OBSERVATIONS ON THE NON-STARCHY BARLEY POLYSACCHARIDES

THESIS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY IN THE UNIVERSITY OF EDINBURGH

Presented by

KENNETH G. MACKENZIE, B.Sc., A.H.-W.C.,A.R.I.C.

HERIOT-WATT COLLEGE 1952

C O N T E N T S

"Experiment is the interpreter of Nature. Experiments never deceive. It is our judgement which sometimes deceives itself because it expects results which experiment refuses."

Leonardo da Vinci.

GENERAL INTRODUCTION

It is well known that profound changes occur in the cell-wall structure of barley during germination. For instance, a feature of the malt grain is its friability as compared with the hard structure of barley, the whole change being known as modification. The properties of the cell-wall and related materials before and after germination have attracted the attention of both the practical brewer and the academic scientist. As with all problems dealing with living tissue, the fact that so many changes - both synthetic and autolytic - are occurring simultaneously, makes it difficult to secure any concise explanation of these changes, and additions to the present state of knowledge must necessarily be slow.

Considerable amounts of isolated data are actually available in the literature regarding the properties of the materials undergoing germination and also of the cytolytic enzyme systems present. That the information does not fit together and make a complete picture is due in part to incompleteness and in part to certain of the data being wrongly interpreted.

Chemical investigations in the field of non-starchy barley polysaccharides were initiated in 1882 by O'Sullivan (1) who, working with aqueous extracts of barley/

barley, isolated two products which he named α- and β- amylan, the former being extracted from barley at 40oC. the latter at 20°C. In this case the choice of name was unfortunate as the products bear little or no relation to starch, and as will be shown in a subsequent section, O'Sullivan's conclusions were largely based on data, which in the light of more recent work, were purely coincidental.

Apart from some isolated observations, an interval of fifty years elapsed before further interest in these amylans was stimulated by Piratzky and Wiecha (2) who showed that the materials, now known as the barley gums, could be precipitated from aqueous extracts by Fehling's solution and alcohol. Their preparations yielded aqueous solutions of high viscosity and one of their gums gave only glucose on acid hydrolysis. Other features of this work included the detection of soluble pentosan material in barley, malt, etc. and the fractionation of a polysaccharide (correspondong to O'Sullivan's α-amylan) into a series having increasing molecular weight and increasing viscosity. The amylans of barley were renamed by Preece, Ashworth and Hunter (3) when it was shown that barley gum B_2 (previously α-amylan) was essentially a glucosan and barley gum C_2 (previously β-amylan) an arabo-xylo-glucosan. This system of nomenclature/

nomenclature was taken from a method of fractionating hemicellulosic material devised by Norris and Preece (4).

It is gradually being accepted that materials such as the barley gums which are water soluble do not differ fundamentally from the hemicelluloses which, in their naturally-occurring state are insoluble in water. The term hemicellulose was originally suggested by Schulze (5) as a name for that part of the plant cell-:wall which was soluble in dilute alkali and on hydrolysis gave a mixture of hexoses and pentoses. Later work (6) showed that hemicelluloses may also yield uronic acid on hydrolysis. The plant cell-wall probably contains mixtures of two types of hemicellulose, namely the encrusting hemicelluloses and the cellulosans. The former are of a polyuronide nature and their function appears to be that of a structural cell-wall constituent; they may have a branched structure similar to certain plant gums. On the other hand, the cellulosans have little or no carboxyl groups within their structure which may be of the straight chain type, and they are firmly associated with the cellulose of the plant (7). In view of the present state of knowledge it would be unwise to try and keep too rigidly to such a definition of the two groups as given above, as there is bound to be a certain overlapping of properties between the two types./

types. The initial insolubility of hemicellulosic material is due partly to association with lignin and other cell-wall constituents (8) and partly to the size of the structural units. Other factors may also be involved (9). The relation between the water-soluble gums and the hemicelluloses may be visualized somewhat as follows:

At one end of the scale there are the gums, consisting of comparatively simple molecular units and which give glucose, xylose and arabinose as the main constituents on hydrolysis. More complex and less soluble are the simpler hemicelluloses built from the same sugar residues and which may be rendered soluble in water by suitable pretreatment with alkali or by chlorination. Finally there are the more complex hemicelluloses which, although they can be extracted from the plant tissues by suitable means remain water insoluble.

Care must be taken in extracting hemicellulosic material to ensure that the methods used are not too damaging to the tissue. Preece (10) has shown for instance that certain of these cell-wall constituents are sensitive to alkaline conditions, and attempts to purify samples using Fehling's solution and acetone have resulted in recoveries of only 55% (3). This low recovery/

recovery may be due to partial degradation of the polysaccharide with the consequence that only a certain percentage of the polysaccharide retains the property of being precipitated by these reagents.

Degradation during fractionating due to alkaline conditions is not accepted by other workers (11) and it is likely that the concentration of the poly-:saccharide solution is the deciding factor. For instance, from a concentrated solution of the polysaccharide, Fehling's solution alone may be sufficient for precip-:itation whereas from a more dilute solution acetone may be required in addition. Finally, if the poly-:saccharide solution is very dilute, incomplete preci-:pitation and hence reduced recovery may occur. Chanda, Hirst, Jones and Percival (11) found that a xylan prepared from esparto grass exhibited no signs of degradation despite the use of alkali. Throughout the various stages in the preparation there was no change in the viscosity of the solution. It is possible then that the incomplete recovery found by Preece et al.(3) was due to either or both of these two possibilities. However, whatever may be the explanation it is not unreasonable to suggest that any vigorous methods of extraction which use strong alkali especially at high temperatures/

temperatures do not leave the final product in the same chemical condition as it possessed in the original tissue.

The distribution of the gums and related materials in barley have been investigated by Enders, Saji and Schneebauer (12). These workers attacked the problem from several angles. A year by year comparison of percentage soluble pentosans available was made over some six different varieties of barley. They found that the gum content was a varietal character, a conclusion recently confirmed by Preece and Prowse (13); Enders et al. further attempted to correlate the variability of gum content within a particular variety with the weather conditions prevailing during the last three months of the plant's growth. If, during this period, a high rainfall had been recorded then a barley was produced which possessed a large amount of soluble pentosan material. The opposite effect was noticed if the rainfall had been slight. These workers also found that if the amount of soluble pentosans was low then this was compensated by a greater percentage of barley husk and vice versa. These facts can be accounted for if it is postulated that a close relationship exists between the soluble gums and the hemicelluloses of the husk - a suggestion made earlier.

It/

It is surprising that such interesting results as were obtained by Enders *et al.* have not been confirmed and extended by other workers. It would be interesting for instance to find out whether any connection could be found between the quality of a brewed beer, the variety of barley used and the weather conditions during the growth of that barley. Any such information is of obvious importance to the brewer, but it would also create interesting studies for the biochemist who is not primarly interested in such mundane matters as beer quality. It is unlikely that the properties of a beer are dependent solely on the amount of soluble pentosans in the barley; nevertheless this may be a significant factor.

Some characteristics of the barley gums and related products have recently been examined by Meredith, Bass and Anderson (14). Their products, prepared directly from barley contained about 3.5% of nitrogen but this amount was removed on treatment of the gum with alkali. Previous work (3) in the same field had afforded products containing no nitrogen so from this point of view and taking into account also the extraction technique used by Meredith *et al.*, it was not surprising that the yields obtained by the last named were considerably higher than those obtained by Preece *et al.*(3). Pre-:cipitation of the gums from aqueous solution with alcohol/

alcohol of varying concentrations gave fractions which were essentially similar as far as viscosity measure-:ments and qualitative chromatographic analysis were concerned (14). This fact led to the conclusion that the gums probably consisted of several arabo-xylo-glucosans of varying complexities.

Of greater significance perhaps is the influence of the non-starchy barley polysaccharides on wort viscosity. Meredith et al. consider the main viscous principle in wort to be a polysaccharide containing pentosan material. Nitrogen was, however, found to be present in the product, whether as protein and associated chemically with the polysaccharide or merely as an impurity is not clear. This wort poly-:saccharide on hydrolysis gave the same sugars as would be obtained from barley gum, although the relative amounts of the sugars had changed - the glucose content of the polysaccharide decreased as the gum under examination was isolated from barley, malt or wort.

A water-soluble polysaccharide associated with the β-amylase of ungerminated wheat was prepared by Ford and Peat in 1941 (15). Their product yielded chiefly xylose and arabinose on hydrolysis although galactose was also detected. On the basis of methylation data a structure was proposed which has recently been challenged/

challenged by Perlin (16) who has examined the soluble pentosans of wheat flour and, although it cannot be expected that strict analogy between two cereals can be made, it is interesting to compare his results with those obtained from an examination of barley pentosans. By an elaborate fractionation of the wheat pentosans followed by methylation of the fractions, Perlin has deduced a structure in which a chain of xylo-pyranose residues joined by 1:4 β-linkages have smaller side chains of arabinofuranose residues, these latter being linked to the primary chain through the C_2 or C_3 position. Perlin visualizes the solubility of the wheat pentosans as dependent on the size of these smaller side chains, and in support of his conclusions cites data supplied by several workers (11,17,18) who have prepared from woods and straws, xylans which consist structurally of units similar to Perlin's wheat pentosans.

A point worth noting in many of these cereal preparations (14,16) is the fact that no inactivation of the enzyme systems present was effected. As so little is known about cytase action it may well be that enzymolysis of these workers' preparations had occurred.

One further theory of cell-wall carbohydrate structure might be mentioned. A tempting idea has been put forward by Isherwood (19) who suggests that each polysaccharide/

polysaccharide present in the cell-wall is built from only one type of monosaccharide unit. Using a Tiselius Electrophoresis apparatus he showed that different polysaccharides had different mobilities and, by reason of this property, a mixture of polysaccharides could be separated into homogenous fractions each of which on hydrolysis yielded one monosaccharide only.

Turning now to another aspect of cell-wall biochemistry, namely the nature of cytase action, apart from a recent publication by Preece and Ashworth (20) information on this topic is scanty. Cytase action has been ill-defined and any enzyme attacking a variety of non-starchy polysaccharides has been given this name. Also included as "cytases" are such enzymes as cellulase, xylanase, and hemicellulase, where enzymic activity has been noted on a particular substrate.

An early investigation on cytase action was provided by Grüss (21) who showed the presence in green malt of an enzyme capable of hydrolysing mannan from date stones and a galactan from gum tragancanth. The observed action was, however, slow and in no case was it complete even after several months. Optimum temperature and pH conditions were not quoted. Baker and Hulton (22) in 1917 showed that an enzyme was present in germinating barley/

barley which hydrolysed pentosan material in the endosperm and that translocation of the pentoses to the embryo had occurred.

A more quantitative examination of this problem has been supplied by Lüers and Volkamer (23) who examined the cytase activity of a green malt extract. The substrates used were xylan preparations from elder pith and barley. The enzyme was found to be most active at a temperature of 45°C. and at a pH of 5.0. In alkaline conditions or at 60°C. activity was destroyed. Purification of the enzyme was effected by adsorption on alumina at pH 5.0 followed by elution using a phosphate buffer at pH 8.3. However, even the purest preparation acting under the most favourable conditions, required two days to produce 75% hydrolysis.

As the inactivation of this cytase system is complete after 15 minutes at 60°C. it is unlikely that cytase activity during mashing would be appreciable and the proportion of water soluble-nonstarchy poly-:saccharides available in the wort is determined by the malting process.

Optimum enzyme activity is generally accepted as being rapid and the results of the above workers (21,23) which showed their cytase factor to be extremely slow acting disclose the danger of using "foreign" substrates. Before <u>in vitro</u> experiments can be said to/

to provide accurate information regarding cytase activity it is essential that suitable pure substrates and corresponding pure enzyme systems should be isolated.

The most recent information on cytase activity has been provided by Preece and Ashworth (20), who demonstrated that at least two enzyme systems were in fact responsible for the attack on cell wall materials during malting. In barley itself is present a cyto-:clastic factor which has two functions: namely that of simplifying the molecular complexity of the initially water soluble materials and also that of rendering soluble, initially insoluble hemicellulosic material. The second system - called the cytolytic factor and which is produced in quantity only after germination - has the property of bringing about a more profound hydrolysis of the substrates.

In the work referred to above (20) attempts were made to fractionate the crude enzyme preparations in order to facilitate enzyme study. Preliminary results showed that extraction with a buffer at pH 8.0 gave a product of enhanced cytolytic activity and reduced cytoclastic action.

It will be noticed that in this idea of two enzyme systems which attack cell-wall materials, there is an analogy to amylase action in that α-amylolysis may/

may be taken as a parallel to cytoclasis and that of β-amylolysis to cytolysis. However, such a comparison cannot be taken any further as it is α-amylase which is produced on germination and not β-amylase, so that strict analogy to the cytase system appears unlikely.

Over 15 years ago it was suggested that cytolysis in the barley grain with its consequent increase in the amount of water soluble pentosans, could be made a measure of modification (24), but unfortunately this increase is masked after the 5th or 6th days germination by synthetic changes taking place in the embryo, so that the method fails (25).

In this review of plant cell-wall biochemistry, it can be seen that although we are far from obtaining a complete picture of the carbohydrate metabolic changes, much progress has been made, especially within recent years. Fundamental enzymic study will make little advance until substrates are available whose constitutions are known so that hydrolytic studies can be carried out under better understood and more easily controlled conditions.

SCOPE OR PURPOSE OF THE PRESENT INVESTIGATION.

In the preceding section there was pointed out on several occasions the need for some method whereby fractionation of the non-starchy water soluble poly-:saccharides of barley could be accomplished. One of the main sections of the experimental work was concerned with the search for a suitable fractionating agent and the development of a successful technique. It was felt that if a suitable method could be worked out there might be the possibility of obtaining homogeneous polysaccharides i.e., ones giving only a single monosaccharide unit on hydrolysis. It was also hoped to apply the method of fractionation to similar polysaccharides present in malted barley.

If it was at all possible, fractionation using physical rather than chemical methods was to be preferred; a non-chemical method of fractionating would increase the probability of obtaining products which were structurally identical to those *in situ*. However, the determination of the structure of such fractionated polysaccharides by methylation or divers other methods was felt to be outside the scope of the present work.

Once fractionation had been achieved, it was hoped/

hoped to carry out sufficient analyses to enable the products to be characterised. Further, a comparison of such analytical figures between corresponding fractions obtained from barley, modified barley and malted barley would help in building up a picture of cytolytic changes during malting.

The source of the barley gums was another problem which remained at least partially unsolved. Enders et al. (12) had made a preliminary survey of the problem but it was clear that only by obtaining relatively pure components of the barley corn could a final answer be given on the subject. Part of the experimental work was accordingly designed to enable a more particular statement on the localisation of the gums to be made.

The position with regard to our knowledge of the cytase system in barley although slightly improved within recent years is still far from completely satisfactory, and so a certain amount of work in this sphere was envisaged. By judicious use of the cyto-:clastic enzyme, increased yields of water-soluble polysaccharides might be obtained. Such a result would be important for two reasons. Firstly, it would supply valuable information regarding cytase action in general, and secondly in a fractionation technique where losses by mechanical and other means are likely to be considerable,

a/

a method whereby the material undergoing fractionation has been increased has obvious advantages.

It may seem at first glance that the topics under consideration are isolated but it is nevertheless true to say that there is a thread connecting the links. The common factor is of course an attempt to determine the precise nature of the non-starchy water soluble polysaccharides present in barley and malt, and how far the use of the word "precise" has been justified will be seen in the subsequent sections.

PRELIMINARY ATTEMPTS AT FRACTIONATION OF THE BARLEY GUM C_2

INTRODUCTION.

As stated in the general introduction, the early investigations of O'Sullivan (1) have largely been superseded by those of later workers viz. Piratzky and Wiecha (2), and Preece *et al*. (3). In the first part of the experimental work the notation devised by Norris and Preece (4) will be adhered to, although later it will become necessary to adopt a new system of nomenclature.

The method employed for preparing a barley gum extract by the above workers (3) was very similar to that used in the present investigation and will be given in full in the experimental section. (The barley used - Pioneer Barley - was not the same as that used by Preece *et al*.). From the extract these workers employed two methods for the preparation of the solid gum:-

(i) The concentrated extract was acidified with hydrochloric acid and an equal volume of acetone was added.

(ii) To the concentrated extract, sodium hydroxide was added to 4%, and an equal volume of mixed Fehling's solution was added, followed by an addition of acetone to 40% of the total volume.

In (ii), which gave a product of lower ash content, the gum was recovered by the method of Norris and Preece/

Preece (loc. cit.).

Chemical analysis of the barley gum C_2:- The following figures give an indication of the composition of the above material,

(i) Yield of the barley gum C_2 on the dry grain varied from 0.2 - 0.4%.

(ii) Furfuraldehyde - 26.1%

(iii) Carbon Dioxide - 0.26%

(iv) Hydrolysis products by partition chromatography - glucose and xylose as major constituents and arabinose as a minor constituent. Galactose was not detected.

Physical properties of the barley gum C_2:-

(i) Appearance - fibrous

(ii) Surface Tension at 25°C. - 72 dynes/sq.cm.(water = 72)

(iii) Viscosity at 25°C. - 190 millipoises (water = 100)

(iv) Specific Rotation at 15°C. - -55°

(ii), (iii), and (iv) were determined on 0.5% aqueous solutions.

From the above data the points to note are, the negative specific rotation, the absence of uronic acid in the barley gum, the almost equal amounts of hexose and pentose residues in the structure, and the fairly high viscosity of the barley gum C_2 solution.

The experimental work which follows was so designed to enable a fractionation of the C_2gum to be carried out, in order to try and obtain several fractions which/

which differed considerably in chemical and physical properties. The presence of three different structural sugars makes the fractionation of the gum a distinct possibility. If a chemical fractionation failed, then a physical fractionation, in which different fractions represented different molecular aggregates of the same sugar structure might be achieved.

EXPERIMENTAL.

Preparation of a barley gum C_2 extract.

Unless a statement is made to the contrary it should be understood that the raw material used in all the preparations was Pioneer Barley. 100 g. of this barley was ground and added slowly to 250 ml. of 80% alcohol at its boiling point. The mixture was then gently refluxed on a water bath for a ½ hr. The extract was filtered at the pump and the extraction repeated with a further 200 ml. of 80% alcohol. This treatment destroys enzymes, extracts the small amount of sugar present and any other soluble material such as protein, and also coagulates some protein. After refiltering and sucking dry at the pump, the barley was spread out and air dried.

The barley residue was extracted three times at room temperature with 300 ml. quantities of distilled water, each extraction occupying about half to one hour. The extract was filtered through cloth and then centri-:fuged. A milky suspension was obtained which, however, contained little or no starch (the latter was removed on centrifuging). These centrifugates were combined and concentrated under reduced pressure to a small bulk - about 30 ml. This concentrate was filtered through acid-washed kieselguhr, the filter-bed washed and the bulk made up to 50 ml. Thus in the now clear solution 1 ml. of the extract = 2 g. of barley. When the extracts were lying/

lying for any length of time a little thymol was always added to inhibit any bacterial or mould growth. In the above manner a barley extract from a kilogram of barley was prepared and treated with acetone as shown in Table 1 below

TABLE 1.

Acetone concentration required for precipitation of barley gum C_2

Mixture used	Result	
	immediate	after 2 hr.
20 ml. extract + 10 ml. acetone	definite precipitate	definite precipitate
" " " + 9 ml. "	very strong turbidity	precipitate (less)
" " " + 8 ml. "	strong turbidity	precipitate (still less)
" " " + 7 ml. "	faint opalescence	faint opalescence
" " " + 6 ml. "	clear solution	clear solution
" " " + 5 ml. "	clear solution	clear solution

TABLE 2. /

TABLE 2.

Physical condition and yields obtained by fractionation of barley gum C_2 with acetone.

Order of precipitation	Ratio of Extract/Acetone required for precipitation	Yield	Physical condition and colour
2nd	103/59	0.07 g.	White; fibrous
3rd	97/86	0.07 g.	White; fibrous
4th	83/117	0.09 g.	White; pulver-:ulent
5th	81/169	0.08 g.	Buff; pulveru-:lent
6th	Xs. Acetone	0.04 g.	Brown; pulver-:ulent

From these preliminary tests it can be seen that the minimum concentration of acetone required for definite precipitation is of the order of almost 30%.

120 ml. of the extract was treated with 48 ml. of acetone (20:8) and the precipitate which came down was centrifuged off. The precipitate was dissolved in warm water and reprecipitated with a volume of acetone in the original ratio of 20:8. After recentri-:fuging, the fraction was dried with alcohol of increasing strength, powdered, and weighed.

To the first centrifugate from the above was added more acetone such that on standing a second precipitate was produced. This was reprecipitated and dried as above. In this manner seven precipitations were made although only six were worked to the dry state - the first precipitate was rejected on account of its dirty/

dirty condition and small bulk (Table 2).

Attempted fractionation of the acidified extract with acetone.

Five 20 ml. portions of the barley extract each containing 7 ml. of acetone (the amount found in the previous experiment which just failed to give precipitation) were adjusted to different pH's using a pH meter and the amount of turbidity produced was noted. Thus from the above tests it can be seen that maximum turbidity has appeared when the extract has been adjusted to a pH of 4.0 (Table 3). Consequently 120 ml. of the extract treated with 42 ml. acetone (29/7) was adjusted to a pH of 4.0. Afterwards the solution was treated with more acetone until the ratio extract acetone was 20:8. The precipitate which came down on standing was collected and dried in the usual way. By gradually increasing the concentration of acetone in the solution, six precipitates were obtained, small in bulk (Table 4).

TABLE 3.

Effect of pH on precipitation of barley gum C_2 with the minimal concentration of acetone.

pH	Result	
	Immediate	After 18 Hr.
3.5	Clear solution	slight turbidity
4.0	" "	turbidity
4.5	" "	clear solution
5.0	" "	" "
5.5	" "	" "

TABLE 4./

TABLE 4.

Physical condition and yields of barley gum C_2 obtained from the acidified extract using increasing concentrations of acetone.

Order of precipitation	Ratio of Extract/Acetone required for precipitation	Yield	Physical condition and colour
1st	179/71	0.04 g.	Grey; fibrous
2nd	146/94	0.06 g.	White; fibrous
3rd	143/167	0.18 g.	White; fibrous
4th	141/224	0.06 g.	Buff; pulver-:ulent
5th	137/288	0.02 g.	Buff; pulver-:ulent
6th	Xs. Acetone	0.02 g.	Buff; pulver-:ulent

Attempted fractionation of the barley extract using ammonium sulphate.

