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The Action of Culture Filtrates of the Fungus *Myrothecium verrucaria* on β -Glucosans

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The soil fungus *Myrothecium verrucaria* (*Metarrhizium glutinosum*) is well known for its ability to decompose cellulose, and it has been used in various investigations of that subject (cf. Siu, 1951). This paper describes some properties of a series of culture filtrates of the fungus and our observations seem interesting from two points of view.

(1) There is at present doubt about the nature of extracellular fungal cellulase. From comparisons of various fungi, including *M. verrucaria*, Reese, Siu & Levinson (1950) and Reese & Levinson (1952) concluded that an enzyme C_1 (cellulase; Siu, 1950) transforms insoluble complex cellulose into single molecules of water-soluble cellulose, and that a second enzyme C_2 , a β -1:4-glucosidase, breaks these glucose chains into fragments mainly of two units, i.e. cellobiose. They sometimes found glucose in

their reaction mixtures, but did not say clearly how they thought it might be formed. On the other hand Whitaker (1953), working with purified preparations from *M. verrucaria*, concluded that there is only a single enzyme, capable of degrading complex cellulose to glucose. Kooiman, Roelofsen & Sweeris (1953), also working with *M. verrucaria*, agreed with Whitaker save that they demonstrated the presence of a distinct cellobiase. Our work confirms that of Kooiman *et al.*

(2) We have found that *M. verrucaria* filtrates also attack β -1:3-glucosides such as laminarin and laminaribiose and, in addition, the β -glucosan isolated from barley by Preece & Mackenzie (1952). This has been analysed chemically by Aspinall & Telfer (1954), who concluded that it contains unbranched chains of β -D-glucopyranose units with approximately equal proportions of 1:3- and 1:4-linkages. The course of its breakdown by our filtrates confirms this view and leads to suggestions about the relative positions of these 1:3- and 1:4-linkages.

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METHODS

Preparation of the culture filtrate

The strain of *M. verrucaria* used was obtained from the Centraal Bureau voor Schimmelcultures (Baarn, The Netherlands) and was maintained on the mineral salt solution detailed below, to which a strip of sterile filter paper was added.

Roux flasks of 1 l. capacity, containing 850 ml. of an inorganic salt solution (KH_2PO_4 , 2.68 g.; K_2HPO_4 , 2.09 g.; NH_4NO_3 , 3.0 g.; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 2.22 g.; distilled water to 1 l.) and 20 g. of cellulose powder (Whatman, standard grade), were sterilized, inoculated from the stock cultures and incubated at 25°. By passing a steady stream of sterile air through the culture, the cellulose powder was kept in suspension and the medium aerated. After 2 weeks, the cultures were filtered and sodium azide (0.1%) was added to the filtrates as preservative. Azide was chosen because it is not volatile. Preliminary experiments showed that the same rate of reaction was obtained when the filtrates were preserved with 0.1% azide, with 0.2% fluoride, or by saturation with toluene or thymol.

The activities of five filtrates (A-E) are described in this paper. Protein-nitrogen contents of filtrates A and C were estimated and were found to be 0.02 and 0.006 mg. of N/ml. respectively. The pH of the filtrates varied only between 6.0 and 6.1; addition of NaN_3 raised the pH to 6.4. The filtrates were stored at 0° until required. No decrease in enzymic activity was found to occur within 2 weeks, nor was any reduction in activity detected after several months when stored at -10° or as an acetone-dried powder prepared by adding 4 vol. of pre-cooled acetone at 0°, and centrifuging and drying.

Heat treatment of the filtrate

One portion of the filtrate was left untreated; a second was heated rapidly over a naked flame, with shaking, to 60°, immersed in a water bath at the same temperature for 10 min. and then cooled rapidly; a third was boiled for 5 min.; and a fourth was boiled for 10 min. The boiled filtrates were used only in viscometric experiments.

Substrates

Cotton wool. Absorbent-cotton wool was boiled for 30 min. with 1% aqueous NaOH to remove residual hemicelluloses, washed and dried. It was found that this material was attacked rather more rapidly than unpurified absorbent-cotton wool, suggesting that slight opening out of the molecule had taken place.

Filter-paper powder. Whatman's cellulose powder for chromatography, standard grade. The degree of polymerization was found to be 160 (by G. van Nederveen, Fibre Research Institute T.N.O., Delft, The Netherlands).

Regenerated cellulose. This was prepared by the method of Hirst, Isherwood, Jermyn & Jones (1949). It is believed to have had a chain length of about 220 anhydroglucose units. The product was not dried but was stored at -5° at a moisture content of 78%; if dried, it rehydrates slowly and is comparatively slowly attacked.

Soluble methylcellulose (I.C.I. Ltd.). This has 0.45-0.5 methyl group/anhydroglucose unit, and its molecule must, presumably, contain a proportion of unsubstituted cellobiose units.

