2. The carbon dioxide ratio, i.e. the ratio of the specific activities of expired carbon dioxide during the infusion of [1-$^{14}$C]acetate and [2-$^{14}$C]acetate, respectively, was $1{\cdot}58 \pm 0{\cdot}17$ (3) for non-pregnant sheep, $1{\cdot}66 \pm 0{\cdot}25$ (4) for sheep in early pregnancy, $1{\cdot}74 \pm 0{\cdot}14$ (4) for sheep in late pregnancy, and $1{\cdot}18 \pm 0{\cdot}11$ (3) for under-fed sheep in late pregnancy. Calculations based on the last two ratios indicate that citric acid-cycle turnover in under-fed pregnant sheep is about one-quarter of the turnover found in well-fed pregnant sheep.

3. The output of carbon dioxide is similar in all four groups of animals, but the percentage of carbon dioxide derived from acetate is low in the poorly-fed sheep in late pregnancy. The amount of acetate converted into carbon dioxide is lower in these animals but, because total acetate utilization is low in this group, the acetate presented to the tissues is oxidized as readily as in the sheep of other groups.

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# Studies on Carbohydrate-Metabolizing Enzymes

## 10. BARLEY β-GLUCOSIDASES*

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Although cereal extracts are well-known sources of amylolytic enzymes (see, for example, Hanes, 1937), the presence of β-glucosidases in such extracts is a more recent finding. Dillon & O'Colla (1950, 1951) reported that amorphous preparations of wheat β-amylase could also hydrolyse laminarin [a polymer of β-(1→3)-linked glucose residues], and Manners (1952) demonstrated the hydrolysis of cellobiose, salicin and laminarin by barley β-amylase preparations. The latter observations have now been extended, and the action of barley preparations on several substrates containing β-glucosidic linkages has been examined. Preliminary accounts of part of this work have been published (Manners, 1955; Anderson, 1958; Cunningham, 1961). During this period (1952–1961) the results of related investigations on barley β-glucosidases were published by Preece and co-workers (see, for example, Preece & Hoggan, 1956) and by Meredith and co-workers (see, for example, Bass & Meredith, 1955). These workers have used barley β-glucosan [an essentially linear polymer of glucose containing both β-(1→3)- and β-(1→4)-linkages] as the major substrate. However, the relative proportion of the two types of linkage is uncertain [methylation studies indicate about 50% of β-(1→3)-linkages whereas periodate-oxidation analysis suggests only 30% (see Aspinall & Telfer, 1954; Parrish, Perlin & Reese, 1960)] and the sequence of linkages has not been rigidly established. Moreover, studies by Parrish *et al.* (1960), confirmed by Cunningham & Manners (1961), have shown that certain 'laminarinase' preparations can, in fact, hydrolyse β-(1→4)-glucosidic linkages. In our studies we have used laminarin and cellodextrin (a water-soluble acid-degraded

* Part 9: Kjolberg & Manners (1963).

cellulose) as substrates, to avoid any possible ambiguity in assessing β-(1→3)- and β-(1→4)-glucosidase activities.

## METHODS

*Analytical methods.* Descending paper chromatograms were prepared as described by Anderson & Manners (1959). Reducing sugars were determined by the iodometric Somogyi (1952) reagent calibrated against glucose. In the presence of cellobiose, glucose was determined by using the Phillips & Caldwell (1951) reagent. Deproteinization, when necessary, was effected with a zinc sulphate–barium hydroxide reagent (Somogyi, 1945). Protein N was determined by the semi-micro-Kjeldahl method of Chibnall, Rees & Williams (1943) or the biuret method of Robinson & Hogden (1940).

Glucose-oxidase assays were carried out as described by Bell & Manners (1952), digest samples of 1·8 ml. being analysed.

*Preparation of enzyme digests.* Weighed amounts of enzyme preparation were suspended in the stated volume of buffer and centrifuged, and any insoluble material was discarded.