100 ml. of the extract was taken and 10 g. of ammonium sulphate added, stirred till dissolved, and the solution allowed to stand; no turbidity was noticed. A further 10 g. of the salt was added as before and on standing, a precipitate came down which was centrifuged off. To the centrifugate was added a further 15 g. of ammonium sulphate in 5 g. portions (35 g. in all). The precipitate which appeared was removed as before and the salt concentration raised to 50 g. when a third precipitate was obtained. At a concentration of 70 g. ammonium sulphate/100 ml. extract the solution was saturated/

saturated with ammonium sulphate but the precipitate obtained at this concentration was ignored.

Purification was effected by dissolving each precipitate separately in hot water and then adding ammonium sulphate to the concentration which had brought the precipitates down previously. After this second precipitation followed by centrifuging, further purifi-:cation was effected by dissolving in water, dialysing, and then adding acetone as a final precipitant. The products were then dried in the usual way.

Fractionation of the barley gum extract using ammonium sulphate, on a larger scale.

The following description outlines the method that was adopted at one time for the fractionation of larger quantities of barley extract i.e., extracts prepared from 1000 g. - 2500 g. of barley.

The method of precipitation was as before but this time precipitates were removed at concentrations of 20 g., 30 g., 40 g., 50 g., 70 g., of ammonium sulphate per 100 ml. of extract i.e. five

TABLE 5. /

TABLE 5.

Physical condition and yields obtained by fractionation using ammonium sulphate.

Order of precipitation	Conc. of ammonium sulphate per 100 ml. extract.	Yield	Physical condition and colour
1st	20 g.	0.01 g.	Black; ?
2nd	35 g.	0.05 g.	Grey; fibrous
3rd	50 g.	0.04 g.	Grey; fibrous

TABLE 6.

Comparison of properties of barley gum C_2 prepared directly and those from an average of a fractionated barley gum C_2.

Property	Direct preparation	Average of first 4 fractions
Furfural	26.1%	21.7%
Viscosity	190	188
Specific Rotation	-55°	-75°

precipitates in all. The saturated solution was then concentrated on a boiling water-bath to a small bulk when most of the ammonium sulphate crystallised out. The latter was filtered off and the filtrate dialysed to remove traces of ammonium sulphate. A sixth precipitate was then obtained from this dialysate on the addition of excess/

excess acetone. In this case no further purification was carried out.

The first five precipitates were then dissolved in water, dialysed and then to each was added ammonium sulphate in the concentration of 20 g./100 ml. of solution. Any precipitate which came down was returned as the 20/100 precipitate. Thus for example the 20/100 precipitate obtained previously was almost completely precipitated at this concentration the second time, the 30/100 precipitate obtained previously gave a small precipitate at the concentration of 20/100, the 40/100 precipitate gave a trace and the 50/100 remained clear. Next, the concentration of each solution was raised to 30/100 and every precipitate produced was returned as the 30/100 precipitate. This procedure was continued until every solution was at saturation. The precipitates were separately dissolved in water, dialysed and the process repeated. Four such exhaustive precipitations ensured that any fraction would be almost completely precipitated by a particular concentration of ammonium sulphate, and contained only small amounts of other fractions carried down by adsorption. Final precipitation of the fractions was carried out by adding slightly more than an equal volume of acetone. The addition of a few drops of 2N hydrochloric acid at this point accelerated the precipitation and helped to lower the ash content of the polysaccharide.

From/

From a kilogram of barley, six such fractions were obtained and subjected to the analysis as shown in Table 7 below. Determination of uronic acid was not carried out due to the smallness of the yields.

The sugars obtained on hydrolysis were detected by the method of partition chromatography (26) using the technique of Partridge (27). The solvents used were either phenol-water or butanol-acetic acid-water mixture. The constituent sugars of the hydrolysate were revealed by spraying the chromatogram with aniline oxalate solution (28) and heating for 10 min. at 100°C. Under these conditions hexoses give a brown spot and pentoses a pink spot. In these preliminary experiments the intensity of the spot on the chromatogram was assumed proportional to the amount of sugar present in the hydrolysate. In later work, quantitative estimation of the constituent sugars was carried out.

The hydrolysate was obtained by refluxing about 50 mg. of the fraction with 25 ml. of N sulphuric acid for $3\frac{1}{2}$ hours. The hydrolysate was then neutralised with barium carbonate, filtered, and concentrated under reduced pressure to about 1 ml.

The percentage of furfural was determined by the usual distillation method (41), the furfural being precipitated by phloroglucinol.

The viscosities were determined using an Ostwald Viscometer. Only yields of the first four fractions/

TABLE 7

Chemical & Physical characters of the fractionated barley gum C_2.

Polysaccharide in order of precipitation	G.$(NH_4)_2SO_4$/100 ml.extract	% Yield Ash + Moisture free	% Furfural on Dry Ash Free Sample	Hydrolysis Products by Partition Chromatography				Colour & Physical Condition	Viscosity at 25°C. on 0.5% Solution	Specific Rotation at 15°C. of 0.5% solution
				G	Ga.	X	A			
First	20	0.038	5.6	+++	-	+	+	Grey; Fibrous	167	Opalescent
Second	30	0.042	5.2	+++	-	+	+	White; Fibrous	161	-24°
Third	40	0.032	29.6	+++	-	+++	+++	White; Fibrous	200	-78°
Fourth	50	0.040	46.2	++	-	+++	+++	Buff;Pulverulent	224	-125°
Fifth	Saturation	0.006	?	?	?	?	?	Grey; Fibrous	?	?
Sixth	Xs.Acetone	0.027	18.8	++	+++	++	++	Buff;Pulverulent	?	?

G = Glucose; Ga. = Galactose;
X = Xylose; A = Arabinose.
+++ = Major Constituent. ++ = Minor Constituent
+ = Trace - = Absent

fractions were sufficient to carry out this determination on a 0.5% aqueous solution. The results are compared against water - 100. On this same solution was determined the specific rotation at 15°C. using a 1 dm. tube as difficulty was experienced in obtaining a clear image with a 2 dm. tube. The opalescent nature of the solution obtained from the first fraction prevented the determination of its specific rotation.

DISCUSSION.

This section may be conveniently divided into two parts:-

(i) In which the results obtained by the fractionation technique are examined.

(ii) Where lines of future work arising from (i) are suggested.

(i) Fractionation of the barley gum C_2.

The results from preliminary experiments that were carried out with acetone and acid/acetone as fractionating agents are not very illuminating. As only small amounts of the extract were taken the yields were necessarily small and no detailed analysis of the fractions could be made. However, even these yields showed that differences in physical condition were obtained - the early fractions being fibrous and the later ones pulverulent. The control of the pH did not seem to have much effect on the sharpness of precipitation and the later experiments showed that useful fractionation can be achieved by means of ammonium sulphate in neutral solution.

It/

It appeared that when using ammonium sulphate a much sharper precipitation of the different fractions was obtained. When using acetone the impression was, that at almost any concentration of acetone some fraction or other would be precipitated from the extract. It was in fact this indefinite and continuous precipitation which stopped the use of acetone and acid acetone as fractionating agents.

When the barley gum C_2 was fractionated on a larger scale using ammonium sulphate in the manner already described the results of the analyses show quite clearly that chemical and physical fractionation had been achieved. Thus the furfural yields varied from 5.6% rising to 46.2% and falling again to 18.8%. This change in furfural content was confirmed by the hydrolysis of the fractions and the identification of the sugars present. The first fractions were almost purely hexosan in character while in the middle fractions pentosan material predominated. The final fraction contained galactose which might possibly have come from pectic material in the barley. The physical conditions of the fractions varied from fibrous and difficultly soluble in cold water, to pulverulent and instantly soluble in cold water. The differences in colour are not significant being probably due to varying amounts of adsorbed colouring matter. The viscosity and specific rotation readings confirmed the fractionation of the gum into different entities. These readings, which should not be taken as being truly accurate, do serve to show that

each/

each fraction has its own physical properties as well as being chemically different.

It is noticeable that the properties of the first two fractions are very similar and it is highly probable that they are in fact, similar glucosans. The fall in viscosity between solutions prepared from these first two fractions can be accounted for if it is assumed that both fractions are similar chemically but differ in their degree of molecular aggregation.

It was unfortunate that the yield of the fifth fraction was insufficient to attempt any analysis. The results from the other fractions indicate that this fifth fraction might be purely pentosan in nature. The final fraction is rather an enigma. The presence of galactose suggests that an entirely different type of material namely pectin, is present in this fraction. The ash content of this fraction, 6.7% (not shown in Table) is almost ten times as high as that found in the other fractions. The explanation of this may be that the inorganic matter in the barley extract escapes precipitation by ammonium sulphate and eventually turns up in a fraction that has been precipitated by excess acetone.

An interesting result may be found by taking the average values of certain of the properties of the first four fractions and comparing with the figures obtained by Preece and Ashworth (3). See Table 6.

It/

It is interesting to find such a close comparison between the two sets of figures. The values for the specific rotations appear to be somewhat different but it must be remembered that the value for the first fraction has not been included in the figure -75° and, as the indications are that the first fraction would exhibit a small negative value this would mean a closer approximation to the figure -55°.

A considerable difference has been found between the yield obtainable by a direct preparation of barley gum C_2 and the sum of the yields of a fractionated gum. A typical yield of barley gum C_2 is 0.3% whereas the combined yield of the fractionated product is of the order of 0.18%.

The explanation for this large difference is not difficult to seek and three factors are probably involved.

(a) In the early development of the fractionation method loss of material due to inexpert technique was inevitable.

(b) All the material that may be precipitated by Fehling's solution and acetone is not recoverable when ammonium sulphate is employed. Further evidence on this point will be given later.

(c)/

(c) The gum content of barley has been shown to be a varietal factor (13) and it may be that Pioneer Barley contains less gum than the average variety.

(ii) Suggestions for further experimental work.

As the fractionation technique with ammonium sulphate had been quite promising, it was clear that the preparation of sufficiently large amounts of each fraction to enable a more complete analysis to be carried out was of primary importance. From the results obtained previously it seemed likely that 10 kg. of barley might provide sufficient of each fraction to make such an analysis possible. As a parallel investigation the fractionation of the barley gum B_2 (O'Sullivan's α-amylan) might be attempted along the same lines using ammonium sulphate as the fractionating agent. A third line of investigation was the fractionation of the water soluble polysaccharides available in a malted barley, preferably a malt prepared from the Pioneer Barley under examination.

It was also evident that as the amounts of workable material encountered in this type of work were small it would be profitable to investigate the possibilities of increasing the yields. Alkali had never been used in any preparations for two reasons:-

(a) Although the yield would undoubtably be increased, entirely different types of compound would additionally be extracted - the hemicelluloses of barley.

(b)/

(b) Use of alkali has been suspected to degrade or depolymerise the polysaccharide.

However, careful use of the cytoclastic enzyme system of barley has been shown to increase the available amount of gum-like material. The investigation which led to the conditions for such an increase will be given in full later on and so in the work to be described below, these conditions are stated but without further comment.

FURTHER ATTEMPTS AT FRACTIONATION OF BARLEY GUMS C_2 and B_2.

INTRODUCTION.

It was decided to apply the technique of fractionation using ammonium sulphate both to the gum B_2 and to the water soluble polysaccharides of malt. The barley gum B_2 had been shown by Preece et al (3) to consist mainly of glucosan material (furfural content 4%) and it was expected therefore that if fractionation could be achieved, the yields of fractions precipitated by high concentrations of ammonium sulphate would be negligible.

The above workers (3) had also shown that it was possible to obtain from malt, two water soluble polysaccharides - one soluble at room temperature, the second soluble at 40°C. These products were described as malt hemicellulose dextrins C_2 and B_2 respectively. They were precipitated from aqueous solution by Fehling's solution and acetone and the recovered products were pulverulent in nature. The C_2 and B_2 dextrins gave furfuraldehyde yields of 44.1% and 12.5% respectively, and both contained small amounts of uronic acid residues. The yield percentage on grain was 0.6 and for the C_2 dextrin and 0.1 for the B_2 dextrin.

EXPERIMENTAL.

A barley extract containing the gum C_2 was prepared exactly as outlined previously. The barley residue was retained and extracted a further three times with thrice its weight of water for ½ hour periods at 40°C.

An/

An extract containing barley gum B_2 was thus prepared. The two extracts were separately concentrated to small bulk and clarified using kieselguhr.

5 kg. of barley was treated in the above manner and a further 5 kg. was extracted for C_2 gum content only.

The two extracts were treated with ammonium sulphate exactly as outlined previously. In the case of the gum C_2, six fractions were obtained; the gum B_2 supplied only three fractions that were workable.

Occasionally during dialysis, the polysaccharide solutions were found to have precipitated some colouring matter and protein. This material was always removed, before re-starting precipitations with ammonium sulphate.

The fractions so obtained were analysed as before and the results of such analyses are collected in Table 8. An additional determination (not quoted in Tables) was that of uronic acid, using the method of Dickson et al. (29). Such an estimation could only be carried out on the largest fractions.

The presence of mannose was detected in certain of the fraction hydrolysates. As there was also present a considerable amount of arabinose which, in the butanol-acetic acid-water mixture has an R_F value close to that of mannose, the presence of the latter sugar was not obvious. It was therefore necessary to re-run the strip containing arabinose and mannose in a different solvent - phenol/

(see photograph page 46)

phenol, where their R_F values are appreciably different.

Fractionation of a modified barley gum C_2 and B_2.

The term "modified" is used throughout the complete investigation in referring to material which has, at one time or another in its preparation, been acted on by an enzyme, usually a cytoclastic enzyme.

500 g. of barley previously inactivated in the usual manner was treated for ½ hour at room temperature with 1200 ml. of a barley extract equivalent to 250 g. barley. It had been found that by treating inactivated barley with a barley extract representing 50% of the substrate for ½ hour, increased the yield of water soluble polysaccharides by about seven times (recovered using Fehling's solution and acetone). The mixture was filtered through cloth, the suspension centrifuged, and the centrifugate boiled for two minutes to prevent further enzyme action. The residue was then re-extracted twice with water in the usual manner. The liquid bulk was concentrated under reduced pressure to 500 ml. and then clarified using kieselguhr. The barley residue was extracted with water (ca. 1500 ml.) a further three times at 40°C. for ½ hour periods. This procedure extracted the modified barley gum B_2.

The above procedure was repeated on a further 500 g. barley and the corresponding extracts combined. The clear extracts were separately made up to a convenient volume - /

volume - 1 litre, with distilled water and then fractionated using ammonium sulphate. A slight change was made in the method of fractionation. Instead of dialysing between each set of precipitations, the crude fractionated polysaccharide, after being dissolved in water was precipitated by adding an equal volume of acetone containing a little acid. This change in technique was thought to have two advantages.

(1) It was much quicker to carry out than dialysis and, although traces of ammonium sulphate were probably retained by the gum when precipitated by acetone it was not felt that such traces would impair the efficiency of the fractionation.

(2) The precipitated polysaccharide left a large part of adsorbed colouring matter in the acid acetone solution.

Resolution of the fractionated polysaccharides was followed by a second precipitation with ammonium sulphate in the usual manner.

After six such precipitations with ammonium sulphate, solutions of the fractionated polysaccharides were dialysed to remove traces of ammonium sulphate, precipitated with acetone and taken to dryness in the usual way.

The fractions were submitted to the usual analysis and the results obtained are collected in Tables 10 and 11. Determination of the surface tension of 0.5% aqueous solutions was also carried out but as no surface activity/

activity was detected in any fractions, the results are not included in the tables.

Fractionation of the hemicellulose dextrins C_2 and B_2 of malt.

One kilogram of malted Pioneer Barley was treated for enzyme inactivation in the usual manner with boiling 80% alcohol. After air drying, the malt residue was extracted first at room temperature and then at 40°C. using each time twice its original weight of water. After concentration and clarification it was noticeable that the hemicellulose dextrin extracts were much less viscous than that encountered at the equivalent stage with barley and also that the solutions had a much darker colour. The concentrated extracts were then submitted to the fractionation procedure using ammonium sulphate similar to that carried out with the modified barley gum C_2 and B_2 extracts. It was found that no 20/100 or 30/100 fractions were obtained from either the C_2 or B_2 extracts. The paucity of the yields was such that only the determination of the hydrolysis products of the fractions could be carried out. The few data supplied by this experiment are collected in Table 12.

In Table 13 is given a comparison of the yields of the malt hemicellulose dextrins C_2 and B_2 obtainable when recovered from the aqueous extracts either by Fehling's solution and acetone, or by saturation of the extract with ammonium sulphate. In this case, no/

TABLE 8

Chemical and physical characters of the fractionated barley gum C_2

Polysaccharide in order of precipitation	GMS. $(NH_4)_2SO_4$ /100 ml.^{4} extract	% Yield Ash + Moisture Free	% Furfural on Dry Ash Free Sample	Hydrolysis Products by Partition Chromatography					Colour & Physical Condition	Specific Rotation at 15°C. of 0.5% solution	Viscosity at 25°C. on 0.5% solution
				G	Ga.	M	X	A			
First C_2	20	0.0036	3.70	+++	-	-	+	+	Grey; Fibrous	-10°	257
Second C_2	30	0.0184	5.09	+++	-	-	++	+	White; Fibrous	-12°	160
Third C_2	40	0.0334	41.0	++	-	-	+++	++	White; Fibrous	-88°	332
Fourth C_2	50	0.0077	45.9	+	-	-	+++	++	Grey; Fibrous	-112°	271
Fifth C_2	70	0.0068	43.4	+	-	++	+++	++	Cream; less fibrous	-116°	181
Sixth C_2	Xs Acetone	0.0089	16.3	++	+++	-	++	++	Buff; Pulverulent	opaque	114

TABLE 9

Chemical and physical characters of the fractionated barley gum B_2

Polysaccharide in order of precipitation	GMS $(NH_4)_2SO_4$ /100 ml. extract	% Yield Ash + Moisture Free	% Furfural on Dry Ash Free Sample	Hydrolysis Products by Partition Chromatography					Colour & Physical Condition	Specific Rotation at 15°C. solution	Viscosity at 25°C. on 0.5% solution
				G	Ga.	M	X	A			
First B_2	20	0.0113	3.95	+++	-	-	+	+	White; Fibrous	-16°	158
Second B_2	30	0.0139	3.88	+++	-	-	+	+	Grey; Fibrous	-36°	147
Third B_2	40	0.0142	43.5	++	-	-	+++	+++	Grey; Fibrous	-100°	321
Fourth B_2	50	0.0052	41.7	+	-	-	+++	+++	White; less Fibrous	-120°	256
Fifth B_2	70	0.0025	?	-	-	+++	+++	+++	Buff; Pulverulent	?	?
Sixth B_2	Xs. Acetone	0.0040	-	+++	-	-	+	+	Buff; Pulverulent	-	-

M = Mannose

TABLE 10

Chemical & Physical Characters of the fractionated modified barley gum C_2.

Polysaccharide in order of precipitation	GMS $(NH_4)_2SO_4$ /100 ml. extract	% Yield Ash + Moisture Free	% Furfural on Dry Ash Free Sample	Hydrolysis Products by Partition Chromatography					Colour & Physical Condition	Specific Rotation at 15°C. of 0.5% solution	Viscosity at 25°C. on 0.5% solution
				G	Ga.	M	A	X			
1st Modified C_2	20	0.0655	3.6	+++	-	-	+	+	White; Fibrous	-8°	190
2nd Modified C_2	30	0.0720	4.1	+++	-	-	+	+	White; Fibrous	-8°	171
3rd Modified C2	40	0.0500	30.7	+++	-	-	+++	+++	White; Fibrous	-70°	186
4th Modified C_2	50	0.0447	44.7	+	-	-	+++	+++	White;pulverulent	-100°	177
5th Modified C_2	70	0.0120	-	+	-	-	+++	+++	White;Pulverulent	-	-

TABLE 11

Chemical and physical characters of the fractionated modified barley gum B_2

Polysaccharide in order of precipitation	GMS. $(NH_4)_2SO_4$ /100 ml. extract	% yield Ash + Moisture Free	% Furfural on Dry Ash Free Sample	Hydrolysis Products by Partition Chromatography					Colour & Physical Condition	Specific Rotation at 15°C. of 0.5% solution	Viscosity at 25°C. on 0.5% solution
				G	Ga.	M	A	X			
1st Modified B_2	30	0.0510	0.0	+++	-	-	-	-	White; less Fibrous	-12°	190
2nd Modified B_2	40	0.0150	-	+++	-	-	++	++	Grey; still less Fibrous	-	-
3rd Modified B_2	50	0.0090	-	+++	-	-	+++	+++	Grey; Paper Like ?	-	-
4th Modified B_2	70	0.0080	-	++	-	-	+++	+++	Grey; Paper Like ?	-	-
5th Modified B_2	Xs. Acetone	0.0180	-	+++	-	-	++	++	Brown; Pulverent	-	-

TABLE 12

Available data on malt fractions.

Polysaccharide Fraction	% Yield	Hydrolysis Products			
		Glucose	Xylose	Arabinose	Galactose
Malt Gum C_2 40/100	0.005	+++	++	++	-
" " C_2 50/100	0.004	+++	++	++	-
" " C_2 Sat.	0.006	+++	+	+	-
" " C_2 Final	0.035	+++	++	++	++
" " B_2 40/100	0.006	+	+++	+++	-
" " B_2 50/100	0.020	+	+++	+++	-
" " B_2 Sat.	0.011	+	+++	+++	-
" " B_2 Final	0.013	+++	-	-	+

TABLE 13

Influence of reagent on % recovery

Malt Gum	% Yield Feh.	% Yield NH_4
C_2	0.80	0.31
B_2	0.36	0.04

Feh. = Recovery by Fehling's solution + acetone

NH_4 = Recovery by saturated ammonium sulphate.

