase, it was incubated with maltose.

Digest. 20.9 mg. maltose

10 ml. distilled water

5 ml. acetate buffer (0.2 M; pH 4.6)

1 ml. β-amylase solution (8 mg./ml.; ca 950 units)

The digest was incubated at 35°, and aliquots (2 ml.) were removed at intervals for reducing power determination.

Time (hr.)	Titre (ml.)
0	4.82
24	4.82
72	4.79

The reducing power did not increase. It was therefore concluded that the β-amylase had no maltase activity.

d) α-Amylase activity of β-amylase.

The presence of α-amylase in the β-amylase preparation would cause random scission of the interior chains of amylopectin β-dextrin, with a resultant decrease in the iodine absorption value of the polysaccharide. The enzyme was therefore incubated with β-dextrin of waxy maize starch (1 mg. polysaccharide/ml.; 90 units β-amylase/mg. polysaccharide); aliquots (1 ml.) were removed at intervals for measurement of the iodine absorption value at 680 mμ (123). Since no significant change in this property occured within 24 hours, it was concluded that the β-amylase was not contaminated with α-amylase.

4. β-Amylolysis of glycogens.

The preparation of β-amylase from the Wallerstein laboratories, New York, which has been shown to be of satisfactorv

activity and purity, was used in this work. 26 glycogens were submitted to the action of β-amylase, each experiment being carried out in duplicate. A typical experiment is reported in detail:

a) Composition of digest and analysis of products.

Before analysis, the glycogen was dried *in vacuo*, over phosphorus pentoxide, for 4 hours at 100°.

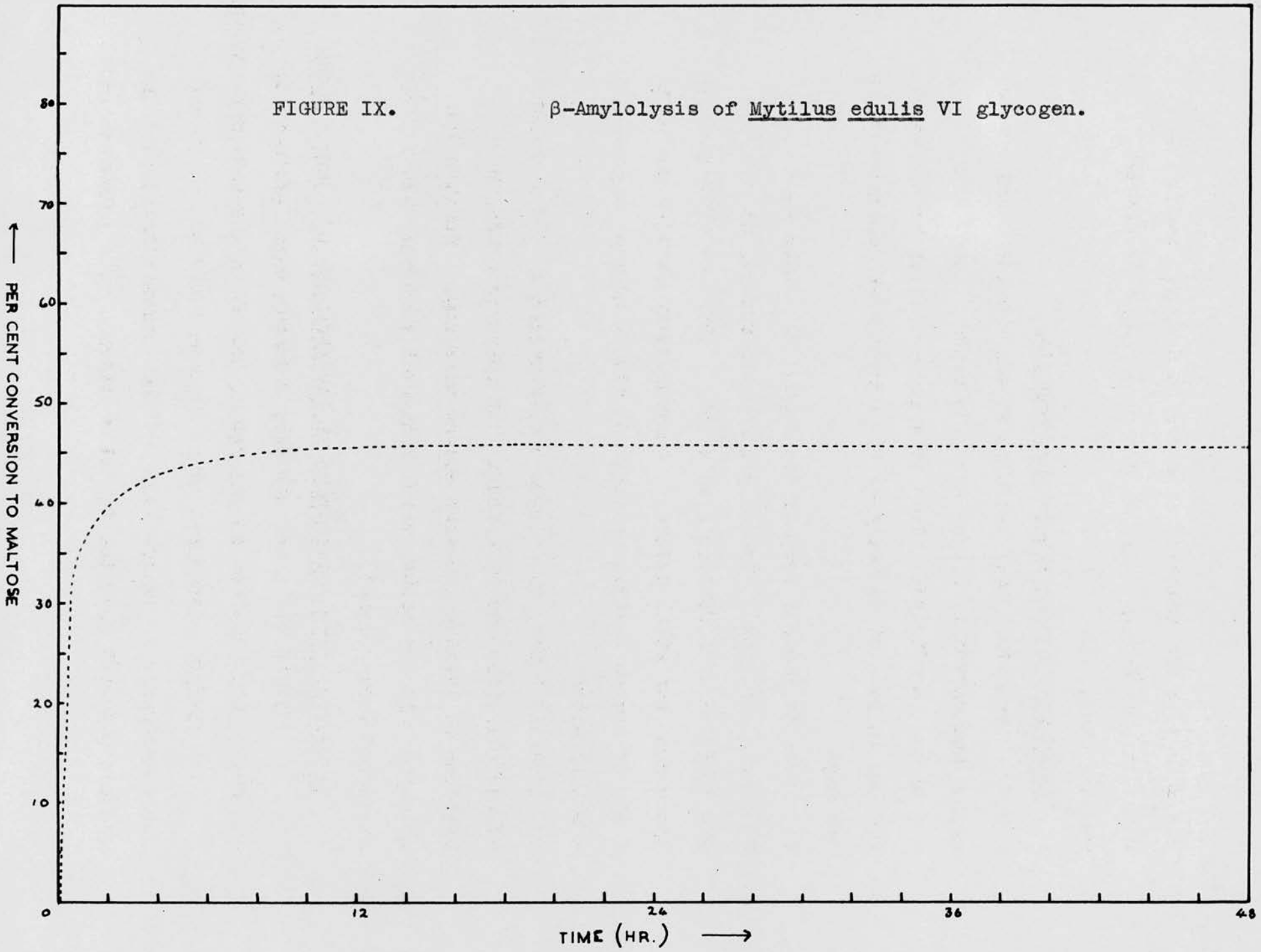
Digest. 45.2 mg. *Mytilus edulis* VI glycogen
25 ml. distilled water
25 ml. acetate buffer (0.2 M; pH 4.6)

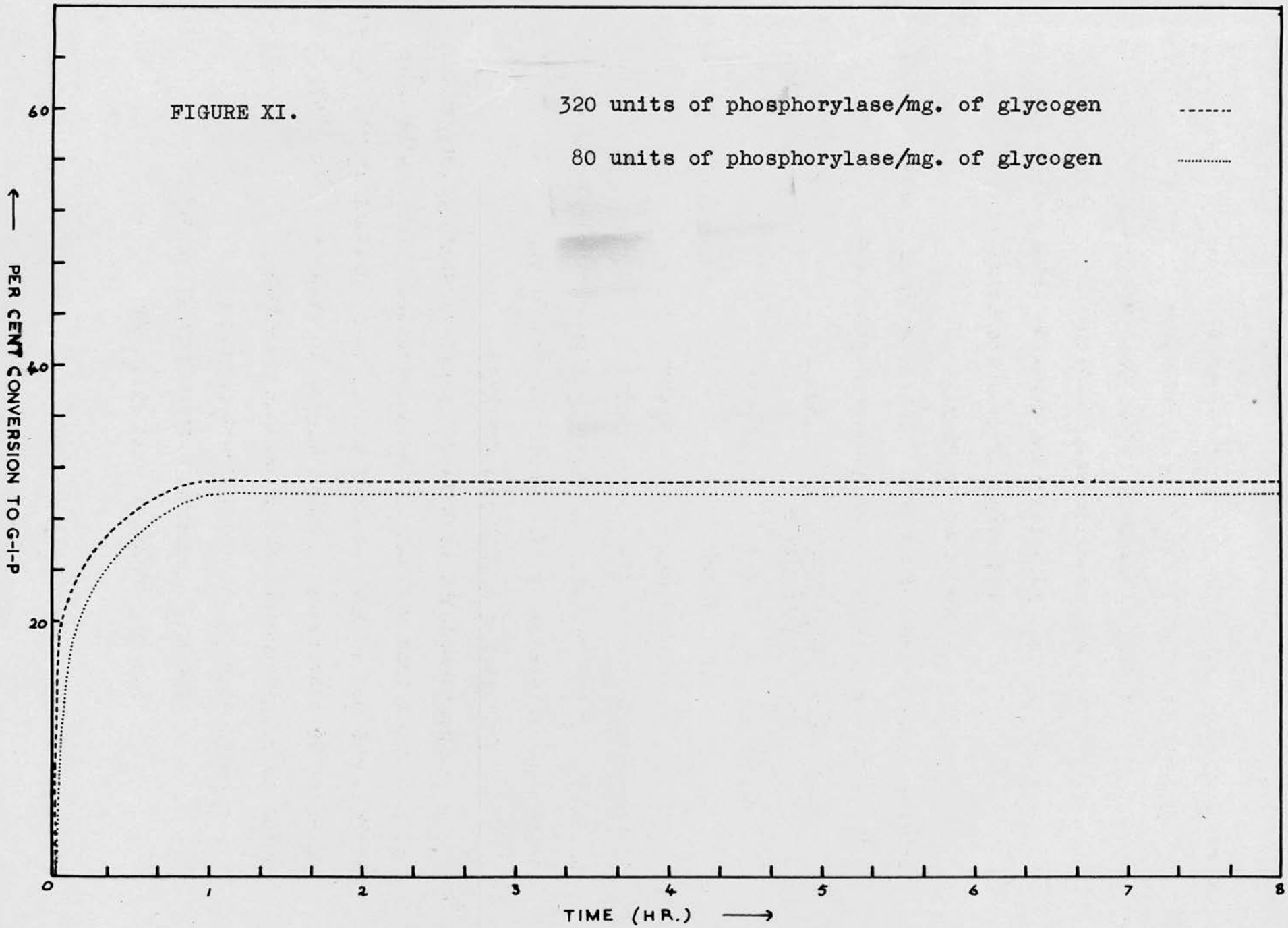
An aliquot (3 ml.) was removed in order to determine the reducing power of glycogen. β-Amylase solution (1 ml.; 16 mg./ml.; *ca* 1900 units) was then added. The final digest therefore contained 42.48 mg. of glycogen in 48 ml.

The digest was incubated at 35°. Aliquots (3 ml.) were removed at intervals for reducing power determination. The titres reported have been corrected for the small reducing power (*ca* 0.05 ml. sodium thiosulphate/3 ml. aliquot) due to glycogen.

Time. (hr.)	Titre. (ml.)	Maltose equivalent. (mg.)	Per cent conversion to maltose.
0.5	1.55	0.88	31
1	1.79	1.01	36
2	1.99	1.12	40
4	2.12	1.20	43
24	2.29	1.29	46
48	2.29	1.29	46

FIGURE IX. β-Amylolysis of Mytilus edulis VI glycogen.





Muscle phosphorolysis of brewer's yeast glycogen.

The course of the reaction is shown in a graph (Fig.IX) in which the percentage β-amylolysis of glycogen is plotted against time.

b) Proof that enzyme action was complete.

From the graph (Fig.IX) it was apparent that enzyme action had virtually ceased after 24 hours. That this was due to the β-amylolysis limit of glycogen having been reached, and not to loss of activity of the enzyme, was demonstrated in two ways:

1) After 48 hours 1 drop of the digest was added to 2 ml. of 1% starch solution. Reducing sugar was formed, as shown by its reaction with Fehling's solution. This was evidence that the enzyme was still active. Controls were carried out with 2 ml. of starch solution alone, and with 1 drop of digest in 2 ml. of water.

2) After 48 hours, the enzyme concentration in the original digest was raised to 90 units/mg. of polysaccharide, by addition of freshly prepared enzyme solution. Further degradation did not occur, which indicated that the β-amylolysis limit had been reached.

c) Chromatographic examination of the products of β-amylolysis.

That maltose was the only reducing sugar produced by the action of β-amylase on glycogens, was shown chromatographically.

Before successful chromatograms could be run it was found necessary to remove both residual polysaccharide and inorganic material from the digest solution. The polysaccharide

was therefore precipitated from a portion of the enzyme digest, by the addition of two volumes of alcohol. After centrifugation, alcohol was removed from the solution by distillation *in vacuo* at 40°. The aqueous solution was then treated alternately with IR-4B(OH) and IR-120(H) ion exchange resins, to remove respectively acetate and sodium ions. Finally the neutral solution was concentrated *in vacuo* and spotted on Whatman No.1 paper. Chromatograms were irrigated with benzene:pyridine: butanol:water (1:3:5:3), and developed by means of a silver nitrate - sodium hydroxide reagent (71).

No glucose was detected. The only reducing sugar present was maltose, which showed a tendency to "trail" on the chromatogram. However, a sample of pure maltose subjected to the same experimental techniques behaved similarly, and it was therefore concluded that the "trailing" effect was due to treatment with ion exchange resins. In a control experiment it was shown that if glucose were present in the digest, it would have been detected by this method.

Results.

Under the conditions which have been detailed (Page 71) namely incubation of digests containing glycogen (*ca* 1 mg./ml.) and β-amylase (*ca* 40-50 units/mg. of glycogen), buffered to pH 4.6, at 35°, β-amylolysis of glycogens was complete in 24 to 48 hours. The enzyme preparation was of satisfactory purity and maltose was the only reducing sugar produced.

The results of β-amylolysis experiments are detailed in Table X.

From the average chain lengths and the β-amylolysis limits of the glycogens, the average exterior and interior chain lengths were calculated. This data is summarised in Table XI.

TABLE X.

β-Amylolysis of glycogens.

Source of glycogen.		Per cent conversion to maltose.				
		1 hr.	4 hr.	24 hr.	48 hr.	72 hr.
Rabbit liver I	a)	18	20	23	-	24
	b)	20	22	25	-	25
Rabbit liver III	a)	41	46	50	51	51
	b)	42	46	50	-	52
Rabbit liver IV	a)	-	-	45	46	-
	b)	-	-	45	45	-
Rabbit liver V	a)	-	-	50	51	-
	b)	-	-	50	51	-
Rabbit liver VI	a)	46	49	52	-	54
	b)	44	49	52	52	-
Rabbit liver X	a)	40	44	47	-	49
	b)	42	-	50	48	-
Rabbit liver XII	a)	-	-	42	43	-

TABLE X (contd.)

Source of glycogen.		Per cent conversion to maltose.				
		1 hr.	4 hr.	24 hr.	48 hr.	72 hr.
Rabbit liver XII	b)	-	-	43	44	-
Rabbit liver XIII	a)	39	44	46	-	48
	b)	37	41	44	-	46
	c)	36	-	46	46	-
Cat liver IV	a)	45	-	53	52	-
	b)	45	-	53	52	-
Cat liver VI	a)	-	-	52	51	-
	b)	-	-	52	53	-
Foetal pig liver	a)	-	-	50	50	-
	b)	-	-	48	48	-
Foetal sheep liver (alkali-treated)		-	-	49	49	-
Rabbit muscle II	a)	-	-	38	38	-
	b)	-	-	39	39	-
Human muscle II	a)	30	-	41	39	-
	b)	30	-	40	40	-
Cock liver	a)	-	-	39	39	-
	b)	-	-	38	38	-
Skate liver	a)	-	-	43	44	-
	b)	-	-	45	46	-
Mytilus edulis I	a)	-	-	40	41	-
	b)	-	-	41	42	-

TABLE X (contd.)

Source of glycogen.	Per cent conversion to maltose.				
	1 hr.	4 hr.	24 hr.	48 hr.	72 hr.
Mytilus edulis IV	-	-	48	51	-
Mytilus edulis V a)	28	34	38	-	41
b)	28	34	38	-	40
Mytilus edulis VI a)	36	43	46	46	-
b)	-	-	46	45	-
Mytilus edulis VII a)	-	-	45	46	-
b)	-	-	45	46	-
Mytilus edulis VIII a)	-	-	44	45	-
b)	-	-	44	45	-
Mytilus edulis IX a)	-	-	51	52	-
b)	-	-	51	51	-
Mytilus edulis X a)	-	-	43	44	-
b)	-	-	45	46	-
Cardium a)	-	-	14	14	-
b)	-	-	13	14	-
Arenicola a)	-	-	43	43	-
b)	-	-	42	43	-

Several digests were incubated for more than 72 hours; β-amylolysis limits were as follows:

Rabbit liver X a) : 49% after 96 hours.

