

STUDIES ON ENZYMES IN MARINE ALGAE

- by -

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INTRODUCTION

The algae which are primitive, chlorophyll-containing plants without true leaves, stems or roots are divided into seven distinct groups (1). In the course of work described in this thesis, studies have been carried out on two marine algae, one from the Rhodophyta and the other from the Chlorophyta. The algae of the Rhodophyta contain chromatophores in which the photosynthetic pigment is masked by phycoerythrin, a red pigment. The alga belonging to this group, Rhodymenia palmata, which is of the class Rhodophyceae, sub-class Florideae and order Rhodymeniales, has a dark red, branched thallus which is parchment-like. The member of the Chlorophyta studied Cladophora rupestris, of the class Chlorophyceae and order Cladophorales, is a green alga with cylindrical multi-nucleate cells joined end to end in branched filaments.

The cells of the algae are essentially similar to those of the more advanced land plants, there being a distinct cell wall and the protoplasm within being divided into cytoplasm and nucleus. The photosynthetic pigments are associated with chromatophores and are usually situated just within the cell wall which normally consists of two layers, an outer layer of pectic material and an inner, firmer layer of cellulose.

A study of the metabolism of any particular living cell is concerned with the different pathways by which the cell synthesises, maintains and degrades the complex organic

molecules which form its structure. In such a study a knowledge of the cell constituents, and their structure, is of prime importance. Considerable work has been carried out on the metabolic pathways of the land plants (2) but very little is known about the metabolism of the marine algae. The metabolism of several fresh water unicellular green algae has been studied and the general metabolism appears to be similar to that of other forms of life, although it is less certain that the actual enzymic steps are the same (3). The multicellular marine algae have been the subject of considerable investigation since the description of alginic acid by Stanford in 1883 (4) and Black (5), who has reviewed present knowledge of the chemical constituents of marine algae, points out that the Rhodophyceae and Chlorophyceae have not been subjected to such intensive investigation as the marine algae of the Phaeophyceae.

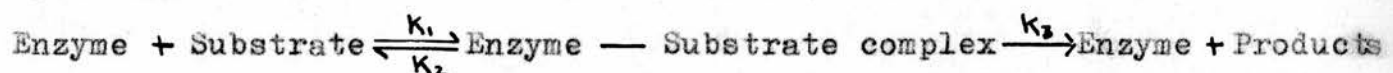
The polysaccharides present in seaweeds have been extensively studied and vary greatly in structure (5). R. palmata contains a xylan in which the D-xylose residues are united by both 1:4- and 1:3- links (6, 7, 8), as well as 15% floridoside (9) which is glycerol α -D-galactoside (10). Ross (11) has reported that it contains 2.4% cellulose and that the ratio of pentose to hexose is approximately 10:1. Coulson (12) has reviewed the literature on the free amino acids in R. palmata and has given the amino acid composition of protein extracted from it.

C. rupestris has a high protein and cellulose content,

a value of 22% having been found for the latter (13). Kylin (14) isolated a water soluble material containing galactose and indicated that it might be a galactan sulphate. Later work has shown, however, that it contains arabinose, galactose, xylose, rhamnose and glucose in the relative molecular proportions of 16:15:4.2:1 and approximately 20% sulphate (15).

Enzymes

The metabolic processes of living organisms are brought about by enzymes,* which are catalytically active proteins capable of accelerating a specific reaction. The protein may be either simple or conjugated, the non-protein portion in the latter case being termed a prosthetic group. Certain enzymes require a dialysable group or co-enzyme for activity and those studied so far have been shown to be vitamins or derivatives of them. In enzyme catalysis, the activation energy of the reaction is lowered; this is achieved by the formation of an enzyme-substrate complex, as first postulated by Michaelis and Menten in 1913 (16).



From this equation it is possible to derive the Michaelis-Menten equation in which the Michaelis constant, $K_M = \frac{K_2 + K_3}{K_1}$, may be

*

The substance which is attacked by an enzyme is termed the substrate of that particular enzyme.

defined as the substrate concentration at half maximum velocity. As enzymes are predominantly protein in nature they exhibit the characteristic properties of proteins, the most important being sensitivity to extremes of hydrogen ion concentration and to temperatures higher than 50°C.

Nomenclature of Enzymes

Unlike chemical catalysts, enzymes are specific for either a particular reaction (e.g. hydrolysis of fats by lipases) or a reaction involving a particular substrate (e.g. catalase), and it is on this specificity that the various systems for the classification and nomenclature of the enzymes have been based. Duclaux (17) was the first to introduce a systematic form of enzyme terminology by naming the enzyme after the substrate upon which it acted followed by the ending "-ase". This method, however, is limited since a particular substrate may be acted upon by two or more different enzymes. Hoffman-Ostenhof (18), in a new system for the classification and nomenclature of the enzymes, has divided the enzymes into five groups, viz.:

- I. Hydrolases,
- II. Transferases,
- III. Oxidoreductases,
- IV. Lyases and Syntheases, and
- V. Isomerases and Racemases.

Groups I, II and III comprise those enzymes whose reaction may be symbolized by the equation:-



Group IV enzymes correspond to the equation:-



and Group V to $A \rightleftharpoons B$.

These five groups are further subdivided according to the chemical nature of the linkage and substrate attacked.

Eight rules are given for the nomenclature of the enzymes but only the rules which have particular application in this thesis are summarised.

"The naming of enzymes by adding the suffix "-ase" to the name of the substrate attacked should be strictly reserved for hydrolysing enzymes".

"Transferases are named in the following way:- the first part of the name should be the name of the donor substrate followed by that of the acceptor. Then comes the syllable "trans" followed by the name of the group transferred and eventually the suffix "-ase". Thus, "phosphorylase" becomes "glucose-1-phosphate $\rightarrow$ amylose transglucosidase".

"The terms peroxidase and catalase remain unchanged".

Enzyme reactions

The progress of an enzymic reaction may be followed by either physical or chemical measurements, in which the rate of disappearance of the substrate or the appearance of the products is determined. For the polysaccharidases the physical methods in most common use are viscometry and measurement of optical rotation whilst chemical methods are based on estimation of the reducing power of the reaction mixture or, for oligosaccharidases the liberation of a chromogenic group. Paper chromatography

is widely used in the qualitative demonstration of many different types of enzymic reactions.

Carbohydrate metabolism

The enzymes of carbohydrate metabolism in animals and higher plants have been extensively studied, and it has been shown that the complete oxidation of carbohydrates to carbon dioxide and water may be effected by three multi-enzyme systems. The first of these, the glycolytic system, contains the enzymes which catalyse the degradation of the polysaccharides to pyruvic acid via glucose and fructose 6- phosphates and fructose 1:6- diphosphate. The pyruvic acid may then be decarboxylated to acetaldehyde, reduced to lactic acid or completely oxidised to carbon dioxide and water by the enzymes of the Krebs or tricarboxylic acid cycle which are found in many animal tissues and higher plants. One of the enzymes of the Krebs cycle, malic dehydrogenase which catalyses the following reaction (Figure 1), has been reported to be present in Ulva lactuca, a marine alga which is a member of the Chlorophyceae (19), thereby providing evidence for the possible existence of this enzyme system in the marine algae.

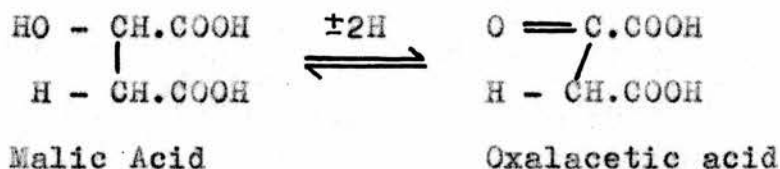


Figure 1.

An alternative metabolic pathway to the glycolytic system is the hexose monophosphate "shunt" (20) by which hexose 6- phosphate

may be converted to carbon dioxide and triose phosphate. Enzymes belonging to this cycle have very recently been found in animals, micro-organisms and plants.

As the monosaccharides may be considered to be the starting point of these cycles the degradation of polysaccharides and glycosides to free sugars is of some importance. This may be effected by the hydrolytic enzymes known collectively as the glycosidases, the general reaction of which is as in Figure 2, where R, the aglycon, may be either a sugar, a group of sugar residues or an alkyl or aryl radical.

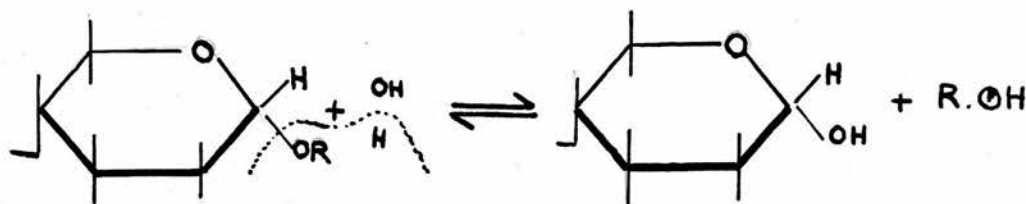


Figure 2.

Where R is a chain of sugar residues the enzymes are known as polysaccharidases, the properties of which have been reviewed by Manners (21). The glycosidases are classified according to the type of sugar to which, and the nature of the linkage by which, the aglycon is attached. The distribution, properties and specificity of these enzymes are considered in section two of this thesis.

Enzymes in marine algae

No comprehensive studies appear to have been made on any enzyme system connected with a particular metabolic pathway in

the marine algae although reports have occasionally been made on the presence of specific enzymes. Bartholomew (22) reported that "diastase" was present in cell-free extracts of several red algae, and the existence of catalase has been observed in many different marine algae (23, 24, 25). Peroxidase activity has been found in a large number of seaweeds (26) and Ronnerstrand (27) has indicated that there is a true and pseudo-peroxidase in many green and red algae, C. rupestris being one of the algae containing both. Two different polyphenolases have also been reported to be present in the marine algae (28). Bean and Hassid (29) have very recently reported that cell-free extracts of a red alga Iridophycus flaccidum contain an enzyme which will oxidise glucose, galactose and several reducing disaccharides to the appropriate aldonic acid.

The work reported in this thesis was undertaken in order to investigate more thoroughly the occurrence of enzymes, in particular the glycosidases, in marine algae.

SECTION ONE

The Preparation of seaweed extracts.

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INTRODUCTION

The problems encountered in preparing cell-free extracts of algae, for enzymic studies, are essentially similar to those of terrestrial plants, in that a high yield of protein will depend on the successful disintegration of the cell wall and the use of a solvent which will disperse the protoplasmic protein.

The extraction can be effected only under mild conditions since the enzyme-proteins will be readily denatured by heat or extremes of pH. Many methods have been evolved for the preparation of cell-free extracts from animal tissue (30), bacteria (31) and the unicellular algae (32). Crook (33) conducted an investigation into the extraction of nitrogenous material from tobacco leaves and described a method in which, by mincing and milling in a triple-roller mill, a 90 - 95% extraction of the leaf nitrogen could be effected. By the use of dilute sodium hydroxide he was able to counteract the "acid-drift" which Holden (34) has shown to be due to the enzymic demethylation of pectin. A similar high yield of nitrogenous material was obtained, on a small scale, by Lugg (35) who ground the leaf in an end-runner mill in the presence of a large volume of borate buffer pH 9.2. Schwimmer and Pardee (36) have reviewed the several methods by which plant cells may be disintegrated; these include grinding in a chilled meat mincer or in a mortar and pestle, with or without the addition of sand. The Waring blender has been frequently used (e.g. 37) although

in certain experiments it has been reported to cause inactivation of the extract (38, 39).

Many different solvents have been used to disperse the liberated protoplasmic protein. Maestrini (40) used 0.0003 M acetic acid and hydrochloric acid to obtain active invertase preparations from germinated barley, whilst Chibnall (41), after plasmolysis of the leaf cell with ether to remove the vacuolar juice, extracted the protein from the minced tissue with distilled water. Much of the protein, however, is associated with the particulate matter of the cell and Wildman and Jagendorf (42) have reviewed the various methods by which the particles may be isolated and their protein extracted. Water saturated with butanol has been used to solubilize the enzymes associated with this particulate matter (43).

Little work has been reported on the extraction of protein from marine algae and the few results which are available indicate that the yields are low. From Fucus vesiculosus, a member of the Phaeophyta, Smith and Young (44) were able to extract only one half of the total protein, by homogenizing the algae in a Waring blender at 55°C in the presence of 0.33% sodium hydroxide, whilst Coulson (42) obtained approximately 2% yields of protein from R. palmata, after double mincing in the presence of a large volume of borate buffer. Authors who have reported the presence of specific enzymes in marine algae (p.7) have mainly used aqueous extracts of the minced algae for activity studies and accordingly, this method of cellular disintegration has been used in the preparation of the seaweed extracts.

EXPERIMENTAL

Methods

Dialysis. In order to remove inorganic material, sugars, amino acids and peptides the extracts were dialysed in cellophane dialysis tubing against running tap water for the quoted times.

Lyophilization. The extracts were concentrated and dried to felt-like solids by freeze-drying in an all-glass apparatus which, when operating at full capacity, removed 110 ml. of water per hour.

Nitrogen. The Kjeldahl nitrogen of the lyophilized extracts was estimated using the method of Chibnall, Rees and Williams (46) and potassium sulphate:copper sulphate:sodium selenate (80:20:1) catalyst.

Centrifugation. All centrifuging was carried out at 0°C.

Effect of solvent on extraction of protein from R. palmata.

The wet weed (North Berwick; 24/9/53) was minced with a small amount of ice in a mechanical meat mincer and portions (50 gm.) shaken overnight with the solvents (100 ml.) listed in Table 1. The weed residues were removed by centrifugation and the extracts and a sample (100 ml.) of the orange coloured liquid which settled out of the original minced residue dialysed for two days. The solutions, on lyophilization, gave reddish-pink products, except that from solvent B (see Table 1) which was white.

<u>Solvent</u>	<u>Extract</u> (gm.)	<u>Percentage</u> <u>Nitrogen</u>	<u>Total</u> <u>Nitrogen</u> (mg.)
1) Distilled water	0.750	3.0	22.5
2) 0.25% Na ₂ CO ₃	0.443	3.7	16.4
3) 3% KCl.	0.690	2.1	14.5
4) 20% Ethanol	1.210	1.6	19.3
5) Water saturated with butanol	1.000	1.4	14.0
6) Boric acid buffer pH9.0	0.410	2.3	9.4
7) Phosphate buffer pH8.0	0.474	3.1	14.6
8) Solvent A (i)	0.631	1.0	6.3
9) Solvent B (ii)	1.100	0.9	9.9
10) Expressed fluid	2.134	2.3	49.1

(i) 100 ml. Borax solution (9.5gm./litre)+40 ml. ethanol+10 ml. ether.

(ii) 50 ml. Boric acid buffer pH9.0+50 ml. water+40 ml. ethanol+
10 ml. n-butanol.

TABLE 1.

Extraction and concentration of protein from R. palmata.

First preparation

Fresh weed (2,600 gm., N. Berwick, 21/10/53) was minced with a small amount of ice and extracted with aqueous sodium carbonate (3,600 ml., 0.25%) by stirring for 2 hours and allowing to stand overnight. The minced residue was removed by centrifugation and the extract (4 l.) dialysed for 4 days.

The dialysed extract was re-centrifuged and ammonium sulphate added to 0.8 saturation; the precipitate, which formed after standing overnight at 0°C, was separated and dissolved in water (395 ml.). This solution was dialysed for 2 days and the precipitate, which formed on addition of ammonium sulphate to 0.75 saturation, was separated by centrifugation, dissolved in water (150 ml.) and dialysed for 2 days.

A precipitate (Fraction A) which formed during dialysis, was separated from the supernatant solution (Fraction B); both fractions were then lyophilized.

<u>Fraction</u>	<u>Yield (gm.)</u>	<u>Kjeldahl Nitrogen (%)</u>
A	0.65	6.9
B	1.74	7.4

Total protein extracted = 1.09 gm.

Second preparation

The fresh weed (660 gm., N. Berwick, 10/3/54) was minced with a small amount of ice and milled with silver sand (Ca. 2 parts weed to 1 part sand) and aqueous sodium carbonate (300 ml., 0.25%) for 45 min. The milled weed was stirred with aqueous sodium carbonate (1,100 ml., 0.25%) and allowed to stand overnight. The residues were separated by filtration, disintegrated cells removed by centrifugation, and the supernatant solution dialysed for 4 days.

Insoluble material was removed by centrifugation and ammonium sulphate added to the supernatant solution to 0.4 saturation. The precipitate (Fraction Y), which formed almost

immediately was separated by centrifugation, dissolved in water (400 ml.), dialysed for 2 days and lyophilized. Ammonium sulphate was added to the above supernatant solution to 0.8 saturation and the precipitate (Fraction Z) separated, dissolved in water (250 ml.), dialysed for 2 days and lyophilized.

<u>Fraction</u>	<u>Yield (gm.)</u>	<u>Kjeldahl Nitrogen (%)</u>
Y	5.1	8.0
Z	1.6	3.2

Total protein extracted = 2.90 gm.

Extraction and concentration of protein from C. rupestris.

First preparation

The fresh, hand-pressed weed (2,900 gm., Dunbar 22/1/54) was minced twice with a little ice and aqueous sodium carbonate (0.25%) and extracted with aqueous sodium carbonate (3 l., 0.25%) by stirring for 3 - 4 hours and allowing to stand overnight. Cell debris was removed by centrifugation, the solution dialysed for 3 days and ammonium sulphate added to 0.71 saturation. The precipitate which settled out overnight was separated by centrifugation, as a slurry (1.6 l.), and dialysed for 1 day. The protein was re-precipitated by the addition of ammonium sulphate to 0.70 saturation, separated as a slurry (1 l.) and dialysed free of ammonium sulphate (3 days).

One portion (1,145 ml.) of the extract was lyophilized to give Fraction A (15.8 gm.) and the remainder (2,500 ml.) fractionated with increasing amounts of ammonium sulphate,

the precipitates being separated, dissolved in water, dialysed and lyophilized after each addition as in Table 2.

<u>Fraction</u>	<u>Saturation</u>	<u>Yield (gm.)</u>	<u>Kjeldahl Nitrogen (%)</u>
A	0.0 - 0.70	15.8	8.8
I	0.0 - 0.18	17.7	7.2
II	0.18 - 0.36	5.7	7.1
III	0.36 - 0.54	2.1	8.5
IV	0.54 - 0.75	0.9	8.1
V	0.75 - 0.80	0.1	5.8

Total protein extracted (calculated from fraction A) = 27.7 gm.

TABLE 2.

Second preparation

Fresh, hand-pressed weed (4,000 gm., Dunbar 24/3/55) was extracted with aqueous sodium carbonate (3.5 l., 0.25%) and precipitated twice with ammonium sulphate as in the first preparation. The dialysed extract (9 l.) was divided into two portions.

To one portion (6 l.) was added ammonium sulphate to 0.70 saturation. The precipitate was separated by centrifugation and filtration, suspended in water (1,300 ml.) and dialysed for 3 days. The supernatant solution (Fraction W) and the insoluble material (Fraction X) were separated and lyophilized.

The other portion (3 l.) was cooled to 0°C and absolute ethanol (6 l.), cooled to -20°C, added slowly, the addition being carried out at -10°C. The precipitate which settled out

after standing overnight at 0°C was separated by centrifugation and dialysed. The supernatant solution (Fraction Y) and the insoluble material (Fraction Z) were separated and lyophilized.

<u>Fraction</u>	<u>Yield (gm.)</u>	<u>Kjeldahl Nitrogen (%)</u>
W	35.0	8.3
X	4.5	7.8
Y	8.5	9.0
Z	2.2	8.2

Total protein extracted (calculated from Fractions W and X)
= 30.5 gm.

Evidence as to the protein nature of extract from C. rupestris.

1. Precipitation by 4% trichloroacetic acid

Fraction W (ca. 40 mg.) was suspended in water (8 ml.) and a sample (2 ml.) analysed for Kjeldahl nitrogen. Trichloroacetic acid (0.24 gm.) was added to the remainder and after standing for 24 hours, the nitrogen content of the supernatant solution (2 ml.) was estimated.

Original suspension = 0.74 mg. N./2ml.

Precipitated solution supernatant = 0.16 mg. N./2ml.

Thus 4% trichloroacetic acid, a typical protein precipitant, precipitates at least 78% of the nitrogen content of the extract.

2. Acid hydrolysis

Fraction W (50 mg.) was hydrolysed with hydrochloric acid (3 ml., 6N.) at 100°C for 48 hours. The hydrolysate was filtered and the acid removed by repeated distillation in vacuo. The hydrolysate was

chromatographed using propanol:water (80:20) and developed with ninhydrin. The chromatogram indicated the presence in the hydrolysate of leucine, valine, proline, glutamic acid and several other amino acids.

DISCUSSION

Extraction of the protoplasm liberated by mechanical disintegration of plant cells may be effected by a large number of solvents, and in the first experiments on the extraction of protein from a marine alga, R. palmata, the effect of different solvents on the yield of nitrogen has been studied.

All the solvents used, except solvent B, gave reddish-pink products showing that the red pigment, phycoerythrin, was also extracted. For the different solvents no correlation was observed between the amount of material extracted and the nitrogen content of the extracts. Aqueous sodium carbonate gave a product with the highest nitrogen content and as this was obtained in a reasonably high yield, it was decided to use this solvent in the large scale extractions.

At all stages during the preparation of the extracts every effort was made to keep the temperature as low as possible to minimise denaturation of the protein and as subsequent work showed, all the extracts contained enzymic activity.

In the first extraction of R. palmata the yield of crude protein was low and in the second extraction an attempt was therefore made to achieve greater cellular disintegration. This was obtained by milling the minced weed with silver sand and aqueous sodium carbonate, the relative ratios being such as to give a mixture which would not overheat on milling. The total yield of protein from this extraction, allowing for the weight of weed used, was a tenfold increase on the first

extraction. The removal of inorganic ions from the extract before precipitation with ammonium sulphate was necessary as the presence of these ions retarded the formation of the protein precipitate.

Two large scale extractions of the green alga, C. rupestris, were made using essentially the same method as in the extraction of R. palmata. The weed, which is filamentous in structure, was more readily disintegrated but, as it occasionally caused a blockage of the mincer, double mincing in the presence of the solvent was necessary. The yield of protein from C. rupestris was approximately twice that from R. palmata, the yields being 8.6 gm. and 4.4 gm. protein per 1,000 gm. weed respectively. This is due to the higher protein content of C. rupestris and not to greater cellular disintegration on double mincing, as the extent of cell rupture of R. palmata on mincing followed by milling appeared to be equally high.

Treatment of an extract of C. rupestris with 4% trichloroacetic acid, a typical protein precipitant, indicated that the nitrogenous material extracted was protein; at least 78% of the nitrogen was precipitated. The same extract on hydrolysis with hydrochloric acid gave several free amino acids which could only have originated from protein; any peptides would have been removed by the dialysis during the preparation.

SUMMARY

1. The effect of different solvents on the extraction of nitrogenous material from R. palmata was studied.
2. With R. palmata it was shown that a tenfold greater yield of protein could be obtained if the cells were disintegrated by mincing followed by milling with silver sand.
3. Two separate extractions of C. rupestris were made, a portion of one being fractionated with ammonium sulphate and a portion of the other precipitated with ethanol.
4. The protein identity of a representative extract from C. rupestris was established by precipitation with trichloroacetic acid and acid hydrolysis.

SECTION TWO

Studies on the hydrolytic activity of whole extracts of Rhodymenia
palmata and Cladophora rupestris

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INTRODUCTION

The work reported in this section is concerned mainly with the hydrolases present in extracts of R. palmata and C. rupestris.

The properties, specificity and distribution of the enzymes which catalyse the hydrolytic degradation of carbohydrates have been extensively studied (47). The different types of glycosidases present in biological extracts may be investigated by chromatographic examination of digests containing the extract and carbohydrates of known structure. By this method the glycosidases may be classified according to the monosaccharide unit and the configuration of the linkage attacked, or by the polysaccharide degraded. The mode of action of the polysaccharidases may be deduced in certain cases, as random hydrolysis of the polysaccharide will produce a series of oligosaccharides whereas step-wise hydrolysis will liberate only mono- or di-saccharides. The individual glycosidases may then be further identified by studies on the general properties, inhibition, purification and substrate specificity of the extract.

Many naturally occurring compounds contain phosphate groups which may be removed by enzymic hydrolysis; the enzymes catalysing this reaction belong to the phosphoesterases which are a subclass of the esterases, a class of hydrolases. The phosphoesterases, the properties of which have been reviewed by Roche (48), have a wide distribution in the plant and animal kingdoms. There are four types of group specific phosphomonoesterases, the types being

differentiated by pH optima and by inhibition and activation studies. The two most widely occurring are types I and II; the former exhibits maximum activity over the pH range 8.5 - 9.5 and is strongly activated by divalent cations, especially magnesium, whilst the other shows maximum activity over the pH range 5.0 - 6.0, is inhibited by fluoride and molybdate, which have little effect on type I, and is not activated by divalent cations. Stein and Koshland (49) using an isotope of oxygen (O^{18}) have shown that phosphomonoesterase type I catalyses the rupture of the oxygen-phosphorous bond as shown in Figure 1.

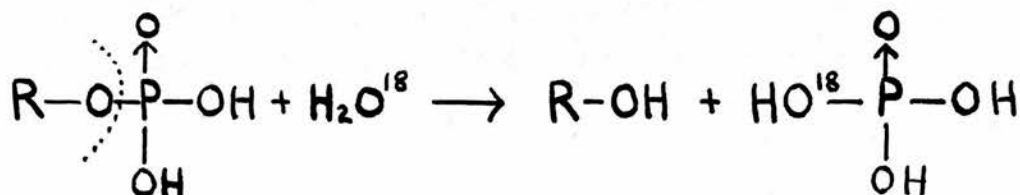


Figure 1.

The hydrolysis of phosphate-phosphate bonds, as in pyrophosphate, is catalysed by enzymes belonging to the class polyphosphatases whose properties have also been reviewed by Roche (48).

EXPERIMENTAL

Methods

Digests. All digests were incubated at 35°C, unless otherwise stated, and sufficient toluene was added to maintain a surface layer.

De-proteinisation (50). Where the amount of protein present in a digest sample was high, the sample was de-proteinised using equivalent quantities of zinc sulphate ($\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$, 5%) and barium hydroxide (0.3N). After the addition of the reagents, the precipitate was separated by centrifugation and the supernatant solution used for reducing sugar estimations.

Estimation of reducing sugars.

1) Iodometric reagents

(A) In this method of Shaffer and Somogyi (51) as modified by Hanes and Cattle (52), the reagent is stored as four separate solutions. Reduction is achieved by heating the copper-tartrate-bicarbonate reagent and the digest sample at 100°C for 15 minutes and the excess reagent is then titrated with sodium thiosulphate (0.01N). Calibration curves were prepared using glucose, xylose and maltose.

(B) The Somogyi reagent (53) containing a phosphate buffer has been used, the potassium iodate being incorporated externally. Calibration curves were prepared using glucose (heated for 10 minutes) and maltose (heated for 20 minutes).

In the estimation of reducing sugars in the presence of the algal extracts a small correction has been applied, for the small but constant reducing power of the extracts. The effect of the different anions used throughout this work on the reducing power of glucose and maltose, as determined by these reagents, was found to be negligible. The two reagents were found to give comparable results. Sodium sulphate, however, precipitated from the more recent reagent unless it was stored at a temperature greater than 20°C; it was therefore advantageous to use the earlier reagent which could be manipulated with equal ease.

2) Colorimetric reagents

(C) The copper reagent prepared by Somogyi (54) was used in conjunction with the chromogenic arsenomolybdate reagent of Nelson (55). The copper reagent (5 ml.) and sample (5 ml.) were heated in a boiling water bath for 10 minutes, cooled and the chromogenic reagent (5 ml.) added. After 10 minutes the solution was diluted to 50 ml. and the colour measured in a 1 cm. cell using filter 603 with the Spekker absorptiometer against a reagent blank. A calibration curve was prepared using glucose.

Paper partition chromatography

Chromatographic identification of mono- and oligo-saccharides and uronides was carried out by spotting the unknown digest, or solution and standards, on Whatman No. 1 paper and irrigating the paper with one of the following irrigants. After a suitable period, the papers were dried at 120°C and treated with one of the following sprays.

Irrigants

With irrigants which formed more than one phase the organic phase was the irrigant and the aqueous layer was placed in a beaker at the bottom of the chromatographic tank.

1. Benzene / n-butanol / pyridine / water - 1 : 5 : 3 : 3, $\frac{V}{V}$. (56).
2. Ethyl acetate / pyridine / water - 10 : 4 : 3, $\frac{V}{V}$. (57).
3. n-Butanol / glacial acetic acid / water - 4 : 1 : 5, $\frac{V}{V}$ (58).
4. Ethyl acetate / glacial acetic acid / formic acid / water - 18 : 3 : 1 : 4, $\frac{V}{V}$.
5. n-Butanol / ethanol / water / ammonia - 40 : 10 : 49 : 1, $\frac{V}{V}$. (59).

With 5, the spot of sugar solution was dried, a spot of benzylamine in methanol (10%, $\frac{W}{V}$) superimposed, and the paper heated at 85°C for 5 minutes before irrigation.

Sprays

1. Aqueous saturated solution of aniline oxalate; the paper was then dried at 130°C until the spots appeared (60).
2. The dried paper was passed through silver nitrate in acetone and after drying sprayed with sodium hydroxide in ethanol, and the excess reagent removed by washing in ammonia and then water (61).
3. Alcoholic solution of naphtharesorcinol (10 ml., 1%) in hydrochloric acid (90 ml., 2N). The paper was developed by drying at 85 - 95°C for 10 minutes (62).

Chromatographic identification of phosphate and phosphate esters was achieved using the method of Hanes and Isherwood (63):-

Irrigant 6. n-Propanol / water / ammonia - 6 : 1 : 3 $\frac{V}{V}$.

Spray 4. Perchloric acid (5ml., 60%, $\frac{W}{W}$), hydrochloric acid

(10 ml., 1.0N) and ammonium molybdate (25 ml., 4%, $\frac{w}{v}$) diluted to 100 ml. with water. The paper was dried and heated at 87°C for 7 minutes and then suspended in an atmosphere of hydrogen sulphide until the characteristic blue spots appeared.

For the separation and identification of amino acids on paper chromatograms the following irrigant and spray was used:-

Irrigant 7. n-Propanol / water - 80 : 20, $\frac{v}{v}$.

Spray 5. Alcoholic solution of ninhydrin (0.2%), the paper being developed by gentle heating. The spots could be preserved indefinitely by spraying with a saturated alcoholic solution of cupric sulphate.

pH measurements. All pH values were checked using a glass-electrode pH meter.