MODIFIED B_2
30/100
HYDROLYSATE

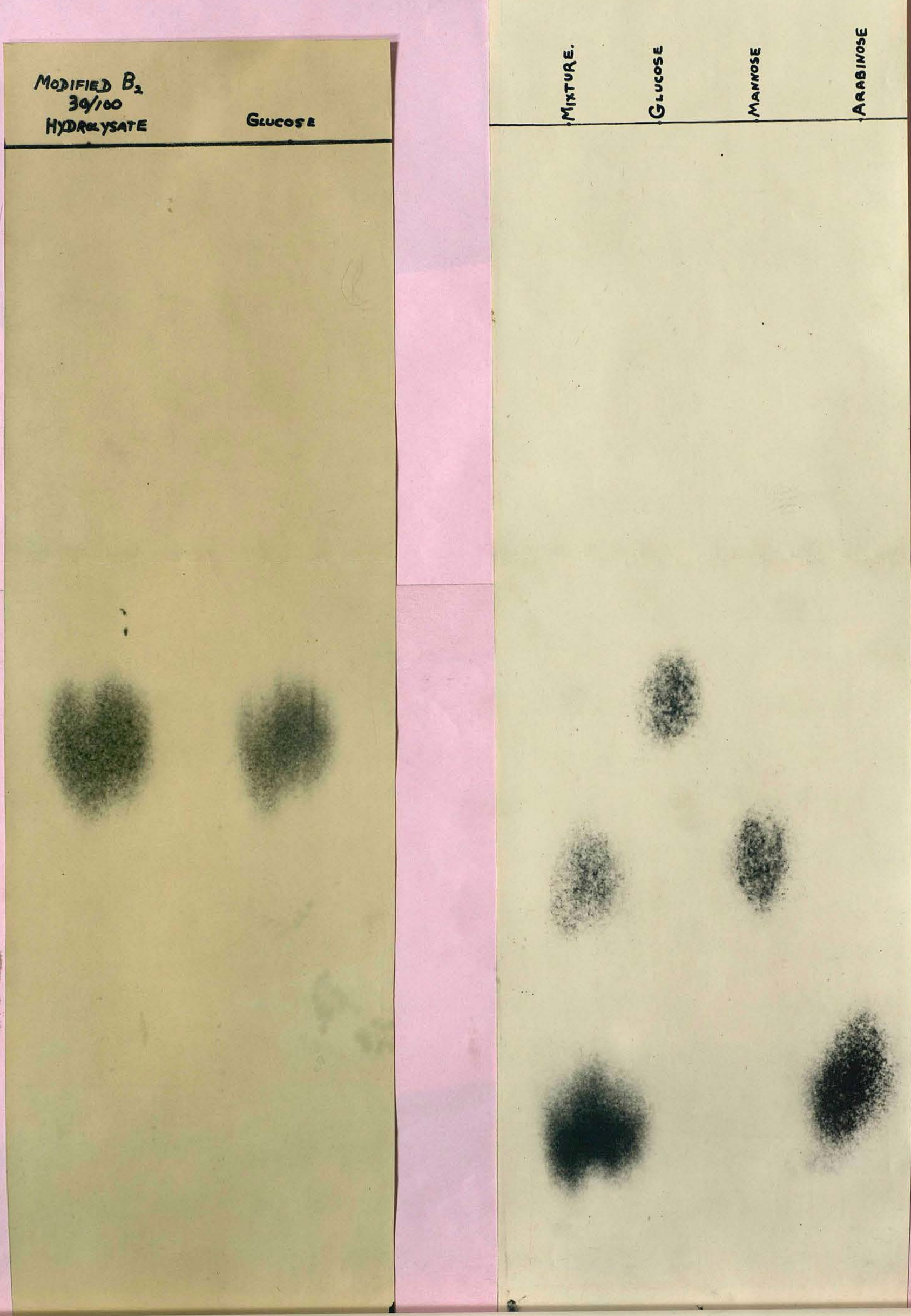
GLUCOSE

MIXTURE.

GLUCOSE

MANNOSE

ARABINOSE



no fractionation was attempted but instead total recovery of the polysaccharide from solution.

DISCUSSION.

The results obtained from the above three sets of preparations reveal both the utility and the limitations of a fractionation technique using ammonium sulphate. These points will be even more noticeable when a discussion on the methods finally adopted for fractionation is given; but briefly, it appears that the main virtue of ammonium sulphate lies in its ability to separate glucosan and pentosan material. Ammonium sulphate fails to resolve the pentosan fractions of barley gum C_2 into material structurally simpler and it lacks the property of precipitating low molecular weight material (Table 13).

In the first large scale preparation of barley gum C_2 the furfural content of the 20/100 fraction was 5.6% (Table 7) and it can be seen that in the later preparation, the percentage of furfural in the corresponding fraction has dropped to 3.7. The indications are that a more exhaustive treatment of the 20/100 and 30/100 fractions with ammonium sulphate would ensure the removal of the last traces of pentosans. Such a state of affairs has evidently been reached in the modified B_2 30/100 fraction. (see photograph)

The fall in viscosity between solutions prepared from the first two C_2 and also the first two B_2 fractions is again observed. It is not unreasonable to suggest/

to suggest that the only difference between such fractions lies in their molecular complexity. The action of the cytoclastic enzyme in disaggregating the material is demonstrated by the fall in viscosity between corresponding fractions prepared from modified barley and unmodified barley. For example the viscosity of asolution prepared from the C_2 20/100 fraction is 257 whereas the corresponding viscosity of the modified barley is 190. A similar difference can be observed between corresponding 40/100 fractions.

The furfural content and the specific rotation shown by the modified fractions are very similar to that exhibited by the analogous unmodified fractions. The close similarity is not surprising considering that the period of enzyme action was limited to a $\frac{1}{2}$ hour.

The fractionation of the barley gum B_2 both modified and unmodified has followed the same lines as that of C_2, and itcan be seen from Tables 8 and 9 that the properties of corresponding fractions of C_2 and B_2 are very close. It is becoming clear that as there is no fundamental difference between say, C_2 20/100 and B_2 20/100, it would be better in future preparations to extract the barley directly at 40°C. This procedure would remove the C_2 and B_2 gums simultaneously.

The presence of mannose in one of the fraction hydrolysates is unusual. Other workers (14) have claimed to have detected mannose residues in barley gums and/

and related products but the precise significance of this sugar is not clear. If the isolation of a pure water soluble mannan is assumed possible then approxi-:mately 100 kg. of barley would have to be treated in order to obtain workable amounts of this polysaccharide - a prodigious task indeed!

From the results available at present it does not seem likely that the fractions rich in pentosan material will be resolved into anything structurally simpler using the present technique at any rate. Filter paper chromatography has shown qualitatively, that the occurrence of xylan is accompanied by virtually an equal amount of araban. The various other methods of fract-:ionation which were applied to this pentosan material will be given in a later section.

The yields of the modified fractions were rather less than was anticipated. The indications were, that yields would be enhanced by about seven times under the conditions used but the final increase was only a three fold one. It is not intended at present to give and comment on a possible explanation of these facts. Later, when more data have been given it will be possible to examine the problem more fully.

Turning now to the few analytical data available regarding the properties of the malt hemicellu-:lose dextrins, in view of the paucity of such data it would be unwise to make more than a few general remarks.

Firstly/

Firstly it would appear that very large quantities of malt will have to be worked in order to obtain yields which can furnish more detailed analytical figures. The malt fraction C_2 40/100 represents a yield of 0.005% on the total grain as compared with 0.033% from the C_2 40/100 barley polysaccharide fraction; 100 kg. of malt would be required for the preparation of 5g. of this fraction.

However, from Table 13 it can be seen that the amount of malt polysaccharide available in solution is of the order of 0.8% (for the C_2 dextrin) which is the amount recoverable by Fehling's solution and acetone. When recovery is attempted with ammonium sulphate this figure drops to 0.31%. Clearly then if precipitation of the polysaccharide once by ammonium sulphate reduces the amount recovered by about 60%, a fractionation technique which involves re-solution followed by a re-precipitation with ammonium sulphate many times will lead to an enormous reduction in the amount of recoverable material. This then, may be the reason for the smallness of the fractionated products.

The pulverulent nature of the products probably indicates that the malt polysaccharides have a comparatively low molecular weight and it may be that the ammonium sulphate is incapable of precipitating such compounds. If it is assumed that the figures in Table 12 are reliable/

reliable then this theory is partly confirmed by the fact that the largest yields were always obtained near the saturation mark. In fact, the largest fraction of all was precipitated from the saturated ammonium sulphate mother liquor (after dialysis) with excess acetone. Again in support of this idea it can be seen that no 20/100 or 30/100 fractions were obtained; considerable amounts of these fractions can be obtained from barley.

The hydrolysis products of the various fractions show that the constituent sugars of the malt polysaccharide fractions are similar although not identical to the analogous barley polysaccharide fractions. The 40/100 fraction of malt has considerable more hexosan material than the corresponding barley fraction, as would be expected if malt modification acts to increase water solubility of the polysaccharides.

Without further comment it can be noted that galactose occurs in the final C_2 and B_2 fractions (cf. the final C_2 and B_2 barley fractions).

A theory covering these above experimental facts can be made as follows:-

On germination of the barley grain, the cytase enzyme system present attacks the barley gums C_2 and B_2 so that their molecular complexity is simplified (3). A large amount of the glucosan is thus degraded and, as the malt grain is produced, is not precipitated/

precipitated from solution by 30% ammonium sulphate. Its molecular size is such that it occurs in the 40/100 and succeeding fractions along with the pentosan material. In other words, it may be that the malt polysaccharide extract contains a fairly large amount of (0.8% yield) low molecular weight material largely incapable of precipitation by ammonium sulphate whereas the barley gum solution contains less material (0.3% yield) but of high molecular weight which can nearly all be precipitated by ammonium sulphate.

The position has now been reached where, as a result of the experience gained in the use of ammonium sulphate as a fractionating agent, the methods that were finally adopted for fractionating the barley and malt gums can be given.

The experimental procedure was similar to that already given for the fractionation of the modified barley gums C_2 and B_2. However, as several important changes were made in the method, it was felt that it would be better at the risk of being accused of unnecessary reiteration to give in some detail the technique adopted.

METHODS FINALLY ADOPTED FOR FRACTIONATION.

EXPERIMENTAL.

(a) From modified barley:- The barley, after being treated for enzyme inactivation in the usual way was extracted for a ½ hr. at room temperature with twice its weight of an extract prepared from live barley - this extract containing barley equivalent to 50% of the substrate. The mixture was filtered through cloth which retained most of the large particles and the milky suspension so obtained was centrifuged. The starch and most of the protein formed a hard layer at the base of the centrifuge bottle. The liquid centrifugate was boiled for 2 min. to stop further enzyme action. The starch and protein were recombined with the main residue and a further two extractions with distilled water (ca. 1000 ml. each time) was carried out. The residue was then thrice extracted at 40°C. with twice its bulk of water to make six aqueous extracts in all. These extracts were all combined and concentrated under reduced pressure to about 1/15 of their original volume. The extract which was highly viscous and opaque, was partially clarified by filtering through acid washed kieselguhr. At this stage, it was not essential that the concentrated extract be water bright/

bright as later, in the actual fractionation, further clarification is effected.

Then to every 100 ml. of extract was added 20 g. of ammonium sulphate with stirring. The precipitate which was thrown down was centrifuged off and the centrifugate treated with a further 10 g. of ammonium sulphate. The precipitate thrown down at this stage was likewise removed and the procedure was repeated at concentrations of 40 g. ammonium sulphate/100 ml. extract, 50/100, and 70/100. The centrifugate remaining after saturation with ammonium sulphate (viz. 70/100) was concentrated to a small bulk when most of the salt crystallised out. The crystals were filtered off and the filtrate dialysed to remove the remainder of the ammonium sulphate. The polysaccharide material present in the dialysate was precipitated by adding an excess of acetone. Apart from taking to dryness in the usual manner, this fraction was not treated further.

The original 20/100, 30/100 etc. fractions were separately dissolved in water and reprecipitated by adding an equal volume of acetone. As stated earlier, this procedure has the advantage of removing much of the colouring matter which the polysaccharide had adsorbed.

After centrifuging and re-solution of the precipitates,/

precipitates, to each was added solid ammonium sulphate to 20%. Any precipitate thrown down by any of the fractions was collected as the 20/100 fraction. It was found that only the 20/100, 30/100, and perhaps the 40/100 fractions showed a precipitate at this concentration. After removal of this precipitate the concentration of each solution including the 20/100 solution was raised to 30% with respect to ammonium sulphate. The procedure was continued until each of the fractions in solution was saturated with ammonium sulphate. At this concen-:tration the first 20/100 and 30/100 fractions gave little or no precipitate.

The combined second 20/100, second 30/100 etc. fractions were separately dissolved in water and treated as before with acetone. The fractionation with ammonium sulphate was then repeated as above but with the following differences.

At this stage it was found that the 20/100 and 30/100 fractions gave little or no precipitate at 40/100 and higher concentrations, and any precipitate so obtained was rejected. Again, the main 40/100 fraction tended to give considerable amounts of a precipitate at a concentration of 30/100 but this precipitate was not included in the main 30/100 fraction. Instead/

Instead it was collected separately and called for want of a better name the 30-40/100 fraction.

Before fractionation with ammonium sulphate for the fourth time, the solutions were all clarified to water brightness by filtration through acid washed kieselguhr. The solutions were then completely clear and practically colourless. Three further fractionations with ammonium sulphate were carried out as outlined above, making six in all. It was felt that after such a fractionation, maximum separation of the polysaccharide components was obtained. The products were then dissolved in water and dialysed to remove traces of ammonium sulphate. The addition of an equal volume of acetone to the solutions containing the 20/100, 30/100 and 40/100 fractions, and slightly more than an equal volume of acetone to the 50/100 and 70/100 fractions, precipitated the polysaccharides. The products were then taken to dryness in the usual way with alcohol, powdered, and weighed.

(b) <u>From unmodified barley</u>:- The enzyme inactivated barley was treated with $2\frac{1}{2}$ times its weight of water for $\frac{1}{2}$ hr. periods at 40°C. Three such extractions were made. Boiling of the centrifugates was unnecessary. The remainder of the procedure was exactly/

exactly as outlined above for the case of the modified barley.

(c) From malted barley:- The enzyme inactivated malt was treated with twice its weight of water for ½ hr. periods at 40°C. Three such extractions were made. Again, the boiling of the centrifugates was unnecessary. The extracts were concentrated to a small bulk and it was observed that although the extract was viscous it was much less so than that obtained at the corresponding stage with barley.

A portion of the extract ≡ 100 g. malt was kept aside and clarified separately. The polysaccharide material in solution was recovered using Fehling's solution and acetone. By this means the potential amount of malt hemicellulose dextrins available in the extract could be measured. The main bulk of the extract was then fractionated in a similar manner to that outlined for the modified barley. However, the malt extract did not contain any material precipitated by 20% ammonium sulphate and practically none by 30% ammonium sulphate. A clear cut fractionation of the malt hemicellulose dextrins was not achieved, but the extract was treated with ammonium sulphate until it was felt that the most efficient separation had been obtained. Throughout the fractionation of the malt extract it was clear that material was steadily being lost and so in view of the information regarding the efficiency

of/

of the fractionation, only four precipitations with ammonium sulphate were carried out. The preparation was completed in the manner already described under (a). The retention of the 30-40 fraction as a separate entity, important when preparing a fractionated barley gum, was not felt to be necessary in the case of the malt gum.

The amount of starting material used in the above three preparations was (a) 6 kg. of barley; (b) 5 kg. of barley; (c) 5 kg. of malt.

Analyses of fractions.

For the first time in preparing barley and malt polysaccharide fractions, sufficient material was available in nearly all cases to enable duplicate determinations to be made. The following determinations were carried out:-

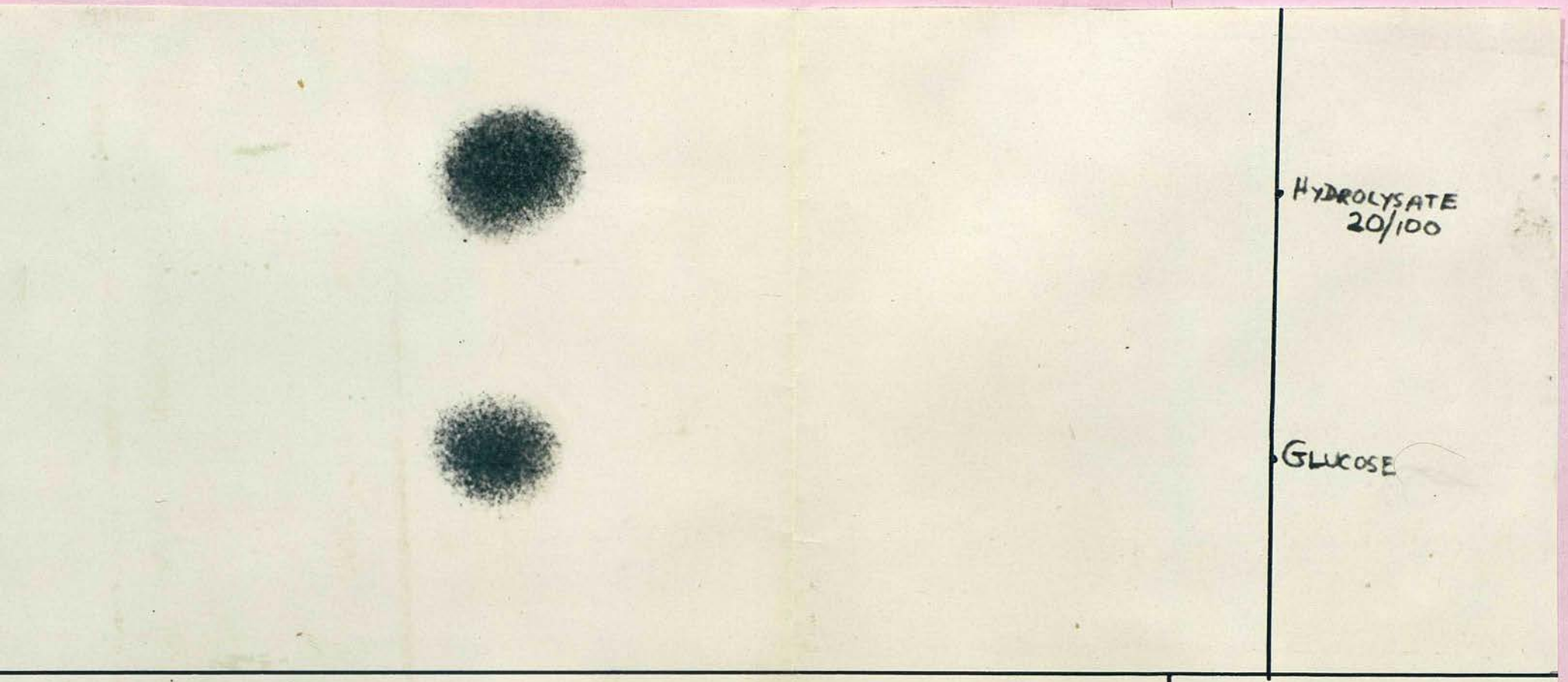
(a) Furfural content. Approximately 100 mg. of each fraction accurately weighed, was distilled under standard conditions with 12% hydrochloric acid, the furfural so obtained was precipitated as the phloroglucide.

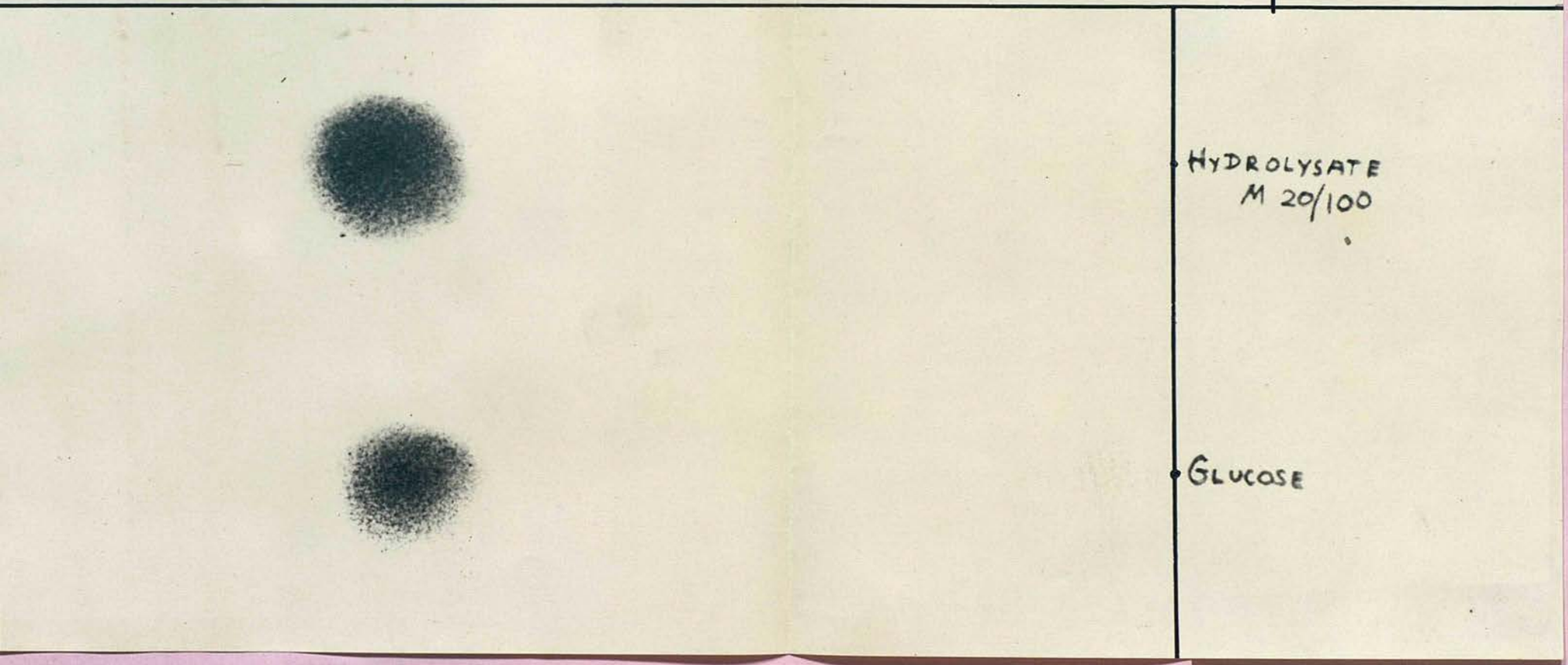
(b) Specific Rotation. The determination was carried out at 15°C. on a 0.5% aqueous solution (moisture and ash free) of each polysaccharide fraction. In the case of the final fractions obtained from modified, unmodified and malted barley the opacity/

opacity of the solution prevented the specific rotation being read.