Rabbit liver XIII a) : 47% after 96 hours.

Mytilus edulis V a) : 39% after 96 hours.

b) : 40% after 96 hours.

Samples a) and b) of rabbit liver XIII glycogen were obtained by aqueous extraction of one half of the liver; sample c) was obtained by extraction of the remainder with potassium hydroxide (c.f. Section II. A.)

TABLE XI

Calculated

average exterior and interior chain lengths of glycogens.

Source of glycogen.	C.L.	β-Amylolysis limit.	Ext.C.L.	Int.C.L.
Rabbit liver I	13*	25	5-6	6-7
Rabbit liver III	13*	51	9	3
Rabbit liver IV	13*	45	8-9	3-4
Rabbit liver V	14	51	9-10	3-4
Rabbit liver VI	18*	52	12	5
Rabbit liver X	12*	49	8-9	2-3
Rabbit liver XII	17	43	9-10	6-7
Rabbit liver XIII	15	46	9-10	4-5

TABLE XI (contd.)

Source of glycogen.	C.L.	β-Amylolysis limit.	Ext.C.L.	Int.C.L.
Cat liver IV	13*	53	9-10	2-3
Cat liver VI	12*	52	8-9	2-3
Foetal pig liver	11*	49	8	2
Foetal sheep liver	13*	49	9	3
Rabbit muscle II	11	39	6-7	3-4
Human muscle II	11	40	7	3
Cock liver	13	39	7-8	4-5
Skate liver	13	45	8-9	3-4
Mytilus edulis I	12*	42	7-8	3-4
Mytilus edulis IV	12*	51	8-9	2-3
Mytilus edulis V	9*	40	6	2
Mytilus edulis VI	13	46	8-9	3-4
Mytilus edulis VII	13	46	8-9	3-4
Mytilus edulis VIII	13	45	8-9	3-4
Mytilus edulis IX	10	51	7-8	1-2
Mytilus edulis X	14	45	8-9	4-5
Cardium	8	14	3-4	3-4
Arenicola	11	43	7-8	2-3

* End group assays carried out by Dr. D.J. Manners and co-workers.

Discussion.

It has been confirmed experimentally (Pages 72-73) that maltose is the only reducing sugar produced when glycogens are degraded by β-amylase. Enzyme action was complete within 24 to 48 hours, and of the twenty-six glycogens which were examined, twenty-four have β-amylolysis limits between 39 and 53%. i.e. of the expected order. The two exceptions (rabbit liver I glycogen and Cardium glycogen) will be discussed later.

β-Amylolysis results provide further evidence that small structural differences exist between one specimen of glycogen and another. Average exterior and interior chain lengths calculated as described on Page 64, show that the former varies from 6 to ca 10 glucose residues, and the latter from ca 1 to ca 7. Although the average exterior chain length is invariably longer than the average interior chain length, the relative position of the branch point glucose residue on the unit chain is not the same in all glycogens. Thus, several specimens of glycogen listed in Table XI have average chain length values of 13 glucose residues; of these rabbit liver III glycogen has a β-amylolysis limit of 51%, indicating that the average position of the branch point is at the tenth glucose residue from the non-reducing end of the chain. The β-amylolysis limit of cock liver glycogen is 39%. and, in this case, the branch point is, on average, at the eighth or ninth glucose residue from the non-reducing end of the chain. This means that rabbit liver III glycogen and cock liver glycogen differ in the relative lengths of their exterior and interior

chains, although from end group assay, they appear to be of similar structure.

In any sample of glycogen, a random structure prevails, and the reported values for chain length, exterior chain length, and interior chain length are statistical averages of the lengths of many chains of varying sizes. This is proved by a further study of β-amylolysis data. β-Amylase is known to remove glucose units, in pairs, as maltose from the non-reducing ends of chains. However, when β-amylolysis limits are used to calculate the number of glucose residues removed per chain, an even number is seldom obtained. This can be explained if the exterior chains are of different lengths; although an even number of glucose residues is in fact removed from each exterior chain, the average number removed per chain is then not necessarily an even number. For example, since cat liver IV glycogen has a chain length of 13 glucose residues and a β-amylolysis limit of 53%, the number of glucose residues removed per chain appears to be 6.9; this must be a statistical average of 4, 6, 8, 10, 12, etc. glucose residues removed from exterior chains of different lengths.

Rabbit liver I glycogen and Cardium glycogen have β-amylolysis limits very much lower than usual. The former has a normal chain length of 13 glucose residues, but only 25% of the molecule is removed by β-amylase. This means that the exterior chains, with an average length of 6 glucose residues, are rather shorter than normal, while the interior chains also average 6 glucose residues in length and are as long as the interior chains

found in a 17- or 18-unit glycogen. Explanations for this unusual structural pattern are merely tentative. It is possible that this glycogen had a chain length of about 18 glucose residues, but that prior to extraction from the animal, active phosphorolysis resulted in the shortening of the exterior chains.

A similar theory could be put forward to account for the short chain length (8 glucose residues), and exterior chain length of Cardium glycogen. It also has a very low β-amylolysis limit (14%); the average interior chain length is therefore the normal length of 3 to 4 glucose residues, but the exterior chains (also averaging 3 to 4 glucose residues in length) are very much shorter than normal.

The results obtained by degrading glycogens with β-amylase will be used again (Section VI) in calculating the degree of multiple branching of the molecules.

SECTION IV.

α-AMYLOLYSIS OF GLYCOGENS.

Introduction.

α-Amylases have been isolated in highly purified, often crystalline forms from a wide variety of sources, including mammalian salivary and pancreatic secretions, barley malt, and several species of bacteria and fungi (124,125). These enzymes catalyse a random hydrolysis of α-1:4 linkages in starch-type polysaccharides and in maltodextrins, and are able to attack both exterior and interior chains. The initial action on polysaccharides is characterised by a rapid decrease in iodine-staining power, viscosity, and turbidity of the substrate, accompanied by a slow increase in reducing power. This "dextrinisation" stage, in which the polysaccharide is broken down into α-dextrins each containing ca 6 - 10 glucose residues, is followed by a "saccharification" stage in which the reducing power continues to increase as the α-dextrins are degraded into progressively smaller oligosaccharides. The enzyme is unable to hydrolyse α-1:6 linkages.

α-Amylases from different sources, which have different chemical compositions, show some variation in action pattern. It has been observed that when amylose is degraded by swine pancreatic α-amylase, the substrate stains with iodine until 23% of the glucosidic linkages have been hydrolysed. When human salivary α-amylase is used, however, the achroic stage of

hydrolysis is reached when only 15% of the linkages have been broken (126). Differences in the affinity of the enzymes for short chain substrates have also been noted; whereas salivary α-amylase hydrolyses maltohexaose at practically the same rate as it hydrolyses starch, with α-amylase from malt, the initial rate of hydrolysis of starch is about six times greater than that of maltohexaose (107).

α-Amylases vary in their ability to attack terminal α-1:4-glucosidic linkages. Pure salivary α-amylase cannot hydrolyse such linkages, and the final products of the salivary amylolysis of amylose are maltose and maltotriose (127). (The glucose observed in some salivary amylolytic digests appears to be produced by the action of a maltotriase which is also secreted by the salivary glands). By contrast, glucose is a primary product of the degradation of amylose by the α-amylases from malt and Bacillus subtilis, indicating that these enzymes are able to attack terminal glucosidic linkages (128).

The action pattern of salivary α-amylase has been defined by Roberts and Whelan, who determined the ratio of maltose to maltotriose produced on complete α-amylolysis of amylose (127); their results indicated that all susceptible linkages (i.e. all non-terminal linkages) were hydrolysed at the same rate, a conclusion which has been confirmed by a study of the α-amylolysis of maltohexaose and other maltodextrins. The products of salivary α-amylolysis of amylopectin and rabbit liver glycogen included, in addition to maltose and maltotriose, α-dextrins

(D.P.>4) which contained one or more α-1:6 linkages, i.e. the intact branch points of the polysaccharide (67). From the structure of the α-dextrins, it was shown that the three α-1:4 linkages which are adjacent to the branch point are not easily hydrolysed by salivary α-amylase. Enzyme action will therefore proceed more quickly at the middle of exterior and interior chains of glycogen.

Other products of the salivary α-amylolysis of rabbit liver glycogen obtained from pregnant does, and of waxy maize starch, were maltulose (4-[α-D-glucopyranosido] -D-fructose), and fructose-containing α-dextrins (67,129). This would suggest that a small amount of fructose is present in both these polysaccharides.

In the course of this work, several glycogens of varying degree of branching were treated with salivary α-amylase. The experiments were designed a) to test for the presence of fructose in those samples of glycogen isolated in the present studies (Section II A), and b) to investigate the relationship between degree of branching and the extent of α-amylolysis of the glycogen.

For comparative purposes, samples of waxy maize starch, β-dextrin of waxy maize starch and β-dextrin of a highly branched glycogen, were included in this study.

Experimental.

1. Preparation of α-amylase.

The method of Fischer and Stein for the preparation of

crystalline salivary α-amylase was generally followed (125); the final crystallisation stage was, however, omitted, and the enzyme was isolated by freeze-drying in citrate buffer (67).

The preparation was carried out in a cold room (0 - 5°). To centrifuged saliva (880 ml.) AnalaR acetone (ca 98% pure; 820 ml.; final concentration 48%) was added dropwise, over a period of 40 minutes, efficient stirring of the solution being maintained during precipitation and for a further 15 minutes. The fine suspension was centrifuged (15 minutes; 1500 g.); to the supernatant solution acetone (1220 ml.; final concentration 69%) was added, the slow precipitation again requiring 40 minutes. After centrifugation (10 minutes; 1500 g.) the supernatant liquid was rejected and the precipitate dissolved in water (150 ml.; pH 6.8).

The following day a second acetone fractionation was carried out. To the enzyme solution (150 ml.) acetone (150 ml.; final concentration 49%) was added (20 minutes); the resulting suspension was centrifuged (15 minutes; 1500 g.) and the precipitate rejected. Sodium acetate (0.6 g.) was dissolved in the solution, and the protein precipitated by the addition of acetone (207 ml.; final concentration 69%; 20 minutes). It was separated by centrifugation (10 minutes; 1500 g.), and dissolved in water (60 ml.; pH 6.5).

The enzyme solution was adjusted to pH 6.8 and the protein precipitated by the rapid addition (20 seconds) of saturated ammonium sulphate solution (60 ml.; pH 7). Efficient

stirring was maintained for a further 30 minutes before the fine precipitate was separated by centrifugation (30 minutes; 4000 g.). It was then dissolved in water (50 ml.), and dialysed overnight against a solution containing calcium acetate (0.05%) and ammonium hydroxide (0.001 N). After addition of citrate buffer (20 ml.; 0.2 M, pH 7) the solution was freeze-dried. The solid preparation weighed 1.14 g.

2. General methods used in α-amylolysis.

a) Analysis of reducing sugars.

Reducing sugars were estimated by the Shaffer-Somogyi copper reductometric technique, as modified by Hanes and Cattle (See page 68) (73,74). Results are expressed as apparent percentage conversion to maltose.

b) Paper chromatography of α-dextrins.

Irrigant. Paper chromatograms were irrigated with benzene: pyridine: butanol: water (1:3:5:3) for a period of 72 hours. Whatman No. 1, or No. 54 paper was used.

Sprays. 1) Sugars gave brown spots when papers were treated with a silver nitrate - sodium hydroxide reagent (71).

2) Ketoses were detected by means of an orcinol reagent (130). Yellow-green spots, fluorescent in ultra violet light, were obtained.

c) Activation of α-amylase and control of pH.

Salivary α-amylase is activated by chloride ions. Sodium chloride (0.05%) was therefore included in all digests.

Since the α-amylase had been freeze-dried in citrate

buffer of pH 7, the optimum pH of the enzyme, it was considered unnecessary to add a further quantity of buffer solution to the enzyme digests. It was desirable to keep the concentration of inorganic material in the digests as low as possible in order to avoid interference when the products of α-amylolysis were analysed by paper chromatography.

3. Examination of the salivary α-amylase.

a) Activity of α-amylase.

One unit of activity is defined as the quantity of enzyme which liberates 1 mg. of "apparent" maltose from a 1% solution of soluble starch in 3 minutes, at 35° (c.f. 125).

Digest. 25 ml. AR soluble starch solution (1.2%) } preheated
3 ml. sodium chloride solution (0.5%) } to 35°
2 ml. α-amylase solution (0.019 mg./ml.)

After 30 minutes, an aliquot (3 ml.) was removed from the digest, and the reducing power determined.

Titre (ml.)	Maltose equivalent (mg.)
2.73	1.60

In the entire digest (30 ml.) 16.0 mg. of "maltose" was liberated by 0.038 mg. of enzyme in 30 minutes. The weight of maltose which would be liberated by 1 mg. of enzyme in 3 minutes was therefore 42.1 mg.; i.e. the activity of the salivary α-amylase preparation was 42 units/mg.

In a duplicate experiment, the activity of the enzyme preparation was found to be 41 units/mg.

b) Reducing power of α-amylase.

In order to determine the reducing power of the enzyme, a digest containing only enzyme solution and sodium chloride was prepared.

Digest. 15 ml. distilled water
2 ml. sodium chloride solution (0.5%)
3 ml. α-amylase solution (5 mg./ml.; ca 630 units)

The digest was incubated at 35°; aliquots (3 ml.) were removed before incubation, and after 24 hours, for reducing power estimation.

Time (hr.)	Titre (ml.)
0	0.04
24	0.06

It was concluded that the reducing power of the enzyme was negligible.

c) Maltotriase activity of α-amylase.

The enzyme was incubated with maltotriose, to determine whether or not it was contaminated with maltotriase.

Digest. 22.9 mg. maltotriose.
15 ml. distilled water
2 ml. sodium chloride solution (0.5%)
3 ml. α-amylase solution (5 mg./ml.; ca 630 units)

The digest was incubated at 35°, and aliquots (3 ml.) were removed at intervals for reducing power estimation.

Time (hr.)	Titre (ml.)
0	2.82
20	5.25
72	5.53

It was concluded that the enzyme was contaminated with maltotriase.

d) Maltase activity of α-amylase.

The enzyme was incubated with maltose, to determine whether or not it was contaminated with maltase.

Digest. 17.2 mg. maltose
15 ml. distilled water
2 ml. sodium chloride solution (0.5%)
3 ml. α-amylase solution (5 mg./ml.; ca 630 units)

The digest was incubated at 35°, and aliquots (3 ml.) were removed at intervals for reducing power estimation.

Time (hr.)	Titre (ml.)	Per cent hydrolysis of maltose.
0	4.24	0
24	4.51	6
72	4.79	14

It was concluded that the α-amylase preparation contained a trace of maltase.

4. α-Amylolysis of α-1:4-glucosans.