Phosphate and Phosphate ester estimation. The methods of Hanes (64) and Cori (65) were used with the reagent of Fiske and Subbarow (66) which had been slightly modified, sodium metabisulphite (14%) being used in place of sodium bisulphite (15%) and the colour intensity being measured in a 1 cm. cell using filter 608 with the Spekker absorptiometer (67). A calibration curve was prepared using potassium dihydrogen phosphate.

Preparation of substrates

Gentiobiose. This was prepared by the synthetic action of almond emulsion on a concentrated solution of glucose, as described by Peat, Whelan and Hinson (68). Separation of the gentiobiose from the other sugars was effected by the use of a

charcoal-celite column (69).

Isomaltose and isomaltotriose. Dextran (55 gm.) was partially hydrolysed by treatment with sulphuric acid (2,750 ml., 1.0N) at 100°C for 100 minutes. The hydrolysate after neutralisation, filtration and concentration in vacuo was fractionated on a charcoal-celite column (69). The products were obtained chromatographically pure.

Maltose. The maltose used was purified by three re-crystallisations from ethanol (80%) and fractionation on a charcoal-celite column (69).

Phenyl- α -glucoside. This was prepared from pentaacetyl- β -glucose as described by Helferich and Smitz-Hillebrecht (70).

A chromatographically pure product was obtained (m.p. 172 - 173°C).

Phenyl- β -glucoside. This was prepared from pentaacetyl- β -glucose by the method of Nath and Rydon (71). The product (m.p. 173°C) was chromatographically pure.

0.01M Phenolphthalein β -glucuronide. Phenolphthalein β -glucuronide cinchonidine salt (395.4mg.) was suspended in water (16 ml.) and ethyl acetate (10 ml.), and concentrated hydrochloric acid (0.6 ml.) added. The solution was quantitatively transferred to a separating funnel by washing with water (2 x 1.5 ml.) and the phenolphthalein β -glucuronide extracted with ethyl acetate (4 x 5 ml.). The ethyl acetate solution was rapidly concentrated to dryness to prevent hydrolysis by traces of acid, the dried product dissolved in water (5 ml.) and sodium hydroxide (2.5 ml., 0.1N), transferred to a beaker with water (3 x 5 ml.) and the pH adjusted to 5.2. The solution was diluted to 50 ml. with distilled water and stored at 1°C.

R. palmata xylan. This was prepared by extraction of the seaweed residue, from the enzyme extraction, with dilute hydrochloric acid according to the method of Barry and Dillon (6). The product which was reprecipitated several times with alcohol was further purified by precipitation with Felling's solution. Chromatographic analysis of a hydrolysate of the product showed the presence of xylose and a very faint trace of galactose.

Preparation of reference glycosidases

Preparation of emulsin. B.D.H. emulsin (10.5 gm.) was suspended in 0.025 M acetate buffer pH 5.0 (200 ml.) and dialysed, to lower the sugar content, for 4 days. After centrifugation, the supernatant solution was lyophilized to yield a powder (3.0 gm.) containing 11.8% nitrogen.

Preparation of barley extract. Spratt Archer barley flour (100 gm) was extracted with potassium chloride (350 ml., 3%) by stirring for 2 hours. After removal of insoluble material, ammonium sulphate was added to the solution to 0.75 saturation and the precipitate which settled out after standing overnight at 0°C separated by centrifugation. The precipitate was dissolved in water, and dialysed for 4 days. Insoluble material was separated by centrifugation and the supernatant solution lyophilized to yield a soluble powder (1.8 gm.) containing 10.1% nitrogen.

The other substrates used in the course of this work were either commercial products or gifts from other workers. In addition to the members of the Chemistry Department, University of Edinburgh, who supplied many of the samples, the author is

indebted to Dr D.J. Bell for butyl and phenyl β -galactosides and levan, Professor C.S. Hanes, F.R.S. for the cellodextrin, Dr L. Hough for the gluco- and galacto-mannans, Dr B. Lindberg for the pustulan, Dr D.H. Northcote for the yeast glucan and mannan and Professor I.A. Preece, F.R.S.E. for the barley β -glucosan.

Studies on the glycosidase activity of extracts of *R. palmata*
and *C. rupestris*

a) The specificity of glycosidase activity

In order to investigate the hydrolytic activity of the extracts towards various carbohydrates, the following digests were set up and incubated at 35°C - carbohydrate (ca. 30 mg.), 0.1 M acetate buffer pH 5.0 (3 - 4 ml.) and extract fraction (ca. 30 mg.). The digests were examined chromatographically at intervals using irrigants 1 and 2, and sprays 1 and 2. Controls, in which the extract fraction and buffer had been heated to 100°C for 10 - 15 min., were set up, and for each different extract fraction a control, without substrate, was used to test for reducing sugars. No reducing sugars were liberated in any control.

In the following tables the appearance of reducing sugars on the paper chromatograms is indicated in the following key:-

t represents a very weak 'spot',

± represents a weak 'spot',

+ represents a moderate 'spot',

++ represents a strong 'spot',

+++ represents an intense 'spot',

N.E. not examined.

Spots due to the original material are not noted.

The reducing sugars liberated by enzymic hydrolysis were identified by comparison of their chromatographic mobility with standards on the same paper chromatogram. Where appropriate

standards were not available, the position of the unknown sugar on the chromatogram is referred to using one of the following standards:-

$$R_G = \frac{\text{Distance travelled by sugar}}{\text{Distance travelled by glucose under identical conditions}}$$

or

$$R_X = \frac{\text{Distance travelled by sugar}}{\text{Distance travelled by xylose under identical conditions}}$$

or

$$R_{Ma} = \frac{\text{Distance travelled by sugar}}{\text{Distance travelled by mannose under identical conditions}}$$

The values quoted in the Tables refer to irrigant 1.

In the abbreviations for the structures of the carbohydrates Fu., Rh., G., F., Ga., M., X. and R represent fucose, rhamnose, glucose, fructose, galactose, mannose, xylose and non-carbohydrate aglycon respectively. R.p. and C.r. represent R. palmata and C. rupestris respectively.

Action of the algal extracts on α -glucosides

TABLE 1

Substrate & linkage	Algae	Fraction	4 days	7 - 9 days	14 days
Methyl α -D-glucoside	R.p.	B	-	-	-
G.1: α :R	C.r.	A	-	glucose t	glucose t
Phenyl α -D-glucoside	R.p.	Z	glucose+	glucose+	glucose+
G.1: α :R	C.r.	A	glucose++	glucose+++	glucose+++
Isomaltose					
G.1: α :6.G	C.r.	A	glucose++	glucose+++	glucose+++
Isomaltotriose					
G.1: α :6.G $\frac{1}{2}$: α :6.G	C.r.	A	glucose+, isomaltose $\frac{1}{2}$	glucose++, isomaltose $\frac{1}{2}$	glucose+++, isomaltose t
Maltose	R.p.	B	glucose+++	glucose+++	glucose+++
G.1: α :4.G	C.r.	A	glucose+++	glucose+++	glucose+++
Sucrose	R.p.	B	-	glucose+, fructose+	glucose++, fructose++
G.1: $\alpha\beta$:2.F	C.r.	A	glucose $\frac{1}{2}$	glucose+, fructose+	glucose++, fructose++
Trehalose	R.p.	B	glucose+	glucose++	glucose+++
G.1: $\alpha\alpha$:1.G	C.r.	A	glucose $\frac{1}{2}$	glucose+	glucose+
	C.r.*	A	glucose t	glucose t	glucose t

* In this digest the extract fraction and buffer were heated to 65°C for 10 min. before the addition of the substrate.

Action of the algal extracts on β -glucosides

TABLE 2

Substrate & linkage	Algae	Fraction	4 days	7 - 9 days	14 days
Methyl β -D glucoside	R.p.	B	glucose†	glucose +	glucose ++
G.l: β :R	C.r.	A	glucose t	glucose †	glucose +
Phenyl β -D glucoside	R.p.	Z	glucose†	glucose +	glucose ++
G.l: β :R	C.r.	A	glucose ++	glucose ++	N.E.
Aesculin	R.p.	Z	glucose +	glucose +	glucose +
G.l: β :R	C.r.	A	glucose +++	glucose +++	glucose +++
Amygdalin	R.p.	B	glucose †	glucose +	glucose ++
G.l: β :R	C.r.	A	glucose +	glucose ++	glucose +++
Arbutin	R.p.	Z	glucose t	glucose +	glucose ++
G.l: β :R	C.r.	A	glucose +++	glucose +++	glucose +++
Cellobiose	R.p.	B	glucose +	glucose ++	glucose +++
G.l: β :4.G	C.r.	A	glucose +++	glucose +++	glucose +++
Gentiobiose					
G.l: β :6.G	C.r.	A	glucose ++	glucose +++	glucose +++
Helicin	R.p.	Z	glucose t	glucose +	glucose +
G.l: β :R	C.r.	A	glucose +++	glucose +++	glucose +++
Phloridzin	R.p.	Z	-	-	-
G.l: β :R	C.r.	A	glucose t	glucose †	glucose †
Salicin	R.p.	B	glucose †	glucose ++	glucose ++
G.l: β :R	C.r.	A	glucose +	glucose ++	glucose ++

Action of the algal extracts on other glycosides and oligo-saccharides

TABLE 3

Substrate & linkage	Algae	Fraction	4 days	7 - 9 days	14 days
Butyl β -D galactoside	R.p.	Z	-	-	-
Ga.1: β :R	C.r.	A	-	-	-
Lactose	R.p.	B	galactose [†]	glucose ⁺ , galactose ⁺	glucose ⁺⁺ , galactose ⁺⁺
Ga.1: β :4.G	C.r.	A	glucose [†]	glucose ⁺ , galactose ⁺	glucose ⁺⁺ , galactose ⁺⁺
Phenyl β -D galactoside	R.p.	Z	-	-	-
Ga.1: β :R	C.r.	A	-	-	-
Methyl α -D galactoside	R.p.	B	-	-	-
Ga.1: α :R	C.r.	A	-	-	-
Melibiose	R.p.	Z	-	glucose [†]	glucose [†] , galactose [†]
Ga.1: α :6.G	C.r.	A	-	glucose [†] , galactose [†]	glucose [†] , galactose [†]
Raffinose	R.p.	B	-	-	-
Ga.1: α :6.G1: α : β :2.F	C.r.	A	-	-	galactose [†]
Hesperidin	R.p.	Z	-	-	-
Rh.1: β :R	C.r.	A	-	-	-
Quercitrin	R.p.	Z	-	-	-
Rh.1: β :R	C.r.	A	-	-	-
Rutin	R.p.	Z	-	-	-
Rh.1: β :6.G	C.r.	A	-	-	-
Methyl α -D mannoside	R.p.	B	-	-	-
M.1: α :R	C.r.	A	-	-	-
Xylobiose	C.r.	A	xylose ⁺	xylose ⁺⁺	xylose ⁺⁺⁺
X.1: β :4.X	C.r.*	A	xylose ⁺	xylose ⁺⁺	xylose ⁺⁺⁺
Xylotriose	R.p.	B	xylose ⁺ , xylobiose ⁺	xylose ⁺ , xylobiose ⁺	xylose [†] , xylobiose [†]
X.1: β :4.X.1: β :4.X					

* In this digest the extract fraction and buffer were heated to 60°C for 10 min. before addition of the substrate.

Action of the algal extracts on polysaccharides

Substrate & repeating unit	Algae	Reaction	4 days	7 - 9 days	14 days
Floridean Starch	R.p.	Z	glucose ++	glucose ++	glucose +++
-G.1: α :4.G-	C.r.	A	glucose ++	glucose ++	glucose +++
Glycogen	R.p.	Z	glucose ++	glucose +++	glucose +++
-G.1: α :4.G-	C.r.	A	glucose ++	glucose +++	glucose +++
Starch	R.p.	Z	glucose +++	glucose +++	glucose +++
-G.1: α :4.G-	C.r.	A	glucose +++	glucose +++	glucose +++
Barley- β -glucosan	R.p.	Z	-	glucose t	glucose $\pm$
-G.1: β :3.G- & -G.1: β :4.G-	C.r.	A	glucose +	glucose ++	glucose ++
Cellodextrin	R.p.	B	glucose +, cellobiose ++	glucose ++, cellobiose +	glucose ++, cellobiose $\pm$
-G.1: β :4.G-	C.r.	A	glucose +++	glucose +++	glucose +++
Laminarin	R.p.	B	glucose +++	glucose +++	glucose +++
-G.1: β :3.G-	C.r.	A	glucose +++	glucose +++	glucose +++
Lichenin	R.p.	Z	glucose t	glucose t	glucose $\pm$
-G.1: β :3.G- & -G.1: β :4.G-	C.r.	A	glucose +	glucose ++	glucose ++
Pustulan	R.p.	Z	-	-	-
-G.1: β :6.G-	C.r.	A	-	-	-
Yeast glucan	R.p.	Z	glucose t	glucose $\pm$	glucose +
-G.1: β :3.G-	C.r.	A	glucose +	glucose + $R_G 0.34t$	glucose ++ $R_G 0.36t$
Ivory nut Mannan	R.p.	B	-	$R_{Ma} 0.05t$	$R_{Ma} 0.05 \pm$
-M.1: β :4.M-	C.r.	A	mannose $\pm$	mannose +, $R_{Ma} 0.42 \pm$, $R_{Ma} 0.11 \pm$	mannose ++, $R_{Ma} 0.41 \pm$, $R_{Ma} 0.10 \pm$
	C.r. [†]	A	mannose +, $R_{Ma} 0.26$	mannose ++, $R_{Ma} 0.28$, 0.11	mannose +++, $R_{Ma} 0.31$, 0.11

TABLE 4

(TABLE 4 contd.)

Substrate & repeating unit	Algae	F e c u l o n	4 days	7 -9days	14 days
Yeast Mannan	R.p.	Z	-	-	-
M.1: α :?M-	C.r.	A	-	-	-
Fucoidin	R.p.	B	-	-	-
-Fu.1: α :2.Fu-	Cr.	A	-	-	-
Inulin	R.p.	B	-	-	-
F.1: β :2.F-	C.r.	A	-	-	-
Levan	R.p.	Z	-	-	-
-F.2: β :6.F-	C.r.	A	-	-	-
Esparto Xylan	R.p.	B	-	xylose t	xylose +
-X.1: β :4.X-	C.r.	A	xylose ⁺ , spots R _x 0.55, 0.39,0.16 0.05	xylose ⁺ , spots R _x 0.56, 0.40, 0.16, 0.06	xylose ⁺⁺ , spots R _x 0.56, 0.37,0.17, 0.05
Rhodymenia Xylan	R.p.	B	-	-	xylose t
-X.1: β :3.X- & -X.1: β :4.X-	Cr.	A	xylose ⁺ , spots R _x 0.58, 0.40,0.17, 0.06	xylose ⁺ , spots R _x 0.56, 0.39 0.16, 0.06	xylose ⁺⁺ , spots R _x 0.57, 0.39, 0.17, 0.06
Galactan					
-Ga.1: :?Ga.- Arabinose	C.r.	A	-	-	-
Glucomannan ex. <u>Lilium candidum</u>	C.r.*	A	mannose ⁺ glucose ⁺	mannose + glucose +	mannose + glucose +
Glucomannan ex. <u>Lilium umbellatum</u>	C.r.*	A	mannose + glucose +	mannose ++ glucose ++	mannose ++ glucose ++
Carob gum	R.p.	Z	-	-	R _{Ma} 0.38t
-M.1: β :4.M-	C.r.	A	mannose ⁺ , galactose t R _{Ma} 0.31t	mannose ⁺ , galactose ⁺ R _{Ma} 0.46 ⁺ , 0.12 ⁺	mannose ⁺ , galactose ⁺ , R _{Ma} 0.40 ⁺ , 0.07 ⁺

Substrate & repeating unit	Algae	Fraction	4 days	7-9 days	14 days
Galactomannan ex.					
<u>Crotolaria intermedia</u>	C.r.*	A	galactose [†]	galactose [†] mannose [†]	mannose [†]
Galactomannan ex.					
<u>Caesalpinia spinosa</u>	C.r.*	A	-	mannose [†]	mannose [†]
Galactomannan ex.					
<u>Cassia leptolarpa</u>	C.r.*	A	-	-	mannose [†]
Galactomannan ex.					
<u>Desmanthus illinoensis</u>	C.r.*	A	-	-	-
Galactomannan ex.					
<u>Gymnocladus dioica</u>	C.r.*	A	-	-	-
Galactomannan ex.					
<u>Delonia regia</u>	C.r.*	A	mannose [†]	mannose [†]	mannose [†]
Galactomannan ex.					
<u>Lucerne seed</u>	C.r.*	A	-	-	-
Galactomannan ex.					
<u>Honey locust bean</u>	C.r.*	A	R _{Ma} 0.16 [†]	mannose [†] R _{Ma} 0.15 [†]	mannose [†] R _{Ma} 0.13 [†]

† Substrate dissolved in sodium hydroxide (1.3 ml., 1.0 N), neutralised with hydrochloric acid and water added to 3 ml., then 0.2 M acetate buffer pH 5.0 (1 ml.) and fraction added.

* Substrate dissolved in sodium hydroxide (0.5 ml., 2 N), neutralised with acetic acid (0.5 ml., 2 N) and 0.2 M acetate buffer pH 5.0 (1 ml.) and water (2 ml.) added before addition of fraction.

b) Studies on the α -glucosidase activity.

For convenience, hydrolytic activity towards maltose will be referred to as maltase activity.

Qualitative determination of the solubility of the maltase

A digest was set up consisting of maltose (10 mg.), C.rupestris fraction A (10 mg.) and 0.04 M acetate buffer pH 5.1 (25 ml.). An aqueous extract of fraction A was prepared by suspending 50 mg. in water (25 ml.); after frequent stirring, it was allowed to stand overnight at 0°C, the insoluble material being separated by centrifugation and the clear, colourless supernatant solution used in the following digest, which contained maltose (10 mg.), soluble extract (5 ml.) and 0.05 M acetate buffer pH 5.1 (20 ml.).

The reducing powers of samples (5 ml.) of both digests were estimated at intervals using reagent B. The results are quoted as percentage hydrolysis and were calculated from a curve based on the reducing powers of maltose and glucose.

Time (days)	0	1	3	13
Whole fraction A	-	34.0	47.0	71.0
Aqueous extract	-	0.1	21.0	47.0
		37.9	26	24

An appreciable amount of the maltase is therefore insoluble in water.

Degradation of maltose

To determine whether phosphorolysis was the mode of degradation of maltose, the following digests were set up and incubated at 35°C.

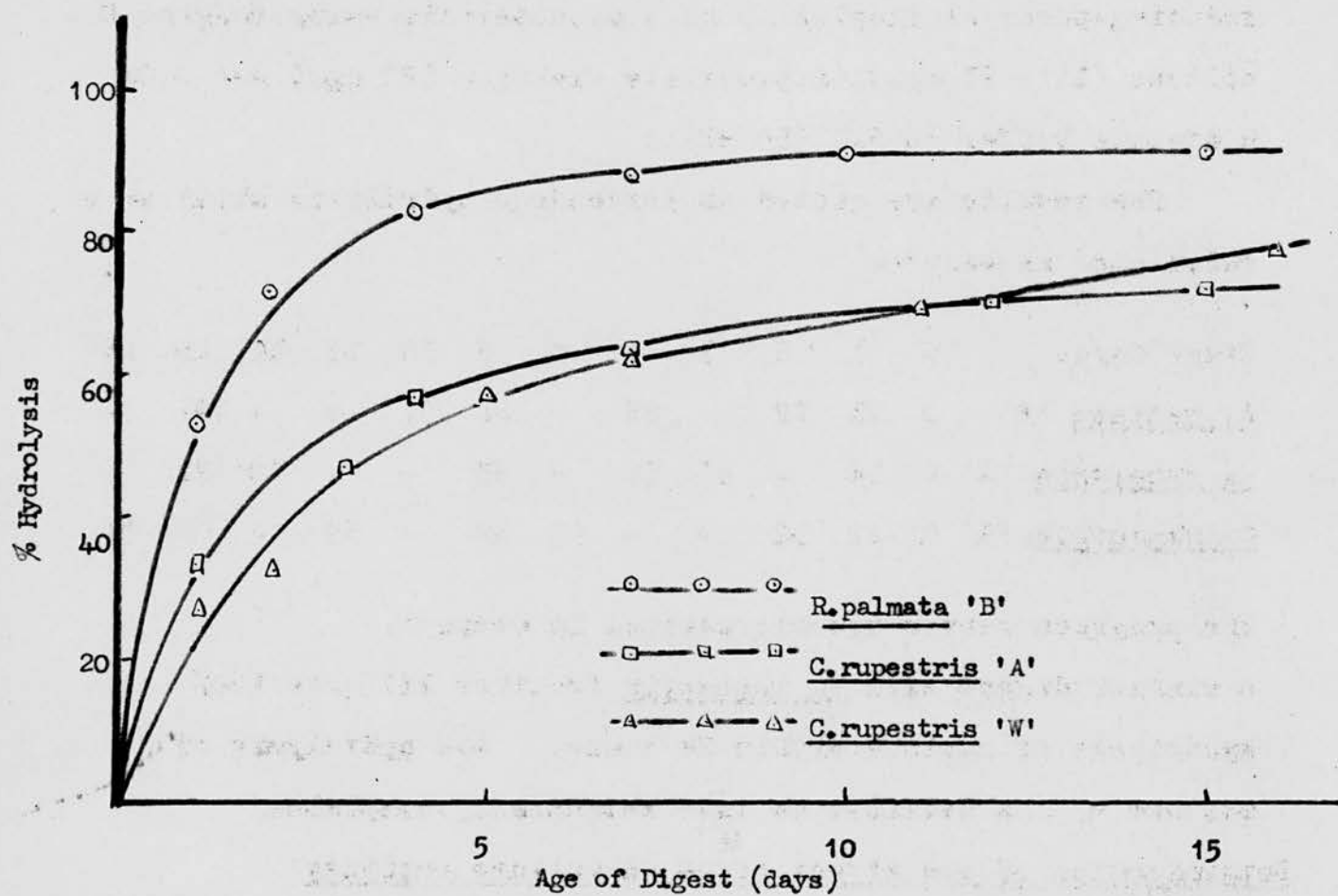
- i) Maltose (70 mg.), 0.5 M phosphate buffer pH 6.6 (4 ml.), water (10 ml.), sodium fluoride (2 ml., 0.1 M), ammonium molybdate (4 ml., 1%) and C. rupestris fraction A (95 mg.).
 - ii) Maltose (30 mg.), 0.5 M phosphate buffer pH 5.3 (1 ml.), water (1.6 ml.), sodium fluoride (0.4 ml., 0.1 M), ammonium molybdate (1 ml., 1%) and R. palmata fraction B (25 mg.).
- Sodium fluoride is an inhibitor for phosphoglucose mutase, and ammonium molybdate an inhibitor for glucose-1-phosphatase. Samples (2 ml.) of digest i) were analysed at intervals for glucose-1- and 6- phosphates using Fiske and Subbarow's reagent whilst digest ii) was examined qualitatively for phosphate esters using irrigant 6, and spray 4.

In digest i) there was no decrease in the amount of inorganic phosphate over 7 days nor was any glucose-1^{or} 6-phosphate detected. Only inorganic phosphate was observed on the chromatograms of digest ii).

Effect of dialysis on the maltase activity

Digests were set up using i) a suspension of C. rupestris fraction A (0.3%) which had been dialysed for 2 days and ii) fraction A at the same concentration as follows:- maltose (3 ml., 0.2%), 0.1 M acetate buffer pH 5.6 (6 ml.) and extract (3 ml.). Samples (5 ml.) were used to estimate the increase in reducing power using method A. Both digests gave identical values.

Graph 1 - Hydrolysis of maltose



The hydrolysis of maltose by extracts of *R. palmata* and *C. rupestris*

Progress curves for the hydrolysis of maltose by *R. palmata* fraction B and *C. rupestris* fractions A and W were obtained by preparing the following digests, and determining the increase in reducing power of samples (5 ml.) at intervals using reagent B - maltose (19 - 21 mg.), appropriate fraction (20 mg.) and 0.04 M acetate buffer pH 5.1 (50 ml.).

The results are quoted as percentage hydrolysis which were calculated as before.

Time (days)	0	1	2	3	4	5	7	10	11	12	15	16
<u><i>R. palmata</i></u> 'B'	0	53	72	-	83	-	88	91	-	-	91	-
<u><i>C. rupestris</i></u> 'A'	0	34	-	47	57	-	63	-	-	70	72	-
<u><i>C. rupestris</i></u> 'W'	0	27	32	-	-	57	62	-	69	-	-	77

The progress curves are represented in Graph 1.

A similar digest with *C. rupestris* fraction III gave 100% hydrolysis of maltose within 24 hours. The hydrolysis of maltose by the extracts is thus essentially complete.

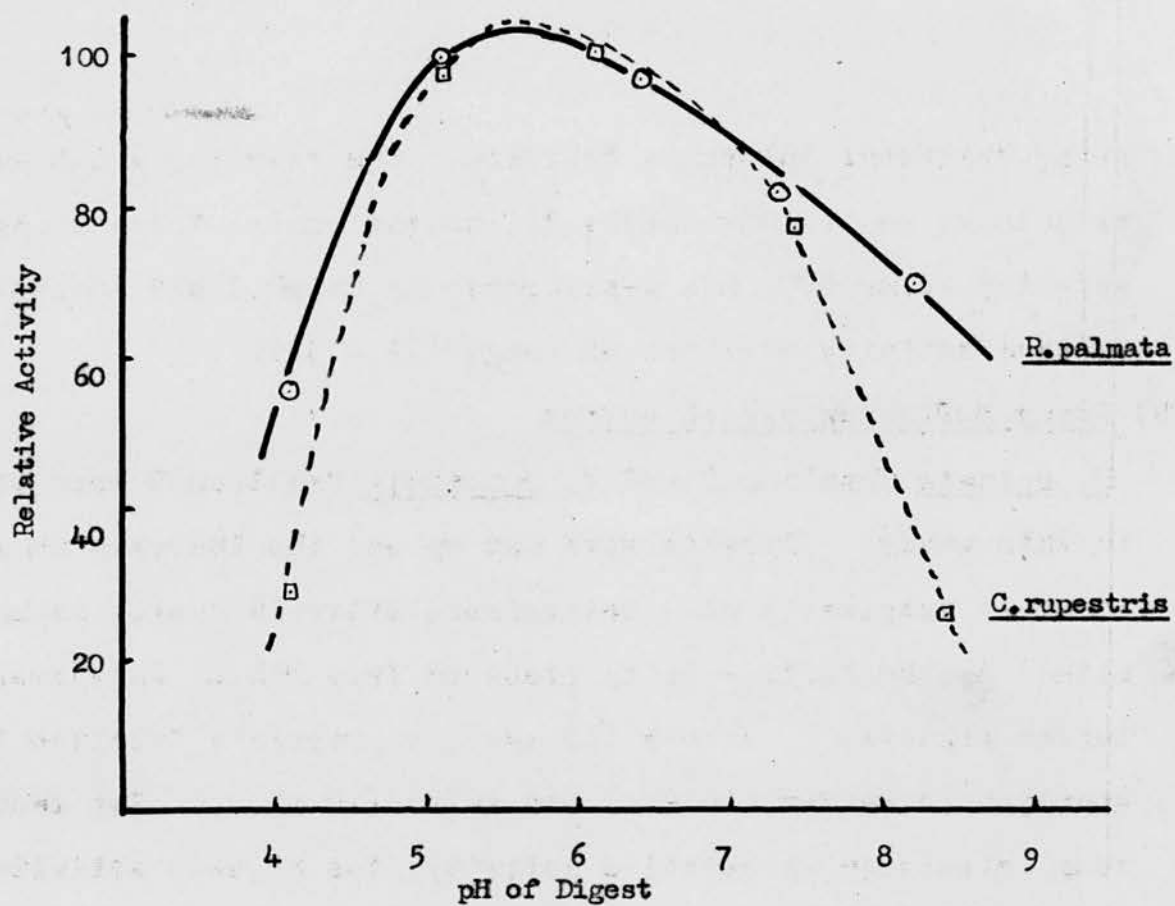
Determination of the effect of pH on maltase activity

a) Using acetate and phosphate buffers

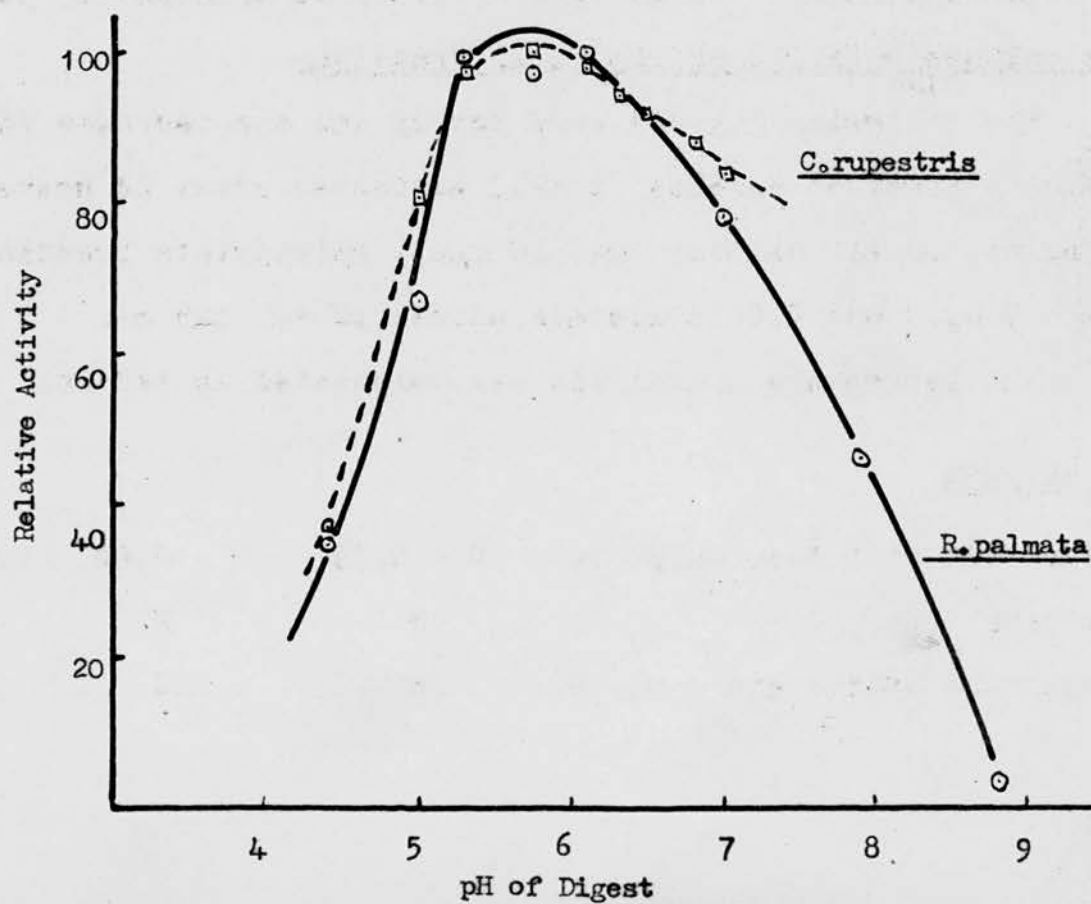
R. palmata fraction B and *C. rupestris* fraction A were used used in this study.