(c) Viscosity. The determination was carried out at 25°C. on the same 0.5% solution using an Ostwald viscometer. The viscosity of pure water was measured under the same conditions and a measure of the viscosity of the polysaccharide solution was given by the expression 100 Ts/Tw where Ts= time of flow in seconds of the solution, Tw= time of flow in seconds of the water.

(d) Qualitative examination of sugars present after hydrolysis. Approximately 50 mg. of each fraction was hydrolysed for 3 hours with N sulphuric acid, the hydrolysate neutralised with barium carbonate and the sugars present in the hydrolysate were detected by the method of filter paper partition chromatography (26, 27, 28). In the main, either butanol-acetic acid-water mixture (40:10:50) or phenol-water mixture were used as suitable irrigating solvents. In certain of the fractions two sugars were present which had similar R_F values in a particular solvent and in such cases it was necessary to re-run the strip containing these sugars in a second solvent. The details of such a technique are given in the section entitled "Micro-determination of the sugars present after hydrolysis of/


HYDROLYSATE
20/100
GLUCOSE


HYDROLYSATE
M 20/100
GLUCOSE

of the polysaccharide fraction". As a first approximation to the concentration of the individual sugars the intensity of the developed spot on the chromatogram (aniline oxalate as developer) was assumed proportional to the concentration of that sugar.

The modified and unmodified barley fraction 20/100 was found to give glucose as the sole constituent of hydrolysis. In addition to the above mentioned solvent mixtures, the hydrolysates from these two fractions were run in a butanol-ethanol-water mixture (50:10:40) and also in a pyridine-benzene-butanol-water mixture (30:10:50:30). In every case only one sugar was detected viz. glucose. (see photographs)

Occasionally a spot on the chromatogram was observed which could not be identified with a known sugar. For instance the modified and unmodified 50/100 fraction hydrolysates gave two faint pink spots (aniline oxalate as developer) having R_G values of about 0.26 and 0.30 in the butanol-ethanol-water mixture. The R_G values for xyloketose and rhamnose respectively (32) are close to the figures quoted above, however, rhamnose under these conditions gives a pale brown spot and the presenceof xyloketose in the/

the hydrolysate is unlikely. Again, the hydrolysate prepared from the final malt polysaccharide fraction contained mainly glucose and galactose which in the butanol-acetic acid-water mixture separate only after a considerable time. The strip containing these two sugars may be re-run in phenol - see under "Micro determinations of the sugars present after hydrolysis of the polysaccharide fraction", for the technique adopted. Instead of two spots appearing viz. glucose and galactose, a third unknown spot with an R_F in phenol of approximately 0.5 was observed.

The results obtained from the above determinations are collected in Tables 14, 15 and 16 which refer to unmodified barley, modified barley and malted barley respectively.

Several additional determinations were carried out but as the results were of a negative character, they are not included in the above tables. Thus:-

The presence or absence of nitrogen in the fractions was determined. A qualitative test was done only, because it was found that the amount of nitrogen present in a fraction was either non-existent or of such a small order that it could not be estimated with reasonable accuracy. The method used for/

TABLE 14

Chemical & Physical Characters of the fractionated Barley Gum

Polysaccharide as Precipitated by Ammonium Sulphate	% Yield, Moisture Ash Free	% Furfural, on Dry Ash Free Sample	Hydrolysis Products by Partition Chromatography					Colour & Physical Condition	Specific Rotation at 15°C. on 0.5% solution	Viscosity at 25°C. on 0.5% solution
			G	Ga.	M	A	X			
Barley Gum 20/100	0.38	Trace	+++	-	-	-	-	White; Fibrous	-11°	288
Barley Gum 30/100+	0.037	3.54	+++	-	-	+	+	White; Fibrous	-10°	166
Barley Gum 30-40/100	0.023	18.4	+++	-	-	++	++	White; Fibrous	-36°	222
Barley Gum 40/100	0.034	56.3	+	-	-	+++	+++	White; Less Fibrous	-128°	452
Barley Gum 50/100	0.035	57.5	+	-	-	+++	+++	White; Pul-:verulent	-141°	290
Barley Gum 70/100x	0.01	47.7	+	-	++	+++	+++	White; Fibrous	-98°	172
Barley Gum Xs Acetone	0.01	6.08	+++	++	-	++	-	Buff; Pul-:verulent	-	110

+ Green colouration with iodine
x Reddish brown colouration with iodine

TABLE 15

Chemical & Physical Characters of the fractionated modified Barley Gum.

Polysaccharide as Precipitated by Ammonium Sulphate	% Yield, Moisture Ash Free	% Furfural, on Dry Ash Free Sample	Hydrolysis Products by Partition Chromato: :graphy					Colour & Physical Condition	Specific Rotation at 15°C. of 0.5% solution	Viscosity at 25°C. on 0.5% solution
			G	Ga.	M	A	X			
Modified Gum 20/100 +	0.240	Trace	+++	-	-	-	-	White; Fibrous	-10°	183
Modified Gum 30/100 +	0.073	2.5	+++	-	-	-	-	White; Fibrous	-11°	149
Modified Gum 30/40 / 100	0.045	12.0	+++	-	-	++	++	White; Fibrous	-30°	162
Modified Gum 40/100	0.075	55.5	+	-	-	+++	+++	White; Less Fibrous	-140°	286
Modified Gum 50/100 ⊠	0.023	48.2	++	-	-	+++	+++	White; Pul- :verulent	-123°	187
Modified Gum 70/100 ⊠	0.008	32.9	++	-	++	+++	+++	White; Pul- :verulent	-57°	152
Modified Gum Xs. Acetone	0.069	17.2	++	+++	-	++	++	Brown; Pul- :verulent	-	109

+ Green colouration with iodine
⊠ Reddish brown colouration with iodine

TABLE 16

Chemical & Physical Characters of the fractionated Malt Gum.

Polysaccharide as Precipitated by Ammonium Sulphate	% Yield, Moisture Ash Free	% Furfural Dry Ash Free Sample	Hydrolysis Products by Partition Chromatography				Colour; Physical Condition	Specific Rotation 0.5% sol. 15°C.	Viscosity 0.5% sol. 25°C.
			G	X	A	Ga.			
Malt Gum 30/100	0.003	-	+++	+++	+++	-	-	-	-
Malt Gum 40/100 +	0.020	40.7	++	+++	+++	-	Buff;Pulverulent	-70°	232
Malt Gum 50/100	0.075	48.4	+	+++	+++	-	White;Pulverulent	-125°	195
Malt Gum 70.100 ⍺	0.027	50.0	+	+++	+++	-	White;Pulverulent	-130°	154
Malt Gum Xs. Acetone	0.110	11.9	+++	+	+	+	Brown;Pulverulent	-	106

\+ Blue colouration with iodine
⍺ Reddish brown colouration with iodine

for detecting the nitrogen was that due to Feigl (43).

The surface tension of a 0.5% solution was measured on the fractions prepared from modified and unmodified barley. Negative results were obtained in that the surface tension of the solution was the same as that of water.

The determination of uronic acid was not carried out for two reasons. Firstly, the results of uronic acid determinations on fractions prepared earlier made it clear that carboxyl groups were present in only minute amount in the barley gums and secondly, qualitative examination of the fraction hydrolysates using filter paper partition chroma-:tography never displayed a spot in a position where it might be attributed to uronic acid.

Micro-determination of the sugars present after hydrolysis of the polysaccharide fractions.

The barley gum fractions analysed in the manner described below were prepared from modified barley, unmodified barley and malted barley.

Each fraction was hydrolysed in the usual way by refluxing 25-30 mg. of the polysaccharide with 25 ml. of N sulphuric acid for three hours. The hydrolysate was neutralised with barium carbonate.

The/

The sugars present in the hydrolysate were separated by filter paper partition chroma-:tography and the following technique was adopted:-

The width of the filter paper was $4\frac{1}{2}$", and across the centre of the paper on the starting line a band of hydrolysate measuring not more than 2" was placed. At the same time two spots of the same hydrolysate were placed on either side of the band and separated from the latter by about half an inch. The paper was dried and more hydrolysate added until the paper held enough sugar to enable fairly accurate analyses to be carried out after separation.

The paper was irrigated with the butanol-acetic acid-water mixture in a suitably enclosed chamber for three days and then removed from the chamber and dried. Two strips containing the sugars which had separated from the two outside spots were removed and developed with aniline oxalate. These strips which now displayed the constituent sugars of the hydrolysate were placed alongside the centre portion of the filter paper thus reforming the original chromatogram.

Lines were drawn across this centre portion indicating the positions to which the constituent sugars/

sugars of the hydrolysate had reached. The bands containing these sugars were marked with an appropriate symbol, removed from the centre portion of the filter paper, and one end of each strip cut to a point. The sugar present on the band was eluted with water using a similar apparatus to that employed by MacLeod (30). The elution flow was increased if two thicknesses of filter paper were placed between the glass slides. The eluted drops were collected in a small crucible. Blank strips were removed from each chromatogram and treated similarly; titre differences found in the analysis were taken into account in the determination of the sugars. The order obtained in a typical blank was that equivalent to about 0.02 mg. of glucose.

The subsequent procedure was dependent on the hydrolysate under consideration. In the case of hydrolysates from fractions M70, 70, MF, F, and the final malt fraction procedure (b) was adopted; in all other cases a straight-forward analysis could be achieved as in procedure (a).

(a) The eluted drops were diluted and transferred to a 150 ml. conical flask so that the total volume of liquid was 5 ml. The sugar present in/

in the flask was determined using the Somogyi reagent (31).

(b) Using the butanol-acetic acid-water mixture as solvent the R_F values for arabinose and mannose are practically identical and those for glucose and galactose are such that little separation could be effected within three days. Now these two pairs of sugars occur in the M70, 70, MF, F, and final malt fractions respectively. In the case of the M70 and 70 hydrolysates the band containing arabinose and mannose was eluted as before and the eluted drops concentrated to about 10/µl. This volume was placed on a second strip of filter paper and control spots of mannose and arabinose placed alongside. When this paper was irrigated in phenol-water mixture separation of arabinose and mannose was achieved. (see photograph). Thereafter the procedure for the determination of the sugars was as outlined above. In a similar manner the glucose-galactose spot from the MF, F, and final malt fractions may be separated.

From standardised curves for glucose, xylose, etc. titre differences obtained in the micro-determination were converted to the appropriate weight of glucose, xylose etc. In the Tables 18, 19, 20, the composition of the original fraction is expressed in terms of single constituent polysaccharides.

Attempted/

TABLE 18.

Percentage composition of the fractionated barley gum.

Fraction	% Yield: Ash Moisture Free	% Glucosan	% Xylan	% Araban	% Mannan	% Galactan
20	0.38	100.0	-	-	-	-
30	0.037	96.0	1.5	2.5	-	-
30-40	0.023	60.0	20.0	20.0	-	-
40	0.034	12.5	56.0	31.5	-	-
50	0.033	7.0	65.0	28.0	-	-
70	0.01	17.0	61.0	9.0	13.0	-
F	0.01	60.0	-	22.0	-	18.0

TABLE 19

Percentage composition of the fractionated modified barley gum.

Fraction	% Yield: Ash Moisture Free	% Glucosan	% Xylan	% $Arab_an$	% Mannan	% Galactan
M20	0.24	100.0	-	-	-	-
M30	0.073	96.0	1.5	2.5	-	-
M30-40	0.045	87.0	6.5	6.5	-	-
M40	0.075	14.0	53.0	33.0	-	-
M50	0.023	13.0	47.0	40.0	-	-
M70	0.008	37.0	37.0	16.0	10.0	-
MF	0.069	44.0	22.0	19.0	-	15.0

TABLE 20

Percentage composition of the fractionated malt gum

Fraction	% Yield: Ash Moisture Free	% Glucosan	% Xylan	% Araban	% Galactan
Malt 30	0.003	49.0	28.0	23.0	-
Malt 40	0.020	26.0	42.0	32.0	-
Malt 50	0.075	9.0	48.0	43.0	-
Malt 70	0.027	9.0	49.0	42.0	-
Malt F	0.110	74.0	9.0	10.0	7.0

Attempted fractionation of a water soluble barley pentosan.

Some further methods of fractionation which were tried with particular reference to a water-soluble barley pentosan will now be outlined. Without further comment at present it can be seen that although ammonium sulphate is an admirable agent for separating glucosan and pentosan material, it does not possess the property of separating the pentosan fraction itself into products structurally simpler. As material for the following investigations a product rich in pentosans, viz. the modified 40/100 fraction, was treated as follows.

(a) Using Fehling's solution:- Previous workers had shown (11) that a water-insoluble arabo-xylan prepared from esparto grass could be freed from arabinose residues by suitable treatment with Fehling's solution. The resulting xylan consisted of a singly branched molecule containing 75 D-xylopyranose units with single branching at the C_3 position.

To 100 ml. of a 1% solution of the modified 40/100 fraction was added 4 g. of sodium hydroxide followed by 100 ml. of mixed Fehling's solution. The heavy copper complex precipitate thrown down was filtered on cloth and washed several times with small quantities of distilled water. The copper complex was then /

then dissolved in the minimum of dilute hydrochloric acid and the polysaccharide precipitated by adding an equal volume of acetone. Let this product be called C_1. To the original filtrate was added just less than a $\frac{1}{2}$ volume of acetone and a second copper complex was thrown down. The latter was decomposed with the minimum of dilute hydrochloric acid and the polysaccharide precipitated by adding an equal volume of acetone - C_2.

The C_1 fraction was washed free from acid with acetone, redissolved in the minimum of water and sodium hydroxide added to 4%. The process outlined above was then repeated. The "C_2" fraction so obtained was designated D_1; a third treatment of the C_1 fraction furnished a third "C_2" fraction - D_2. The four products - original C_2, final C_1, D_1 and D_2 were recovered in the usual way and 50 mg. portions of each were hydrolysed with dilute sulphuric acid. The sugars present in the hydrolysate were detected by the method of filter paper partition chromatography and estimated qualitatively by the intensity of the developed spot. Contrary to expectation the C_1 fraction, which corresponded to the xylan of Chanda et al. (11) was found to contain virtually the same proportion of arabinose residues as the original 40/100 fraction.

In/

In fact as far as could be ascertained from the chroma-:tographic evidence, all the fractions - C_1, C_2, D_1 and D_2 appeared to be very similar as regards composition. The combined yields of the four fractions amounted to only 63% of the original material calculated on a dry weight basis (Table 21). The experiment was not carried any further along these lines.

(b) Using differential acid hydrolysis. The work of Bywater, Haworth, Hirst and Peat (33) had shown that acid hydrolysis under controlled conditions was very effective in removing arabinose residues from a xylan prepared from esparto grass. The modified barley fraction 40/100 is a simpler type of material to that examined by the above workers so it was anticipated that extremely mild hydrolysing conditions would have to be found to bring about the removal of the arabinose residues without simultaneously degrading the xylan portion of the fraction.

TABLE 21./

TABLE 21.

Recovery of the modified 40/100 fraction after treatment with Fehling's solution and acetone.

FRACTION	YIELD
M 40/100	1.00 g.
C_1	0.143 g.
C_2	0.220 g.
D_1	0.122 g.
D_2	0.141 g.
$C_1 + C_2 + D_1 + D_2$	0.626 g.

TABLE 17

Influence of reagent on percentage recovery of malt gum.

Method of recovery from extract	% Yield
Fehling's Solution + Acetone	0.84
Saturated Ammonium Sulphate	0.24[X]

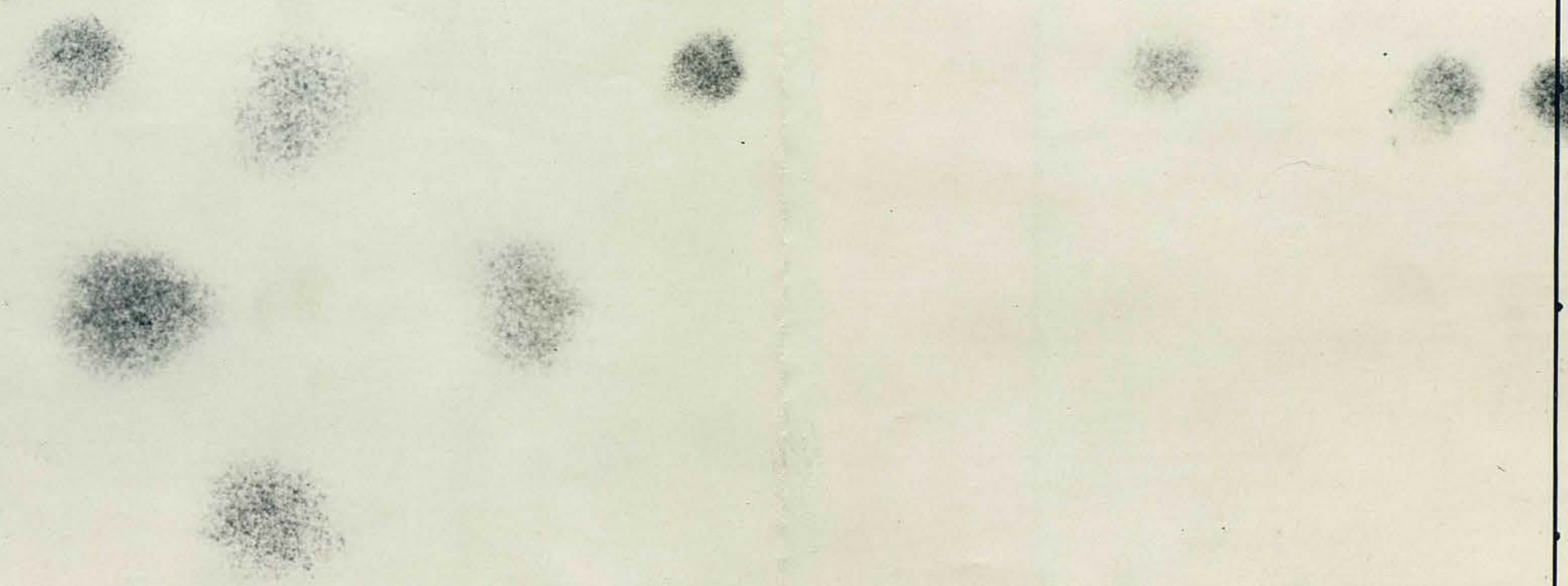
X Figure obtained by summation of % yields in Table 16.

The experimental method was as follows:- Several aqueous solutions each containing 10 mg. of the pentosan fraction were digested at 50°C. for ½ hour periods with 20 ml. portions of oxalic acid of known concentration. The treatment was repeated on fresh samples of the pentosan material using sulphuric acid as the hydrolytic agent. By varying the time of digestion, the temperature of digestion, and the strength of acid used, a series of results were obtained which are collected in Table 22. For the detection of the sugars present in the hydrolysed and unhydrolysed polysaccharide, the following technique was adopted:-

The acid solution was cooled and treated with an equal volume of ethyl alcohol when the unhydrolysed polysaccharide was precipitated. The latter was removed by centrifugation and submitted to vigorous hydrolysing conditions viz. boiling N sulphuric acid for 3 hrs. The sugars so liberated were identified by the method of filter paper partition chromatography. The acid alcohol, which contained the sugar residues split off under the mild hydrolysing conditions, was neutralised with barium carbonate and the sugars present identified in a like manner.

It was noticed in certain cases that the cooled acid solution of the partially hydrolysed polysaccharide had/


HYDROLYSATE
M40 N/100 60MIN.
GLUCOSE
XYLOSE
ARABINOSE


HYDROLYSATE
M40 N/10 10MIN.
GLUCOSE
XYLOSE
ARABINOSE

had deposited a white residue. The phenomenon was most noticeable under conditions whereby most, or all of the arabinose residues had been removed (16). Again, on certain of the chromatograms displaying the sugars obtained from the partially hydrolysed polysaccharide several pink spots were observed possessing low R_F values which could only be attributable to pentose oligosaccharides. (see photographs).

(c) Using a modified electrophoresis apparatus. A simple apparatus has recently been designed by Durrum (34) which is capable of producing on a micro-scale the same effect as that of a Tiselius Electrophoresis apparatus. The work of Isherwood (19) had revealed the possibility of separating a mixed polysaccharide into homogeneous units by means of the latter instrument and, as the simpler form of apparatus was available, it was decided to use it in an attempt to fractionate the barley pentosan.

A strip of filter paper about 2" broad was treated with an aqueous solution of the 40/100 fraction by drawing bands of liquid across the breadth, half way down the length of the paper, the technique being similar to that employed in preparing a paper for filter paper partition chromatographic analysis. The strip was soaked in a buffer solution of pH 10. The centre

of/

TABLE 22

Influence of acid concentration, etc. on decomposition of the modified 40/100 fraction

Acid used and concentration	Temperature	Time	Hydrolysis Products			Hydrolysis Products of Residue after Recovery		
			Glucose	Xylose	Arabinose	Glucose	Xylose	Arabinose
Oxalic Acid $\frac{N}{100}$	50°C	½ hr.	-	-	-	+	+++	+++
" " $\frac{N}{40}$; $\frac{N}{20}$; $\frac{N}{10}$	50°C	½ hr.	-	-	-	+	+++	+++
Sulphuric Acid $\frac{N}{100}$	50°C	½ hr.	-	-	-	+	+++	+++
" " " $\frac{N}{40}$; $\frac{N}{20}$; $\frac{N}{10}$	50°C	½ hr.	-	-	-	+	+++	+++
" " " $\frac{N}{100}$	75°C	½ hr.	-	-	x	+	+++	+++
" " " $\frac{N}{40}$	75°C	½ hr.	-	-	xx	+	+++	+++
" " " $\frac{N}{20}$	75°C	½ hr.	-	-	xxx	+	+++	++
" " " $\frac{N}{10}$	75°C	½ hr.	-	-	xxx	+	+++	++
" " " $\frac{N}{10}$	75°C	1½ hr.	-	-	xxx	+	+++	++
" " " $\frac{N}{10}$	75°C	2 hr.	-	-	xxx	+	+++	++
" " " $\frac{N}{10}$	75°C	3 hr.	-	+	+++	+	+++	+
" " " $\frac{N}{10}$	75°C	4 hr.	-	+	+++	+	+++	*
" " " $\frac{N}{10}$	80°C	1 hr.	-	-	xxx	+	+++	+
✱ " " " $\frac{N}{10}$	100°C	5 min.	-	-	xxx	+	+++	+
✱ Sulphuric Acid $\frac{N}{10}$	100°C	10 min.	-	X +	+++	+	+++	-
" " " $\frac{N}{100}$	100°C	5 min.	-	-	xx	+	+++	+++
" " " $\frac{N}{100}$	100°C	10 min.	-	-	xxx	+	+++	+++
" " " $\frac{N}{100}$	100°C	20 min.	-	-	xxx	+	+++	++
" " " $\frac{N}{100}$	100°C	30 min.	-	-	xxx	+	+++	+
✱ " " " $\frac{N}{100}$	100°C	45 min.	-	-	xxx	+	+++	+
✱ " " " $\frac{N}{100}$	100°C	1 hr.	-	X +	+++	+	+++	+

x = Trace present (only constituent) + = Trace

xx = Appreciable amount (only constituent) ++ = Minor Constituent

xxx = High concentration (only constituent) +++ = Major constituent

✱ = White deposit in acid solution of partially hydrolysed polysaccharide

X = Pentose oligosaccharides on chromatogram

of the paper was suspended over a glass rod and the free ends of the paper dipped into separate dishes each containing the buffer solution. A current of 1.5 mA was passed for four hours. The strip was dried and the position of the polysaccharide band determined by means of an ultraviolet lamp. The band was not observed either to move as a whole or to show any tendency to separate into two or more parts.