Standard digests contained substrate solution (3 mg./ml.; 5 ml.), 0·2M-sodium acetate buffer, pH 5 (2 ml.), and enzyme solution (1 ml.), and were incubated with toluene at 35° or 37°. With 2 hr. incubation periods, there was a linear relation between enzyme concentration and the production of reducing sugars when salicin (the most common substrate for the assay of β-glucosidase activity) and laminarin were the substrates. With cellodextrin there was some departure from linearity, although this was not sufficient to invalidate most of the comparative studies, but strict linearity was observed with a 30 min. incubation period. The pH range used in the digests is based on previous experiments (Manners, 1952). Enzyme controls without substrate were always prepared, to correct for any autolysis of contaminating polysaccharide.

For the viscometry experiments described in Table 3, the digests consisted of equal volumes of Cellofas B (0·25% solution) and 0·2M-sodium acetate buffer, pH 5·0, in which the enzyme preparation had been dissolved, and were incubated at 25°. The flow-time of 20 ml. samples was measured; the substrate control had a specific viscosity ($\eta_{sp.}$) of 0·500. In other experiments, the digests contained 5 ml. of enzyme solution and 10 ml. of Cellofas B solution and were prepared in Ostwald or modified Ubbelohde viscometers from solutions which had been filtered through G4 sintered-glass filters, and the flow-time was measured at intervals. Enzyme activity was calculated from the relation (see Hultin, 1947):

$$\text{Activity} = c^2 \mathrm{d}\frac{\left(\frac{1}{\eta_{sp.}}\right)}{\mathrm{d}t}$$

where $c$ is the substrate concentration (g./ml.) and $t$ is the time of incubation (hr.). With Cellofas B as substrate, there was a linear relationship between activity and enzyme concentration.

*Fractionation with ammonium sulphate.* Several fractionations were carried out from extracts prepared from 1 kg. of barley flour and 3·5 l. of 0·2M-sodium acetate buffer, pH 5·0; one is described in Table 1 where fractions of similar weight were obtained. Each precipitate was dissolved in 0·2M-sodium acetate buffer, pH 5·0 (150 ml.), dialysed for 2 days at 0° against water to remove inorganic salt and the products of any autolysis, and then freeze-dried. About 100 mg. of each freeze-dried powder was dissolved in 0·2M-sodium acetate buffer, pH 5·0 (10 ml.), and then analysed. In some experiments, the protein precipitated by 20–40% saturation with ammonium sulphate was used.

## MATERIALS

*Substrates.* Laminarin was the sample B.B. 2 examined by Anderson, Hirst, Manners & Ross (1958). Cellodextrin, a soluble dextrin from cellulose, had a degree of polymerization of 7–10 and was kindly provided by Professor C. S. Hanes, F.R.S. Lichenin was the sample II described by Chanda, Hirst & Manners (1957). Cellobiose and the various β-glucosides were commercial samples whose purity was checked by paper chromatography. Laminaribiose was isolated from a partial acid hydrolysate of laminarin. Cellofas B (sodium carboxymethylcellulose) was a gift from Imperial Chemical Industries Ltd.

*Enzyme source.* Flour from ungerminated barley grain (var. Spratt-Archer, 1954 harvest) was used.

*Nomenclature of enzymes.* Cellodextrinase activity denotes the formation of various reducing sugars from cellodextrin, and is regarded as the result of combined β-(1→4)-glucosanase activity (random hydrolysis) and cellobiase activity (stepwise hydrolysis). Laminarinase activity is similarly regarded as the sum of random β-(1→3)-glucosanase activity and laminaribiase activity. Lichenase activity is assessed by the production of reducing sugar from lichenin by the combined action of cellodextrinase and laminarinase. β-(1→4)-Glucosanase activity can be selectively assayed from the random hydrolysis of sodium carboxymethylcellulose; this activity is referred to as 'cellulase' activity by some workers, but we prefer to restrict the term 'cellulase' to the enzymic degradation of insoluble native cellulose.

## RESULTS

*Extraction of β-glucosidases.* From a series of preliminary experiments in which barley flour was extracted at different temperatures for various periods with either 3% (w/v) potassium chloride solution or 0·2M-sodium acetate buffer, pH 5·0, it was concluded that extraction with 0·2M-sodium acetate buffer, pH 5·0, at 18–20° for 2 hr. gave extracts with high salicinase, laminarinase and cellodextrinase activities. The protein was isolated by precipitation with ammonium sulphate followed by dialysis and freeze-drying; some loss (about 20%) in activity occurred if precipitation by acetone was used in the final stage.