The α-amylase preparation had a satisfactorily high activity, but it was contaminated with maltotriase, and a very slight trace of maltase. In digests in which maltotriose and maltose had been incubated with the enzyme preparation, the ratio of enzyme to substrate was, however, extremely high (ca 31.5 units/mg.). In the salivary α-amylolysis of polysaccharides, the digests contained a lowered ratio of enzyme to substrate (ca 4-5 units/mg.); the effect of the maltase would thus be considerably reduced. The R_M values (the apparent percentage conversion to

maltose by salivary α-amylase) of polysaccharides will inevitably be increased by the presence of maltotriase; however, since identical conditions were used in salivary α-amylolysis experiments, differences in the observed R_M values should reflect differences in polysaccharide structure.

Nine samples of glycogen, one sample of waxy maize starch, β-dextrin of Helix pomatia glycogen and β-dextrin of waxy maize starch were submitted to the action of salivary α-amylase. In all experiments the digest contained polysaccharide (ca.50 mg.) sodium chloride solution (10 ml.; 0.5%), α-amylase solution (1 ml.; 5.5 mg./ml.; ca 230 units) and distilled water to 100 ml. Digests were incubated at 35°, and aliquots (5 ml.) were removed at intervals for reducing power estimation.

From all digests, aliquots (10 ml.) were removed after 48 hours, boiled to inactivate the enzyme, concentrated in vacuo, and analysed chromatographically.

Results.

R_M values obtained for the twelve polysaccharides which were degraded by α-amylase, are reported in Table XII..

Chromatograms sprayed with the silver nitrate-sodium hydroxide reagent showed that glucose and maltose were produced, in quantity, when branched α-1:4-glucosans were treated with this salivary α-amylase preparation. Only traces of maltotriose were present, and maltotetraose was absent. Two higher dextrins (R_G values 0.10 and 0.05 respectively) were present, together with other sugars of still lower R_G values.

TABLE XII.

α-Amylolysis of branched α-1:4-glucosans.

Source of polysaccharide.	C.L.	Apparent per cent conversion to maltose.		
		2 hr.	6 hr.	24 hr.
Glycogens.				
Cock liver	13	57	72	76
Skate liver	13	59	75	80
Mytilus edulis VII	13	60	73	80
Mytilus edulis VIII	13	61	76	80
Mytilus edulis IX	10	62	78	83
Mytilus edulis X	14	61	75	80
Cardium	8	39	49	54
Arenicola	11	56	71	76
Helix pomatia	7*	51	64	67
Amylopectin.				
Waxy maize starch	20*	78	93	95
β-Dextrins				
Helix pomatia	4-5	21	26	29
Waxy maize starch	10*	48	59	66

* Values determined by Dr. D. J. Manners.

When duplicate chromatograms were sprayed with the orcinol reagent neither maltulose nor fructose-containing α-dextrins were detected. An authentic sample of maltulose was easily detected, and had R_G 0.48 under these conditions.

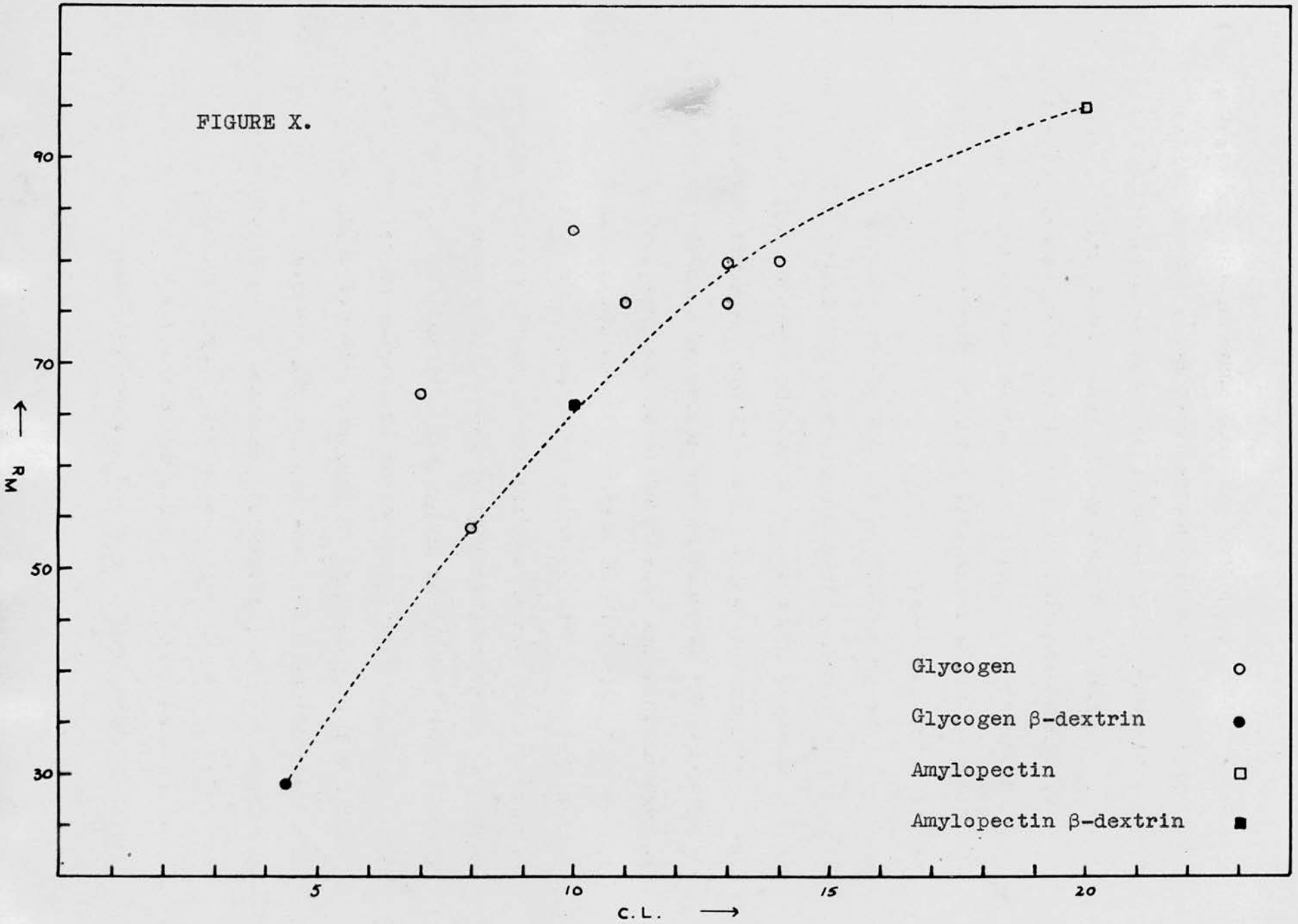
Discussion.

The fructose content of the rabbit liver glycogen isolated from pregnant does was ca 5%, and that of a sample of waxy maize starch was rather lower (67). In this study, paper chromatographic techniques, which were similar to those used by Roberts, failed to detect the presence of ketoses among the products of salivary α-amylolysis of any of the polysaccharides listed in Table XII. It is therefore concluded that significant quantities of fructose are absent from these polysaccharides. It is of interest that samples of glycogen (from cat, rabbit and foetal sheep livers) failed to react with the acid resorcinol (ketose) reagent (118).

By chromatographic examination of the reducing sugars present in the α-amylase digests, maltose (R_G 0.49) and sugars of R_G 0.10 and less (branched α-dextrins) were detected. Maltotriose was virtually absent, but glucose was present, showing that the enzyme preparation contained sufficient maltotriase to hydrolyse the maltotriose resulting from α-amylase action. Maltotetraose was absent, in accordance with the postulated action pattern of α-amylase.

Since the same experimental conditions were used in treating 12 α-1:4-glucosans with α-amylase it was considered that

FIGURE X.


90
70
50
30
R_M ⟶
5
10
15
20
C. L. ⟶
Glycogen
Glycogen β-dextrin
Amylopectin
Amylopectin β-dextrin

the values of R_M (apparent per cent conversion to maltose) should be comparable. The amylopectin, waxy maize starch, is most readily hydrolysed, an R_M value of 93 being attained within 6 hours. During the subsequent 18 hours the R_M value increased by only 2, indicating that maltase action is relatively unimportant. It is therefore considered that the 24 hour values of R_M reported in Table XII are a reasonably accurate measure of the α-amylolysis of the polysaccharides.

The proportion of the polysaccharide molecule in the vicinity of branch points increases as the degree of branching of the molecule increases (i.e. as the average chain length of the polysaccharide decreases). It was therefore expected that R_M values would decrease as the degree of branching increased. In general this has been found to be the case, and in Fig. X values of R_M are plotted against average chain length of the polysaccharide. The R_M value of β-dextrin of Helix pomatia glycogen, which has an average chain length of 4 - 5 glucose residues, is 29, whilst that of waxy maize starch, with an average chain length of 20 glucose residues, is 95. 11 - 13 unit glycogens have R_M values in the region of 76 - 80; the value of 83 for Mytilus edulis IX glycogen (average chain length 10 glucose residues) therefore appears slightly high. The β-amylolysis limit of this glycogen is, however, 51, indicating that while the interior of the molecule is very highly branched (average interior chain length, 1 - 2 glucose residues), the average exterior chain length is 7 - 8 glucose residues. An unusually

large proportion of the molecule is thus comprised of exterior chains where there are no barriers to α-amylolysis; a high R_M value would therefore be expected.

Waxy maize starch β-dextrin also has an average chain length of 10 glucose residues, but the R_M value is only 66. The exterior chains of this molecule are, however, only 2 - 3 glucose residues in length, and therefore α-amylolysis is limited to the interior chains.

The fact that the R_M value of Helix pomatia glycogen (average chain length 7 glucose residues) is more than 10% greater than that of Cardium glycogen (average chain length 8 glucose residues) can again be explained by considering the average position of the branch point on the unit chain. Helix pomatia glycogen has longer exterior chains than Cardium glycogen, and therefore the percentage of the molecule in which there are no barriers to α-amylolysis is proportionately greater.

From this preliminary survey it may be concluded that the extent of degradation of branched α-1:4-glucosans is related to the degree of branching of the molecule.

SECTION V.

POTATO PHOSPHOROLYSIS OF GLYCOGENS.

Introduction.

The first indication that the enzymic synthesis and degradation of α-1:4-glucosans might occur by a phosphorolytic mechanism, came in 1936, when it was reported that, in muscle tissue, glycogen was degraded yielding a mixture of hexose phosphates (131). Subsequently, C.T. and C.F. Cori isolated glucose-1-phosphate, which was shown to be the primary product of the phosphorolytic breakdown of glycogen (132,133). Further work indicated that the reaction "glycogen + inorganic phosphate → glucose-1-phosphate" was reversible and that when phosphorylase acted upon glucose-1-phosphate, a polysaccharide was synthesised (134-136). That synthesis and degradation of plant starch, occured by a similar mechanism, was shown by Hanes, when he isolated a phosphorylase from peas (137).

Since 1936, phosphorylase has been found in animal muscle, heart, liver, and brain (135), in higher plants (139, 140), in yeasts (135,138,141,142), and in bacteria (143). In this section only plant phosphorylases will be considered.

Subsequent to the isolation of pea phosphorylase by Hanes, phosphorylases have been obtained from other plants, including potatoes, beans, bananas and corn (140). Improvements in methods of extraction and purification have also been made (144-148), and recently, potato phosphorylase has been crystallised

(149,150). The potato enzyme has been more extensively studied than other plant phosphorylases.

Potato phosphorylase is not activated by adenylic acid or by reducing agents (c.f. muscle phosphorylase, Section VI) (144). The pure enzyme is inhibited to a slight extent by mercuric ions, but is not affected by molybdate ions (149,151); these substances inhibit α-1:4-glucosanases and R-enzyme respectively. Glucose appears to act as an inhibitor of potato phosphorylase (149).

The phosphorolytic reaction may be written:

$$(C_6H_{10}O_5)_n + \text{G-1-P} \rightleftharpoons (C_6H_{10}O_5)_{n+1} + \text{P}$$

where $(C_6H_{10}O_5)_n$ and $(C_6H_{10}O_5)_{n+1}$ represent chains of α-1:4-linked glucose residues. When synthesis takes place, the glucosyl radical of a glucose-1-phosphate molecule is added to the non-reducing end of a glucosan chain, and inorganic phosphate is liberated. Degradation occurs in the presence of excess inorganic phosphate, a glucosyl radical being removed from the non-reducing end of a chain as glucose-1-phosphate.

Theoretically, whether the reaction proceeds in the direction of synthesis or degradation, the number of polysaccharide end groups does not change. At equilibrium, therefore, the ratio of inorganic phosphate to ester phosphate should be constant and, within large limits, independent of the polysaccharide concentration. In practice, this is found to be the case, although, because inorganic phosphate and ester phosphate have different acid strengths, the position of the equilibrium is dependent upon the hydrogen

ion concentration. For potato phosphorylase, it is found that as the pH value is varied from 5.0 to 7.0, the ratio of inorganic phosphate to ester phosphate at equilibrium falls from 10.8 to 3.1 (139).

Potato phosphorylase cannot synthesise polysaccharide material from glucose-1-phosphate unless some α-1:4-glucosan, in the form of either amylopectin, glycogen, amylose, or maltosaccharide DP>3, is present to act as a "primer" (152-159). The synthetic action consists of the lengthening of already existing polyglucosan chains; hence the more non-reducing end groups that the primer possesses, the greater will be the rate of polysaccharide synthesis. For this reason, amylopectin is a much more efficient primer than amylose; on acid hydrolysis of amylose, however, the number of end groups, and hence the priming ability, is increased. The efficiency of the primer is also dependent upon its chain length, which must be a least four glucose units. (Maltotriose has a extremely low priming ability, whereas maltose has none at all). Cori and colleagues suggest that priming ability of acid-hydrolysed amylose is at a maximum when the average chain length of the maltosaccharides is five glucose units (158). They also point out that the β-dextrins from glycogen and amylopectin have negligible priming ability; the chain length of the stub containing the non-reducing end group is less than four glucose units (158). It is interesting to note that jack bean phosphorylase appears to be more efficiently primed by amylose than by amylopectin (160).

The polysaccharide which is synthesised by pure plant phosphorylase is of the amylose type (139,161,162). Phosphorylase itself does not synthesise α-1:6 linkages, although crude preparations are generally contaminated with a branching enzyme, and therefore appear to synthesise branched polysaccharides. The branching enzyme of plants, referred to as Q-enzyme, is a transglucosidase, which catalyses the scission of an α-1:4 linkage with the almost simultaneous synthesis of an α-1:6 linkage (163,164).

On degradation of α-1:4-glucosans in the presence of excess inorganic phosphate, glucosyl radicals are removed from the non-reducing chain ends by the phosphorolytic mechanism, until a branch point or other anomaly is reached. Reports that amylose is completely degraded by plant phosphorylases appear in the literature (165); Peat, Whelan and Thomas suggest, however, that pure potato phosphorylase, which is free of β-glucosidases, effects about 70% degradation of amylose, and that complete degradation occurs only in the presence of β-glucosidases (111). It is to be expected that pure phosphorylase would be unable to degrade or to by-pass β-glucosidic linkages.