DISCUSSION.

Perhaps the most outstanding feature of the fractionated barley gums is the fact that the modified and unmodified 20/100 fractions are built solely from glucose residues. Both the chromatographic evidence and the negligible furfural content of the fractions (Tables 14 and 15) support this hypothesis. The small negative specific rotation given by aqueous solutions of these two polysaccharides suggests that the glucose residues are joined by β-linkages and the complete structure may be likened to a short chain cellulosan. The fibrous nature of the polysaccharide is in accordance with this view. It must be remembered that extractions were carried out directly at 40°C. and consequently it is no longer strictly logical to speak in terms of barley gum C_2 or B_2. However, it is clear that the glucosan fraction corresponds with what might be regarded as a pure barley gum B_2 for previous workers had/

had shown that the furfural content of the barley gum B_2 was only 4% (3).

The glucosan material is present in by far the largest quantity and represents almost 70% of the total gum recovered (unmodified) and 45% (modified). If to these figures are added the amounts of the 30/100 fractions which too are virtually pure glucosans, the figures increase to 77% (unmodified) and 59% (modified).

The advantage of retaining the so-called 30-40/100 fractions as separate entities can be seen from a glance at Tables 14 and 15. If this had not been done, material containing 25% of pentosans would have contaminated not only the 30/100 fractions but, because of the nature of the fractionation technique the 20/100 fractions also. The properties of this intermediate fraction indicate that it is probably composed of a physical mixture of the two types of material viz. glucosan and pentosan.

It may be observed that the 40/100 fractions from all three sources although mainly pentosan in nature invariably contain a percentage of glucosan material. Quantitative estimation of the hexosan content of the 40/100 fractions puts the figure at 12.5% (unmodified), 14.0% (modified), and 26.0% (malt). It is not likely that/

that the glucose residues are linked by normal chemical bonds to the pentosan; rather is it thought that the occurrence of glucose residues in the 40/100 fraction implies the presence of a short chain glucosan which is structurally simpler than the 20/100 and 30/100 fractions and is not precipitated by low concentrations of ammonium sulphate. In other words, if a suitable fractionating agent could be found, the glucosan material could be separated by physical means from the main constituent of the 40/100 fractions. The appearance of glucose residues in later fractions may be attributed to the occurrence of still simpler glucosan material. On the other hand, traces of soluble starch dextrins could account for the appearance of glucose residues in the 50/100 and 70/100 fractions, as reddish brown colourations were sometimes observed when aqueous solutions of these fractions were treated with iodine (Tables 14, 15 and 16). Broadly speaking the 50/100 and 70/100 fractions from all three sources may be regarded to bear much the same relation to the 40/100 fractions as the 30/100 fractions bear to the main glucosan fraction.

Additional evidence for the hypothesis that there is present in the barley and malt gums two distinct types of polysaccharide material - glucosan and pentosan each/

each capable of existing at several degrees of molecular aggregation, is supplied by the viscosity measurements summarised in Tables 14 and 15. The lower viscosity of the 30/100 fractions as compared with the corresponding 20/100 fractions and a similar state of affairs with the pentosan fractions is felt to be in line with the above hypothesis. Another interesting aspect of viscosity measurements lies in the comparison of the values obtained from corresponding fractions of modified and unmodified barley and it may be seen that in every case the viscosity of solutions prepared from the fractionated unmodified barley are higher than those of the modified series. A probable explanation to account for these viscosity changes has been outlined before but a more detailed statement must await further experimental data to be given in a later section. In particular, the unmodified 40/100 fraction has an extremely high viscosity (452, water = 100) whereas the viscosity of the corresponding modified and malt fractions has been more than halved. As a practical application of these facts it can be seen that pentosan material will only slightly influence wort viscosity if such wort has been prepared solely from malted barley. If however, unmalted adjuncts such as/

as barley flakes which, by virtue of their mode of preparation will have no enzyme activity, are added to the mash tun, extracts containing this highly viscous principle will be incorporated in the wort. The desirability or otherwise of such a state of affairs is outwith the scope of the present investigation.

The absence of nitrogen from the fractionated barley and malt polysaccharides is highly significant. The products obtained by Meredith et al.(14) contained considerable quantities of nitrogen although its presence could be eliminated by treatment with alkali. These workers were unable to decide whether such nitrogen was associated chemically or not with the polysaccharide, but the absence of nitrogen in the fractionated products strongly supports the view that nitrogen if present is merely an impurity.

An examination of the characters of the fractionated malted barley displays some interesting features. The main glucosan fraction so characteristic of barley has disappeared and hexosan material appears only in the later fractions. The malt fraction 30/100 although containing 50% glucosan (Table 20) is obtained in such small yield that it may be ignored as contri-:buting greatly to the glucosan content of the malt hemicellulose dextrins. However, as was pointed out in/

in a previous discussion the low yields are not due to lack of water soluble material for Table 17 indicates that the amount of malt gum available is of the order 0.84%. The recovery of workable quantities of the malt fractions depends on the extraction of a large amount of malt and restricting the number of precipitations with ammonium sulphate. If such precipitations are continued, the utility of the fractionation is outweighed by the considerable loss of material.

As there is no comparable fraction to the 20/100 fraction of barley present in malt, the fraction-:ation technique is useless for preparing a polysaccharide which will yield on hydrolysis one sugar unit only. The value of such a fractionation lies in the compari-:sons which can be made between corresponding fractions prepared from unmodified, modified, and malted barley and several such comparisons have already been made. It was unnecessary to retain separately a 30-40/100 fraction for obvious reasons. It is rather significant that the recovery of the fractionated malt gums is only about 30% of that potentially available and that this figure includes the large amount of the final fraction composed of less interesting material. Again, in the malt fractions there is the possibility of contamination by traces of starch dextrins in view of the iodine colourations obtained with certain of the solutions.

The presence of mannose residues has been

confirmed/

confirmed in the penultimate fractions prepared from modified and unmodified barley. However, this sugar unit is no longer present in the malt fractions but this is perhaps not surprising as the amount of mannan material in the unmodified barley is only 0.0013% which falls to 0.0008% in the modified barley. Galactose is present in all three final fractions but these fractions were always the least interesting. The specific rotation of their solutions could not be determined because of the large amount of colouring matter which was always present and it is interesting to note that it was these fractions which had a fairly high ash content ca.10% (not quoted in Tables). Whether the ash was present as an integral part of the structure or merely due to inorganic impurity is not clear. The ash was not examined for its chemical nature.

An examination of the Tables 18,19, and 20 shows that in the main the analytical figures confirm the amounts of furfural obtainable from each fraction. More significant is the fact that as the series is ascended the proportion of araban relative to the xylan becomes less and less. According to Perlin (16) the most soluble arabo-xylan should contain the highest proportion of arabinose residues and as the 70/100 fractions are certainly the most soluble, the figures quoted in the above Tables would appear to be contrary to Perlin's views, although strict analogy between

wheat/

wheat and barley cannot of course be made. It may be that in these barley pentosans there are three types of structure viz. an arabo-xylan similar to that visualized by Perlin, a homogenous xylan, and a homo-:geneous araban and that the fractionated polysaccharides 40/100, 50/100 and 70/100 consist of physical mixtures of all three types in varying proportions.

The discussion may now be conveniently brought to bear on an examination of the data summarised in Tables 21 and 22.

The results of the attempted fractionation using Fehling's solution are not very helpful in deciding whether the pentosan fraction is an arabo-xylan, a physical mixture of a xylan and araban, or a combination of the two possibilities. No change in the relative amounts of the two pentose sugars was observed and although it is not true to say that no such change had occurred, as quantitative estimation of the sugars present was not carried out, at least no significant change was noticed. The production of the fractions designated D_1 and D_2 is probably due to the fact that in the re-solution of the fraction C_1 progressively more dilute solutions were obtained and an important factor in precipitations with Fehling's solution is the concentration of the polysaccharide solution. The recovery of only 63% of the starting material, however, suggests degradation of the product at some step in the fractionation.

Some/

Some workers are reluctant to lay this charge against alkali and it may be that solution of the product in acid at certain stages induces degradation.

Much more interesting results can be observed from the differential acid hydrolysis experiments. It must be emphasised that the experimental procedure was essentially of a preliminary nature but Table 22 should provide a valuable stepping stone for any future work designed to enable a pure xylan to be isolated from the barley pentosans and related materials. It is worth bearing in mind that whereas fractionation with ammonium sulphate could have effected little change in the structure of the barley and malt gums, a method using acid hydrolysis immediately produces material considerably different from that in situ and in addition, even although one compound may be recoverable - the xylan, the nature of the araban remains obscure.

At the outset it was clear that the arabinose residues were much more freely liberated than those of xylose and in fact before the latter sugar could be detected on a paper chromatogram, comparatively strong hydrolysing conditions had to be employed. Ten minutes boiling with N/10 sulphuric acid had removed virtually all the arabinose residues leaving the xylan portion practically untouched although even under these conditions the xylan is becoming degraded and this fact is underlined by the appearance on the chromatograms prepared from the partially hydrolysed polysaccharides of/

of pink spots with low R_F values which are most easily attributable to pentose oligosaccharides.

A ten fold dilution of the acid produced a more even breakdown and it required 1 hour at 100^0 C. with N/100 sulphuric acid to produce approximately the same result. The trace of glucosan material which is present in the modified 40/100 fractions is remarkably stable in that even under the most vigorous hydrolysing conditions employed, glucose residues were not detected in the partially hydrolysed polysaccharide.

The white deposit in the partially hydrolysed solution referred to in the experimental section is in direct agreement with Perlin's findings and he puts this phenomenon forward as providing important analytical evidence for his structure of the arabo-xylan of wheat flour. However, before the structure of the arabo-xylan of barley can be fully worked out, a methylation technique similar to that carried out by the above worker may have to be attempted as insufficient evidence is available at present.

Finally a word on the attempted fractionation of the modified fraction 40/100 using the micro-electro-:phoresis apparatus. The indications are that the polysaccharide not only is incapable of being separated under the conditions specified, but also that it possesses no electric charge and hence no movement, even as an inseparable band, can occur.

The Barley Polysaccharides of O'Sullivan in the light of more recent work.

It is convenient at this point in view of the analytical data presented in the preceding pages to re-:examine the findings of O'Sullivan (1) who in 1882 prepared two water soluble polysaccharides from barley which he named β-amylan (soluble at room temperature) and α-amylan (soluble at 40°C.).

An interesting question arises. How was it that O'Sullivan did not detect the presence of xylose and arabinose in these polysaccharides, especially in β-amylan which may contain 40-50% of mixed pentose residues?

The methods of preparation of α and β-amylan by O'Sullivan (loc cit.), by Preece and Ashworth (loc.cit.) and in this investigation do not differ fundamentally in procedure, so that analytical figures obtained by all three sets of workers may be taken as comparable. In this review, in order to facilitate comparison, all specific rotations have been returned as $[\alpha]_D$ although in O'Sullivan's original work he used the expression $[\alpha]_J$ as a measure of optical activity. The relation-:ship between these two expressions is $[\alpha]_J = [\alpha]_D \times 1.111$.

The β-amylan of O'Sullivan was found to give a specific rotation of $[\alpha]_D = -67^\circ$. It has now been shown (Table 14) that these barley polysaccharides consist mainly of a glucosan with an $[\alpha]_D$ of -10° and

a/

a mixed pentosan with $[\alpha]_D$ of - 130°. These two fractions constitute not less than 80% of the total yield of water soluble barley polysaccharides. The mean value of these two specific rotations is -70° which is close to that obtained by O'Sullivan. A comparison in this way is justified when it is remembered that β -amylan consists of approximately 50% glucosan and 50% pentosan.

A second interesting coincidence is seen when the specific rotation of the β -amylan is examined after hydrolysis. The figure quoted by O'Sullivan is $[\alpha]_D = +51°$. As the specific rotation of D-glucose is +52.5° O'Sullivan assumed that he was dealing with a pure glucosan. L-Arabinose and D-xylose have specific rotations of $[\alpha]_D = +106°$ and +18° respectively and the mean value of these figures is +62°. It can therefore be seen that although we have a polysaccharide which on hydrolysis gives a specific rotation of $[\alpha]_D = +51°$ it does not necessarily follow that it is a pure glucosan as a solution containing equal amounts of xylose, arabinose, and glucose will show $[\alpha]_D = +59°$ and if it so happens that the percentage of xylose is slightly greater than that of the other two sugars (Table 18), the figure obtained will be even closer to that quoted by O'Sullivan.

The same type of argument can be applied to α -amylan. This body contains about 10% mixed pentosan and the figures obtained by O'Sullivan before and after hydrolysis were $[\alpha]_D =$ -22° and +50° respectively. Using the more recent data the specific rotation/

rotation of the mixed polysaccharide becomes $[\alpha]_D = -22^\circ$ and that of the hydrolysate $[\alpha]_D = +53^\circ$ assuming that we are dealing with a polysaccharide composed of 90% glucosan and 10% mixed pentosan.

It can be seen therefore that the specific rotation figures obtained by O'Sullivan are purely fortuitous in that they appear to show the presence of a pure glucosan. The more recent data show that the presence of pentosan can be accounted for, even although at first sight the polysaccharide appears to consist of one sugar unit only.

It is interesting to note that O'Sullivan attempted a fractionation of his α and β-amylan by precipitating the polysaccharides from solution with increasing concentrations of alcohol. From the α-amylan he obtained two fractions which showed the same optical activity as the original polysaccharide. He inferred from this fact that he was dealing with a simple substance. As the α-amylan contains not more than 10% pentosan and one fractionation only with alcohol was effected, it is perhaps not surprising that no difference in specific rotation was found. However, O'Sullivan observed that β-amylan could be separated with alcohol into two fractions yielding specific rotations of $[\alpha]_D = -131^\circ$ and $[\alpha]_D = -65^\circ$. As more recent work has shown, this former reading indicates that O'Sullivan had in his hands a polysaccharide substantially free from glucosan material. It is significant/

significant that he apparently made no attempt to hydrolyse this component or it is probable that he would have detected the presence of arabinose and xylose.

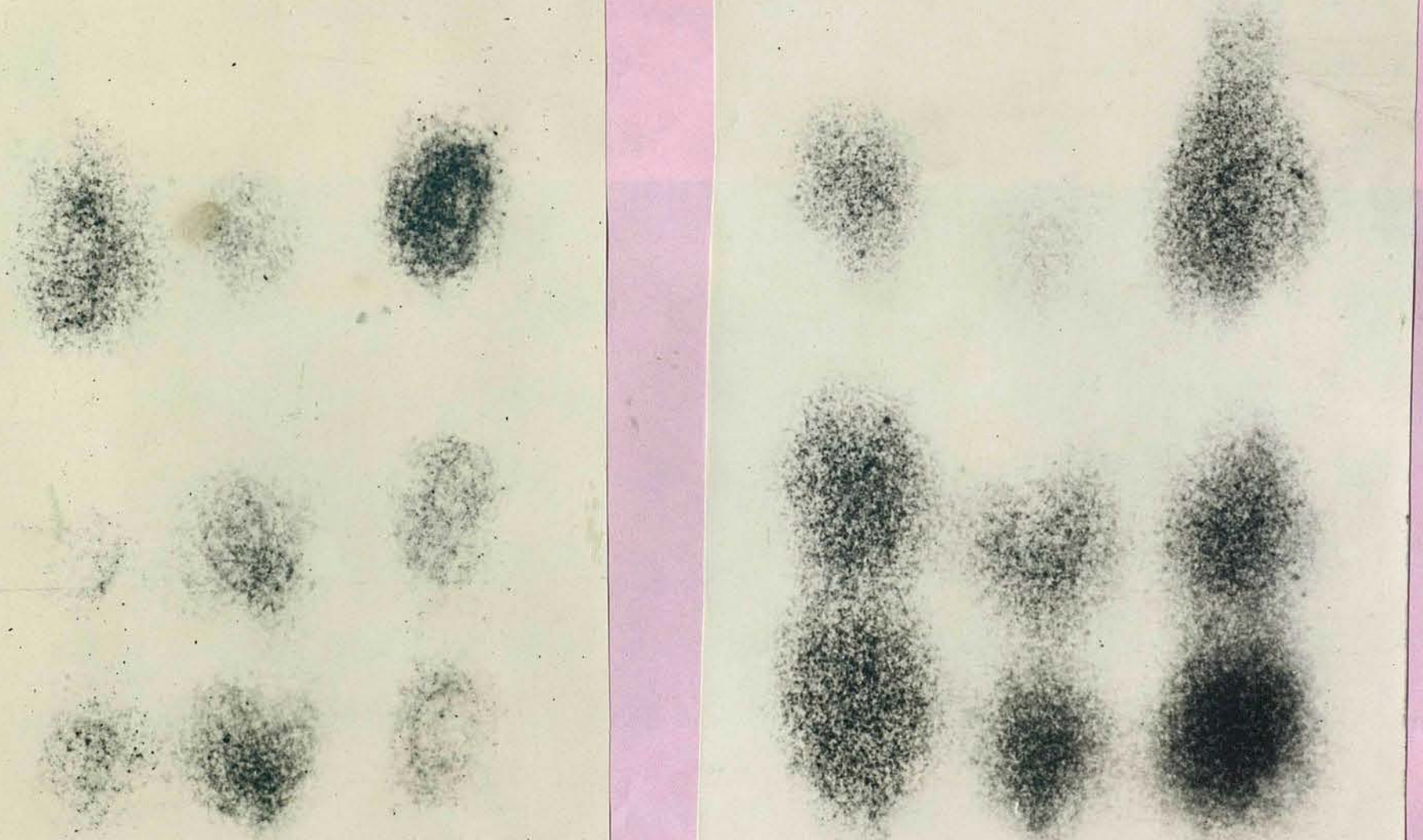
O'Sullivan also found that his hydrolysates from α-and β-amylan differed in their ability to cry-:stallise. The α-amylan, he found, gave a hydroly-:sate which crystallised within a few days whereas the β-amylan hydrolysate did not give crystals even on long standing and remained as a syrup. Such a differ-:ence might have suggested that the two amylans differed fundamentally in composition so that the uncrystallisable hydrolysate contained units other than glucose.

Attention may be drawn to one further point. The β-amylan submitted to combustion gave C = 44.35% and H = 6.28% which was close enough to the % composi-:tion of starch (C = 44.44 H = 6.17) for O'Sullivan to suggest that he was dealing with a pure glucosan. Theoretically, however, the carbon figure is rather low if it is assumed that β-amylan contains 40% pentosan material. Such a mixture requires C = 44.8%. However, as this difference is within the limits of experimental error it is perhaps not surprising that the existence of pentose residues was not suspected by O'Sullivan.

It may be mentioned in support of the above arguments that samples of O'Sullivan's original α-and β-amylan have been made available by courtesy of Mr. J.H. St. Johnston of Bass, Ratcliff and Cretton Ltd., Burton-on-Trent and partition chromatographic analysis of/

α-amylan | Glucose, Arabinose, Xylose | Barley Gum B_2

β-amylan | Glucose, Arabinose, Xylose | Barley Gum C_2



of the hydrolysates obtained from the amylans reveals the presence of arabinose and xylose in similar quantity to that obtained from more recent preparations. (See photographs).

It can therefore be seen that the criteria which O'Sullivan chose for characterising polysaccharides were unfortunate in that they would also apply superficially to the facts as known to-day. That O'Sullivan did not detect the presence of pentose residues can be simply explained by the fact that his evidence did not, for reasons which were largely coincidental, imply the presence of pentosan material; hence such material was not looked for.

THE SOURCE OR LOCALISATION OF THE NON-STARCHY WATER SOLUBLE POLYSACCHARIDES OF BARLEY AND WHEAT.

INTRODUCTION.

Of the several groups of workers who have provided evidence for or against a particular part of the cereal grain as being the source of the water soluble polysaccharides, perhaps only Enders et al.(12) attacked the problem mainly from this point of view. These workers stated that 1/6 of the total soluble pentosan material of barley was to be found in the husk and the remaining 5/6 in the endosperm; a similar state of affairs existed in malted barley. The source of the laevorotatory glucosan, which, in barley is present in appreciable amounts, was obscure.

Other workers (16, 35,36) have provided evidence that the soluble pentosans of wheat are present in the endosperm. The importance of the glucosan material referred to above which is present also in maize (37), and to a lesser extent in wheat (38), has until recently been ignored, possibly because in industry the presence of pentosans appeared to have adverse effects on certain commercial products (36) and hence from the academic point of view the importance of the pentosans was exaggerated.

A final statement regarding the source of these/

these materials could most easily be made if workable amounts of the separated cereals could be obtained and analysed for their cereal gum content. Fortunately, commercial samples of separated wheat flour, germ and bran were made available through the courtesy of Mr. R. T. Potter of R. Hutchison & Co.Ltd., Millers, Kirkcaldy. A different problem was presented with barley as similar samples were not available. However, as will be described in the experimental section a sample of barley was subjected to a pearling process and the various "rubbings" so obtained were analysed.

EXPERIMENTAL.

1. Source of the Barley Gums.

(a) Preparation of barley husk; the presence or absence of water soluble polysaccharides in the husk.

The problem of separating the husk from the remainder of the grain proved simpler than was at first expected. It was decided at the outset to avoid the use of solvents wherever possible and instead to use a mechanical method. By trial and error an apparatus was constructed which if worked carefully was capable of giving a remarkably good separation of the husk from the endosperm and germ.

