THESIS SUBMITTED TO THE
UNIVERSITY OF EDINBURGH
FOR THE DEGREE OF DOCTOR OF PHILOSOPHY
BY
ARTHUR S. ASHWORTH.
MAY 1949.

CYTOLYSIS AND RELATED ENZYMATIC CHANGES
IN GERMINATING BARLEY.

CONTENTS.

I. GENERAL INTRODUCTION.

It is widely accepted that changes in the cell walls of the barley corn, and of seeds in general, play a vital part in germination, but despite this, very little information is available on the subject. The problem of the elucidation of the changes in the cell wall and the agencies responsible for them, finds interest in practical as well as academic circles, since there is little doubt that changes in the physical and chemical nature of the cell wall play a principal part in the conversion of the hard barley corn to the characteristic, friable corn of malt.

Ling discussing this subject (1904) noted that according to Brown and Morris there is a gradual dissolution of the cell walls of the barley endosperm during germination, this change being accompanied by a softening of the contents of the endosperm. Grüss however had later produced evidence to show that these workers were in error to suppose that the cell wall was completely dissolved, a residual wall being left which was only visible after staining. From his own results Ling was also of the opinion that the dissolution of the cell walls was not complete, and proceeded to show that hydrolysis was greatest at the germ end and in the peripheral portion of the endosperm, rather than in the centre and at the distal end. Hopkins and Krause (1937) state the prevalent opinion that diffusion of amylases from the scutellum to the endosperm is facilitated by the action of hemicellulases on cell wall materials, to leave a skeleton permeable to amylases.

It was therefore felt that information of both practical and theoretical value would accrue from an investigation of the enzymic processes which lead to the hydrolysis of cell wall and related materials, and from a study of the enzyme or enzymes concerned, usually referred to as cytase.

Enzymes capable of hydrolysing cell wall materials have been isolated from various sources, and in some cases have been given names according to their various substrates, e.g. Sumner and Somers (1947) describe an araban from takadiastase, a lichenase from *Aspergillus oryzae* and a xylanase from the digestive juices of the snail, *Helix pomatia*; these hydrolyse araban, lichenin and xylan respectively. In other cases the enzymes have been included under the general names cytase or hemicellulases, of which the former is to be prefered, since it is by no means certain that all the substances attacked by these enzymes are true hemicelluloses.

The presence of cytase in many seeds has been noted by Waksman and Davison (1926) the most abundant sources including the seeds of barley, oats and rye, in which cytase are apparently located in the aleurone layer; the enzyme lichenase, mentioned above, is stated by them to be present in germinating barley.

The assumption that each of the different actions arise~~s~~ from a specific enzyme is not justifiable at present, and it would be equally unjustifiable to suppose that these actions are different aspects of the activity of the same enzyme. It seems likely that a more limited number of cy~~s~~tases exist, capable, in different combinations, of producing the various actions observed.

Preliminary investigations on the cytase of barley have been made by various workers (Grüss 1902, Baker and Hulton 1917, Lüers and Volkamer 1928, Lüers and Malsch 1929) using green malt as the source of cytase; comment on their results will be more conveniently made below, in the introductions to subsequent sections.

The cell wall constituents attacked by cytase are commonly accepted to be hemicelluloses and hemicellulose-like substances, and in the past such substances from various sources other than barley have been used as substrates for barley cytase (Grüss, <u>loc.cit</u>; Lüers and Volkamer, <u>loc.cit</u>: Lüers and Malsch, <u>loc.cit</u>:) leaving the results open to the criticism that the cytase under investigation was not acting on its natural substrate. In the present work therefore an attempt has been made to study the cytolysis of hemicellulosic substances from barley.

The term hemicellulose was originated by Schulze as a group name ~~fro~~ for substances, which, although in some respects similar to cellulose, differ from it in being soluble in dilute alkali, and readily hydrolysed by boiling, dilute mineral acids. Earlier workers were of the opinion that hemicelluloses were hexo-pentosan in nature, the principal sugars obtained on hydrolysis being glucose, mannose, xylose and arabinose. Subsequent workers have however shown that uronic acids enter into the constitution of some hemicelluloses, and although the nature of the uronic acids present has not been definitely decided in all cases, where uronic acids are associated with xylose and arabinose they are thought to be glucuronic and galacturonic acids respectively. Detailed information is given in reviews on this subject by Norman (1937) and more recently by Preece (1948); information relative to the subjects of the present studies appears in the relative sections below.

II. PREPARATION OF NON STARCHY POLYSACCHARIDES FROM BARLEY AND WILLOW WOOD

Before beginning the discussion of the methods of preparation used in this section, it would be well to survey briefly the nature of the non-starchy polysaccharides and polysaccharide-like materials of the barley grain. The classification of these materials is in a somewhat confused state, but the materials fit broadly into the following classes.

A. Cell Wall Materials.
  I. Cellulose.
  II. Pectic substances.
  III. Hemicelluloses.
    (a) Cellulosans, intimately associated with cellulose, and possibly structurally related to it.
    (b) Encrusting hemicelluloses, initially more soluble than cellulosans, and possibly having some structural analogy with the pectic substances and plant gums.

B. Other Polysaccharide-like Materials.
  I. The barley gums.
    (a) amylan, soluble in hot water 40°C
    (b) amylan, soluble in cold water 15-20°C

possible inter-relationships of these materials have been discussed in detail in a recent paper by I.A.Preece (1948).

Cellulose and the pectic substances need not be further discussed, as they are outside the scope of investigation at this stage, but some knowledge of the remaining classes is vital as a preliminary to the study of cytolysis, since these are possible substrates for cytase. Exclusion of the pectic substances can not be regarded as final, as it will become clear as description of the present work proceeds that cytolysis and pectolysis are not necessarily unrelated.

The functions of the hemicelluloses are not precisely understood, but it appears that the cellulosans are essentially structural materials, whilst the encrusting hemicelluloses, which may serve as structural material especially when in association with lignin (see e.g. Norman, 1937), may also serve as reserve carbohydrate. Thus the hemicelluloses of the barley husk will be considered as structural carbohydrates, whilst those of the endosperm may combine both structural and reserve functions.

Preece (1944) has shown that as yet no reliable methods for the separate extraction of the two classes have been developed; consequently extractions of variable proportions of the two together are made with dilute alkali (usually 4% NaOH) in accordance with the definition that hemicelluloses, although initially insoluble in water in the natural state, are soluble in dilute alkali. Numerous workers have proceeded along these lines (O'Dwyer 1923; Norris and Preece 1930, 1931a, 1931b; Norman loc.cit.) A method for the fractionation of hemicelluloses has been described by Norris and Preece (loc.cit.), by which the following fractions are distinguished: hemicellulose A, precipitated by acidification of the alkaline extract, hemicellulose B, from the filtrate from this by the addition of a half volume of acetone, and hemicellulose C, from the second filtrate by the addition of more acetone. Fractions B and C may further be divided into B and C (precipitated from alkaline solution by Fehling's solution) and B_2 and C_2 (precipitated from alkaline solution by Fehling's solution in the presence of acetone). A given tissue may yield one or more of these fractions, the spent grains from all malt mash yielded hemicelluloses B_1 B_2 C_1 and C_2 (Preece 1931a). Norris and Preece (loc.cit) are careful to point out that it is not claimed that these fractions represent chemically homogeneous entities, but rather that they must be regarded as aggregates of molecules of similar properties. Attention must be drawn to the fact that hemicelluloses, although insoluble in water in their natural state, are frequently water soluble after extraction by the methods described, others however are only water soluble as their sodium salts; whether this post-preparative water solubility is due to rupture of associations of hemicellulose with substances such as lignin, or to depolymerisation of the hemicelluloses is not clear.

It appears from the recent work of Preece (1944) that extraction of hemicelluloses is influenced by four factors:

A. Penetration of the tissue by the solvent.
B. Freedom from association with other substances (e.g. lignin).
C. Conversion to e.g. their sodium salts.
D. Depolymerisation.

NaOH would be effective under all four headings, extracting a proportion of all classes of hemicelluloses; alcoholic NaOH also satisfies all four of the conditions, but does not result in hemicellulose extraction in cases where alcohol concentration is greater than 80%, however if the tissue is extracted with boiling water subsequent to treatment with alcoholic NaOH, hemicellulose extraction is achieved, the extract again containing a mixture of both classes of hemicelluloses.

At present it therefore appears that workers must remain content to use mixed hemicellulose preparations, and further investigation in the field of separation is clearly most desirable, one approach possibly being the search for a solvent which would achieve one or more of the conditions listed above, but leaving others unsatisfied.

Turning to the barley gums, let it be said at the outset that the term "barley gum" is prefered to "amylan" since the latter term implies a close relationship to starch; indeed the term was originated with that object in view (see O'sullivan 1882). There has been little useful work carried out on these substances since O'Sullivan's original communication, though some advance was achieved by Preece and Hunter (private communication), who prepared barkey gums by a modification of O'Sullivan's method. These workers extracted barley gum with water at room temperature after inactivating the enzymes of the grain by refluxing with boiling alcohol; barley gum was then extracted with water at 40°C. Whereas O'Sullivan found that the two gums were both essentially hexosan in nature, Preece and Hunter (<u>loc.cit.</u>) whilst showing that the gum approached this structure, yielding only a small percentage of furfuraldehyde, possibly due to impurities, showed that the gum was quite different, giving approximately 25% furfuraldehyde, and appearing to consist in the main part of urono-xylan. Moreover both gums are precipitated by Fehling's solution in the presence of acetone, and Preece (1948) has proposed that the and gums should be regarded as analogous to hemicelluloses B_2 and C_2 respectively; these substances will thus be subsequently referred to as barley gum B_2 and barley gum C_2.

It would appear therefore that great similarity exists between these gums and the hemicelluloses, and that they differ only in that former are initially water soluble, whereas the latter are initially insoluble. Preece and Hunter (<u>loc.cit.</u>) have further shown that the yield of barley gum C_2 can be increased by pretreatment of the barley with alcoholic NaOH, and this without any apparent change in the nature of the gum. Hence initially insoluble material is apparently present in the grain, which differs little if at all from the soluble material. It would be illogical therefore to regard the hemicelluloses and gums as materials of widely different nature.

Methods for the preparation of representatives of both classes from barley, and hemicelluloses from willow are considered below. Preece (1941) has shown that one of the most abundant sources of hemicelluloses is willow wood, and it is for this reason that preparations of hemicelluloses from willow wood were made.

EXPERIMENTAL

Preparation of Barley Gums:- The method used for the preparation of barley gums is based on that of Preece and Hunter (loc. cit); the cold water and hot water soluble barley gums, (barley gums C and B respectively) were however extracted in one operation with water at 40°C.

200g. of barley, ground in the coffee mill, were extracted twice, for 30 minute periods, with boiling 80% alcohol under reflux, to inactivate enzymes and remove sugars etc., the residues being filtered off through cloth at the pump and the extract discarded. After removing the alcohol as completely as possible, the residues were extracted twice with 600ml. quantities of water at 40°C., filtration being effected through cloth at the pump. The combined extracts were filtered through a layer of Kieselguhr on paper, giving a water-clear filtrate, which was then reduced to small volume by distillation under reduced pressure.

To the concentrated extract was added rather more than half its volume of acetone, containing a little HCl, and the precipitate of barley gum B allowed to settle; the decanted supernatant liquid was poured into a volume of acetone equal to that previously used, and the precipitate, barley gum C, allowed to settle. The precipitates were individually re-dissolved in water, and the precipitation repeated; the gums were then washed with acid-80% alcohol, and taken to dryness by washing with increasing strengths of alcohol, with final drying in a vacuum desiccator.

A further preparation of barley gum C_2 was made in the following manner:

500g. of barley, pre-treated as before with boiling alcohol, were extracted thrice with with 1,250ml. quantities of water at room temperature, for 20 minutes each extraction, filtering after each extraction through cloth at the pump. The combined ~~extractes~~ extracts were filtered bright through a layer of Kieselguhr on paper, and reduced to small volume by distillation under reduced pressure. The concentrated extract was seen to contain suspended matter, presumably coagulated protein, and was consequently filtered clear through paper at the pump. The clear concentrate was poured into an equal volume of acetone, to which a little HCl, had been added, and the precipitate obtained separated. The precipitate was dissolved in water, reprecipitated by pouring into an equal volume of acid acetone, and taken to dryness in the usual manner.

It had been reported by Preece (1948) that a separation of the barley gums could be achieved through the copper complex; taking into consideration the fact that this method tends to give a preparation of less ash content than the above, an attempt was made to isolate barley gum C_2 by this method.

A cold water extract of barley was prepared and concentrated using the method described in the previous experiment. To the filtered concentrate was added sufficient NaOH to give a final concentration of 4%, mixed Fehling's solution was then added until a blue colouration could be seen in the liquid, and the resulting viscous fluid was poured into slightly less than half its volume of acetone. The gelatinous precipitate formed was filtered off through muslin, redissolved in the minimum of dilute HCl, and reprecipitated by pouring into one and a half volumes of acetone. The precipitate was was washed with 60% acetone containing a little HCl, and taken to dryness in the usual manner after washing with 60% acetone.

It is of interest to note that barley gums B_2 and C_2 are snow-white, fibrous substances of very low density, which show electrostatic properties on grinding in a mortar if sufficiently dry; this description corresponds to that given by O'Sullivan (loc. cit) for his α and β amylans. Also solutions of these substances appeared to be very viscous, and to posses some surface activity, the extracts being very difficult to filter, giving on boiling a very stable foam.

Preparation of Barley Hemicelluloses:- The main problem to be faced during these preparations was the removal of starch prior to the extraction of hemicelluloses, and several attempts to achieve this had to be made before a successful method was evolved.

100 g. of coffee-mill ground barley, in which the enzymes had been inactivated in the usual manner, were stirred with water at 37°C. To this mix were added 100 ml. of a 5% malt extract, in which β amylase and cytase had been inactivated by heating at 70°C. in the presence of 0.2% calcium acetate; the mix was then incubated at 37°C for 4 hours to allow α amylase activity to proceed, and at the end of this time the residue was filtered off and tested for starch. As starch was shown to be present the procedure was repeated, but again starch persisted, and the preparation was not proceeded with.

Again, 100 g. of barley, pretreated as usual, were added to 250 ml. of a 5% malt extract, β amylase free and cytase free, at 70°C., and the temperature maintained for 15 min. The mixture wasthen diluted by the addition of 250 ml. of water, and hydrolysis allowed to proceed for 36 hours at 37°C. Again starch was not eliminated.

In view of the failure of the above experiments it was decided to gelatinise the starch prior to the addition of the malt extract.

100 g. of ground barley were added to 400 ml. of boiling water at such a rate that the water stayed on the boil; after cooling to 75°C., 250 ml. of a 5% malt extract were added, and a temperature of 70°C. maintained for 2 hours. No inactivation of β amylase or cytase was attempted as it was considered that the temperature of the mash would be sufficient to achieve this. On testing, it was found that only a slight trace of starch remained, and this was removed by a further similar treatment.

The starch free residues were filtered off through cloth at the pump, and washed thoroughly with hot water to remove the dextrins produced by the enzyme hydrolysis. The residues were then extracted once with 500 ml. and twice with 250 ml. of 4% NaOH in the cold for twenty minute periods, filtering after each extraction through cloth at the pump; the combined extracts were filtered bright through paper pulp, and the hemicelluloses recovered in the manner described for the alkaline, concentrated extracts of barley gum C_2. Two hemicelluloses were isolated, one of series one, this fraction is designated S_1, precipitated on the addition of Fehling's solution alone, and one of series 2, designated S_2, on the addition of acetone to the filtrate from the above.

During the course of subsequent work a further preparation of hemicelluloses was made from a different sample of barley, using precisely the same method as described above, but in this case no hemicellulose S_1 could be separated, though it must be noted that a great increase in viscosity and turbidity occurred on the addition of Fehling's solution. A second preparation was therefore made from the same sample of barley, and the absence of hemicellulose S_1 again noted.

The barley hemicelluloses are very similar in appearance to the barley gums, being white, fibrous substances, but not showing such pronounced electrostatic properties as the barley gums.

Both barley hemicellulose S_1 and S_2 were fractionated on a small scale by the method of Norris and Preece (loc. cit.). Hemicellulose S_1 yielded fractions A, B, and C, and hemicellulose S_2 fractions B and C; this latter is in conformity with the results of previous workers, no hemicellulose A_2 being obtained from any source to date. The barley hemicellulose S_2 which was unaccompanied by a hemicellulose S_1, was also fractionated yielding again fractions B and C. The above fractionation must only be regarded as a preliminary experiment, as no attempt was made to isolate the fractions for drying and weighing.

Preparation of Willow Hemicelluloses;- Hemicelluloses were prepared from willow sawdust by the method of Norris and Preece (1930), the treatment with 1% NaOH being omitted since Preece has subsequently shown that treatments involving the use of hot NaOH tend to cause the loss of furfuraldehyde-yielding material. A hemicellulose of series 1 was isolated.

A second preparation was made using a method based on an observation by Preec (1944), that a proportion of the willow hemicelluloses can be rendered soluble in boiling water by pretreatment of the wood with cold alcoholic NaOH.

100g. of willow sawdust were treated in the cold with 4% NaOH in 80% alcohol for 24 hours and, after filtering, the residues were washed with 80% alcohol containing a little H_2SO_4 until free from alkali, and then with 80% alcohol until free from acid. After removing the alcohol as completely as possible, the residues were extracted with one litre of boiling water under reflux for one hour, and the extract separated through cloth at the pump. The filtrate was then reduced to small volume by distillation under reduced pressure, and the hemicelluloses precipitated by the addition of a half volume of acetone, i.e. the precipitate comprised a mixture of hemicelluloses A and B; a slight precipitate was obtained on raising the concentration of acetone in the filtrate to one volume, i.e. hemicellulose C, but this was too small to allow recovery.

All the preparations described above, and in addition some repeated preparations, were subject to analysis for moisture, ash and uronic acid contents and furfuraldehyde yield. The latter was determined by distillation of the substance with 12% HCl, and precipitation as the phlorglucide by Kröber's method (Brown and Zerban 1941); the uronic acid content was determined by distillation with 12% HCl and adsorption of the carbon dioxide evolved to standard Ba $(OH)_2$, as described by Dickson, Otterson and Link (1930). Results will be found in table 1. together with figures for yield.

Table I. Yield and composition of various polysaccharide preparations.

Substance.	Yield as % of starting material.	Dry, ash-free.		
		Ash as % of substance.	Uronic acid as % of substance.	Furfural yield as as % of substance.
Barley gum B. *	0.7	0.32	1.16	3.96
Barley gum C. *	0.3	1.12	----	26.10
Barley gum C. ※	0.4	7.56	----	25.05
Barley gum C_2. #	0.2	0.0	1.04	25.61
Barley gum C_2. #	0.3	0.0	----	28.81
Barley gum C_2. #	0.4	0.0	----	25.33
Barley hemicellulose S_1. #	1.4	0.20		47.94
Barley hemicellulose S_2. #	0.~~8~~9	0.44		36.48
Barley ~~hemic~~ hemicellulose S_1. ⁜	0.6	----	----	-----
Barley hemicellulose S_2. ⁜	1.1	0.0		45.50
Willow hemicellulose S_1.	9.5	1.91	----	59.32
Willow hemicellulose (A&B)	0.85	1.8	----	52.98

* Jointly extracted at 40°C.

※ Precipitated by acetone.

\# Precipitated by Fehling's solution and acetone.

\# Preparation 1.

⁜ Preparation 2.

DISCUSSION

The suggestion made by Preece (1948) that the cold and hot water soluble gums of O'Sullivan are analogous to hemicelluloses C_2 and B_2 respectively has been confirmed. The figures for furfuraldehyde yield from the barley gums described herein, agree quite well with those obtained by Preece and Hunter (loc.cit) for their preparations (table 11), and little doubt can remain that pentosans do enter into the constitution of the barley gums. It will be seen however that barley gum B_2 is essentially hexosan in constitution, and the possibillity that a pentosan is present as a contaminating material can not be dismissed. There would appear to be very little, if any uronic acids present in, either barley gum B_2or C_2.

Table II. Furfuraldehyde yields from barley gums prepared by different workers.

Substance.	Furfural yield as % of dry, ash-free substance.	
	Prepared by Preece and Hunter.	Present preparation.
Barley gum B_2.	9.4.	3.9
Barley gum C_2.	21.1	26.1

The isolation of two, hitherto unprepared, hemicelluloses from whole barley has been described, and some information as to their constitution obtained. The problem of the removal of starch proved very difficult, and the methods which had eventually to be used were rather drastic; whether the yield or nature of the hemicelluloses is affected by this treatment is not known, and information on this point would be of value.

For purposes of comparison, the furfural yields from the hemicelluloses obtained by Preece (1931) from spent brewers grains are given in table III., along with those of the hemicelluloses under discussion. Although no strict comparison may be made, it is of interest to note that both in the case of spent grains, and in the case of whole barley, where fractionation was possible, the furfuraldehyde yield of the series 2 hemicellulose is less than that of the corresponding S_1 hemicellulose.

Table III. Furfuraldehyde yields from various hemicellulose preparations.

Substance.	Source.	Furfural yield as % of substance.
Hemicellulose B_1.	Spent grains.	57.76
Hemicellulose B_2.	Spent grains.	19.60
Hemicellulose C_1.	Spent grains.	56.45
Hemicellulose C_2.	Spent grains.	48.68
Hemicellulose S_1.	Whole barley.	47.94
Hemicellulose S_2.	Whole barley.	36.48
Hemicellulose S_2. *	Whole barley.	45.50

* - no S_1 isolated.

The sample of barley, from which both series 1 and series 2 hemicelluloses were isolated, yielded five hemicellulose fractions, namely hemicelluloses A_1, B_1, C_1, B_2 and C_2, and the barley from which only series 2 hemicelluloses were obtained gave two fractions, hemicelluloses B_2 and C_2. As was pointed out in the experimental section, the fractionation experiment can only be regarded as a preliminary to future work on these hemicelluloses, and it is clearly most important that this fractionation should be carried further, with particular reference to the yields and compositions of the individual fractions, before any great advance in the knowledge of these hemicelluloses is possible.

The absence of hemicellulose A_1 from the hemicelluloses of spent grains will be noted (table III), but no conclusions of any value may be drawn from this since it is possible that this fraction was absent from the barley from which the malt was prepared. Preece(1940b) investigated the effect of germination on the furfuraldehyde-yielding constituents of barley, and showed that a substantial increase in these substances occurs on germination; the methods used did not involve the isolation of the substances, furfuraldehyde determinations being carried out directly on the grains. Clearly therefore it would be of great interest and value to study the influence of germination, and consequently malting, on the individual hemicelluloses of barley.

There can be two possible explanations for the absence of hemicellulose S_1 from the second preparation that was made. The first is that hemicellulose S_1 was absent from the grain, since the second preparation was carried out on a different sample of barley. The second possible reason is that the treatment to which the hemicellulose was subjected caused it to be depolymerised, or otherwise modified, to the extent that it is no longer precipitated by the addition of Fehling's solution alone, and appears in the hemicellulose S_2 fraction, on addition of acetone. The second possibility has been intimated by Preece (1944), who showed that treatments involving the use of cold aqueous and alcoholic NaOH caused degradation of hemicelluloses. Some indication that this may be the cause of the absence of hemicellulose S_1, is the fact that a great increase in viscosity and turbidity is noted on the addition of Fehling's solution. It is possible that the S_1 materials are of such a composition as to be near a critical point in respect of precipitation by Fehling's solution; thus, slight (perhaps un-noticed) modifications of procedure, e.g. a somewhat longer contact with NaOH, might suffice to take them beyond the precipitation point. The series of changes precipitation - high viscosity - no precipitation, would appear to be consistent with this.

The great similarity between the barley gums and the hemicelluloses, which has been commented upon in the introduction, is further stressed by the work described in the present section; it therefore appears that a strong case can be made for the classification of the barley gums as hemicelluloses, or on the other hand a modification of the definition of hemicelluloses so as to include such materials as the barley gums.

SUMMARY.