The action of pure phosphorylase (free of R-enzyme, which catalyses the hydrolysis of α-1:6 linkages) on amylopectin, is confined to the exterior chains of the molecule, although there is little agreement in the literature on the extent of degradation of the exterior chains. Several workers report that the potato phosphorolysis limit of amylopectins is at least

10% less than the β-amylolysis limit, and that the phosphorylase limit dextrin is further degraded by β-amylase (159,111,166). Meyer, however, carried out the degradation with inorganic arsenate in place of inorganic phosphate (167). It was presumed that an unstable glucose-1-arsenate was formed, which immediately decomposed to glucose and inorganic arsenate. The extent of degradation of the amylopectin was therefore followed by measuring the amount of glucose liberated. When arsenolysis had ceased, the solution was dialysed to remove glucose; on addition of fresh enzyme and inorganic arsenate, further arsenolysis took place. After two repetitions of this procedure, the arsenolysis limit reached the β-amylolysis limit; the limit dextrin could then be degraded no further by either β-amylase or potato phosphorylase. Meyer reported that the rate of arsenolysis was about ten times slower than that of phosphorolysis, and about a thousand times slower than that of β-amylolysis (167).

Results obtained by phosphorolysis and arsenolysis of amylopectins are summarised in Table XIII. It is unlikely that phosphorolysis and arsenolysis limits would be different, but whether or not phosphorylase can degrade amylopectin to the same extent as β-amylase is uncertain. It appears that the affinity of potato phosphorylase for the exterior chains of amylopectin decreases rapidly as they are shortened by enzyme action.

Very little work has been done on the potato phosphorolysis of glycogens. Swanson reports that about 20% of rabbit liver glycogen is converted to glucose-1-phosphate (166), but Fischer suggests that the enzyme has practically no affinity

towards muscle or oyster glycogens (149).

In the work to be reported, potato phosphorolysis of several glycogens was carried out, in an attempt to relate the extent of degradation to the details already known about their molecular structure, and hence to use potato phosphorylase as a structural tool. A sample of waxy maize starch, and three samples of Floridean starch (a glycogen-type polysaccharide isolated from the seaweed, Dilsea edulis) were also subjected to the action of potato phosphorylase.

TABLE XIII.

Degradation of amylopectins by potato phosphorylase.

Source of amylopectin	Per cent phosphorolysis	Per cent arsenolysis	Per cent β-amylolysis	A	Ref.
Corn	ca 40	-	60	+	166
Tapioca	58	-	-		165
Tapioca	-	62	62	-	167
Waxy maize	-	70	70	-	167
Potato	58	54	-		165
Potato	39	-	53	+	159
Potato	48	-	59		111
Potato	-	70	70	-	167

A. Ability of β-amylase to attack potato phosphorylase limit dextrin.

Experimental.

1. Estimation of inorganic phosphate.

Inorganic phosphate was estimated by the method of Allen (168).

Reagents.

AnalaR reagents were used where possible.

Perchloric acid. A 60% solution was prepared.

Amidol. Sodium metabisulphite (10 g.) was dissolved in glass distilled water (ca. 45 ml.). Amidol (2:4-diamino-phenol hydrochloride) (0.5 g.) was added and dissolved as completely as possible. Insoluble material was removed by filtration, the volume made up to 50 ml., and the reagent stored in a dark bottle. It was rather unstable, and was renewed frequently.

Ammonium molybdate. A 10% solution of ammonium molybdate in distilled water was prepared. To facilitate solution, a small quantity of concentrated ammonium hydroxide was added.

Allen suggested the use of an 8.3% solution of ammonium molybdate, but, during the course of this work, it was found that more satisfactory results were obtained if the concentration was increased to 10%. This modification eliminated a tendency for the extinction coefficient of the colorimetric solution to increase gradually, without ever reaching a constant value.

Estimation.

An aliquot of solution containing inorganic phosphate was placed in a 25 ml. volumetric flask, and diluted to ca 19 ml.

Perchloric acid (2 ml.), amidol reagent (2 ml.), and ammonium molybdate reagent (1 ml.) were added, in the order given, the solution being shaken after each addition. The volume was made up to 25 ml., and, after allowing five minutes for the blue colour to develop to the full, the extinction coefficient was read against a reagent blank on a Hilger-Spekker photoelectric absorptiometer with a red filter (Ilford 608; ca 680 mμ).

The reagent was calibrated by means of a standard potassium dihydrogen phosphate solution (1 mg.P/ml.). The extinction coefficients of aliquots containing from 0.01 to 0.20 mg. P were measured, and a straight line graph obtained. The calibration was repeated periodically, but the slope of the graph was found to be fairly constant.

2. Estimation of glucose-1-phosphate.

After hydrolysis of glucose-1-phosphate to glucose and inorganic phosphate, the latter was estimated by the method detailed above. Normally, however, it was necessary to determine the amount of glucose-1-phosphate in a solution which also contained inorganic phosphate. Before glucose-1-phosphate was hydrolysed, inorganic phosphate was removed by magnesia mixture precipitation.

An aliquot (2 ml.) of solution containing glucose-1-phosphate and inorganic phosphate (normally the content of inorganic phosphate was about 0.1 M.) was diluted with distilled water (2 ml.). Dilute magnesia mixture (10 ml.; 8.60 g. magnesium chloride hexahydrate, 13.50 g. ammonium chloride, 35 ml. concentrated ammonium hydroxide, to 1000 ml.) was added

slowly, the temperature of the mixture being kept between about 40° and 60°. After allowing the mixture to stand for 30 to 60 minutes, the volume was made up to 25 ml., and the magnesium ammonium phosphate precipitate was filtered off. To an aliquot (5 ml.) of the filtrate, concentrated hydrochloric acid (0.65 ml.) was added, and the solution heated in a boiling water bath for 7 minutes to hydrolyse the glucose-1-phosphate. After cooling, the solution was transferred to a 25 ml. volumetric flask, and the content of inorganic phosphate determined by the normal method. To correct for any unprecipitated inorganic phosphate in the magnesia mixture filtrate, the phosphate content of an aliquot (5 ml.) containing unhydrolysed glucose-1-phosphate, was measured.

Control experiments carried out using this method showed that no adsorption of glucose-1-phosphate on to the magnesium ammonium phosphate precipitate occured. It was also verified that the presence of 0.5% to 1% of glycogen in the glucose-1-phosphate -inorganic phosphate solutions did not affect the estimation of glucose-1-phosphate. Glucose-6-phosphate was not hydrolysed under these conditions.

If inorganic phosphate is precipitated from an undiluted aliquot (2 ml.) of glucose-1-phosphate - inorganic phosphate solution by concentrated magnesia mixture (2.5 ml.; above quantities of reagents in 100 ml. of solution), co-precipitation of ca 5% to 10% of the glucose-1-phosphate on the magnesium ammonium phosphate occurs. This effect is completely eliminated by carrying out the precipitation in more dilute conditions, as

detailed above.

3. Activity of potato phosphorylase.

The method of Green and Stumpf was used in determining the activities of phosphorylase solutions (144).

One unit of phosphorylase activity is defined as that amount of enzyme which catalyses the liberation of 01 mg. of inorganic phosphate from glucose-1-phosphate in 3 minutes, when the following digest is incubated at 35°.

Digest. Solution A. 1 ml. glucose-1-phosphate solution (0.1 M)

Solution B. 0.5 ml. citrate buffer (0.5 M; pH 6.0)
0.2 ml. soluble starch solution (5%)
1.0 - 1.8 ml. enzyme solution
Distilled water to 2.5 ml.

Solutions A and B were preheated separately to 35°, then were mixed and incubated at 35° for exactly 6 minutes. The reaction was stopped by the addition of 5% trichloracetic acid (5 ml.). Precipitated protein was centrifuged off, and the inorganic phosphate in an aliquot (usually 1 to 3 ml.) of the centrifugate was estimated.

At the same time a blank digest was set up. It differed from the above digest only in that the enzyme solution was not added until after the trichloracetic acid solution, thus preventing enzyme action from occuring. The inorganic phosphate in a aliquot of the centrifugate was estimated, and the resulting figure was subtracted from the value which was obtained when enzyme action had occured. The activity of the enzyme could then be calculated.

When determining the activity of crude phosphorylase preparations, interfering enzymes (e.g. phosphatase and phosphoglucomutase) were inhibited by including ions such as molybdate (0.2%) and fluoride (0.01 M) in the digest.

4. Preparation of potato phosphorylase.

The method employed for the preparation of potato phosphorylase was personally communicated to Dr. D. J. Manners by Dr. G. A. Gilbert and colleagues.

It involved precipitating phosphorylase from 11% ethanol, in the cold, as a phosphorylase - amylose complex, extracting the phosphorylase from the amylose, reprecipitating, re-extracting, and finally crystallising it from ammonium sulphate. Gilbert and colleagues obtained 20 to 30 units of crystalline enzyme from 100 ml. of potato juice.

Reagents.

50% ethanol: 50% (v/v) ethanol solution, pH 6.0, containing 0.01 M citrate (1074 ml. water, 1000 ml. absolute ethanol, 4.64 g. sodium dihydrogen citrate, pH to 6.0 with solid sodium hydroxide. The pH was tested at 20° after five-fold dilution of a sample with 0.01 M potassium chloride).

11% ethanol: 11% (v/v) ethanol solution, pH 6.0, containing 0.01 M citrate, (0.282 ml. of 50% ethanol/ml. of 0.01 M citrate, pH 6.0).

Amylose: Potatoes were peeled, washed, thinly sliced and pulped in a "Waring" blender for 1.5 minutes in sodium chloride

solution (1% w/v). The starch suspension was expressed through a muslin bag (after prior washing of the muslin to remove any starch added during manufacture), and the residual pulp returned to the blender in fresh salt solution for a further 15 minutes. The starch was washed several times by decantation with salt solution, and then with distilled water to remove the salt. It was stored under water at 0°.

Wet starch (40 g.) was suspended in water (100 ml.) and the suspension poured into boiling hydrochloric acid (4 l.; 0,001 N). After boiling for 30 minutes, the solution was cooled slightly, and filtered through a layer of keiselguhr. Excess n-butanol (ca 600 ml.) was added and the mixture heated to 65 - 70°. It was then allowed to cool to room temperature, while being vigorously stirred to maintain saturation with butanol. The amylose - butanol complex separated on standing overnight, and was removed by centrifugation. It was dissolved in cold distilled water (2 l.), and the solution left at room temperature for 30 - 40 minutes. Excess n-butanol (ca 300 ml.) was added, and the solution heated and cooled as before. The amylose-butanol complex was allowed to settle overnight, then separated by centrifugation, and its content of amylose determined by drying down a portion. It was normally about 12%.

When a solution of amylose was required, sufficient of the complex to provide 2 g. of amylose was dissolved in water (350 ml.). To facilitate solution, the water was added gradually with stirring. Citrate buffer (4 ml.; 0.5 M; pH 6.0) was added, and the solution was boiled down in an open beaker to a volume

of 200 ml. to remove butanol. The resulting solution (1% w/v amylose; 0.01 M citrate) was then cooled to 0°, with stirring, in a refrigerated ethanol bath at -8°.

Experimental method.

a) Preparation of freeze-dried potato juice: Peeled potatoes (King Edward or Golden Wonder) were thinly sliced into sodium hydrosulphite solution (7 g./l.) at room temperature, and left to soak for 30 minutes. The slices were then washed well with distilled water, cooled to 0°, and pulped in a hand mincer. The juice was expressed through muslin, clarified by centrifugation at 0°, and, unless being used immediately, was freeze-dried. The freeze-dried product could be stored indefinitely at 0° over phosphorus pentoxide, without deterioration.

b) Preparation of phosphorylase: All operations were done in a cold room at 0°.

Stage 1. When necessary, freeze-dried potato juice was reconstituted with distilled water to the original volume. Juice, or reconstituted juice (100 ml.), containing between 200 and 300 Green and Stumpf units of phosphorylase, was stirred gently at -1° while "50% ethanol" (28.2 ml.) was slowly added from a burette, to bring the concentration of ethanol to 11% v/v. After centrifugation at 0° (8 minutes; 2800 g.), the precipitate was discarded and the supernatant liquid poured into a portion of amylose solution (20 ml.). The mixture was brought back to 11% ethanol concentration by slowly adding "50% ethanol" (5.6 ml.) with gentle stirring. It was then centrifuged (5 minutes;

2800 g.) and the precipitate, which contained the whole of the Q-enzyme in the original potato juice, was discarded. The supernatant liquid was poured into the remainder of the amylose solution (180 ml.), which by this time was very opalescent, and the mixture was again brought to 11% ethanol concentration by adding "50% ethanol" (50.8 ml.) slowly at -1°. After being stirred for 10 minutes, the mixture was centrifuged (5 minutes; 900 g.) and the precipitate was washed with "11% ethanol" (280 ml.). The precipitate was separated by centrifugation (5 minutes; 900 g.), and the washing repeated a further three times.

The precipitate was extracted with gentle stirring for 30 to 60 minutes with 0.05 M citrate solution pH 7.0 (120 ml.). After centrifugation (5 minutes; 2800 g.) the supernatant liquid was filtered carefully through a sintered glass filter, porosity 3. The precipitate was re-extracted with 0.05 M citrate (80 ml.) for 5 to 10 minutes, and the second extract was filtered and combined with the first extract. Gilbert and colleagues indicated that this filtrate should contain 100 to 120 Green and Stumpf units of phosphorylase.

Stage. 2. Amylose solution (200 ml.) was stirred while the enzyme solution was added slowly, and the mixture was then brought to 11% ethanol concentration by carefully adding "50% ethanol" (113 ml.) at -1°. After being stirred for 10 minutes, the mixture was centrifuged (5 minutes; 900 g.), and the precipitate washed twice, as before, with"11% ethanol" (280 ml.),

The precipitate was again extracted with 0.05 M citrate pH 7.0 (120 ml., followed by 60 ml.) and the extracts filtered carefully through a grade-3 filter. This filtrate should contain 60 to 70 Green and Stumpf units of phosphorylase.

Stage 3. The enzyme is crystallised by addition of ammonium sulphate. As this step was not found necessary in the present work experimental details are omitted.

Enzyme preparation.

Gilbert and colleagues obtained only 20 to 30 units of crystalline phosphorylase from 100 ml. of potato juice. By carrying the procedure as far as the second re-extraction, and then concentrating the solution by freeze-drying, it was hoped to obtain a higher yield of phosphorylase of a sufficient degree of purity for phosphorolysis experiments.

In the first preparation of phosphorylase, King Edward potatoes were used; freeze-dried potato juice (100 ml.) contained 240 units of phosphorylase. The volumes of citrate solution used at the end of stage 1 to extract phosphorylase from the phosphorylase - amylose complex, were slightly modified; a total of 180 ml. of solution containing 173 units of phosphorylase was obtained. At this point phosphorylase was contaminated by traces of Q-enzyme and R-enzyme.

In stage 2, it was hoped that by adding the enzyme solution to a small portion of the amylose solution (10 ml.), adjusting the concentration of ethanol to 11%. and discarding the precipitate, to eliminate contaminating enzymes. The supernatant from this step was added to a further volume of amylose

solution (170 ml.), and the concentration of ethanol adjusted to 11%. Phosphorylase (70 units) was extracted from the complex with citrate solution (total volume 160 ml.), and found to be free of Q-enzyme and R-enzyme.

The activity per ml. of this extract was too low for it to be used in phosphorolysis experiments. A portion of the extract (60 ml.) was therefore freeze-dried; the product was dissolved in a small volume of distilled water, and dialysed against distilled water to remove excess inorganic material. (It had previously been found that the activity of phosphorylase was not affected by dialysis.) An activity determination showed that a high percentage of phosphorylase was destroyed by freeze-drying.

According to Barker, Bourne, Wilkinson and Peat (147), phosphorylase is partially deactivated when it is freeze-dried from a citrate solution of molarity less than 0.2. The concentration of citrate in a further portion of the extract (45 ml.) was therefore increased to 0.2 M, and the solution was freeze-dried. Again, the enzyme lost practically all of its activity.