The method was as follows:- 200 g. of Pioneer Barley was fairly finely ground and then sieved through two meshes (10/in.,20/in.). The coarsest sample contained most/

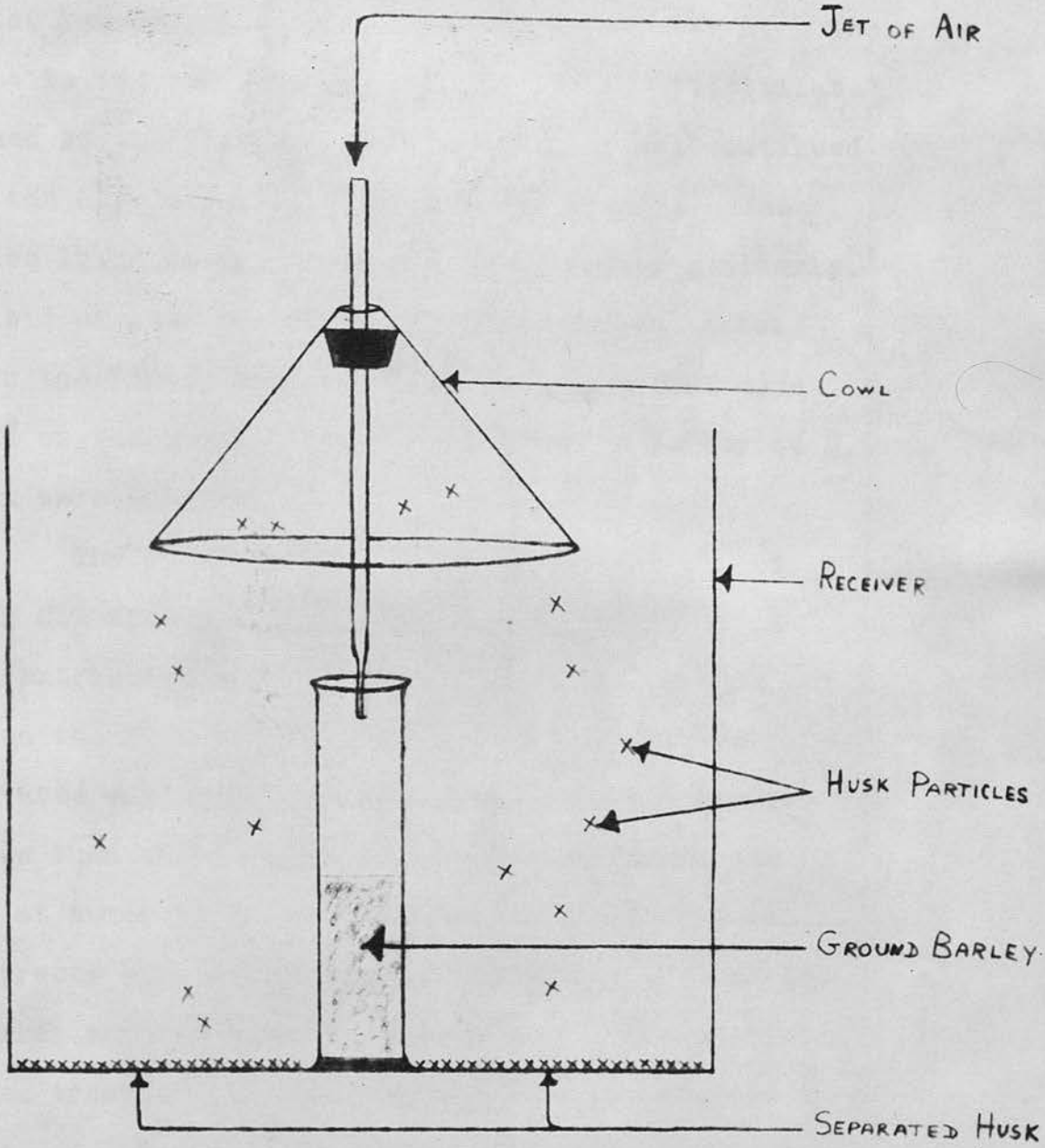


Diagram I

Apparatus For Preparation Of Barley Husk

most of the husk and was transferred to a glass cylinder (see Diagram I). When a jet of air was blown down the cylinder the lighter husk particles were blown over into the receiver along with some germ and starch. The residue in the cylinder was removed and the "distillate" returned to the cylinder when the blowing was continued until the husk was free from germ and starch. The fraction lying on the finer mesh was treated similarly. The yield of husk was about 5% of the grain; total husk in the barley examined could not have been more than 7% of the grain. From a kilogram of barley 54 g. of husk were obtained.

The yield of husk was added to 250 ml. of boiling 80% alcohol and extracted for a ½ hour. A second extraction with boiling alcohol was carried out and then the husk was air dried. This procedure inactivated any enzyme systems present in the husk. The husk was then thrice extracted with three times its weight of water at room temperature for ½ hour periods. The extracts were combined, concentrated and clarified by passing through a bed of kieselguhr. The extract was then treated with ammonium sulphate as outlined on page 24 . However, no precipitates were obtained at concentrations of 20, 30, 40, 70 g. ammonium sulphate/100 ml. extract. At 50 g. ammonium sulphate/100 ml. extract/

extract a slight precipitate (unworkable) was obtained. Dialysis of the mother liquor followed by addition of excess acetone failed to give any appreciable product. It was noticeable that the viscosity of the extract before the addition of ammonium sulphate was low.

A further 1000 g. of barley was dehusked in the manner described above and the husk so obtained was finely ground (to ensure maximum penetration). The extraction process was repeated but again no workable yields were obtained.

(b) Examination of fractions of barley obtained by a pearling process for the barley gum content.

Although the absence of precipitable water soluble material in barley husk was important it was a negative result in that the actual source of the gums was still undetermined. No simple method of isolating pure barley germ or endosperm presented itself but through the courtesy of Mr. Sloan of the S.C.W.S. laboratories, Leith, samples of fractionated Spratt-Archer barley were made available. In the manufacture of Pearl Barley the whole corn is subjected to what may be described as an abrasive action whereby the outer coatings (husk, aleurone layer, etc.) are gradually removed leaving finally the Pearl which is virtually pure endosperm. Samples of the "rubbings" taken at certain time intervals of the pearling process were analysed/

analysed for the barley gum content. The first fraction consisted mainly of husk, the second, husk, aleurone layer and traces of endosperm, the remaining fractions contained increasing amounts of flour and the sixth fraction was the Pearl Barley. A sample of unpearled Spratt-Archer was also treated in the manner described below.

The procedure was to inactivate in the usual way known weights (ca. 50 g.) of each fraction with 150 ml. boiling 80% alcohol, air dry, and extract three times with thrice its weight of water at 40°C. The extracts were clarified by passing through a bed of kieselguhr, combined and concentrated to 150 ml. The coagulated protein and other material which had been precipitated during the concentration was left in the solution to which 6 g. of sodium hydroxide was now added (4%). An equal volume of Fehling's solution followed by just less than a $\frac{1}{2}$ volume of acetone (120 ml.) precipitated the gum as the copper complex. The latter was centrifuged off, dissolved in the minimum of cold dilute hydrochloric acid and any material remaining undissolved was filtered off. The polysaccharide in solution was precipitated by adding an equal volume of acetone whereafter it was washed free from acid with aqueous acetone and then taken to dryness in the usual way with increasing concentrations of alcohol. The products were then powdered and weighed. It was noticeable

that/

that the dried products were not easily soluble in water even in the hot and it appeared that a retrogradation effect had occurred. This fact will be commented on in the appropriate discussion section.

The results of the experiment which give the yields of gum obtained from the various fractions and which are expressed as a percentage of the whole grain are collected in Table 23. It will be observed that the sum of the yields obtained from the fractions is considerably higher than that obtained from a direct extraction of the unpearled barley. Contamination of the gums by starch dextrins was suspected and this fact was confirmed by the intense blue colourations which certain of the products gave with iodine (Table 23). The high percentage of glucosan material in the gums was evinced when samples were hydrolysed and the sugars present, (glucose, arabinose and xylose), after separation on the paper chromatogram, estimated by means of the Somogyi reagent (31). The results are not quoted but the amount of glucose varied from 78 - 90%.

2. Source of the Wheat Gums.

When the experiments to be outlined below were carried out it was still thought advisable to retain the nomenclature of Norris and Preece (4) to describe the products and consequently the wheat gums C_2 and B_2 were separately prepared. As will be shown, recovery of the/

the gums from the extracts was effected (a) with Fehling's solution and acetone, and (b) with saturated ammonium sulphate. In this way a further comparison of the efficiency of the two methods could be made.

In all, five wheat fractions and the whole grain were analysed for the wheat gum content. The fractions were, the wheat germ representing 2% of the whole grain, the wheat bran representing 9.75%, the parings representing 7.25%, and the winter flour represent-:ing 81% of the whole grain. It is seen that the per-:centage sum of these four fractions is equivalent to the total wheat. The other fraction examined was a sample of C flour which is the purest form of endosperm obtainable in a modern Mill. The quantities used were - 100 g. bran, parings and germ, 200 g. winter flour, C flour and the whole grain.

Each fraction was enzyme inactivated by treating with twice its weight of boiling 80% alcohol for two $\frac{1}{2}$ hr. periods. In addition,the germ fraction was extracted in a soxhlet apparatus with petroleum ether to remove germ oil. The residues after being air dried were thrice extracted with water at 20°C. in the usual manner. The extracts were filtered bright through kieselguhr and concentrated to about 250 ml. when after a second filtration through kieselguhr the concentrates were made up to 300 ml. with water and then divided into two equal portions. Meanwhile three

further/

further extractions were made on the residue at 40°C. thus removing the wheat gum B_2 from the fractions. The extracts so obtained were similarly treated and divided likewise into two portions.

The water soluble material present in the extracts was recovered by the following methods:-

(a) The 150 ml. of extract was treated with Fehling's solution and acetone in exactly the same manner as described on page 97 for the isolation of the barley gum.

(b) To the second 150 ml. of solution was added ammonium sulphate to saturation (ca. 120 g.) and after stirring vigorously the precipitated wheat gum was centrifuged off, care being taken to remove any solid ammonium sulphate present. The gum was then dissolved in warm water and reprecipitated with an equal volume of acetone. The solution was centrifuged, the gum redissolved in hot water and the solution dialysed to remove traces of ammonium sulphate. Finally the wheat gum was recovered by precipitating with an equal volume of acetone and drying in the usual way when it was powdered and weighed.

The furfural content and hydrolysis products of the gums obtained from several of the fractions were determined (Table 24).

The products were found to be difficultly

soluble/

TABLE 23

Percentage Yield etc. of the gum obtained from the pearl barley and "rubbings".

		Preparation I			Preparation II			
Fraction	Time in Pearling Machine	% Yield of fraction on Whole Barley	Actual Yield Gum/100 g.fraction	% Yield Gum on Whole Grain	% Yield of fraction on Whole Barley	Actual Yield Gum/100 g.fraction	% Yield Gum on Whole Grain	Colouration with Iodine
1	25 sec.	11.5	0.20 g.	0.02	11.0	0.16 g.	0.02	Faint Purple
2	22 sec.	3.9	2.86 g.	0.11	3.9	1.65 g.	0.06	Faint Blue
3	60 sec.	10.9	4.23 g.	0.47	12.9	2.45 g.	0.32	Intense Blue
4	75 sec.	14.6	1.26 g.	0.18	19.8	2.62 g.	0.52	Intense Blue
5	120 sec.	15.2	3.37 g.	0.54	14.5	3.80 g.	0.55	Intense Blue
Pearl		43.9	0.84 g.	0.29	37.9	0.90 g.	0.34	Blue
Sum	-	100.0	-	1.61	100.0	-	1.81	
Whole Barley		100.0	0.64 g.	0.65 g.				

TABLE 24

Some characters of the wheat gums prepared from various parts of the grain.

Fraction	Method of Recovery	% Furfural	Hydrolysis Products		
			Glucose	Xylose	Arabinose
Whole Grain C_2	Fehling's Solution	50.4	+	+++	+++
Whole Grain B_2	" "	26.0	+++	+++	+++
C Flour C_2	" "	31.0	++	+++	+++
C Flour B_2	" "	8.65	+++	++	++
Winter Flour C_2	" "	36.2			
Winter Flour B_2	" "	23.4			
Bran C_2	" "	40.6			
Bran B_2	" "	23.4			
Parings C_2	" "	49.8			
Parings B_2	" "	20.8			
Germ C_2	" "	32.5			
Germ B_2	" "	23.7			

TABLE 25.

Percentage yields etc. of the Wheat gum obtained from the separated Wheat; recovery using Fehling's solution and acetone or saturated ammonium sulphate.

Test Material	% of whole grain represented ‡	Recovery by Fehling's solution				Recovery by Ammonium Sulphate			
		C_2 % yield portion	B_2 % yield portion	C_2 % yield whole grain	B_2 % yield whole grain	C_2 % yield portion	B_2 % yield portion	C_2 % yield whole grain	B_2 % yield whole grain
Whole Grain	100	0.44	0.52	0.44	0.52	0.37	0.38	0.37	0.38
Winter Flour	81	0.52	0.70	0.42	0.57	0.49	0.47	0.40	0.38
Bran	9.75	0.22	0.30	0.02	0.03	0.18	0.19	0.02	0.02
Parings	7.25	0.46	0.64	0.03	0.05	0.42	0.58	0.03	0.04
Germ	2	0.40	0.14	0.01	0.003	0.26	0.15	0.005	0.003
C Flour	-	0.69	0.35	-	-	0.43	0.23	-	-

‡ Figures kindly supplied by R. T. Potter of R. Hutchison & Co.Ltd., Millers, Kirkcaldy.

soluble in water yielding colloidal viscous solutions and with iodine they gave a faint blue colour which faded after standing for 15 minutes.

The percentage yields of the various products have been collected in Table 25; the figures have been corrected for moisture and ash.

DISCUSSION

In view of the conflicting evidence presented in the experimental section it would be unwise to make dogmatic statements regarding the source of the barley and wheat gums. However, certain interesting facts have come to light and may be commented on.

It is at least certain that barley husk is not the source of the gum like materials as no workable products were obtained when the husk was extracted in the usual way. This fact is perhaps not surprising when it is considered that during germination it is the endosperm which undergoes modification and as the barley gums and related hemicellulosic materials are modified in the process of germination they are most likely to be found near to or in the endosperm.

It would be expected therefore that the samples of pearled barley and "rubbings" which were made available would in fact clinch this problem as analysis could be made on selected "rubbings" of the barley corn and also on the Pearl Barley itself which is virtually/

virtually pure endosperm. Unfortunately the products obtained from the various fractions contained large amounts of soluble starch as can be seen by the fact that the sum of the percentage yields of gum prepared from the six fractions exceeds that of a direct preparation from unpearled barley by about three times (Table 23). How has this anomaly arisen? An explanation may be found by an examination of the findings of Lampitt, Fuller and Goldenberg (39) who, in 1941, published a series of papers on the properties of wheat starch when subjected to grinding for prolonged periods. These workers found that wheat starch gave fractions soluble in cold water and in hot water, and such solutions gave the usual intense blue colouration with iodine. They also found that on drying with alcohol, their fractions suffered retrogradation (40), and became only partially soluble in water. These features were found to be characteristic of the products extracted from the pearled barley and "rubbings" so it may well be that during the pearling process sufficient degradation of the starch molecules occurs to furnish appreciable quantities of soluble starch. This may or may not be the explanation but the fact that contamination has occurred, nullifies any rigorous conclusions that may be drawn as to the source of the gum-like materials.

Although/

Although it is likely that the barley gums are confined to the endosperm, whether they are present in the outermost layers of same, or distributed throughout the whole endosperm remains at present unsolved.

A further problem arises. The fractionation of barley gum extracts with ammonium sulphate reveals the presence of two physically separable non-starchy water-soluble polysaccharides. Do the two types of material - glucosan and pentosan have a common source or do they exist in different parts of the barley grain? It may be recalled from the work of Enders, Saji and Schneebauer (12) that these workers postulated a dynamic equilibrium between the insoluble pentosans of barley husk and the water soluble pentosans. To enable such transformations to be carried out easily, it would be most convenient if the soluble pentosan material lay close to the husk - either in the aleurone layer or the outermost part of the endosperm. On the other hand the glucosan material which disappears to a large extent on malting may be intimately connected with the inner endosperm as it is this part of the grain which undergoes the most substantial modification.

It must be emphasised that although the evidence supporting the above speculations is scanty, any reasonable hypothesis is better than none at all and it/

it is going to be an extremely difficult task to obtain further experimental evidence which will either substantiate or invalidate the above theory.

Turning now to an examination of properties of gums prepared from the various parts of the separated wheat grain, it can be seen from Table 25 that the combined yields of gum recovered from the fractions is substantially the same as that obtained from the whole grain. Contamination by soluble starch in this case would be surprising as the workers referred to previously (39,40) found that degradation of the wheat starch occurred only after a minimum of 500 hours grinding.

The general properties of the wheat gums are in agreement with those obtained by Pence, Elder and Mecham (35). Although these workers used different methods for extraction their yields were in the range 0.5 - 0.8% of the flour examined agreeing with the values of 0.5 - 0.7% obtained from winter flour. The pentosan content of the gum according to Pence et al. was of the order of 75%, again similar to that obtained in the above experiments. It is noticeable that this figure is much higher than that obtained from a similar determination on gums derived from barley, about 45% for barley gum C_2 and 8% for barley gum B_2 (3). The dangers of arguing directly from one cereal to another are here underlined,/

underlined, as the experimental results indicate that even between similar types of material in the two grains, wide differences in properties can occur.

As to the source of the wheat gums it is fairly clear after examining Table 25 that the winter flour is the only part of the wheat grain which supplies an amount of wheat gum equivalent to what would be obtained from the total wheat. It must be remembered that although the yield of gum C_2 from the parings is 0.48%, when the fraction of the total wheat that the parings represents is taken into account, the figure comes down to that of 0.03%.

The important point is that recovery from the winter flour fraction - representing the endosperm materials - is 86 - 88% of the total recovery of the cereal gums from the whole grain. It may also be noted that C flour representing the purest form of endosperm available gives the highest yield of any of the fractions. It is perhaps natural that the parings should contain some water soluble polysaccharides, as it represents the aleurone layer and that part of the wheat grain closest to the endosperm. Mechanical separation is bound to lead to some contamination of the parings with endosperm material.

In this investigation there was no equivalent fraction to barley husk as the miller receives the wheat grain without this fraction. In the absence of signi-

:ficant amounts of wheat gum in the embryo it is tempting/

tempting to suggest by analogy that barley germ too is devoid of this type of material thus further supporting the view that the gums are confined in the main to the endosperm.

As to the efficiency of the two methods of recovery, Table 25 shows that in every case recovery using Fehling's solution and acetone was the higher. Further evidence is thus given for the view that the latter reagent has a property of precipitating low molecular weight polysaccharides which is not possessed by ammonium sulphate. An important line for future work would be to seek means of recovering those materials which are precipitated by Fehling's solution but which are lost by the ammonium sulphate fraction: :ation. The loss of this type of material appears to be dependent on the extent of modification of the barley corn (see Tables 13 and 17), and although such material must have a small effect on the viscosity of brewery wort, the inevitable presence of these low molecular weight fractions in the latter medium implies that the study of their properties would be of more than academic interest.

OBSERVATIONS ON THE ACTION OF A CYTOCLASTIC BARLEY ENZYME

INTRODUCTION.

In the general introduction, some of the literature pertaining to the enzyme or enzyme systems which hydrolyse cell-wall materials was reviewed and although in the main our knowledge of such processes is scanty and empirical, recent investigations (20) have shown the presence in barley of two cell-wall hydrolysing enzyme systems viz:-

(a) A cytoclastic system present in raw barley capable of simplifying pre-formed water soluble non-starchy polysaccharides and also of rendering soluble initially insoluble hemicellulosic material.

(b) A cytolytic system first formed in substantial amount only after steeping and growth which exerts a saccharifying action and brings about a more profound hydrolysis of the substrates.

The purpose of the experimental work to be described below was two-fold. In the first place for reasons which have been stated elsewhere, it was hoped to evolve a method whereby the yields of the water soluble non-starchy barley polysaccharides could be substantially increased by the judicious use of the aforesaid cytoclastic enzyme. Secondly, as such a method would arise only by trial and error, much information on the nature of the cytoclastic enzyme action would be forth-

:coming/

coming, which in view of the paucity of such information, would stimulate further investigation.

In a previous experimental section (page 38) certain conditions were specified which enabled an extract of modified barley to be made. It was as a result of the following work that optimum conditions for increased yield of the barley gums could be stated.

EXPERIMENTAL.

A barley extract representing 10% of the barley used as substrate was prepared by extracting 20 g. of ground barley with 600 ml. of distilled water at 20°C. overnight. The extract was centrifuged and 300 ml. of the clear extract (≡ 10 g. of barley) was added to 100 g. of enzyme inactivated barley and the mixture was allowed to stand with occasional stirring for a ½ hr. at 20°C. The mixture was centrifuged and the centrifugate passed through a bed of kieselguhr as quickly as possible. The filtrate was then boiled for two minutes to inhibit further enzyme action. Meanwhile the residue was re-extracted for a ½ hr. with 300 ml. of distilled water again at 20°C. recentrifuged, refiltered and the residue extracted again with a third quantity of 300 ml. of water for a similar period. The second and/

and third filtrates were also boiled for two minutes.

The combined filtrates were then concentrated to 100 ml. and centrifuged to remove coagulated protein. Solid sodium hydroxide was added to 4%, then 100 ml. of mixed Fehling's solution and 80 ml. of acetone. The copper complex of the gum C_2 was centrifuged off, dissolved in dilute hydrochloric acid and the gum precipitated by adding an equal volume of acetone. The modified barley gum C_2 was then taken to dryness in the usual way and weighed on a sintered glass crucible.

The experiment was repeated, steeping the barley in the active extract for preliminary periods of 1 hr., 2 hr., 4 hr., and 24 hr. Second and third extractions occupied only a $\frac{1}{2}$ hr. each time. A control experiment was carried out each time, following the same procedure but omitting the inactive substrate, in order to find out how much barley gum there was in the active extract. This amount was then subtracted from the yield obtained from experiments when the dead barley was used.

A second and third series of experiments were carried out steeping the barley in extracts equivalent to 25% and 50% of the substrate for similar time intervals as before.

The yields obtained were very interesting and were sufficient in most cases to enable analyses to be carried/

carried out. (See Table 26).