Digests were set up as follows and the increase in reducing power of samples (5ml.) determined, after 48 hours, using reagent B - maltose (10 mg.) appropriate fraction (10 mg.), appropriate buffer (4 ml.) and water (16 ml.). The pH values 4.1 - 5.1 were obtained using 0.2 M acetate buffer and the others

Graph 2 - Acetate and Phosphate Buffers



Graph 3 - B.D.H. Universal Buffer



using McIlvaine phosphate buffers. The results, which were calculated as relative activity, on the basis of the highest activity being 100, are represented in Graph 2 and indicate maximum activity over the pH range 5.4 - 5.6.

b) Using B.D.H. Universal buffer

R. palmata fraction Z and C. rupestris fraction W were used in this study. Digests were set up and the increase in reducing power of samples (5 ml.) determined, after 46 hours, using method A, the buffers being prepared from B.D.H. Universal buffer mixture.- maltose (10 mg.), appropriate fraction (12 mg.), appropriate buffer (10 ml.) and water (10 ml.). The results were calculated as relative activity, the highest activity being 100, and are represented in Graph 3.

In both algae, the maltase activity is greatest at pH 5.8.

The maltase activity of the algal fractions

The following digests were set up and the increase in reducing power of samples (5 ml.) estimated after 24 hours using method A; maltose (ca. 10 mg.), appropriate fraction (ca. 10 mg.) and 0.04 M acetate buffer pH 5.1 (25 ml.).

The percentage hydrolysis was calculated as before.

R. palmata

Saturation with Amm. sulphate	0 - 0.75	0 - 0.40	0.40 - 0.80
Fraction	B	Y	Z
Percentage hydrolysis / mg. N.	76	10	59

C. rupestris

1st extraction

Saturation with Amm. sulphate	0-0.75	0-0.18	0.18-0.36	0.36-0.54	0.54-0.75	0.75-0.80
Fraction	A	I	II	III	IV	V
Percentage hydrolysis / mg. N.	19	11	24	59	76	65

2nd extraction

Fraction	W	X	Y	Z
Percentage hydrolysis / mg. N.	16	4	1	2

c) Studies on the specificity of β -glucosidase activity

The hydrolytic activity towards laminarin will be referred to as laminarinase.

Qualitative determination of the solubility of the laminarinase

A digest was set up consisting of laminarin (12 mg.), C. rupestris fraction A (10 mg.) and 0.04 M acetate buffer pH 5.1 (25 ml.). A second digest was set up using an aqueous extract of fraction A prepared as before (p.40) - laminarin (12 mg.), soluble extract (5 ml.) and 0.05 M acetate buffer pH 5.1 (20 ml.). The reducing powers of samples (5 ml.) of both digests were estimated at intervals using reagent B. The results are quoted as percentage hydrolysis to glucose.

Time (days)	0	1	2	3
Whole fraction A	-	49	68	75
Aqueous extract	-	20	38	57

Approximately one-half of the laminarinase is insoluble in water.

Degradation of laminarin

To determine whether phosphorolysis was the mode of degradation of laminarin the following digests were set up and samples (2 ml.) analysed at intervals for glucose-1- and 6-phosphates using Fiske and Subbarow's reagent. Controls using fraction A and buffer heated to 100°C for 10 minutes were also examined.

	(i)	(ii)
Laminarin	100 mg.	70 mg.
<u>C. rupestris</u> fraction A	100 mg.	95 mg.
0.5 M phosphate buffer pH 6.6	4.0 ml.	4.0 ml.
Distilled water	11.8 ml.	10.0 ml.
Ammonium molybdate (1%)	4.0 ml.	4.0 ml.
Mercuric chloride (0.0014 M)	0.2 ml.	-
Sodium fluoride (0.1 M)	-	2.0 ml.

Ammonium molybdate is an inhibitor of glucose 1- phosphatase, mercuric chloride and sodium fluoride of phosphoglucose mutase.

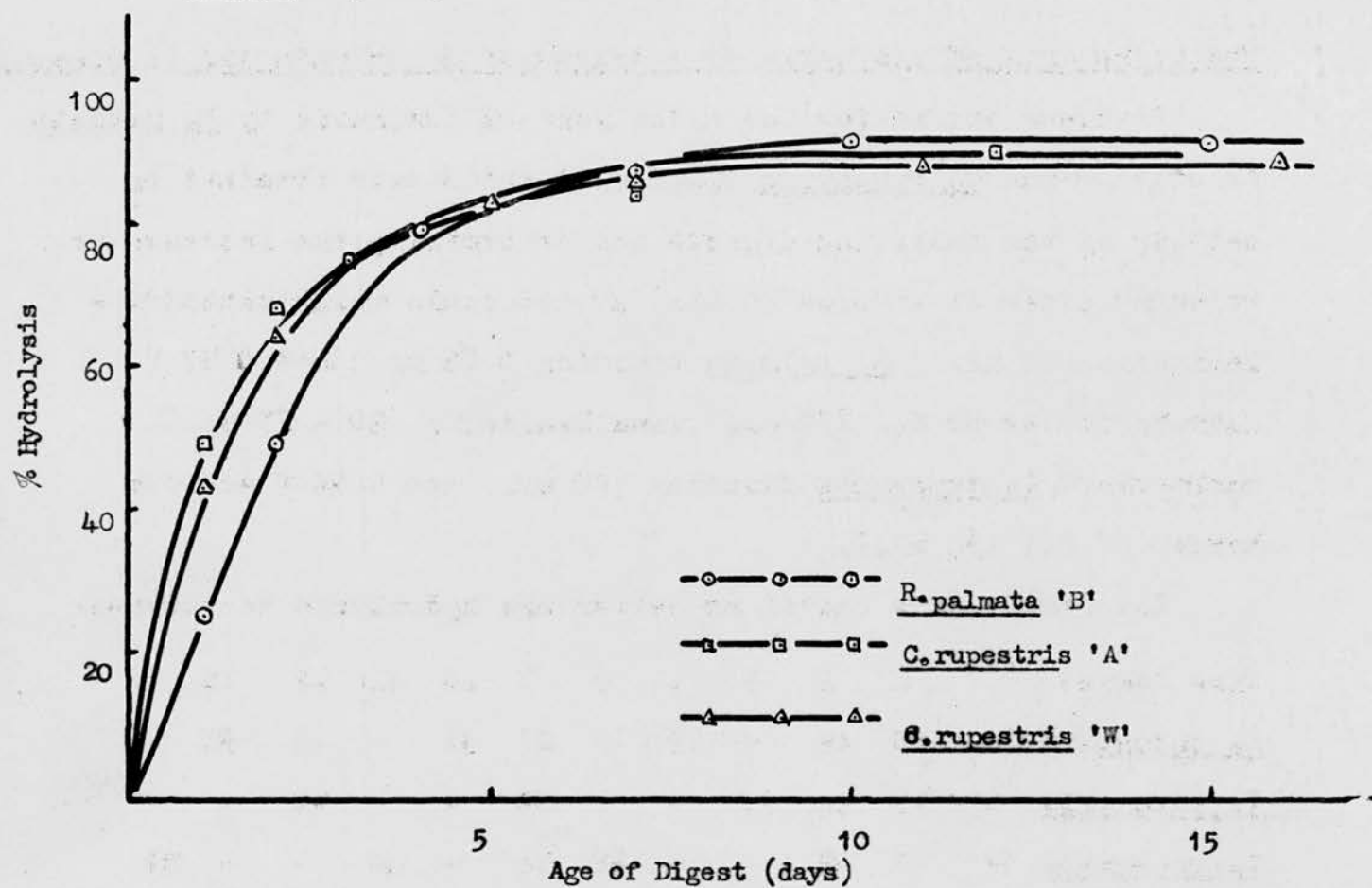
In neither case was any glucose-1- or 6- phosphate detected, indicating that phosphorolysis was not the mode of degradation.

Effect of dialysis on the β -glucosidase activity

Digests were set up as follows using i) a suspension of C. rupestris fraction A (0.3%) which had been dialysed for 2 days and ii) fraction A at the same concentration:-

- a) Laminarin (3 ml., 0.2%), 0.1 M acetate buffer pH 5.6 (6 ml.) and extract (3 ml.).
- b) Salicin (3 ml., 0.5%), 0.2 M acetate buffer pH 5.6 (3 ml.) and extract (6 ml.).

Graph 4 - Hydrolysis of laminarin



The reducing powers of samples (5 ml.) were estimated at zero time and after 24 hours using method A. The increases in reducing power were the same with the dialysed and undialysed extracts.

The hydrolysis of laminarin by extracts of *R. palmata* and *C. rupestris*

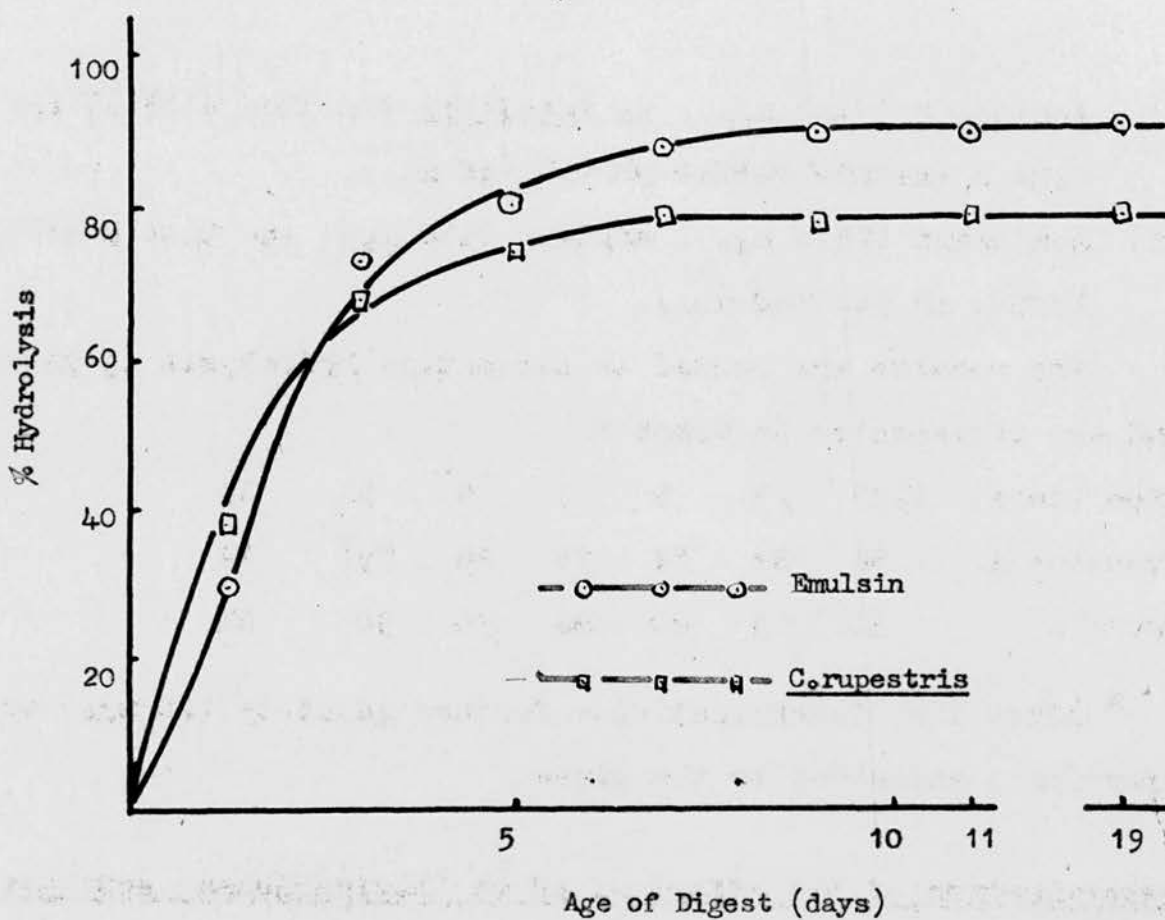
Progress curves for the hydrolysis of laminarin by *R. palmata* fraction B and *C. rupestris* fractions A and W were obtained by setting up the following digests and determining the increase in reducing power of samples (5 ml.) at intervals using method 'A' - laminarin (10 mg.), *R. palmata* fraction B (5 mg.) and 0.02 M acetate buffer pH 5.1 (50 ml.), and laminarin (20 - 23 mg.), appropriate *C. rupestris* fraction (20 mg.) and 0.04 M acetate buffer pH 5.1 (50 ml.).

The results are quoted as percentage hydrolysis to glucose.

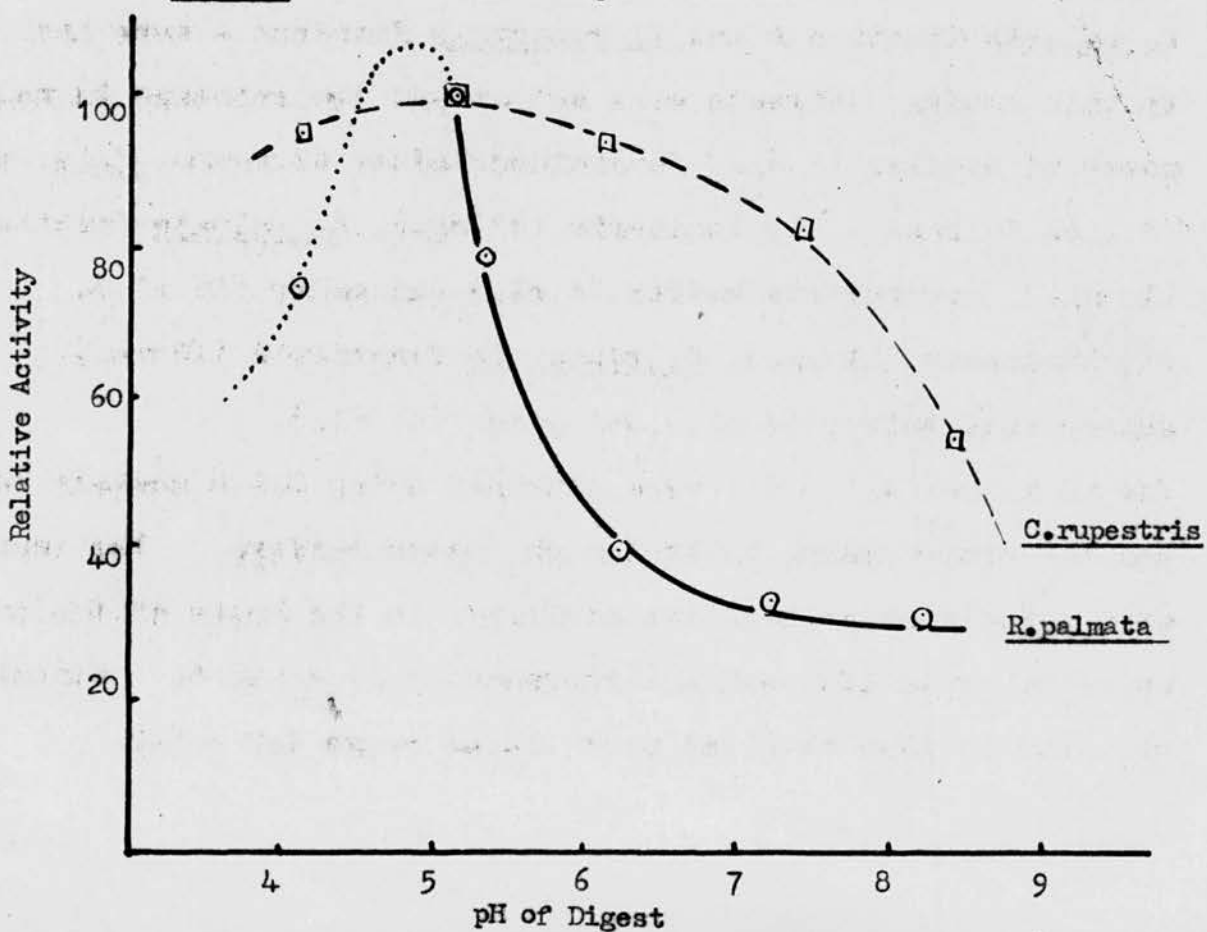
Time (days)	1	2	3	4	5	7	10	11	12	15	16
<u><i>R. palmata</i></u> 'B'	25	49	-	79	-	87	91	-	-	91	-
<u><i>C. rupestris</i></u> 'A'	49	68	75	-	-	84	-	-	90	-	-
<u><i>C. rupestris</i></u> 'W'	43	64	-	-	83	86	-	88	-	-	89

The progress curves are represented in Graph 4 and indicate almost complete hydrolysis of laminarin. A sample of laminarin, kindly supplied by Dr W.J. Whelan, was used in the following digests, samples (5 ml.) of which were withdrawn at intervals, boiled and de-proteinised using barium hydroxide (1.0 ml., 0.3 N) and zinc sulphate (1.0 ml., 5.0%). The reducing power of the supernatant solution (5 ml.) was estimated using method 'A'.

Graph 5 - Hydrolysis of laminarin



Graph 6 - Acetate and Phosphate Buffers



- i) Laminarin (31.5 mg.), C. rupestris fraction A (30.0 mg.) and 0.04 M acetate buffer pH 5.1 (50 ml.).
- ii) Laminarin (28.0 mg.), emulsin (150 mg.) and 0.04 M acetate buffer pH 5.1 (50 ml.).

The results are quoted as percentage hydrolysis to glucose and are represented in Graph 5.

Time (days)	1.25	3	5	7	9	11	19
Fraction A	38	68	74	79	78	79*	79
Emulsin	30	73	80	88	90	90	91

* After this determination a further quantity (10 mg.) of fraction A was added to the digest.

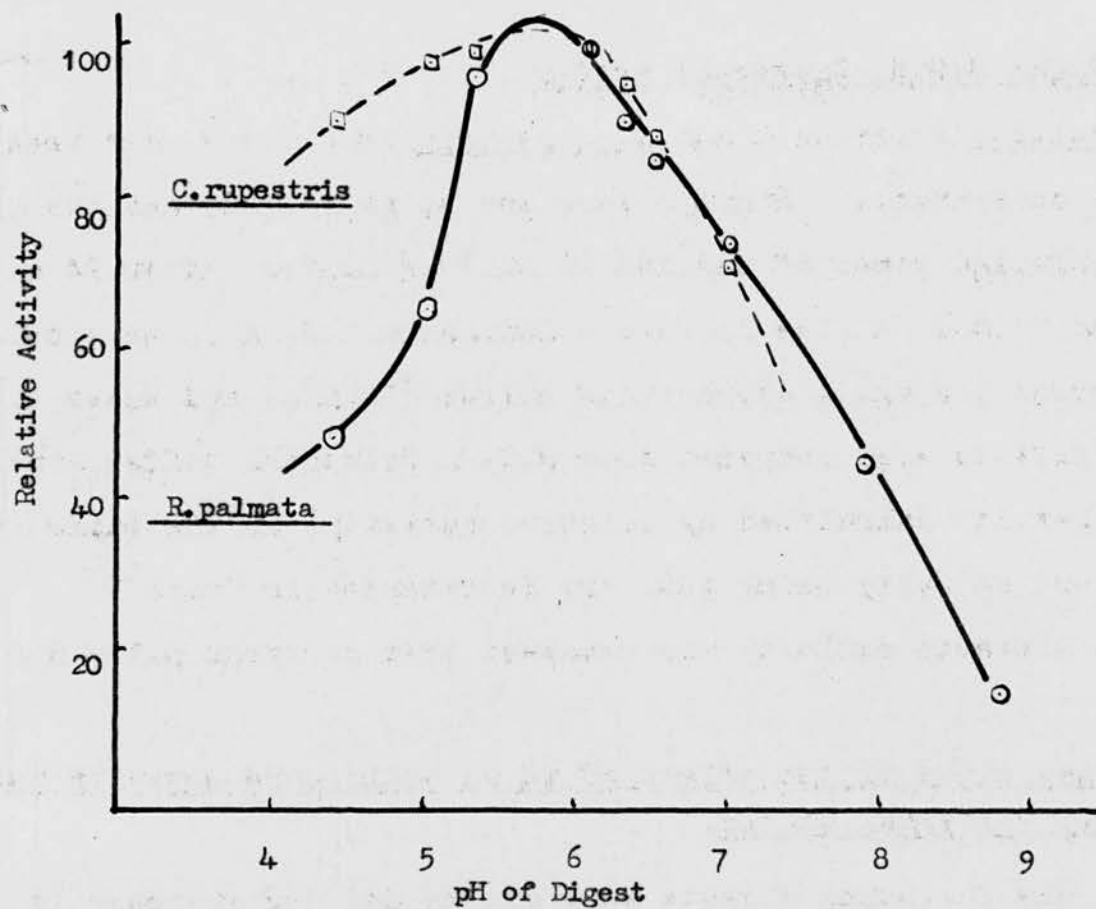
Determination of the effect of pH on 'laminarinase' activity

a) Using acetate and phosphate buffers

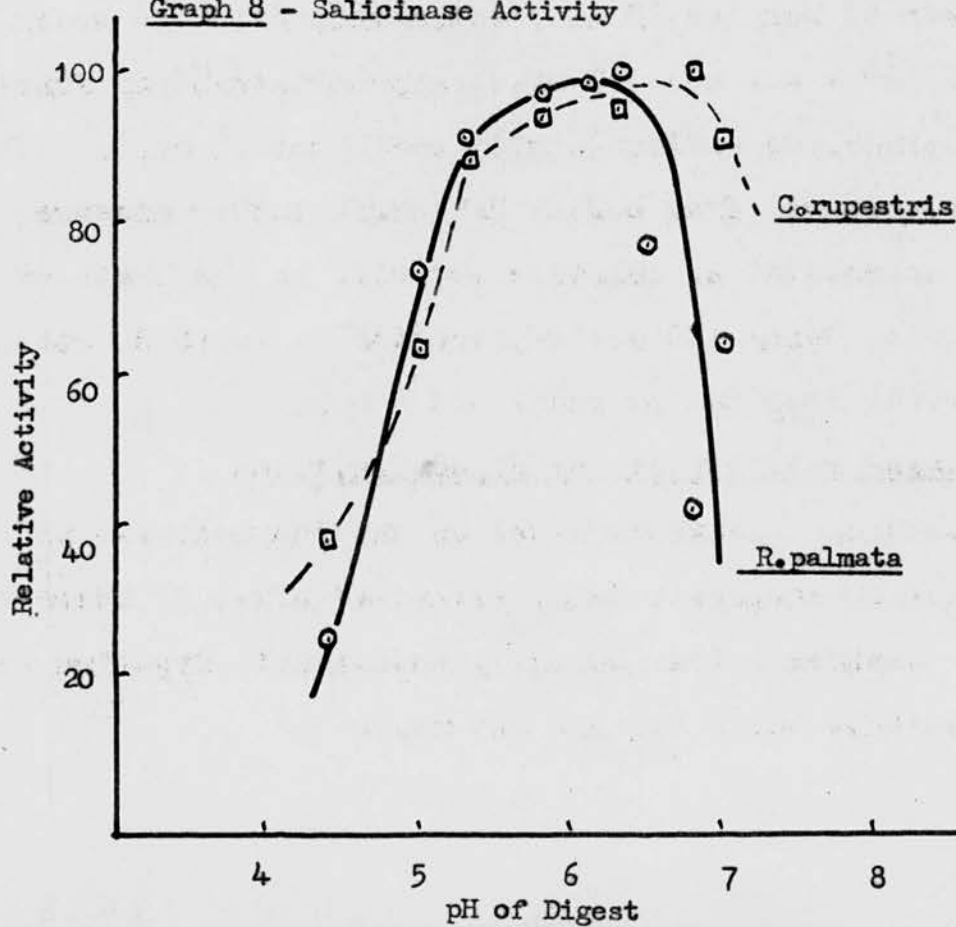
R. palmata fraction B and C. rupestris fraction A were used in this study. Digests were set up and the increase in reducing power of samples (5 ml.) determined, after 48 hours, using method 'A', as follows - i) laminarin (22 mg.), R. palmata fraction B (15 mg.), appropriate buffer (4 ml.) and water (16 ml.), ii) laminarin (11 mg.), C. rupestris fraction A (12 mg.), appropriate buffer (4 ml.) and water (16 ml.).

The pH values 4.1 - 5.1 were obtained using 0.2 M acetate buffer and the others using McIlvaine phosphate buffers. The results were calculated as relative activity, on the basis of the highest activity being 100, and are represented in Graph 6. Maximum activity is thus obtained over the pH range 4.8 - 5.2.

Graph 7 - B.D.H. Universal Buffer



Graph 8 - Salicinase Activity



b) Using B.D.H. Universal buffer

R. palmata fraction Z and C. rupestris fraction W were used in this experiment. Digests were set up as follows and the increase in reducing power of samples (5 ml.) estimated, after 24 hours, using method 'A', as follows - laminarin (10 mg.), appropriate fraction (12 mg.), appropriate buffer (10 ml.) and water (10 ml.). The buffers were prepared from B.D.H. Universal buffer mixture. The results, calculated as relative activity, on the basis of the highest activity being 100, are represented in Graph 7. In both extracts activity was greatest over pH range 5.7 - 5.8.

Determination of the effect of pH on salicinase activity using B.D.H. Universal buffer.

The following digests were set up and the increase in reducing power of samples (5 ml.) estimated, after 24 hours, using method 'A' - salicin (15 mg.), appropriate algal fraction (30 mg.), appropriate buffer (5 ml.) and water (7 ml.). The buffers were prepared from B.D.H. Universal buffer mixture. The results, calculated as relative activity on the basis of the highest activity being 100, are represented in Graph 8, and indicate maximum activity over the pH range 6.3 - 6.8.

The laminarinase activity of the algal fractions

The following digests were set up and the increase in reducing power of samples (5 ml.) estimated after 24 hours using method 'A' - laminarin (ca. 10 mg.), appropriate fraction (ca. 10 mg.) and 0.04 M acetate buffer pH 5.0 (25 ml.).

R. palmata

Fraction	B	Y	Z
Percentage hydrolysis / mg. N.	68	6	20

C. rupestris

1st extraction

Fraction	A	I	II	III	IV	V
Percentage hydrolysis / mg. N.	24	15	77	44	38	30

2nd extraction

Fraction	W	X	Y	Z
Percentage hydrolysis / mg. N.	26	5	5	4

Comparison of the laminarinase activity with that of a barley extract

The laminarinase activity of C. rupestris fraction A was compared with that of a laminarinase preparation from barley (p.³⁰). Digests were set up as follows and samples (6 ml.) withdrawn at intervals, heated to 100°C, filtered and the reducing power of the filtrate estimated using method 'A'.

	(i)	(ii)
Laminarin	16 mg.	16 mg.
0.15 M acetate buffer pH 5.1	35 ml.	35 ml.
Fraction A	20 mg.	-
Barley preparation	-	5 mg.

Time (days)	<u>Results</u>	
1 mg. glucose liberated / mg. N.	0.80	3.50
2 mg. glucose liberated / mg. N.	1.13	4.36
3 mg. glucose liberated / mg. N.	1.38	-
4 mg. glucose liberated / mg. N.	-	4.61

The barley preparation had no reducing power. The laminarinase activity is therefore ca. 0.25 that of the barley preparation.

The salicinase activity of *C. rupestris* fraction A

The β -glucosidase activity of the extract was measured by comparing its action on salicin with that of an emulsin preparation (p.30). Digests were set up as follows and samples (5 ml.) analysed for glucose using the colorimetric method of Somogyi and Nelson.

	(i)	(ii)	(iii)
Salicin	32 mg.	32 mg.	32 mg.
Fraction A	32 mg.	-	-
Emulsin	-	32 μ gm.	16 μ gm.
0.1 M acetate buffer pH 5.0	20 ml.	20 ml.	20 ml.
% hydrolysis after 22 hours	3.1	4.5	1.5
% hydrolysis after 46 hours	5.8	8.1	3.0

Control digests of salicin and buffer, and extract/emulsin in water were also set up.

The salicinase activity of the extract is therefore ca. 6×10^{-4} that of the emulsin preparation.

The presence of more than one β -glucosidase in *C. rupestris*

Digests were set up as follows;- carbohydrate (200 mg.), *C. rupestris* fraction A (15 mg.), McIlvaine phosphate buffer of various pH values (2 ml.) and water (2 ml.). With digests (5) and (10) the buffer, water and fraction A were heated at 60°C for 15 minutes before addition of carbohydrate. The digests were examined chromatographically at intervals using irrigant 1 and spray 2.