*Ammonium sulphate fractionation of extract.* To an extract of barley flour, prepared under optimum conditions, solid ammonium sulphate was added at 0°. The protein material precipitated at 0–35, 35–50, 50–65 and 65–80% saturation was col-

Table 1. *Distribution of β-glucosidases in the fractions obtained by precipitation with ammonium sulphate*

Experimental details are given in the text. The incubation times were 2 hr. Activities are expressed as percentage hydrolysis/mg. of protein.

| | β-Glucosidase activity | | | |
|---|---|---|---|---|
| Saturation with $(NH_4)_2SO_4$ ... | 0–35 % | 35–50 % | 50–65 % | 65–80 % |
| Yield (from 1 kg. of flour) ... | 2·6 g. | 2·3 g. | 2·3 g. | 2·0 g. |
| Protein N content ... ... | 2·8 % | 5·9 % | 4·5 % | 1·7 % |
| Activity | | | | |
| Cellobiase | 8·7 | 30·2 | 54·5 | 69·3 |
| Salicinase | 3·1 | 3·1 | 6·1 | 7·5 |
| Phenyl β-glucosidase | 3·2 | 2·4 | 3·8 | 8·8 |
| Cellodextrinase | 5·4 | 10·1 | 13·3 | 33·6 |
| Laminarinase | 9·6 | 15·2 | 28·5 | 28·3 |
| Lichenase | 2·4 | 2·9 | 10·6 | 16·2 |

lected, dialysed, isolated and analysed. The results (Table 1) show that there was no complete separation of the various activities of each fraction, but indicate that different enzymes are involved in the hydrolysis of the six substrates; in particular, the distribution of cellobiase, salicinase and phenyl β-D-glucosidase activities are different.

Solutions containing 0·10, 0·20 and 0·25 % of the fraction precipitated by 50–65 % saturation with ammonium sulphate were incubated with Cellofas B. The relative activities, determined viscometrically, were 0·60, 1·22 and 1·57 units respectively, indicating a linear relationship between enzyme concentration and activity. That random degradation of the substrate had occurred was confirmed by measurements of the increase in reducing power of the digests, and by paper chromatography which showed the formation of glucose and small quantities of oligosaccharides.

*Effect of temperature on β-glucosidase activities.* The protein precipitated from an extract by 20–40 % saturation with ammonium sulphate was dissolved in 0·2 M-sodium acetate buffer, pH 5·0, and portions were heated for 15 min. at five temperatures in the range 45–65°. A comparison of the salicinase, laminarinase and cellodextrinase activities of these heated solutions with a control extract is given in Table 2, and show appreciable differences in their temperature-stability ranges.

*Removal of amylase from barley extract.* Since plant β-amylases are known to be acid-labile (cf. Peat, Thomas & Whelan, 1952), portions of barley extract were treated: (*a*) at pH 3·4 and 0°, for 18 hr, the pH then being readjusted to 5·0; (*b*) at pH 3·0 and 37°, for 90 min., followed by cooling to 20°, and adjustment to pH 5·0; (*c*) procedure (*a*) followed by (*b*). The solutions were centrifuged to remove insoluble material before dialysis and isolation. The relative β-amylase activity, determined by the method of Hobson, Whelan & Peat (1950), and β-glucosidase activities are reported in Table 3.

Table 2. *Effect of heat on β-glucosidase activities*

Experimental details are given in the text. The composition of the digests was standard, 5 mg. of enzyme preparation being used in each digest. Incubation times were 2 hr. for salicinase and laminarinase, and 0·5 hr. for cellodextrinase. Activities are expressed as percentage hydrolysis/mg. of protein.

| Temperature of treatment | Salicinase activity | Laminarinase activity | Cellodextrinase activity |
|---|---|---|---|
| Control digest | 3·6 | 7·5 | 6·0 |
| 45° | 2·3 | 5·3 | 5·3 |
| 50 | 1·7 | 4·1 | 4·5 |
| 55 | 1·1 | 3·2 | 2·8 |
| 60 | 0·4 | 2·3 | 0·8 |
| 65 | 0·2 | 0·0 | 0·3 |