From Table 26 which gives yields of gum obtained using different concentrations of barley extract it is seen that an optimum time and concentration occurs when using a 50% extract of barley for a $\frac{1}{2}$ hr.

It was, therefore, decided to use the above concentration of active extract to try and prepare a fractionated modified barley gum C_2. The experiments outlined in this section were of course carried out before the fractionation outlined on page 53 could be attempted. The experience gained here was invaluable in altering the experimental procedure adopted when working with larger quantities of substrate.

200 g. of enzyme inactivated barley was treated with 600 ml. of barley extract containing an equivalent of 100 g. barley for a $\frac{1}{2}$ hr. The procedure outlined above was followed through and then the combined extracts and washings were concentrated to 500 ml. filtered, and then put through the fractionation procedure with ammonium sulphate as was done in the early development of the technique as shown on page 24 .

It was found that the major fractions occurred at concentrations of 40 g. ammonium sulphate and 50 g. ammonium sulphate per 100 ml. extract. The fraction 20/100 /

20/100 was absent. The final yields were disappointing but the smallness of the yields was apparently due to the fact that after each dialysis, considerable quantities of material was left in the dialysing tubing. This type of precipitation had not occurred before and unfortunately this material was always rejected. There was also the possibility of actual loss by dialysis.

Table 26 collects the analytical data obtained from the yields of the modified gum; Table 27 gives the little information that was supplied by the fraction-:ated modified gum. The variation of percentage yield with time of steeping is also shown graphically (Graph I).

A discussion of the significance of the above results will be deferred until some further experimental work has been outlined.

Two kilograms of Ymer Barley was treated for enzyme inactivation in the usual manner and the residue treated as follows:-

100 g. of the inactive barley was extracted at 20°C. for a $\frac{1}{2}$ hr. with 300 ml. of a barley extract equivalent to 10% of the substrate. After this period, the mixture was centrifuged and the centrifugate passed through a kieselguhr bed whereafter the extract was boiled for two minutes to inhibit further enzyme action. The residue in the centrifuge bottle was re-extracted a further/

further twice with 300 ml. quantities of distilled water at 40°C. The clarified centrifugates were then boiled, combined and concentrated to small bulk. The concentrate was made up to a convenient volume, either 100 ml. or 150 ml. and the modified barley gum present in solution was recovered using Fehling's solution and acetone exactly as described on page 97 . The alcohol dried gum was collected on a sintered glass crucible, heated at 100°C. for 4 hrs., and weighed. The extractions etc., were repeated on fresh 100 g. samples of the inactive barley allowing contact with the 10% live extract for 1 hr., 2 hr., 4 hr., and 24 hr. periods. The second and third extractions in each case were always carried out at 40°C. for ½ hr. to 1 hr. periods.

Two further series of preparations were carried out on 100 g. samples using as a preliminary extraction medium (a) 300 ml. of a barley extract equivalent to 25% of the substrate, (b) 300 ml. of a barley extract equivalent to 50% of the substrate. Subsequent treatment of the residues and extracts was exactly as outlined above for the case of the 10% barley extract.

In addition, 100 g. of the enzyme inactivated barley was extracted once at 20°C. and twice at 40°C. with 300 ml. quantities of distilled water. The recovered/

recovered gum in this case gave a measure of the amount of barley gum C_2 + B_2 present in the unmodified grain. Further, the amount of barley gum in the live extracts was determined by treating 300 ml. quantities of the appropriate extract, after boiling and concentration of same, with Fehling's solution and acetone and recovering the precipitated material in the usual way. The figure so obtained when subtracted from the appropriate yield of modified barley gum gave a true measure of the amount of gum-like material made available by the action of the enzyme system.

Table 28 summarises the data collected by the above preparations and also gives some of the chemical and physical characters of the products.

As the actual yields of the modified barley gum B_2 + C_2 was of the order 1 g., it was possible to carry out several analyses on the various products:-

(a) The amount of furfural liberated from 0.1 g. samples of the gums was determined using the standard distillation method (41).

(b) 100 ml. of 0.5% solutions of the gum samples (expressed on a dry weight basis) was carefully made up in a graduated flask. Using this solution, viscosity and specific rotation measurements were made at/

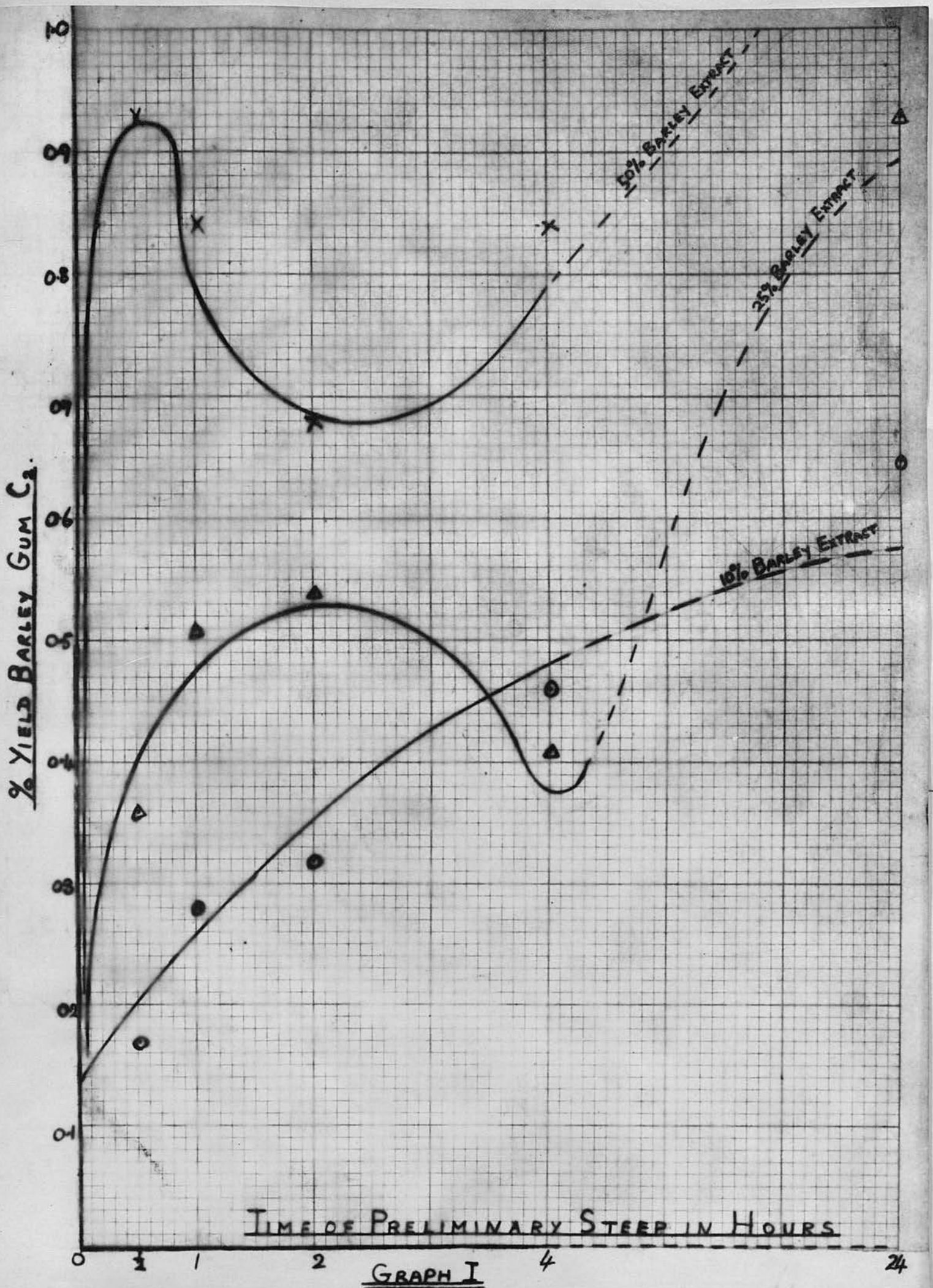
at 25°C. and 15°C. respectively in the usual way.

(c) 50 ml. of the above solution was pipetted into a beaker and 15 g. of solid ammonium sulphate was added with stirring. The barley gum which was precipitated (by previous work this precipitate has been shown to be substantially pure glucosan material) was centrifuged off, dissolved in warm water and dialysed to remove traces of ammonium sulphate. After 48 hrs. dialysis the material in solution was precipitated by adding an equal volume of acetone. Thereafter, the gum was taken to dryness with increasing concentrations of alcohol, collected on a sintered glass crucible and weighed.

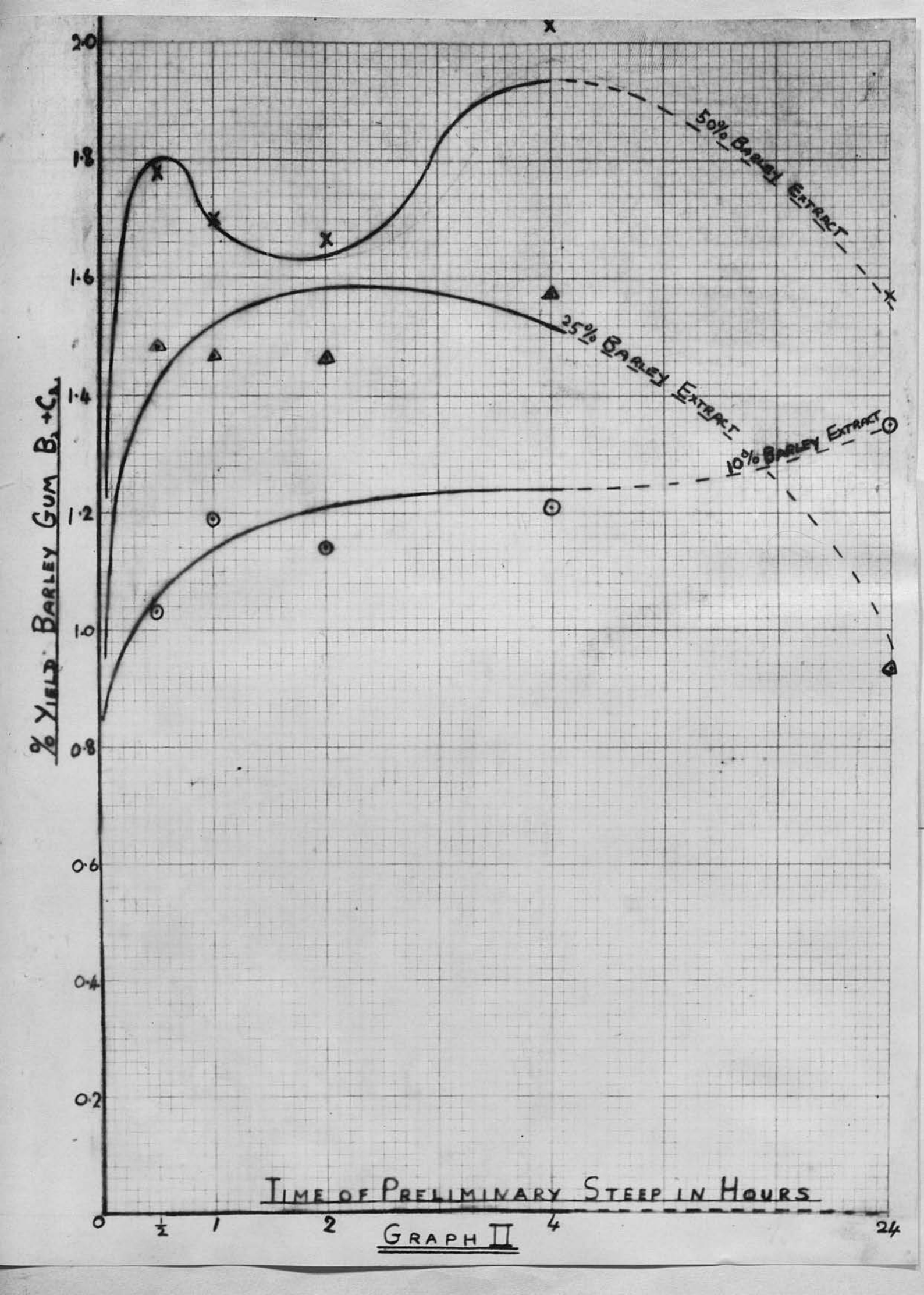
Certain of the results have been expressed graphically in order to bring out more clearly the variations in the characters of the products which can occur.

Graph II shows variation of percentage yield with time of preliminary steep in the active extract.

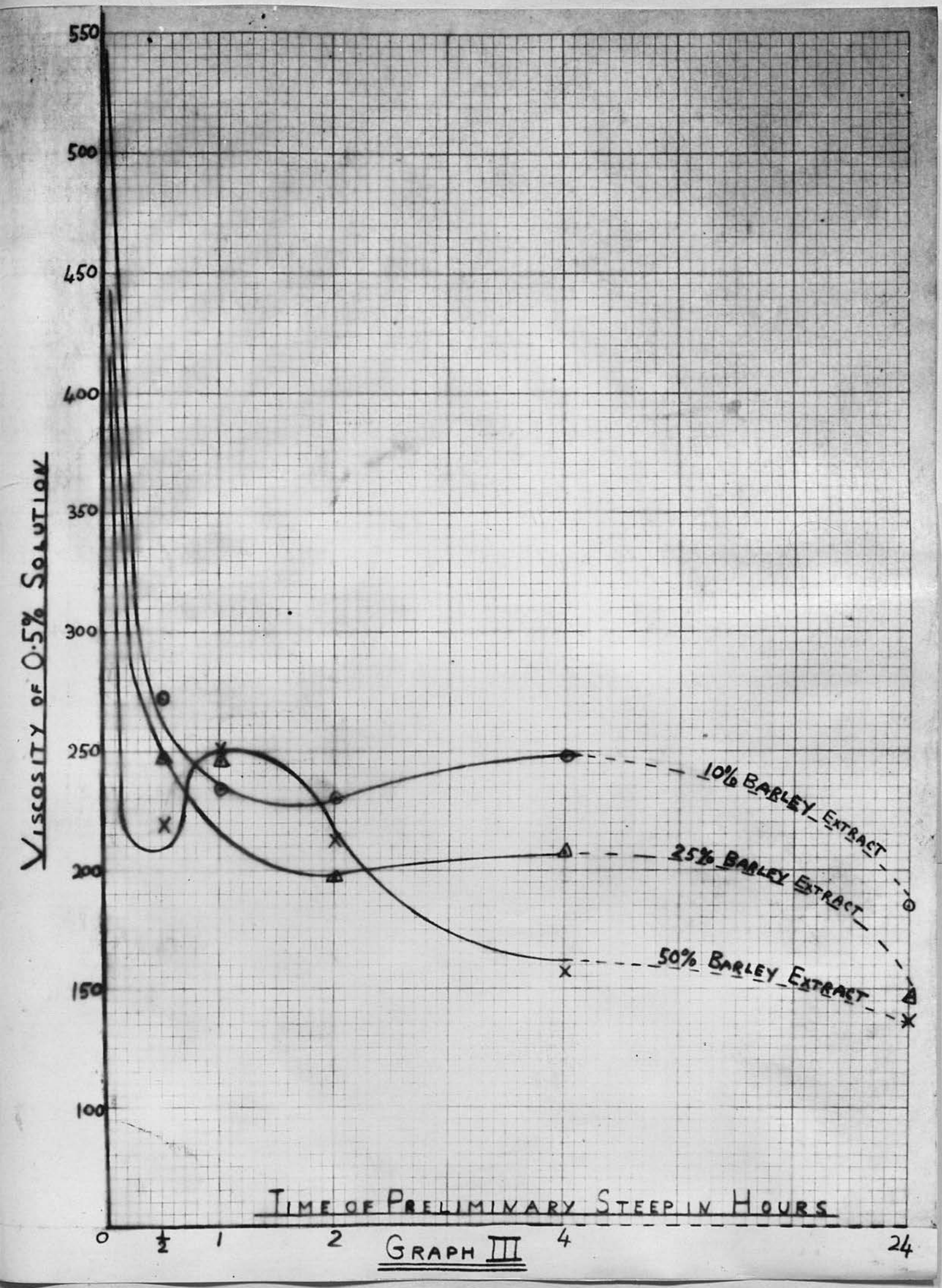
Graph III shows variation in viscosity with time of preliminary steep.


% Yield Barley Gum C_2.
3
2.8
2.6
2.4
2.2
2
1.8
1.6
1.4
1.2
1
50% Barley Extract
25% Barley Extract
10% Barley Extract
Time of Preliminary Steep in Hours
0
½
1
2
4
24

Graph I


% Yield Barley Gum B, +C,
2·0
1·8
1·6
1·4
1·2
1·0
0·8
0·6
0·4
0·2
0
½
1
2
4
24
50% Barley Extract
25% Barley Extract
10% Barley Extract
Time of Preliminary Steep in Hours

Graph II


VISCOSITY OF 0·5% SOLUTION
550
500
450
400
350
300
250
200
150
100
10% BARLEY EXTRACT
25% BARLEY EXTRACT
50% BARLEY EXTRACT
TIME OF PRELIMINARY STEEP IN HOURS
0
½
1
2
4
24

GRAPH III

TABLE 26

Some characters of the modified barley gum C_2 prepared using various concentrations of enzyme extract.

Amount of Barley Extract used	Time of steeping	% Yield Dry, Ash Free and with Control Subtracted	Actual Yield per 100 g. Barley	% Furfural Dry, Ash Free	Colour and Physical Con-:dition of Fraction	Specific Rotation at 15°C on 0.5% solution	Viscosity at 25°C on 0.5% solution (Water = 100)
10%	½ hr	0.17	0.21	23.5	White; Fibrous	-	234
10%	1 hr	0.28	0.33	22.3	White; Fibrous	-36°	229
10%	2 hrs	0.32	0.36	20.3	White; Fibrous	-30°	222
10%	4 hrs	0.46	0.56	19.6	Cream; less Fibrous	-24°	165
10%	24 hrs	0.65	0.68	20.0	White; still Fibrous	-40°	154
10%	48 hrs	1.04	1.03	19.6	Cream; Pulverulent	-30°	137
25%	½ hr	0.36	0.47	18.6	Cream; Fibrous	0°?	117?
25%	1 hr	0.51	0.67	19.6	White; Fibrous	-34°	211
25%	2 hrs	0.54	0.51	18.6	White;less Fibrous	-24°	134
25%	4 hrs	0.41	0.46	26.0	White; less Fibrous	-60°	202
25%	24 hrs	0.93	0.96	20.0	White; Pulverulent	-17°	122
50%	½ hr	0.93	0.99	21.2	White; less Fibrous	-45°	218
50%	1 hr	0.84	0.89	24.2	White; less Fibrous	-45°	158
50%	2 hrs	0.68	0.69	24.5	White; still Fibrous	-45°	174
50%	4 hrs	0.85	1.05	21.6	White; Pulverulent	-45°	164
50%	24 hrs	1.13	1.18	21.9	Cream; Pulverulent	-41°	136
10% control	-	-	0.28	27.1	White; Pulverulent	-	-
25% control	-	-	0.37	30.4	Cream; Pulverulent	-	-
50% control	-	-	0.45	37.8	Cream; Pulverulent	-	-

TABLE 27

Some characters of an early preparation of a fractionated modified barley gum C_2.

Polysaccharide in Order of Precipitation	Gms. $(NH_4)_2SO_4$ per 100 mls. Extract	% Yield	% Furfural
First	30	0.01	-
Second	40	0.065	19.7
Third	50	0.060	56.3
Fourth	Saturation	0.025	-
Fifth	XS.Acetone	Trace	-

TABLE 28

Yields of the barley gum $B_2 + C_2$ prepared by using varying concentrations of active barley extract; some chemical and physical characters of the products. All results expressed on a dry-ash free basis.

Product; Extract used and time of steep.	% Yield ¥ by Fehling's Solution.	Viscosity 0.5% Sol.	Specific Rotation 0.5%	% Furfural	% Recovery by 30% Ammon. Sulph.	% Recoverable Glucosan
From unmod. barley	0.85	550	-22°	9.10	63.0	0.536
10 % ½ hr.	1.03	273	-10°	8.20	69.5	0.716
10% 1 hr.	1.19	236	-18°	8.57	66.0	0.785
10% 2 hr.	1.14	231	-22°	12.5	55.5	0.633
10% 4 hr.	1.21	248	-22°	10.7	59.0	0.714
10% 24 hr.	1.35	185	-20°	12.6	56.0	0.756
25% ½ hr.	1.48	248	-12°	10.9	62.5	0.925
25% 1 hr.	1.47	246	-10°	12.0	60.0	0.882
25% 2 hr.	1.47	196	-18°	10.5	53.0	0.779
25% 4 hr.	1.57	208	-14°	12.1	60.0	0.942
25% 24 hr.	0.93	148	-10°	13.1	46.0	0.428
50% ½ hr.	1.78	221	-14°	11.9	51.5	0.917
50% 1 hr.	1.70	250	-16°	11.7	57.0	0.969
50% 2 hr.	1.67	214	-16°	13.6	55.0	0.918
50% 4 hr.	2.06	156	-40°	20.1	35.0	0.721
50% 24 hr.	1.57	139	-40°	16.3	32.0	0.502
From 10% extract	0.10			13.6		
" 25% "	0.15			33.0		
" 50% "	0.35			20.5		

¥ Figures have been corrected for gum in actual live extract.

DISCUSSION.

From Table 26 it is seen immediately that there is a considerable variation in the percentage yields of the gum obtainable under the conditions specified. Thus a 10% extract of barley acting for a ½ hr. produces a yield of barley gum C_2 of 0.17% whereas a 50% extract acting for a similar period produces a yield of nearly 1.0%. Also it is noted that the furfural figures never vary more than ± 4% from an average of 22%. It is therefore reasonable to suggest that the increased yield is made up of similar material to that extracted from barley by water alone. However, it may be that a higher furfural content due to the presence of a larger percentage of pentosan material is compensated by contamination of the products with starch dextrins.

At low concentrations of this disaggregating enzyme (i.e. when using a 10% extract of barley), it is seen that only after 4 hr. steeping are appreciable quantities of the initially insoluble material made water soluble, whereas using a 50% extract, a ½ hr. is sufficient. This would seem to indicate that the disaggregating enzyme of barley has to be present in a fairly high concentration before appreciable breakdown of the/

the hemicellulosic material occurs.

However, when the percentage yields obtained using 25% and 50% extracts of barley are examined, these percentage yields are not proportional to the time of steeping. This phenomenon is perhaps seen more clearly on the Graph I.