Laminarin

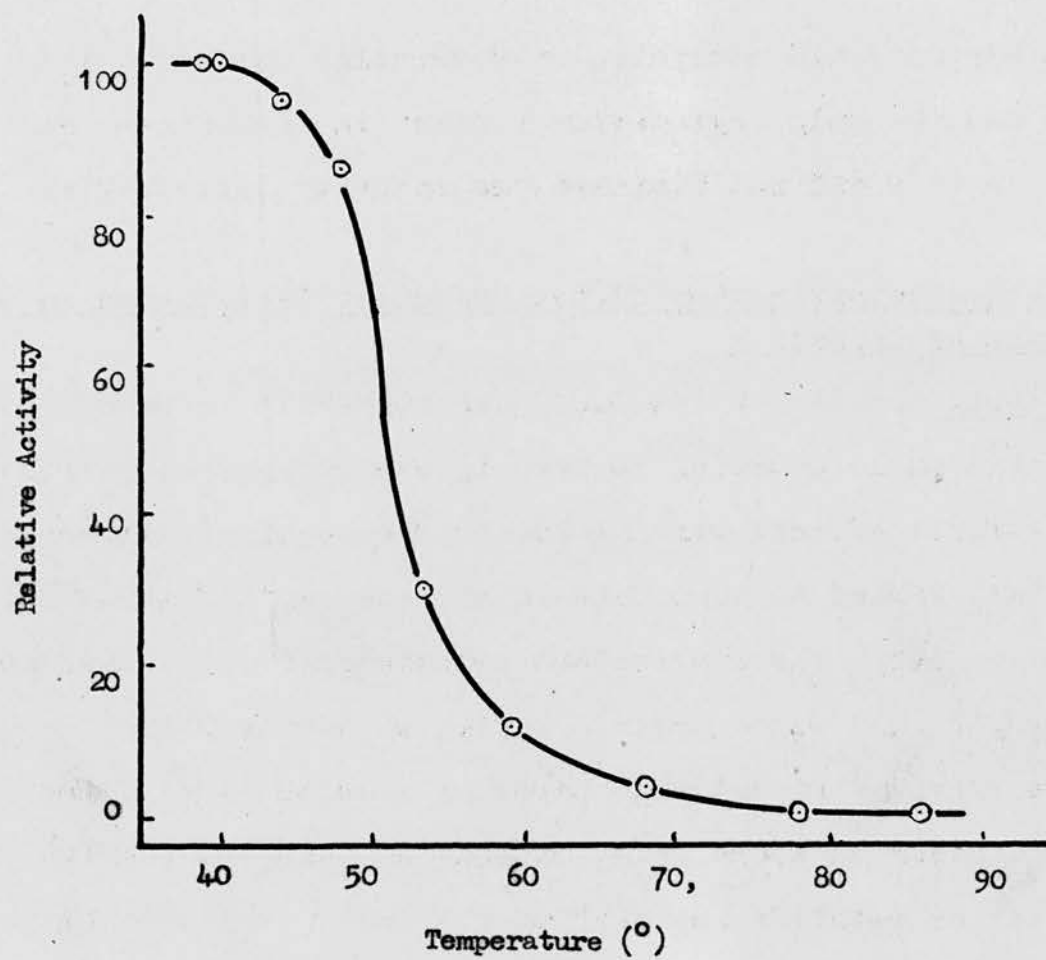
Time (days)	1	2	7	14
(1) pH 2.9	glucose t	glucose $\pm$ $R_G 0.65$ & 0.33 t	glucose + $R_G 0.65$ & 0.34 +	glucose ++ $R_G 0.55$ +, 0.20 $\pm$
(2) pH 5.1	glucose $\pm$	glucose + $R_G 0.65$ & 0.33 $\pm$	glucose ++ $R_G 0.65$ +, 0.34 $\pm$	glucose ++ $R_G 0.56$ +, 0.20 $\pm$, 0.40 t
(3) pH 7.0	glucose t	glucose $\pm$ $R_G 0.63$ t	glucose + $R_G 0.65$ & 0.34 $\pm$	glucose ++ $R_G 0.56$ & 0.20 $\pm$, 0.40 t
(4) pH 9.0	—	glucose t	glucose $\pm$ $R_G 0.65$ & 0.34 $\pm$	glucose ++ $R_G 0.56$ & 0.20 $\pm$
(5) pH 5.1	glucose t	glucose $\pm$	glucose +	glucose ++ $R_G 0.56$ & 0.20 t

Cellodextrin

Time (days)	1	2	7
(6) pH 2.9	glucose $\pm$ $R_G 0.46$ & 0.08 $\pm$	glucose + $R_G 0.46$ & 0.08 +	glucose +, $R_G 0.50$ & 0.15 +, $R_G 0.71$ t
(7) pH 5.1	glucose $\pm$ $R_G 0.46$ & 0.08 $\pm$	glucose ++ $R_G 0.46$ & 0.08 ++	glucose ++, $R_G 0.50$ & 0.15 ++, $R_G 0.71$ $\pm$
(8) pH 7.0	glucose $\pm$ $R_G 0.46$ & 0.08 $\pm$	glucose + $R_G 0.46$ & 0.08 +	glucose +, $R_G 0.50$ & 0.15 +, $R_G 0.71$ t
(9) pH 9.0	glucose $\pm$ $R_G 0.46$ & 0.08 $\pm$	glucose $\pm$ $R_G 0.46$ & 0.08 $\pm$	glucose $\pm$, $R_G 0.50$ & 0.15 $\pm$, $R_G 0.71$ t
(10) pH 5.1	glucose $\pm$ $R_G 0.46$ & 0.08 $\pm$	glucose + $R_G 0.46$ & 0.08 +	glucose +, $R_G 0.50$ & 0.15 +, $R_G 0.71$ t



Graph 9 - Effect of temperature



Digests were set up with lichenin or barley β -glucosan (30 mg), C. rupestris fraction A (15 mg.) and 0.1 M acetate buffer pH 5.0 (4 ml.). Similar digests were set up in which the buffer and fraction A were heated to 60°C for 10 minutes before addition of the substrate.

Chromatographic analysis, at intervals, indicated that glucose was the only degradation product in each digest and that heating to 60°C did not diminish the activity appreciably.

Quantitative determination of the effect of temperature on the hydrolysis of laminarin

C. rupestris fraction W (300 mg.) was suspended in water (25 ml.) and the insoluble material removed by centrifugation. Portions of the soluble extract were heated at the required temperature for 15 minutes, cooled to room temperature and centrifuged. Digests were set up using the supernatant solutions (2 ml.), laminarin (4 ml., 0.3%) and 0.2 M acetate buffer pH 5.6 (4 ml.).

The increase in reducing power of samples (4 ml.) was estimated after 24 hours using method 'A', and the results calculated as relative activity, on the basis of the highest activity being 100.

Temperature (°C)	39.0	40.0	44.0	48.0	53.5	59.0	68.0	78.0	86.6
Activity	100	100	95	86	30	12	4	0	0

From Graph 9 it can be seen that the laminarinase is decreased at temperatures greater than 41°C.

Hydrolysis of yeast glucan by *C. rupestris* fraction A

The following digest was set up and samples (3 ml.), withdrawn at intervals, heated to 100°C and de-proteinised using barium hydroxide (2.5 ml., 0.3 N) and zinc sulphate (2.5 ml., 5%)- yeast glucan (28 mg.), fraction A (280 mg.) and 0.1 M acetate buffer pH 5.0 (21 ml.). The reducing power of the supernatant solution (5ml.) was determined using method 'A'. The results are quoted as percentage hydrolysis to glucose.

Time (days)	0	1	3	6	8
Percentage hydrolysis	-	51.1	73.1	76.0	76.1

Hydrolysis of Cellofas 'B' by *C. rupestris* fraction A

This demonstrates the hydrolysis of β ,1'-4- glucosidic linkages.
Enzyme: fraction A (75 mg.) in buffer (30 ml.) and filtered.

Substrate: cellofas B - sodium carboxymethyl cellulose - (250 mg.)
in water (100 ml.) and filtered.

Buffer: 0.2 M acetate pH 5.05.

A digest was set up using substrate (20 ml.) and enzyme (20 ml.), and a control using substrate (20 ml.) and buffer (20 ml.).

The degradation of the substrate was followed viscometrically at 20°C using an Ostwald viscometer with a flow time for the solvent, water (10 ml.) and buffer (10 ml.), of 226 seconds.

Relative viscosity = $\frac{\text{flow time for solution}}{\text{flow time for solvent}}$

Specific viscosity = $\text{Rel. } \eta - 1$

Time (mins.)	0	5	10	15	20	25	30	18 hr.
Control Spec. η	0.57	-	-	-	-	-	-	0.55
Digest Spec. η	-	0.38	0.33	0.29	0.26	0.25	0.23	0.03

At 18 hour samples (5 ml.) were withdrawn and the reducing power estimated using method 'A'. A difference of 2.06 ml. of 0.01 N thiosulphate in reducing power between the control and digest at 18 hour was obtained indicating approximately 8% hydrolysis (as glucose). Chromatographic analysis using irrigant 1 and spray 1 showed a streak extending from the position of glucose to the starting line.

d) Studies on the specificity of other glycosidase activity

Degradation of xylan from *R. palmata*

To determine whether phosphorolysis was the mode of degradation of the xylan extracted from *R. palmata* the following digest was set up and chromatographed at intervals - xylan (30 mg.), *R. palmata* fraction A (130 mg.), McIlvaine phosphate buffer pH 5.3 (2 ml.) and water (2 ml.).

The presence of xylose and a series of oligosaccharides in the digest was indicated, after 12 days incubation, using irrigant 1 and spray 1.

To detect the possible presence of phosphate esters the digest was chromatographed using irrigant 6, and a control strip from the paper on development with spray 4 indicated the presence of only inorganic phosphate. The unsprayed part of the paper was, however, cut into three strips corresponding to (a) the spot, which appeared to be inorganic phosphate, (b) the portion from the spot to the starting line, and (c) the starting line. The strips were eluted with cold water and the eluates concentrated and hydrolysed with

hydrochloric acid (1.0 N) at 100°C for 2 hours. The hydrolysates were neutralised, concentrated and chromatographed using irrigant 1 and spray 1. Only the hydrolysed eluate from strip (c) contained xylose indicating that no xylose-phosphates were present in the digest.

The hydrolysis of xylan by extracts of *C. rupestris*

The following digests were set up and the increase in reducing power of samples (5 ml.) estimated after 20 hours using method 'A' - *R. palmata* xylan (12.5 mg.), appropriate *C. rupestris* fraction (20 mg.) and 0.04 M acetate buffer pH 5.0 (30 ml.). The results were calculated as percentage hydrolysis to xylose, although all digests on chromatographic analysis indicated the presence of xylose and xylose-oligosaccharides.

Fraction	A	I	II	III	IV	V
Percentage hydrolysis / mg. N	3.4	0.7	3.5	9.2	7.0	3.7

The digests containing fractions A and III were further analysed, the results being quoted as percentage hydrolysis.

Time (days)	0.8	3	5	11
Fraction A	6	12	18	29
Fraction III	15	35	43	58

Demonstration of β -glucuronidase activity

Enzymes: Nutritional Biochemical Co-operation glucuronidase (6 mg.)
in water (1 ml.)

C. rupestris fraction W (78 mg.) in water (2.5 ml.) and
filtered.

Substrate: 0.01 M phenolphthalein β -glucuronide (p. 29).

Buffer: 0.1 M acetate buffer pH 5.2.

Digests were set up containing substrate (1 ml.), appropriate enzyme (1 ml.) and buffer (8 ml.). Controls were set up using enzyme heated to 100°C for 10 minutes. Samples (1 ml.) were withdrawn at intervals, trichloroacetic acid (1 ml., 5%) added and the solution centrifuged. The supernatant solution (1.5 ml.) was withdrawn and the residue washed with water (2 x 1 ml.), the washings (2 ml.) being added to the original supernatant solution. To this solution (3.5 ml.) was added glycine buffer pH 10.4 (2 ml.) and sodium hydroxide (0.5 ml., 0.5 N) and the absorption measured in a 1 cm. cell, using filter 605 on the Spekker absorptiometer, against water.

In the results the Spekker absorptiometer readings for the controls have been subtracted from the readings for the digests.

Time (days)	0.05	1	3	4	11	18
Glucuronidase	0.568	-	0.935	-	-	-
<u>C. rupestris</u>	-	0	-	0	0.202	0.246

The extract of C. rupestris thus contains weak β -glucuronidase activity.

The hydrolysis of sodium alginate

Enzyme: C. rupestris fraction A (75 mg.) in buffer (30 ml.) and filtered.

Substrate: sodium alginate (200 mg.) in water (80 ml.) and filtered.

Buffer: 0.2 M acetate pH 5.05.

A digest was set up using substrate (20 ml.) and enzyme (20 ml.), and a control using substrate (20 ml.) and buffer (20 ml.). The degradation of the substrate was followed viscometrically, at 20°C, using the same Ostwald viscometer as before.

Time (days)	0	2	3	4	5	7
Control, spec. η	0.86	0.83	0.80	0.78	0.82	0.78
Digest, spec. η	0.84	0.28	0.22	0.18	0.16	0.13

At 7 days samples (5 ml.) were withdrawn and the reducing power estimated using method 'A'. The reducing powers of the digest and control differed by 0.82 ml. of 0.01 N thiosulphate indicating a low degree of hydrolysis. On chromatographic analysis, using irrigant 3 and spray 1, no low molecular weight oligouronides were detected.

The extract of C. rupestris therefore contains very weak alginase activity.

Detection of pectic enzymes

Enzyme: C. rupestris fraction A (125 mg.) in water (25ml.) and filtered.

Substrate: apple pectin (2 gm) dissolved in water (100 ml.) and buffer (100 ml.) added.

Buffer: 0.2 M acetate pH 4.6.

A digest was set up consisting of substrate (20 ml.) and enzyme (20 ml.), and a control consisting of substrate (20 ml.) and water (20 ml.). The digest and control were each divided into two portions, one of which, in each case, was heated at 100°C for 5 minutes.

The degradation was followed viscometrically, at 20°C, using an Ostwald viscometer with a flow time of 224 seconds for the solvent, buffer (15 ml.) and water (5 ml.).

Time (days)	0	1	7
Digest, spec. η	1.83	1.41	0.82
Heated digest, spec. η	1.67	1.58	1.37
Control, spec. η	1.85	1.73	1.48
Heated control, spec. η	1.63	1.56	1.34

Chromatographic analysis using irrigant 3 and spray 1 failed to show any degradation products.

A similar digest and control were set up using takadiastase (Parke, Davis & Co. Ltd., 0.025%) as enzyme.

Time (days)	0	1	2
Digest, spec. η	1.68	1.25	1.00
Control, spec. η	1.75	1.63	1.56

The extract of C. rupestris thus contains a pectic enzyme the activity of which is ca. 5×10^{-2} that of takadiastase.

Studies on the esterase activity of extracts of C. rupestris

Detection of phosphomonoesterase activity

Enzyme: C. rupestris fraction A (619.8 mg.) in water (150 ml.).
 Substrate: sodium β -glycerophosphate (225.2 mg.) in water (80 ml.).
 Buffers: 0.2 M acetate pH 4.1 ; 0.2 M acetate pH 6.0 ; borate pH 7.4 ; borate pH 9.2.

Digests were set up as follows and the free orthophosphates in samples (5 ml.) determined, at intervals using Fiske and

Subbarow's reagent - substrate (10 ml.), enzyme (10 ml.), buffer (10 ml.) and water (40 ml.). An enzyme blank was set up using enzyme (10 ml.), buffer (10 ml.) and water (50 ml.). Controls were set up using enzyme which had been heated to 100°C for 20 minutes. The results are quoted as percentage hydrolysis to orthophosphate.

Time (days)	1	2	8
pH 4.1	0.0	0.0	3.0
pH 6.0	15.2	26.3	32.4
pH 7.4	18.7	28.3	35.4
pH 9.2	12.6	19.7	28.3

After 13 days a further quantity of enzyme (10 ml.) was added to the digest at pH 7.4 and after a further two days incubation, the percentage hydrolysis had increased to 81.8. These results indicate the presence of a phosphomonoesterase type I.

Detection of glucose-6-phosphatase activity

Enzyme: C. rupestris fraction A (125 mg.) in water (30 ml.)

Substrate: potassium salt of glucose-6-phosphate (101.2 mg.) in water (25 ml.).

Buffer: 0.2 M citrate pH 6.8.

A digest was set up containing substrate (8 ml.), enzyme (10 ml.), buffer (10 ml.) and water (42 ml.). A control was set up in a similar fashion using enzyme which had been heated to 100°C for 20 minutes. Free phosphate was determined in samples (5 ml.) as before. The results are quoted as percentage hydrolysis to phosphate. No phosphate was liberated in the control.

Time (days)	1	3	8
Digest	21.6	18.8	20.2

The extract of C. rupestris thus contains hydrolytic activity towards glucose-6-phosphate.

Detection of glucose-1-phosphatase activity

Enzyme: C. rupestris fraction A (413 mg.) in water (100 ml.).

Substrate: potassium salt of glucose-1-phosphate (158.2 mg.)
in water (50 ml.).

Buffer: 0.2 M citrate pH 6.8.

A digest, and a control, using enzyme heated to 100°C for 20 minutes, were set up as follows:- substrate (7 ml.), enzyme (10 ml.), buffer (10 ml.) and water (43 ml.). Samples (5 ml.) were withdrawn at intervals and free phosphate estimated as before. The results are quoted as percentage hydrolysis to phosphate. No phosphate was liberated in the control.

Time (hours)	5	18	42
Digest	37.7	48.6	65.2

The digest was chromatographed using irrigant 2 and spray 1 and the presence of free glucose observed. This indicates the presence of either a glucose-1-phosphatase or phosphoglucose mutase and glucose-6-phosphatase; the following digest was therefore set up with sodium fluoride as an inhibitor of phosphoglucose mutase - potassium salt of glucose-1-phosphate (100 mg.), C. rupestris fraction A (260 mg.), sodium fluoride (2.0 ml., 0.1 M), 0.2 M citrate buffer pH 6.8 (10.0 ml.) and water (8.0 ml.).

Orthophosphate in samples (1 ml.) was determined as before, as well as glucose-1-phosphate (G -1- P) and total ester phosphate (G - 1 - P + G - 6 - P), the latter being a control on the inhibitor. The results are quoted as mg. estimated phosphorus.

<u>Time (hours)</u>		<u>Orthophosphate</u>	<u>G - 1 - P</u>	<u>G - 1 - P + G - 6 - P</u>
0	mg. P.	0	0.445	0.440
26	mg. P.	0.197	0.235	0.235
Percentage hydrolysis		44.8		
50	mg. P.	0.241	0.170	0.170
Percentage hydrolysis		55.2		

These results indicate hydrolytic activity in the extract of C. rupestris towards glucose-1-phosphate.

Attempted demonstration of sulphatase activity

Enzyme: C. rupestris fraction W (150 mg.) in water (10 ml.) and filtered.

Substrate: potassium salt of p-nitrophenyl sulphate (13.6 mg.) in water (10 ml.).

Buffer: 0.5 N acetate pH 5.6.

The following digest and controls were set up:-

Digest: substrate (4 ml.), enzyme (4 ml.) and buffer (12 ml.).

Controls 1. substrate (4 ml.), water (4 ml.) and buffer (12 ml.).

2. enzyme (4 ml.), water (4 ml.) and buffer (12 ml.).

Samples (ca. 4 ml.) were withdrawn at intervals, heated to 100°C (to inactivate the enzymes) and centrifuged. To the supernatant (3 ml.) was added sodium hydroxide (3 ml., 1.0 N) and the absorption

measured in a 1 cm. cell using filter 601, against control 2, on the Spekker absorptiometer.

The amount of p-nitrophenol liberated, calculated from a calibration curve, after 20 days incubation corresponded to less than 2% hydrolysis.

Studies on the other enzymic activity of extracts of C. rupestris

Detection of proteolytic activity

Digests were set up as follows:-

substrate (30 mg.), C. rupestris fraction A (30 mg.) and 0.15 M citrate buffer pH 6.8 (3 ml.). An enzyme control was set up in a similar fashion but without substrate. The digests were chromatographed on Whatman No. 1 paper which had been pre-washed with 8-hydroxy-quinoline in acetone, using irrigant 7 and spray 5. In the results allowance is made for intensity of the spots due to autolysis of the extract.

Time (days)	10	13	20
Fraction A	glycine	glycine, alanine, valine, leucine	glycine, alanine, valine leucine
Gelatin	glycine, hydroxy-proline, alanine, proline, valine	glycine +, hydroxyproline, alanine, valine leucine	glycine +, histidine, leucine, hydroxyproline, alanine, valine glutamic acid
Casein	valine t, leucine t	glycine t, valine t, leucine t	glycine, proline, valine t, leucine t, glutamic acid

In a similar digest containing ovalbumin, no free amino acids were observed within 20 days.

Detection of pyrophosphatase activity

The enzymes catalysing the hydrolysis of pyrophosphate are not classified with the esterases because the linkage hydrolysed is not an ester bond.

Enzyme: C. rupestris fraction A (413 mg.) in water (100 ml.).

Substrate: sodium pyrophosphate (445.1 mg.) in water (100 ml.).

Buffers: 0.2 M acetate pH 6.0 ; borate pH 7.5.

Activator: 0.1 M magnesium chloride.

Digests were set up as follows and the free phosphate in samples (5 ml.) determined, as before -

(a) substrate (4 ml.), enzyme (10 ml.), buffer (10 ml.) and water (46 ml.).

(b) substrate (4 ml.), enzyme (10 ml.), buffer (10 ml.), activator (2 ml.) and water (44 ml.).

Controls containing activator and enzyme heated to 100°C for 20 minutes were also prepared. The results are quoted as percentage hydrolysis to phosphate.

No phosphate was liberated in the controls.

Time (days)	1	2	4
(a) pH 6.0	96.2	96.2	101.9
(b) pH 6.0	78.7	90.5	88.3
(a) pH 7.5	44.7	52.1	65.6
(b) pH 7.5	100.0	99.6	93.9

The magnesium chloride was shown to have no effect on the pH of the digests. These results indicate the probable presence of two pyrophosphatases.

Detection of catalase activity

Digests were set up in Warburg manometer cups using C. rupestris fraction W and minced tissue which had been stored at -10°C , under toluene, for 9 months since collection of the fresh seaweed (24/3/55). Sodium azide is a catalase inhibitor.

	1	2	3	4	5
McIlvaine phosphate buffer pH 7.0 (ml.)	1.0	1.0	1.0	1.0	1.0
0.1 M sodium azide (ml.)	-	1.0	-	1.0	-
<u>C. rupestris</u> fraction W (mg.)	10	10	-	-	-
<u>C. rupestris</u> minced tissue (ml.)	-	-	0.5	0.5	-
Water (ml.)	1.8	0.8	1.3	0.3	1.8

The side arm of each manometer flask contained hydrogen peroxide (0.2 ml., 20 vol.). These digests were duplicated, one set being incubated at 35°C . and the other at 13°C .

No oxygen evolution was observed in manometers 2, 4 and 5. The oxygen output from manometer 1 was ca. 0.17 that of manometer 3, the values for those incubated at 35°C being ca. 0.5 the output of those incubated at 13°C .

These results indicate the presence of weak catalase activity in the extract of C. rupestris.

DISCUSSION

Evidence has been obtained for the presence of several glycosidases and various other hydrolytic enzymes in the algal extracts.

Toluene has been used as a germicide in all the enzymic digests and no evidence has been obtained of the contamination of any digest containing a surface layer of toluene.

α -glucosidase activity

The enzymic hydrolysis of methyl and phenyl α -glucosides and maltose has been attributed to a group specific α -glucosidase (72), the chemical identity of which appears to depend on the source of the enzyme (73). It has been shown that α -glucosidases from yeasts and moulds differ in their substrate specificity, pH optima and ability to synthesise glucosides from a mixture of glucose and an alcohol. The degradation of maltose by the algal extracts is due to enzymic hydrolysis and not phosphorolysis since no glucose phosphates could be detected in a maltose digest buffered by phosphate. The enzyme responsible for the hydrolysis of maltose contains no dialysable co-factor since the activity before and after dialysis was constant. The algal extracts possess an α -glucosidase which shows decreasing activity towards maltose, phenyl α -glucoside and methyl α -glucoside, activity towards the latter being detected only with C. rupestris (Table 1). Whole extracts of C. rupestris hydrolysed maltose to an extent of 70 - 80% whilst that of R. palmata caused 90% hydrolysis. A more active preparation of C. rupestris however was able to hydrolyse maltose completely;

it thus seems likely that the lower values were due to slow inactivation of the enzyme on prolonged incubation at 35°C. The α -glucosidase pH - activity curves, using McIlvaine phosphate buffer and B.D.H. Universal buffer are very similar for both algae and show maximum activity over the pH range 5.5 - 5.8. Considerable increase in the α -glucosidase activity of the algal extracts can be obtained by precipitation of other material with increasing concentration of ammonium sulphate whilst the use of alcohol as a precipitant results in considerable inactivation, a property which has been observed by other authors (e.g. Gottschalk, 72) using yeast α -glucosidase.

The algal α -glucosidase, although having the same substrate specificity as that of Aspergillus oryzae resembles that from yeast in the characteristics of optimum pH, inactivation by alcohol and, as will be shown in section III, the inability to synthesise glucosides from glucose.

Sucrose is the biological substrate for two different glycosidases, one of which, sucrose α -glucosidase, attacks the molecule through the glucose moiety while the other, β -h-fructosidase establishes contact with the fructose part of the molecule. Ishizawa and Miwa (74) separated these enzymes by adsorption on an alumina gel and differentiated between them by transglycosylation to methanol and identification of the products. They may also be identified by the observation that only β -h-fructosidase will degrade raffinose to yield fructose, and it is by this test that the absence of β -h-fructosidase in the algal extracts has been confirmed (Table 3). Gottschalk (72) has

discussed the theory that sucrose α -glucosidase and α -glucosidase are identical and concludes that the available evidence is insufficient to prove their identity; in the light of present knowledge both algal extracts contain sucrose α -glucosidase.

The enzymic hydrolysis of the non-reducing α -glucoside, trehalose, may be catalysed by a specific enzyme, trehalase, which occurs mainly in fungi and has been differentiated from α -glucosidase by its greater thermolability (72). No quantitative estimation has been made of the thermolability of the algal trehalase but it has been observed that heating the extract to 65°C for 10 minutes results in an appreciable decrease in activity.

The ability of cell-free extracts to hydrolyse α ,1:6-glucosidic linkages has been reported in only a limited number of cases e.g. hog intestinal mucosa (75, 76), Clostridium acetobutylicum (77) and brewer's yeast (78), the ability of the extract of C. rupestris to cause appreciable hydrolysis of isomaltose and to a lesser extent, of isomaltotriose, is therefore of some interest.

The three α -glucosans used as substrates (Table 4) were hydrolysed by the algal extracts indicating the presence of an enzyme, or enzymes, with amylolytic activity; this observation has been further investigated using the preparations from C. rupestris and the results are presented and considered in section IV.

β -glucosidase activity

Qualitative examination of the algal extracts has indicated the presence of β -glucosidase activity with a wide range of

specificity (Table 2) and the ability to hydrolyse 1:3- and 1:4-glucosidic linkages in β -glucosans (Table 4).

From the results of this investigation it is not yet possible to state whether the hydrolysis of the aromatic β -glucosides and disaccharides is due to a group specific β -glucosidase or several closely related enzymes with a more limited specificity, cf. Zechmeister and his co-workers (79) who have reported the separation of a β -glucosidase preparation into fractions which are each active towards only one β -glucoside. The activity of the whole extract of C. rupestris has been found to be approximately 6×10^{-4} that of an emulsin preparation under identical conditions. The β -glucosidase pH - activity curves, using salicin as substrate and B.D.H. Universal buffer, for both algal extracts were similar in shape, with maximum activity over the pH range 6 - 7.

β -glucosanase activity

The enzymic hydrolysis of cellulose and other β -glucosans has been reviewed by Manners (21) who has considered the literature published since the review of Pigman (80).

The degradation of glucosans containing β ,1:3- linkages has been attributed to a hydrolytic mechanism since the presence of glucose phosphates could not be detected when laminarin, a seaweed polysaccharide containing β ,1:3- linkages (81), was degraded in the presence of phosphate buffer and phosphoesterase inhibitors. It has also been shown that the algal extracts undergo no loss of activity on dialysis indicating the absence of any dialysable co-factor.

Insoluble laminarin, from Laminaria cloustoni, on hydrolysis with whole extracts of both algae gave a ca. 90% yield of glucose whereas soluble laminarin, from the same seaweed, was hydrolysed by the extract of C. rupestris to 80%; using emulsin, a 90% conversion was obtained. The difference in the results must be attributed to different structural features in the two laminarin samples. The latter results are considerably higher than those reported by Dillon and O'Colla (82, 83) who, using cell-free extracts of cereals obtained only a 70% hydrolysis of soluble laminarin, and Peat, Thomas and Whelan (84) who found that the same sample of laminarin had an apparent hydrolysis limit of 73% using emulsin. It has been observed that emulsin, unless removed by de-proteinisation, interferes with the estimation of glucose by the Somogyi reagents and this may account for the value of 73% using emulsin where no de-proteinisation was effected.

A study of the effect of pH on the hydrolysis of laminarin indicated that the pH at which maximum activity was obtained depended on the buffer used; the optimum pH in acetate buffer was ca. 5.0 whilst in Universal buffer it was ca. 5.8. Different shaped pH - activity curves were obtained for the extracts of the two algae indicating the possible non-identity of the laminarinase systems in the two algae. In general the extract of R. palmata exhibited greater sensitivity to pH values more acid and alkaline than the optimum pH.

By heating the extract of C. rupestris to 60°C it has been shown that the degradation of laminarin may be attributed to two enzymes, one of which is heat labile. Using the normal extract,

glucose and a series of oligosaccharides are the degradation products of laminarin whilst the heated extract produces only glucose, thus indicating that the two enzymes not only differ in heat stability but also in their mode of attack. The heat stable enzyme exerts a step-wise or exo-action by removing single glucose units from the molecule, possibly from the non-reducing end of the chains, whilst the heat labile enzyme degrades the laminarin by random or endo-hydrolysis.

Cellodextrin, a β ,1:4- linked glucosan, is degraded by random hydrolysis, the endo β ,1:4- glucosidase being heat stable. The hydrolysis of lichenin and barley β -glucosan must be due to an exo- β -glucosidase which is heat stable as both the normal and heated extracts of *C. rupestris* degrades these polysaccharides to glucose.

Quantitative estimation of the thermolability of the enzymes hydrolysing laminarin has shown that at temperatures greater than 41°C the activity is diminished, so that at 53°C, 70% of the original activity is destroyed. This observation confirms that of Dillon and O'Colla (83) who reported that undried wheat was three times more active towards laminarin than wheat which had been air-dried at 50°C.

The activity of the extract of *C. rupestris* towards laminarin has been found to be ca. 0.25 that of a barley preparation.

Fractional precipitation has shown that the most active preparation is that precipitated between 0.18 and 0.36 saturation with ammonium sulphate; precipitation with ethanol results in loss of activity.