*Action of acid-treated barley preparation on various β-glucosides.* Digests were prepared from various substrates (5 % solution; 2 ml.) and acid-treated barley preparation (40 mg./ml. in 0·1 M-sodium acetate buffer, pH 5·5; 1 ml.), and examined at intervals by paper chromatography. With laminarin, the products were glucose, laminaribiose and a homologous series of oligosaccharides; with cellodextrin, glucose, cellobiose and a different series of oligosaccharides were produced; and with cellobiose, glucose and several oligosaccharides were formed. [The characterization of the oligosaccharides produced under these conditions was described by Anderson & Manners (1959).] Since glucose alone was not polymerized or degraded, these results show the presence of both β-(1→3)- and β-(1→4)-glucosanase enzymes in addition to cellobiase.

In further digests, six aromatic β-glucosides (2·5 % solution; 1 ml.) were incubated with barley preparation (20 mg./ml.; 1 ml.) at 37°, and the formation of D-glucose was shown by paper chromatography. In quantitative experiments, the digests contained β-glucoside (25–40 mg. in 10 ml. of water), 0·1 M-sodium acetate buffer,

Table 3. *Effect of acid-treatment on the β-amylase and β-glucosidase activities of a barley preparation*

Experimental details are given in the text; 5 mg. of barley preparation being used in each digest. The incubation times were 2 hr. β-Glucosidase activities are based on measurements of reducing power except that for β-(1→4)-glucosanase which was assayed viscometrically with Cellofas B as substrate. The results are expressed as percentage hydrolysis of substrate/mg. of protein, or percentage decrease in specific viscosity.

| Enzyme preparation | β-Amylase activity | Salicinase activity | Laminarinase activity | Cellodextrinase activity | β-(1→4)-Glucosanase activity |
|---|---|---|---|---|---|
| Control | 9·58 | 2·1 | 5·0 | 4·7 | 42 |
| Procedure (*a*) | 7·80 | 1·4 | 3·8 | 3·9 | 42 |
| Procedure (*b*) | 6·10 | 0·8 | 2·8 | 2·1 | 35 |
| Procedures (*a*) and (*b*) | 0·08 | 0·5 | 2·0 | 1·0 | 14 |

Table 4. *Effect of mercuric chloride on β-glucosidase activities*

Experimental details are given in the text. The composition of the digests was standard, 10 mg. of enzyme preparation (a 20–40% ammonium sulphate fraction) being used in each digest. The incubation times were 2 hr., at 35°. Results are expressed as percentage hydrolysis of the substrate/mg. of protein.

| Concn. of $HgCl_2$ (mM) | Salicinase activity | Laminarinase activity | Cellodextrinase activity |
|---|---|---|---|
| 0 | 3·8 | 7·9 | 5·8 |
| 0·001 | 3·7 | 7·3 | 5·6 |
| 0·01 | 1·6 | 4·0 | 4·5 |
| 0·1 | 1·2 | 2·6 | 4·2 |
| 1·0 | 0·5 | 1·4 | 2·4 |

Table 5. *Effect of glucono-(1→4)-lacton on barley β-glucosidases*

Experimental details are given in the text. The composition of the digests was standard, the lactone solution was freshly prepared, and the enzyme preparation was a 50–65% ammonium sulphate fraction.

| | Inhibition of β-glucosidase activity (%) | | | | |
|---|---|---|---|---|---|
| Concn. of lactone (mM) | 1 | 2 | 4 | 8 | 12 |
| Activity | | | | | |
| Laminaribiase | 80 | 91 | 99 | 100 | 100 |
| Cellobiase | 97 | 99 | 100 | 100 | 100 |
| Laminarinase | 67 | 72 | 78 | 79 | 78 |
| Cellodextrinase | 77 | 88 | 94 | 100 | 100 |

pH 5·5 (5 ml.), and barley preparation (20 mg./ml.; 5 ml.). After 76 hr., the results for the percentage hydrolysis of aesculin, arbutin, amygdalin, helicin and salicin determined with D-glucose oxidase were 75, 52, 71, 71 and 50 respectively. After 100 hr., the results were 85, 71, 73, 80 and 74 respectively. Phlorrhizin gave only a trace of glucose, presumably owing to enzyme inhibition by the aglycone phloretin.