10% extract curve:- This resembles that for the action of a normal enzyme. Thus, up to 4 hr. steeping there is a fairly quick increase of yield then the curve flattens out to the value obtained after steeping for 24 hrs. The increase after this point may be due to the action of other enzyme systems. This latter action is seen more clearly when the other two curves are examined.

25% extract curve:-This rises very quickly to a first maximum of 0.54% after 2 hr. steeping; the yield drops somewhat at 4 hr., then the curve rises to the value obtained after 24 hr. steeping. Although the part of the graph between 4 hr. and 24 hr. is hypothetical, it is not thought that it would vary much from this form.

50% extract curve:-An even quicker rise is seen here where the first maximum yield is obtained after only a ½ hr. steeping. This is followed by a quick fall up to 2 hr. steeping and then a second quick rise at 4 hr. Finally the curve flattens out and gradually reaches the value of 1.13% yield at 24 hr. steeping.

Why/

Why should these curves take this apparently anomalous form? Thus, the first rise of the 50% extract curve is due to the action of the disaggregating enzyme. The high concentration used, means that the action of the enzyme will be swift. After a ½ hr. this action slows down as no more material is available for rapid breakdown. The second enzymic system found in barley viz. the cytolysis factor, then begins breaking down the water soluble polysaccharides to such an extent that they no longer form copper complexes with Fehling's solution and acetone. This type of action is seen in the graph by the drop in percentage yield up to 2 hr. steeping.

The second rise in the curve is a little more difficult to explain but two possibilities are considered:-

(a) the action of a third hydrolytic enzyme,

(b) the continued slow action of the dis-:aggregating enzyme

(a) The second rise in the curve could possibly be due to the action of an entirely different type of enzyme. That is, one which is breaking down different material from cell-wall constituents. A possible enzyme is of course α-amylase which, after prolonged steeping would tend to make available for precipitation, quantities of dextrin. Against this idea/

idea is the fact that none of the polysaccharide solutions showed a positive specific rotation which would be expected if appreciable quantities of dextrin were present. Certain of the polysaccharide products were tested for starch dextrins by means of iodine solution, and although faint blue colourations were observed, it was not felt that α-amylase action is a major factor in this second increase of yield.

(b) A more likely explanation may be found in the continued slow action of the disaggregating enzyme. This action, which is continuing throughout the whole period of steeping, is masked during the stage when the cytolytic enzyme is active and appears later on when the saccharifying action itself has slowed down. The continued slow action of the disaggregating enzyme suggests that more resistant hemicellulosic material is being attacked. The chances are too, that the cytolytic enzyme is still acting although much more slowly, hence the production of pulverulent material, but the shape of the graph is determined by the dominating action, in this case the disaggregating enzyme.

The viscosity of the polysaccharide solutions and the physical appearance of the dry products both tend to confirm the view that the second enzyme action greatly simplifies the molecular complexity. The products after short contact with the enzyme were all fibrous, whereas after 24 hrs. steeping were pulverulent.

The/

The viscosities of the aqueous solutions showed an overall decrease but the fall was not regular. These fluctuations in the viscosity measurements could not be satisfactorily accounted for at first, but the results summarised in Table 28 tend to support the view that such apparent anomalies are in accordance with the idea of a complex enzyme system which has several actions when acting on cell-wall material. The specific rotations did not vary very much although the average value was rather less than might be expected (3).

The data obtained from the modified fractionated polysaccharides although slight gave an indication of the type of result to be expected when the experiment was repeated on a larger scale. The reason for using a 50% extract of barley for a period of a $\frac{1}{2}$ hr. was because it was felt that it would be better to have a quick disaggregating action, rather than have a product which had been in contact with the saccharifying enzyme for a long period.

In the experimental section it was stated that considerable quantities of material were lost after each dialysis. The extent of this loss can be seen from the fact that under the conditions specified, the total yield of the fractions from 200 g. of barley should have amounted to about 2 g. The actual yield was 0.32 g.

(Table/

(Table 27). The precipitation, in the dialysing tubing, of quantities of material which were rejected before continuing the fractionation was obviously a major factor in this loss. It may be that some of the newly water soluble material was slightly salt soluble but in any case it was clear that a different technique for the fractionation of the modified polysaccharide would have to be developed before much success could be attained. These conclusions led to the development of a fractionation technique involving a combination of ammonium sulphate and acetone precipitations without dialysis.

When the results obtained from the second series of enzyme experiments are examined, it must be borne in mind that as a different variety of barley was employed the figures are not strictly comparable with those of Table 26.

An examination of Table 28 shows that the differences in percentage yield recoverable under the conditions specified do not vary so much as in the earlier experiments. Apart from the use of a different variety of barley, it must be remembered that in the second case the B_2 and C_2 gums were extracted simultaneously. Now if it is postulated that the first action of the cytoclastic enzyme is to make pre-formed water soluble material more soluble, then if a modified C_2 gum is extracted alone,

it/

it will contain considerable quantities of material originally soluble at 40°C. As the average variety of barley contains about twice as much gum B_2 as C_2, big increases in yield may be expected as shown in Table 26. On the other hand more resistant tissue will have to be attacked before material extracted at 40°C. is to show any increase. Such an action will be slower than the solubilisation of the B_2 gum and hence the relatively smaller increases summarised in Table 28 may be accounted for.

It is again evident that, broadly speaking, the higher the enzyme concentration, the larger is the resultant yield. The increase in barley gum yield under the influence of a 10% barley extract is straightforward and, as can be seen from Graph II, its shape resembles that obtained when using the corresponding extract on barley at 20°C (see Graph I). Although the product after treatment with a 10% extract for 24 hrs. is pulverulent, the cytolytic action does not appear to be sufficiently strong to decrease appreciably the amount of recoverable material and hence the graph shows a steady if gradual increase.

Up to 4 hr. preliminary steep, the form of the other two curves resemble those obtained before. Certain of the results appear anomalous at first sight but this may/

may not be so. Our knowledge of the action of these enzyme systems is scanty and that actions are going on simultaneously and which vary in speed, has been confirmed by autolysis experiments carried out by Preece and Gregson (42).

These workers found that when barley was allowed to autolyse at 40°C. for varying lengths of time, different amounts of barley gum were extractable and the figures when plotted graphically took a similar form to that shown by the 50% extract curve. To try and expand the theory as outlined on page 123 which accounts for these changes in barley gum yields would, in view of the still insufficient data available, be unwise. For instance the yield of gum obtained after a preliminary steep in the 25% or 50% extract of 24 hr. may be due in part to the influence of other factors e.g., bacterial action.

Similar results were obtained by measurement of the amount of barley gum C_2 and B_2 recoverable from a germinating barley (44). Details of this work (42,44) have not yet been fully worked out but a preliminary survey would seem to indicate that the actions of the various enzyme systems are even more complex than might be expected from an examination of Graphs I and II.

Several fresh points of interest emerge from the/

the viscosity measurements. Again it is noticeable that the fall in viscosity is not regular; the viscosity of solutions, the gum of which had been prepared under the influence of a 10% and a 25% barley extract for 4 hrs. and also of a 50% extract for 1 hr., are higher than those of solutions immediately preceding and following (see Table 28). At the time of preliminary steep specified, the barley extract may be bringing into solution some of the more resistant type of material - the insoluble gums or the simple hemicelluloses - which, still possessing a complex structure would tend to increase the viscosity of the solution. That simplification of pre-formed water soluble material does occur can be seen from the fact that the viscosity of an unmodified Ymer barley gum is 550 (higher than might be expected) but that this figure is more than halved by the action for only a $\frac{1}{2}$ hr. of a 10% barley extract.

Neither the specific rotation nor the furfural figures vary very much although material which has had prolonged contact with a 50% barley extract displays a specific rotation of -40° (Table 28). This may be due to the presence of a greater proportion of pentosan material which has been shown to possess a high negative specific rotation. In other words, here we may have the beginnings of an action similar to modification.

The/

The physical appearance of the products (not quoted in Table) were very similar to those obtained previously (Table 26). Material which had been in contact with the live extract for short periods were fibrous in nature whereas progressively longer enzyme action produced a more pulverulent product.

Throughout this investigation as a whole, emphasis has been laid on the property of Fehling's solution and acetone to recover more polysaccharide material from solution than ammonium sulphate especially if the original substrate material has been modified by the action of the cytase enzyme system. If, on modification of the barley corn, one of the results is to decrease the amount of glucosan material available for precipitation by normal methods, then the modified barley gums obtained in this experiment should possess progressively less material which may be precipitated from solution by 30% ammonium sulphate, previous work having shown that at this concentration of ammonium sulphate, glucosan material is largely precipitated.

Table 28 shows that material which has been subjected to the action of cell-wall hydrolysing enzymes does differ in the manner suggested above, and that the amount recoverable depends on the concentration of enzyme extract used and the length of preliminary steep. The most/

most striking difference is to be seen from action by a 50% barley extract where the amount of glucosan recoverable by 30% ammonium sulphate drops from 0.917% to 0.502%. Malted barley has been subjected to a far more prolonged enzyme action than the examples cited above and it is therefore easy to see why little or no glucosan material of the type found in an unmodified barley is present in malt.

In passing it may be observed that solutions which display the apparently anomalous high viscosity are also the ones which, relatively speaking, possess a greater amount of material precipitable by 30% ammonium sulphate than either the solutions above or below it in Table 28. If these results are significant then it would fit in with the view that the action of the enzyme at that particular concentration and time of action has brought into solution initially insoluble material, rather than the degradation of pre-formed water soluble material.

Although many of the ideas expressed in this discussion have by no means been rigidly confirmed, it has been said elsewhere that some working hypothesis is better than none at all, and if the ideas serve no other purpose than to provide a stimulus for further investigation, enough will have been achieved.

GENERAL DISCUSSION.

It is intended in this section to review briefly the results of the investigation as a whole and to correlate where possible the results obtained in the various experimental sections. Difficulties in the interpretation of certain of the data will be emphasised and the lines along which future work could most usefully be made will be outlined, with particular reference to the use of the results of this investigation as a stepping stone.

Broadly speaking, the present investigation has been sub-divided as follows:-

(a) The main section dealing with the development of a method for the fractionation of the barley and malt gums.

(b) A section which was concerned with the distribution and source of the wheat and barley gums.

(c) A section which attempted to increase the present state of knowledge concerning the cytase system of barley.

It will have been noticeable that many of the early fractionation methods were soon discarded in favour of the more precise technique which was gradually evolved. It is hoped that the picture presented in that experimental section will show, how, as each change was made in the method/

method a more efficient fractionation resulted until methods were perfected whereby a pure laevorotatory glucosan in substantial yield was obtained. As will have been noticed, in the early development of the fractionation technique the yields were poor and there was always a fair percentage of pentosan material to be found in the main glucosan fractions.

The water soluble pentosan of barley presented a different problem. It was never obtained completely free from glucose residues but for reasons given elsewhere it was felt that such residues were present merely as an impurity, and not linked chemically to the main pentosan structure. The evidence presented in this thesis was insufficient to decide whether the pentosan was indeed a physical mixture of two distinct polysaccharides or possessed a structure similar to that put forward by Perlin (16) for the pentosan of wheat flour. If we assume for the sake of argument that it is possible to separate the two constituent units by physical means then it is unlikely that one factor, namely solubility in ammonium sulphate would be sufficient to effect such a separation. It is interesting to find that similar pentosan material occurs in other cereals but that the percentage yield varies considerably from cereal to cereal (37). However, each cereal pentosan appears

to/

to contain approximately equal amounts of xylose and arabinose residues, and so closely similar pentosans to those described by Perlin may be widely distributed in the plant kingdom.

With regard to the use of ammonium sulphate as an agent for the fractionation of the non-starchy water soluble barley and malt polysaccharides it is felt that the utility of such a technique has been fully explored. It may be that other substances possess similar properties to ammonium sulphate and also exercise a greater degree of efficiency in fractionation. Clearly a final answer on the nature of these polysaccharides can only be obtained by recourse to further attempts at fractionation and also to the now standard methylation techniques.

However, the physical separability of water soluble pentosan and glucosan material is highly significant. Not only is it possible that the two types of polysaccharide are to be located in different parts of the barley corn but it is also likely that they are concerned with different metabolic processes in the plant.

This was the type of problem which was hoped to be solved by definite information regarding the source of the water soluble polysaccharides. However, as was demonstrated/

demonstrated in the appropriate section, precise information was difficult to secure and apart from obtaining fairly satisfactory evidence that the husk of barley was deficient in these materials and probably the germ also, little advance was made. It would have been highly satisfactory from the point of future preparative work if the source of even one of the barley gum components could have been determined. For instance how simple it would be to prepare large quantities of the water soluble barley pentosan if the latter had been located in the husk. Modern pearling methods produce a husk which is virtually free from endosperm and the extraction of the pentosan could have gone on unhindered by extraneous matter.

As far as is known, the pearling process is the only one available which is of any practical use in achieving a separation of the barley corn. But as has been pointed out previously the production of appreciable quantities of soluble starch makes the process useless if interest lies in the non-starchy polysaccharides. It was possible to devise a fairly simple mechanical method in the laboratory to separate barley husk from the remainder of the grain but further attempts at separation of the embryo or endosperm were defeated, either by the fact that contamination with other members of the barley corn/

corn would invalidate any results so obtained or because no simple method of separation presented itself.

Now it is a comparatively simple matter to interpret the experimental data concerned with either the fractionation technique or with the question of the distribution of the barley gums. On the other hand it is extremely difficult to account satisfactorily for certain of the results of the enzyme experiments due both to insufficiency of data and also because what evidence is available cannot be pieced together in a simple manner. There can be little doubt as to the presence of the two types of cell-wall hydrolysing enzymes, the properties of which have already been defined elsewhere, and the results of this investigation have been confirmed to a large extent by certain autolysis and germination experiments (42, 44). It is when details of the enzyme action are asked for that a ready answer is unobtainable.

Clearly, here is a field where important advances in our knowledge of the modification process could most usefully be made. In the general introduction it was stated that both the preparation of pure substrate material and a pure cytase system were of primary importance before much advance could be made in the subject. This/

This investigation has provided methods whereby two different types of substrate material in a fairly high state of purity may be obtained. Several lines of future work can be seen.

Firstly it will be necessary to devise means whereby pure and active preparations of the cytoclastic and cytolytic enzymes can be obtained. The ever increasing field of chromatography suggest itself immediately as a convenient tool for the purpose. Once suitable preparations have been made, it will then be a much more simple task to follow the course of enzyme action especially as suitable substrates are now available. The use of substrates foreign to the enzyme system under examination has been deplored elsewhere but as the substrates have been obtained from barley, such experiments should furnish a true picture of at least one part of the process of modification.

What has been achieved in this investigation? In the section entitled "Scope or Purpose of the Present Investigation." some lines were given along which it was hoped the practical work would proceed and also the type of information which such work might well afford. There is no need to recapitulate either the essence of that section or the main results of the investigation, but/

but a certain advance has been made and, like most research, the more information which is obtained, the larger are the fields still remaining to be conquered. Generally speaking the implications of new information are never fully appreciated immediately after their discovery and so perhaps certain of the data obtained here which has not been particularly emphasised may provide a starting point for future work.

It is often difficult to transpose the results of an investigation of this nature to the needs of the industry. In this country the use of barley as a raw material is confined largely to the brewing, malting and distilling industries and in recent times complaints have been made to farmers regarding the quality of the barley grown. The brewer alleges that he is unable to brew satisfactorily with the varieties of barley which are popular with the farmer. On the other hand, the farmer naturally wishes to grow a barley which is a heavy cropper and feels in times of food shortages that it is in the national interest to do so. Reconciliation between the two opposing camps can only come about by a better understanding of the complex processes occurring in the cereal itself. There seems no logical reason why one variety/

variety of barley should be more acceptable to industry than another and surely if more was known about the metabolic changes which occur, not necessarily just on modification, each variety of barley could be treated according to its own characteristics.

The yield of gum obtainable from a particular variety of barley is dependent both on the variety and on the conditions prevailing throughout the growth of the plant and it is also highly likely that the amounts of the other components of the barley grain vary in a similar fashion. It is therefore not surprising that a wort prepared from a particular variety of barley will display different properties from a second wort prepared from an entirely different variety of barley and grown under different conditions.

We are still a long way from the position in which a brewer or distiller confronted with a particular sample of barley, which, according to the older methods of assessment, would be useless for his purpose, will be able, by making certain alterations in his general procedure to obtain a satisfactory product.

The problems which remain will not be solved completely by the methods of biochemistry alone, but by a combination and pooling of the results found by workers in several branches of science.

SUMMARY.

1. Methods have been developed whereby an extract of barley containing the non-starchy water soluble polysaccharides may be fractionated. The most successful fractionating agent used was ammonium sulphate.

2. At least two distinct polysaccharides have been isolated. One, a pure glucosan which possesses a small negative specific rotation and may be likened to a short chain cellulose; two, a pentosan which gives arabinose and xylose on hydrolysis and which is suspected to be a mixed polysaccharide.

3. The precise source of the water soluble non-starchy polysaccharides remains undetermined. The husk and embryo would appear to be deficient in these materials and the most probable location seems to be some part of the endosperm.

4. The nature of action of the cell-wall hydrolysing enzyme systems of barley has been examined. The results obtained confirm previous work which postulated two enzyme systems - one, which had a disaggregating action, and the second which had a saccharifying action. Such data as is available indicate that the precise nature of the combined action of the two systems is more complex than at first suspected.

REFERENCES.

1. O'Sullivan, C., J. Chem. Soc. 1882, 41, 24.

2. Piratzky, W. and Wiecha, G. Woch. Brau. 1938,55,97.

3. Preece, I.A., Ashworth, A.S., and Hunter, A.D., J. Inst. Brew. 1950, 56, 33.

4. Norris, F.W., and Preece, I.A., Biochem, J. 1930, 24, 59.

5. Schulze, E., Ber. 1891, 24, 2277.

6. O'Dwyer, M.H., Biochem, J. 1926, 20, 656.

7. Preece, I.A., Chem. and Ind. 1948, 611.

8. Norman, A.G., Biochemistry of Cellulose, Polyuronides, Lignin etc. Oxford: Clarendon Press, 1937.

9. Preece, I.A., Recent Advances in Fermentation Industries, Royal Institute of Chemistry, 1949,16.

10. Preece, I.A., Biochem. J. 1940, 34, 251.

11. Chanda, S.K., Hirst, E.L., Jones, J.K.N., and Percival, E.G.V., J. Chem. Soc. 1950,1289.

12. Enders, C., Saji, T. and Schneebauer, F., Woch. Brau. 1938, 55, 121.

13. Preece, I.A., and Prowse, A.B., unpublished.

14. Meredith, W.O.S., Bass, E.J., and Anderson, J.A., Cereal Chem. 1951, 28, 177.

15. Ford, L.H., and Peat, S., J. Chem. Soc. 1941, 856.

16. Perlin,A.S., Cereal Chem. 1951, 28, 370, 382.

17. Hampton, H.A., Haworth, W.N., and Hirst, E.L., J. Chem. Soc. 1929, 1739.

18. Haworth, W.N., Hirst, E.L., and Oliver, E. ibid. 1934, 1917.

19./

19. Isherwood, F.A., Abs. 1st International Congress Biochemistry, Cambridge. 1949, 515.

20. Preece, I.A., and Ashworth, A.S., J. Inst. Brew. 1950, 56, 40.

21. Grüss, J., Woch Brau. 1902, 19, 243.

22. Baker, J.L., and Hulton, H. F. E., J. Chem. Soc. 1917, 121.

23. Lüers, H., and Volkamer, W., Woch. Brau. 1928, 45, 83, 95.

24. Fink, H., and Hartman, J., ibid., 1934, 51, 273.

25. Fink, H., ibid., 1935, 52, 265.

26. Consden, R., Gordon, A.H., and Martin, A.J.P., Biochem. J. 1944, 38, 224.

27. Partridge, S.M., ibid. 1948, 42, 238.

28. Partridge, S.M., Biochem. Soc. Symposia No. 3. Cambridge: University Press 1949.

29. Dickson, A.D., Otterson, H., and Link, K.P., J. Amer. Chem. Soc. 1930, 52, 775.

30. MacLeod, A.M., J. Inst. Brew. 1951, 163.

31. Somogyi, M., J. Biol. Chem. 1945, 160, 61.

32. Hirst, E.L., and Jones, J.K.N., Discuss. Faraday Soc. No. 7. 1949, 268.

33. Bywater, R.A.S., Haworth, W.N., Hirst, E.L., and Peat, S., J. Chem. Soc. 1937, 1983.

34. Durrum, E.L., J. Amer. Chem. Soc. 1950, 72, 2943.

35. Pence, J.W., Elder, A.H., and Mecham, D.K., Cereal. Chem. 1950, 27, 60.

36. Clendenning, K.A., and Wright, D.E., Canad.J.Res. 28F, 1950, 390.

37./

37. Preece, I.A., and Henderson, G., Unpublished.

38. Preece, I.A., and Crow, N.C., Unpublished.

39. Lampitt, L.H., Fuller, C.H.F., and Goldenberg, N. J. Soc. Chem. Ind. 1941, 60, 1.

40. Lampitt, L.H., Fuller, C.H.F., and Goldenberg, N. ibid. 1941, 60, 175.

41. Browne, C.A., and Zerban, F.W., Physical and Chemical Methods of Sugar Analysis pp. 904 - 910, New York, John Wiley & Sons, Inc., 1941.

42. Preece, I.A., and Gregson, A. Unpublished.

43. Feigl, F. Qualitative Analysis by Spot Tests, Trans. by R. E. Oesper, New York, Elsevier Publishing Co., Inc. 1947.

44. Preece, I.A., and Mackinlay, W., Unpublished.

ACKNOWLEDGEMENTS.

It is a pleasure to express my thanks to Professor I. A. Preece, D.Sc., F.R.S.E., Professor of Brewing and Industrial Fermentation, the Heriot-Watt College, Edinburgh, under whose direction the work described in this thesis was carried out. Best thanks are also due to Professor E. L. Hirst, M.A., D.Sc., F.R.S., Professor of Organic Chemistry, Edinburgh University, who gave much help and guidance during the course of the work.

The writer is grateful to certain Edinburgh Breweries for generous supplies of barley and malt, to the Research Foundation of the Institute of Brewing for a grant which made the work possible, and to Mr. A. MacDonald of the Heriot-Watt College, Edinburgh, who carried out several qualitative nitrogen determinations.