1. The preparation and fractionation of barley gums B_2 and C_2 have been described.
2. Barley hemicelluloses have been prepared by hitherto unpublished methods.
3. Preliminary fractionation of the barley hemicelluloses has been described, and the following fractions shown to be present in one sample of barley: A_1, B_1, C_1, B_2 and C_2, whereas from another sample only hemicelluloses B_2 and C_2 were obtained.
4. Two willow hemicelluloses have been prepared, one of them by a hitherto unpublished method.
5. The furfuraldehyde yields of the barley gums and hemicelluloses and the willow hemicelluloses have been determined, and the uronic acid content of the barley gums.

III. PRELIMINARY EXPERIMENTS ON THE ACTION OF CYTASE, INVOLVING THE USE OF EXTRACTS AND A PRECIPITATED FORM OF CYTASE.

Credit for the first work on cytase is due to Grüss (loc.cit), who extracted the enzyme from green malt, and showed that it hydrolysed a galactan from gum tragacanth and a mannan from date stone, to produce galactose and mannose respectively.

Baker and Hulton (loc.cit.) showed that the furfuraldehyde-yielding substances of barley embryos increased during germination, whilst a corresponding decrease occurred in the endosperm. This they took to be evidence for the existence of a system of enzymes, which, acting in a manner analogous to that of diastase, allowed the translocation of furfuraldehyde-yielding material to the embryo. Again, they showed, if green malt and barley are extracted together, more furfuraldehyde-yielding material is extracted than if they are extracted separately, suggesting action by cytolytic enzymes on soluble materials to render them soluble. Further information given by these workers is that the enzyme may be precipitated by alcohol in an active form, and that it is capable of hydrolysing furfuraldehyde-yielding polysaccharides to sugars.

Probably the most important contribution to the knowledge of cytase is that of Lüers and Volkamer (loc.cit.) who used green malt as a source of cytase, and two xylan preparations as substrates, one from elder pith and the other from barley. The optimum pH and temperature were found to be 5.0 and 45°C., activity being lost at pH 9.0, and after heating at 60°C. for 15 minutes; the workers cited showed that the enzymes could be precipitated by alcohol without losing its activity, and that by adsorption on aluminium hydroxide at pH 5.0 and elution with phosphate buffer at pH 8.3, a great increase in purity was attained. Xylose was identified as one of the products of substrate hydrolysis, and it is stated that excess of this sugar retarded the action of cytase. An important feature of their investigation, is the extreme slowness of the action; two days were necessary to achieve a hydrolysis of 70%, even under the most favourable conditions.

Lüers and Malsch (loc.cit.) followed up this work by an investigation into the effect on cytase of the germination and kilning of malting. By studying the xylan hydrolysing activity of extracts of barley at various stages during malting, they showed that although cytase activity decreases during steeping, it increases later to a value approximately 2.5 times that of the original barley; on kilning however cytase is inactivated, until in the final malt its activity is less than that of the original barley.

The work described herein, which was intended to serve as a foundation for future and more detailed investigation included: a search for a convenient source of cytase, a study of the cytolytic activity of extracts, the preparation of a precipitated form of cytase, a preliminary examination of the pH and temperature relationships of cytase and the testing of several possible substrates, the preparation of which has been described in the previous section.

EXPERIMENTAL

The Search for a Source of Cytase

Several possible sources of cytase were examined, including those used by previous workers, namely, germinated barley and ungerminated oats, (Grüss loc.cit. Baker and Hulton loc.cit. Lüers and Volkamers loc.cit.); in addition the cytolytic activity of a specially prepared, low-dried malt was tested.

Green Malt. 25g. of barley were steeped in water for 24 hours and subsequently germinated in the dark on moistened paper for 5 days; it will be convenient henceforth to refer to barley treated thus as " green malt".

The green malt was ground in the mincing machine, further ground with sand to a pulp, and extracted with

150ml. of water at 21°C. for $1\frac{1}{2}$ hours. Two ml quantities of the filtered extract were tested against 100ml. quantities of 0.10% (dry, ash-free equivalent) solution of the following sunstances: willow hemicellulose S_1, willow hemicellulose (A and B), barley gum B_2, and barley gum C_2, a control being carried out on water. The substrate solutions were buffered with sodium acetate/acetic acid buffer solution to pH 4.6 prior to the addition of extract, and the conversions were carried out at 37°C. The course of the hydrolysis was followed by the determination of the reducing groups, liberated, using the Hanes-Somogyi method (Hockenhull and Herbert, 1945), the results being expresses as apparent xylose, after correction fo~~z~~r the reducing groups indroduced in the enzyme preparation. The results will be found in table IV.

The pssibility that some of the action produced was due to amylases, was examined by repeating the experiment on barley gums B_2 and C_2, the 2 ml. of green malt extract being replaced by 2 ml. of a 5% malt extract in which β amylase, and presumably cytase, had been inactivated. The results will be found in table V, and will be seen to confirm that barley gum B_2 is contaminated with starch, as was suspected from other evidence. Barley gum C_2 is free from starch, and apparently from dextrins susceptible to the action of amylase.

<u>Table</u> <u>IV</u>. The cytolytic action of 2 ml. of a 16.6% extract of green malt on various substrates at pH 4.6 and 37°C.

Time.	Corrected reducing groups liberated (as xylose) as % of substrate.			
	Willow hemicellulose S_1.	Willow hemicellulose (A and B).	Barley gum B_2.	Barley gum C_2.
30 min.	0.0	0.0	2.3	5.1
60min.	0.0	0.0	3.6	9.3
90 min.	0.0	0.0	4.2	9.5
48 hrs.	0.0	0.0	--- *	14.9

* - outside range of determination.

<u>Table</u> <u>V</u>. The action of the α amylase of 2 ml. of a 5% malt extract on barley gums B_2 and C_2, at pH 4.6 and 37°C.

Time in min.	Corrected reducing groups liberated (as xylose) as % of substrate.	
	Barley gum B_2.	Barley gum C_2.
30	1.6	0.0
60	2.0	0.0
90	4.2	0.0
120	6.6	0.0

Oats:- 25 g. of ground, ungerminated oats were extracted with 70 ml. of water at 21°C. for 3 hours, and the extract filtered through paper. 2 ml. of the extract were tested for cytolytic activity against barley gum C_2 by the method described above.

Low-driedmalt:- The cytolytic activity of a specially prepared low-dried malt, which had been dried by heating at 50 - 53°C. for 24 hours, and in which therefore cytase might be extected to have survived with little or no deterioration, was tested in the manner described for oats.

The results of the last experiment showed that both oats and low-dried malt possessed some activity, but the action was so slow that no more than 4% hydrolysis was achieved after 4 hours conversion. Attempts were made to increase the hydrolysis by increasing the volume of extract used, but this introduced too great a concentration of reducing groups and substances susceptible to the action of the enzymes of the extract, and the results were accordingly of doubtful value. The effect of long period conversion was therefore tried.

Long Period Conversion:- 25 g. of green malt were ground in a mincing machine, and extracted with 150 ml. of water at 21°C. for 3 hours. 2 ml. of the filtered extract were allowed to act on 50 ml. of 0.10% (dry, ash-free equivalent) solutions of each of the following substrates, adjusted to pH 4.6 with sodium acetate/acetic acid buffer solution; barley hemicellulose S_1, barley hemicellulose S_2, barley gums B_2 and C_2, willow hemicellulose S_1, and willow hemicellulose (A and B); the reducing groups liberated were determined after 18 and 66 hours conversion (table VI). The possibility that some of the action was due to amylases was tested in the manner described previously. Only in the case of barley gum B_2 was starch found to be present.

Table VI. Cytolytic action of 2 ml. of a 16.6% green-malt extract, at pH 4.6 and 37°C., over long period conversion.

Time in hours.	Corrected reducing groups liberated (as xylose) as % of substrate.				
	Willow hemicellulose S_1.	Willow hemicellulose (A & B)	Barley gum C_2.	Barley hemicellulose S_1.	Barley hemicellulose S_2.
18	0.0	0.0	16.0	4.1	7.9
66	0.0	0.0	25.1	4.8	17.1

Since starch was again shown to be present in barley gum B_2, the figures for this substance have been omitted from the table of results.

The cytolytic activity of a 5% low-dried malt extract was tested in the same manner against barley gum C_2 and the barley hemicelluloses S_1 and S_2; the results will be found in table VII.

Table VII. The cytolytic activity of 2 ml. of a 5% low-dried malt extract, acting on 50 ml. quantities of 0.10% solutions of various substrates, at pH 4.6 and 37°C.

Time in hours.	Corrected reducing groups liberated (as xylose) as % of substrate.		
	Barley gum C_2.	Barley hemicellulose S_1.	Barley hemicellulose S_2.
1	2.0	0.9	2.8
18	7.9	0.9	3.0
42	13.5	1.9	4.1

From the above experiments it is clear that green malt and low-dried malt possess some cytolytic activity, the extracts of the latter being the more active, and it is highly probable that cytase is also present in oats, but this remains to be confirmed.

There can be little doubt that neither of the willow hemicelluloses is attacked by these preparations of cytase, and only slight hydrolysis of barley hemicellulose S_1 has been achieved. All the remaining substrates tested, viz. barley gum B_2, barley gum C_2 and barley hemicellulose S_2, have been shown susceptible to cytase action, but only barley gum C_2 and barley hemicellulose S_2 are suitable as substrates, since starch has been shown to be present in barley gum B_2, and attempts to eliminate it have so far failed. However it is doubtful whether amylase action on the starch in barley gum B_2 can be responsible for all the hydrolysis produced by the green malt extract.

Preparation of a Crude Precipitated Form of Cytase.

25 g. of low-dried malt, ground in the malt-mill, were extracted with 500 ml. of water at 21°C. for three hours, the extract was then filtered through fluted paper, and having removed a portion to be tested immediately for cytolytic activity, the remainder was dialysed through parchment against water for 18 hours, at the end of which time the reducing group content was substantially nil.

The cytolytic activity of the dialysed extract was tested by adding 2 ml. quantities to 50 ml. of 0.10% (dry, ash-free equivalent) barley gum C_2 solution, and to 50 ml. of water to serve as a control, both buffered to pH 4.6 with sodium acetate/acetic acid buffer solution. For purposes of comparison the undialysed extract had been tested on the day of preparation in the same manner. Conversion was carried out at 37°C., and the reducing groups determined after 1. 18, and 42 hours.

Table VIII. The cytolytic action of 2 ml. of a 5% low-dried malt extract, before and after dialysis; substrate barley gum C_2, pH 4.6, 37°C.

Time in hours.	Corrected reducing groups liberated (as xylose) as % of substrate.	
	Undialysed extract.	Dialysed extract.
1	0.0	1.8
18	7.8	9.2
42	9.2	11.3

Although at the time of testing the dialysed extract had been necessarily been kept 24 hours, it will be seen that there has been no loss of activity; the activity of solutions of cytase is therefore apparently unchanged by keeping at room temperature for 24 hours, or on dialysis.

Workable quantities of precipitated enzymes were not obtainable from this extract, due to its low concentration.

In a second experiment, 200 g. of low-dried malt were extracted with 500 ml. of water at 21°C. for 3 hours; the extract was filtered through a fluted paper, and poured into 4 volumes of 95% alcohol. The precipitate was allowed to settle, the supernatant liquid decanted off, and the precipitate taken to dryness in the usual manner, the whole process being carried out in as short a time as possible to avoid undue inactivation by the alcohol. This preparation will be referred to below as "crude precipitated malt enzymes".

Table IX. Yield and ash content of crude precipitated malt enzymes.

Dry, ash-free yield.	1.8% of malt.
Ash content.	11.2%

Influence of pH and Temperature on Cytolysis.

Long period conversion:- The influence of pH and temperature on cytolysis was studied over long period conversion , at temperatures 33, 41 and 45°C., and at pH values 4.6, 5.0 and 5.4. The investigation of pH effects at 33°C. was combined with a study of the effect of increasing amounts of enzyme, and was carried out using 1, 2, 4, 5 and 10 ml. quantities of a 0.10% (dry, ash-free equivalent) solution of crude precipitated malt enzymes added to 50ml. quantities of a 0.10% (dry, ash-free equivalent) barley gum C_2 solution, controls on water being carried out for each quantity of enzyme. The conversion mixtures were adjusted to pH 4.6, using sodium acetate/acetic acid buffer solution, reducing groups liberated being determined after 20 and 44 hours.

Cytolysis at 41 and 45°C. was studied in the following manner: 1 and 3 ml. quantities of 0.10% (dry, ash-free equivalent) crude precipitated malt enzymes solution were added to 50 ml. quantities of 0.10% (dry, ash-free equivalent) barley gum C_2 solution and to 50 ml. quantities of water to serve as controls; the conversions were adjusted to the appropriate pH value with sodium acetate/acetic acid buffer solutions, and incubated at the required temperature , the reducing groups liberated being determined after 20 hours conversion. The results of the above experiments will be found in tables X and XI.

<u>Table X.</u> The cytolysis of 50 ml. of 0.10% barley gum C_2 solution by varying amounts of crude precipitated malt enzymes at 33°C.

mg. of enzyme added.	Corrected reducing groups liberated (as xylose) as % of substrate.					
	pH 4.6		pH 5.0		pH 5.4	
	20 hrs.	44 hrs.	20 hrs.	44 hrs.	20 hrs.	44 hrs.
1	3.8	6.0	6.65	10.3	6.7	11.6
2	7.5	8.5	13.3	19.4	11.3	18.3
4	17.3	18.6	18.7	31.4	14.6	19.9
5	22.5	25.5	25.5	35.2	17.0	20.1
10	34.6	36.0	36.1	41.0	21.0	----

Table XI. 20 hour cytolysis of 50 ml. of 0.10% barley gum C_2 solution by crude precipitated malt enzymes at various pH values and temperatures.

Temp.	Corrected reducing groups liberated (as xylose) as % of substrate.					
	1 mg. of enzyme.			3 mg. of enzyme.		
	pH 4.6	pH 5.0	pH5.4	pH 4.6	pH 5.0	pH 5.4
33	3.8	6.65	6.7	13.1 *	17.5 *	12.7 *
41	8.4	8.9	8.9	23.3	22.8	18.7
45	4.4	7.9	6.6	11.2	14.9	14.9

* - interpolated from fig. 1

The results are graphed in figs. 1-4, and the figures for hydrolysis by 3 mg. of enzyme at 33°C. were read off the appropriate curve in fig. 1. It will be seen from the curves that hydrolysis is substantially proportional to enzyme concentration up to figures of about 20% hydrolysis in thecases of the lower temperature and pH values; in the case of hydrolysis at ph 5.4 and also at 45°C. proportionality between hydrolysis and enzyme concentration is rapidly lost and hydrolysis tends to stop after a figure of 20% hydrolysis is reached.

No hydrolysis/pH or hydrolysis/temperature curves have been drawn, but from an examination of the figures in table XI, the respective maxima appear to be in the regions of pH 5.0 and 40°C., though considerable variation will be noted, especially that of optimum pH with temperature. It may well be considered

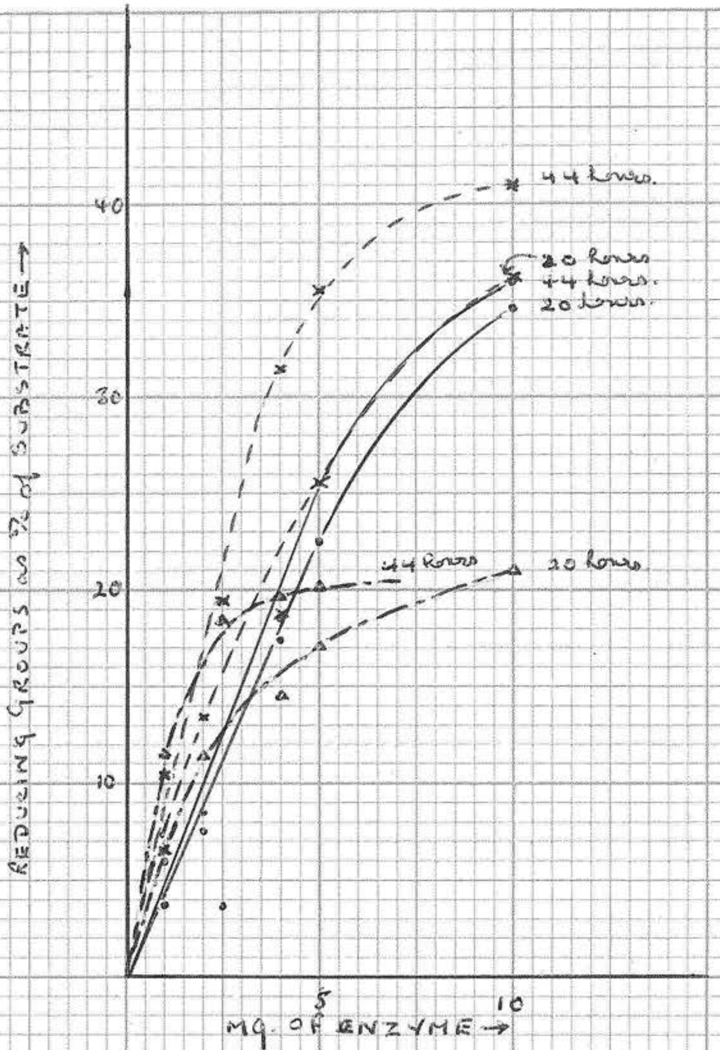


Fig. 1. Influence of enzyme concentration on long period cytolysis of barley gum C_2. Long period conversions, at 33°C.

—o——o——o—— pH 4.6

--x---x-- x--- pH 5.0

—Δ—·Δ—·Δ—· pH 5.4

that more than one enzyme is concerned, the components of the system showing different time/temperature/pH relationships.

Short Period Conversions:- It was considered desirable to examine cytolysis over short periods rather than long, and to this end a standard method was designed, laying down strict conditions of working in order that figures obtained from different experiments might be comparable, and that figures after 1-5 hours conversion might be considered reliable.

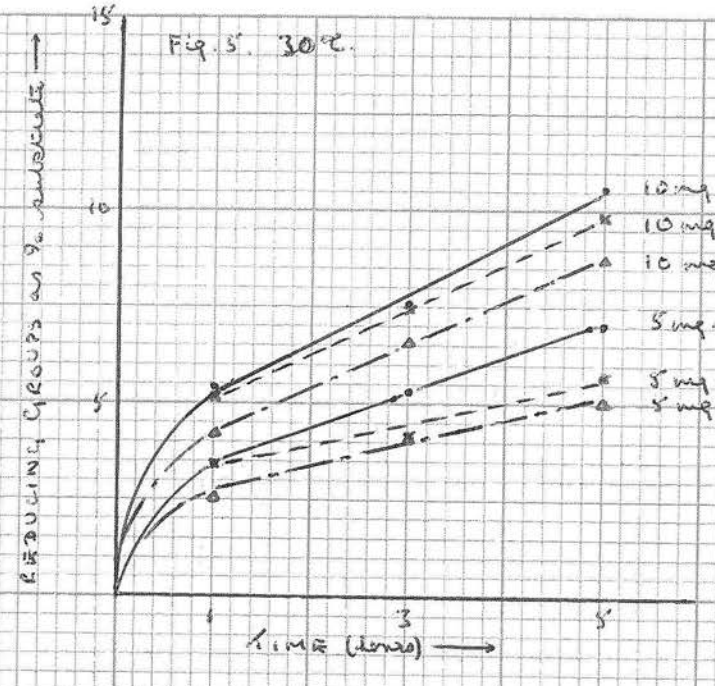
In the "standard short period method", the volume of the substrate solution was adjusted so that when the enzyme solution has been added, the total conversion liquid was in all cases 60 ml., containing the equivalent of 60 mg. of dry, ash-free substrate, and including the appropriate sodium acetate/acetic acid buffer solution. The concentration of enzyme solution used was commonly 1 mg. (dry, ash-free equivalent) of enzyme per ml., and 5 or 10 ml. of this were added to the conversion, the substrate being adjusted to the required temperature before adding the enzyme. Controls on enzyme - buffer - water mixtures were run throughout the duration of all conversions, and for the purpose of determining the initial reduction of the conversion mixtures, the reduction of the substrate itself was determined. Samples for the determination of reducing group content were removed immediately on adding the enzyme so.

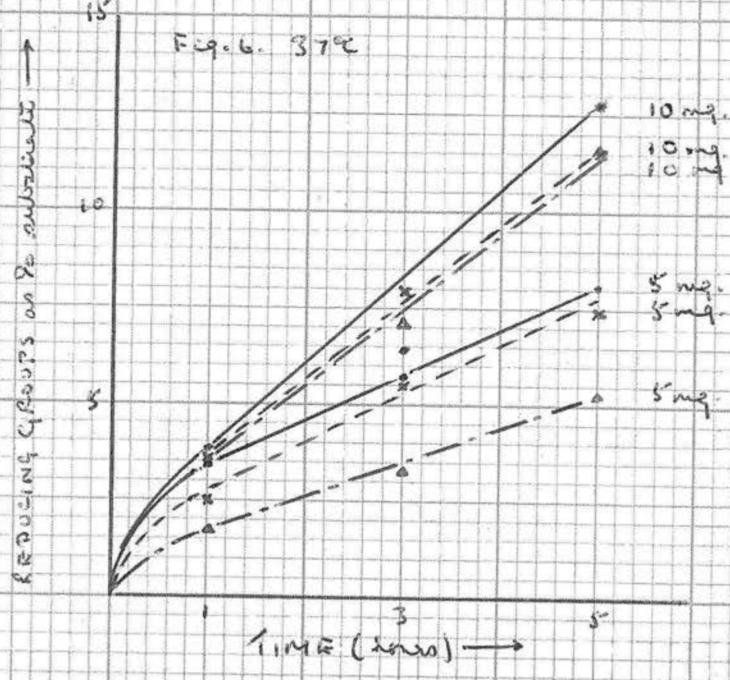
solution, and after 1, 3, and 5 hours of conversion.

The above method was used to examine the influence of pH and temperature on the cytolysis of barley gum C_2. The temperatures used were 30, 37, 44 and 51°C.; the pH values 4.6, 5.0 and 5.4 were tested at each temperature, and the source of the enzyme was crude precipitated malt enzymes. The results will be found in table XII, and the curves drawn from them in figs. 5-8.

Table XII. The influence of pH and temperature on the cytolysis of barley gum C_2, using short period (1-5 hours) conversion.

Temp. °C.	Corrected reducing groups liberated (as xylose) as % of substrate.								
	pH 4.6			pH 5.0			pH 5.4		
	1 hr.	3 hr.	5 hr.	1 hr.	3 hr.	5 hr.	1 hr.	3 hr.	5 hr.
	5 mg. of enzyme.								
30	3.4	5.3	7.0	3.4	4.2	5.7	2.5	4.1	5.0
37	3.4	5.7	8.1	2.5	5.5	7.4	1.7	3.3	5.3
44	2.2	5.0	7.0	2.9	5.3	7.6	1.7	2.1	3.1
51	2.4	2.9	4.9	2.5	3.9	6.4	1.8	2.1	2.9
	10 mg. of enzyme.								
30	5.4	7.6	10.6	5.2	7.4	9.8	4.2	6.6	8.7
37	3.8	6.4	12.7	3.6	7.9	11.5	3.8	7.1	11.6
44	3.1	8.4	8.5	4.2	8.1	12.6	2.9	5.7	8.0
51	2.9	5.3	7.4	3.1	6.7	8.7	2.2	3.1	4.9





Figs 5 and 6. Hydrolysis/time curves for cytolysis of barley gum C_2 by 5 and 10 mg. quantities of crude precipitated malt enzymes, at various pH values. Short-period conversions 1-5 hrs.

——•——•—— pH 4.6

– – ✱ – – ✱ – – pH 5.0

—·—▲—·—▲— pH 5.4.

The above experiment was repeated on barley hemicelluloses S_1 and S_2, using the same pH values and temperatures. During the determination of reducing groups at 0 time, a starch-iodine colour was noticed with both hemicelluloses; the action of α amylase on these substances was therefore tested as before (page 30). The presence of starch in both was confirmed.