*Effect of various inhibitors on β-glucosidase activities.* (*a*) Mercuric chloride. Since this substance is an inhibitor of several carbohydrases, the effect on salicinase, laminarinase and cellodextrinase activities was examined. The results (Table 4) indicate further differences between the two polysaccharase systems.

(*b*) Phenylmercuric acetate and phenylacetate. Preece & Hoggan (1956) reported that phenylmercuric nitrate and phenylacetic acid had a marked effect on the pattern of degradation of barley β-glucosan by barley enzymes. In both cases, exo-β-glucosanase activity was either decreased or destroyed. A barley preparation obtained by precipitation with 50–65% saturation with ammonium sulphate (2 mg./ml. in 0·1 M-sodium acetate buffer, pH 5·6; 3 ml.) was incubated with substrate (3 mg./ml.; 4 ml.) and inhibitor (5 mg./ml.; 1 ml.). After 2 hr. at 37°, samples were deproteinized and their reducing powers determined. The activities, expressed as percentages of that of a control digest, were: (*a*) with phenylmercuric acetate: laminarinase, 40; cellodextrinase, 39; lichenase, 50; (*b*) with phenylacetate: laminarinase, 94; cellodextrinase, 95; lichenase, 87. These inhibitors do not therefore distinguish between the cellodextrinase and laminarinase activities.

(*c*) Glucono-(1→4)-lactone. This lactone has a marked effect on several carbohydrases (see, for example, Conchie & Levvy, 1957; Reese & Mandels, 1959). With a shorter incubation period of 1 hr. to minimize hydrolysis of the lactone to the less inhibitory gluconic acid [the half-life at pH 4·8 and 4° is 145 min. (Reese & Mandels, 1959)] the cellobiase activity of a barley preparation was decreased by 97% by 2 mM-glucono-(1→4)-lactone. Results obtained with a range of lactone concentrations are shown in Table 5, and indicate that at a concentration of 8 mM the lactone completely inhibits the cellodextrinase and β-glucosidase activities, though 22% of the laminarinase activity is retained.

## DISCUSSION

The present results indicate that the β-glucosidase system in barley is even more complex than that envisaged by other workers. Sandegren & Enebo (1952) showed that extracts of barley caused random degradation of ethylhydroxyethylcellulose, activity being optimum at pH 5. This activity, which was ascribed to 'cellulase' and corresponds to our β-(1→4)-glucosanase, increased markedly during germination (Enebo, Sandegren

& Ljungdahl, 1953); evidence for the presence of a trans-β-glucosylase was also obtained.

Since the enzymic activity of malted barley is greater than that of the ungerminated grain, Bass, Meredith & Anderson (1953) and Bass & Meredith (1955) examined the action of malt preparations on barley β-glucosan and concluded that they contained an endo-β-glucosanase, an exo-β-glucosanase that could liberate cellobiose from the ends of β-glucosan chains, and cellobiase. Independently, Preece, Aitken & Dick (1954) and Preece & Hoggan (1956) studied the degradation of barley β-glucosan by barley extracts and also concluded that endo-β-glucosanase, exo-β-glucosanase and cellobiase activities were involved. The exo-β-glucosanase activity was selectively inhibited by phenylacetic acid and phenylmercuric nitrate. However, with both groups of workers, the nature of the linkages hydrolysed by the polysaccharases was not specified.

The presence of separate β-(1→3)- and β-(1→4)-glucosanases in barley extracts is shown by the results in Tables 1–5, although the quantitative assessment of these observations must await the separation of the individual enzymes. As yet we have no evidence for the presence of an enzyme hydrolysing, in stepwise fashion, alternate linkages in cellodextrin (cf. β-amylase action on starch); a cellulolytic enzyme of this type is present in *Irpex lacteus* (Nishizawa & Kobayashi, 1953); for the reasons stated in the introduction section, it is not possible to correlate our results completely with those of Preece and co-workers and of Meredith and co-workers. Moreover, we conclude that barley contains cellobiase and a separate laminaribiase (Table 5), although the maximum size of their substrates is not known. Almond emulsin has also been shown to contain separate cellobiase and laminaribiase enzymes (Cunningham, 1961). The general pattern of a polysaccharase and oligosaccharase for a particular type of glucosidic linkage follows that already established in starch, dextran and pectic acid metabolism, and for laminarin and cellodextrin in their degradation by algal enzymes (Duncan, Manners & Ross, 1956).