Cellobiose (Thomas Kerfoot and Co. Ltd.). This was chromatographically free from glucose and oligosaccharides.

Laminarin and laminaribiose. These were the respective gifts of Mr F. T. Walker and Dr W. A. P. Black of the Institute of Seaweed Research, Inveresk.

Barley β -glucosan. This polysaccharide was first isolated by Preece & Mackenzie (1952) and was given by Professor I. A. Preece (Heriot-Watt College, Edinburgh).

Reductometric determinations

Reaction mixtures consisted of culture filtrate containing 1% (w/v) of substrate, except with cellobiose, where the concentration varied. In earlier experiments they were incubated, unshaken, at 30° but in later experiments, when shaking was adopted, they were incubated at 37°. In controlled experiments, unshaken, no difference in reaction velocity was observed at the higher temperature. Samples were filtered and total reducing power was determined on portions (diluted if necessary) by the method of Somogyi (1952). It was found that 1 ml. of 5 mM- $\text{Na}_2\text{S}_2\text{O}_3$ solution was equivalent to 0.143 mg. of anhydrous glucose or 0.212 mg. of anhydrous cellobiose. When it was desired to estimate glucose and cellobiose separately, glucose was removed by fermentation with baker's yeast (not fermenting cellobiose) and then calculated as the difference between the initial and residual reducing powers.

Chromatography

All solutions to be chromatographed were de-ionized by successive passage over columns of Zeo-Karb 225 and Deacidite G. They were then evaporated to dryness and the residue was taken up in a volume of 10% isopropyl alcohol to give a solution roughly equivalent in reducing power to 2% glucose, or in 1 ml., whichever was the greater. Where the resultant extract fell below the desired concentration, proportionately more spots were put on the papers. All chromatograms were run on Whatman no. 1 paper. The papers were irrigated with ethyl acetate-propan-1-ol-water (57:32:13, by vol., or 57:42:20, by vol., a mixture giving better separation) at 20°, and developed either by spraying with a solution of aniline phthalate in water-saturated butanol and heating or with alkaline AgNO_3 by the method of Trevelyan, Proctor & Harrison (1950).

Viscometric determinations

Culture filtrate was added to 0.7% (w/v) aqueous methylcellulose at 25°. Viscosity measurements were made immediately and at suitable intervals by timing the fall of the meniscus between two points on a 5 ml. bulb pipette. All solutions and apparatus were kept overnight at 25° before use.

RESULTS

Cellobiose

The five culture filtrates differed in their action on cellobiose. With three (A-C), the cellobiose was hydrolysed almost completely to glucose, an activity which was totally absent from the corresponding part of the filtrate which had been heated for 10 min. at 60°. This was observed both reductometrically (Fig. 1), and chromatographically

(Fig. 2) with both filtrate and acetone-dried powder, and indicates the occurrence of heat-labile cellobiase.

On the other hand, with two filtrates (*D* and *E*) acting on cellobiose, no increase in reducing power could be detected. However, chromatograms of the reaction products of filtrate *E* revealed the presence of three reducing ingredients, one of which ran more slowly than cellobiose and might be cellotriose. This spot did not appear when heated filtrate was used, nor was any glucose produced.

Fig. 1. Hydrolysis of cellobiose by culture filtrate *A* of *M. verrucaria*. Unheated (●) and heated (×) filtrate (30 ml.) acting on cellobiose (200 mg.) at 30° without shaking. Arrow indicates 100% conversion.

Fig. 2. Chromatograms of products of action of culture filtrate *C* of *M. verrucaria*, unheated (*u*) and heated (*h*), on cellobiose, methylcellulose and filter-paper powder at 30°: *a* with culture filtrate, *b* and *c* with acetone-dried powder (1% solution in 0.05M succinate-borate buffer, pH 5.6). Solvent composition, 57:32:13; sprayed with aniline phthalate (see Methods section).

Soluble methylated cellulose

When culture filtrates were added to a methylcellulose solution, a rapid fall in viscosity ensued. Heating a filtrate (*E*) at 60° for 10 min. reduced the rate of this change by about half (Fig. 3); but it was not completely abolished by boiling for several minutes, as Kooiman *et al.* (1953) also observed. In experiments with another filtrate (*C*) the change was followed virtually to completion. The viscosity approached that of water (specific viscosity < 1.1), and chromatographic examination of the reaction mixture revealed glucose and traces of cellobiose. Heating the filtrate for 10 min. at 60° had two effects: the reaction was again slowed down but not abolished, and the reaction products contained a higher ratio of cellobiose to glucose (Fig. 2). This difference corresponded with the previously observed inactivation, by such heating, of the system attacking cellobiose. Since, however, some glucose was present (Fig. 2) it must have been produced by the system attacking the cellulose chain. This system, largely heat-stable and apparently capable of degrading cellulose chains at least in large part into cellobiose plus a little glucose, was evidently distinct from the cellobiase in the filtrates.