Since freeze-drying could not be used for concentrating potato phosphorylase solutions, it was apparent that, if the enzyme were not of sufficient purity at the end of stage 1 (where the concentration was ca 1 unit/ml.), the preparation would have to be carried beyond stage 2 to the crystallisation step.

The experiment was therefore repeated using potato

juice (100 ml.) freshly prepared from Golden Wonder potatoes, and containing 350 units of phosphorylase. The quantities of 0.5 M citrate solution used in stage 1 were again reduced; the total volume of citrate extract (150 ml.) contained 143 units of phosphorylase.

Examination of this extract (see below), showed that it was free of α-amylase, Q-enzyme, and phosphatase, but that it contained a trace of R-enzyme. However, as the activity of phosphorylase per ml. was at a high level, it was decided to use this preparation in phosphorolysis experiments, adding ammonium molybdate to inhibit R-enzyme, when necessary.

5. Tests for enzymic impurities.

The solution of potato phosphorylase prepared from Golden Wonder potatoes was examined, in order that any traces of α-amylase, Q-enzyme, R-enzyme, or phosphatase, which would interfere with phosphorolysis of α-1:4-glucosans, might be detected.

a) Q-enzyme and α-amylase.

Amylose is stained blue by iodine, but the intensity of blue colour (and therefore the "absorption value" at ca 680 mμ) of solutions containing constant quantities of amylose and iodine, decreases as the chain length of the amylose decreases. In the absence of inorganic phosphate, phosphorylase has no action upon amylose, but both Q-enzyme, (branching enzyme), and α-amylase, (which effects random hydrolysis of the molecule), cause the chain length of amylose to decrease.

A solution of amylose (ca 2 mg./ml.) was prepared:

amylose (ca 50 mg.) was dissolved in 0.5 N sodium hydroxide (4 ml.) by heating in a boiling water bath for 3 to 5 minutes. The solution was neutralised to phenolphthalein with approximately 0.25 N hydrochloric acid, and diluted to 25 ml.

A digest containing amylose solution (2 ml.) and enzyme solution (2 ml.) was incubated at 35°. At intervals, aliquots (1 ml.) of the digest were removed, and stained with iodine - potassium iodide solution (1 ml.; 0.2% iodine, 2% potassium iodide) in a total volume of 100 ml. The iodine absorption value (A.V.) was determined by reading the extinction coefficient of this solution against a blank containing iodine and potassium iodide (0.002% iodine, 0.02% potassium iodide), in a Hilger-Spekker photo-electric absorptiometer, using a red filter (Ilford 608; ca 680 mμ). The initial A.V. was obtained by measuring the A.V. of an aliquot (1 ml.) of a "blank" digest in which enzyme solution was replaced by citrate buffer (0.05 M; pH 7.0).

Time. (hr.)	A.V.
0	1.117
1.25	1.101
3	1.107

The A.V. was constant within the limits of experimental error; it was therefore concluded that neither Q-enzyme, nor α-amylase was present.

b) R-enzyme and α-amylase.

α-Amylase lowers the iodine absorption value of amylopectin

β-dextrin, by causing random scission of the interior chains of the molecule. R-enzyme, by hydrolysis of α-1:6 linkages, causes an increase in the length of the exterior chains, and hence an increase in the iodine absorption value.

β-Dextrin of waxy maize starch (ca 20 mg.) was dissolved in distilled water (10 ml.), the concentration of the resulting solution being approximately 2 mg./ml. A digest containing β-dextrin solution (2 ml.) and enzyme solution (2 ml.) was incubated at 35°; at intervals, aliquots (1 ml.) were removed for determination of A.V. A blank containing citrate buffer (0.05 M; pH 7.0) in place of enzyme solution, was used for measuring the initial A.V.

Time (hr.)	A.V.
0	0.076
1.25	0.078
3	0.082
24	0.106

The A.V. increased gradually, indicating that phosphorylase was contaminated with a trace of R-enzyme.

c) Phosphatase.

Phosphatase causes hydrolytic liberation of inorganic phosphate from glucose-1-phosphate and from glycerophosphate. Digests containing sodium β-glycerophosphate and enzyme were incubated at 35°, and the content of inorganic phosphate determined at intervals.

Enzyme digest.

3 ml. sodium β-glycerophosphate solution (0.1 M)

3 ml. enzyme solution

Blank digest.

3 ml. sodium β-glycerophosphate solution (0.1 M)

3 ml. citrate buffer (0.05 M; pH 7.0)

Aliquots (2 ml.) were removed for determination of inorganic phosphate. To correct for any hydrolysis of glycerophosphate by the reagents used in the analytical method, an aliquot of the blank digest was treated in the same manner, and the extinction coefficient of the enzyme digest sample was read with the blank digest sample in place of a reagent blank.

The percentage hydrolysis of sodium β-glycerophosphate was calculated from the content of inorganic phosphate in the digest solution.

Time (hr.)	Per cent hydrolysis of glycerophosphate.
4	0.16
20	0.29

It was concluded that the phosphatase activity was negligible.

6. Phosphorolysis of amylopectins and glycogens.

The solution of phosphorylase prepared from Golden Wonder potatoes, has been shown to be of satisfactory activity and purity, and was used in the phosphorolysis of α-1:4-glucosans.

Experiments were carried out at pH 6.0. the optimum pH of the enzyme (139), and, to prevent contamination by moulds, toluene was added to the digests.

The inorganic phosphate content of digests at the

commencement of the phosphorolysis reaction was usually 0.1 M. In most cases the polysaccharide concentration was ca 2 mg./ml. Assuming that the phosphorolysis limit was reached when 50% of the polysaccharide molecule had been converted to glucose-1-phosphate (a higher phosphorolysis limit than was ever found experimentally), the ratio of inorganic phosphate to ester phosphate at the end of the reaction would be 15.1. This figure is greatly in excess of the inorganic phosphate to ester phosphate ratio reached at equilibrium of the phosphorolytic reaction. When the pH is 6.0, this value is 6.7 . Hence the cessation of phosphorolysis could not be due to the equilibrium point of the reaction having been reached.

a). Phosphorolysis of amylopectin.

A sample of amylopectin was degraded in order to show that the enzyme preparation was sufficiently active to reach a phosphorolysis limit within 24 hours.

To inhibit R-enzyme, ammonium molybdate (0.2%) was added to the digest. Bailey noted that both ammonium molybdate and mercuric chloride inhibit R-enzyme, that ammonium molybdate is without effect on potato phosphorylase, and that mercuric chloride causes only slight inhibition of phosphorylase (169). However, control experiments showed that, although ammonium molybdate (0.2%) caused no inhibition of phosphorylase, the enzyme exhibited only 19% of its activity in the presence of mercuric chloride (M/72,000). It is suggested that the purer enzyme preparations are more sensitive to the presence of

inorganic ions.

Before analysis, the amylopectin was dried, *in vacuo*, over phosphorus pentoxide, for 4 hours at 100°.

Digest. 20.9 mg. waxy maize starch
2 ml. phosphate buffer (0.5 M; pH 6.0)
1 ml. ammonium molybdate solution (2%)
7 ml. phosphorylase solution (6.7 units; 0.32 units/mg. of polysaccharide)

The digest was incubated at 35°.

Aliquots (2 ml.) were removed at intervals for estimation of glucose-1-phosphate.

Time (hr.)	Extinction coefficient.	mg. P.	Per cent phosphorolysis.
2	0.223	0.050	31
4	0.269	0.061	38
24	0.318	0.072	45

That phosphorolysis was complete within 24 hours was proved by increasing the phosphorylase content of the original digest to 0.55 units/mg. of polysaccharide: no further degradation occured in the following 24 hours.

The phosphorolysis limit of 45% for this sample of waxy maize starch was 5% lower than the β-amylolysis limit (50%), a result which was of the same order as those obtained by other workers (see Table XIII). This indicates that the conditions which were used for phosphorolytic degradation of amylopectins were satisfactory. Glycogens were therefore degraded using similar conditions.

b) Phosphorolysis of glycogens.

Phosphorolysis of several glycogens was carried out. A typical experiment is reported in detail:

Before analysis, the glycogen was dried, in vacuo, over phosphorus pentoxide, for 4 hours at 100°.

Digest a. 20.0 mg. Mytilus edulis VI glycogen
2 ml. phosphate buffer (0.5 M; pH 6.0)
7 ml. phosphorylase solution (6.7 units; 0.33 units/mg. of polysaccharide.)
1 ml. distilled water

(Since R-enzyme has no effect on glycogen, ammonium molybdate was not included in digests in which glycogen was submitted to the action of potato phosphorylase). The digest was incubated at 35°. Aliquots (2 ml.) were removed at intervals for estimation of glucose-1-phosphate.

Time. (hr.)	Extinction coefficient	mg. P.	Per cent phosphorolysis.
2	0.041	0.009	6
4	0.043	0.010	7
24	0.060	0.014	9

After 48 hours the phosphorylase content of the original digest was increased to 0.57 units/mg. of polysaccharide, by the addition of 2 ml. of phosphorylase solution. After a further 24 hours, an aliquot (3 ml, equivalent in polysaccharide content to 2 ml. of the original digest) was removed for estimation of glucose-1-phosphate. The per cent phosphorolysis had increased to 11; i.e. phosphorolysis had not reached a limiting value

within 24 hours.

It appeared that phosphorolysis of glycogens was slower than that of amylopectins. In a second experiment in which the same specimen of glycogen was submitted to the action of phosphorylase, the amount of enzyme per mg. of polysaccharide was therefore increased. However, this could only be done by increasing the volume and thus the dilution of the digest. To ensure that the effect obtained by using a larger quantity of enzyme was not nullified by the greater dilution, two digests were set up. These contained equal quantities of glycogen, inorganic phosphate, and enzyme, but the total volume of digest b was 15 ml., while that of digest c was 20 ml.

Digest b. 20.9 mg. Mytilus edulis VI glycogen
2 ml. phosphate buffer (0.5 M; pH 6.0)
13 ml. phosphorylase solution (12.4 units; 0.59 units/mg. of polysaccharide.)

The digest was incubated at 35°. Aliquots (3 ml.) were removed at intervals for estimation of glucose-1-phosphate.

Time (hr.)	Extinction coefficient	mg. P.	per cent phosphorolysis.
4	0.040	0.009	6
10	0.055	0.013	8
24	0.064	0.014	9
96	0.128	0.025	16

Digest c. 20.0 mg. Mytilus edulis VI glycogen
2 ml. phosphate buffer (0.5 M; pH 6.0)

13 ml. phosphorylase solution (12.4 units; 0.62 units/mg. of polysaccharide.)

5 ml. distilled water.

The digest was incubated at 35°. Aliquots (4 ml.) were removed at intervals for estimation of glucose-1-phosphate.

Time (hr.)	Extinction coefficient	mg. P.	per cent phosphorolysis.
4	0.033	0.008	5
10	0.059	0.013	9
24	0.061	0.014	9
96	0.114	0.022	14

Comparing the results of digests b and c, it is apparent that phosphorolysis is not appreciably affected by dilution.

When the results of digest a are compared with those of digests b and c it is seen that increasing the amount of enzyme per mg. of polysaccharide does not affect the rate of phosphorolysis of glycogen.

Results.

Five glycogens, of varying chain lengths and structure, were submitted to the action of potato phosphorylase, the conditions being similar to those in either digest a or digest b (see above). The experiments were normally carried out in duplicate and are summarised in Tables XIV and XV.

An additional series of phosphorolysis experiments was carried out with a preparation of potato phosphorylase obtained from the Bios Laboratories, Inc., New York. The enzyme had an activity of ca 60 units/g., and the amount of enzyme used,

in terms of activity units, was only slightly less than in the experiments reported in Table XIV, but the rate of phosphorolysis of glycogens was considerably slower. The results obtained with this preparation are reported in Table XVI, and include data on Floridean starches I, II and III.

TABLE XIV.

Potato phosphorolysis of glycogens (Series I).

Source of glycogen.		U.	Per cent phosphorolysis.			
			4 hr.	10 hr.	24 hr.	96 hr.
Rabbit liver I		0.40	-	3	3	-
Rabbit liver V	a)	0.40	8	9	11	14
	b)	0.65	7	8	10	-
Mytilus edulis VI	a)	0.33	7*	-	9	11†
	b)	0.59	6	8	9	16
	c)	0.62	5	9	9	14
Helix pomatia	a)	0.39	2	5	5	7
	b)	0.36	2	5	3	5
Trichomonas foetus	a)	0.64	12	14	16	19
	b)	0.37	16	15	18	-

U. Units of phosphorylase per mg. of glycogen.

* 6 per cent phosphorolysis was attained after 2 hours.

† After 72 hours incubation.

TABLE XV.

A comparison of phosphorolysis and other data.

Source of Polysaccharide	Per cent phosphorolysis (24 hr.)	C.L.	β-Amylolysis Limit	Ext. C.L.
Glycogens				
Rabbit liver I	3	13*	25	5-6
Rabbit liver V	11	14	51	9-10
Mytilus edulis VI	9	13	46	8-9
Helix pomatia	5	7*	37*	5
Trichomonas foetus	16	15	60	11-12
Amylopectin				
Waxy maize starch	45	20	50	12-13

* Experiments carried out by Dr. D. J. Manners and colleagues.

Discussion.

It has been found that glycogens are degraded by potato phosphorylase (c.f. 149,166); the reaction is, however, extremely slow, and, even when high concentrations of enzyme are used, phosphorolysis does not cease within 72 to 96 hours. This

TABLE XVI.

Potato phosphorolysis of glycogens and related polysaccharides (Series II).

Polysaccharide.	U	Per cent phosphorolysis.			
		10 hr.	22 hr.	40 hr.	112-240 hr.
Glycogens.					
Rabbit liver IV	0.29	-	5	6	9
Rabbit liver (B.D.H.)	0.32	-	6	8	-
Cat liver VI	0.32	3	3	5*	9
Trichomonas foetus	0.28	6	7	10*	10
Floridean starch I	0.24	-	33	35	37
Floridean starch II	0.26	23	24	24*	-
Floridean starch III	0.27	-	27	28	29

U. Units of phosphorylase per mg. of glycogen.

* After 70 hours incubation.

meant that potato phosphorolysis limits of glycogens could not be obtained, and the original intention of relating phosphorolysis limits to known structural features of the glycogens, and possibly using potato phosphorylase as a structural tool, had therefore to be abandoned.

When the data presented in Table XIV was examined, it was realised that although degradation limits were not attainable, different glycogens were not degraded to the same extent. It was considered that, when one enzyme preparation was used throughout a series of experiments, and the experimental conditions were not altered, the extent of phosphorolysis of different glycogens at any particular time, should be comparable. The experiments reported in Table XIV fulfilled these conditions, and in Table XV, the average percentage of each glycogen converted to glucose-1-phosphate in 24 hours (per cent phosphorolysis) is summarised.

Five glycogens were examined, and the per cent phosphorolysis varied from 3 to 16. In order to determine whether this variation could be attributed to known differences in the structure of these glycogens, figures for chain length, exterior chain length, and interior chain length (See Section III) are included in Table XV. It will be noted that the per cent phosphorolysis figures increase in much the same order as the average exterior chain lengths, but that they are in no way related to either the average chain lengths or the average interior chain lengths. This is not unexpected, and must be interpreted as indicating that the longest exterior chains are the most readily attacked.