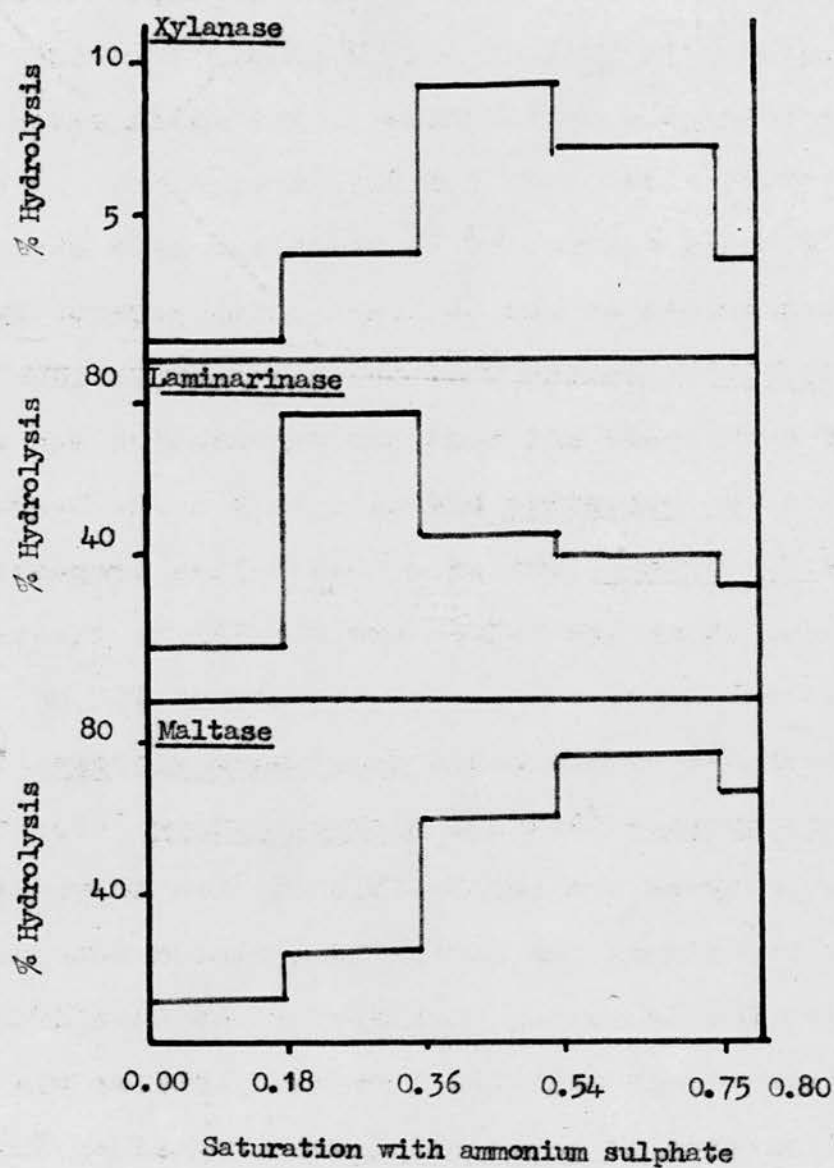
Yeast glucan, a branched glucosan containing β ,1:3- and approximately 11% β ,1:2- linkages (85), gave a 76% yield of glucose on incubation with a whole extract of C. rupestris; a similar high value was obtained by Manners (86) using a barley preparation whilst Dillon and O'Colla (83) were unable to detect any degradation using a wheat extract, which however gave 20% hydrolysis of an oxidised preparation of the polysaccharide.

The presence of an endo β ,1:4-glucosidase has been confirmed by following the degradation of sodium carboxymethyl cellulose viscometrically and by reducing power estimations. The viscosity of the solution decreased rapidly and reached a minimum value in 18 hours at which time reducing power estimations indicated only 8% hydrolysis; results of this type can only be attributed to random hydrolysis. This enzyme appears to be similar to the C_x enzyme which has been described by Levinson and Reese (87).

The algal extracts therefore contain a heat labile endo β ,1:3- glucosidase, an endo β ,1:4-glucosidase and an exo β -glucosidase, which may be responsible for the group specific β -glucosidase activity. This complement of enzymes is very similar to that of cereals, in particular barley, and confirms the separate identity of the enzymes responsible for the hydrolysis of lichenin and cellulose (88).

Estimations of the activity of the whole extract of C. rupestris towards maltose and laminarin has shown that on lyophilization part of the activity becomes insoluble. Aqueous extracts of the lyophilized product were found to have lower activity, although the β -glucosidase was more readily re-extracted than the α -glucosidase.

Distribution of enzymes in ammonium sulphate fractions
of Cladophora rupestris



Xylanase activity

The present investigation has shown that the algal extracts contain hydrolytic activity towards β -xylosidic linkages. This activity could be concentrated by fractional precipitation, the precipitate collected between 0.36 - 0.54 saturation with ammonium sulphate being the most active. The xylan extracted from R. palmata (with β ,1:4- and β ,1:3- linkages p.2) on degradation with a whole extract of C. rupestris gave the same series of oligosaccharides as was obtained using esparto xylan (a β ,1:4 xylan (89)) indicating that the β ,1:3- xylosidic linkages were more readily hydrolysed and that the degradation was random. The algal extract of C. rupestris effected only a 29% hydrolysis of the xylan from R. Palmata, whilst a more active preparation gave 58% hydrolysis, these low values are similar to the value of 56% reported of Holden and Tracy using a snail extract (90). Sørensen using cell-free extracts of the mould Chaetomium globosum (91) and species of Streptomyces (92) and Micromonospora (92) has indicated that two enzymes are responsible for the degradation of xylan by these organisms, one acting in a random fashion and the other which is heat labile (being destroyed at 68°C) hydrolysing xylobiose. No decrease in activity towards xylobiose was detected when the extract of C. rupestris was heated to 60°C. Xylanase activity has been reported in extracts from many different sources (21) and it is significant that it has been reported in barley.

Other glycosidase activity

The algal extracts show weak activity towards lactose, a

β -galactoside, an activity which is frequently found in association with β -glucosidase preparations from plant sources (93). The apparent inability to hydrolyse aromatic β -galactosides may be attributed to the low order of β -galactosidase activity. Melibiose, which has been described as the typical substrate for α -galactosidase (93), is hydrolysed by the algal extracts, although no activity could be detected using methyl α -galactoside and only very weak activity towards raffinose. The presence of a very weak α -galactosidase in the algal extracts is however indicated.

No activity has been detected towards any rhamnoside or methyl α -mannoside.

Hydrolytic activity towards mannans, containing β ,1:4-mannosidic linkages, has been found in the extracts of C. rupestris and, to a lesser extent, A. palmata. No explanation can be advanced for the production of different oligosaccharides from ivory nut mannan when it is dispersed by treatment with alkali. The inability to hydrolyse yeast mannan is attributed to the absence of α -mannanase activity as this mannan has recently been shown to be composed of branched α -mannosidic linkages (94). The occurrence of mannanases in biological extracts has not been widely investigated, but no plant source of this enzyme has been reported although it has been observed to occur in many moulds (21, 95).

Few reports are available on the enzymic degradation of fructosans other than inulin, the hydrolysis of which has been mainly studied using extracts from moulds (21.). The algal extracts are inactive towards inulin and levan, the two fructosans studied, and also towards fucoidin, a polyfucose, the enzymic hydrolysis of which does not appear to have been reported.

The hydrolysis of galacto- and glucomannans by biological extracts has not been studied to any extent (21). Qualitative examination of a whole extract of C. rupestris (Table 4) has indicated that it contains weak hydrolytic activity towards the galactomannan from carob bean, which is mainly composed of β ,1:4-linked mannose units, one-fifth of which have a single galactose residue attached at C₍₆₎ (21), and to a lesser extent towards two glucomannans and several other galactomannans from varied sources. Two oligosaccharides were produced by the degradation of the carob gum and a comparison of their chromatographic mobilities with those obtained from ivory nut mannan indicates that they are possibly β ,1:4-linked mannosaccharides. As galactose was detected within four days it seems highly probable that the galactose is attached to the mannose by a β -linkage as the extract contains a more active β -galactosidase than an α -galactosidase even although no activity could be detected towards " ξ -galactan" from larch which appears to be a β -galactan (96).

Various β -glucosidase preparations have been found to contain activity towards β -glucuronides and similar activity has been found in an extract of C. rupestris. The activity was estimated, by an agluconometric method described by Fishman (97) and compared with a commercial preparation, the activity of which was very considerably greater.

Several reports have been made on the enzymic hydrolysis of alginic acid which consists essentially of β ,1:4-linked mannuronic acid radicals with a chain length of ca. 100 (98). The first observation of an alginase was by Oshima (99) who found it in

aqueous extracts of the intestines of Haliotus giganteus (a marine Gastropoda) and Sphaerechinus pulcherrimus (an Echinoderm) but not in Aspergillus oryzae. Since then it has been reported to be present in various gram negative soil and marine bacteria (100, 101, 102), the liver of shipworm (103) and the mollusc Dolabella scapula (103). The degradation products have been reported to be mannuronic acid and oligomannuronides (102). Waksman and Allen (104) however had found that, using a marine bacterium, very little mannuronic acid was formed. Very weak alginase activity has been observed in C. rupestris using the viscosity technique as no degradation products could be detected by partition chromatography.

Several enzymes are responsible for the degradation of the pectic substances, which are polymers of galacturonic acid. The distribution and specificity of these enzymes has been recently reviewed by Manners (21) who has considered the advances made since the review by Kertesz (105).

The decrease in viscosity of apple pectin produced by the extract of C. rupestris over a given period of time was found to be equivalent to that produced by approximately one-twentieth the concentration of takadiastase, no small weight oligouronides being detected after 7 days. Thus it seems probable that the algal extract contains a pectic enzyme.

Other hydrolytic activity

Four different types of phosphomonoesterases have been described, the types being differentiated by pH optima and behaviour in presence of certain inorganic ions especially magnesium and manganese (48). The action of a whole extract of C. rupestris on

sodium β -glycerophosphate, a standard substrate for phosphomonoesterases, at different pH values has indicated an activity optimum at a pH greater than 7. Although further study is required, it appears that this activity must be due to a type I enzyme, i.e., an alkaline phosphatase, which rarely occurs in plants.

In addition to the phosphomonoesterases, described by Roche (48), which have a wide substrate specificity, enzymes have been reported which are specific for the hydrolysis of glucose-1-phosphate and glucose -6-phosphate. The blood of silkworm has been found to contain an enzyme which will hydrolyse only glucose- and galactose-1-phosphates and p-nitrophenyl phosphate to any appreciable extent (106), whilst Swanson has described the preparation of an extract from liver which will hydrolyse glucose-6-phosphate but not glucose-1-phosphate (107). The extract of C. rupestris shows hydrolytic activity towards glucose-6-phosphate but from the results of this preliminary investigation it is not possible to determine whether this is due to a specific enzyme or to the phosphomonoesterase type I which has been shown to be present. Incubation of glucose-1-phosphate with C. rupestris extract in the presence and absence of fluoride (a phosphoglucose mutase inhibitor) resulted in the liberation of inorganic phosphate in both experiments indicating that the extract contained an enzyme which could hydrolyse glucose-1-phosphate without the mediation of phosphoglucose mutase and glucose-6-phosphatase. If this activity is due to a specific glucose-1-phosphatase, the enzyme is not chemically identical with that from the silkworm which is strongly inactivated by fluoride.

Naganna and Sripathi (108) have reported the presence in potato and wheat extracts of both an acid and an alkaline pyrophosphatase. The pyrophosphatases which have been described so far are apparently dependent upon the presence of magnesium for their ability to catalyse the hydrolysis of pyrophosphate. It has been suggested that the function of the magnesium is to combine with the pyrophosphate so that the actual substrate is not $\text{Na}_4\text{P}_2\text{O}_7$ but the complex ion $[\text{MgP}_2\text{O}_7]^-$ (109). The activity of C. rupestris extract towards pyrophosphate was estimated at pH 6.0 and 7.5 in the absence of, and presence of, magnesium ions; in the former case activity was found to be greater at pH 6.0 and in the latter at pH 7.5. These results indicated the probable presence of an alkaline pyrophosphatase which is activated by magnesium ions and an acid pyrophosphatase, the activity of which appears to be diminished slightly by magnesium ions.

Many different extracts, from a variety of biological sources have been described which show hydrolytic activity towards proteins (110). The extract of C. rupestris shows appreciable proteolytic activity towards gelatin and, to a lesser extent, towards casein and the proteinⁱⁿ/the extract; no activity was detected towards ovalbumin.

An attempt to demonstrate phenol sulphatase activity was unsuccessful.

Hydrogen peroxide is catalytically decomposed to water and oxygen by catalase which has an extremely wide distribution in living organisms. The in vivo function of catalase is as a catalyst for coupled oxidations in which hydrogen peroxide is

one of the reactants rather than the destruction of toxic hydrogen peroxide (111). The biochemistry of catalase, which is a metalloprotein, has been reviewed by Burris and Little (112). Catalase activity which has been reported in many marine algae (p. 8) was detected in C. rupestris but the results using minced tissue indicated that the activity had been considerably decreased during the preparation of the lyophilized extract.

In this preliminary investigation into the enzymic activity of extracts of R. palmata and C. rupestris the results have indicated that both of these seaweeds contain essentially the same complement of glycosidases.

SUMMARY

1. Whole extracts of the marine algae R. palmata and C. rupestris have been shown to contain hydrolytic activity towards α -glucosides (maltose, isomaltose, sucrose, trehalose), many different β -glucosides, lactose, melibiose, xylobiose, α ,1:4-glucosans, β ,1:3- and β ,1:4-glucosans, β -mannans, and β ,1:3 and β ,1:4-xylans.
2. Further investigation of the β -glucosidase activity of C. rupestris has indicated the presence of at least three β -glucosidases viz., a group specific β -glucosidase, and endo β ,1-3 and an endo β ,1-4 glucosidase.
3. The extract of C. rupestris also contains a β -glucuronidase, an alginase and a pectic enzyme.
4. A study of the esterase activity has indicated hydrolytic activity towards glucose-1- and 6- phosphates as well as the presence of a phosphomonoesterase type I.
5. Proteolytic, pyrophosphatase and catalase activities have also been observed in C. rupestris.

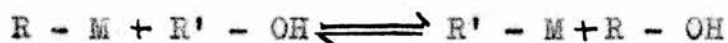
SECTION THREE

Studies on the transglycosylase (transglycosidase) activity of
extracts of R.palmata and C.rupestris

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INTRODUCTION

Recent investigations have shown that a number of enzymes which normally catalyse a hydrolytic or phosphorolytic breakdown of carbohydrates can, in certain conditions, catalyse the transfer of a monosaccharide residue from a carbohydrate molecule to an acceptor. Rabaté(113) was the first to recognise that hydrolases can catalyse reactions of the type -

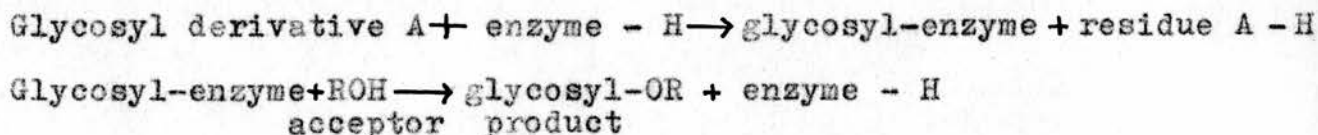


where M is the monosaccharide residue and R and R' are alkyl or aryl radicals, or a chain of one or more sugar residues. These enzymes have been described as "transferases", "transglycosidases" or "transglycosylases". It was originally believed that enzyme action involved the transfer of a glycosidic radical. However, in the case of sucrose $\rightarrow$ phosphate transglucosidase (sucrose phosphorylase), by the use of inorganic phosphate containing P^{32} the transfer of glucosyl rather than a glucosidic radical has been established; the term transglucosylation rather than transglucosidation has therefore been used (114). In the present discussion the more widely accepted terms transglycosidation and transglucosidation will be used, since the detailed mechanism of action of the algal enzymes is unknown.

Barker and Bourne (115), in reviewing the enzymic synthesis of polysaccharides, have discussed two types of transglucosidase activity which are responsible for the synthesis of α -glucosans of the starch and dextran classes. The first, glucose-1-phosphate $\rightarrow$ amylose transglucosidase (phosphorylase), catalyses the transfer of a

glucose residue from glucose-1-phosphate to an acceptor glucosaccharide, containing $\alpha,1:4$ - links, thus forming a linear $\alpha,1:4$ -glucosan. In the second, the polysaccharides are formed by transglucosidic reactions, which are completely independent of phosphate, from disaccharides. For example, linear $\alpha,1:4$ -glucosans may be synthesised from maltose, whilst sucrose is the primary substrate in dextran synthesis by Leuconostoc mesenteroides (115).

The synthesis of oligosaccharides has been reported from maltose (116, 117, 118), sucrose (119), isomaltose (120) and cellobiose (121, 122) the reaction mechanism being apparently transglucosidation. Pazar and French (123), from studies on transglucosidation from maltose, have represented the reaction as occurring in two steps, the first being the transfer of a glucosyl residue from maltose to the enzyme, and the second, from the glucosyl-enzyme complex to the acceptor substrate. Reactions of this type may be summarised in the form of an equation (124) -



In one case of transglucosidation from maltose, residue A is glucose, maltose the acceptor and maltotriose the product.

Bourquelot and his co-workers (125, 126) prepared various alkyl glucosides by enzymic synthesis from glucose and alkyl alcohols. Miwa and Takano (127, 128) using cell-free extracts from green leaves, were able to effect the transfer of a glucose residue from an aryl glucoside to an alcohol; in the course of

purification studies, they observed that the hydrolase and trans-glucosidase activities appeared to be due to the same enzyme. During the enzymic hydrolysis of p-nitrophenyl- β -glucoside, Jermyn (129) observed the non-equivalence of the glucose and p-nitrophenol produced. Later, Jermyn and Thomas (130) estimated the liberation of glucose and p-nitrophenol using an extract of Aspergillus oryzae in the presence of methanol and butanol, and found that the amount of glucose was greatly reduced, whilst the p-nitrophenol was scarcely affected. This indicated that trans-glucosidation to the appropriate alcohol was taking place. Similar activity has been observed by the Japanese workers (131) using aryl galactosides.

This section contains an account of the qualitative demonstration of transglycosidase activity in algal extracts and of the separation, on a large scale, of the oligosaccharides synthesised from maltose by an extract from C. rupestris.

EXPERIMENTAL

Methods

The methods used in this section are identical with those used in section II (p. 25).

Studies on the transglycosidase activity of extracts of *R. palmata* and *C. rupestris*

The algal extracts were examined qualitatively for transglycosidase activity using two types of digests; in the first, the donor and acceptor substrates were the same; in the second, they were different.

One substrate

The following digests were set up and examined at intervals for the presence of oligosaccharides using irrigant 1 and spray 2:-

<u>Digest number</u>	<u>Weight of substrate</u> (mg.)	<u>Weight of appropriate</u> <u>algal fraction</u> (mg.)
1 and 2	30	15
3 - 8	220 - 260	10 - 20
9	50	3
10	300	10

In addition each digest contained 0.07 M acetate buffer pH 5.1 (4 ml.) except digest 10 which contained 0.1 M acetate buffer pH 5.6 (1 ml.).

The results in Tables 1 and 2 are reported using the key detailed in section II (p.32); degradation products are not noted.

Action on one substrate

TABLE 1

Substrate	Algae Fraction	4 days	7 - 9 days	14 days
1) Glucose	R.p. B	-	-	-
2) Glucose	C.r. A	-	-	-
3) Maltose	R.p. B	$R_G 0.27, \pm$	$R_G 0.47, 0.39, 0.18, \pm$	$R_G 0.49, 0.44, 0.21, \pm$
4) Glucose	C.r. A	-	-	-
5) Xylose	C.r. A	-	-	-
6) Isomaltose	C.r. A	$R_G 0.14, t$	$R_G 0.15, t$	$R_G 0.14, \pm$
7) Maltose	C.r. A	-	$R_G 0.49, 0.26, \pm$	$R_G 0.49, 0.26, \pm$
8) Sucrose	C.r. A	-	-	-
9) Maltose	C.r. A	-	-	-
10) Cellobiose	C.r. W	-	$R_G 0.75t, 0.35, 0.23, 0.13, \pm$	$R_G 0.75t, 0.35, 0.22, 0.12, 0.08, 0.06, \pm$

Two substrates

Digests were set up consisting of a glucose donor and suitable acceptor, C. rupestris fraction A or W (see Table 2) and sufficient 0.1 M acetate buffer pH 5.6 to make the total volume 2 ml. Irrigant 1 and sprays 1 and 2 were used for the chromatographic analysis. Although the digests were examined at intervals, only one typical result is reported.

Action on two substrates

TABLE 2

Donor	Acceptor	Fraction, mg.	Incubation (days)	Results
Maltose, 32 mg.	Fructose, 10 mg.	A, 25	16	R _G 0.38
Maltose, 32 mg.	Galactose, 10 mg.	A, 25	16	R _G 0.38
Maltose, 32 mg.	Methanol, 2 ml.	A, 25	16	-
Maltose, 400 mg.	Sucrose, 40 mg.	W, 20	15	R _G 0.50, 0.41, 0.35, 0.24, 0.20, 0.13, 0.07
Glucose, 25 mg.	Methanol, 0.5 ml.	W, 10	19	-
Glucose, 25 mg.	Methanol, 0.5 ml.	W, 30	19	-
Glucose, 25 mg.	Methanol, 2 ml.	W, 20	19	-
Phenyl α - glucoside, 25 mg.	Methanol, 0.5 ml.	W, 10	19	-
Phenyl β - glucoside, 25 mg.	Methanol, 0.5 ml.	W, 10	3	spot with same mobility as a methyl-glucoside
Cellobiose, 300 mg.	D-Xylose, 300 mg.	W, 20	10	R _G 1.02, 0.70
Cellobiose, 300 mg.	L-Xylose, 300 mg.	W, 20	7	R _G 0.72, 0.37
Cellobiose, 300 mg.	N-acet. gluc., [‡] 300 mg.	W, 20	15	-
Cellobiose, 300 mg.	L-Sorbose, 300 mg.	W, 20	15	-

[‡] N-acetyl glucosamine

Evidence for transglucosidation from cellobiose to D- and L-xylose

The digests containing cellobiose and D- and L-xylose (Table 2) were chromatographed using irrigant 4 and the positions of the two oligosaccharides, in each case, determined by development of control strips with spray 1. The unsprayed parts of the papers were cut into strips corresponding to the oligosaccharides, which were then eluted with cold water. The eluates were concentrated in vacuo, hydrolysed with sulphuric acid (0.5 ml., 2N) for 2 hours at 100°C, neutralised and concentrated. The hydrolysates of the more mobile oligosaccharide in each digest contained glucose and xylose in apparently equivalent quantities as determined by qualitative paper chromatography using irrigant 2 and spray 1. The hydrolysates of the less mobile oligosaccharides also contained xylose.

These results indicate that D- and L-xylose can act as acceptors for the glucose liberated by hydrolysis of cellobiose, with the formation of glucose-xylose saccharides.

Attempted demonstration of glucose-1-phosphate → amylose trans-glucosidase (phosphorylase) activity

a) Synthetic activity

Enzyme: C. rupestris fraction A (413 mg.) in water (50 ml.)

Substrate: potassium salt of glucose-1-phosphate (158 mg.) in water (50 ml.)

Buffer: 0.2 M citrate pH 6.8.

Inhibitors: for glucose-1-phosphatase -1% ammonium molybdate,
and phosphoglucose mutase -0.0014 M mercuric chloride.

Primers: starch (5 mg.) in water (10 ml.),
laminarin (5 mg.) in water (10 ml.).

Digests, and controls, using enzyme heated to 100°C for 10 minutes, were set up as follows:- substrate (7 ml.), enzyme (5 ml.), buffer (10 ml.), primer (3 ml.), molybdate (14 ml.), mercuric chloride (0.7 ml.) and water (30.3 ml.). A digest and control were also set up without primer.

Inorganic phosphate (P) was determined in samples (5 ml.), as before. Iodine staining power was determined by adding 0.02% iodine in 2% potassium iodide (5 ml.) to a sample (2 ml.) and estimating the absorption in a 1 cm. cell against water using filter 603 on the Spekker absorptiometer. The iodine stains are quoted as the Spekker absorptiometer readings after subtraction of the appropriate control.

Time of incubation (days)	<u>Starch</u>		<u>Laminarin</u>		<u>Control</u> (without primer)	
	<u>% P liberated</u>	<u>Iodine Stain</u>	<u>% P liberated</u>	<u>Iodine Stain</u>	<u>% P liberated</u>	<u>Iodine Stain</u>
1	6.8	0.044	8.4	0.030	8.4	0.039
3*	7.6	0.035	8.4	0.034	9.2	0.039
4	6.1	-	13.7	-	-	-

* After this period a further portion of substrate (11.7 mg.) was added to the starch digest and a further portion of enzyme (21.8 mg.) to the laminarin digest.

b) Degradative activity

Enzyme: C. rupestris fraction A (400 mg.) in water (20 ml.)

Substrate: soluble starch (300 mg.) in water (15 ml.)

Buffer: 0.5 M phosphate pH 6.7

Inhibitors: for glucose-1-phosphatase - 1% ammonium molybdate,
and phosphoglucose mutase - 0.0014 M mercuric chloride.

A digest, and control, using enzyme heated to 100°C for 10 minutes, were set up as follows:- substrate (5 ml.), enzyme (5 ml.), buffer (4 ml.), molybdate (4 ml.), mercuric chloride (0.2 ml.) and water (1.8 ml.). Samples (2 ml.) were withdrawn at intervals and analysed for glucose-1-phosphate. After 4 days no significant amount of glucose-1-phosphate was detected.

It was concluded that the extract was devoid of glucose-1-phosphate → amylose transglucosidase activity.

Separation and identification of the oligosaccharides produced by the action of *C. rupestris* fraction A on maltose

a) Separation

The following large scale digest and control were set up:- maltose (32.5 gm.), fraction A (1.25 gm.) and 0.1 M acetate buffer pH 5.0 (250 ml.); maltose (8.1 gm.) and 0.1 M acetate buffer pH 5.0 (63 ml.). They were incubated at 35°C for 16 days before being heated to 100°C to inactivate the enzyme; the digests were then concentrated in vacuo.

The control digest was applied to a charcoal:celite column (21 x 4.5 cm.) and the sugars eluted in the normal fashion with aqueous ethanol (69). No significant amount of sugar other than maltose was obtained.

The digest on chromatographic analysis using irrigant 1 and spray 2 was shown to contain several oligosaccharides with the following R_F values:- 0.38, 0.27, 0.21, 0.15, 0.10 and 0.07, in addition to glucose and maltose. To facilitate the separation of the oligosaccharides, the digest was initially chromatographed

on a charcoal:celite column (79 x 4.5 cm.) and the glucose and maltose eluted with water and aqueous ethanol (4%). The oligosaccharides were then eluted with aqueous ethanol (30%) and the eluate concentrated in vacuo. The syrup was applied to a charcoal:celite column (70 x 4.5 cm.) and the oligosaccharides eluted with increasing concentration of aqueous ethanol as in the following table. The fractions collected were approximately 700 ml. each.

The oligosaccharides are referred to as M_0 , M_1 , M_2 etc. in order of elution from the column; in the preliminary published account of this work (132), the sugars were originally named in order of paper chromatographic mobility so that M_1 and M_2 referred to in ref. 132 are re-designated M_2 and M_1 respectively.

Separation of oligosaccharides from maltose and glucose

TABLE 3

<u>Solvent</u>	<u>Fractions</u>	<u>Sugars</u>
Water	1 - 5	Glucose
Water	6 - 11	Maltose + M_0^*
Water	12 - 15	Maltose
5% Ethanol	16 - 19	Maltose
5% Ethanol	20 - 21	Maltose and M_1^*
5% Ethanol	22 - 26	M_1 (1.38 gm)
5% Ethanol	27 - 31	M_1 , M_2 and traces maltose
10% Ethanol	32 - 34	M_1 , M_2 and traces maltose
10% Ethanol	35 - 45	M_2 and maltose*
20% Ethanol	47 - 53	Mixed higher oligosaccharides
50% Ethanol	55 - 57	Mixed higher oligosaccharides

* faint trace

Fractions 35 - 45 were recolumned and chromatographically pure M_2 (1.5 gm.) obtained. Fractions 47 - 57 were combined and concentrated to a syrup (3.6 gm.). This syrup was applied to a water-washed charcoal-celite column (67 x 3 cm.) prepared from B.D.H. activated charcoal (90 gm.) and celite "545" (90 gm.) according to a method communicated to Dr D.J. Manners by Dr J.S.D. Bacon. Elution, in a thermostatically controlled room (20°C), was effected in the usual manner. Fractions of ca. 700 ml. each were collected until the concentration of the alcohol was 6%, from which point onwards fractions of ca. 7 ml. each were collected on an automatic fraction collector. The results are presented in Table 4.

Separation of oligosaccharides

TABLE 4

<u>Solvent</u>	<u>Fractions</u>	<u>Sugars (mg.)</u>
Water, 1% and 2% ethanol	1 - 4	Glucose
5% Ethanol	7	M_0 (25)
<u>Tube numbers</u>		
6% Ethanol	71 - 360	Maltose } 132
7% Ethanol	361 - 460	Maltose)
7% Ethanol	480 - 549	M_1)
8% Ethanol	550 - 819	M_1 } 683
9% Ethanol	820 - 970	M_1)
9% Ethanol	1031 - 1329	M_2)
10% Ethanol	1330 - 1560	M_2 } 613

Table 4 continued.

10% Ethanol	1561 - 1880	M ₂ , M ₃	110
11% Ethanol	1901 - 1990	M ₁ , M ₂ , M ₄	40
11% Ethanol	1991 - 2429	M ₄)	300
12% Ethanol	2430 - 2610	M ₄)	
12% Ethanol	2611 - 2729	M ₄ , M ₅	28
12% Ethanol	2730 - 2999	M ₅)	452
13% Ethanol	3000 - 3869	M ₅)	
14% Ethanol	3870 - 4099	M ₅)	
15% Ethanol	4100 - 4410	M ₅)	
15% Ethanol	4411 - 4459	M ₅ , M ₆)	75
16% Ethanol	4460 - 4680	M ₅ , M ₆)	
17% Ethanol	4681 - 4749	M ₅ , M ₆)	
17% Ethanol	4750 - 4890	M ₆)	15
18% Ethanol	4891 - 4960	M ₆)	

Pure M₃ (10 mg.) was obtained by separation on a paper chromatogram (3MM).

b) Characterisation of oligosaccharides

(1) Paper Partition chromatography

Free sugars. The R_G values of the sugars were obtained usings irrigants 1 and 2, and spray 2.