It was also found in these experiments with barley hemicelluloses, which were carried out before the full short method was adopted, that in the case of barley hemicellulose S_1 and S_2 there was a difference in the titre figures at 0 hours between the substrate solutions containing 5 mg. and those containing 10 mg., at all temperatures and pH values (table XIII), which was greater than the corresponding difference in the controls. The only possible explanation seems to be that there is an almost instantaneous liberation of reducing groups on adding the enzyme, and that this liberation is greater with 10 mg. than with 5 mg. The inconsistency of the varation probably arises from slight, unavoidable differences in time between adding the enzyme and withdrawing the solution for determination. It was for this reason that the method already described, involving the examination of substrate and enzyme alone, was adopted. The results in table XIV were calculated using the full method of control, so avoiding the errors outlined above,

Table XIII. "0 hour" titre figures for barley hemicelluloses S_1 and S_2, obtained when substrate and enzyme solution were mixed and samples taken as rapidly as possible.

Temp. °C.	ml. N/100 $Na_2S_2O_3$.					
	pH 4.6		pH 5.0		pH 5.4	
	5 mg. of enzyme.	10 mg. of enzyme.	5 mg. of enzyme.	10 mg. of enzyme.	5 mg. of enzyme	10 mg. of enzyme.
	Barley hemicellulose S_1.					
30	9.8	9.7	9.8	9.75	9.9	9.7
37	8.45	8.0	8.9	8.3	9.25	8.7
44	9.8	9.4	9.8	9.4	9.8	9.5
51	9.35	8.9	8.6	8.9	9.65	8.9
	Barley hemicellulose S_2					
30	10.0	9.6	10.0	9.7	9.9	9.8
37	9.25	9.2	9.3	9.3	9.4	9.4
44	9.6	9.35	9.55	9.15	9.6	9.3
51	9.5	9.2	9.45	9.15	9.5	9.2

<u>Table XIV.</u> The influence of pH and temperature on the cytolysis of barley hemicelluloses S_1 and S_2, using short period (1-5 hour) conversion.

Temp.	Corrected reducing groups liberated (as xylose) as % of substrate.								
	pH 4.6			pH 5.0			pH 5.4		
°C.	1 hr.	3 hr.	5 hr.	1 hr.	3 hr.	5 hr.	1 hr.	3 hr.	5 hr.
	Barley Hemicellulose S_1.								
	5 mg. of enzyme.								
30	5.8	6.9	7.2	6.2	6.5	7.4	5.5	5.9	6.7
37	8.7	9.5	10.7	8.4	9.6	9.9	8.3	9.4	7.6
44	6.8	7.1	8.4	6.0	7.0	8.1	6.4	6.7	7.4
51	6.7	6.8	7.4	6.4	7.0	7.8	6.1	6.8	7.4
	10 mg. of enzyme.								
30	6.9	8.4	9.9	6.5	8.0	9.9	6.2	7.4	8.6
37	9.6	10.4	12.8	10.1	11.6	12.2	9.4	10.9	11.6
44	8.1	8.4	9.3	7.4	8.4	9.6	7.2	8.1	9.6
51	7.4	8.8	9.1	8.4	9.0	9.7	6.7	7.8	8.2
	Barley Hemicellulose S_2.								
	5 mg. of enzyme.								
30	4.1	5.7	7.0	4.6	6.5	8.0	4.6	5.8	8.0
37	5.2	6.4	7.5 ~~6.6~~	4.9	6.5	8.4	5.4	7.4	8.4
44	6.2	7.0	7.5	5.9	7.5	8.3	5.8	7.5	8.7
51	5.1	5.5	5.9	5.4	6.7	6.7	5.8	7.2	7.2
	10 mg. of enzyme.								
30	5.5	9.1	10.4	5.9	8.7	10.6	5.9	9.0	12.2
37	6.7	10.4	12.8	6.1	9.2	13.6	6.5	10.6	14.5
44	7.2	9.9	11.3	6.8	10.1	12.3	7.1	10.3	13.3
51	6.4	7.8	8.1	6.8	8.4	9.0	6.7	8.4	9.4

None of the figures for reducing groups liberated from either of the hemicelluloses by the action of crude precipitated malt enzymes can be considered truly representative of cytolytic action, due to the presence of starch in these substances and the consequent amylase action.

It is interesting to note that whereas in long period cytolysis of barley gum C_2 the relationship between hydrolysis and enzyme concentration appeared substantially linear from the start of the action, the expected linearity hydrolysis and time was not observed in the case of short period cytolysis, there being a rapid liberation of reducing groups at the start of the action, followed by a slower liberation of reducing groups, the latter being proportional to time (figs. 5-8). Similar actions will be noted in the case of the barley hemicelluloses, but here some of this action is probably due to amylase action. Hydrolysis/pH and hydrolysis/temperature curves have not been drawn for these results, but from an examination of the figures for the cytolysis of barley gum C_2 (table XII), it would appear that the optimum conditions for this action are at pH 5.0 and 44°C.

The Preparation of a Precipitated Enzyme from Ungerminated Barley and Oats.

100 g. each of ungerminated barley and ungerminated oats were extracted with 250 ml. of water at room temperature for 2 hours; the filtered extracts were poured into 4 volumes of 95% alcohol, and the precipitates obtained taken to dryness in the usual manner. The dried precipitates were tested for cytolytic activity against barley gum C_2 by the standard short period method at pH 5.0 and 37°C.; a conversion using crude precipitated malt enzymes was also run for purposes of comparison.

Table XV. The hydrolysis of barley gum C_2 by crude precipitated enzymes from various sources at pH 5.0 and 37°C.

Source of enzyme.	mg. of enzyme added.	Corrected reducing groups liberated (as xylose) as % of substrate.		
		1 hour.	3 hour.	5 hour.
Ungermind. barley.	5	1.2	2.1	2.0
	10	1.5	3.3	3.2
Ungermind. oats.	5	2.4	3.6	5.6
	10	3.3	6.2	9.0
Low-dried barley malt	5	3.5	5.3	7.7
	10	4.1	9.6	13.8

As will be seen in table XV and fig 11, the hydrolysis of barley gum C_2 by the precipitated enzymes from oats is substantially similar to that of crude precipitated

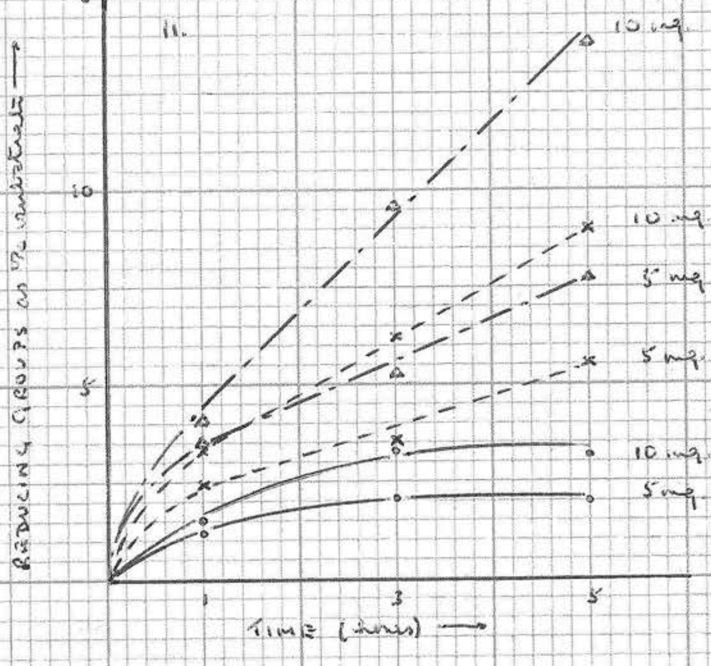


Fig 11. Hydrolysis of barley gum C_1 by precipitated enzymes from low-dried malt, ungerminated barley and ungerminated oats. pH 5·0, 37°C.

——o——o—— Barley

- - -x- - -x- - - Oats

—·—Δ—·—Δ—·— Low-dried malt.

malt enzymes, the only difference lying in the lower activity of the oat preparation. The action of the ungerminated barley preparation however is entirely different, substantially ceasing after 1-3 hours action, and it would appear that an enzyme of entirely different nature has been extracted.

DISCUSSION.

The use of extracts for the study of cytolysis has proved of little value, principally because of the low activity of the extracts, allied with the large number of contaminating substances introduced into the conversions by the extracts. However the presence of cytase in green malt and low-dried malt has been clearly shown, and its presence in ungerminated oats indicated, by the use of extracts.

A comparison of the activities of green malt and low-dried malt will be found in table XVI, where it will be seen that the low-dried malt is considerably more active than the green malt extract in the case of the cytlolysis of barley gum C_2; this however is not the case for the barley hemicelluloses, particularly with barley hemicellulose S_1, where it will be seen that the green malt extract was the more active. Whether this greater activity, where barley gum C_2 is concerned, is inherent in the malt itself cannot be definitely stated, since it may be due to the more efficient extraction possible in the case of low-dried malt, the green malt being extremely difficult ot grind. Accordingly the use of green-malt as a source of cytase was discontinued, and attention concentrated on low-dried malt.

Table XVI. Comparison of the cytolytic activities of extracts of green malt and low-dried malt, at pH 5.0 and 37°C.; 2 ml. of extract used in each case.

Substrate.	Corrected reducing groups liberated (as xylose) as % of substrate, after 18 hours conversion. Green malt. extract.		Low-dried malt extract.	
	Found 16%.	Calculated 10%.	Found 5%.	Calculated 10%.
Barley gum C_2.	16.0	10.0	7.9	15.8
Barley hemicellulose S_1.	4.1	2.5	0.9	1.8
Barley hemicellulose S_2.	7.9	5.0	3.0	6.0

Of the substrates examined, although four of these were susceptible to cytase action, namely barley gum B_2, barley gum C_2, barley hemicellulose S_1 and barley hemicellulose S_2, only one, barley gum C_2, has proved suitable for work in the immediate future, the remainders being contaminated to an appreciable extent with starch. The hydrolysis of the barley hemicelluloses was extremely slow, especially in the case of hemicellulose S_1, when only 4.8% hydrolysis was achieved after 66 hours conversion (table ~~III~~ VI); no measurable action could be observed in the cases of willow hemicelluloses S_1 and (Aand B).

The findings of previous workers that cytase is precipitable in an active form by alcohol has been confirmed, and the use of a precipitated form of cytase, necessarily contaminated with amylases etc., has provided some interesting results.

That cytase does not depend on the presence of dialysable material for its action is apparent, since the dialysed extract was active without the addition of such substances other than buffer materials (table VIII); the possibility that sodium or acetate ions activate cytase cannot be dismissed however, as these materials were present in the buffer solutions used to adjust the pH value of the conversion solutions. The age of the dialysed extract on testing was of necessity 24 hours, but its cytolytic activity was unimpaired; it seems safe to assume therefore, that no inactivation of cytase occurs over a period of 24 hours, at least in dialysed extracts.

The crude precipitated is stable in the dry form for appreciable lengths of time; the same preparation has been used over periods of several months, with no appreciable deterioration.

<u>Long Period Conversions of Barley gum C_2</u>:- The curves hydrolysis/enzyme concentration (figs. 1-4) for long period cytolysis of barley gum C_2, show that the two bear a substantially linear relationship at first. This is most

marked at pH 4.6, in which case hydrolysis in constant time and enzyme concentration were proportional at each of the temperatures tested; with increase in pH value linearity is lost, particularly at 45°C. With higher enzyme concentrations linearity is speedily lost, particularly at the higher pH values; extension of the reaction time to 44 hours at pH 5.4 gives substantially no increase in hydrolysis with five fold increase in enzyme concentration. Under optimum conditions hydrolysis and enzyme concentration show proportionality up to 20% hydrolysis, but beyond this point even under optimum conditions linearity is rapidly lost; it is possible that the point thus reached includes the end point of a particular phase of cytolysis for this substrate. From the above it may be concluded that there is a substantial decrease in the stability of cytase with decrease in acidity, even at the lower temperatures; figs. 2-4 illustrate clearly this decrease in stability with increase in pH value.

The influence of pH varies with temperature; at a temperature of 37°C. hydrolysis is greatest in the range 5.0 - 5.4, but with increase in temperature the optimum shifts to a value between 4.6 and 5.0. For temperature a more definite optimum may be stated, viz. 40°C. or lower, pH having little influence on its position.

Assuming the existence of two types of linkage in the substrate molecule, each attacked by a different enzyme, these observations may be taken to indicate the presence in the cytase preparation used, of two cytolytic factors. Thus factor X with an optimum pH value near 5.4 gives a rapid hydrolysis of the substrate, but its action ceases after the total hydrolysis reaches 10 - 20%, whilst factor Y with an optimum pH value near 4.6 and little or no stability at pH 5.4, gives a more prolonged hydrolysis at the lower pH values. The optimum temperature for both factors would appear to be in the region of 40°C. Possible reasons for the absence of any action by factor X after the stage of hydrolysis represented by a total liberation of 20% of reducing groups has been reached are: firstly that the enzyme has been inactivated, and secondly that, by the time this stage has been reached, all the linkages susceptible to the action of this enzyme have been broken, and consequently no further action is possible.

Further support for the suggestion that two enzymes are involved comes from the observation made above, that whilst the low-dried extracts are more active in the case of barley gum C_2 than green malt extracts, in the case of barley hemicellulose S_1 the latter is the more active, and in the case of barley hemicellulose S_2 their activities are approximately equal; this would be understandable if more than one cytolytic factor was concerned attacking different linkages, the distribution of which would be expected to differ in different substrates.

<u>Short Period Conversions of Barley Gum C_2</u>: (1-5 hours.):- The hydrolysis/time curves for short period conversions of barley gum C_2 are extremely interesting, and it must be noted that this material is free from starch. These curves are not

at all the usual type for enzyme actions; instead an extremely rapid hydrolysis~~m~~is indicated at the commencement of the action, subsequently the curve flattens out, and thereafter shows marked proportionality between hydrolysis and time. As was pointed out in the experimental section, the initial action is sufficiently rapid to be noticeable in a fraction of a minute. Turning to the phase during which hydrolysis and time bear a linear relationship; if the reducing groups liberated during this period are plotted against enzyme concentration it will be seen that the relationship is, in the majority of cases, substantially linear (fig.9).

The relationships between pH and hydrolysis and temperature and hydrolysis are extremely complex, and curves to illustrate these relationships have not been drawn; however from a study of table ~~VIII~~ XII certain inferences may be drawn. The influence of pH clearly varies with temperature of conversion and length of conversion, being of greater importance at higher temperatures; at low temperatures (30 and 37°C.) and short conversions (i.e. up to 1 hour) the optimum pH value is 4.6 or lower, but with rise in temperature and increase in conversion time the optimum pH rises to a value in the region of 5.0. Similarly the influence of temperature varies with pH and length of conversion; for conversions up to 1 hour the optimum temperature is 30°C. or lower at all pH values, but for longer conversions the optimum temperature will be seen to vary

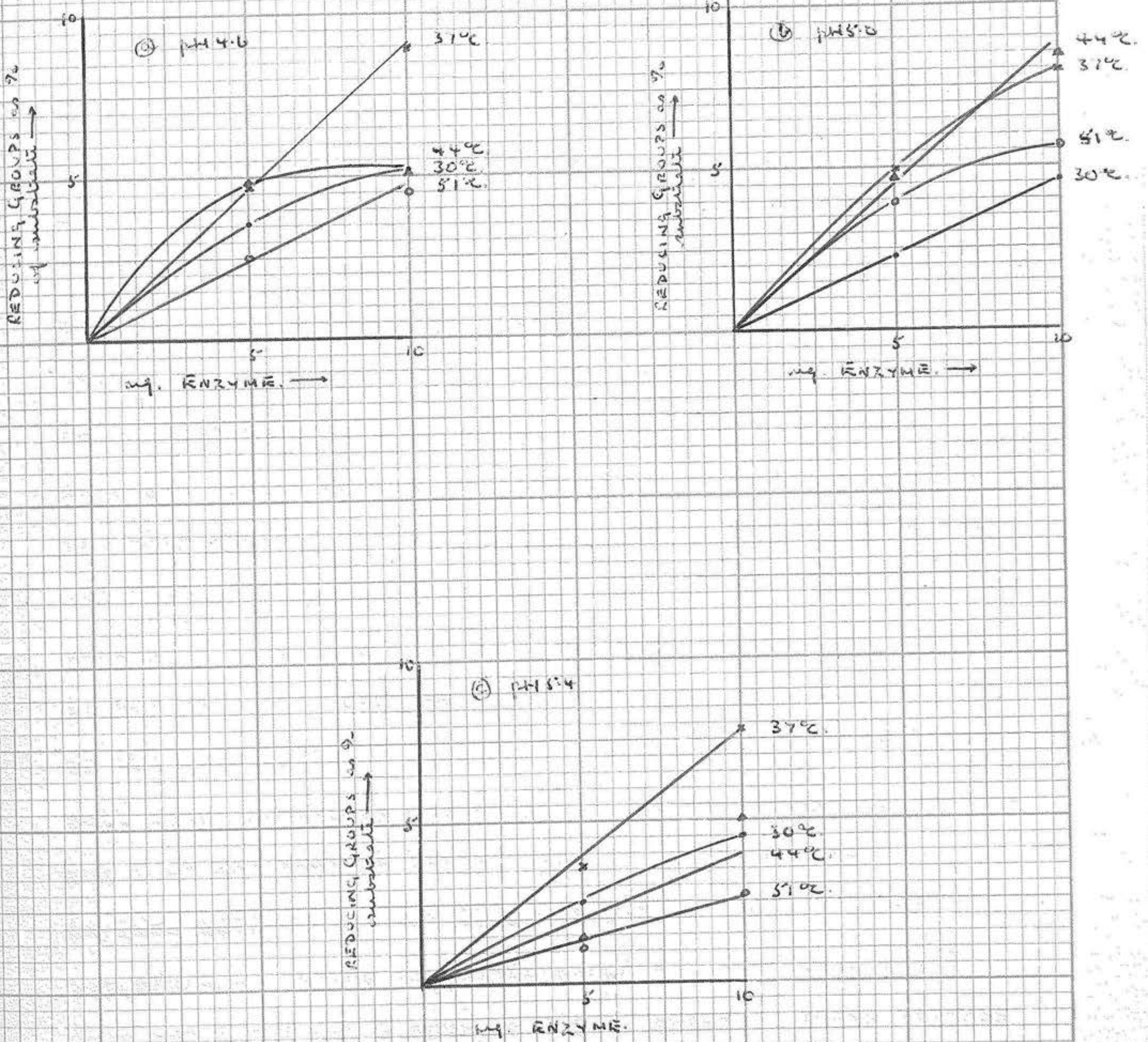


Fig. 9. Hydrolysis/enzyme concentration, for linear phase of cytolysis of barley gum c_2, (5 hr.–1 hr. figures).

with the pH value, being near 44°C. at pH 5.0, but near 44°C. at pH values 4.6 and 5.4, apparently inconsistent with the findings for long period conversions.

Short Period Conversions of Barley Hemicelluloses S_1 and S_2:-

The presence of starch in the barley hemicelluloses has unfortunately caused the figures for the hydrolysis of these by crude precipitated malt enzymes to be unreliable as a measure of total cytolysis. An attempt has been made to correct these figures for amylase action. It is well recognised that 80% of the breakdown of starch by amylases occurs within the first hour of their action, if their concentration is high (as it is relatively here), and that hydrolysis beyond this point is extremely slow, taking up to 40 hours or more to reach completion. Thus it may be safely assumed that by the end of the first hour of the hydrolysis of barley hemicelluloses by crude precipitated malt enzymes, amylase action will have substantially ceased, and that any further action will be due to cytase. Amylase action has been eliminated from these figures therefore, by taking the differences between the 1 and 3 hour and the 1 and 5 hour as due to cytase; these figures will be found in table XVII and the curves drawn from them in fig 10b. It thus appears legitimate to use these figures for a study of the second, linear phase of cytolysis.

Table XVII. Corrected figures for the cytolysis of barley hemicelluloses S_1 and S_2 by crude precipitated malt enzymes.

Barley hemicellulose S_1.

mg. of enzyme.	Temperature. °C.	Corrected reducing groups liberated (as xylose) as % of substrate.					
		pH 4.6		pH 5.0		pH 5.4	
		3-1 hr.	5-1 hr.	3-1 hr.	5-1 hr.	3-1 hr.	5-1 hr
5	30	1.1	1.4	0.3	1.2	0.4	1.1
	37	0.8	2.0	1.1	1.4	1.1	1.2
	44	0.3	1.6	1.0	2.0	0.3	1.1
	51	0.3	0.8	0.5	1.4	0.7	1.3
10	30	1.5	3.0	1.5	3.4	1.2	2.4
	37	0.3	3.2	1.4	2.7	1.5	2.2
	44	0.3	1.2	1.0	2.2	0.9	1.8
	51	1.5	1.7	0.6	1.3	0.9	1.6
				Barley hemicellulose S_2.			
5	30	1.6	2.9	1.9	3.4	1.2	3.4
	37	1.2	2.3	1.6	3.5	2.0	3.0
	44	0.8	1.3	1.6	2.4	1.7	2.9
	51	0.4	1.3	1.6	2.4	1.7	2.9
10	30	3.6	4.9	2.8	4.7	3.1	6.3
	37	3.7	6.1	3.1	7.5	4.1	8.0
	44	2.7	4.1	3.3	5.5	3.2	6.2
	51	1.4	1.7	1.6	2.2	1.7	2.7

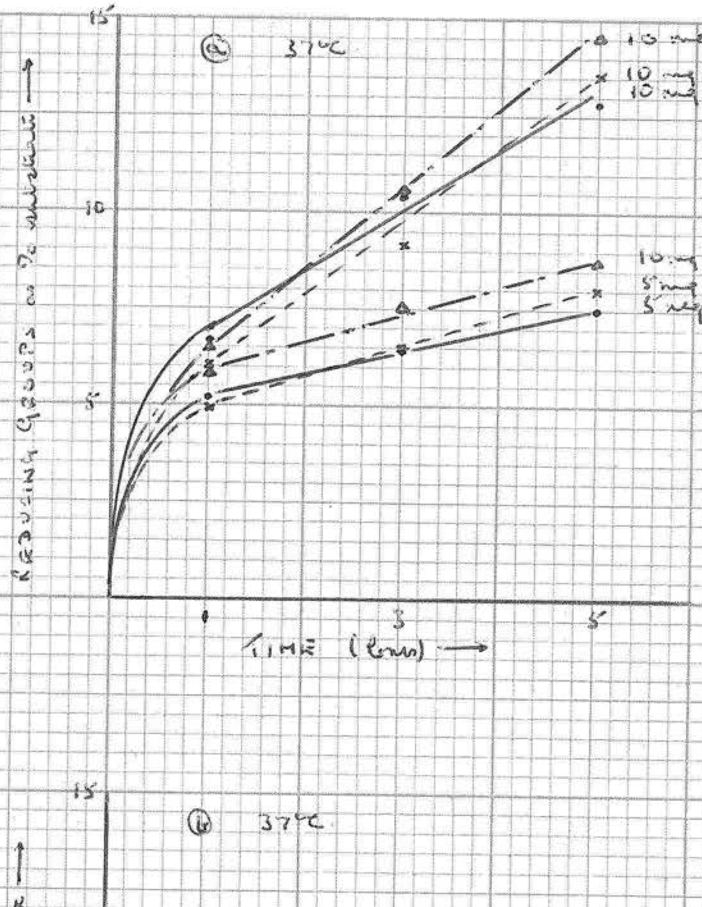


Fig. 10 Hydrolysis/time curves for the action of 5 and 10 mg. quantities of acid precipitated malt enzymes on barley hemicellulose S_W at various pH values. ⓐ Uncorrected figures ⓑ Figures corrected for amylase action.

——o——o—— pH 4.6
- - - -x- - - -x- - - pH 5.0
— · —Δ— · —Δ— · — pH 5.4

An example typical of the hydrolysis/time curves for both substrates for the corrected figures has been given in fig. 10b, and the corresponding one for the uncorrected figures in fig 10a. Both of these curves show the broadly linear relationship between hydrplysis and time during the second phase. It is of interest to note that in the cases of the hemicelluloses the first phase is more marked than in the case of barley gum C_2 (tables XII and XIV, figs. 5-8 and 10a), and there seems little doubt that, though some of this greater action is due to the hydrolysis of starch by amylases, the initial rapid action is present during the hydrolysis of these substrates by cytase. In the case of barley hemicellulose S_1 both pH and temperature have little influence on cytolysis; conditions seem to be most favourable at a pH value of 5.0 and a temperature of 30°C. or lower. The effects of temperature and pH on the cytolysis of hemicellulose S_2 are more pronounced; the optimum pH value tends to be a little higher than in the case of barley gum C_2, being at least 5.4, and the optimum temperature is around 37°C. It must be noted, that as yet there is no evidence that the same actions are involved in all the three cases under discussion, and it is possible that the shift in optimum pH observed with hemicellulose S_2 may be due to a different type of action being involved.