Later experiments, with different filtrates and better chromatographic technique, gave chromato-

Fig. 3. Diminution of viscosity of methylcellulose solution by culture filtrate *E* of *M. verrucaria*. The digest contained 0.5 ml. of filtrate and 30 ml. of 0.7% (w/v) aqueous methylcellulose at 25°. ●, Unheated filtrate; ○, filtrate heated at 60° for 10 min.; ▲, filtrate boiled for 5 min.; △, filtrate boiled for 10 min.; ■, water (control).

grams in which there were present several slower-running reducing compounds, besides cellobiose. This too was observed by Kooiman *et al.* (1953).

Insoluble cellulose and derivatives

The actions of three different filtrates (*A*, *D* and *E*), and of a solution of the acetone-dried powder of filtrate *C*, on filter-paper powder were followed reductometrically or chromatographically. All the reductometric measurements agreed in showing a rapidly falling rate of breakdown, which practically ceased when only a small fraction of the cellulose had appeared as reducing compounds (Fig. 4). If glucose were the only reducing substance produced, it would correspond to less than 2% of the original cellulose; if cellobiose, to less than 4%. The actual percentage breakdown is therefore probably of this order.

In other respects there were differences. With the first filtrate (*A*), 10 min. heating at 60° stopped the production of reducing power almost, but not quite, completely (Fig. 4 α). This filtrate contained an active cellobiase (that of Fig. 1). With the acetone-dried powder from a filtrate *C*, chromatograms (Fig. 2) showed the chief product with an unheated solution to be glucose; with the heated solution it was cellobiose, traces of glucose appearing only after incubation for 120 hr. This confirmed the previous indication, with methylcellulose, that glucose can be formed by cellulase alone, but only slowly.

With later filtrates (*D* and *E*) the standard heating did not diminish the production of reducing substances to anything like the same degree. Filtrate *D* (used after 3 months' storage at -10°) showed only about 50% inactivation (Fig. 4 β) and, in an attempt to explain this difference, further experiments were made with the same and with a

further filtrate (*E*) in which the reaction mixture was shaken continuously. This considerably accelerated the action of the unheated filtrates and somewhat raised the proportion of filter-paper powder broken down, especially in the last experiment where the shaking was vigorous. But the inactivation by heating was still variable, being roughly three-quarters and two-thirds in the last two experiments (Figs. 4 γ , δ). These differences are still unexplained.

The low degrees of conversion and the variable results of heating, in these experiments, raised doubts about the suitability of the substrate. Hence experiments were made with other forms of cellulose: cotton wool, and a regenerated cellulose, prepared as described above. Stored filtrate *D*, mentioned above in Fig. 4 γ , was used and the mixtures were shaken at a moderate speed. The reducing powers of the mixtures after different times are given in Fig. 5. With cotton wool, the course of the reaction resembled that for filter-paper powder with the same filtrate in that it slowed down greatly after about 12 hr., though the conversion here slightly exceeded that attained with filter-paper powder (cf. Fig. 4 γ). The inactivation as a result of heating appeared greater when the heated filtrate was applied to cotton wool. With the more soluble regenerated cellulose, the reaction was much more rapid and proceeded much further, while the degree of inactivation appeared slightly less than when acting on filter-paper powder.

Fig. 4. Action of unheated (—) and heated (---) culture filtrates of *M. verrucaria* on filter-paper powder (1%, w/v) under different conditions. α , Filtrate *A*, 30°, unshaken; β , filtrate *D*, 30°, unshaken; γ , filtrate *D*, 37°, shaken moderately; δ , filtrate *E*, 37°, shaken vigorously. Arrow indicates 1% conversion (calculated as glucose).

Fig. 5. Action of culture filtrate *D* of *M. verrucaria*, unheated (—) and heated (---), on regenerated cellulose (●) and cotton wool (x) at 37° with moderate shaking. Arrow indicates 1% conversion (calculated as glucose).

Comparison of actions on insoluble cellulose, soluble-cellulose derivatives, and cellobiose

After these experiments we wished to confirm the results by comparing simultaneously the actions of a single filtrate, unheated and heated, on all the various substrates. To accelerate the reaction and avoid premature stasis, the reaction mixtures were shaken vigorously. This filtrate (*E*) had a small reducing power of its own which did not change during incubation; hence this has been deducted from the other determinations which are presented in Fig. 6.