In the Series II experiments reported in Table XVI, four glycogens and three samples of Floridean starch were degraded with the commercial preparation of potato phosphorylase. The amount of enzyme used (units/mg. of polysaccharide) was very

slightly less than in the experiments of Series I, yet the rate of phosphorolysis of the glycogens was greatly decreased. As the maximum difference in extent of phosphorolysis of the four different samples was only 4% after 24 hours, no attempt has been made to relate the per cent phosphorolysis to the exterior chain length.

The difference in the pattern of degradation of amylopectins and glycogens by potato phosphorylase cannot be explained solely by differences in the lengths of their exterior chains. The sample of waxy maize starch which was treated with potato phosphorylase under the same conditions as the glycogens listed in Table XIV, had a chain length of 20 glucose residues, and a β-amylolysis limit of 50%. The average exterior chain length was therefore between 12 and 13 glucose residues, a figure which is closely comparable to the average exterior chain length of Trichomonas foetus glycogen (11 - 12 glucose residues). Nevertheless, while 45% of waxy maize starch was converted to glucose-1-phosphate in 24 hours, the corresponding figure for Trichomonas foetus glycogen was only ca 16%. It is tentatively suggested that the potato phosphorylase molecule is large, and that it is able to attack only those chains of branched α-1:4-glucosans which do not lie in close proximity to other chains. As amylopectins have longer interior chains than glycogens, they must be less compact in structure, and will therefore be more readily attacked.

The increase in extent of phosphorolysis with increasing exterior chain length of glycogens, can be explained

in a similar manner. The longer the average exterior chain length of a sample of glycogen, the less compact will be the structure at the perimeter of the molecule, and consequently phosphorolytic attack will occur more easily. If this hypothesis is correct, the number of sites on the glycogen molecule which are available to the enzyme, is limited. Enzymic attack will therefore be slow, and will not be accelerated by increasing the enzyme concentration.

R-enzyme, which hydrolyses α-1:6 linkages, resembles potato phosphorylase in that it readily attacks amylopectins, but has a very limited action on glycogens. This may also be attributed to the size of the enzyme molecule.

The uncertainty as to the true potato phosphorolysis limits of amylopectins can also be explained by this theory. Although the amylopectin molecule has a more open network of chains than the glycogen molecule, the compactness of the structure again increases towards the interior of the molecule; it is suggested that certain of the branch point glucose residues are situated too close together to allow potato phosphorylase to effect, with ease, complete degradation of the corresponding exterior chains.

The potato phosphorolysis of Floridean starch is of interest. Initial rapid degradation occurs (See Table XVI), and thereafter a slow creep in per cent phosphorolysis is again noted. Evidence from periodate oxidation (Floridean starches I, II and III have average chain length values of 9, 12 and 13

respectively), and from β-amylolysis (β-amylolysis limits are respectively 46, 37 and 37%), indicates that these polysaccharides are not significantly different from normal glycogens (170). Certain differences in physico-chemical properties have, however, been noted. Floridean starch has a higher reducing power and lower turbidity than glycogen, indicating that it has a lower molecular weight. The specific rotation is lower than that of glycogen, and the position of the iodine absorption maximum is higher. These observations, supported by the higher per cent phosphorolysis, suggest that the structure of Floridean starch is not identical to that of a normal glycogen.

SECTION VI.

MUSCLE PHOSPHOROLYSIS OF GLYCOGENS.

Introduction.

The animal phosphorylase which has been studied in greatest detail is that from the leg and back muscles of rabbits. It differs from plant phosphorylases in several ways, notably in activator and primer requirements, and in action pattern towards branched glucopolysaccharides.

Cori and Green report that muscle phosphorylase exists in two forms, denoted a and b (171). Although these are equally active in the presence of muscle adenylic acid (adenosine 5'-phosphate), in the absence of adenylic acid, phosphorylase b is inactive, whereas phosphorylase a exhibits about 60% of its maximum activity. These authors have isolated an enzyme which they consider is responsible for conversion of phosphorylase a to phosphorylase b. Since the b form has a molecular weight about half that of the a form, Keller and Cori suggest that this enzyme, the PR, or phosphorylase rupturing enzyme, catalyses the scission of the phosphorylase a molecule into two parts (172). Keller reports that trypsin can also effect the conversion of phosphorylase a to b, but that trypsin and PR enzyme are not identical (173).

Workers in the Cori school found that the greater part of the phosphorlase extracted from rabbit muscles in a rested

condition, was in the a form, but that, if the muscle had been stimulated, the b form of the enzyme prevailed (171). They suggested that conversion of phosphorylase a to b *in vivo* was a device which prevented exhaustion of muscle glycogen supplies during excessive stimulation (174). More recently however, Krebs and Fischer have reported that the Cori procedure for the preparation of rabbit muscle phosphorylase, yields the bulk of the enzyme in the b form, and that phosphorylase b is readily converted to phosphorylase a in the presence of divalent cations and adenosine triphosphate (175). This indicates that the *in vivo* state of rabbit muscle phosphorylase is still uncertain and that the form of phosphorylase which is isolated is dependent upon small differences in experimental technique.

Like potato phosphorylase, muscle phosphorylase can catalyse the synthesis of α-1:4-glucosidic bonds, but the two enzymes differ in their primer requirements. Both are primed to a small extent only by amylose, but, whereas amylopectin is more effective than glycogen as primer for potato phosphorylase, in the case of muscle phosphorylase, the reverse is true (158). Moreover, muscle phosphorylase requires the presence of polysaccharide material before synthesis takes place; unlike the potato enzyme, it is not primed by short chain oligosaccharides.

In the presence of excess inorganic phosphate, amylose, amylopectin and glycogen are degraded by muscle phosphorylase. Degradation of amylose is slow and stops when about 70% of the

molecule has been converted to glucose-1-phosphate (176); it is probable that the barriers to further enzyme action are the anomalous linkages which have already been mentioned (Pages 59 and 98). Phosphorolysis of glycogen reaches a limit when between ca 28% and 49% of the molecule has been removed (117); the corresponding values for amylopectin are higher (ca 36% to 53%) (176,65).

The action pattern of muscle phosphorylase when branched gluco-polysaccharides are degraded, has been studied by Cori and Larner (177). When the muscle phosphorylase limit dextrins of glycogen and amylopectin (Ø-dextrins) are submitted to the action of amylo-1:6-glucosidase, the debranching enzyme of rabbit muscle, glucose is produced. Since this glucose must have been joined by an α-1:6 linkage to the rest of the limit dextrin molecule, it could have originated only from the A-chain stubs. It is thus proved that the A-chain stubs of Ø-dextrins contain only one glucose unit.

Hestrin degraded the Ø-dextrins of glycogens and amylopectins with β-amylase and calculated that the amount of maltose produced was equivalent to one molecule per exterior chain (176). Since β-amylase would be unable to attack the A-chain stubs of the Ø-dextrin, Cori and Larner, assuming that the polysaccharide molecule contained equal numbers of A- and B-chains, suggested that β-amylase removed two molecules of maltose from each of the B-chain stubs of the Ø-dextrin (177). They concluded that the B-chain stub of the Ø-dextrin was 4 glucose units longer than the corresponding B-chain stub of the β-dextrin; if the length of

the B-chain stub of the β-dextrin is 2 to 3 glucose units, that of the ∅-dextrin must average 6 to 7 glucose units.

The differences between muscle and potato phosphorylase have already been considered. Phosphorylase from brewer's yeast has also been studied (142). While it is similar to potato phosphorylase in that it is not activated by adenylic acid, it resembles muscle phosphorylase in that it is more effectively primed by glycogen than by amylopectin, and is not primed by maltosaccharides. Yeast phosphorylase also differs from both the animal and plant enzymes in the extent to which it degrades branched α-1:4-glucosans. Although its action is confined to the exterior chains of the molecule, the yeast phosphorolysis limits of both glycogens and amylopectins are similar to the β-amylolysis limits (i.e. ca 50%). It is suggested that, as in the case of β-amylolysis, the exterior chain stubs are two to three glucose units in length. It is interesting that these three enzymes from different sources catalyse the same basic reaction, yet differ in their detailed mode of action.

Experimental.

1. Preparation of muscle phosphorylase.

Muscle phosphorylase was prepared by the method of Green and Cori (178), the preparation being carried out eleven times. It was essential to work at 0°, but since no cold room was available for the earlier experiments, the solutions were kept cold by means of an ice-bath.

The leg and back muscles of two rabbits were removed and chilled. They were minced, and an equal volume of ice-cold water added to the mince. The mixture was allowed to stand for 10 minutes with frequent stirring, and the extract strained off through muslin. The extraction procedure was then repeated.

After adjusting the pH of the combined extracts to between 6.0 and 6.2 with dilute hydrochloric acid (the acid being added slowly to avoid foaming), the slightly turbid solution was dialysed against cold running water (5° to 10°) for 3 hours. The pH was then brought to between 5.8 and 5.9 with 0.03 N hydrochloric acid. The precipitate which formed contained PR enzyme (171), and was removed by centrifugation.

The clear supernatant solution was brought to pH 6.8 by the addition of sodium β-glycerophosphate (ca 1 g. per 100 ml. of solution). Saturated ammonium sulphate solution (0.7 vol.) which had been prepared at room temperature, and adjusted to pH 6.8, was added, thus making the final solution 41% saturated with ammonium sulphate. The pH was checked, and, if necessary, adjusted to 6.8.

A small precipitate settled out overnight, and was collected by centrifugation. (About 25% to 30% of the protein in this precipitate was phosphorylase). It was dissolved in water (15 to 20 ml.) and dialysed against cold running water for 60 to 80 minutes, during which time a precipitate appeared in the dialysis bags. Dialysis was continued against cysteine-glycerophosphate buffer solution of pH 6.8 (1 ml. 0.3 M cysteine

hydrochloride to 39 ml. sodium β-glycerophosphate), the solution being changed three to four times during the course of 20 hours.

After 20 hours, the contents of the dialysis bags were centrifuged at high speed, and the precipitate which contained phosphorylase and some cystine, was well drained. The phosphorylase was extracted from the precipitate by triturating for 5 minutes with cysteine - glycerophosphate solution (10 ml.; 0.03 M cysteine hydrochloride, 1% sodium β-glycerophosphate, adjusted to pH 6.8) at 30° to 35°. This solution of phosphorylase was then placed in a refrigerator at 0°.

With the earlier experiments, in which refrigeration facilities were inadequate, it was difficult to obtain an enzyme preparation sufficiently active to be used in phosphorolysis experiments. Although this problem was overcome, by the use of a cold room, and the phosphorylase activity of the later preparations was higher, the enzyme was difficult to crystallise. This may have been because the greater part of the phosphorylase was in the b form which does not crystallise so readily as the a form. Fischer and Krebs suggested that phosphorylase b could be converted to phosphorylase a by adding manganous acetate (3×10^{-3} M) and adenosine triphosphate (4×10^{-4} M) to the crude extract and allowing it to stand for 2 hours at 0°, before proceeding with the next stage of the preparation (179); this technique was tried, but crystalline phosphorylase a was not formed. However, it was found that the solution obtained by extracting the precipitate formed during the final dialysis stage, with cysteine -

glycerophosphate buffer, had a sufficiently high phosphorylase content for it to be used in phosphorolysis experiments.

The preliminary experiments which are reported were carried out on a typical preparation of muscle phosphorylase. Other phosphorylase solutions which had activities of the same order, were similarly free from contaminating enzymes.

2. General methods used in muscle phosphorolysis.

a) Estimation of inorganic phosphate and glucose-1-phosphate.

The methods are detailed in Section V.

b) Precautions against mould contamination.

Toluene was used in all digests, to prevent the growth of moulds.

c) Control of pH

All digests were buffered to pH 6.8, the optimum pH of the enzyme (180).

3. Examination of a typical preparation of muscle phosphorylase.

a) Activity of muscle phosphorylase.

The method used was that of Cori, Cori and Green (180), who incubated the enzyme with glucose-1-phosphate in the presence of glycogen, under conditions in which the synthetic reaction was kinetically first order. They showed that, over a considerable range of enzyme dilutions, the rate of the reaction was proportional to the enzyme concentration, and based the unit of activity on the equilibrium constant of the reaction.

For a first order reaction

$$k = \frac{1}{t} \log_{10} \frac{x_e}{xe-x}$$

where x_e is the percentage of glucose-1-phosphate converted to inorganic phosphate and polysaccharide at time t (in minutes). Cori, Cori and Green showed that x_e varied with pH, and that at pH 6.8, $x_e = 78$. They measured the value of x for three values of t (5, 10, and 15 minutes), were then able to calculate k, and arbitrarily multiplying k by 1000, obtained the total number of enzyme units present. This figure multiplied by the appropriate dilution factor (see below) gave the number of units of phosphorylase present in the original enzyme solution.

The following solutions were prepared:

1) 4% glycogen solution.
2) 0.08 M sodium β-glycerophosphate (pH 6.8).
3) 0.06 M cysteine hydrochloride freshly neutralised to pH 6.8. Solutions 2 and 3 were mixed in equal volumes and used for dilution of the enzyme.
4) 0,064 M crystalline dipotassium salt of glucose-1-phosphoric acid, brought to pH 6.8.
5) To solution 4, adenylic acid was added to bring the concentration to 0.004 M.

Experimental Procedure.

0.1 ml. of the stock solution of phosphorylase was diluted with cysteine -glycerophosphate buffer, the operation being carried out in two steps when the dilution required was greater than 25 fold.

To dilute enzyme solution (0.4 ml.), solution 1 (0.2 ml.) was added, and the mixture incubated at 35° for 20 minutes. Solution 4 or 5 (0.2 ml.), prewarmed to 35°, was then added to start the reaction. After exactly 5, 10 and 15 minutes, aliquots (0.2 ml.) were pipetted into perchloric acid (10 ml.; 1.2%) which stopped the enzymic reaction. From the content of inorganic phosphate in these aliquots, the percentage (x) of glucose-1-phosphate converted to inorganic phosphate and polysaccharide, was obtained, and k was calculated. When the stock solution had been diluted the correct amount, k was found to be constant.

Results.

The phosphorylase a activity of the enzyme solution was determined by incubating the enzyme with glucose-1-phosphate and glycogen in the absence of adenylic acid. The stock solution of phosphorylase was diluted 1:10 by cysteine - glycerophosphate buffer. Since, in the digest it was further diluted 1:1, the "dilution factor" was 20.

Time (min)	x (%)	k x 1000	No of units/ml. of stock phosphorylase solution.
5	28	38.6	772
10	46	38.7	774
15	53	32.9	658

The total phosphorylase activity of the enzyme solution was determined by incubating the enzyme with glucose-1-phosphate and glycogen in the presence of adenylic acid. The stock solution of the phosphorylase was diluted 1:140 by cysteine - glycerophosphate buffer. Since, in the digest it was further diluted 1:1, the "dilution factor" was 280.

Time (min.)	x (%)	k x 1000.	No. of units/ml. of stock phosphorylase solution.
5	19	24.2	6776
10	33	23.9	6692
15	43	23.2	6496

It may be concluded that the bulk of the phosphorylase in the stock solution was in the b form. All phosphorolysis experiments were therefore carried out in the presence of 0.001 M adenylic acid.

b) Purity of muscle phosphorylase.

The enzyme with which muscle phosphorylase preparations are most frequently contaminated, and which would interfere with the course of phosphorolysis of glycogens, is the debranching enzyme, amyb-1:6-glucosidase. Its presence was tested for, by incubating the enzyme solution with a naturally occuring polysaccharide which was virtually a phosphorylase limit dextrin. (This substance was the "glycogen" obtained from the liver of a human female suffering from von Gierke's disease (181).)