Cytase as a System of Enzymes;- The hydrolysis of barley gum C_2 by the precipitated cytase preparation from barley is very interesting, as it is apparent from an examination of the hydrolysis/time curves (fig.11) that a different type of action is involved, the action of the enzyme ceasing after 1-3 hours conversion. The hydrolysis achieved (approximately 3%) corresponds to that of the initial rapid action of the malt enzyme. The presence of cytase in ungerminated oats has been confirmed by the preparation of a precipitated mixture of enzymes from this source. The action of this enzyme preparation on barley gum C_2 is similar to that of crude precipitated malt enzymes (table XIV, fig.11), although the preparation is less active.

Clearly cytolysis by crude precipitated malt enzymes involves two distinct actions: firstly a rapid hydrolysis corresponding to production of 3-5% of reducing groups, and secondly a more prolonged hydrolysis which is proportional to time. It seems highly probable that these two phases arise from the action of more than one enzyme, the initial phase arising from the action of an enzyme which attacks the substrate by breaking it down to molecules of still large size, e.g. depolymerisation, the second being due to an enzyme, or group~~s~~ of enzymes, which hydrolyses the breakdown products of the action of the first enzyme, and possibly the unchanged substrate, to (eventually) simple saccharides. It is possible in view of the complex constitution of the barley gums and hemicelluloses that different types of linkage are involved in these changes.

The suggestion that more than one enzyme is involved in cytolysis fits in admirably with the observation made above on the action of crude precipitated enzymes from barley, since it would appear that only one of these, namely that responsible for the initial rapid action, is present in barley.

Further evidence in favour of the above suggestion is provided by the temperature and pH effects noted with barley gum C_2. Here it will be noted that two groups of optima are present, one ~~fro~~ for conversions up to 1 hour, in which the respective optima are pH 4.6 and 30°C., and a second group ~~fro~~ for conversions up to 5 hours, in which the relationships are more complex.

If the linear portions of the hydrolysis/time curves for barley gum C_2 are extended to cut the hydrolysis axis, it will be seen that the intersections of the majority fall indiscriminately within a close range of hydrolysis from 3-5%. It would appear therefore that the initial rapid action is limited in its extent, and that this limit can not be exceeded by increasing the enzyme concentration, thus supporting the contention that this action comprises depolymerisation or disaggregation, rather than a continuous hydrolysis.

It was noted whilst discussing the long period ~~by~~ a-ction of cytase on barley gum C2. that the available evidence pointed to the presence of two enzyme actions, and since the action must correspond to that of the "enzyme" responsible for the linear phase of short period conversions, this may be taken to indicate that this second enzyme itself is a system of enzymes.

This possibility is further supported by the complex nature of the pH and temperature effects of the linear phase. The absence of any indication of the existence of the initial rapid phase during long period actions is not surprising, since the conversion was allowed to proceed 20 hours before the reducing groups liberated were determined, and consequently departure from linearity would be masked by the large number of reducing groups liberated in that time.

Thus it appears that cytase comprises a system of enzymes and not a single enzyme as previous workers appear to have assumed. As far as it is possible to state at present, the enzymes or systems of enzymes have the following properties:

Cytase 1:- An enzyme capable of producing a rapid hydrolysis of hemicellulosic substances from barley immediately the two are brought in contact, without large scale liberation of reducing groups; this enzyme may be conveniently termed cytoclastase, and its a-ction cytoclasis. It is apparently little affected by changes in temperature or pH value, but conditions are best for its action at pH 5.0 and 30°C. The possible mode of its action is to hydrolyse the substrate to molecules of still complex nature, which are the acted upon by cytase 2.

Cytase 2:- An enzyme, or more likely a system of enzymes acting on the products of "cytoclastase" action or on the substrate itself, at a much slower rate than "cytoclastase", the action being long lasting. This enzyme or enzyme system may be termed "cytolytase", and its action "cytolysis". The optimum pH of this "enzyme" is 5.0, and at this, the optimum pH, the optimum temperature is 44°C.; at pH values 4.6 and 5.4 the optimum temperature is 37°C.

SUMMARY.

1. Cytase has been shown to be present in green malt, low-dried malt, ungerminated barley and ungerminated oats, low-dried malt being the most convenient source. A precipitated form of cytase has been prepared which is stable over long periods of time in the dry state.

2. Of six substances tested as substrates for cytase, only one, barley gum C_2, has proved fully suitable. No measurable hydrolysis was observed in the cases of the willow hemicelluloses, but barley gum B_2 and the barley hemicelluloses have been shown susceptible to cytase action, but the use of these is complicated by their contamination with starch.

3. The action of cytase comprises two distinct phases, the total action not being proportional to time from the start of the action. The first phase is an extremely rapid action of short duration, producing few reducing groups; a linear relationship exists during the second phase, up to a liberation of 20% reducing groups. The pH and temperature relationships of the two phases have been examined.

4. It is suggested that cytase is a system of at least two enzymes, "cytoclastase" and "cytolytase" (itself possibly a mixture), and the properties of these have been described as far as is possible at the present stage. Whilst both are present in low-dried malt and ungerminated oats, it appears that substantially only cytoclastase is present in ungerminated barley.

IV. OBSERVATIONS ON THE COMPLEX NATURE OF CYTOLYSIS.

In the previous section evidence was given which showed that the action of cytase involved two phases, and led to the conception of cytase as a system of at least two enzymes, i.e. the cytolytic and cytoclastic factors.

Three possible methods by which these actions might be separately studied suggest themselves: firstly the separation of the enzymes responsible for the two phases; secondly the preparation of substrates susceptible to only one of the factors; the use of methods for following the particular aspects of cytase action other than that of determination of reducing groups. In this section it is only proposed to deal with the second of these methods.

A possible method by which the preparation of a modified substrate might be achieved was indicated when the cytase of ungerminated barley was examined, the results of which examination led to the conclusion that substantially only the cytoclastic factor was present (Section III). It was hoped that by allowing the precipitated preparation from barley to act on the substrate prior to the action of crude precipitated malt enzymes, the substrate would be sufficiently modified to eliminate the cytoclastic action normally observed, and thus produce a substrate suitable for the study of cytolysis.

During the preparation of modified substrates it will be shown that if an extract of barley is incubated at

37°C. for a sufficient length of time, no precipitate can be obtained on the addition of Fehling's solution and acetnne, indicating presumably a complete hydrolysis of barley gum C_2. This initiated a study of the water soluble polysaccharides of malt, since it would appear that the constitution of these substances changes radically during germination. An increase in the soluble pentosans of barley during germination has been reported by Windisch and van Waveren (1909), and an increase in the total pentosans i.e. soluble and insoluble, has been reported by various workers (Schöne and Tollens, 1901; van Laer and Masschelein, 1923; Preece 1940b.) Several of these workers (Baker and Hulton, <u>loc. cit.</u>; Preece, 1940b.) have studied the change in the pentosans of various sites in the kernel, i.e. embryo, endosperm and husk; the method used was the determination of furfuraldehyde yield from the respective sources, and as yet no attempt to isolate these substances has been reported. It was therefore felt that information of considerable interest would be obtained if the water-soluble polysaccharides of malt could be isolated and subjected to analysis.

EXPERIMENTAL.

In view of the very low yeilds of barley gum C_2 etc., it was considered advisable to carry out preliminary experiments to test the possibility outlined in the introdudtion, before commencing large scale preparations of modified substrates directly from barley.

100 ml. of a solution containing the equivalent of 75 mg. of dry, ash-free barley gum C_2 per 40 ml. were prepared, and 40ml. of this were pipetted into each of two conversion flasks. The substrate solutions were adjusted to pH 4.6 with sodium acetate/ acetic acid buffer solution and to 30°C., and 10 ml. of a 0.10% (dry, ash-free equivalent) solution of precipitated barley enzymes were added to each; the conversions were allowed to proceed for two hours when the solutions were boiled. Controls on water were prepared in a similar manner. After boiling, the controls and conversions were made up to 50 ml. with the prior addition of 1 ml. of pH 5.0 sodium acetate/acetic acid buffer solution, and 10 ml. of each withdrawn for reducing group determination. 15 ml. of water were added to each of one control and one conversion, and 10 ml. to each of the other pair, the former to receive 5 ml. of 0.10% (dry, ash-free equivalent) crude precipitated malt enzymes solution and the latter 10ml., making the total conversion volume 60 ml. in all cases; the flasks were brought to 37°C., and the requisite amount of enzyme solution added. Reducing groups were determined after 1,3 and 5 hours conversion, the 0 hour figure being taken as that of the substrate solution after boiling, but prior to the addition of crude precipitated malt enzymes solution. It is important to note that the final enzyme/ substrate relationships are the same as in the standard conversion method previously used.

Table XVIII. Action of crude precipitated malt enzymes on 0.10% barley gum C_2 solution previously acted on by precipitated barley enzymes; pH 5.0, 37°C.

mg. of enzyme added.	Corrected reducing groups liberated (as xylose) as % of substrate.		
	1 hour.	3 hour.	5 hour.
5	1.3	3.45	5.4
10	2.4	7.1	10.95

As will be seen in table XVIII and fig. 12, a considerable measure of success has been achieved, and although cytoclasis has not been completely eliminated, its extent has been considerably reduced. In this experiment however no control was exercised over the action of the precipitated barley enzymes, and consequently it may not have reached completion in the time allowed; in order to eliminate this possibility as far as possible the experiment was modified as below.

1.0 g. of barley gum C_2 was dissolved in water, 1 ml. of sodium acetate/acetic acid buffer solution added, and the volume made up to 100 ml. The solution was placed in a conversion flask and immersed in a water bath at 30°C.; 0.050 g. (dry, ash-free eqiuvalent) of precipitated barley enzymes were dissolved in water, and added to the barley gum C_2 solution. Reducing groups were determined after 1 hour, 2 hour and thereafter at suitable intervals until the action had ceased, and the barley gum was then

64a

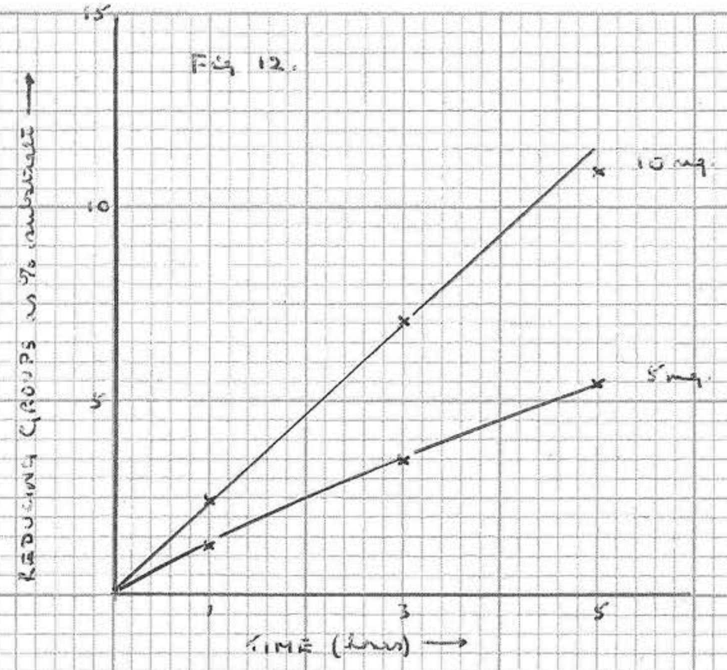


Fig 12. Action of crude precipitated malt enzyme (5 and 10 mg) on a solution of barley gum C_2 which had been previously acted on by precipitated barley enzymes. pH 5.0, 37°C

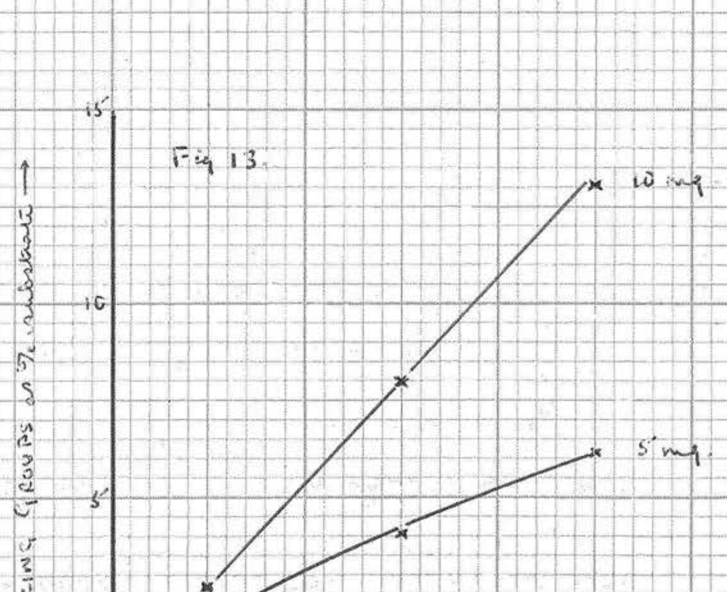


Fig 13. Action of crude precipitated malt enzymes (5 and 10 mg). on modified barley gum C_2 prepared from a preparation of barley gum C_2. pH 5.0, 37°C

recovered in the usual manner, the treatment stopping the enzyme action. The action of crude precipitated malt enzymes on the product, modified barley gum C_2, was tested using the standard short period method, the temperature used being 37°C. and the pH value 5.0; the results will be found in table XIX and the corresponding graphs in fig. 13.

Table XIX. Action of crude precipitated malt enzymes on a modified barley gum C_2 prepared from a solution of barley gum C_2; pH 5.0 and 37°C.

mg. of enzyme.	Corrected reducing groups liberated (as xylose) as % of substrate.		
	1 hour.	3 hour.	5 hour.
5	1.8	4.05	6.15
10	2.7	7.95	13.05

From the results it appeared that the attempt to prepare a substrate susceptible only to cytolysis had been largely successful, consequently large scale preparations of modified substrates were attempted.

Preparation of Modified Barley Gum C_2:- ~~100~~ 500 g. of barley were ground and extracted three times with 1,000 ml. quantities of water without previous inactivation of enzymes, filtering through cloth after each extraction. The combined extracts were raised to 30°C., and held at that temperature for three hours. Note that the action of barley enzymes was necessarily proceeding for several days during this preparation. The temperature of 30°C. was chosen as earlier work (SectionIII) had indicated that the cytoclastic factor was most active at this or lower temperatures. The extract was then filtered through paper covered with a layer of Kieselguhr, and the volume reduced to approximately 300 ml. by distillation under reduced pressure. The extract was then filtered through paper pulp, and NaOH added to a concentration of 4%. Fehling's solution was added until a blue colouration was visible in the extract: no precipitate was obtained, as was expected, and acetone was added but again no precipitate was obtained. It seems apparent that long contact between the barley gum and the barley enzyme has modified the g-um to the extent that it can not be precipitated by the addition of Fehling's solution and acetone.

To eliminate the long contact between the gum and the enzyme the following method was adopted;

500 g. of garley were ground and extracted twice with boiling 80% alcohol under reflux for 20 min. to inactivate the enzymes. The residues were filtered off through cloth at the pump, and extracted thrice with 1,000 ml. quantities of water at room temperature, filtering through cloth at the pump after each extraction. The extracts were combined, raised to 30°C., and 300 ml. of a 20% barley extract were added. The extract was maintained at 30°C. until a

constant titre was obtained by the usual reducing group determination. The extract was then boiled for a short time to inactivate the enzymes, filtered through a layer of Kieselguhr on paper, and the volume reducedto approximately 300 ml. by distillation under reduced pressure. The concentrate was filtered through paper pulp, and NaOH added to a concentration of 4%; Fehling's solution was then added followed by half a volume of acetone, and the precipitate obtained, modified barley gum C_2, taken to dryness in the usual manner.

Preparation of Modified Barley Hemicelluloses:- In this first method it was hoped that the action of the enzymes of the barley under treatment, with the addition of an α amylase containing extract would prove sufficient both to modify the hemicelluloses, and to remove starch.

100 g. of ground barley were mixed with 500 ml. of water at 30°C., and the temperature maintained for 5 hours; 200 ml. of a 5% low-dried malt extract, in which β amylase and cytase had been inactivated in the usual manner, were added, and the temperature raised to 57°C. overnight. On testing, starch was found to be still present in the extract. The residues were filtered off through cloth at the pump, mixed with 500 ml. of a 5% barley extract, and the temperature raised to and maintained at 30°C. for 2 hours. At the end of this time 200 ml. of a 5%, β amylase free and cytase free, malt extract were added and the temperature raised to 57°C. overnight. Starch was still present after this further treatment , and the

preparation was discontinued.

In view of the failure of the preceeding experiment , it was decided to gelatinise the starch before attempting the modification of the hemicelluloses. Accordingly, 100 g. of ground barley were added slowly to 70^0 ml. of boiling water so that the water did not go off the boil, and the mash formed was boiled for a short time. The gelatinous mixture was then cooled to 80°C., 150 ml. of a low-dried malt extract added, and the temperature held at 75°C. for 20 min. to liquefy starch. The mash was cooled to 30°C., 300 ml. of a 20% barley extract added, and the temperature held at that temperature for 1 hour. At the end of this time the temperature was raised to 57°C., 150 ml. of a~~n~~ β amylase free and cytase free malt extract added,and the mash incubated at that temperature overnight. Starch was still present after the treatmant, but after a further similar treatment starch was completely removed. The residues were filtered off through cloth at the pump, and thouroughly washed with hot water to remove dextrins etc.

The residues were extracted with 500 ml. and two further 250 ml. quantities of 4% NaOH, filtering after each extraction through cloth at the pump. Fehling's solution was then added, but no precipitate was obtained, i.e. hemicellulose S_1 was absent. Half a volume of acetone was therefore added giving a precipitate of hemicellulose S_2, which was recovered in the usual manner.

It will be noted that no hemicellulose S_1 was obtained, whereas this fraction was present in previous preparations. A further preparation of modified barley hemicelluloses, and also two normal hemicellulose preparations were made from the same barley, but in no case was a hemicellulose S_1 obtained.

The furfuraldehyde yields of the modified substrates were determined, and the results will be found in table XX.

Table XX. Yields of, and furfuraldehyde yields from modified substrates.

	Dry, ash-free.	
Substance.	Yield as % of barley.	Furfural as % of substance.
Modified barley gum C_2.	0.5	25.55
Modified barley hemicellulose S_2. *	3.4 3.7	46.90

* - 2 preparations

The action of crude precipitated malt enzymes on the modified substrates was tested by the standard short period method, at pH 5.0 and 37°C. In order that the action of cytase on the modified substrates could be more accurately compared with its action on normal substrates, conversions on barley gum C_2 and barley hemicellulose S_2 were run concurrently. The results are recorded in table XXI and graphed in fig. 14.

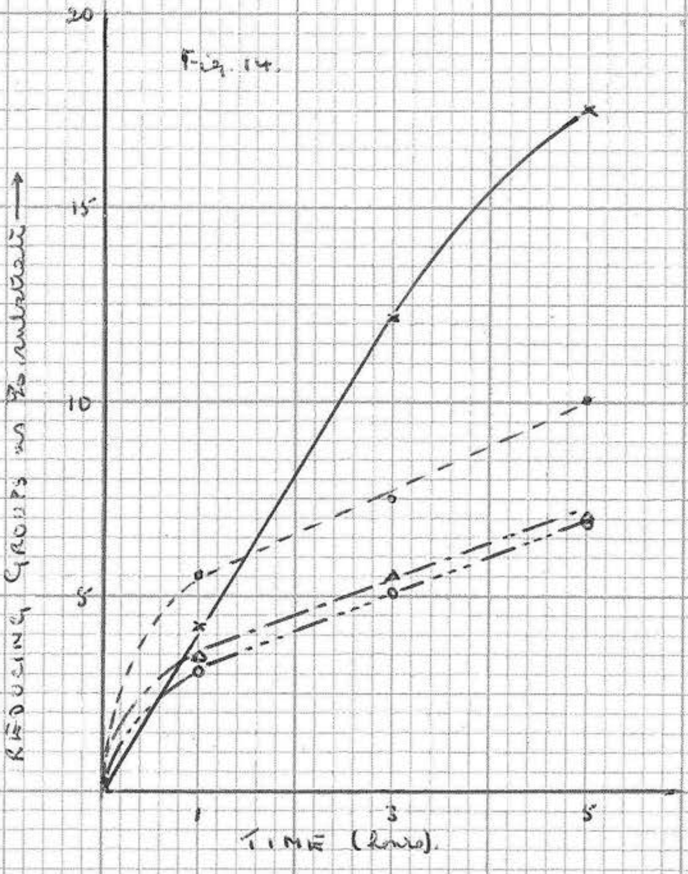


Fig 14. Hydrolysis of modified and unmodified substrates by 10 mg. of crude precipitated malt enzymes. pH 5.0; 37°C

——×————×—— Modified barley gum C_2.

- - - ∘ - - - - - ∘ - - - Barley gum C_2

—·—Δ—·—·—Δ—·— Modified barley hemicellulose S_2

——· · ·o——· · ·o· · ·— Barley hemicellulose S_2

Table XXI. Action of 10 mg. of crude precipitated malt enzymes on modified and unmodified substrates at pH 5.0 and 37°C.

Substrate.	Corrected reducing groups liberated(as xylose) as % of substrate.		
	1 hour.	3 hour.	5 hour.
Modified barley gum C_2.	4.2	12.15	17.55
Barley gum C_2;	5.55	7.5	10.05
Modified barley hemicellulose S_2.	3.45	5.55	7.05
Barley hemicellulose S_2.	3.15	5.1	6.9

It would appear ffom the results that, in the case of modified barley gum C_2, the search for a substrate susceptible to the action of the cytolytic factor alone has been successful, the action of the cytoclastic factor being almost completely eliminated. In the case of modified hemicellulose S_2 however, a considerable measure of cytoclasis remains. It must be noted that slight traces of starch were detected in both of the modified substrates and in barley hemicellulose S_2 durimg the determination of reducing groups before the start of the action, but this had completely disappeared at the end of one hours conversion. The slight deviation from linearity between hydrolysis and time at 1 hour may, in the case of modified barley gum C_2, be due to the action of amylases on this small amount of starch; this is less likely to be the case with modified barley hemivellulose S_2. A point worthy of note is that the action of cytase on modified barley gum C_2 is more rapid than its action on the unmodified substance.

The Preparation of Water-soluble Polysaccharides from Malt:- 500 g. of a normal brewers malt were treated in the manner described for the preparation of barley gum C_2, and a polysaccharide was isolated through its copper complex, being precipitated by the addition of Fehling's solution and acetone, i.e. a series 2 polysaccharide.

The first striking feature about this substance is its entirely different physical nature from the corresponding, fibrous barley gum C_2.; the dried substance from malt is a white, comparatively hard, granular substance, of much greater density than barley gum C_2.

Since it had been shown possible to isolate a cold-water-soluble polysaccharide from malt, it was considered advisable to examine the malt for the presence of a hot-water-soluble polysaccharide, i. e. one corresponding to barley gum B_2.

Accordingly, 500 g. of malt after pretreatment in the usual manner to inactivate enzymes, were extracted with successive 1,000 ml. quantities of water until little or no further extraction occurred, and the first three of these extracts were reserved for the preparation of, the cold-water-soluble polysaccharide, (above), the subsequent cold-water extracts being discarded. The malt residues were then extracted thrice with 1,000 ml. quantities of water at 40°C., the extracts being separated through cloth at the pump after each extraction, and the polysaccharide was recovered from the extracts in the manner described for barley gum C_2. This polysaccharide again proved to be of the second series, and was of very similar nature to the cold-water-soluble polysaccharide.

The substances were subjected to the usual analytical determinations, the results of which will be found in table XXII.

Table XXII. Analytical data on the water-soluble polysaccharides of malt.