It is not yet possible to state the number of β-glucosidases in barley. The salicinase activity is clearly differentiated from cellobiase (Table 1) and, surprisingly, from phenyl β-glucosidase, since the distribution of the two activities in the four fractions is significantly different. Whether or not the other aromatic β-glucosides are hydrolysed by a single group-specific enzyme has not been established. Attempts to separate the β-glucosidase activities by acetone fractionation at −12° were unsuccessful. In unreported experiments, six protein fractions were collected over the range 0–50% (v/v) acetone, but the yields of protein were low, and the distribution of salicinase and laminarinase activities was almost identical. Attempts to selectively adsorb β-glucosanase enzymes on powdered cellulose or on laminarin also gave no significant change in enzyme activity. In general, β-glucosidases exist in Nature as complex mixtures of enzymes as, for example, in almond emulsin and various *Aspergillus* moulds, and separation is extremely difficult (cf. Gascoigne & Gascoigne, 1960).

Glucono-(1→4)-lactone has proved valuable in selectively inhibiting certain activities. The sensitivity of cellobiase to the lactone is similar to that observed by Festenstein (1958), who used 2·5 mM-glucono-(1→4)-lactone to selectively inhibit the cellobiase activity in cellulolytic preparations from sheep-rumen micro-organisms. The results in Table 5 distinguish between four enzyme activities, and since 8 mM-glucono-(1→4)-lactone inhibits all but the β-(1→3)-glucosanase activity, this provides a convenient method for studying its random action. This technique has been applied to the degradation of lichenin by β-glucosidase preparations from malted barley, and resulted in the production of oligosaccharides including $4^3$-β-glucosyllaminaribiose (Cunningham & Manners, 1961).

Dillon & O'Colla (1950) suggested that the ability of wheat β-amylase preparations to hydrolyse (1→3)-linkages might be significant in starch metabolism. This is now considered to be unlikely since: (*a*) the configuration of the (1→3)-linkages in laminarin is opposite to that suggested for amylopectin (Wolfrom & Thompson, 1956); (*b*) a critical re-examination of the latter work suggests that there are, in fact, no (1→3)-linkages in starch (Manners & Mercer, 1963*a*, *b*).

## SUMMARY

1. Extracts of ungerminated barley show hydrolytic activity towards salicin, phenyl β-glucoside, aesculin, arbutin, amygdalin, helicin, cellobiose, laminaribiose, lichenin, laminarin, cellodextrin and sodium carboxymethylcellulose.

2. On the basis of studies involving ammonium sulphate fractionation, temperature-stability and inhibition by mercuric chloride, it is concluded that the salicinase, laminarinase and cellodextrinase activities are due to different enzymes.

3. It is probable that barley contains separate cellobiase, laminaribiase, β-(1→3)-glucosanase and β-(1→4)-glucosanase enzymes, since these activities are inhibited to different extents by glucono-(1→4)-lactone.

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# Studies on the Acylation of Lysolecithin by Rat Brain

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Evidence has been obtained recently indicating that lysolecithin is a normal minor constituent of nervous tissue (Thompson, Niemiro & Webster, 1960; Webster & Thompson, 1962). Previous reports from several other Laboratories have described the presence of small amounts of this lipid in extracts from various other mammalian tissues (see Webster & Thompson, 1962, for references).

In view of the structural and functional changes produced by adding lysolecithin or other lysophosphatides to tissue preparations, which have been the subject of numerous studies in recent years (see Webster, 1961, for references), the origin, further metabolism and physiological significance of lysolecithin in tissues is of considerable interest. The question whether pathological disturbances such as certain diseases of the nervous system might result from abnormalities in the metabolism of lysophosphatides *in vivo* is a further reason for obtaining more information about the biochemistry of these lipids.

Phospholipase A-like activity has recently been demonstrated in preparations of human and of rat brain which can hydrolyse added diacylphosphatides with the liberation of a free fatty acid and the

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