Digest. 1 ml. von Gierke glycogen solution (6.8 mg./ml.)
0.4ml. phosphate buffer (0.5 M.; pH 6.8)
0.2ml. adenylic acid solution (0.01 M.)
0.1ml. phosphorylase solution.
Distilled water to 2 ml.

After 16 hours, the glucose-1-phosphate content of the complete digest, was determined, and the extent of phosphorolysis found to be 2.7%. This indicated that, although slight phosphorolysis of von Gierke glycogen had occured, no amylo-1:6-glucosidase

was present in the phosphorylase solution. Even a trace of amylo-1:6-glucosidase would rupture several 1:6 linkages, causing the extent of phosphorolysis to be greatly in excess of 3%.

Moreover, had amylo-1:6-glucosidase been present, phosphorolysis of normal glycogens would not have reached a limit when less than 50% of the molecule had been degraded, but would have continued slowly beyond this point. It was always found that phosphorolysis stopped within about 3 hours, and, in one case, it was shown that the degradation limit did not increase when the amount of phosphorylase (and therefore the amount of debranching enzyme, if any were present) was increased fourfold. This is further proof of the absence of amylo-1:6-glucosidase.

4. Muscle phosphorolysis of glycogens.

It has been shown that the muscle phosphorylase used in the course of this work was of satisfactory activity, and was free of debranching enzyme. A typical phosphorolysis experiment is reported in detail.

At the commencement of the reaction the inorganic phosphate content of all digests was 0.01 M. This high concentration of inorganic phosphate ensured that phosphorolysis did not cease because equilibrium between inorganic phosphate and glucose-1-phosphate was reached. (see Section V, Page 115) The inorganic phosphate : glucose-1-phosphate equilibrium ratio at pH 6.8 is 3.5.

a) Composition of digest and analysis of products.

Before weighing out, the glycogen was dried in vacuo,

over phosphorus pentoxide for 4 hours, at 100°.

Digest. 46.0 mg. brewer's yeast glycogen.

4.0 ml. phosphate buffer (0.5 M; pH 6.8)

2.0 ml. adenylic acid (0.01 M)

0.5 ml. phosphorylase solution (3750 units; ca 80 units/mg. of polysaccharide

Distilled water to 20 ml.

The digest was incubated at 35°. Aliquots (2 ml.) were removed at intervals for estimation of glucose-1-phosphate.

Time (hr.)	Extinction coefficient	mg. P.	Per cent phosphorolysis.
0.02	0.043	0,009	5
1.0	0.238	0.053	30
5.0	0.241	0.053	30
24.0	0.239	0.053	30

The course of the reaction is shown by a graph (Fig.XI) in which percentage phosphorolysis is plotted against time.

b) Proof that enzyme action was complete.

Phosphorolysis of brewer's yeast glycogen ceased within 1 hour. That this was due to the degradation limit having been reached, and not to insufficient enzyme being present in the digest, was shown by setting up a second digest in which the ratio of enzyme to polysaccharide was increased fourfold.

Digest. 47.0 mg. brewer's yeast glycogen.

4.0 ml. phosphate buffer (0.5 M; pH 6.8)

2.0 ml. adenylic acid (0.01 M).

2.0 ml. phosphorylase solution (15,000 units: ca 320 units/mg. of polysaccharide.

Distilled water to 20 ml.

The digest was incubated at 35°. Aliquots (2 ml.) were removed at intervals for estimation of glucose-1-phosphate.

Time (hr.)	Extinction coefficient	mg. P.	Per cent phosphorolysis.
0.02	0.155	0.034	19
1.0	0.253	0.056	31
5.0	0.253	0.056	31
24.0	0.238	0.053	29

The course of the reaction is plotted on the graph (Fig.XI).

Increasing the ratio of enzyme to polysaccharide did not alter significantly the phosphorolysis limit of brewer's yeast glycogen. It was therefore assumed that the amount of enzyme used in the first digest (sub-section a, above) was sufficient to cause the true phosphorolysis limit of the glycogen, to be reached.

In a subsequent experiment, in which glycogen from Helix pomatia was subjected to the action of muscle phosphorylase (110 units/mg. of polysaccharide) degradation reached a limit of 20% within 1 hour. After 4 hours the enzyme concentration was increased to 250 units/mg. of polysaccharide, but no further degradation occured in the subsequent 20 hours. It was thus apparent that the lower concentration of enzyme was sufficient to cause complete phosphorolysis of glycogen.

Results.

Phosphorolysis of glycogens was complete within 2 to 3 hours, when the amount of enzyme used was about 80 to 100 units per mg. of polysaccharide. Similar conditions to those detailed above were used to degrade a series of polysaccharides; most experiments being carried out in duplicate. The results are given in Table XVII.

Results obtained with an earlier, and less active preparation of phosphorylase are reported in Table XVIII.

TABLE XVII.

Muscle phosphorolysis of α-1:4-glucosans (Series I).

Source of polysaccharide.		U.	Per cent phosphorolysis.			
			1 - 1.5 hr.	3 hr.	5 hr.	24 hr.
Glycogens.						
Rabbit liver V	a)	100	33	32	-	-
	b)	95	32	32	-	-
Rabbit liver XIII	a)	100	29	30	-	-
	b)	100	31	30	-	-
Cat liver IV	a)	105	37	36	-	-
	b)	95	36	36	-	-
Cat liver VI	a)	100	34	34	-	-
	b)	105	34	34	-	-

Source of polysaccharide		U.	Per cent phosphorolysis.			
			1 - 1.5 hr.	3 hr.	5 hr.	24 hr.
Foetal sheep liver		75	-	-	28	29
Human muscle II	a)	105	21	22	-	-
	b)	90	20	21	-	-
Mytilus edulis V	a)	95	19	20	-	-
	b)	110	20	21	-	-
Mytilus edulis VI	a)	105	27	31	-	28
	b)	85	28	28	-	-
Ascaris lumbricoides	a)	85	29	31	-	31
	b)	130	29	30	-	-
Helix pomatia	a)	95	22	22	-	-
	b)	110	20	21	-	20*
Trichomonas foetus		75	-	-	36	35
Brewer's yeast	a)	80	30	-	30	30
	b)	320	31	-	31	29
Amylopectins.						
Waxy maize starch II	a)	75	41	41	-	-
	b)	85	41	41	-	-
Waxy sorghum starch	a)	80	40	40	-	-
	b)	90	39	39	-	-
Rumen protozoa	a)	80	42	45	-	46
	b)	75	43	44	-	-

* After 4 hours enzyme concentration was increased to 250 units/mg. of polysaccharide.

TABLE XVIII.

Muscle phosphorolysis of α-1:4-glucosans (Series II).

Source of polysaccharide.	Per cent phosphorolysis.				
	4 hr.	6 hr.	24 hr.	48 hr.	75 hr.
Glycogens.					
Rabbit liver I	-	12	13	14	-
Rabbit liver III	-	29	31	31	-
Rabbit liver VI	-	32	33	33	-
Foetal sheep liver	-	27	28	28	-
Rabbit muscle	-	23	24	25	-
Helix pomatia	18	-	20	-	21
Trichomonas foetus	30	-	34	-	35
Tetrahymena pyriformis	28	-	30	-	31
Brewer's yeast	25	-	27	-	28
Amylopectins					
Waxy maize starch I	39	39	40	41	-
Waxy maize starch II	-	42	43	43	-

Discussion.

1. Muscle phosphorolysis of α-1:4-glucosans.

Muscle phosphorolysis limits of seventeen glycogens were obtained in the course of this work. They vary from 14 to 36%, although in the majority of cases, degradation ceased when between 28 and 36% of the glycogen molecule had been converted to glucose-1-phosphate.

In similar experiments carried out on other samples of glycogen, by Cori and colleagues, phosphorolysis limits were generally between 30 and 40% (117), and on average, were somewhat higher than those obtained in this work. Dr. G. T. Cori, in a personal communication to Dr. D. J. Manners, indicated that, for maximum activity of phosphorylase, the addition of cysteine to the enzymic digests was essential. In the published experiments of this author, however, there appears to be little consistency in the cysteine content of phosphorolytic digests. Although the activity of the enzyme was measured in the presence of 0.015 M cysteine (180), the complete degradation of glycogen by phosphorylase plus amylo-1:6-glucosidase (debranching enzyme) was carried out in digests to which no cysteine was added other than that present in the enzyme solutions (177). The final cysteine content of these digests was not greater than 0.005 M. In two subsequent publications in which a) glycogen was exhaustively degraded with phosphorylase in order to determine the phosphorolysis limit (117), and b) was degraded alternately by phosphorylase and amlyo-1:6-glucosidase, for the purpose of studying the internal

structure of the molecule (65), there is no mention of the addition of cysteine.

Hestrin had earlier indicated that addition of cysteine to phosphorolytic digests interfered with the determination of phosphate esters of glucose by reductometric methods. Cori reports that, subsequent to the removal of protein and polysaccharide from aliquots of the enzymic digest, glucose-1-phosphate was estimated by hydrolysis and then reducing power measurement (177). Accordingly, control experiments (unreported) in which this technique was followed have been carried out, and it was shown that cysteine interfered markedly with glucose-1-phosphate estimation. It was thus obvious that, if cysteine were added to phosphorolytic digests, and glucose-1-phosphate estimated in this manner, large blank values would result, and hence, the accuracy of the estimation would decrease. Cori, however, makes no mention of this.

In view of the fact that, in the experiments of Cori and colleagues no constant quantity of cysteine was added to phosphorolytic digests, it is suggested that the presence of cysteine is not essential, and that, provided sufficient phosphorylase is present, true phosphorolysis limits will be attained. In the control experiments carried out with brewer's yeast and Helix pomatia glycogens (Pages 137, 138 and 139), phosphorolysis ceased within 1 to 3 hours, and neither addition of a further quantity of enzyme, dissolved in cysteine - glycerophosphate buffer, nor an increase in the initial amount of enzyme added to the digest, raised the

phosphorolysis limit. It is therefore considered that under the conditions used in this work, virtually complete phosphorolysis of glycogens was achieved.

The results reported in Table XVIII were obtained with a less active phosphorylase preparation, than was used for the experiments of Table XVII. However, when duplicate experiments were carried out, the phosphorolysis limits obtained with the less active preparation were only 1% - 2% less than those obtained with a more active enzyme preparation. This difference is considered barely significant, and therefore, for those glycogens listed in Table XVIII which were not also submitted to the action of a highly active phosphorylase preparation, the maximum per cent phosphorolysis obtained after 48 - 75 hours with the less active enzyme is considered to be the phosphorolysis limit of the glycogen.

Several amylopectins were also submitted to the action of muscle phosphorylase; the results obtained are included in Tables XVII and XVIII.

2. Multiple branching of glycogens.

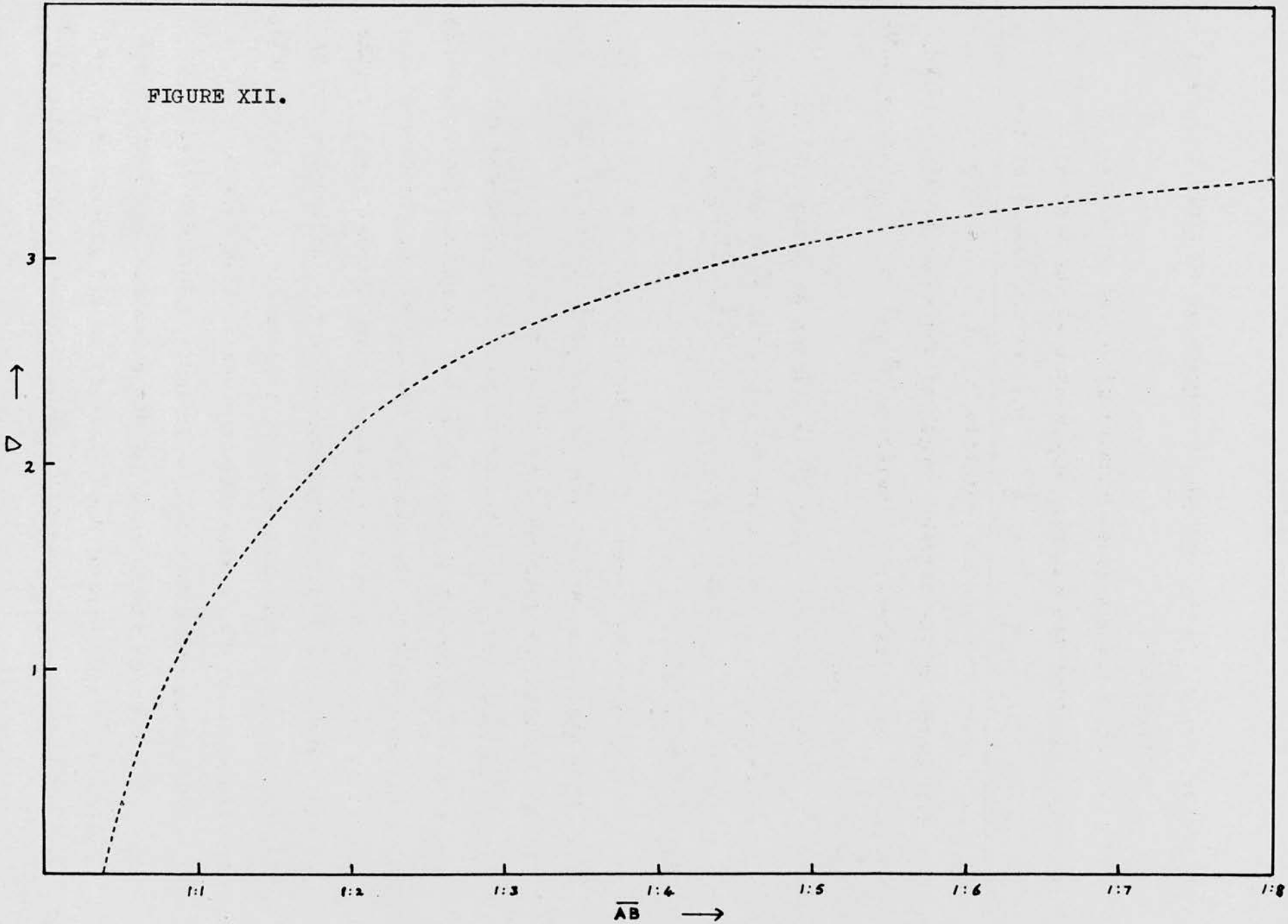
Although Meyer suggested, in 1941, the glycogen had a multiply-branched structure (45), experimental evidence was not obtained until comparatively recently. Larner, Illingworth, Cori and Cori degraded two samples of glycogen and two samples of amylopectin by the alternate action of rabbit muscle phosphorylase and amylo-1:6-glucosidase, and estimated the percentage conversion to glucose-1-phosphate or glucose after

each enzymic reaction had reached completion (65). Several successive degradations were carried out; between 30% and 40% of the polysaccharide or corresponding limit dextrin molecule was removed at each phosphorylase step, and ca 3% to 6% by each glucosidase reaction. These results can be explained only if the polysaccharides are multiply-branched.

It has already been shown that samples of glycogen vary in degree of branching and in the average position of the branch point residue on the unit chain, and it was thought unlikely that there would be uniformity in degree of multiple branching (ratio of A-chains to B-chains, designated $\overline{AB}$.) A method for determining approximate values of $\overline{AB}$ was accordingly devised.