		Dry, ash-free.	
Substance.	Ash as % of substance.	Yield as % of barley.	Furfural as % of substance.
Cold-water-soluble malt polysaccharide.	0.0	0.5	44.05
Hot-water-soluble malt polysaccharide.	0.0	0.1	12.50

The action of cytase on the malt polysaccharides was studied using the method described for the modified substrates; The results will be found in table XXIII and the corresponding curves in fig. 15.

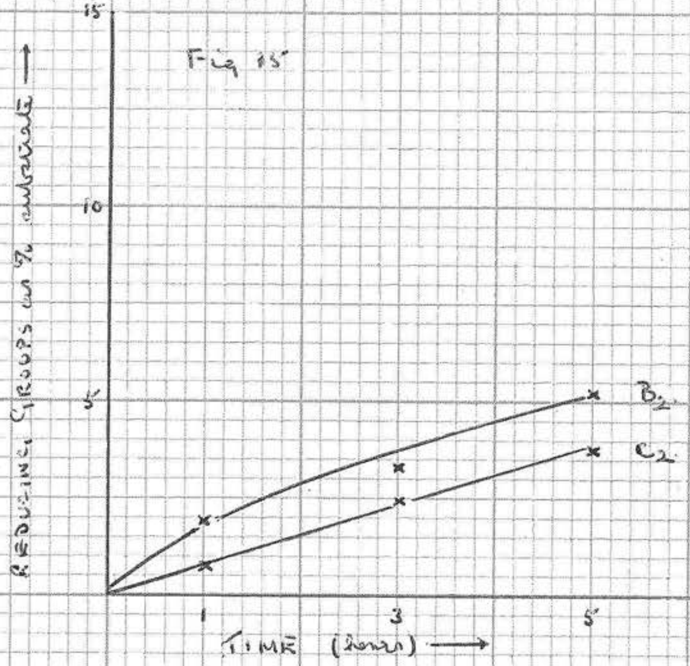


Fig. 15. Hydrolysis of malt hemicellulose - fractions B_2 and C_2 by crude precipitated malt enzymes (10 mg.). pH 5.0, 37°C.

Table XXIII. The action of 10 mg. of crude precipitated malt enzymes on the water-soluble polysaccharides of malt, at pH 5.0 and 37°C.

Time in hours.	Corrected reducing groups liberated (as xylose) as % of substrate.	
	Cold-water-soluble polysaccharide	Hot-water-soluble polysaccharide.
1	0.75	1.95
3	2.4	3.3
5	3.75	5.2

From the results it is clear that the water-soluble malt polysaccharides are hydrolysed at a much slower rate than are the corresponding barley gums; the hydrolysis of the cold-water-soluble substance is proportional to time, whereas that of the hot-water-soluble substance is not. This latter substance gives a purple-brown colour with iodine, possibly due to the presence of starch-dextrins.

It is interesting to note that extracts of the malt polysaccharides, in distinction from extracts of the barley gums, filtered quite easily.

DISCUSSION.

The Preparation of Modified Substrates:- The attempted elimination of cytoclasis has given some very satisfactory results, all the variations of method being successful to a greater or lesser extent.

The first of these methods, i.e. the modification of a standard barley gum C_2 solution, though the action of barley enzymes was uncontrolled, gave a substrate which when acted on by cytase showed linearity between hydrolysis and time from the start of the action (table XVIII, fig 12).

The success of the first experiment led to the hope that the preparation of modified substrates on a large scale i.e. directly from barley, would be possible, so obviating the necessity for the preliminary hydrolysis with barley enzymes each time a study of cytolysis was to be made. A preliminary experiment on the precipitation of modified barley gum C_2 was therefore carried out ,using a preparation of barley gum C_2 as the starting point. This again proved successful, and the hydrolysis/time curves (fig 13) for the action of cytase on this preparation are straight lines. Also as was noted in the previous Section for the second phase of normal cytase action, the gradients of the curves are proportional to enzyme concentration.

The first and unsuccessful attempt to prepare modified barley gum C_2 from barley yielded valuable information. This was that sufficiently long contact (4-5 days) between barley gum C_2 and the cytase of barley, i.e. the supposed cytoclastic factor, breaks down the gum to the extent that it can no longer be precipitated by Fehling's solution and acetone. The second attempt to prepare modified barley gum C_2 from barley, in which the action of the barley enzymes was controlled, came up to expectation, giving a reasonable yield (0.5%), and a substrate which when acted on by crude precipitated malt enzymes gave a straight line curve for hydrolysis/time (fig. 14). It is most interesting to note the increased susceptibility of modified barley gum C_2 to the action of crude precipitated malt enzymes; the gradient of the hydrolysis/time curves is much steeper than the gradient of the straight portion of normal actions.

When the first preparation of modified barley hemicelluloses was completed, it appeared that a radical change had occurred in the nature of the hemicelluloses, i.e. the presence of series 2 only, where previously both series 1 and series 2 hemicelluloses had been obtained. However when a normal preparation of hemicelluloses was made from a sample of the same barley, and again only series 2 obtained, it became apparent that the absence of hemicellulose S_1 was not due to the action of barley enzymes, but was due to either its absence from the barley, or to the method of preparation; these last two possibilities were discussed in Section II. The action of crude precipitated malt enzymes on the modified hemicellulose did not provide such promising results as the modified gum, the hydrolysis/time curve (fig. 14) being very much the usual shape, and the hydrolysis attained no greater than with barley hemicellulose S_2.

Table ~~XXIII~~ XXIV. Comparison of yields and furfuraldehyde yields from modified and normal barley gum C_2 and barley hemicellulose S_2 preparations.

Substance.	Dry, ash-free. Yield as % of barley.	Furfural. yield as % of substance.
Barley gum C_2.	* 0.2, 0.3, 0.4	25.60
Modified barley gum C_2.	0.5	25.55
Barley hemicellulose S_1.	1.0	47.94
Barley hemicellulose S_2.	0.9	36.48
Barley hemicellulose S_2. ✱	1.3	45.50
Modified barley hemicellulose S_2. #	3.4, 3.7	46.90

* - three preparations.

✱ - no hemicellulose S_1 isolated.

\# - two preparations.

By comparing the yields of barley gum C_2 with that of modified barley gum C_2, it is clear that modification has resulted in no substantial loss of material, and from a comparison of the furfuraldehyde yields of the substances, it would appear that modification has not led to any drastic change in the chemical nature of the gum, though the absence of any change in furfuraldehyde yield from the two substances does not exclude the possibility that some depolymerisation or disaggregation has occurred.

Turning to,modified hemicellulose S_2, it will be seen that modification has led to a great increase in the yield of hemicellulose; it must be noted that a similar increase could not occur in the case of modified barley gum C_2, since modification was carried out subsequent to the extraction of the gum, whereas with the hemicellulose it was carried out in the presence of the grains. This increase in yield must presumably be due to the action of barley enzymes on the insoluble hemicelluloses of the barley to render a larger proportion of them soluble. This observation confirms those of previous workers, who, using the cytase from green malt not barley, noted that it brought about an increase in the soluble furfuraldehyde yielding material when allowed to act on barley grains (Section III)

There is an apparent slight increase in furfuraldehyde yield in the modified hemicellulose over that yielded by the hemicellulose S_2 isolated along with a hemicellulose S_1, but the validity of such a comparison is very doubtful, since the hemicellulose S_2 isolated in the absence of any S_1 fraction gave a furfuraldehyde yield which is essentially the same as that of modified hemicellulose S_2. Thus, as in the case of the gum, modification would appear to have caused no change in character of the hemicellulose; however it must not be overlooked that the modified hemicellulose contains material not present in the normal preparation, which might confer a change in character on the substance undetectable determination of furfuraldehyde yield.

<u>The Water-soluble Polysaccharides of Malt</u>;- Two water-soluble polysaccharides, analogous to barley gums B_2 and C_2

in so far as their solubilities are similar, have been isolated from malt. These substances have proved to be entirely different from the barley gums both in physical appearance and texture, and also in chemical constitution. The barley gums are both very light, fibrous substances, whereaas the substances are denser, compact, granular bodies, and do not posses any electrostatic properties. The yield of furfuraldehyde from the two groups of substances are vastly different (table ~~XXIV~~ XV), the malt polysaccharides giving much larger yields; however it must be noted that in this respect, the two groups show a similarity, namely the yield of furfuraldehyde from the cold-water-soluble substances is the greater in both.

<u>Table ~~XIV~~</u> XV. Furfuraldehyde yields from water-soluble p polysaccharides of barley and malt.

Substance.	% of dry, ash-free material.	
	Furfural yield.	Uronic acid.
Barley gum C_2.	26.10	1.04
Barley gum B_2.	3.96	1.16
Cold-water-soluble malt polysaccharide.	44.05	7.64
Hot-water-soluble malt polysaccharide.	12.50	2.05

There seems little doubt that the malt polysaccharides are in type entirely differen t from the barley gums, and that consequently they should not be classified as gums.

It has tentatively decided to name these substances "hemicellulose-dextrins" This name has been chosen since these substances, though excluded from the class "hemicelluloses" through the initial water solubility, are much more hemicellulosic in nature than are the barley gums (higher furfuraldehyde yield, and physical appearance of preparation); the term "dextrin" has been introduced to indicate their initial w-ater solubility and their possible origin as break down products of hemicelluloses, in analogy with starch (c.f. Preece, 1948.). The cold-water-soluble substance will thus be know as "hemicellulose-dextrin" C_2 and the hot water soluble substance as "hemicellulose-dextrin B_2".

The action of cytase on malt hemicellulose- dextrins further enphasises their difference from barley gums. The most striking feature about the action of cytase on these substances is their much greater resistance to its action (table XXIII), only 3.5% hydrolysis being achieved in the case of hemicellulose-dextrin C_2, and 5.2% in the case of hemicellulose-dextrin B_2, whereas figures for the corresponding barley gums excede 10%. Again in the case of hemicellulose-dextrin C_2, no cytoclastic action was observed (fig,15), and in the case of hemicellulose-dextrin B_2, though this action was present, its extent was considerably reduced. Little doubt can therefore remain that the w-ater-soluble constituents of malt are entirely different from those of barley, and consequently results have confirmed the predictions made in the introduction, namely that radical changes in the constitution of the substances susceptible to cytase occur during germination.

The work described in this section strengthens the conviction that cytase comprises at least two enzymes, or enzyme systems, and had no other useful purpose been served, this would prove ample justification for the work. The results have shown that although a separation of the enzymes of the system has not been achieved, it is possible to separate the actions involved; the cytoclastic phase in the action of precipitated barley enzymes on barley gum C_2, and the cytolytic phase in the action of crude precipitated malt enzymes on modified barley gum C_2 and hemicellulose-dextrin C_2. There seems little doubt therefore that the theories developed to explain the action of cytase, so far as they have gone, have a firm foundation in fact.

SUMMARY

1. The preparation of substances, modified barley gum C_2 and modified barley hemicellulose S_2, possibly suitable as substrates for the study of cytolysis, has been described; the action of cytase on these substances has been examined and one of them, modified barley gum C_2, proved susceptible only to cytolysis.
2. Modification, i.e. the action of precipitated barley enzymes, has been shown to result in an increased yield of barley hemicelluloses, and a possible explanation for this suggested.
3. Water-soluble polysaccharides, described as hemicellulose-dextrins, have been prepared from malt, and the action of cytase on them studied. these substances have been shown to be quite different from barley gums, and the suggestion that radical changes in the hemicellulosic constituents of barley, occur during germination, has been confirmed.
4. Several of the earlier suggestions as to the nature of cytase have been confirmed.

V. THE FRACTIONATION AND PURIFICATION OF CYTASE.

During the course of the work described in the previous sections, evidence has accumulated which, it would appear, is consistent with the assumption that cytase is not a single enzyme, but a system of enzymes. However, no matter how great the volume of indirect evidence for the presence of more than one enzyme, the ultimate acceptance of this suggestion will require the separation of individual enzymes from the system.

The problem of separating these enzymes, and incidentally the purification of cytase, has been approached from three directions; firstly fractional precipitation by alcohol, secondly adsorption, and lastly extraction with buffer solutions of varying pH value.

The adsorption of an enzyme by its substrate has been demonstrated, in the case of α-amylase, by Hockenhull and Herbert (1945), who carried out the adsorption on a suspension of starch in the presence of 50% alcohol at 0°C., and eluted with phosphate buffer of pH value 5.8. It was therefore decided, since the substrate viz. barley gum C_2, is not suitable for this purpose owing to its solubility, to attempt the adsorption on a water insoluble hemicellulose, viz. willow hemicellulose S_1.

In considering methods which might lead to a higher yield, a purer preparation or a fractionation of cytase, the possibility that the pH value at which extraction was carried out might have some influence arose. The pH value of extraction could be effective in two ways; firstly by increasing the solubility of one or more of the components of the enzyme system, or secondly by decreasing the solubility of the usually contaminating substances. That both of these possibilities exist will be seen when it is appreciated that both cytase, and much of the contaminating material are protein in nature, and as such will have isoelectric points at which they will be in their most insoluble state. An examination of the influence of pH on the extraction of cytase has therefore been made.

Lüers and Malsch (1929), in a study of the effect of malting on cytase, concluded that although a loss of cytase occurred on steeping, cytase was produced during germination to an amount 2.5 times that of the original barley. These workers were of the opinion that cytase was a single enzyme, whereas it has been suggested that cytase is in fact a system of enzynes, and that only one of these, the cytoclastic factor, is present in ungerminated barley. It seems imperative therefore that the behaviour of cytase during germination should be re-examined, both to test the findings of the above workers, and more important still to determine the stage at which the cytolytic factor appears.

EXPERIMENTAL.

The Adsorption of Cytase on Willow Hemicellulose S_1:- The method, used for the attempted adsorption of cytase is based on that of Hockenhull and Herbert (loc. cit.), outlined in the introduction to this section. Willow hemicellulose S_1 was chosen because of its similarity to the barley gums (see Section II), and its insolubility in water. A preliminary experiment had shown that the highest concentration of alcohol which would not cause any precipitation from a solution of crude precipitated malt enzymes was ~~45%~~ 36%, i.e. 3 ml. of 95% alcohol/5 ml. of solution, and prior to adsorption alcohol to this concentration was added to the enzyme solution.

125 ml. of crude precipitated malt enzymes solution, containing the equivalent of 3.2 mg. of dry, ash-free enzyme per ml. were prepared; to 50 ml. of this solution 30 ml. of water were added, to give the control enzyme solution of concentration 2 mg./ml., and to a further 25 ml. of the solution 15 ml. of alcohol were added, giving the enzyme plus alcohol control solution of concentration 2 mg./ml.

To the remaining 50 ml. of solution, 30 ml. of 95% alcohol were added, and the solution well mixed; 12.5 ml. quantities of the mixed solution were added to each of four centrifuge tubes, which already contained 0.1 g. of willow hemicellulose S_1, and contact between the enzyme and hemicellulose maintained for a short time.

The tubes were centrifuged until the supernatant liquid was clear, and the residual solutions decanted and reserved in a clean, dry beaker. The hemicellulose in each tube was then washed with a mixture of 50 ml. of water and 30 ml. of 95% alcohol; the washings were centrifuged bright and discarded. Elution was then attempted, by adding 12.5 ml. of water, allowing to stand for a short time and centrifuging; the eluates were decanted and combined in a clean, dry beaker. Control enzyme solution, enzyme plus alcohol control solution, residual solution after adsorption, and eluate were tested for cytase activity against barley gum C_2, using the standard short period method at pH 5.0 and 44°C.; 10 ml. of each solution were used, i.e. equivalent to 10 mg. of enzyme, and the results were thus comparable.

Table XXVI. The hydrolysis of barley gum C_2 by solutions of cytase before and after attempted adsorption and elution; pH 5.0, 44°C.

Enzyme.	Corrected reducing groups liberated (as xylose) as % of substrate.		
	1 hour.	3 hour.	5 hour.
(a) Control enzyme solution.	4.5	8.3	9.9
(b) Enzyme & alcohol control soution.	3.1	4.3	4.3
(c) Residual solution after adsorption.	2.3	3.3	3.3
(d) Eluate	0.7	0.7	0.7

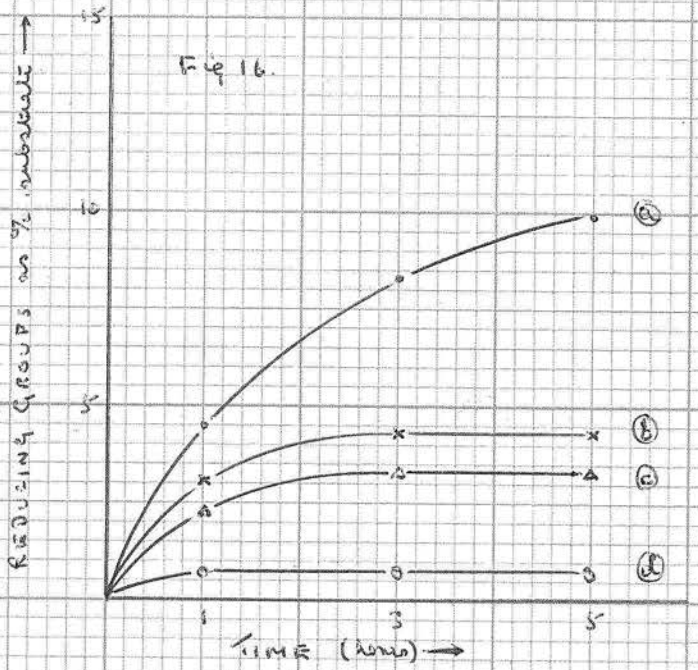


Fig 16. Hydrolysis of barley gum C_2 by solutions of cytase before and after attempted adsorption and elution. Enzyme equivalent to 10 mg. crude precipitated malt enzymes; pH 5·0, 44°C.

ⓐ Control enzyme solution.
ⓑ Enzyme + alcohol control solution.
ⓒ Residual solution after adsorption.
ⓓ Eluate.

From the results, recorded in table XXVIand graphed in fig. 16, it will be seen that, since the sums of (c) and (d) agree within limits with the value of (b) for the same length of conversion, to a certain extent the attempted adsorption and elution has been successful. Attention must be drawn to the influence of alcohol on the action of cytase; it will be seen that in the case of the enzyme and alcohol control solution (b), the action of cytase is less at one hour than in the control enzyme solution (a), and that this action ceases completely after three hours conversion, suggesting that inactivation of cytase has occurred.

<u>The Influence of pH Value on the Nature of the Cytase Extracted</u>;- 100 g. samples of low-dried malt were ground and extracted with 200 ml. quantities of sodium phosphate/citric acid buffer solutions of the following pH values: 2.2, 3.0, 4.0, 5.0, 6.0, 7.0 and 8.0; at the end of 3 hours the extracts were filtered, and their pH values measured using a glass-electrode. The extracts were then dialysed against running tap water until substantially free from salts; it was observed that during dialysis a precipitate was thrown down, presumably protein, and in all cases this was filtered off. The dialysed extracts, after their volumes had been measured, were poured into 4 volumes of 95% alcohol, and taken to dryness in the usual manner.

The activities of the preparations were tested against barley gum C_2 using the standard short period method, 10 and 20 mg. quantities of the preparations being used, at pH 5.0 and 37°C.

Figures for the final pH values of the extracts, and yields and ashes of the preparations will be found in Table 27, and the results of the hydrolysis of barley gum C_2 by the preparations will be found in table 28.

Table XXVII. Various data on the extracts of low-dried malt at various pH values and the preparations obtained from them.

Original pH value of extract.	pH value after extraction.	Volume of extract ml.	Yield mg. (dry, ash-free) of enzyme.	Ash as % of preparation.
2.2	3.5	100	247	0.3
3.0	3.75	100	237	1.5
4.0	4.45	120	676	1.7
5.0	5.2	120	743	1.8
6.0	5.9	140	516	2.4
7.0	6.8	140	411	2.4
8.0	7.3	90	403	1.9

Table XXVIII. The hydrolysis of barley gum C_2 by enzymes precipitated from extracts of low-dried malt, of the orinal pH values indicated; pH 5.0 and 37°C.

Original pH of extract.	Corrected reducing groups liberated (as xylose) as % of substrate.					
	10 mg. of enzyme.			20 mg. of enzyme.		
	1 hour.	3 hour.	5 hour.	1 hour.	3 hour.	5 hour.
2.2	2.1	3.0	3.9	3.15	5.1	7.2
3.0	6.9	15.6	22.5	12.15	28.35	41.1
4.0	6.6	12.3	17.7	9.0	21 ~~12~~.9	33.6
5.0	5.7	11.7	17.1	9.0	20.4	32.7
6.0	4.8	12.3	18.9	8.7	19.5	31.8
7.0	4.5	12.45	18.45	8.8	19.5	32.7
8.0	4.0	11.0	17.05	8.7	18.4	27.0

Fig. 17.

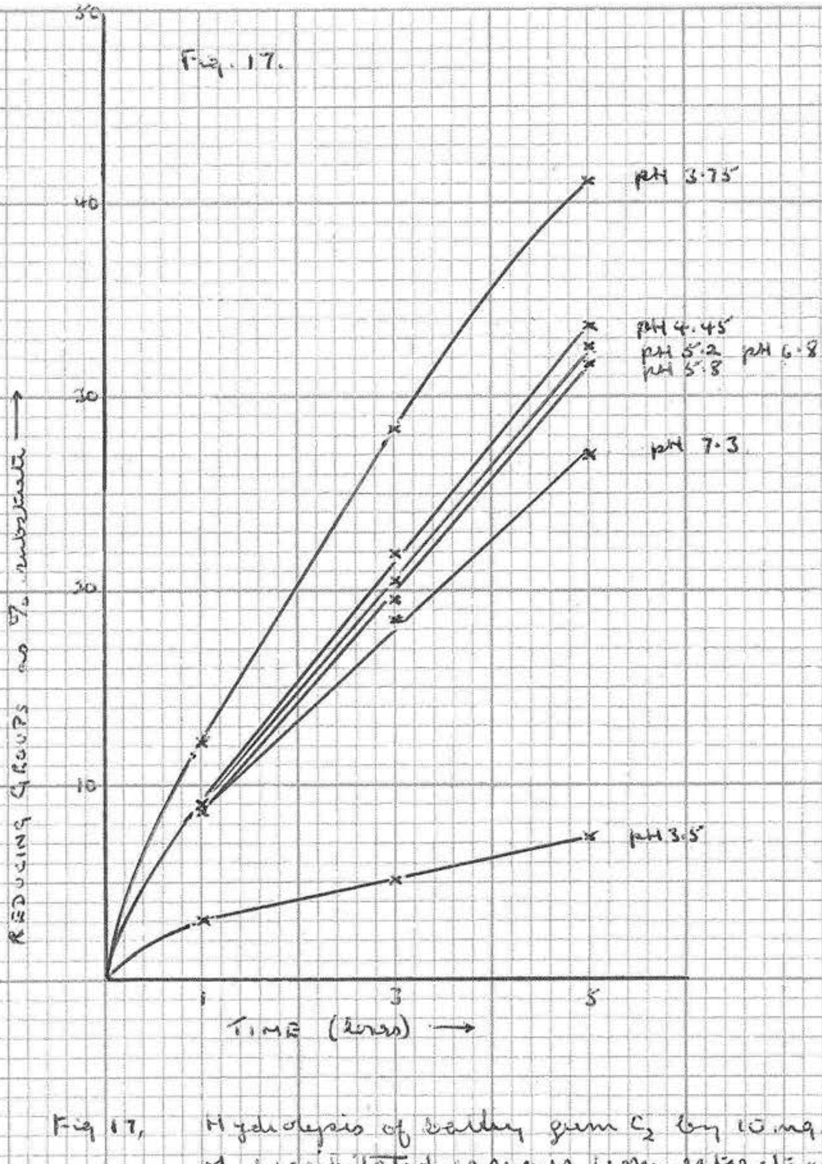


Fig 17, Hydrolysis of barley gum C_2 by 10 mg. quantities of precipitated enzymes from extracts of low-dried malt of varying pH values. pH 5·0, 37°C.