R_G values of oligosaccharides

<u>Irrigant</u>	M ₀	M ₁	M ₂	M ₃	M ₄	M ₅	M ₆
1	0.38	0.18	0.35	0.07	0.11	0.19	0.03
2	0.50	0.28	0.41	0.14	0.17	0.26	0.09

R_G values of reference sugars

<u>Irrigant</u>	<u>Maltose</u>	<u>Maltotriose</u>	<u>Isomaltose</u>	<u>Isomaltotriose</u>
1	0.55	0.33	0.38	0.12
2	0.60	0.41	0.48	0.20

The R_G values of these sugars were obtained using separate chromatograms; authentic maltotriose was kindly supplied by Dr W.J. Whelan. M₀ and M₂ thus have the same R_G values as authentic isomaltose and maltotriose respectively.

N-benzyl glycosylamine derivatives

The chromatographic mobilities of M₁ and M₂ were compared with those of authentic samples of maltose and maltotriose using the method of Bayly and Bourne (p.27).

R_G values of N-benzyl glycosylamine derivatives

<u>Maltose</u>	<u>Maltotriose</u>	<u>M₁</u>	<u>M₂</u>
0.57	0.31	0.29	0.31

(ii) Estimation of degree of polymerisation

The sugars (ca. 5 mg.) were dissolved in water (10 ml.) and the reducing power of samples (2 ml.) estimated using reagent A; M₀ was heated for 20 minutes and the other sugars for 60 minutes. The reducing powers were calculated as equivalents of maltose. Samples (2 ml.) were hydrolysed with sulphuric acid (0.12 ml., 36 N) at 100°C for 2 hours, neutralised with sodium hydroxide using phenolphthalein as indicator, diluted to 5 ml. and the glucose estimated using reagent A. The degrees of polymerisation were calculated as follows:-

$$\text{Weight of oligosaccharide / 2 ml.} = \frac{\text{weight of glucose in hydrolysate} \times 162}{180}$$

$$\text{Degree of polymerisation} = \frac{\text{weight of oligosaccharide} \times 342}{\text{equivalent weight of maltose} \times 162}$$

	M_0	M_2	M_3	M_4	M_5	M_6
Degree of polymerisation	1.94	3.04	2.63	2.86	3.95	3.69

M_2 , M_3 and M_4 would appear to be trisaccharides and M_5 and M_6 tetrasaccharides.

(iii) Characterisation of M_1 as panose

Partial acid hydrolysis. M_1 (1ml., 1%) and sulphuric acid (0.6 ml., 0.5 N) were heated at 100°C for 1 hour, neutralised and concentrated. Paper chromatography using irrigant 2 and spray 2, indicated the presence of M_1 , isomaltose, maltose and glucose.

Oxidation followed by partial acid hydrolysis (133). M_1 (5 ml., 1%), iodine (30 mg.) and potassium hydroxide (0.2 ml., 1.0 M) were shaken together and allowed to stand for 30 minutes before the addition of a further portion of alkali (0.1 ml.). After standing overnight, a sample (1 ml.) was partially hydrolysed as above. Chromatographic analysis indicated the presence of isomaltose and glucose.

Reduction and acetylation (134). To M_1 (150 mg.) in water (3 ml.) was added potassium borohydride (1 ml., 4.5%) and after 2 hours at room temperature, the excess reagent was destroyed by acidification with acetic acid. The solution was neutralised with sodium hydroxide and concentrated to dryness. The product was acetylated using sodium acetate (162 mg.) and acetic anhydride (3 ml.) by heating to 135°C for 20 minutes. The product (120 mg.), after separation by

pouring into ice-water, and recrystallisation from ethanol had m.p. $148^{\circ} - 148.5^{\circ}\text{C}$: mixed m.p. (with authentic sample of panitol dodecaacetate kindly supplied by Dr W. J. Whelan) $148^{\circ} - 148.5^{\circ}\text{C}$ and $[\alpha]_{\text{D}}^{15} +119^{\circ}$ (c. 1.0 in chloroform), in agreement with the value of $[\alpha]_{\text{D}}^{27} +120^{\circ}$ (c. 4.0 in chloroform) reported by Wolfrom, Thompson and Galkowski (135) for panitol dodecaacetate.

Optical rotation

M_1 had $[\alpha]_{\text{D}}^{15} +148.3^{\circ}$ (c. 3.7 in water); Pazur and French (123) give $[\alpha]_{\text{D}} +150^{\circ}$ for panose.

(iv) Characterisation of M_2 as maltotriose

Partial acid hydrolysis. M_2 (1 ml., 1%) and sulphuric acid (0.6 ml., 0.1 N) were heated at 100°C for 1 hour, and the hydrolysate treated in the same way as that of M_1 (p.94). Chromatographic analysis indicated the presence of M_2 , maltose and glucose in the hydrolysate.

Oxidation followed by partial acid hydrolysis. The oxidation was effected using the same method as for M_1 and the hydrolysis as above. Maltose and glucose were the degradation products.

α -Amylolysis. A digest was set up consisting of M_2 (20 mg.), unpurified salivary amylase (0.2 ml.), sodium chloride (4 mg.) and 0.1 M acetate buffer pH 5.0 (0.2 ml.). Chromatographic analysis using irrigant 1 and spray 1 after 1.5 hours showed the presence of M_2 , maltose and glucose and after 2 days of maltose and glucose.

β -Amylolysis. A digest was set up as follows, using a barley β -amylase preparation from the Wallerstein Laboratories

(New York) - M_2 (13 mg.), β -amylase (0.5 ml., 30 units), 0.2 M acetate buffer pH 5.0 (1.5 ml.) and water (9.5 ml.). After 2 hours incubation, a portion (7 ml.) was withdrawn and added to fresh β -amylase (6 mg., 600 units). Samples (1 ml.) were withdrawn at intervals and the reducing power estimated using method A by heating for 20 minutes. The results are quoted as ml. of 0.01 N thiosulphate.

Time (hr.)	0	0.5	1.0	2.0	4.0	23.0	48.0	123.0
Reducing power	0.91	0.96	1.05	1.04	1.08	1.08	1.16	1.19

M_2 is therefore slowly hydrolysed by the β -amylase preparation; it is possible that this activity is due to the presence of maltotriase, rather than the β -amylase itself (see p.100).

Optical rotation

The concentration of a solution of M_2 was determined by acid hydrolysis and estimation of the glucose produced by method A. M_2 had

$[\alpha]_D^{15} +164^\circ$ (c. 2.8 in water), Sugihara and Wolfrom (136) and Whelan, Bailey and Roberts (137) have reported that maltotriose has $[\alpha]_D^{23} +160^\circ$ (c. 2.36 in water).

(v) Action of α -and β -amylases on M_4 and M_5

Digests were prepared consisting of M_4 or M_5 (6 mg.) sodium chloride (0.05 ml., 0.6 M), phosphate-citrate buffer pH 7.0 (0.05 ml.) and unpurified salivary amylase (0.1 ml.), and M_4 or M_5 (6 mg.), water (0.15 ml.), 0.2 M acetate buffer pH 5.0 (0.05 ml.) and barley β -amylase preparation (3 mg., 300 units).

Chromatographic analysis after 1.5 hours incubation indicated that M_4 had not been degraded; M_5 on treatment with α -amylase was completely hydrolysed to maltose and glucose, and with β -amylase to maltose. This indicates the presence of α ,1:4-glucosidic linkages in M_5 .

DISCUSSION

The algal extracts, which contain considerable α - and β -glucosidase activity, have now been shown to catalyse the transfer of glucose from phenyl β -glucoside, maltose, isomaltose and cellobiose to various acceptor substrates.

Synthesis from monosaccharides

The use of glucose as the donor substrate failed to produce transglucosidation to either other glucose molecules or to methanol; similar absence of synthetic activity was observed using xylose as the donor and acceptor substrate. These results thus confirm the conclusion that the algal β -glucosidase differs from those of almond emulsin and yeast, which are capable of catalysing transglucosidation with glucose as the donor substrate and glucose or methanol as the acceptor molecule, resulting in the production of β -linked disaccharides or methyl β -glucoside respectively (68, 73).

Transglucosidation from α -glucosides

Several reports have been made of transglucosidation reactions from substrates containing α -glucosidic links (114 - 120). Pazur (138) using C^{14} labelled glucose and an extract from A. oryzae has demonstrated the reversibility of the reaction -

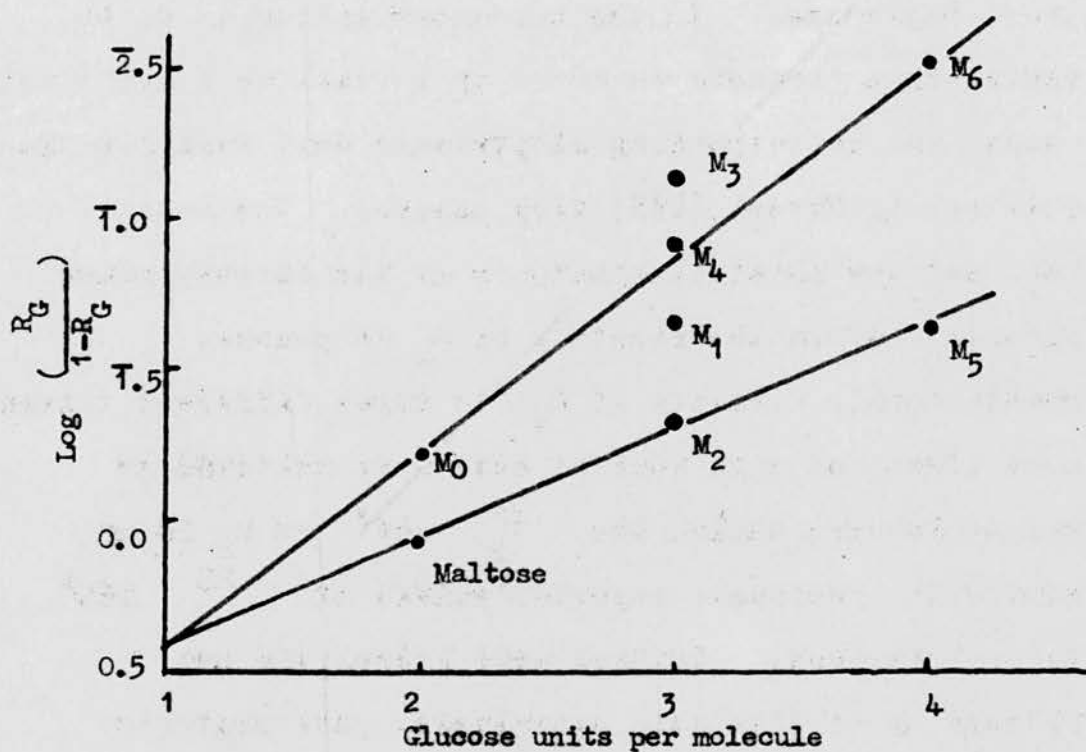


The extract of C. rupestris was unable to catalyse transglucosidation using sucrose and phenyl α -glucoside as donor substrates although with isomaltose as donor, an oligosaccharide with the same chromatographic mobility as isomaltotriose was formed thus indicating the ability of the extract to catalyse the synthesis of

α ,1:6-glucosidic linkages. The results in Table 2 indicate that transglucosidation from maltose to fructose, galactose or sucrose has not taken place, since the R_G values of the oligosaccharides produced were similar to those obtained when maltose served as both donor and acceptor substrate. This is further substantiated by the observation that on spraying the chromatograms of the sucrose-maltose digest with spray 3, a ketose reagent, sucrose was the only ketose present. Both the algal extracts show strong transglucosidase activity with maltose as both donor and acceptor substrate. In this reaction water acts as a competitive acceptor since a digest (Table 1, digest 9) containing the same ratio of maltose : extract as another (Table 1, digest 7), but in a more dilute solution, failed to show any transglucosidase activity.

To identify some of the oligosaccharides produced, a large scale digest and control were set up and the sugars partially separated by use of charcoal : celite columns following the method of Whistler and Durso (69). The oligosaccharides were then further separated using a charcoal : celite column in which the charcoal and celite had not been pre-washed. Estimation of the degrees of polymerisation of the oligosaccharides thus obtained indicated that one disaccharide, four trisaccharides and two tetrasaccharides had been formed; no oligosaccharides were found in the control experiment showing that non-enzymic oligosaccharide synthesis did not occur. The disaccharide, M_0 , of which only 25 mg. was obtained, has been tentatively identified as isomaltose since it exhibited the same chromatographic mobility as authentic isomaltose. The oligosaccharides M_1 and M_2 which were obtained in the highest yields (2.06 gm. and 2.11 gm. respectively) have been identified

Graph 1



Correlation of carbohydrate structure with chromatographic
mobility using the method of French and Wild (45).

as panose and maltotriose.

Pan, Andreassen and Kolachov (116) isolated a trisaccharide, later called panose, which was produced by the action of A. niger on maltose. This sugar was later characterised (133, 135) as 4- α -isomaltosyl-D-glucose. In the characterisation of M_1 as panose the degradation products obtained by partial acid hydrolysis of the free sugar and corresponding aldotrionic acid were identical with those obtained by French (133) from panose. The optical rotation of M_1 , and the physical constants of the corresponding acetylated alcohol confirm the identity of M_1 as panose.

The chromatographic mobility of M_2 , in three different solvents, was found to be identical with that of authentic maltotriose (4- α -maltosyl-D-glucose) whilst the $[\alpha]_D^{15} +164^\circ$ for M_2 is in close agreement with previously reported values of $[\alpha]_D^{23} +160^\circ$ (136, 137) for maltotriose. Partial acid hydrolysis and oxidation followed by partial acid hydrolysis, gave products identical with those obtained from maltotriose. Incubation of M_2 with salivary amylase produced maltose and glucose. Whelan and Roberts (139), using β -amylase preparations from soya beans and sweet potatoes, observed a slow hydrolysis of maltotriose only when a high concentration of enzyme was used. This may be due to maltotriase activity in the preparations. A similar slow hydrolysis has been found with M_2 , which shows the absence of glucosidic linkages other than those of the $\alpha,1:4$ - type. M_2 is therefore maltotriose.

M_4 , a trisaccharide, has the same chromatographic mobility as isomaltotriose and is resistant to hydrolysis by α - and β -

amylase preparations, an observation which confirms the presence of at least one linkage which is not $\alpha,1:4$.

M_5 is identified as maltotetraose from the degree of polymerisation, the paper chromatographic mobility (see Graph 1, p.100), hydrolysis by β -amylase giving maltose and no other sugar, and hydrolysis by α -amylase yielding glucose and maltose.

M_6 is tentatively identified as isomaltotetraose since it is a reducing tetrasaccharide, and a graph of the logarithm of the partition function against molecular size shows a linear relationship with those of isomaltose and isomaltotriose.

From Graph 1, M_1 is shown to have a partition function intermediate in value between the series of the $\alpha,1:4$ - and the $\alpha,1:6$ -glucosaccharides, in agreement with the evidence that M_1 is panose. In contrast, the partition function of M_3 shows no obvious relationship to those of the other oligosaccharides; the constitution of M_3 , a reducing trisaccharide, is, as yet, unknown. Characterisation of the oligosaccharides, by the preparation of crystalline derivatives, is being continued.

Several workers have reported the presence of an enzyme in certain moulds (116, 118, 123) which is capable of synthesising branched-chain oligosaccharides from maltose. The ability of the extract of C. rupestris to synthesise both oligosaccharides of the maltose and isomaltose series, and panose is therefore possibly due to two enzymes one of which catalyses by trans-glucosidation, the synthesis of $\alpha,1:4$ - links and the other of $\alpha,1:6$ - links.

Transglucosidation from β -glucosides

The extract of C. rupestris catalysed the transfer of a glucose residue from phenyl β -glucoside to methanol, a reaction similar to that of the β -glucosidase obtained from various green leaves (128). The product is tentatively identified as a methyl glucoside by its chromatographic mobility and inability to be detected by aniline oxalate on a chromatogram; it seems probable that it is methyl β -glucoside.

It has been shown that transglucosidation may be effected from cellobiose to either D- or L-xylose but not to N-acetyl glucosamine or L-sorbose although all four have been reported to act as acceptors using a β -glucosidase preparation from A. niger (122). In the digest with cellobiose alone several oligosaccharides were formed. One of these, with the highest R_G value, corresponded to laminaribiose, 3- β -glucosyl-D-glucose; during the later stages of the enzymic degradation of cellodextrin by an extract of C. rupestris (p. 51) the occurrence of a similar oligosaccharide was observed. It thus seems likely that this was not a degradation product but was formed by transglucosidation. It is of interest that Crook and Stone (121) observed, during the action of fungal extracts or a preparation from the gut of Helix pomatia on cellobiose, the formation of a similar oligosaccharide, together with a series of oligosaccharides which corresponded to those produced by acid hydrolysis of cellodextrin. More recently Barker and his colleagues (122) using cellobiose as both donor and acceptor substrate and an extract from A. niger observed the formation of a series of trisaccharides which appear to contain β ,1:6-, 1:4-, 1:3- and 1:2- links.

Glucose-1-phosphate $\rightarrow$ amylose transglucosidase

An attempt to demonstrate synthetic activity in the extract of C. rupestris using glucose-1- phosphate as donor substrate was unsuccessful even in the presence of starch and laminarin as reaction primers. On incubation of starch with inorganic phosphate, glucose-1-phosphate was not produced thus indicating that the extract of C. rupestris did not contain glucose-1-phosphate $\rightarrow$ amylose transglucosidase. This type of activity has recently been demonstrated in two types of green algae viz. Oscillatoria princeps (140) and Chlorella vulgaris (141).

SUMMARY

1. The algal extracts were unable to catalyse transglycosidation reactions using monosaccharides as the donor substrates.
2. The extract of C. rupestris catalyses the synthesis of α 1:4- and 1:6- links, using maltose and isomaltose as substrates.
3. Panose (4- α -isomaltosyl-D-glucose), maltotriose (4- α -maltosyl-D-glucose) and (?) maltotetraose (4- α -maltotriosyl-D-glucose), synthesised by the action of C. rupestris extract on maltose, have been separated and characterised; other oligosaccharides have also been isolated.
4. Cellobiose is a suitable donor substrate for transglucosidation to cellobiose, D- and L-xylose in the presence of an extract of C. rupestris.
5. An attempt to demonstrate the presence of glucose-1-phosphate $\rightarrow$ amylose transglucosidase in the extract of C. rupestris was unsuccessful.

SECTION FOUR

Studies on the purification and properties of the amylase in the extract of C. rupestris

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INTRODUCTION

The structures of starch, the main reserve carbohydrate of the higher plants, and glycogen, which has a similar function in the animal kingdom, have been studied using both chemical and biochemical techniques. Starch, a heterogeneous glucopoly-saccharide, contains two components, amylose and amylopectin, the proportions of which vary according to the botanical source of the starch. Amylose, usually the minor component, has an essentially linear structure of several hundred glucose residues united by α ,1:4- links, although evidence for the presence of a few anomalous linkages in certain samples of amylose has recently been obtained (142). The other component, amylopectin, a branched molecule of high molecular weight, contains chains of α ,1:4- linked glucose residues (average chain length 20 - 25) which are interlinked by α ,1:6-glucosidic linkages.

Glycogen has a structure generally similar to that of amylopectin but is more highly branched since it possesses, in the majority of samples, fewer glucose residues (ca. 12) per chain.

The enzymic hydrolysis of these polysaccharides is catalysed by the amylases which degrade the α ,1:4-glucosidic linkages. The mode of action and occurrence of these enzymes have been reviewed by Myrbäck and Neumüller (143) and Manners (142, 21). α -Amylases degrade these α -glucosans by random hydrolysis whilst β -amylases and amylo-glucosidases exert a step-wise action commencing at the non-reducing ends of the chains of glucose residues. The amylases

appear to be exclusively degradative, with the exception of that from Bacillus macerans which converts starch into a mixture of cyclic dextrans without the production of reducing sugars (115).

α -Amylolysis of amylose produces a series of linear α -dextrans (6 - 10 glucose units) which may then be slowly hydrolysed to fermentable sugars. The degradation of amylopectin and glycogen by α -amylase produces branched dextrans, the smallest of which is a pentasaccharide (21, 144).

Amylose, unless it contains anomalous linkages, is completely degraded by β -amylase yielding maltose. Unlike α -amylase, β -amylase cannot by-pass branch-points in the molecule, with the result that β -amylolysis of amylopectin and glycogen produces maltose and a β -limit dextrin of high molecular weight in which all the $\alpha_{1:6}$ - linkages of the original molecule are intact.

The α - and β -amylases may be differentiated by measurement of the rate of change of iodine absorption value and the increase in reducing power of the polysaccharide-enzyme digest. A rapid decrease in iodine absorption value with a small apparent percentage hydrolysis is typical of the α -amylases, and takes place during the first (dextrinisation) stage of the reaction. Saccharification takes place at the commencement of β -amylolysis whilst with α -amylolysis it occurs only after dextrinisation. The ratio saccharification value: dextrinisation value (PS : PD) has been used to identify α - and β -amylases; the value for α -amylases is ca. 10 and for β -amylases ca. 60 (e.g. 145). The purification and subsequent crystallisation of α - and β -amylases from many different sources has been achieved (142); some of these enzymes have then been used in studies on the fine structures of the $\alpha_{1:4}$ -glucosans (21).

In this section, an attempt has been made to obtain an extract from C. rupestris with a high amylase activity and a decreased maltase activity. The low isomaltase activity of the extract has been neglected. The effect of various anions on the amylase activity has been studied and the mode of action of the amylase determined.

EXPERIMENTAL

The whole extract of C. rupestris has been shown to contain hydrolytic activity towards α ,1:4-glucosans (Table 4, page 37); the only degradation product observed was glucose. In addition, the extract contains an α -glucosidase which hydrolyses maltose and an enzyme catalysing the hydrolysis of isomaltose (α ,1:6- links).

Methods

The colorimetric and paper chromatographic reagents used in this section are the same as those reported in section II. An additional cuprimetric reagent has been used to estimate glucose in the presence of maltose. This is the reagent of Phillips and Caldwell (146) which has been slightly modified, the potassium iodate solution being used at half the recommended strength and the whole estimation, including titration, being performed in Quickfit and Quartz test tubes (12 x 2 cm.). A calibration curve was prepared using mixtures of glucose and maltose which corresponded to different degrees of hydrolysis of maltose (3 mg.).

Attempted purification of amylase activity

Units of amylase activity

Digests of the following composition were used in activity determinations:- soluble starch (4 ml., 0.32%), 0.2 M acetate buffer pH 5.6 (3 ml.), sodium chloride (1 ml., 0.6 M) and extract fraction (2 ml.). Glucose was estimated in samples (4 ml.) after 30 minutes incubation at 15°C, using reagent A.

The unit of activity is defined as the amount of enzyme liberating 10 μ gm. glucose from the above digest, within 30 minutes.

Specific activities are quoted as units of activity per mg. protein-N. The activity was shown to be directly proportional to the concentration of the algal extract.

Units of maltase activity

The extract solution (1 ml.) at 35°C was added to a mixture of maltose (2 ml., 0.375%) and 0.2 M acetate buffer pH 5.6 (2 ml.), also at 35°C. The digest was incubated for 3 hours and the glucose produced estimated on samples (2 ml.) using Phillips and Caldwell's reagent.

A unit of maltase activity is defined as the amount of enzyme liberating 100 μ gm. glucose from the above digest within 3 hours. The activity was directly proportional to the concentration of the algal extract.

Separation of several activities of *C. rupestris* fraction III using electrophoresis

Fraction III (60 mg.) was dissolved in McIlvaine phosphate-citrate buffer pH 3.3 (0.5 ml.) and spotted as a band on Whatman 3MM paper (10 x 57 cm.) which had been saturated with the buffer. The electrophoresis apparatus was similar to that described by Woods and Gillespie (147). After an equilibration period of one hour, approximately 110 v. was applied for 44 hours. Part of the paper, after drying at room temperature, was cut into 1 cm. wide longitudinal strips which were sprayed with the appropriate substrate (125 mg. dissolved in 10 ml. water and 5 ml. 0.2 M acetate buffer pH 5.0). The strips were incubated for 4 days in a moist atmosphere at 28°C before development with spray 1 (2% phosphoric acid was incorporated in the spray). To determine the position of the maltase, strips, 3 cm. wide cut from the paper parallel to the

starting line, were eluted with water (0.5 ml.) and the eluate incubated with maltose (0.5 ml., 1% in acetate buffer pH 5.0). The digests were examined chromatographically using irrigant 2 and spray 1. The following movements were indicated.

Substrate	Anode	Origin
Maltose	15 cm. ← 9 cm.	
Starch	16.5 cm. ← 15 cm.	
Trehalose	20 cm. ← 16 cm.	
Phenyl β -glucoside		6 cm. ← 2 cm.

Optimum conditions for the extraction of the amylase activity from *C. rupestris* fraction W

Portions (250 mg.) of fraction W (whole extract, p. 16) were stirred at 1°C with the undernoted solvents (40 ml.) and samples (3 ml.) removed after 2, 4 and 6 hours, centrifuged and the supernatant solution (2 ml.) used for estimation of amylase activity. The activity digests were similar to the standard digest except that the sodium chloride was replaced by buffer (1 ml.). After stirring for 6 hours the extracts (25 ml.) were centrifuged and the supernatant solutions dialysed overnight before lyophilizing. The insoluble residues were dried with acetone and weighed.

The results are quoted as increase in reducing power (ml. 0.01 N thiosulphate) of the digests after 24 hours incubation at 35°C.

S O L V E N T S

<u>Sample</u>	<u>0.2 M acetate buffer pH 5.6</u>	<u>0.25% Na₂CO₃</u>	<u>1% KCl</u>	<u>Distilled Water</u>
Stirring for 2 hours	1.69	0.71	4.22	1.09
Stirring for 4 hours	1.79	0.52	4.94	1.66
Stirring for 6 hours	2.75	0.76	5.32	1.87

- - - - -

<u>Sample</u>	<u>0.2 M acetate buffer pH 5.6</u>	<u>S O L V E N T S</u>		<u>Distilled Water</u>
		<u>0.25% Na₂CO₃</u>	<u>1% KCl</u>	
Weight of insoluble residue (mg.)	49.9	118.1	42.2	52.5
Weight of lyophilized extract (mg.)	28.0	63.8	30.0	38.6

The high activity of the potassium chloride extract, as is shown later, is due to activation of the amylase by chloride ions. The results indicate that re-extraction of amylase activity from the lyophilized extract requires stirring, with distilled water or acetate buffer pH 5.6, for several hours.

Preparation of extract

Fraction W (2.0 gm.), 0.2 M acetate buffer pH 5.6 (160 ml.) and distilled water (160 ml.) were stirred together for 18 - 20 hours at 1°C. The residual material (0.96 gm., 7.6% protein-N) was separated by centrifugation, the supernatant solution dialysed for 2 days and lyophilized to yield a readily soluble product (0.54 gm., 6.9% protein-N). This extraction procedure was repeated as required, similar results being obtained. The soluble product contained 23 amylase units/mg. protein-N.

Attempted precipitation of inactive material from the extract of fraction W.

The soluble extract of fraction W (0.54 gm.) was dissolved in water (320 ml.) and divided into portions (35 ml.). To each portion was added 0.2 M acetate buffer of appropriate pH value (5 ml.) and the solutions allowed to stand at 1°C for 24 hours before centrifugation in weighed tubes. The pH values 6.1, 6.3 and 6.8 were obtained by adding sodium hydroxide to the solutions. Digests

were prepared using soluble starch (4 ml., 0.32%) or maltose (4 ml., 0.125%), 0.2 M acetate buffer pH 5.6 (4 ml.) and supernatant solutions (2 ml.). The results are quoted as increase in reducing power of samples (4 ml.) after incubation for 24 hours.

Solution pH	3.5	4.0	4.4	4.8	5.3	5.6	6.1	6.3	6.8
Inactive precipitate (mg.)	4.0	4.1	3.0	3.1	3.1	3.2	3.0	2.2	2.1
Turbidity	+++++	++++	+++	+++	++	+	+	+	+
Amylase activity	0.28	0.53	0.99	1.12	1.15	1.21	1.10	1.15	1.13
Maltase activity	0.85	1.33	1.65	1.67	1.71	1.85	1.72	1.65	1.70

These results indicate that there is no preferential precipitation of either the maltase or amylase activities.

Fractionation with ammonium sulphate

The extracts of *C. rupestris* prepared by fractional precipitation with ammonium sulphate (p.¹⁵) were examined for amylase activity.

Fraction	I	II	III	IV
Saturation with amm. sulphate	0.0 - 0.18	0.18 - 0.36	0.36 - 0.54	0.54 - 0.75
Yield (gm.)	17.7	5.7	2.1	0.9
Protein-N (%)	7.2	7.1	8.5	8.1
Amylase units / mg. protein-N	6	16	94	90
Total number of amylase units	7,647	6,477	16,780	6,560

The maltase activity was also found to increase from fraction I - IV (p. 44). These results indicate considerable increase in amylase activity by precipitation of other proteins with 0.36 saturated ammonium sulphate. The recovery is, however, low.

Precipitation with ethanol

A comparison of the amylase activity of fractions W, X, Y and Z (p. 16) indicated that considerable loss of activity occurred on precipitation of the whole extract with ethanol.

Precipitation with isopropanol

Fraction II contained 15.8 amylase units/mg. N and 7.7 maltase units/mg. N. Fraction II (2 gm., 2,244 amylase units and 1,093 maltase units), suspended in water (160 ml.) and 0.2 M acetate buffer pH 5.6 (40 ml.), was cooled to 1°C and isopropanol (86 ml.), pre-cooled to -5°C, added, over a period of 10 minutes, with constant stirring. The precipitate, fraction 30, was separated by centrifugation, dialysed for 2 days and lyophilized. The supernatant solution (250 ml.) was treated with isopropanol (250 ml.) as before to yield a precipitate, fraction 65 which was dialysed and lyophilized. The residual solution (470 ml.) was precipitated with a further portion of isopropanol (470 ml.) to yield a third precipitate (fraction 90). The latter weighed less than 10 mg. and was not examined further.

<u>Fraction</u>	<u>Yield (gm.)</u>	<u>Protein-N(%)</u>	<u>Amylase</u>		<u>Maltase</u>	
			<u>Specific activity</u>	<u>Total</u>	<u>Specific activity</u>	<u>Total</u>
30	1.126	8.2	4.6	422	4.3	401
65	0.431	7.8	54.5	1822	20.2	679

Fraction 65 represents a 3.5-fold increase in amylase activity with 81% recovery; the maltase activity increased 2.6-fold with a 63% yield.

Fraction 65 (300 mg., 1268 units amylase and 437 units maltase) was fractionally precipitated with isopropanol to yield three

fractions, the method being similar to that above. The third fraction weighed less than 10 mg. and was not examined further.