Calculations of the degree of multiple branching are based on the differences between the experimentally determined chain lengths of the β-amylase limit dextrins (β-dextrins) and muscle phosphorylase limit dextrins (Ø-dextrins), and on the accepted action patterns of these enzymes. The average length of the A-chain stub of glycogen β-dextrins is 2.5 glucose residues (Page 62); that of the B-chain stub is considered to be n glucose residues (the most probable value of n being 2.5). It has been proved that the A-chain stub of Ø-dextrins contains 1 glucose residue, and, on the basis of work by Hestrin and Cori (176, 177), it is assumed that the length of the B-chain stub is (n + 4) glucose residues (Page 129). Variations in the difference in average length of the exterior chain stubs of β- and Ø-dextrins from one sample of glycogen to another, must

FIGURE XII.



therefore reflect variations in the degree of multiple branching of the glycogens.

If there are equal numbers of A- and B-chains, the average length of the exterior chain stub of the ∅-dextrin (E.C.L.∅.) is $\frac{1 + n + 4}{2} = \frac{n+5}{2}$. The average length of the exterior chain stub of the β-dextrin (E.C.L.β.) is $\frac{2.5 + n}{2}$. The difference $_{\Lambda}^{(\Delta)}$ in the average length of the exterior chain stubs of the ∅- and β-dextrins is therefore $\frac{n + 5}{2} - \frac{n + 2.5}{2} = 1.25$ glucose residues.

The value of Δ when $\overline{AB}$ is 1:2 can be calculated in a similar manner. Since there is 1 A-chain to every 2 B-chains, E.C.L.∅. = $\frac{1 + 2(n + 4)}{3}$, and E.C.L. = $\frac{2.5 + 2n}{3}$; thus $\Delta = \frac{2n + 9}{3} - \frac{2n + 2.5}{3} = 2.17$.

A complete series of calculations for $\overline{AB}$ = 1:0.5, 1:1. 1:2, 1:3, etc., was carried out; in Fig. XII values of Δ are plotted against the corresponding values of $\overline{AB}$.

Since only the exterior chains of the glycogen molecule are attacked by muscle phosphorylase and β-amylase, the difference (Δ) in the lengths of the exterior chain stubs of the ∅- and β-dextrins, is equal to the difference in the average chain lengths of the dextrins. Experimental values of this difference can be readily obtained from muscle phosphorolysis and β-amylolysis data. For example, rabbit liver V glycogen has a chain length of 14, a muscle phosphorolysis limit of 32 and a β-amylolysis limit of 51. The average chain length of the ∅-dextrin is therefore 9.5 glucose residues, that of the β-dextrin is 6.9 glucose residues, and the difference, Δ , is 2.6 glucose residues. The value of

is dependent upon the degree of multiple branching of the molecule, and from the graph (Fig. XII) it is seen that $\Delta = 2.6$ corresponds to an $\overline{AB}$ value of 1:2.9. In a similar manner, values of $\overline{AB}$ were calculated for the other glycogens for which muscle phosphorolysis data was obtained (see Tables XVII and XVIII). The results are summarised in Table XIX.

A second method for calculating values of $\overline{AB}$ has been developed, and will be illustrated by the case of human muscle II glycogen. After enzyme action (muscle phosphorylase and β-amylase only the exterior chain stubs of the A-chains remain; these are respectively 1 and 2.5 glucose residues in length. Both enzymes leave intact the interior parts of the B-chains; if the average interior chain length is i glucose residues, and the exterior chain stub of the β-dextrin contains n glucose residues, the average length of the B-chain after β-amylase action is (i + n + 1) glucose residues. (The branch point glucose residue is not included in either the exterior chain length or the interior chain length). Similarly the average length of the B-chain after muscle phosphorolysis is (i + n + 1 + 4) glucose residues. (i + n + 1) is common to both, and is therefore denoted by m. It is considered that the value of $\overline{AB}$ is 1:x.

Human muscle II glycogen has an average chain length of 11 glucose residues, a β-amylolysis limit of 40 and a muscle phosphorylysis limit of 22. After β-amylolysis has reached completion, the total number of glucose residues remaining, per 1 A-chain and B-chains is 60% of $11(x + 1) = 6.6\ (x + 1)$.

However, $6.6(x + 1) = 2.5 + xm$

i.e. $x(m - 6.6) = 4.1$ --------- (1)

After muscle phosphorylase action has reached completion, the total number of glucose residues remaining per 1 A-chain and x B-chains is 78% of 11 $(x + 1) = 8.6(x + 1)$.

$$8.6(x + 1) = 1 + x(m + 4)$$

$$\text{i.e.} x(m - 4.6) = 7.6 \quad \text{---------- (2)}$$

Solving equations (1) and (2),

$$\frac{m - 6.6}{m - 4.6} = \frac{4.1}{7.6}$$

$$3.5m = 31.3$$

$$m = 8.9$$

Substituting in (1).

$$2.3x = 4.1$$

$$x = 1.8$$

By both methods of calculation human muscle II glycogen has an $\overline{AB}$ value of 1:1.8.

From Table XIX it is seen that with two exceptions values of $\overline{AB}$ for glycogens vary from 1:0.9 to 1 : 2.9. It is thus concluded that glycogens show some variation in degree of multiple branching. For comparative purposes, two samples of amylopectin were examined; the data obtained is also recorded in Table XIX. Waxy maize starch I does not differ significantly from glycogens in degree of multiple branching, although waxy sorghum starch, which has a higher average chain length appears to have a higher $\overline{AB}$ value.

Two glycogens from rabbit liver VI and Trichomonas foetus have $\overline{AB}$ values of greater than 1:8. It is interesting

TABLE XIX.

Multiple branching of α-1:4-glucosans.

Source of polysaccharide.	C.L.	A	C.L.Ø	B	C.L.β	Δ	$\overline{AB}$
Glycogens.							
Rabbit liver I	13*	14	11.2	25	9.8	1.4	1:1.1
Rabbit liver III	13*	31	9.0	51	6.4	2.6	1:2.9
Rabbit liver V	14	32	9.5	51	6.9	2.6	1:2.9
Rabbit liver VI	18*	33	12.1	52	8.6	3.5	> 1:8
Rabbit liver XIII	15	30	10.5	46	8.1	2.4	1:2.4
Cat liver IV	13*	36	8.3	53	6.1	2.2	1:2.1
Cat liver VI	12*	34	7.9	52	5.8	2.1	1:1.9
Foetal sheep liver	13*	29	9.2	49	6.6	2.6	1:2.9
Rabbit muscle I	13*	25	9.8	45*	7.2	2.6	1:2.9
Human muscle II	11	22	8.6	40	6.6	2.0	1:1.8
Mytilus edulis V	9*	21	7.1	40	5.4	1.7	1:1.5
Mytilus edulis VI	13	28	9.4	46	7.0	2.4	1:2.4
Ascaris lumbricoides	12*	31	8.3	49*	6.1	2.2	1:2.1
Helix pomatia	7*	22	5.5	37*	4.4	1.1	1:0.9
Trichomonas foetus	15*	36	9.6	60*	6.0	3.6	> 1:8
Tetrahymena pyriformis	13*	31	9.0	44*	7.3	1.7	1:1.5
Brewer's yeast	13*	30	9.1	44*	7.3	1.8	1:1.5
Amylopectins.							
Waxy maize starch I	20*	41	11.8	50*	10.0	1.8	1:1.6
Waxy sorghum starch	25†	40	15.0	52†	12.0	3.0	1:6.3

A. Muscle phosphorolysis limit (per cent)

B. β-amylolysis limit (per cent)

*. Experiments carried out by Dr. D. J. Manners and colleagues.

C.L.Ø. Average chain length of Ø-dextrin.

C.L.β. Average chain length of β-dextrin.

†. Values reported by Hirst and colleagues; Ref. 182.

to note that both these samples have longer than average chain lengths.

The values of $\overline{AB}$ presented in Table XIX are not claimed as absolute, since it is virtually impossible to ensure that each of the several thousand exterior chains in a glycogen molecule is completely degraded by the enzymes, whilst the analytical procedures, despite every precaution, are barely accurate to within ±1%. Small differences in Ø- and β-limits result in relatively large differences in $\overline{AB}$. The results in Table XIX are however considered to show that significant differences in degree of multiple branching of different samples of glycogen and amylopectin exist.

Comparable calculations based on data published by Cori and colleagues are detailed in Table XX. Degrees of multiple branching of both glycogens and amylopectins are of the same order of magnitude as those reported in Table XIX.

3. Acetylation of hydroxyl groups in glycogen.

In 1952, Illingworth, Larner, Cori and Cori reported that three samples of glycogen, which had been purified by precipitation from 80% acetic acid, were not completely degraded

by the simultaneous action of muscle phosphorylase and amylo-1:6-glucosidase (117). When these glycogens were treated with hot 15% sodium hydroxide, and the experiments repeated, complete degradation occured. The authors postulated that the barriers to enzyme action were acetyl groups which were subsequently removed by alkali treatment.

TABLE XX.

Degree of multiple branching calculated from data published by Cori and colleagues (65).

Source of polysaccharide.	C.L.	C.L. ∅-dextrin	C.L. β-dextrin	Δ	$\overline{AB}$
Glycogen.					
Rabbit liver	15	9.7	7.9	1.8	1:1.6
Amylopectins.					
Wheat	18	9.8	7.2	2.6	1:2.9
Corn	24	11.1	8.4	2.7	1:3.2

In the course of this work, a sample of foetal sheep liver glycogen (purified by acetic acid treatment) was digested with 0.1 N alkali at 100° for 0.5 hour in order to remove any acetyl groups which might be present. If precipitation from acetic acid caused acetylation of a significant number of hydroxyl

groups, differences in the extent of enzymic degradation of acetic acid precipitated glycogen before and after treatment with alkali, should occur. The relevant experimental data is summarised in Table XXI.

TABLE XXI.

A comparison of the enzymic degradation of glycogens purified by different methods.

Glycogen.	β-Amylolysis limit	Muscle phosphorolysis limit	α-Amylolysis[d] limit
Foetal sheep liver[a]	49[c]	28	82
Foetal sheep liver[b]	49	28	81

a. Purified by precipitation from acetic acid

b. Acetic acid-precipitated sample treated with alkali.

c. Value determined by Dr. D. J. Manners.

d. Experiments carried out with crude saliva.

β-Amylolysis and muscle phosphorolysis experiments indicate that acetylation of glucose residues of the exterior chains has not occured. From the results of the α-amylolysis experiments it is concluded that, unless the acetyl groups are situated, not at random, but solely at or near the branch points, no acetylation has taken place.

4. Comparison of glycogens and amylopectins.

In the work described in this thesis, a large number of glycogens have been subjected to the action of α-1:4-glucosanases. The data obtained has been used, along with that from chemical studies, in elucidating certain details of the structure of these polysaccharides. It has been shown that glycogens vary in degree of branching, in the average position of the branch point residue on the unit chain, and in degree of multiple branching. Samples obtained from different individuals of the same species are liable to differ from one another in structure to as great an extent as samples from different species. It appears that, in general, glycogens from vertebrate and invertebrate sources have a similar type of structure, although mammalian glycogens differ radically from other glycogens in the characteristics of their iodine absorption spectra. This indicates that mammalian glycogens are in some respect different from glycogens from lower animals, but the nature of this difference is not at present obvious.

True glycogens have been isolated only from animal sources. However, in 1939, Morris and Morris discovered that a glycogen-type polysaccharide could be extracted from the seeds of the sweet corn plant (14), and more recently Meyer and Fuld have shown that this "phytoglycogen" is similar to a normal animal glycogen in both chain length (12 glucose residues) and β-amylolysis limit (47.5%) (18). Like Floridean starch, discussed in Section V, "phytoglycogen" from sweet corn differs from true animal glycogens in certain physico-chemical properties, which indicates that structural differences may also be present.

The glucopolysaccharides elaborated by protozoa are interesting in that they may resemble either glycogens, amylopectins or normal starches. The polysaccharide synthesised by the flagellate Polytomella coeca has both linear and branched components, which are analogous to normal amylose and amylopectin (182). The ciliate Cycloposthium and a holotrich ciliate present in the rumen of the sheep, both produce homogeneous branched polysaccharides, of the amylopectin type (184, 185). However, the polysaccharides elaborated by other organisms, for example, Tetrahymena pyriformis, Trichomonas foetus, and Trichomonas gallinae are glycogens, with unit chain lengths of 13, 15 and 9 glucose residues, respectively (10,11). Another protozoan, Chilomonas paramecium has been found to synthesise a polysaccharide of chain length 18 which appears to be associated with a small percentage of amylose (186).

Amylopectins resemble glycogens in that they have multiply-branched structures in which the average exterior chain length is longer than the average interior chain length. Variations in degree of multiple branching occur from one sample of amylopectin to another, these variations apparently being of the same order as those found in the glycogen series. Whether glycogens and amylopectins form one homologous series of branched glucopolysaccharides differing only in degree of branching, or whether there is a more fundamental difference in structure, is not at present obvious. It is believed that in both animals and plants, the linear portions of the polysaccharides are elaborated by the enzyme phosphorylase, and the α-1:6 linkages by branching enzymes.

The latter are transglucosidases, which cause the scission of an α-1:4 linkage with the immediate transference of part of the chain to position 6 of another glucose residue. The phosphorylases and branching enzymes of plants and animals differ considerably in specificity and action pattern, and thus it is not surprising that the polysaccharides which they synthesise are structurally dissimilar.

SUMMARY.

1. The degradation of glycogens from a variety of biological sources by the enzymes barley β-amylase, salivary α-amylase, potato phosphorylase and rabbit muscle phosphorylase has been studied.

2. Twenty-six samples of glycogen were degraded by β-amylase. In the majority of cases, the percentage conversion to maltose varied from 39% to 53%, but for glycogens from one sample of rabbit liver and from Cardium, unusually low values of 25% and 14% respectively, were obtained. The average length of the exterior chains of the glycogen molecules was shown to be normally longer than that of the interior chains.

3. Nine glycogens were subjected to the action of salivary α-amylase, and the products of enzyme action examined chromatographically. Neither maltulose nor fructose-containing α-dextrins were detected, indicating that fructose is not present in these glycogens. The apparent percentage conversion to maltose, in general, decreased as the degree of branching of the glycogen increased.

4. The potato phosphorolysis of glycogens was investigated. The reaction was very slow, between 3% and 16% conversion to glucose-1-phosphate occuring within 24 hours. The percentage phosphorolysis increased as the exterior chain length of the glycogen increased.

5. Muscle phosphorolysis of glycogens was studied. With the exception of one sample of rabbit liver glycogen (which had a phosphorolysis limit of 14%) phosphorolysis limits between 21% and 36% were obtained. Calculations based on muscle phosphorolysis and β-amylolysis data showed that the degree of multiple branching of glycogens varied from one specimen to another.

6. For comparative purposes, samples of amylopectin were also subjected to the action of these four enzymes.

7. In the course of this work, six samples of glycogen were isolated from various tissues. These polysaccharides have been characterised with respect to glucose content, specific rotation, and absorption spectra of iodine complex. In addition, the average chain length of these, and several other samples of glycogen have been determined by potassium periodate oxidation. The majority of samples had a chain length of 12 ± 2 glucose residues, although specimens with chain lengths of 8, 15, 16 and 17 glucose residues were encountered.

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