The figures in table XXVIII, as they stand, and the curves in fig. 17, can only provide information relating to the purity of the enzyme preparations, and the relative proportions of the cytolytic and cytoclastic factors present; no definite conclusions on the actual yield of cytase can be made at this stage, and comment will be subsequently made in the discussion. Let it be said at the start that no fractionation of cytase has been achieved, as the hydrolysis/time curves all show the characteristic shape to a greater or lesser extent, and although in the case of the curve for the preparation from the pH 3.0 extract the departure from linearity is only slight, this is more probably due to the increased cytolytic activity masking the cytoclastic action , than to decreased cytoclastic activity; that this is correct will be seen if the straight line portion of this curve is extended to cut the hydrolysis axis,when it will be seen that the point of intersection coincides with that of each of the other curves (3-4% hydrolysis). pH has little influence on the relative extraction of either of the cytase factors, within the range 3.0 - 8.0, although at the highest value there would appear to be some slight inactivation of the cytolytic factor; at pH 2.2 however the extraction of both factors is considerably reduced. The most active preparation is clearly that prepared from the extract of original pH value 3.0, the slope of the straight portion of the hydrolysis/time curve for this preparation being much greater than those of the other curves; the activities of the preparations extracted in the pH range 4.0 - 7.0 are for all practical purposes identical.

Fractional Precipitation of Cytase by Alcohol:- It will be shown during the discussion, that the greatest yield of cytase from low-dried malt is obtained by extraction with a buffer solution of original pH value 5.0. For this reason it was decided to prepare the extract for use in the fractionation experiment with a buffer solution of the original pH value above.

Accordingly, 150 g. of low-dried malt were extracted with 300 ml. of a sodium phosphate/citric acid buffer solution of pH value 5.0 for 3 hours at room temperature; the extract was filtered through paper at the pump and dialysed through cellophane tubing against running water for 20 hours. The dialysed extract was centrifuged to remove any precipitated material, and poured into an equal volume of 95% alcohol; the precipitate was separated in the centrifuge and reserved. The fractionation was carried out in a like manner, i.e. by the addition of volumes of alcohol equal to the volume of original extract present, until no further precipitates could be obtained. Separable precipitates were obtained at alcohol concentrations of 49% (1 volume), 65% (2 volumes), 74% (3 volumes) and 79% (4 volumes), and these were taken to dryness in the usual manner.

The activities of 10 mg. quantities of the preparations were tested against barley gum C_2 and barley hemicellulose S_2 by the standard short period method, at pH 5.0 and 37°C.

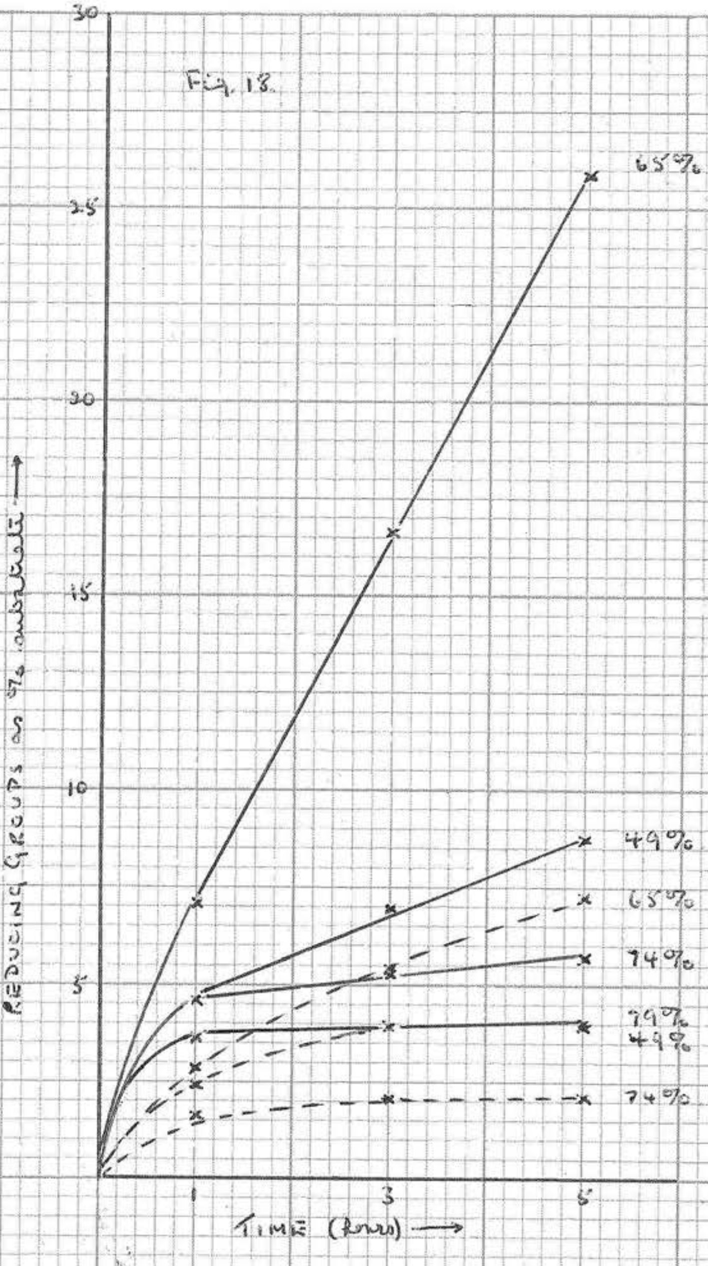


Fig 18 Hydrolysis of barley gum C_2 and barley hemicellulose S_2 by 10 mg. quantities of fractionally precipitated cytase preparations, using alcohol concentrations given in the graph. pH 5.0, 37°C.

——x——x—— barley gum C_2

- - - - - - - barley hemicellulose S_2

the results will be found in table IXXX, and the curves drawn from them in fig. 18.

Table IXXX. The hydrolysis of barley gum C_2 and barley hemicellulose S_2 by fractionally precipitated preparations of cytase from low-dried malt, at pH 5.0 and 37°C.

Corrected reducing groups liberated (as xylose) as % of substrate.

Time in hours.	Alcohol concentration used for precipitation.							
	49 %.	65 %.	74 %.	79 %.	49 %.	65 %.	74 %.	79 %.
	Barley gum C_2.				Barley hemicellulose S_2.			
1	4.65	7.05	4.65	3.6	2.4	2.85	1.65	---
3	6.9	16.65	5.25	3.9	3.9	5.4	2.1	---
5	8.7	25.8	5.7	4.05	3.9	7.2	2.1	---

The results of the experiment have proved very satisfactory, and it is possible that a partial separation of the two factors of cytase has been accomplished, since since it will be seen that the fraction precipitated by 79% of alcohol when acting on barley gum C_2 shows substantially no hydrolysis after 1 hours conversion; it seems highly probable therefore that this fraction consists solely of the cytoclastic factor. If the figures for the hydrolysis of barley gum C_2 by the fraction precipitated by 65% alcohol are compared with the figures for the hydrolysis of the same substrate by the preparation precipitated from the pH 3.0 extract (table XXVIII), it will be seen that the former is the more active, and is as a consequence the most active preparation of cytase isolated to date. The hydrolysis of barley hemicellulose S_2 by these preparations is in all cases much less than that of barley gum C_2, and from the results it would appear that the cytoclastic factor only is present in the fraction precipitated by 74% alcohol; however is purely tentative owing to the very limited action of these preparations on this substrate.

The Behaviour of Cytase During the Germination of Barley:-

The original intention was to study the changes in cytase during the germination of barley by directly examining the activities of extracts of barley at various stages of germination, however this proved unsatisfactory due to the presence of substances susceptible to cytase in the extracts themselves. The study had therefore to be carried out using precipitated enzymes prepared from the extracts.

Four 25 g. samples of barley were steeped in water for 48 hours at room temperature; the steep-water was then decanted off, and the grains washed with water. 3 samples of steeped grain were then placed in a dark chamber on blotting paper provided with a sufficient supply of water to keep it moist, and allowed to germinate at room temperature. An unsteeped 25 g. sample of barley, a steeped, ungerminated sample, and samples of barley removed after 2, 4 and 7 days germination were tested for examination of their cytase activity in the following manner:

The sample of barley was ground in a mortar with sand, n.b. the steeped sample was ground in the coffee-mill, and extracted with water to 125 g., (sand excluded), for 1½ hours at 21°C. The extract was filtered through paper at the pump, and crude precipitated enzymes were prepared from it by pouring into 4 volumes of 95% alcohol, and taking to dryness in the usual manner. The volumes of the extracts, and the yields, moistures and ashes were determined, and are recorded in table XXXI. The activities of 5 and 10 mg. quantities of the precipitated enzymes were tested against barley gum C_2 by the standard short period method at pH 5.0 and 37°C., controls being run in all cases. Little or no liberation of reducing groups occurred in the controls, and figures for these have therefore been omitted from the table of results (table XXXII); allowance however has been made for the controls, where necessary, in calculating the hydrolysis due to cytase.

Table XXX. The mean conditions prevailing in the dark chamber.

Maximum temperature.	Minimum temperature.	Relative humidity.
61.5°C.	47.0°C.	100 %.

Table XXXI. The yields and ash contents of precipitated enzymes prepared from extracts of barley at various stages of germination.

Number of days germination.	Volume of extract.ml.	Ash as % of preparation.	Yield mg. (dry, ash-free)
Unsteeped - 0	200	10.8	657
Steeped - 0	120	10.3	304
2	120	7.3	354
4	190	6.9	252
7	100	10.1	477

Table XXXII. The hydrolysis of barley gum C_2 by precipitated enzymes from extracts of barley at various stages of germination, at pH 5.0 and 37°C.

	Corrected reducing groups liberated (as xylose) as % of, substrate.				
Time in hours.	Unsteeped.	Steeped.	2 day.	4 day.	7 day.
			5 mg. of enzyme.		
1	0.9	1.2	1.8	1.95	2.1
3	0.9	1.65	2.25	2.55	3.9
5	0.9	1.95	2.7	3.3	5.4
			10 mg. of enzyme.		
1	1.5	1.8	2.55	2.55	2.85
3	1.95	3.15	3.15	3.6	6.15
5	2.1	4.65	4.8	5.55	9.3

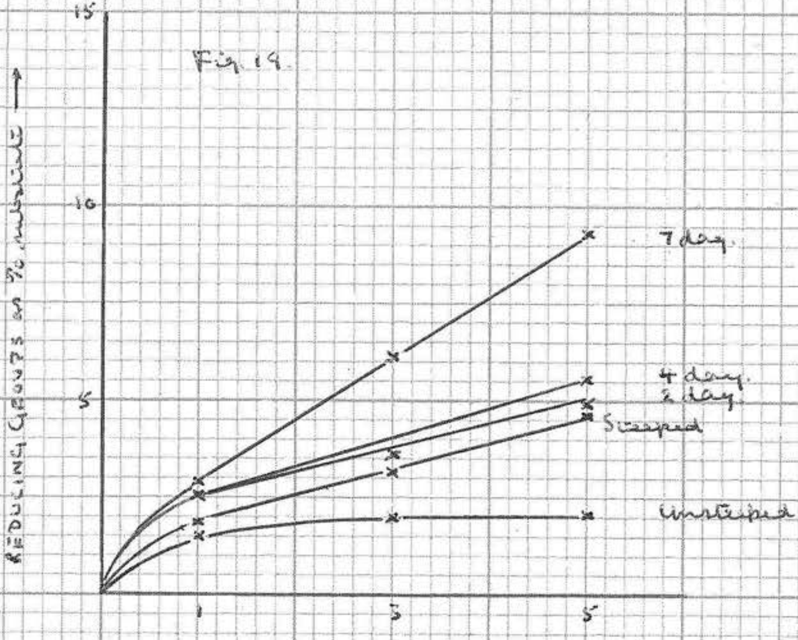


Fig 19. Hydrolysis of barley gum G_2 by 10 mg of precipitated enzymes from extracts of barley at various stages of germination. pH 5.0, 37°C.

It is clear that production of cytase starts even during steeping (fig. 19), and that this production continues throughout the 7 day period investigated. There appears to be little or no production of the cytoclastic factor above that present in the original barley.

Extraction of Cytase with 20% Alcohol:- 100 g. of low-dried malt were ground, and extracted with 125 ml. of 20% alcohol for 3 hours at room temperature. The extract was filtered, and poured into a sufficient volume of alcohol to bring the concentration up to 79%, and the precipitate obtained was taken to dryness in the usual manner. The yield and ash content of the precipitated enzyme will be found in table XXXIII.

Table XXXIII. The yield and ash content of the precipitated cytase preparation from a 20% extract of low-dried malt.

Ash as % of preparation.	Dry, ash-free.	
	Yield mg.	Yield as % of malt.
6.1	772	0.9

The hydrolysis of barley gum C_2 by 10 mg. quantities of the preparation was examined at pH 5.0 and 37°C., by the standard **short period method; a control conversion** with crude precipitated malt enzymes was carried out under the same conditions, for purposes of comparison. The results are recorded in table XXXIV, and graphed in fig. 20.

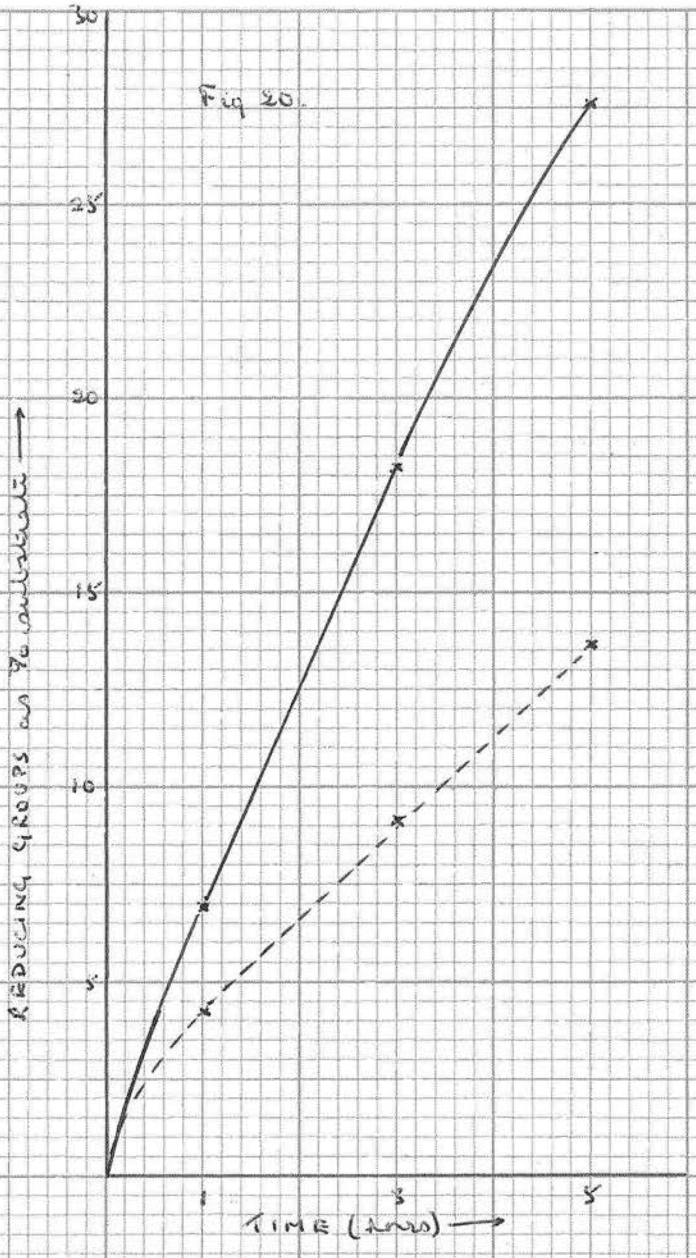


Fig. 20. Hydrolysis of barley gum C_2 by 10 mg. quantities of an enzyme precipitated after extraction with 20% alcohol and crude precipitated malt enzymes, at pH 5.0, 37°C.

——x——x—— Cytase precipitated from 20% alcohol extract.

– – –x– – –x– – – Crude precipitated malt enzymes.

Table XXXIV. The hydrolysis of barley gum C_2 by 10 mg. quantities of crude precipitated malt enzymes, and an enzyme prepared from a 20% alcohol extract of low-dried malt; pH 5.0 and 37°C.

Time in hours.	Corrected reducing groups liberated (as xylose) as % of substrate.	
	Crude precipitated malt enzymes.	Prepared from 20% alcohol extract.
1	4.2	6.9
3	9.15	18.2
5	13.65	27.6

Clearly extraction with 20% alcohol leads to a much more active preparation than does extraction with water.

DISCUSSION.

The Adsorption of Cytase:- It seems clear that some measure of adsorption and elution of cytase has been achieved when the results of this experiment (table XXVI) are examined. It will be seen that the sum of the hydrolysis achieved by the residual enzyme solution after adsorption and that by the eluate agrees, as well as might be expected, with the hydrolysis achieved by the enzyme plus alcohol control, which would be the anticipated result had adsorption followed by elution been successful. The curves for hydrolysis/time for the action of the solutions (fig. 16) show that the action of the eluate ceases after 1 hours conversion, indicating that only the cytoclastic factor was eluted, and since elution was complete (see above), it would appear that only the cytoclastic factor was adsorbed. However it must be noted that the hydrolysis/time curve for the enzyme plus alcohol control is not of the usual shape for cytase actions, and hydrolysis will be seen to cease after 3 hours; it must be concluded therefore that, at the temperature of conversion, a concentration of 36% alcohol inhibits or inactivates at least part of the cytase complex. Consequently it cannot be conclusively claimed that only one factor is adsorbed, since the absence of any action after 1 hour in the hydrolysis by the eluate may be due to either the inhibiting effect of slight traces of alcohol present in the eluate, or the very low activity of the eluate.

<u>The Influence of pH on the Extraction of Cytase</u>:- The results of the study of the influence of pH on the extraction of cytase were a little disappointing, in that no clear separation of the factors of cytase was achieved. From the hydrolysis/time curves (fig 17), it is clear that the most active preparation, and presumably therefore the purest, is that extracted at pH 3.75. N.B. the pH value to which cytase is subjected for the major part of the extraction is not that of the buffer solution before extraction, but that of the final extract, and consequently the pH value

quoted

quoted above, and similar figures quoted in the future, will be those of the final extract.

If the portions of the curves representing cytolysis are extended to cut the hydrolysis axis, it will be seen that their points of intersection coincide, at a figure of approximately 3%; it seems quite conclusive therefore that there is no variation in cytoclastic activity with the pH of extraction. The absence of any increase in cytoclastic activity is not unexpected, since it confirms an earlier suggestion that cytoclasis reaches a limiting value for each substrate, which is independant of the amount of the cytoclastic factor present. It therefore cannot be definitely stated whether there is any increase in the extraction of the cytoclastic factor or not.

Since no change in the total cytoclasis occurs, the quantity of cytase present in the preparations can be expressed in terms of cytolytic activity, and to this end a unit for this factor has been defined. One cytolytic factor unit is taken to be that amount of enzyme which produces reducing groups equivalent to 1% of the substrate in 1 hour, during the linear phase of its action on barley gum C_2. Using this unit a numerical measure of the amount of cytase extracted, and the purity of the preparations is possible, and figures for this purpose will be found in table XXXV.

Fig 21.

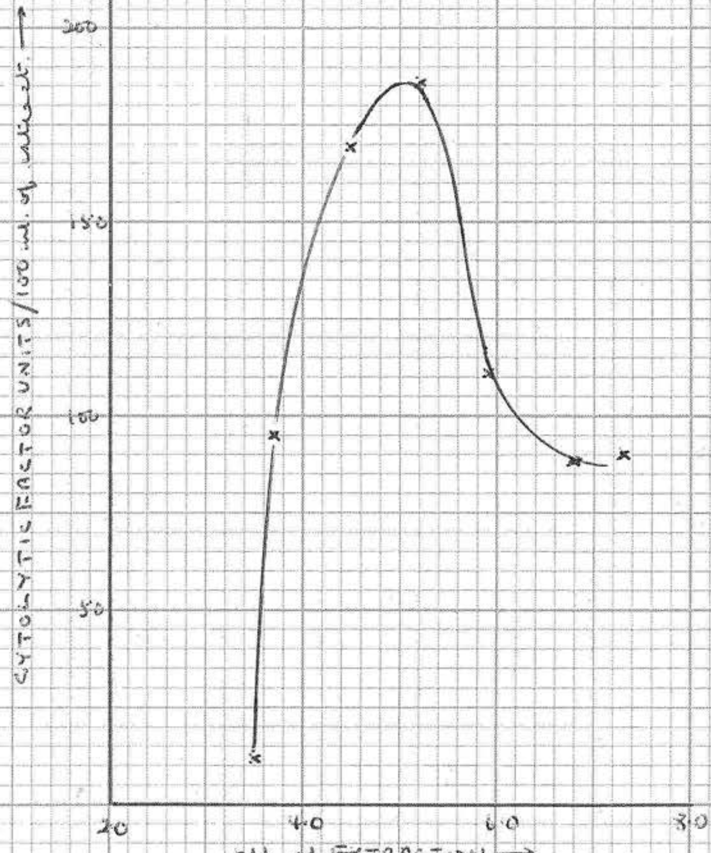


Fig. 21. Influence of pH on the extraction of the cytolytic factor of cytase

Table XXXV. The yields of cytase precipitated from extracts of barley of various pH values, calculated from the cytolytic activities of the preparations.

Original pH of extraction.	Final pH of extraction.	Yield (dry, ash-free) of preparation from 100 ml. of extract.	Cytolytic factor units/ per	
			mg. of preparation.	100 ml. of extract.
2.2	3.5	247 mg.	0.05	12
3.0	3.75	237 mg.	0.4	95
4.0	4.45	563 mg.	0.3	169
5.0	5.2	619 mg.	0.3	186
6.0	5.9	369 mg.	0.3	111
7.0	6.8	293 mg.	0.3	88
8.0	7.3	448 mg.	0.2	90

The figures for cytolytic factor units/mg. of preparation confirm the observations made from a study of the hydrolysis/time curves, that the most active preparation is that precipitated after extraction at pH 3.75, and that the activities of preparations extracted at pH values between 4.45 and 6.8 are identical. The greatest yield of cytase is obtained by extraction at pH 5.2, although extraction at pH 4.45 gives only a slightly lower yield.

The Fractional Precipitation of Cytase by Alcohol:- The study of the fractional precipitation of cytase by alcohol has given some extremely interesting and valuable results, and has led to the most efficient separation of the cytolytic and cytoclastic factors yet achieved. From a study of the hydrolysis/time curves (fig. 18) for the preparations it will be seen that although although some of the cytolytic factor is precipitated by an alcohol concentration of 49%, the bulk of this factor is precipitated by an alcohol concentration of 65%, and the preparation so obtained is the most active preparation of cytase isolated up to the time of this experiment, as will be seen if the hydrolysis of barley gum C_2 by this preparation is compared with those of past preparations; the activity of the preparation precipitated by 65% alcohol may be expressed numerically as 0.47 cytolytic factor units/ mg. However, no preparation of the cytolytic factor free from the cytoclastic was obtained, since it will be seen that the most active preparation with respect to cytolysis shows some departure from linearity between hydrolysis and time at 1 hour.

The shapes of the hydrolysis/time curves for all the preparations show that the cytoclastic factor is precipitated continously throughout the range of alcohol concentrations used, and, for the reason given whilst discussing the influence of pH on the extraction of cytase, the alcohol concentration giving the greatest yield of this factor cannot be stated. However, hydrolysis by the fraction precipitated 79% of alcohol ceases after 1 hours action, and it seems therefore not unlikely that this fraction contains the cytoclastic factor free from the cytolytic. The action of the fraction precipitated by 74% of alcohol is very limited after 1 hours conversion,

and this

and this fraction presumably only contains a slight amount of the cytolytic factor.

It is pleasing to note that again the extent of cytoclasis is in all cases approximately equal, and further that this extent agrees very well with that reached by past preparations, confirming once more that cytoclasis reaches a maximum value for each substrate.

It will be convenient at this point to discuss the cytase preparation from the 20% alcohol extract of low-dried malt, since the purpose of this, and the preceeding experiment was to prepare a more active form of cytase.