<u>Fraction</u>	<u>Yield (mg.)</u>	<u>Protein-N(%)</u>	<u>Amylase</u>		<u>Maltase</u>	
Percentage isopropanol (v/v)			Specific Activity	Total	Specific Activity	Total
0 - 40	140	8.8	8.0	98	-	-
40 - 59	86	9.0	91.4	708	27.2	211

For fraction 59 this represents a 1.8-fold increase in, and 55.8% recovery of, amylase activity, and a 1.3-fold increase in maltase activity with a 48.3% recovery compared with the first isopropanol fractionation. However, as the total recovery was much lower on this second fractionation it appears as though appreciable inactivation had occurred. The two stage isopropanol fractionation thus yields a 5.8-fold purification of the amylase from the original extract.

Partial purification of the amylase

The soluble extract (5.07 gm., 8,000 amylase units and 7,700 maltase units) was dissolved in water (800 ml.) and 0.2 M acetate buffer pH 5.6 (200 ml.) and ammonium sulphate added to 0.2 saturation. The precipitate, fraction 1, and the supernatant solution, fraction 2, were separated by centrifugation, dialysed and lyophilized.

Fraction 2 (1.75 gm.) was dissolved in water (160 ml.) and 0.2 M acetate buffer pH 5.6 (40 ml.) and a deep green product (<20 mg.) precipitated with isopropanol (50 ml.). A precipitate, fraction 3, which formed on addition of isopropanol (350 ml.) to the supernatant solution (250 ml.) was separated, dialysed and lyophilized. The supernatant solution from this precipitation contained the green pigment.

<u>Fraction</u>	<u>Yield</u> (gm.)	<u>% N</u>	<u>Amylase</u>		<u>Maltase</u>	
			<u>Specific</u> <u>Activity</u>	<u>Total</u>	<u>Specific</u> <u>Activity</u>	<u>Total</u>
<u>Ammonium sulphate fractionation</u>						
soluble extract	5.07	6.9	22.8	8,000	22.1	7,700
1	1.48	8.4	8.0	1,000	3.1	387
2	1.82	6.8	46.4	5,740	33.6	4,164
<u>Isopropanol fractionation</u>						
2	1.75	6.8	46.4	5,520	33.6	4,000
3	1.34	7.3	50.0	4,860	25.4	2,485

These results indicate that the maltase activity has increased by a factor of only 1.1 with a 32% recovery, whilst the amylase (62% yield) has shown a 2.2-fold increase in activity.

Attempted inhibition of maltase activity

In view of the reported inhibition of oligosaccharidases by the corresponding aldono-1:4-lactones (148, 149), an attempt was made to selectively inhibit the maltase present in the C. rupestris extract. A similar experiment was also devised using methyl α -glucoside which in high concentrations would be expected to competitively inhibit the maltase.

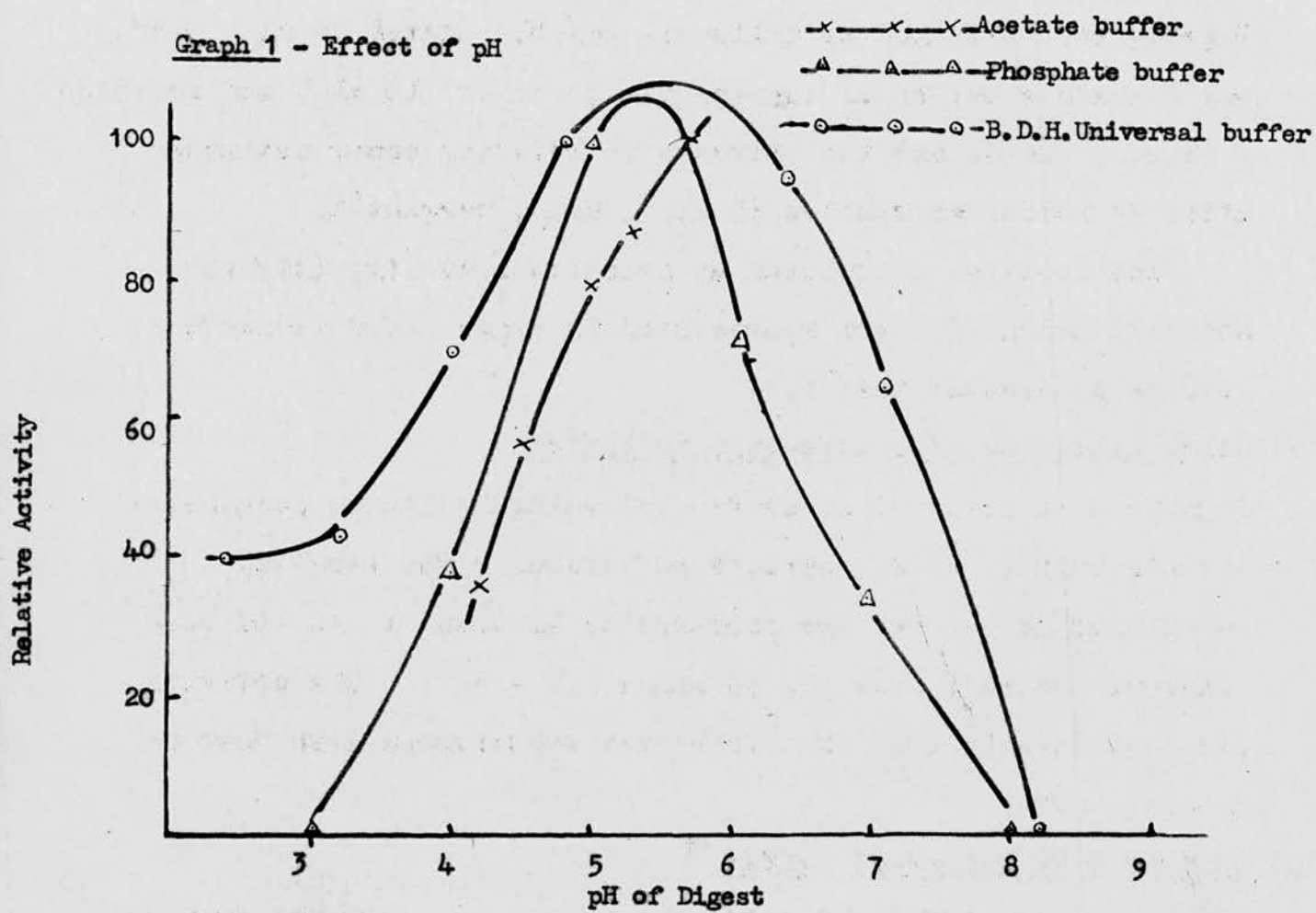
Substrate:- maltose (0.375%)
 Enzyme:- fraction 3 (2.4%)
 Buffer:- 0.2 M acetate pH 5.6
 Inhibitors:- glucurono-1:4-lactone (0.1 M) and methyl α -glucoside (0.1 M).

Digests were prepared as follows, and the increase in reducing power of samples (2 ml.) estimated after 3 hours incubation using Phillips and Caldwell's reagent.

Digest	1	2	3	4	5
Substrate (ml.)	2.0	2.0	2.0	2.0	2.0
Buffer (ml.)	2.0	2.0	2.0	2.0	2.0
Enzyme (ml.)	0.5	0.5	0.5	-	-
Lactone (ml.)	-	0.5	-	0.5	-
Methyl α - glucoside (ml.)	-	-	0.5	-	0.5
Water (ml.)	0.5	-	-	0.5	0.5
Increase in reducing power (ml. of 0.01 N thiosulphate)	3.79	3.13	3.42	-0.09	0.05
% Inhibition	-	17.4	9.8	-	-

The low degree of inhibition and high reducing power of glucurono-1:4-lactone make it unsuitable as an inhibitor in this work.

Graph 1 - Effect of pH



Properties of the amylase of *C. rupestris*

Determination of the effect of pH on amylase activity

a) Using acetate buffer

Digests were prepared as follows:- soluble starch (5 ml., 0.3%), 0.2 M acetate buffer of appropriate pH value (10 ml.) and fraction W (5 ml., 0.6%), and the increase in reducing power estimated after 24 hours, on samples (5 ml.), using reagent A.

The results, calculated as relative activity, (highest activity being 100) are represented in Graph 1 and indicate an optimum pH greater than 5.7.

b) Using McIlvaine phosphate-citrate buffer

Digests were prepared as above, but using McIlvaine phosphate-citrate buffers of appropriate pH values. The results, calculated as before, are represented in Graph 1 and indicate maximum activity over the pH range 5.2 - 5.4. The activity was considerably less in this buffer as compared with that in acetate buffer.

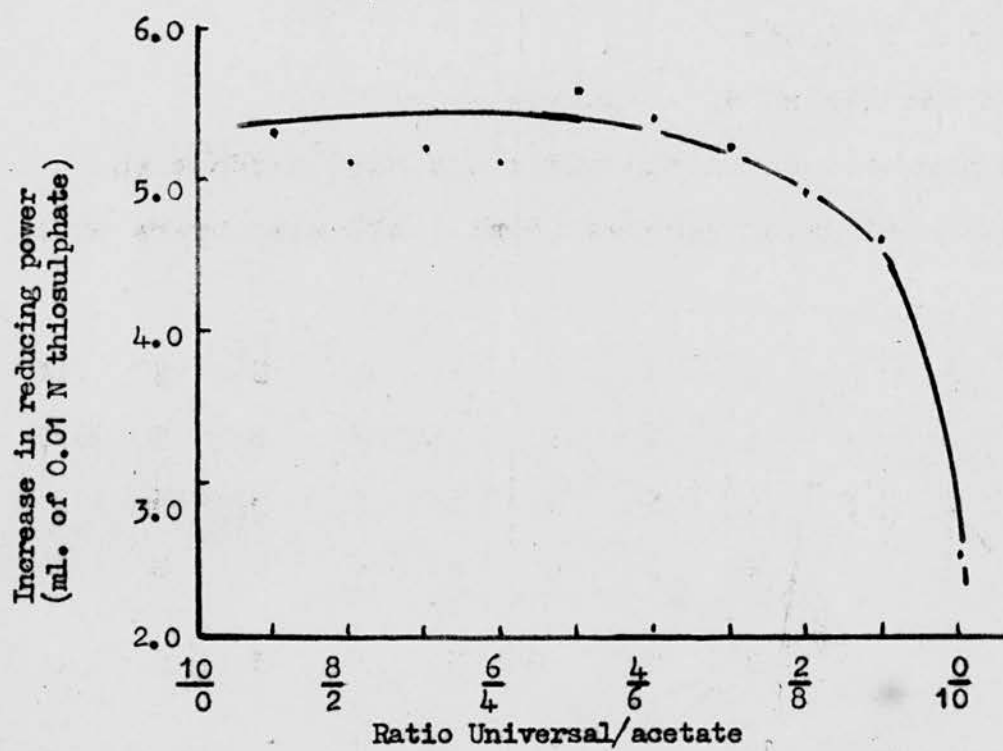
c) Using B.D.H. Universal buffer

The effect of buffers of different pH values, prepared from B.D.H. Universal buffer mixture, on the amylase activity was studied using digests similar to those above. The results, Graph 1, indicate maximum activity over the pH range 5.4 - 5.8.

d) Using a mixed buffer

Buffers of different pH values were prepared containing B.D.H. Universal buffer and 0.2 M acetate in equal proportions and incorporated in digests as above. The increase in reducing power of each digest was greater than the value obtained using either buffer by itself.

Graph 2 -Effect of varying proportions of Universal/acetate



pH	4.6	5.0	5.3	5.7
Relative activity	9.7	58.3	84.5	100

In the pH range 4.6 - 5.7 maximum activity is therefore obtained at pH 5.7; this is similar to the result observed using acetate buffer alone.

Effect of using varying proportions of Universal buffer to acetate buffer at pH 5.7

Substrate:- soluble starch (0.3%)

Enzyme:- fraction W (0.6%)

Buffers:- 0.2 M acetate pH 5.7; Universal pH 5.7

Digests were prepared as in the table and the increase in reducing power estimated using samples (5 ml.) after 24 hours, with reagent A.

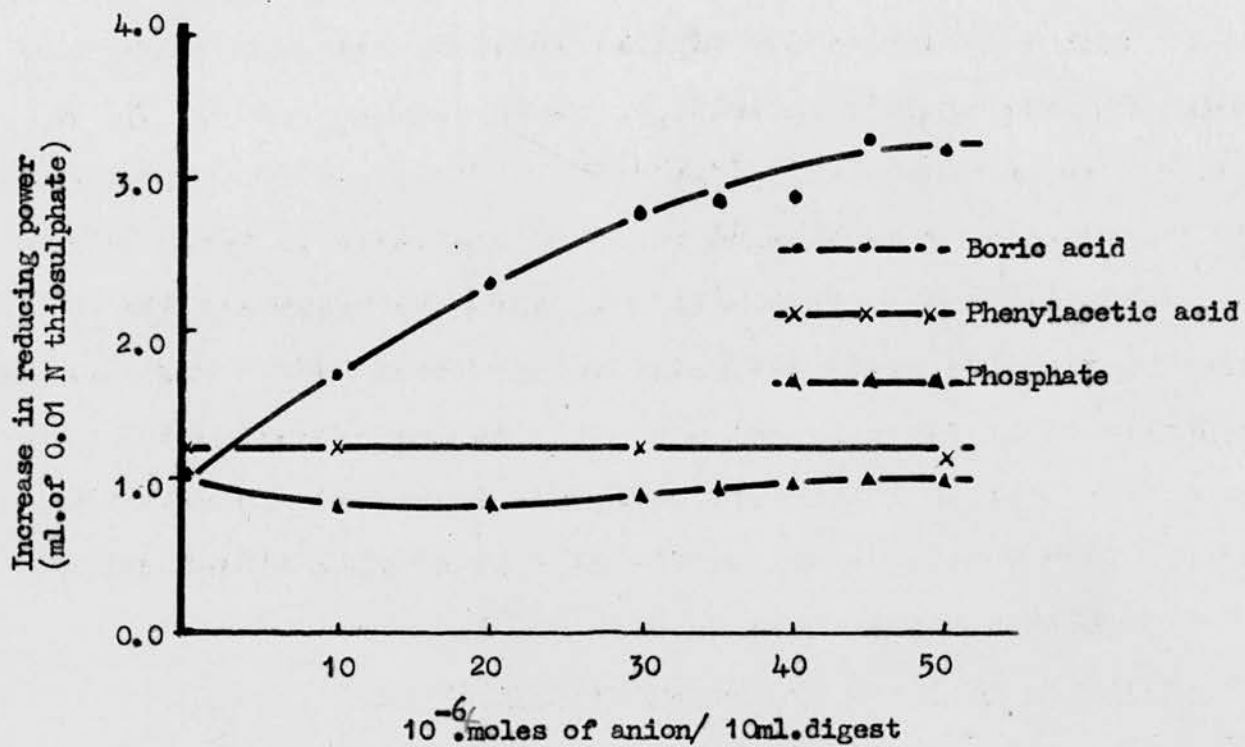
Digest	1	2	3	4	5	6	7	8	9	10	11
Substrate (ml.)	5	5	5	5	5	5	5	5	5	5	5
Enzyme (ml.)	5	5	5	5	5	5	5	5	5	5	5
Acetate (ml.) buffer	0	1	2	3	4	5	6	7	8	9	10
Universal (ml.) buffer	10	9	8	7	6	5	4	3	2	1	0
Increase in reducing power (ml. of 0.01 N thiosulphate)	5.1	5.3	5.1	5.2	5.1	5.6	5.4	5.2	4.9	4.6	2.5

These results indicate that Universal buffer has an activating effect on the amylase activity (Graph 2).

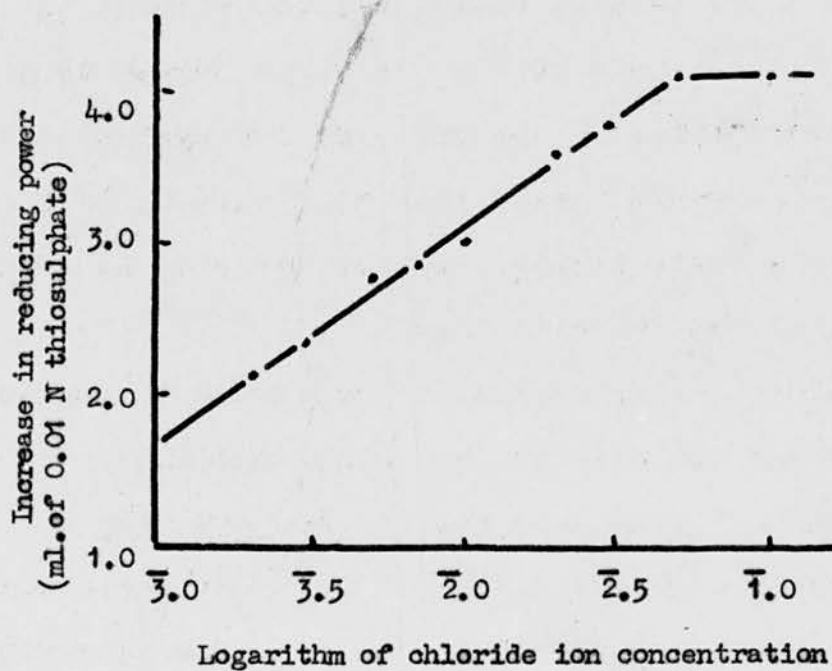
The amylase activating constituent(s) of Universal buffer

B.D.H. Universal buffer consists essentially of a mixture of sodium dihydrogen phosphate, phenylacetic acid and boric acid. The

Graph 3 - Activating constituent of Universal buffer



Graph 4 - Effect of sodium chloride



effect of each of these on the amylase activity has been studied by preparing digests containing soluble starch (2 ml., 0.32%), 0.2 M acetate buffer pH 5.6 (5 ml.), fraction W (2 ml., 0.6%), phosphate or phenyl acetic acid or boric acid (0.05 M) and water to total volume 10 ml. Samples (4 ml.) were withdrawn after 24 hours and the increase in reducing power estimated using method 'A'. The results are represented in Graph 3, and show that whereas phenylacetic acid had no effect, sodium dihydrogen phosphate depressed the activity slightly. Boric acid had a considerable activating effect which was maximum at a concentration of $45 \times 10^{-4} \text{M}$.

Effect of sodium chloride on the amylase activity

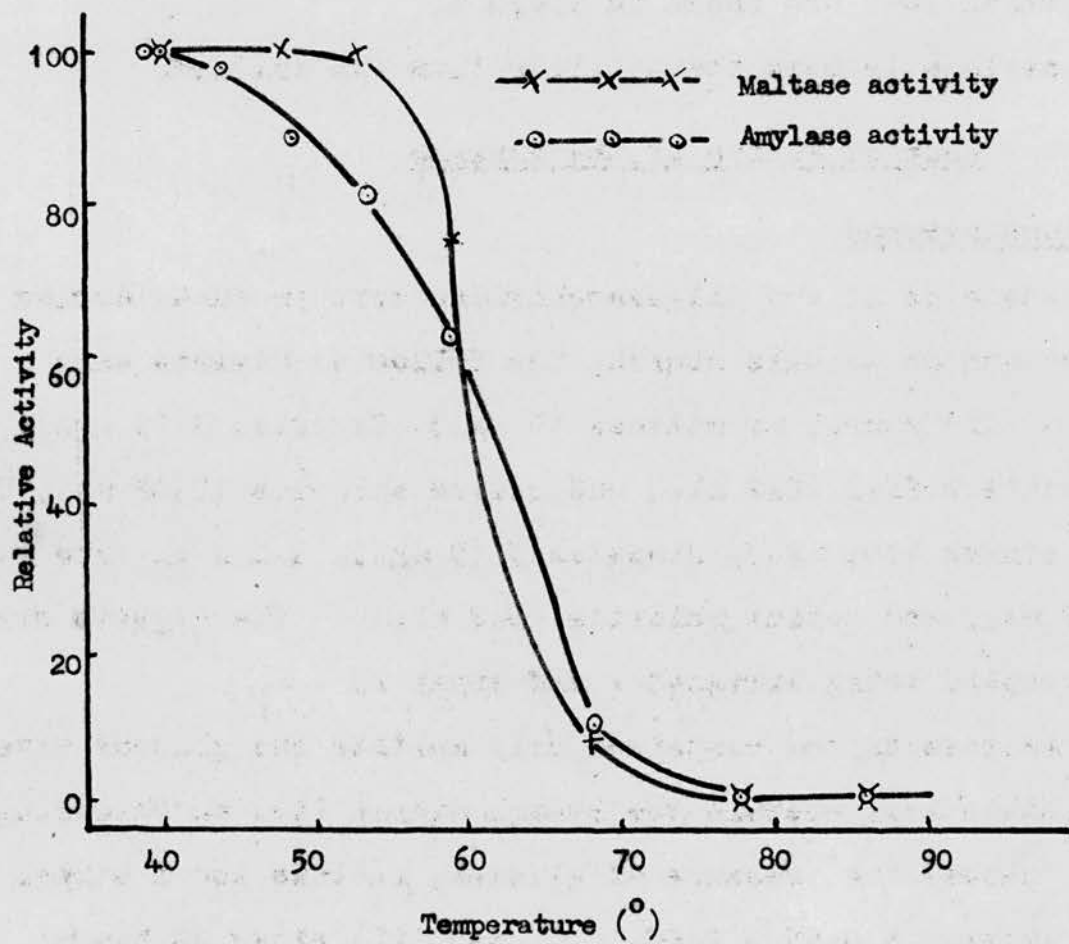
Digests were prepared as follows, and the increase in reducing power of samples (4 ml.) estimated, after 24 hours, using reagent A:- soluble starch (4 ml., 0.32%), 0.2 M acetate buffer pH 5.6 (3 ml.), fraction W soluble extract (2 ml., 0.2%), sodium chloride (0.1 M or 1.0 M) and water to total volume 10 ml. A graph (Graph 4) of the logarithm of the concentration of the chloride ion against the increase in reducing power was linear over the range 0.001 M - 0.05 M indicating that, within these limits, amylase activity is proportional to the concentration of the chloride ions.

A similar experiment using maltose as substrate indicated that the maltase activity was not affected by sodium chloride.

Effect of temperature on the amylase and maltase activity

The soluble extract of fraction W (78.5 mg.) was dissolved in water (25 ml.) and portions heated at the required temperature for 15 minutes, cooled to room temperature and centrifuged. Digests were prepared, consisting of soluble starch (4 ml., 0.32%) or

Graph 5 - Effect of temperature



maltose (4 ml., 0.125%), 0.2 M acetate buffer pH 5.6 (4 ml.) and the supernatant solutions (2 ml.). The increase in reducing power of samples (4 ml.) was estimated after 24 hours using method A, and the results calculated as relative activity (the highest activity being 100) are shown in Graph 5.

The amylase is more thermolabile than the maltase.

Mode of action of the amylase

Paper chromatography

To determine if any oligosaccharides were produced during amylase action on soluble starch, the following digests were prepared - (i) starch or maltose (5 mg.), fraction 3 (5 mg.), 0.1 M acetate buffer (0.2 ml.) and sodium chloride (0.02 ml., 0.6 M), and (ii) starch (100 mg.), fraction 3 (3 mg.), 0.1 M acetate buffer pH 5.6 (2 ml.) and sodium chloride (0.2 ml.). The digests were chromatographed using irrigant 2 and spray 2.

The maltose digest contained only maltose and glucose after 5 hours incubation, whereas the starch digest (i), on chromatographic analysis, showed the presence of glucose, maltose and a streak of reducing sugars, R_f 0.55 - 0.00. Digest (ii) after 24 hours incubation also contained glucose, maltose and higher oligosaccharides.

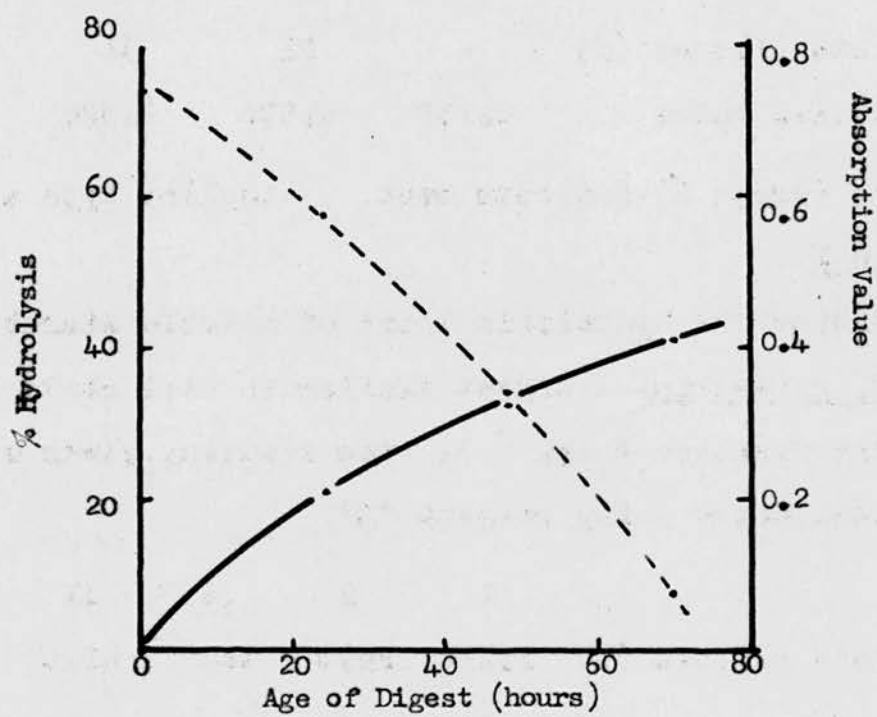
These results indicate that glucose is not the primary product of amylase action.

Hydrolysis of soluble starch

(i) Fraction A

A digest containing soluble starch (39 mg.), fraction A (60 mg.) and 0.075 M acetate buffer pH 5.1 (40 ml.) was prepared and samples (ca. 7 ml.) withdrawn at intervals, heated to 100°C and filtered.

Graph 6 -Degradation of starch



The liberated glucose ~~was~~ estimated using the filtrate (4 ml.) and reagent 'A'; the iodine absorption value of the filtrate (1 ml.) and 0.02% iodine in 0.2% potassium iodide (5 ml.) was measured in a 1 cm. cell with filter 608 on a Spekker absorptiometer. Iodine absorption values are quoted as the Spekker absorptiometer readings.

Time (hours)	0	24	48	70
Conversion into glucose (%)	-	21	34	41
Iodine absorption value	0.739	0.578	0.328	0.076

These results (Graph 6) indicate weak α -amylase type action.

(ii) Fraction W

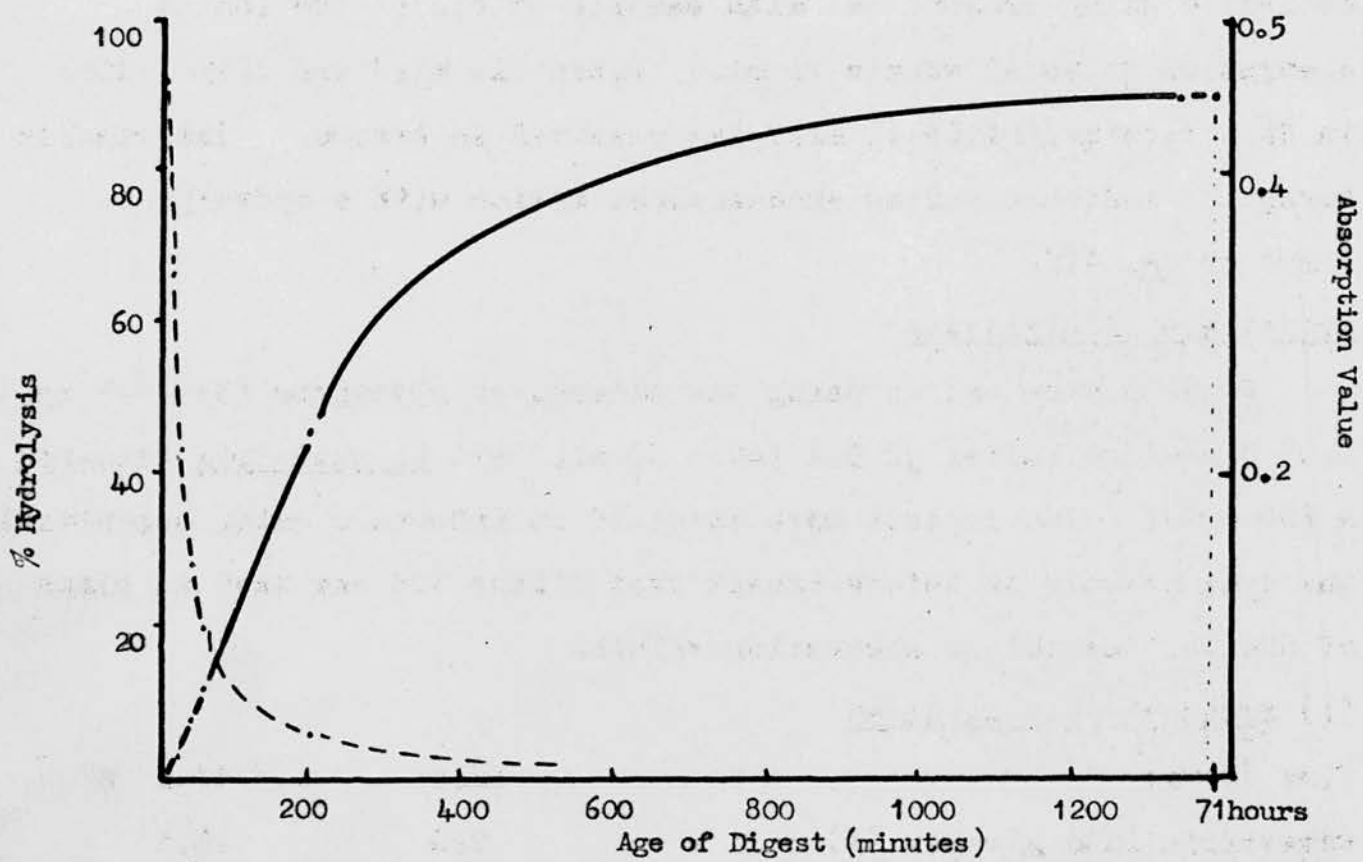
To determine the hydrolysis limit of soluble starch by a whole extract of C. rupestris, a digest similar to that above was prepared using fraction W (p. 16); the reducing power of samples (4 ml.) was estimated using reagent 'A'.

Time (days)	1	2	4	11	19	25
Conversion into glucose (%)	15.5	29.0	46.0	62.0	70.0	70.0

After 25 days incubation, chromatographic analysis indicated that glucose was the only degradation product. The digest was concentrated in vacuo, dialysed and a further portion of fraction W (12 mg.) added. This digest after 4 days incubation contained a further 0.21 mg. of glucose / 4 ml. indicating that the hydrolysis limit of 70%, observed in the main digest, was not a true limit; this was probably caused by slow inactivation of the amylase at 35°C.