The cytase activity of this preparation, and the total amount of cytase yielded may be most conveniently expressed in terms of cytolysis, and figures to this end will be found in table XXXVI; similar figures for crude precipitated malt enzymes, the preparation precipitated directly by 79% alcohol from a low-dried malt extract at pH 3.75, and the preparation fractionally precipitated by 65.5% alcohol after extraction of low-dried malt at pH 5.2 have been included in the table for purposes of comparison. From the contents of table XXXIV and the hydrolysis/time curve for the preparation under discussion, it will be seen that both of the factors of cytase are present, and that no fractionation has occurred.

Table XXXVI. Cytase yields and activities of preparations from low-dried malt.

Description of enzyme preparation.	Cytolytic factor units.	
	per mg. of preparation.	Yield from 100 g. of malt
Crude precipitated malt enzymes.	0.25	450
Precipitated by 79% alcohol after extraction at pH 3.75.	0.41	95
Fractionally precipitated after extraction at pH 5.2	0.47	---
Precipitated by 79% alcohol after extraction with 20% alcohol.	0.57	450

The figures in table XXXVI make it clear that the most active preparation, and the greatest yield of cytase is obtained by extraction with 20% alcohol.

Extraction at pH 3.75, extraction with 20% alcohol, and precipitation by 65% alcohol with prior removal of the precipitate obtained by 49% alcohol, all lead to a purer preparation of cytase, and it is possible that by a combination of these methods a very active preparation of cytase would be achieved. A possible line of approach to the problem of purifying cytase might be to dissolve the precipitate obtained after extraction with 20% alcohol in a buffered solution of pH value 3.75, and after maintaining contact between the enzyme and buffer for a suitable time, add alcohol to a concentration of 49%;

after removing any precipitated or undissolved material, cytase might then be precipitated by raising the alcohol concentration to 65%.

The Behaviour of Cytase During the Germination of Barley:- Several interesting points arise from the results of the study of the change in cytase during germination. From the shapes of the hydrolysis/time curves it is clear that the production of cytase occurs during germination. It had been previously shown that ungerminated barley contains substantially only the cytoclastic factor, and it is pleasing to note that this is confirmed by the present experiment. Contrary to the findings of Lüers and Malsch (*loc. cit.*), who were of the opinion that a loss of cytase occurs during steeping, it is quite clear that production of the cytolytic factor starts even during steeping, since the action of the steeped-ungerminated sample continues throughout the whole conversion period. As in the pH extraction and fractional precipitation studies, there is no substantial increase in cytoclastic action over the stages of germination studied, though, as explained above, this does not exclude the possibility that some production of the cytoclastic factor occurs, or that germination facilitates the extraction of this factor, as is the case with βamylase; again therefore, production of cytase may be measured in terms of increase in cytolysis. Figures for the production of cytase during germination will be found in table XXXVII.

Table XXXVII. The yields and activities of cytase preparations from barley at various stages of germination, in terms of cytolytic factor units.

Stage of germination.	Yield (dry, ash-free) of preparation from 100 ml. of extract.mg.	Cytolytic factor units per mg. of preparation.	Cytolytic factor units per 100 ml. of extract.
Unsteeped.	329	0.00	0.0
Steeped.	253	0.06	15.2
2 days.	295	0.07	20.6
4 days.	280	0.08	22.7
7 days.	477	0.16	76.3

From the figures for cytolytic factor units/100 ml. of extract it is apparent that the cytase present in the barley increased 5 times within 7 days germination. Further, this increase is accompanied by an increase in the activity of the preparations, i.e. the preparations contain less inactive material as germination proceeds. This increase in purity may be accounted for in two ways: first that during germination there is some enzymic degradation of c contaminating material, e.g. by proteolysis, and by cytoclasis of barley gums etc., to a point where they are not precipitated by the concentration of alcohol used to precipitate cytase, and secondly that cytase is present in ungerminated barley and consequently in the enzyme preparations in an inactive form, which is activated during germination, possibly by the removal of an inhibiting group.

The results of the present work do not agree with those of Lüers and Malsch (loc. cit.) on two points, firstly whereas these workers reported a loss of cytase during steeping, the work under discussion indicates a gain, and secondly the increase in cytase observed above is double that which they observed. However it must be noted that Lüers and Malsch carried out their investigation using extracts of the barley to study the change in cytase, whereas during the present work precipitated enzymes have been used; the results may therefore not be strictly comparable. It is not improbable that the loss of cytase observed by the above workers during steeping is an apparent, not true loss, arising out of the decreased efficiency of grinding, and hence extraction, involved in the use of wet grain. It is also important to note that Lüers and Malsch used a different substrate than the one in present use.

After consideration of the results herein, there can be little doubt that cytase comprises a system of enzymes, since in addition to providing further indirect evidence, it has been shown possible to effect partial separation of the factors of cytase; indeed by one method, i.e. fractional precipitation with alcohol, it would appear that a preparation of the cytoclastic factor free from the cytolytic factor has been achieved. The present work has further confirmed in more than one instance the very limited nature of cytoclasis.

SUMMARY.

1. The adsorption and elution of cytase on willow hemicellulose has been studied.
2. The pH value leading to the most active extraction of cytase has been shown to be 3.75, and that giving the maximum extraction to be 5.2
3. The fractional precipitation of cytase has been studied, and the results show that the bulk of the cytolytic factor is precipitated by a concentration of 65% alcohol, and it is highly probable that the fraction precipitated by 79% alcohol contains only the cytoclastic factor.
4. The most active, and presumably therefore the purest, preparation of cytase isolated to date, is that precipitated from by 79% alcohol from a 20% alcohol extract of low-dried malt.
5. The change in cytase during the germination of barley has been studied, and it has been shown that production of the cytolytic factor starts even during steeping, and continues up to at least seven days. Production of the cytoclastic factor has not been demonstrated.
6. The present work has provided ample confirmation for the suggestion that cytase is a complex enzyme system, comprising at least two enzymes.

Appendix to Section V.

In view of the high activity of the precipitated cytase preparation from the extract of pH value 3.75, it was considered that a greater measure of success might be achieved with the adsorption experiment if it were repeated using this preparation. The experiment was however modified, and alcohol was not added to the enzyme solution prior to adsorption, since it had been shown to have an inhibiting influence on cytase action.

Willow hemicellulose S_1 was added to a solution of the above enzyme preparation of concentration 2 mg./ml., and after standing for a short time the hemicellulose was separated by centrifuging; the enzyme solution was decanted, and the hemicellulose washed with water. After separating by centrifuging, it was noted that the bulk of the hemicellulose had disappeared, and since the supernatant liquid after each centrifuging was clear, it can only be assumed that the hemicellulose had gone into solution.

It must be noted that this hemicellulose preparation is very insoluble in water under normal circumstances, being difficult to dissolve even in boiling water; it would appear that the cytase preparation used is capable of attacking the willow hemicellulose S_1, to render it soluble in water.

VI. FURTHER STUDIES ON HEMICELLULOSES.

In the introduction to Section IV, possible methods by which the actions of individual factors of cytase might be separately studied were suggested, two of which, viz. the preparation of modified substrates and the fractionation of cytase, have now been examined (see Sections IV and V). In this Section it is proposed to deal with the last of these suggestions, namely, the use of methods other than that of determination of reducing groups for following the particular aspects of cytase action.

By definition, cytoclasis is taken to be the breakdown of the substrate to molecules of still comparatively large size, i.e. to sizes of the order of ten sugar residues; that is to say, the molecular size of the substrate should be rapidly reduced to the extent that the normal hemicellulose prepipitation methods are not effective. That it is possinle for cytoclasis to proceed to this extent was shown in Section IV., where it was observed that if the enzymes of barley, which contains substantially only the cytoclastic factor, are allowed to on a barley extract for a sufficient length of time, no precipitate is obtained on adding Fehling's solution and acetone.

During the course of the work to be described in this Section, it will be shown that the hemicellulose precipitation using Fehling's solution and acetone, although excellent for the purpose of pure, ash-free materials, does not give a quantitive yield of hemicellulose, and an alternative method, that of Angell and Norris (1936), was examined.

It was observed during the preparation of modified barley hemicelluloses, that whereas previously hemicelluloses of series 1 and 2 had been isolated, a fresh sample of barley yielded only hemicellulose S_2, even in an unmodified preparation; possible reasons for the absence of hemicellulose S_1 were suggested in Section II. Since the Fehling's solution/acetone method of hemicellulose precipitation has been shown to be unreliable with respect to quantitative yield, a study has been made of the hemicelluloses from different varieties of barley, in the hope that it might be shown whether the method of preparation, or the barley itself was responsible for the absence of hemicellulose S_1.

During the preparation of various water-soluble polysaccharides of barley and malt, it was noted that whereas the barley polysaccharides gave very viscous solutions, their extracts proving very difficult to filter and giving a very stable froth whilst boiling under reduced pressure, the malt polysaccharides gave much less viscous solutions, e.g. their extracts filtered quite easily through paper. Accordingly it was thought that information of interest might be obtained if the viscosities and surface tensions of these polysaccharides were determined; the optical rotation of these substances has also been determined

EXPERIMENTAL.

Methods for Following the Course of Cytoclasis:- It was proposed to use two methods for the study of cytoclasis, but before proceeding with them it was necessary to know whether the quantitative precipitation and recovery of hemicelluloses was possible using the Fehling's solution and acetone method of precipitation .

5, 4, 3 and 2 ml. quantities of a solution of barley gum C_2, of concentration 10 mg. (dry equivalent) per ml., were pipetted into centrifuge tubes containing 1 ml. of 24% NaOH; the volume of the liquid in each tube was made up to 6 ml. with water, i.e. to give a concentration of 4% NaOH in each case, and three ml. of Fehling's solution and 4 ml. of acetone were added, and the precipitates obtained were separated by centrifuging. The precipitates were washed once with acid (HCl) 60% acetone, twice with 60% acetone, and three times with 95% alcohol, centrifuging after each washing to deposit the precipitate, and the precipitates were then transferred quantitatively to dried, weighed, cinter-glass crucibles; the precipitates were washed on the crucibles with absolute alcohol, and after drying overnight in a vacuum desiccator, dried in a 100°C. oven for 2 hours. The dried crucibles were weighed rapidly in a stoppered bottle. The experiment was repeated on barley hemicelluloses S_1 and S_2, and willow hemicellulose S_1, omitting the addition of acetone in the case of series 1 hemicelluloses.

Table XXXVIII. The recovery of hemicelluloses etc. using the Fehling's solution/acetone method of precipitation

Weight of substance taken. mg.	Recovery as % of original substances			
	Barley gum C_2.	Barley hemicellulose S_1.	Barley hemicellulose S_2.	WILLOW ~~Barley~~ hemicellulose S_1.
50	83.0	59.8	81.4	67.0
40	83.0	58.0	80.2	67.0
30	85.8	52.1	79.9	66.7
20	83.0	53.0	84.5	61.0
Mean recovery	83.7	55.7	81.5	65.4

From the results (Table XXXVIII) it seemed safe to assume that reasonably reproducible results for the recovery of hemicelluloses and barley gum C_2 could be attained, and a method for studying cytoclasis based on the above results was designed. Recovery of series 2 products is considerably better than series 1, doubtless due to the influence of the added acetone.

First Method for Following Cytoclasis:- 0.60 g. (dry, ash-free equivalent) of barley gum C_2 were dissolved in water, 1 ml. of pH 5.0 sodium acetate/acetic acid buffer solution added, and the volume made up to 50 ml.; the solution was transferred to a suitable conical flask, and placed in a water bath at 37°C. After allowing the substrate to reach the temperature of the bath, 5 ml. of a solution containing 1.2 mg. of crude precipitated malt enzymes per ml., and 5 ml. of water were added, and the time carefully noted.

After 5, 15, 30, 60, 180 and 300 min. conversion, 5 ml. quantities of conversion solution were pipetted into 1 ml. of 24% NaOH in a centrifuge tube; 3 ml. of Fehling's solution and 4 ml. of acetone were added, and the precipitates obtained were treated exactly as in the previous experiment. It is important to note that the 5 ml. of conversion solution contain 0.5 mg. of crude precipitated malt enzymes, and that the results will be in error to the extent of any hemicellulose present in the crude precipitated malt enzymes, but as the total of the latter is only 1% of the barley gum, this error will be of little significance, and consequently no control was run.

The experiment was repeated on barley hemicellulose S_2, the results for which, with those for barley gum C_2 will be found in table IXL , in calculating the percentage of residual barley gum the recovery was taken as 85%, and for barley hemicellulose S_2 80.3%. Curves drawn from the results will be found in fig. 21.

Table IXL. Cytoclasis of barley gum C_2 and barley hemicellulose S_2 by crude precipitated malt enzymes at pH 5.0 and 37°C.

Time in minutes.	Corrected residue as % of original substance.	
	Barley gum C_2.	Barley hemicellulose S_2.
5	100	79
15	90	78
30	79	76
60	78	76
300	81	70

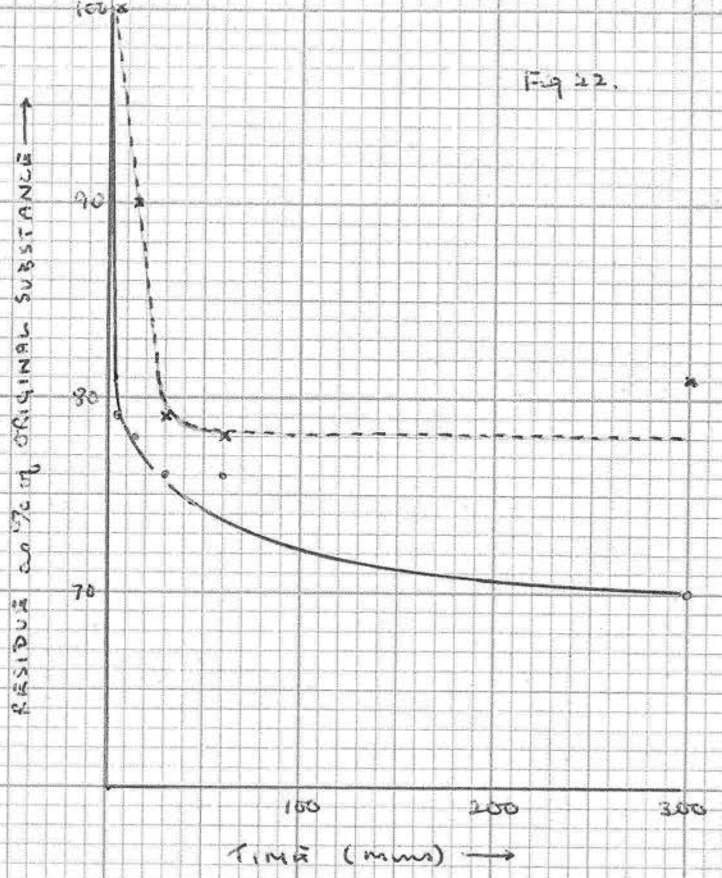


Fig 22 Cytoclasis of barley gum C_2 and barley hemicellulose S_2 by crude precipitated malt-enzymes. pH 5·0, 37°C

- - - -x- - - -x- - - - barley gum C_2

———o————o——— barley hemicellulose S_2

Second Method for Following Cytoclasis:- The intention behind this experiment was to measure cytoclastic activity by the time taken by the enzyme to break down the substrate to the extent that it could no longer be precipitated by Fehling's solution and acetone.

The substrate solution was prepared exactly as in the first method, and adjusted to 37°C.; 5 ml. of water, and 5 ml. of a solution containing 12 mg. of crude precipitated malt enzymes per ml. were added to the substrate solution, and the time carefully noted. 5 min. after the addition of the enzyme, 5 ml. of conversion solution were pipetted into 1 ml. of 24% NaOH in a centrifuge tube; to this were then added 3 ml. of Fehling's solution and 4 ml. of acetone, and the presence or absence of a precipitate noted after centrifuging. This procedure was repeated after 15, 30, 60, and 180 min., but at no stage did the addition of the reagents fail to give a precipitate.

It would appear that this second method can be put to little use at present. The results of the first experiment are more encouraging, and it will be seen that they confirm the suggestion that the action of the cytoclastic factor is extremely rapid. The points of inflection of the curves would correspond to the end of cytoclasis, and are reached in 20 - 30 min. under the conditions described. Since ~~the~~ material precipitable by Fehling's solution and acetone is still present at the end of cytoclasis, it would appear that the products of cytoclasis are still of relatively large molecular size. However it is clear that the method does not give sufficiently reproducible results, since it is clear that some of the figures quoted are in error, and an alternative method, that of Angell and Norris, for the precipitation of hemicelluloses was therefore examined, in the hope that it might give more reproducible results.

5, 4, 3, and 2 ml. quantities of barley gum C_2 solution of concentration 10 mg./ml. were pipetted into centrifuge tubes containing 1 ml. of 24% NaOH and 0.25 ml. of glycerol, and the volumes made up to 6 ml. with water. To each of the tubes was added 3 ml. of acetone, and the precipitates obtained were separated in the centrifuge. The supernatant solutions were decanted, and the precipitates treated exactly as in the recovery experiment above. The experiment was repeated on barley hemicellulose S_1 and willow hemicellulose S_1, with the omission of the addition of acetone; results will be found in table XL.

Table XL. Recovery of hemicelluloses etc. using Angell and Norris's method

Weight of substance taken. mg.	Recovery as % of original substance.		
	Barley hemicellulose S_1.	Barley gum C_2.	Willow hemicellulose S_1.
50	31	85	60
40	31	86	60
30	30	90	64
20	32	82	62

Here again it will be noted that the method is not sufficiently reproducible to justify its use under the present conditions, and consequently work along these lines was discontinued. Recovery by the last method is very similar to that obtained using Fehling's for barley gum C_2 and willow hemicellulose S_2, but markedly inferior for barley hemicellulose S_1 (cf. table XXXVIII).

The Preparation of Hemicelluloses from Different Varieties of Barley:- The purpose of this experiment was to try to discover whether the yield and types of hemicelluloses isolated from barley are varietal characters, or are dependent on the treatment to which they have been subjected.

Accordingly hemicelluloses were prepared from a selection of barleys by the method described in Section II. On the addition of Fehling's solution alone to the extracts three different phases could be noted;- (1) a definite precipitate, (2) a great increase in viscosity and turbidity, (3) no appreciable change; the changes observed on the addition of Fehling's solution alone will be found in table XLI., together with the yields of the hemicelluloses isolated.

Table XLI.. The yield of hemicelluloses from a selection of barleys.

Barley.	Change on adding Fehling's Solution	Yield (dry, ash-free) as % of barley.		
		Hemicellulose S_1.	Hemicellulose S_2.	Total.
Piomeer.	x	0.00	2.46	2.46
Californian.	x x	1.71	0.84	2.55
Prefect.	x	0.00	2.09	2.09
Cyprian.	x x	1.62	0.93	2.55
Plumage Archer.	-	0.00	1.40	1.40
Spratt Archer.	-	0.00	1.90	1.90
Australian.	x	0.00	2.22	2.22
Scotch Variety unknown.	x	0.00	2.68	2.68

x x - definite precipitated
x - increased viscosity and turbidity.
\- - no appreciable change.

The preparation was repeated on those barleys from which hemicelluloses of both series were isolated, i.e. Californian and Cyprian, but in neither could a hemicellulose S_1 be isolated a second time. FRom a preliminary survey of the results it would appear that the isolation of hemicellulose S_1 from barley is influenced by the method of extraction, though varietal differences of character cannot be excluded.

Various Physical Constants for the Barley Bolysaccharides:-

The viscosity and surface tension of 0.50% (dry, ash-free equivalent) solutions of each of the various polysaccharides isolated from barley and malt were measured, using the following methods;

Viscosity:- The apparatus used was the "Ostwald Viscometer", and the method was that described by Hatschek (1928). The viscosities of the various solutions were calculated relative to that of water, the viscosity of water being taken as 100.

Surface Tension:- Surface tension was measured by the "Capillary rise" method, using the apparatus described by Sugden (1930); the surface tensions were calculated by comparison with water, the surface tension of water being taken as 72.

All measurements were carried out in a water bath at 25°C. (± 0.05°).

The optical rotation of the substances was also measured, where possible, and $[\alpha]_D$ for, the substances calculated.

The results and a brief description of the appearance of the substances will be found in table XLII.

Table XLII. Physical constants for barley and malt non-starchy polysaccharides.

		Determined on 0.5% solutions.		
Substance.	Appearance.	$[\alpha]_D$ 15.5°C.	Surface tension 25°C.*	Viscosity 25°C. #
Barley gum B_2.	Fibrous.	✱	54	128
Barley gum C_2.	Fibrous.	-54.8°	72	190
Modified barley gum C_2.	Fibrous.	-63.6°	72	147
Malt hemicellulose-dextrin B_2.	Pulverulent.	+148.8°	72	114
Malt hemicellulose-dextrin C_2.	Pulverulent.	-88.0°	72	156
Barley hemicellulose S_1.	Fibrous.	✱	71	107
Barley hemicellulose S_2.	Fibrous.	✱	71	167
Barley hemicellulose S_2. ##	Fibrous.	✱	71	421
Modified barley hemicellulose S_2.	Fibrous.	✱	70	372

✱ - solution opalescent.
\# - water 100 .
* - water 72.
\## - no hemicellulose S_1 isolated.

DISCUSSION.

Recovery Experiments:- The first method for following cytoclasis is essentially a gravimetric method for the determination of hemicellulosic substances, and would be of interest in that respect alone, if it had proved of no value for the purpose for which it was designed. It will be seen from the results (tables XXXVIII and IXL) and more clearly from the curves drawn from them (fig.21), that the method is not, as reliable as could be wished. Nevertheless the results support the suggestion that cytoclasis comprises a rapid depolymerisation or disaggregation of the substrate, and that the action is complete within 20 - 30 mins. The curves drawn from these results show the extremely rapid nature of cytoclasis, the point of inflection being reached in 30 min. in both cases, by which time only 79% and 76% respectively of barley gum C_2 and barley hemicellulose S_2 were recoverable; the action would seem to, be more rapid in the case of barley hemicellulose S_2, when a 20% fall in recovery occured in 5 min. The results obtained by the second method for following cytoclasis indicate that this method is of little use at present, though this does not exclude its possible use in the future, when more active preparations should be available.

Turning to, the recovery of hemicellulosic substances in the absence of enzyme action, it will be seen that in no case was a complete recovery possible using the Fehling's solution/acetone method of precipitation, and that in all cases the figures were not very reproducible. It is interesting to note that the recovery of series 1 substances, viz. barley hemicellulose S_1 and willow hemicellulose S_1, is much lower than that of the series 2 substances, viz. barley gum C_2 and barley hemicellulose S_2. The recovery of hemicellulosic substances by Angell and Norris's method (loc. cit.) has proved no more reproducible than recovery by the Fehling's solution/acetone method, and the use of gravimetric methods for following cytoclasis has therefore been temporarily discontinued. It is intereting to note that again the recovery of series 1 substances by Angell and Norris's method is lower than that of series 2 substances.

The Hemicelluloses of Different Varieties of Barley:-

As was pointed out in the introduction to this Section, two possible explanations for the absence of hemicellulose S_1 have been advanced: (a) that this hemicellulose was absent from the barley, and (b) that the treatment to which the hemicellulose has been subjected has caused it to be depolymerised so that it can not be precipitated by the normal method, i.e. the addition of Fehling's solution alone.

Although the two possibilities are not mutually exclusive, from the results it seems that of the two, (b) is a more correct explanation, since it was found that on a second preparation from tha barleys from which a hemicellulose S_1 was obtained previously, no hemicellulose S_1 could be isolated; however the possibility that hemicellulose S_1 is absent from some of the barleys cannot be excluded.

The series of changes from the presence of a precipitate through high viscosity and turbitity to no change on adding Fehling's solution to the alkaline extracts noted in Section II, has again been observed, thus confirming the suggestion that hemicellulose S_1 is in a critical condition with respect to precipitation with Fehling's solution, in which condition very slight modifications of method might result in no precipitate being obtained on adding Fehling's solution.

The Physical Characters of Non-starchy Barley Polysaccharides:-

It is of some satisfaction to note that the $[\alpha]_D$ value for barley gum C_2 (table XLII) is quite close to that obtained by O'Sullivan for his β amylan, viz. 65.8°; no comparison of the optical rotation of barley gum B_2 with this worker's figure for his α amylan can be made, as the solution of barley gum B_2 was too opalescent. Modification of barley gum C_2 causes little change in its optical properties.