The extent of degradation of soluble starch by the algal preparation is therefore greater than that with β -amylase; the latter hydrolyses only 53 - 60% of the starch molecules.

Graph 7 - Degradation of starch



(iii) Fraction 3

A digest containing soluble starch (32 mg.), fraction 3 (40 mg.), 0.07 M acetate buffer pH 5.6 (45 ml.) and sodium chloride (5 ml., 0.6 M) was prepared. The percentage hydrolysis to glucose was estimated using reagent 'A' with samples (3 ml.); the iodine absorption value of sample (1 ml.), water (10 ml.) and 0.2% iodine in 2% potassium iodide (1 ml.) was measured as before. The results (Graph 7) indicate strong endo-amylase action with a hydrolysis limit of ca. 90%.

Hydrolysis of glycogens

Digests were set up using the undernoted glycogens (39 - 47 mg.), 0.05 M acetate buffer pH 5.1 (40 - 50 ml.) and C. rupestris fraction A (60 mg.). The digests were examined at intervals using essentially the same methods as before except that filter 603 was used in place of 608 for the iodine absorption values.

(i) Brewer's yeast glycogen

Time (hours)	0	16.5	40.5
Conversion into glucose (%)	-	7.0	15.5
Iodine absorption value	1.297	0.173	0.114

(ii) Glycogen from Ascaris lumbricoides

Time (days)	0	1	2	3	5	8	10	14
Conversion into glucose (%)	-	6.0	9.0	13.0	20.0	27.5	30.0	33.5
Iodine absorption value	0.082	0.043	0.022	0.015	0.005	0.000	0.000	-

(iii) Comparison with α - and β -amylase preparations

The following digests were prepared, and samples (2 ml.) withdrawn at intervals and the reducing power estimated using reagent 'A'. For digest (1) the results are quoted as percentage

hydrolysis to glucose, and for digests (2) and (3) as apparent conversion to maltose.

<u>Digest</u>	(1)	(2)	(3)
Rabbit liver glycogen (mg.)	46.8	48.4	48.4
0.2 M Acetate buffer pH 5.0 (ml.)	10.0	10.0	10.0
Water (ml.)	40.0	17.0	18.0
Fraction A (mg.)	150.0	-	-
Soya bean β -amylase (ml.)	-	3.0	-
Unpurified salivary α -amylase (ml.)	-	-	2.0

Time (days)

1	17.8	43.7	84.4
2	27.4	46.6	88.5
3	36.1	46.1	97.3
5	43.3	-	-
11	50.0	-	-
14	51.4	-	-
21	52.4	-	-

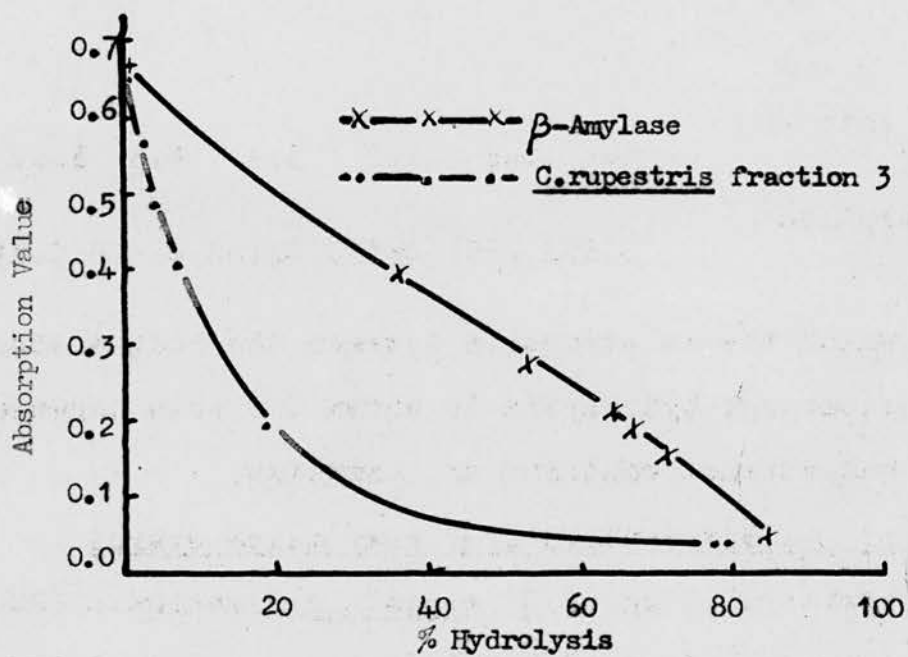
The algal amylase thus causes a greater degradation of glycogen than β -amylase.

Hydrolysis of potato amylose

Substrate:- potato amylose (42.0 mg., blue value 1.33) was dissolved in sodium hydroxide (10 ml., 0.2 N) at 100°C. The solution was neutralised with acetic acid, 0.2 M acetate buffer pH 5.6 (10 ml.) added and the solution diluted to 50 ml. with water. Sodium chloride (5 ml., 0.6 M) was added to the substrate solution, before dilution, which was used for the C. rupestris digest.

Enzymes:- Wallerstein Laboratories (New York) β -amylase preparation (19.2 mg., 1,920 units) in water (4 ml.); C. rupestris fraction 3 (56.0 mg.) in water (4 ml.).

Graph 8 - Comparison of Algal and β -Amylases



Digests were prepared containing solutions of substrate (47 ml.) and enzyme (3 ml.). The reducing power and iodine absorption value for each digest were estimated as described on p423.

Time (minutes)	0	15	30	45	60	120	21 hrs.	46 hrs.
<u>β-Amylase</u>								
Conversion into maltose (%)	-	36.0	52.8	64.0	65.6	71.2	84.8	84.8
Iodine absorption value	0.672	0.398	0.273	0.213	0.189	0.150	0.044	-

Fraction 3

Conversion into glucose (%)	-	0.8	2.3	3.4	6.5	18.2	77.1	78.2
Iodine absorption value	0.672	0.654	0.568	0.484	0.408	0.190	0.038	-

Graph 8 in which the relationship between the iodine absorption value and percentage hydrolysis is shown for both digests indicates that the algal extract contains an α -amylase.

Hydrolysis of β -limit dextrin from waxy maize starch

β -Limit dextrin (19.1 mg.), C. rupestris fraction A (20.0 mg.) and 0.07 M acetate buffer pH 5.0 (40 ml.) were incubated together. Chromatographic analysis indicated that glucose was the only degradation product and this was estimated on samples (5 ml.) using reagent 'A'.

Time (days)	1	2	3	5	10	13
Conversion into glucose (%)	6.4	10.9	14.3	19.3	30.9	31.0

DISCUSSION

The extract of C. rupestris has been shown to contain an α -amylase, the activity of which is considerably affected by various anions. The absence of glucose-1-phosphate $\rightarrow$ amylose transglucosidase (phosphorylase) in the algal extract (see Section III) indicated that the degradation of α ,1:4-glucosans (Table 4, p. 37) must be amylolytic rather than phosphorolytic, and this section has therefore been devoted to a preliminary study of the properties of the amylase.

Many of the methods for activity determinations of amylases are based on measurement of the liberated reducing sugars, as maltose. The end product of the algal extract action on starch was however, glucose; this, allied to a low activity, necessitated the introduction of a different activity unit. Using the devised conditions, the liberation of glucose was shown to be proportional to the concentration of the extract.

Attempted purification of amylase

The separation of proteins including enzymes by electrophoresis has been reported by several workers. Wallenfells and Peckmann (150) have reported the separation of amylase, phosphatase and a proteinase by filter paper electrophoresis whilst the separation of α - and β -glucosidases by this method has also been observed (151). Essentially the same technique has been used with the algal extract and different electrophoretic mobilities were observed for the amylase and maltase. The results also confirmed the separate identity of the trehalose and maltose hydrolysing enzymes.

An investigation of the re-extraction of amylase activity from lyophilized whole extracts has shown that the activity is only re-extracted by prolonged stirring with the solvent; acetate buffer and distilled water were the most effective of the solvents studied. The precipitation of protein at different pH values showed that the amylase and maltase behaved in a similar fashion. The introduction of this procedure into a scheme for the purification of the algal amylase would therefore have no advantage.

The fractionation and subsequent purification of many enzymes has been reported using inorganic salts and organic solvents as precipitants. Ammonium sulphate has been used in the majority of enzyme purifications, whilst acetone and ethanol are the most widely used organic solvents. Newton and Naylor (152) reported the use of ethanol as a precipitant for soya bean β -amylase whilst Sherman and Schlesinger (153) used it for the preparation of pancreatic α -amylase. Gates and Kneen (154) studied the effect of isopropanol, acetone, ethanol and methanol as precipitants of the amylase from A. oryzae bran; they concluded that isopropanol and acetone are the most useful, as denaturation is considerably less than with either ethanol or methanol.

The amylase activity of the algal fractions precipitated with higher concentrations of ammonium sulphate were found to be considerably greater than that of the whole extract. The yield was, however, low and there was no preferential precipitation of the maltase. Precipitation with ethanol appeared to cause inactivation of the amylase; similar results were observed for the maltase (section II, p. 66). Examination of the fraction collected by

precipitation with isopropanol between 30 and 65% concentration showed that the amylase activity had been preferentially precipitated with a good recovery. Refractionation, of this fraction, resulted however in a greater loss of activity, although the specific activity had increased. Application of these methods, on a large scale, resulted in a 2.2-fold purification of the amylase (62% yield) whereas the maltase activity increased by a factor of only 1.1 with a 32% recovery.

A study of the thermolability of the amylase and maltase showed that the amylase, the activity of which was decreased by temperatures greater than 40°C, was more labile than the maltase, which showed no appreciable loss of activity until the temperature was higher than 53°C. The subsequent elimination of maltase activity from the amylase preparation would thus appear to be difficult.

Conchie (148) has reported that glucurono-1:4- and 1:5-lactones are effective competitive inhibitors for rumen β -glucosidase. In a further study it was observed that these lactones would also act as inhibitors for other oligosaccharidases, including α -glucosidase (149). The inhibitory effect of glucurono-1:4- lactone on the algal α -glucosidase was however found to be only 17%; that of methyl α -glucoside was only 10%.

Properties of the amylase

The algal amylase resembles those obtained from animal sources in that it ^{is} activated by anions (143). In Universal and phosphate-citrate buffers, the optimum pH is in the range

5.2 - 5.8, values similar to those of crystalline A. oryzae α -amylase (145) and Clostridium butyricum amylase (155). The results obtained with acetate buffer indicated an optimum pH greater than 5.7, the highest pH obtainable using that buffer. Universal buffer exerted an activating action on the amylase and further study showed that this was due to the boric acid constituent of the buffer mixture. This effect was maximal in 45×10^{-4} M boric acid; phenylacetic acid had no effect whilst phosphate decreased the activity slightly. These results are not artefacts caused by interference of the anion with the Somogyi copper reagent.

The amylase activity of the algal extract increased by 720% on addition of 0.05 M sodium chloride and a graph of the logarithm of the chloride ion concentration against increase in reducing power of the digests showed that over the concentration range 0.001 - 0.05 M the activity was proportional to the concentration of chloride ions. Animal and bacterial α -amylases are similarly activated by chloride ions and the concentration required for maximum activation and the increase in activity are similar to those observed with the algal amylase (143).

Mode of Action

α -Amylolysis of α ,1:4-glucosans is characterised by the production of oligosaccharides and a rapid decrease in the iodine absorption value of the digest. The extent of degradation of amylose, amylopectin and glycogen by α -amylase is high, an apparent conversion to maltose of 80% or more being observed. The step-wise hydrolysis of α ,1:4-glucosans, catalysed by β -amylase or amyloglucosidase, is indicated by the production of only

one sugar (maltose or glucose) and a slow change in iodine absorption value; a high degree of hydrolysis of amylose is required before the iodine colouration reaches the achroic stage. The number of branch points in the molecule has a considerable effect on the extent of degradation; amyloses, essentially linear molecules, undergo a 70 - 100% conversion to reducing sugar using β -amylase, whereas only ca. 50% hydrolysis of amylopectins and glycogens is obtained.

The contamination of a preparation of either α - or β -amylase with maltase would not affect the changes in iodine absorption value, since this is a measure of the amount of unattacked substrate. If maltase is present, the calculation of reducing power estimation in terms of the apparent conversion into glucose will give hydrolysis limits similar to those obtained, using pure α - and β -amylases, for the apparent conversion into maltose. The maltase would also have little effect on the liberated reducing sugars, in the early stages of amylolysis; an α -amylase - maltase digest would contain the normal series of oligosaccharides and glucose, whilst a β -amylase - maltase digest would contain maltose and glucose (both digests would, of course, mainly contain glucose in the final stages of enzyme action).

The action of the algal extracts on glycogen and amylose has been compared with that of β -amylase preparations, and observed to be different. With the C. rupestris preparation a greater apparent conversion of the glycogen to reducing sugar was obtained. A study of the iodine absorption values of two different glycogens, in the presence of the algal extract, showed that the glycogens

became achroic at ca. 20% hydrolysis, indicating random action. A comparison of the decrease in iodine absorption value with apparent conversion of amylose (Graph 8, p.¹²⁵) shows that the algal extract catalyses a more rapid decrease in absorption value than β -amylase. The colour changes observed, in presence of the algal extract, were from blue, through purple and red to the achroic stage, changes which are typical of α -amylase action.

Paper chromatographic analysis of two different starch-extract digests showed that oligosaccharides were produced, in agreement with the postulated α -amylase activity of the algal preparations.

The whole extract of C. rupestris shows low amylolytic activity which is inactivated on prolonged incubation at 35°C. Soluble starch, after 25 days incubation, gave a 70% conversion into glucose, but the residual material was further hydrolysed by the addition of fresh enzyme. Incubation of soluble starch with a partially purified preparation gave ca. 90% conversion into glucose and a rapid decrease in iodine absorption value was observed.

These studies indicate that the algal amylase is of the α -type.

The hydrolysis of the β -limit dextrin from waxy maize starch could not be catalysed by a β -amylase, unless acting in the presence of a de-branching enzyme. The extent of degradation of the β -limit dextrin, in conjunction with the above evidence, is therefore attributed to α -amylase action, and the low apparent conversion to the low activity of the whole extract.

Further purification of the amylase activity, free from maltase, isomaltase and possible de-branching enzymes is desirable before the mode of action of this enzyme, including specificity towards α ,1:4-linkages adjacent to reducing groups and α ,1:6-linkages, can be studied in greater detail.

SUMMARY

1. The amylase activity of an extract of C. rupestris has been increased by precipitation with ammonium sulphate and isopropanol.
2. The effect of different buffers on the optimum pH indicated that the amylase was sensitive to anions.
3. Boric acid (45×10^{-4} M) and sodium chloride (5×10^{-2} M) are effective activators.
4. The degradation of α ,1:4-glucosans, followed by measurement of iodine absorption value and increase in reducing power, indicates that the mode of action is that of α -amylolysis.
5. Chromatographic analysis of soluble starch digests using low enzyme concentrations or after a short reaction period indicated that glucose was not the primary degradation product.

BIBLIOGRAPHY

1. Fritsch, F.E. (1945). Structure and Reproduction of the Algae, Cambridge University Press.
2. Bonner, J. (1950). Plant Biochemistry, New York : Academic Press.
3. Fogg, G.E. (1952). The Metabolism of Algae, Methuen & Co. Ltd., London.
4. Stanford, E.C.C. (1883). Chem. News, 47, 254.
5. Black, W.A.P. (1953). Rep. Progr. Chem. 50, 322.
6. Barry, V.C. & Dillon, T. (1940). Nature, Lond., 146, 620.
7. Percival, E.G.V. & Chanda, S.K. (1950). Nature, Lond., 166, 787.
8. Barry, V.C., Dillon, T., Hawkins, B. & O'Colla, P. (1950). Nature, Lond., 166, 788.
9. Kylin, H. (1918). Hoppe-Seyl. Z. 101, 236.
10. Collin, H. (1937). Bull. Soc. chim. Fr. 4, 277.
11. Ross, A.G. (1953). J. Sci. Fd. Agric. 4, 333.
12. Coulson, C.B. (1955). J. Sci. Fd. Agric. 6, 674.
13. Ross, A.G. (1956). Private communication.
14. Kylin, H. (1944). K. fysiogr. Sällsk. Lund Förh. 14, 221.
15. Fisher, I.S. & Percival, E.E. (1955). Abs. Second International Seaweed Symp.
16. Michaelis, L. & Menten, M.L. (1913). Biochem. Z. 49, 333.
17. Duclaux, E. (1883). Traité de Microbiologie, Paris.
18. Hoffmann-Ostenhof, O. (1953). Advanc. Enzymol. 14, 219.
19. Watanabe, A. (1949). Acta phytochim., Tokyo, 15, 129.
20. Neillands, J.B. & Stumpf, P.K. (1955). Outlines of Enzyme Chemistry, p. 258. London, Chapman & Hall Ltd.
21. Manners, D.J. (1955). Quart. Rev. chem. Soc. 9, 73.
22. Bartholomew, E.T. (1914). Bot. Gaz. 57, 136.
23. Hampton, H.C. & Baas-Beeking, L.G.M. (1920). J. gen. Physiol. 2, 635.

24. Marks, G.W. (1934). J. biol. Chem. 107, 623.
25. Takagi, M. (1953). Bull. Jap. Soc. sci. Fish. 19, 798.
26. Peterrson, S. (1940). K. fysiogr. Sällsk. Lund Förh. 10, 171.
27. Ronnerstrand, R. (1946). K. fysiogr. Sällsk. Lund Förh. 16, 117.
28. Peterrson, S. (1939). Bot. Notiser, p. 193.
29. Bean, R.C. & Hassid, W.Z. (1956). J. biol. Chem. 218, 425.
30. Potter, V.R. (1955). Methods in Enzymology, vol. 1, p. 10. Ed. by Colowick, S.P. & Kaplan, N.O. New York : Academic Press.
31. Hugo, W.B. (1954). Bact. Rev. 18, 87.
32. Punnett, T.R. (1955). Dissert. Abs. 15, 45.
33. Crook, E.M. (1946). Biochem. J. 40, 197.
34. Holden, M. (1945). Biochem. J. 39, 172.
35. Lugg, J.W.H. (1939). Biochem. J. 33, 110.
36. Schwimmer, S. & Pardee, A.B. (1953). Advanc. Enzymol. 14, 375.
37. Evans, H.J. & Nason, A. (1953). Plant Physiol. 28, 233.
38. Stern, R. & Bird, L.H. (1949). Biochem. J. 44, 635.
39. Staubs, E. & Vestling, F.S. (1951). J. biol. Chem. 191, 395.
40. Maestrini, D. (1919). Atti Accad. Lincei, 28, 509.
41. Chibnall, A.C. (1923). J. biol. Chem. 55, 333.
42. Wildman, S.G. & Jagendorf, A.T. (1952). Ann. Rev. Pl. Physiol. 3, 131.
43. Morton, R.K. (1950). Nature, Lond., 166, 1092.
44. Smith, D.G. & Young, E.G. (1953). J. biol. Chem. 205, 849.
45. French, D. & Wild G.M. (1953). J. Amer. chem. Soc. 76, 2612.
46. Chibnall, E.C., Rees, M.W. & Williams, E.F. (1943). Biochem. J. 37, 354.
47. Sumner, J.B. & Myrback, K. (1950). The Enzymes, New York : Academic Press.

48. Roche, J. (1950). The Enzymes, vol. 1, p. 473. Ed. by Sumner, J.B. & Myrback, K. New York : Academic Press.
49. Stein, S.S. & Koshland, D.E. (1952). Arch. Biochem. Biophys. 39, 229.
50. Somogyi, M. (1945). J. biol. Chem. 160, 69.
51. Shaffer, P.A. & Somogyi, M. (1933). J. biol. Chem. 100, 695.
52. Hanes, C.S. & Cattle, M. (1938). Proc. Roy. Soc. B125, 387.
53. Somogyi, M. (1945). J. biol. Chem. 160, 61.
54. Somogyi, M. (1952). J. biol. Chem. 195, 20.
55. Nelson, N. (1944). J. biol. Chem. 153, 375.
56. de Whalley, H.C.S., Albon, N. & Gross, D. (1951). Analyst. 76, 287.
57. Whistler, R.L. (1953). unpublished.
58. Partridge, S.M. (1948). Biochem. J. 42, 238.
59. Bayly, R.J. & Bourne, E.J. (1953). Nature, Lond., 171, 385.
60. Partridge, S.M. (1949). Symp. biochem. Soc. 3, 57.
61. Trevelyan, W.E., Procter, D.P. & Harrison, J.S. (1950). Nature, Lond., 166, 444.
62. Forsyth, W.G.C. (1948). Nature, Lond., 161, 239.
63. Hanes, C.S. & Isherwood, F.A. (1949). Nature, Lond., 164, 1107.
64. Hanes, C.S. (1940). Proc. Roy. Soc. B129, 174.
65. Cori, G.T., Colowick, S.P. & Cori, C.F. (1938). J. biol. Chem. 124, 543.
66. Fiske, C.H. & Subbarow, Y. (1925). J. biol. Chem. 66, 375.
67. Khin Maung. (1956). Ph.D. Thesis, Edinburgh University.
68. Peat, S., Whelan, W.J. & Hinson, K.A. (1952). Nature, Lond., 170, 1056.
69. Whistler, R.L. & Durso, D.F. (1950). J. Amer. chem. Soc. 72, 677.
70. Helferich, B. & Smitz-Hillebrecht, E. (1933). Ber. dtsh. chem. Ges. 66, 378.
71. Nath, R.L. & Rydon, H.N. (1954). Biochem. J. 57, 1.
72. Gottschalk, A. (1950). The Enzymes, vol. 1, p. 551. Ed. by Sumner, J.B. & Myrback, K. New York : Academic Press.

73. Hestrin, S. (1940). *Enzymologia*. 8, 193.
74. Ishizawa, K. & Miwa, T. (1954). *Symp. enzyme chem. (Japan)*. 2, 40.
75. Larner, J. & McNickle, C.M. (1954). *J. Amer. chem. Soc.* 76, 4747.
76. Larner, J. (1955). *J. Amer. chem. Soc.* 77, 6385.
77. French, D. & Knapp, D.W. (1950). *J. biol. Chem.* 187, 463.
78. Manners, D.J. & Khin Maung. (1955). *Chem. & Ind.* p. 950.
79. Zechmeister, L., Tóth, G., Furth, P. & Barsony, J. (1940).
Enzymologia. 2, 155.
80. Pigman, W. (1950). *The Enzymes*, vol. 1, p. 728. Ed. by Sumner
J.B. & Myrback, K. New York : Academic Press.
81. Percival, E.G.V. & Ross, A.G. (1951). *J. chem. Soc.* p. 720.
82. Dillon, T. & O'Colla, P. (1950). *Nature, Lond.*, 166, 67.
83. Dillon, T. & O'Colla, P. (1951). *Chem. & Ind.* p. 111.
84. Peat, S., Thomas, G.W. & Whelan, W.J. (1952). *J. chem. Soc.* p. 722.
85. Bell, D.J. & Northcote, D.H. (1950). *J. chem. Soc.* p. 1944.
86. Manners, D.J. (1952). *Ph.D. Thesis, Cambridge University.*
87. Levinson, H.S. & Reese, E.T. (1950). *J. gen. Physiol.* 33, 601.
88. Manners, D.J. (1955). *Biochem. J.* 61, XIII.
89. Chanda, S.K., Hirst, E.L., Jones, J.K.N. & Percival, E.G.V. (1950).
J. chem. Soc. p. 1289.
90. Holden, M. & Tracey, M.V. (1950). *Biochem. J.* 47, 407.
91. Sørensen, H. (1952). *Physiol. plant.* 5, 183.
92. Sørensen, H. (1953). *Nature, Lond.*, 172, 305.
93. Veibel, S. (1950). *The Enzymes*, vol. 1, p. 621, Ed. by Sumner,
J.B. & Myrback, K. New York : Academic Press.
94. Northcote, D.H. (1956). *Unpublished.*
95. Mayeda, M. (1922). *J. Biochem. Tokyo.* 1, 131.
96. Ramstad, E. (1956). *Ph.D. Thesis, Edinburgh University.*
97. Fishman, W.H. (1950). *The Enzymes*, vol. 1, p. 642, Ed. by
Sumner, J.B. & Myrback, K. New York :
Academic Press.

98. Chanda, S.K., Hirst, E.L., Percival, E.G.V. & Ross, A.G. (1952).
J. chem. Soc. p. 1833.
99. Oshima, K. (1931). J. Agr. chem. Soc. Japan, 7, 328.
100. Thjøtta, T. & Kass, E. (1945). Avh. norske Vidensk Akad. 1, 20.
101. Waksman, S.A., Carey, C.L. & Allen, M.C. (1934). J. Bact. 28, 213.
102. Kooiman, P. (1954). Biochim. biophys. Acta, 13, 338.
103. Hashimoto, Y. & Onama, K. (1949). Bull. Jap. Soc. sci. Fish.
15, 253.
104. Waksman, S.A. & Allen, M.C. (1934). J. Amer. chem. Soc. 56, 2701.
105. Kertesz, Z.I. (1950). The Enzymes, vol. 1, p. 745. Ed. by
Sumner, J.B. & Myrback, K. New York :
Academic Press.
106. Faulkner, P. (1955). Biochem. J. 60, 590.
107. Swanson, M.A. (1950). J. Amer. chem. Soc. 184, 647.
108. Naganna, B. & Sripathi, C.E. (1954). Nature, Lond., 174, 593.
109. Block-Frankenthal, L. (1954). Biochem. J. 57, 87.
110. Sumner, J.B. & Somers, G.E. (1953). Chemistry and Methods of
Enzymes, p. 166, New York :
Academic Press.
111. Keilin, D. & Hartree, E.F. (1945). Biochem. J. 39, 293.
112. Burris, R.H. & Little, H.N. (1950). Respiratory Enzymes, p. 158.
Burgess Publishing Co.
113. Rabaté, M.J. (1935). Bull. Soc. Chim. biol., Paris, 17, 372.
114. Doudoroff, M., Barker, H.A. & Hassid, W.Z. (1947). J. biol.
Chem. 168, 725.
115. Barker, S.A. & Bourne, E.J. (1953). Quart. Rev. chem. Soc. 7, 56.
116. Pan, S.C., Andreasen, A.A. & Kolachov, P. (1950). Science, 112, 115.
117. Barker, S.A. & Bourne, E.J. (1952). J. chem. Soc. p. 209.
118. Saroja, K., Venkataraman, R. & Giri, K.V. (1955). Biochem. J.
60, 399.
119. Killey, M., Dimler, R.G. & Cluskey, J.E. (1955). J. Amer. chem.
Soc. 77, 3315.

120. Pazur, J.H. (1955). J. biol. Chem. 216, 531.
121. Crook, E.M. & Stone, B.A. (1953). Biochem. J. 55, XXV.
122. Barker, S.A., Bourne, E.J., Hewitt, G.C. & Stacey, M. (1955).
J. chem. Soc. p. 3734.
123. Pazur, J.H. & French, D. (1952). J. biol. Chem. 196, 265.
124. Isbell, H.S. & Frush, H.L. (1953). Ann. Rev. Biochem. 22, 113.
125. Bourquelot, E. & Aubry, A. (1913). C.R. Acad. Sci., Paris,
158, 70.
126. Bourquelot, E. & Bridel, M. (1913). C.R. Soc. Biol., Paris,
73, 267.
127. Miwa, T. & Takano, K. (1950). J. Biochem. Tokyo. 37, 435.
128. Miwa, T. & Takano, K. (1950). Symp. enzyme chem. (Japan). 4, 76.
129. Jermyn, M.A. (1952). Aust. J. Sci. Res. B5, 409.
130. Jermyn, M.A. & Thomas, R. (1953). Aust. J. biol. Sci. 6, 70.
131. Takano, K. & Miwa, T. (1953). J. Biochem., Tokyo. 40, 471.
132. Duncan, W.A.M., Manners, D.J. & Ross, A.G. (1956). Biochem. J.
63, 44.
133. French, D. (1951). Science, 113, 352.
134. Peat, S., Whelan, W.J. & Edwards, T.E. (1955). J. chem. Soc.
p. 355.
135. Wolfrom, M.L., Thompson, A. & Galkowski, T.T. (1951). J. Amer.
Chem. Soc. 73, 4093.
136. Sugihara, M.L. & Wolfrom, M.L. (1949). J. Amer. chem. Soc. 71,
3357.
137. Whelan, W.J., Bailey, J.M. & Roberts, P.J.P. (1952). J. chem. Soc.
p. 1293.
138. Pazur, J.H. (1954). Biochim. biophys. Acta, 13, 158.
139. Whelan, W.J. & Roberts, P.J.P. (1953). J. chem. Soc. p. 1298.
140. Fredrick, J.M. (1951). Physiol. plant. 4, 621.
141. Bailey, J.M. & Neish, A.C. (1954). Canad. J. Biochem. Physiol.
32, 452.
142. Manners, D.J. (1953). Rep. Progr. Chem. 50, 288.

143. Myrbäck, K. & Neumüller, G. (1950). The Enzymes, vol. 1, p. 653.
Ed. by Sumner, J.B. & Myrback, K. New
York : Academic Press.
144. Whelan, W.J. & Bines, B.J. (1955). Biochem. J. 61, i.
145. de Montmollin, R. (1951). Helv. chim. acta, 34, 1987.
146. Phillips, L.L. & Caldwell, M.L. (1951). Analyt. Chem. 23, 1172.
147. Woods, E.F. & Gillespie, J.M. (1953). Aust. J. biol. Sci. 6, 130.
148. Conchie, J. (1954). Biochem. J. 58, 552.
149. Conchie, J. & Levvy, G.A. (1955). Biochem. J. 61, XVI.
150. Wallenfells, K. & Peckmann E. v. (1951). Angew. Chem. 63, 44.
151. Nystrom, C.W., Williams, B.L. & Wendor, S.H. (1954). J. Amer. Chem.
Soc. 76, 1950.
152. Newton, J.M. & Naylor, N.M. (1939). Cereal Chem. 16, 71.
153. Sherman, H.C. & Schlesinger, M.D. (1912). J. Amer. chem. Soc.
34, 1104.
154. Gates, R.L. & Kneen, E. (1948). Cereal Chem. 25, 1.
155. Whelan, W.J. & Nasr, H. (1951). Biochem. J. 48, 416.

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