It is interesting to note that whilst the $[\alpha]_D$ values for barley gum C_2 (-54.8) and malt hemicellulose-dextrin C_2 (-88.0°) are negative, the value for malt hemicellulose-dextrin B_2 is positive (+148.8°). It has been previously noted that malt hemicellulose-dextrin B_2 is chiefly hexosan in nature (Section IV), and it does not seem improbable that the small quantity of furfuraldehyde obtained from this substance originates in a contaminating pentosan; this possibility is further strengthened by the discrepancy between the $[\alpha]_D$ of this substance and those of barley gum C_2 and malt hemicellulose-dextrin C_2. The $[\alpha]_D$ of malt hemicellulose-dextrin B_2 corresponds more to that of the starch-dextrins, typical figures for which are those given by Baker and Hulton (1938) for malto-dextrin, and Baker (1941) for stable-dextrin, viz. $+178.2^\circ$ and $+195.1^\circ$ respectively. It would therefore appear that the substance designated malt hemicellulose-dextrin B_2 is actually a starch-dextrin, or a mixture of dextrins, contaminated to a certain extent with pentosan material.

Unfortunately no figures are available for the values of the barley hemicelluloses, as solutions of these substances were too opalescent to allow polarimeter readings to be taken.

Clearly none of the materials under discussion, other than barley gum B_2, which causes an appreciable lowering of the surface tension of water, possesses surface activity (see table XLII), but all of these substances are viscous to a greater or lesser degree, with the exception of barley hemicellulose S_1. Modification will be seen to have caused a decrease in the viscous properties of both barley gum C_2 and barley hemicellulose S_2, and the suggestion that the malt hemicellulose-dextrins are less viscous than the barley gums has been confirmed.

Whilst appreciating that the results may be in error to some extent, since the validity of certain assumptions was not proved, it was considered that information of interest might be obtained by calculating the molecular weights of barley gum C_2, modified barley gum C_2 and malt hemicellulose-dextrin C_2 from both their viscosities and their reducing powers, assuming that the polymeric unit was in each case xylose, and that the substances in question had straight chain molecules, with only one reducing group.

To calculate the molecular weights from viscosity the equation given by Kraemer (1943) was used:-

$$M = \frac{\eta_{sp}}{C_{gm}} \times 10K_{cel}.$$

Where M = molecular weight.

η_{sp} = specific viscosity increment. $= \dfrac{\text{relative viscosity of substance} - \text{viscosity of water}}{\text{Viscosity of water}}$

C_{gm} = concentration as molarity of polymeric unit.

K_{cel} = the constant for cellulose. = 100

The reducing powers of 0.10% solutions of barley gum C_2, modified barley gum C_2 and malt hemicellulose-dextrin C_2 are, 0.3, 0.45, and 1.65% as xylose of substrate.

Table XLIII. Molecular weights of various barley polysaccharides.

Molecular weight calculated from.	Barley gum C_2.	Modified barley gum C_2.	MAlt hemicellulose-dextrin C_2.
Viscosity.	46,460	24,700	26,800
Reducing power.	44,000	29,300	8,000

The figures for molecular weight can only be accepted as minimum figures since it has been assumed that the molecules are built up from pentose, i.e. xylose, residues alone, whereas there can be no doubt that the substances contain a considerable proportion of hexose residues.

The agreement between the molecular weights calculated by the two methods is excellent in the cases of barley gum C_2 and modified barley gum C_2, and it would therefore appear that the assumptions made above are justifiable for these two substances. It seems probable therefore that barley gum C_2 and modified barley gum C_2 have straight chain molecules, as was suspected from their fibrous appearance, and, in view of their very high viscosities, it does not seem improbable that modified barley hemicellulose S_2 and barley hemicellulose S_2 have a similar molecular structure.

Turning to malt hemicellulose-dextrin C_2, the molecular weight calculated from viscosity is over three times greater than that calculated from reducing power, throwing suspicion on the validity of the assumptions in this particular case. It would thus appear that this substance is not a straight chain polymer, but has a branched molecular structure, which would be in accord with its amorphous appearance, and such a structure with three,free reducing groups would give agreement with the molecular weights calculated by the two methods; however great confidence cannot be placed on this latter conclusion since the equation used to calculate the molecular weight from viscosity is derived for straight chain polymers, and does not give reliable results for branched polymers.

Piratzki (1936) showed that malt kilned after three to four days on the floor gave worts of abnormally high viscosity, of the value of 1,300 compared with 1,060 for a normal malt, taking water as 1,000; the minimum time on the floor to give a malt which on mashing gave a wort of normal viscosity was quoted as 5 days. Piratzki was therefore led to conclude that the viscosity of wort depends to a large extent on the degree of modificacation of the malt from which it was prepared. Piratzki and Wiecha (1937) showed that the action of crude amylase or pectic enzyme preparations greatly reduced the viscosity of abnormal malts, whilst the action of proteolytic enzymes was ineffective in this respect, and they concluded that the action responsible for the decrease was that of cytase on hemicellulosic substances. They further showed that whereas the maximum viscosities of a large number of worts showed wide variation, the minimum values fell within a narrow range, and they were of the opinion that the minimum values represented a condition in which the hydrolysis of hemicellulosic substances by cytase had ceased.

The present results do not confirm the above workers conclusions, since it has been shown that although the viscosity of the malt hemicellulose-dextrins is less than that of the corresponding barley gums, the decrease is not sufficient to account for the above changes, when the enhanced total yield of the malt products is taken into account.

Meredith and Sallans (1945), have shown wort viscosity to be closely correlated with the salt soluble nitrogen of barley and with wort nitrogen, it would therefore appear that the solution to this problem must be sought in proteolysis.

Turning to the barley hemicelluloses , it will be noted thatthe hemicellulose S_2 isolated in the absence of a hemicellulose S_1 has a much higher viscosity than the hemicellulose S_2isolated along with a hemicellulose S_1; this observation, which is the opposite of that which would be expected since hemicellulose S_1 has a much lower viscosity than hemicellulose S_2,and is thought to appear with the hemicellulose S_2 when absent from the preparation,requires further investigation before it can be explained.

Since modification is considered to result from the action of the cytoclastic factor, the decrease in viscosity observed as a consequence of it indicates a method by which cytoclasis might be followed, namely by studying the change in viscosity of substrates subjected to this action.

SUMMARY.

1. Two methods for following cytoclasis have been designed, but their use did not prove entirely satis-factory. However the results obtained seemed to confirm the observations on the very rapid and limited nature of cytoclasis.
2. The isolation of hemicelluloses from different varieties of barley has been studied, and it would appear that the possibility of isolation of hemicellulose S_1 is influanced to a large extent by slight variations in the method used.
3. $[\alpha]_D$ values for the barley and malt polysaccharides have been determined, and it seems that "malt hemicellulose-dextrin B_2" is largely of the nature of a starch-dextrin.
4. The viscosity and surface tension of the barley and malt polysaccharides have been measured; modification has been shown to lead to a decrease in viscosity of the material treated.
5. Molecular weights have been calculated for the barley and malt cold-water-soluble polysaccharides.

VII. GENERAL DISCUSSION.

A full discussion has been made in each section of the work therein, but it is now necessary to collect the material together in such a manner that a full appreciation of the conclusions reached can be made, and lines for future work indicated.

The fact of cytolysis has been amply confirmed, both by direct and indirect evidence. Probably the most striking indirect evidence is the great increase in yield of extractable barley hemicellulose on modification; here barley enzymes were allowed to act on starch-free barley grains prior to the extraction of hemicelluloses, with the result that the yield of hemicellulose was more than doubled, with little or no apparent change in the type of hemicellulose extracted. There can be no doubt that this increased yield finds its origin in initially insoluble hemicelluloses, which were rendered soluble by the action of a cytase in barley. A parallel increase in yield of barley gum C_2 on modification could not be expected in the method used, since the barley enzymes were allowed to act on the gum subsequent to extraction; modification of barley gum C_2 did however provide indirect evidence of cytase action, namely the failure of Fehling's solution and acetone to produce a precipitate from a barley gum C_2 extract, which had been in long contact with barley enzymes. Here again it must be assumed that the absence of a precipitate was due to the action of cytase on barley gum C_2, the gum being broken down to the extent that the usual method of precipitation was not effective. Further indirect evidence for the presence of a cytolytic system in barley is the radical change in nature of the water soluble polysaccharides on malting.

Turning to the direct evidence of cytase action; all the hemicellulose-like substances prepared from barley have been shown susceptible to the action of an enzyme, or system of enzymes present in germinated barley, and a precipitated form of this enzyme, presumably cytase, has been prepared. The hydrolysis of the hemicellulosic substances from barley by this precipitated enzyme has been studied, and it is clear from the results that the problem of cytase action is not so simple as previous workers seem to have assumed.

The hydrolysis of unmodified substrates, with the exception of malt hemicellulose-dextrin C_2, is not the normal type for simple enzyme actions, and there can be no doubt that this hydrolysis involves two distinct actions, namely, a rapid liberation of reducing groups (an action of limited duration), followed by a slower but prolonged action which is proportional to time. It has been found convenient to describe these actions as "cytoclasis" and "cytolysis" respectively. Malt hemicellulose-dextrin C_2 and modified barley gum C_2 are not susceptible to cytoclasis, and show proportionality between hydrolysis and time from the start.

It will be noted that the pH and temperature relationships for the action of cytase on barley gum C_2 and barley hemicelluloses S_1 and S_2, show appreciable variation. With barley gum C_2 and barley hemicellulose S_1 the optimum pH value for cytolysis is near 5.0, whilst with hemicellulose S_2 the optimum pH value is in the region of 5.4. Even greater variation will be seen in the optimum temperature for the cytloysis of these substrates, the values being 44°C. for barley gum C_2 at the optimum pH, 30°C. for barley hemicellulose S_1, and 33°C. for barley hemicellulose S_2. Further, the rate of cytolysis of different substrates by the same enzme preparation and under the same conditions varies considerably, that of malt hemicellulose-dextrin C_2 being extremely slow compared with that of barley gum C_2, whilst the hydrolysis of the barley hemicelluloses is intermediate to the two.

The above observations, and the hydrolysis of barley gum C_2 by a precipitated enzyme from barley, which was shown to correspond to the cytoclastic action only, are consistent with the presence in cytase of at least two enzymes, viz. cytoclastase and cytolytase, and it seems inconceivable that the wide range of properties noted for cytolytase arise from the action of a single enzyme. Indeed the evidence of long period conversions of barley gum C_2 may be taken to indicate the presence of two enzymes in cytolytase, one of which is responsible for the major part of hydrolysis up to a total liberation of reducing groups of approximately 20% under optimum conditions, and a second which gives a slower and more prolonged hydrolysis. The possibility that enzymes other than the above are present in the cytase of barley cannot be eliminated.

The system of enzymes which it has been suggested are present in cytase, even supposing, as seems highly probable, that each enzyme is specific for one type of linkage, would be capable of producing the wide range of properties which has been observed. As was pointed out in the General Introduction and in the Introduction to Section II, the polysaccharides under discussion are built up from a limited number of mono-saccharides, and presumably a limited number of linkages will be involved. A particular enzyme, specific for one type of linkage, would therefore be capable of attacking variety of substrates, though with varying efficiencies according to the extent to which the particular linkage was present in the substrates. Thus with two or more enzymes, the varying pH and temperature optima and types of action observed for the action of cytase on these materials are what would be expected.

The action of cytoclastase has been shown to be extremely rapid, and it seems probable that it comprises a depolymerisation or disaggregation of the molecule. Such a suggestion fits very well the conception of cytase action outlined above, since if the linkage for which cytoclastase is specific was present to only a slight extent in the substances investigated, hydrolysis by this enzyme would cease after a short time, and only result in the break down of the substrate to molecules of still complex nature.

The absence of cytoclasis from the hydrolysis of malt hemicellulose-dextrin C_2 could also be easily explained by the absence of the specific linkage from this substance.

During the General Introduction it was intimated that cytolysis and pectolysis are not necessarily unrelated, and this possibility is further strengthened by certain peculiarities in the hydrolysis of pectinous substances by polygalacturonase noted by Phaff and Joslyn in a recent review of literature relating to the pectic enzymes (1947). As presented by them these peculiarities were quite unconnected, but on further examination they would appear to be closely related, as cause is to effect. The first peculiarity noted is a rapid drop in the viscosity of the substrate during the initial stages of polygalacturonase action, and the second is that the hydrolysis consists of two stages, (a) an initial rapid hydrloysis of 4 - 5% of the glycosidic bonds, and (b) a slow stage in which the residual bonds are hydrolysed. The rapid fall in viscosity would be the expected result of a partial disaggregation or depolymerisation of the substrate molecule, which need only be accompanied by the fission of relatively few linkages, as was observed. It is tempting to suggest that, like cytase, polygalacturonase is a system involving two enzymes.

Clearly great similarity exists between the actions of barley malt cytase and polygalacturonase; indeed the actions are almost identical in that they both consist of an initial rapid phase succeeded by a slower phase.

Though the progressive change in viscosity of substrate solutions during the action of cytase has not been measured, it would appear that a fall in viscosity, corresponding to that observed during the action of polygalacturonase, occurs during the action of cytoclastase, since it has been shown that modified substrates, i.e. substrates which have been subjected to the action of ~~cyt~~ cytoclastase, have considerably lower viscosities than the initial, unmodified materials.

Taking into consideration the great similarity between the two actions, the possibility that the action under investigation is that of ~~cytase~~ polygalacturonase, or the converse, cannot be lightly dismissed, and it is important that this point should be examined. A valuable contribution to the elucidation of this problem would be more detailed information on the constitution of the barley gums and hemicelluloses than is available at present.

A major part of the present work has been the preparation of hemicellulosic substances from barley, which might serve as substrates for cytase action, or in themselves contribute to a closer understanding of the problem on hand; in the course of these preparations several new substances have been isolated, namely, the barley hemicelluloses and the malt hemicellulose-dextrins.

In the introduction to Section II comment was passed on the great similarity between the barley gums and the hemicelluloses, and the work described herein on these substances has confirmed this. Indeed it appears that the only essential difference between the gums and the hemicelluloses lies in their initial water solubilities.

Clearly the water-soluble malt ppolysaccharides, the malt hemicellulose-dextrins, are of different chemical and physical nature from the barley gums: the latter are fibrous substances with little or no uronic anhydride content, yielding 25 -28% of furfuraldehyde in the case of barley gum C_2, and approximately 4% in the case of barley gum B_2; hemicellulose-dextrin $C_2$is a granular, pulverulent substance with 7 - 8% of uronic anhydride, and a furfuraldehyde yield of 44%. It seems probable that malt hemicellulose-dextrin B_2, having a low (2%) uronic anhydride content. and a furfuraldehyde yield of 12%, is not hemicellulosic in nature but is principally a starch-dextrin. There can be no doubt therefore that the barley gums disappear during germination, and are replaced by substances of somewhat different nature; whether these substances are produced by the action of cytase on previously insoluble materials, or are the result of enzymic synthesis, cannot be said, but of the two possible origins the former seems the more probable.

From the review of the published data on hemicelluloses etc. given in the General Introduction, and in the introduction to Section II, it is clear that little precise information on the constitution of such substances is available; indeed it is doubtful whether past preparations of these substances, and the preparations de described herein, are chemically homogeneous. ~~Clearly~~

Clearly such a state of affairs is extremely undesirable, and there can be no doubt that before any great advance in the knowledge of cytase is possible, a detailed examination of the substances on which it acts must be made, primarily with respect to the preparation, if possible, of chemically homogeneous samples, and subsequently to an examination of their chemical constitution.

It can not be claimed that any great advance in the knowledge of cytase has been achieved, nor was the work described primarily intended to serve such a purpose. The aim of the present work has been to provide a general survey of the problem of cytase action, to form a foundation for more detailed investigation, and to indicate possible future lines of work; it would seem justifiable to claim that the end to which the work was designed has been achieved.

Possible methods for the purification of cytase have been examined, and from the results it would appear that several of these show promise, namely, extraction of cytase with buffer solutions, extraction with 20% alcohol, and fractionation with alcohol. The adsorption of ~~of~~ cytase on willow hemicellulose S_1 has been studied, and although this work did not give such satisfactory results as the above, it does not seem improbable that a cytase is adsorbed by this substance.

The results of the three successful methods are summarised in Table XXXVI (p. 101) ,and it will be seen that the same degree of purification was attained by each of them, i.e. the precipitated cytase preparation obtained by each method was approximately twice as active as crude precipitated malt enzymes, the standard cytase preparation then in use. A promising approach to the problem of the purification of cytase would seem to be the use of a combination of these methods, possibly in the manner indicated in Section V

The final acceptance of the suggestion that cytase is a system of enzymes must inevitably depend on the separation of the individual enzymes. An attempt was made to achieve this object during the course of the present work; it was hoped that one of the methods of purification might also lead to the separation of the enzymes, and it would appear that some measure of success has been achieved. The results of the adsorption experiment indicate that of the two factors, only the cytoclastic factor has been adsorbed and subsequently eluted, but in view of the very low activity of the cytase solutions as a result of the adsorption etc. and the inactivating action of the alcohol used, these results must be treated with some suspicion. More concrete results were obtained by fractional precipitation with alcohol. Clearly the bulk of the cytolytic factor is precipitated by a concentration of 65% alcohol, although it is possible that some of this factor is left in solution at this concentration. The cytoclastic factor would appear to be precipitated continuously over the range of alcohol concentrations studied, but from the shape of the hydrolysis/time curves (Section V, fig. 18) it seems likely that the final fraction, i.e. that precipitated by a concentration of 79% alcohol, contains the cytoclastic factor free from the cytolytic. Extraction with buffer solutions or with 20% alcohol seems to exercise no selective action.

The separation of the cytoclastic and cytolytic factors by fractional precipitation with alcohol shows considerable promise of success, and this method could without doubt be used to great advantage; a parallel method which must not be overlooked is fractionation using $(NH_4)_2SO_4$ as the precipitating agent. Though the results of the adsorption experiment were rather unreliable, this method clearly calls for investigation, and a greater measure of success might be achieved if this method were applied to a more active preparation of cytase, or to the cytase preparation precipitated by 65% alcohol after removal of the fraction precipitated by 49% alcohol.

As stated above, too great emphasis cannot be laid on the importance of a more complete knowledge of the materials on which cytase acts, as a preliminary to a closer appreciation of the problem on hand. Of prime importance is the preparation of chemically homogeneous substrates, since a possible alternative explanation, which however unlikely cannot be overlooked, of the two fold nature of cytase action e.g. on barley gum C_2, is that this substance is actually a mixture of two compounds and that the two phases of the action arise from the hydrolysis of these compounds. An obvious step towards accomplishing this is the search for methods of precipitation and fractionation alternative to that involving the use of Fehling's solution and acetone, since this method, though excellent in many respects, has not proved completely satisfactory. A knowledge of the number of different types of linkage present in substances susceptible to cytase action would be of great assistance, should the suggestion made in the present work on the complex nature of cytase prove correct; here however it may prove that the mechanism of cytase action will eventually provode the key to this problem, as appears to be the case with starch and amylase action.

Before turning to the more practical applications of the material presented, an attempt must be made to indicate the probable physiological function of cytase, as far as is possible with the available information. Cytase has been shown to be capable of an extensive hydrolysis of the hemicellulosic constituents of the cell wall of barley, and this preseumably will be its principal function during germination, thus rendering the cell walls permeable to larger molecules such as those of other enzymes, and facilitating the translocation of materials essential to germination from one part of the seed to another. In the absence of an action such as this the amylases would presumably not be capable of carrying out their function of hydrolysing the starch of the endosperm, since these enzymes originate in the scutellum, and could not be brought in contact with their substrate had the cell walls not been made permeable to them. Similarly cytase might be expected to influence other enzyme actions occurring during germination, by facilitating the free passage of the enzymes and their products through the cell walls of the seed.

The work undertaken leaves no doubt of the importance of a greater knowledge of cytase as an aid to a closer understanding of the malting changes of barley. The change from the hard corn of barley to the friable corn of malt is clearly closely connected with the action of cytase, since this action must inevitably lead to a considerable decrease in the physical strength of the cell walls, and hence of the barley corn. The influence of cytase in producing materials of modified character which might be expected to affect the properties of wort, and hence probanly of beer cannot be overlooked, since cytase has been shown to produce drastic changes in the water-soluble polysaccharides during germination. There can be no doubt that the action of cytase leads to the presence of a larger proportion of water-soluble polysaccharides in malt than in the original barley, and that these substances, the malt hemicellulose-dextrins, though of lower molecular size than the barley gums, are molecules of still complex nature; however the effect of such substances on the properties of wort can not be precisely stated. Piratzki (*loc. cit.*) and Piratzki and Wiecha (*loc. cit.*) were of the opinion that the lowering of wort viscosity was dependent on the degree of modification of the malt, especially with respect to cytase action; this however has not been confirmed, as, although the viscosities of the malt hemicellulose-dextrins are lower than those of the corresponding barley gums, the larger quantity of the henicellulose-dextrins present in malt largely offsets the effect of their lower viscosities. It would appear therefore that cytase has little influence on the decrease in wort v viscosity associated with increase in the degree of modification of the malt, and consequently the solution to this particular problem must be sought elsewhere, e.g. in proteolysis.

VIII. BIBLIOGRAPHY.

Angell S. and Norris F.W., 1936;

Biochem. J., 30, 2155.

Baker J.L., 1941;

J. Inst. Brew., 47, 344.

Baker J.L. and Hulton H.F.E., 1917;

J. Chem. Soc., 111, 124.

1938; J. Inst. Brew., 44, 514.

Browne C.A. and Zerban F.W., 1941;

"Sugar Analysis", Wiley, New York.

Dickson A.D., Otterson H. and Link K.P. 1930;

J.A.C.S. 52, 775.

Grüss J., 1902;

Woch. Brau., 19, 243.

Hatschek E., 1928;

"The Viscosity of Liquids", Bell, London.

Hockenhull D.J.D., and Herbert D., 1945;

Biochem.J., 39, 1o2.

Hopkins R.H. and Krause B., 1937;

"Biochemistry Applied to Malting and Brewing", Allen and Unwin, London.

Kräemer E.O., 1943;

"The Chemistry of Large Molecules", Interscience Publishers New York.

Ling A.R., 1904;

J. Inst. Brew., 40, 741.

Lüers H. and Volkamer L., 1928;

Woch. Brau., 45, 244.

Lüers H. and Malsch L., 1929;

Woch. Brau., 46, 163.

Meredith W.O.S. and Sallens H.R., 1945;

Can. J. Research, 23F, 132.

Norman A.G., 1937;

"Chemistry of Cellulose, the Polyuronides, Lignin etc.", Oxford.

O'Dwyer M.H., 1923;

Biochem. J., 24, 973.

Norris F.W. and Preece I.A., 1930;

ibid., 24, 59.

O'Sullivan C., 1882;

J. Chem. Soc., 41, 24.

Piratzki W., 1936;

Woch. Brau., 53, 105.

Piratzki W. and Wiescha G., 1937;

ibid., 54, 145.

Preece I.A., 1930; Biochem. J., 24, 973.

1931a; J. Inst. Brew., 37, 409.

1931b; Biochem. J., 25, 1304.

1940a; ibid., 34, 251.

1940b; J. Inst. Brew., 46, 38.

1941; Biochem. J., 35, 659.

1944; ibid., 38, 402.

1948; Wallerstein Lab. Commun., 11, 119.

Schöne A. and Tollens B., 1901;

J. Landwirts., 48, 493.

Sugden S., 1930;

"The Parachor and Valency", Routledge, London.

Sumner J.B. and Somers G.F., 1947;
"Enzymex", Academic Press, New York.

Van Laer M. and M.H. and A. Masschelein, 1923;
Bull Soc. Chim. Belg., <u>32</u>, 402.

Waksman S.A. and Davison W.C., 1926;
"Enzymes", Balliere, Tindall and Cox, London.

Windisch W. and Van Waveren G., 1909;
Woch. Brau., <u>26</u>, 581.

ACKNOWLEDGEMENT.

The practical work described herein was carried out in the Chemistry Department of the Heriot-Watt College, Under the guidance of Dr. I.A. Preece. The author's best thanks are due to Prof. Marrian and Dr. I.A. Preece for their guidance and encouragement.