Compared with the filtrate *D* over the first 12 hr., the activity of filtrate *E*, unheated, was about the same on cotton wool, about two-thirds as great on filter-paper powder, and twice as great on regenerated cellulose. Corresponding with the more vigorous shaking, however, the breakdown of cotton wool (like that of filter-paper powder in Fig. 4*δ*) did not slow down noticeably after the first few hours. The inactivation by heating, on cotton wool, filter-paper powder or regenerated cellulose, was less in proportion than with the previous filtrate. The two soluble derivatives were much more rapidly attacked than the insoluble ones, methylcellulose especially; but the breakdown of methylcellulose ceased abruptly at roughly 30% conversion, conceivably because the remaining fragments were too highly methylated to be attacked.

After 53 hr., besides total reducing powers, reducing powers after fermentation of the glucose by baker's yeast were determined. The difference between these values can be ascribed with some confidence to the glucose present, which the yeast ferments; the unfermented residue might include higher oligoses besides cellobiose. These results (Table 1) show clearly that the proportion of cello-

biose to glucose was greater with heated than with unheated filtrate. This in itself would account for part of the apparent inactivation on heating shown in Fig. 6. Hence another estimate making allowance for this difference in composition has been calculated in Table 1, though this estimate too is clearly

Fig. 6. Action of culture filtrate *E* of *M. verrucaria*, unheated (—) and heated (---), on cotton wool (●), filter-paper powder (○), regenerated cellulose (×) and methylcellulose (▲) at 37° with vigorous shaking.

Table 1. Action of *M. verrucaria* culture filtrate on cellulosic substrates

Action of filtrate *E*, unheated (*u*) and heated (*h*) at 60° for 10 min., on cotton wool, filter-paper powder, regenerated cellulose, methylcellulose and cellobiose (all 1%, w/v) after 53 hr. at 37°. Glucose and unfermentable sugars (cellobiose, etc.) were determined as in the Methods section. Weights refer to 1 ml. of digest.

Substrate	Filtrate	Glucose (mg.)	Cellobiose, etc. (mg.)	Glucose + cellobiose (mg. of anhydroglucose)	Approx. breakdown of substrate (%)
Cotton wool	<i>u</i>	0.33	0.36	0.64	6.4
	<i>h</i>	0.09	0.35	0.42	4.2
Filter-paper powder	<i>u</i>	0.12	0.05	0.16	1.6
	<i>h</i>	0.015	0.055	0.065	0.65
Regenerated cellulose	<i>u</i>	1.1	3.35	4.2	42
	<i>h</i>	0.35	2.9	3.06	30.6
Methylcellulose	<i>u</i>	1.28	1.85	2.9	29
	<i>h</i>	0.32	2.25	2.43	24.3
Cellobiose	<i>u</i>	1.45	*	*	*
	<i>h</i>	Nil	12.0	Nil	0

* Incalculable because of production of cellotriose.

approximate, because the influence of oligoses is undetermined and it is unlikely that the proportions of the various constituents were the same throughout.

With all forms of cellulose, relatively small amounts of glucose were produced by the heated filtrate, which was unable to produce any glucose from cellobiose. Hence it is clear here, as in earlier experiments, that this glucose must have been produced by the cellulase system.

In general, the chromatograms confirmed and extended the results of the reductometric experiments. In particular the changes in the proportion of glucose and cellobiose were clearly visible. Methylcellulose, both with heated and unheated filtrate, showed much less cellobiose chromatographically than the reductometric figures in Table 1 indicate. The presence of reducing compounds in addition to cellobiose and glucose was evident in slower-running spots on the chromatogram, conceivably due to higher, possibly methylated, saccharides (see above).

This filtrate was one of those which gave no significant change in reducing power in the presence of cellobiose. The fermentation experiments nevertheless revealed about 1.5 mg. of glucose/ml. of reaction mixture after 53 hr., and both these experiments and the chromatograms revealed greater quantities of cellobiose with the heated (12 mg.) than with the unheated filtrate (< 10 mg.). This difference was clearly not the result simply of cellobiase activity, and with unheated filtrate and cellobiose the chromatogram revealed the existence of a reducing compound running more slowly than cellobiose. Similar observations led Kooiman *et al.* (1953) to suggest a transglycosidation reaction of the type

on which basis the production of 1.5 mg. of glucose from an initial 12 mg. of cellobiose/ml. corresponds to the progress of about half the cellobiose through

the reaction. This system awaits further investigation, but it is clear that whatever the reaction may be, it was wholly abolished when the filtrate was heated, so that the enzyme involved must be different from that attacking cellulose.

Laminarin and laminaribiose

The filtrate (*D*) used in this and the following reaction lacked cellobiase. Its action, unheated, on laminarin was to produce glucose and laminaribiose, whereas, heated, it produced only very small amounts of glucose and no laminaribiose. Both heated and unheated filtrates produced glucose from laminaribiose, no difference in amount being detected chromatographically. It appears therefore that the activity of the filtrate towards laminarin was largely destroyed by heating at 60° for 10 min., whereas that towards laminaribiose was unaffected. These reactions to heating are the opposite of those of the corresponding steps in cellulose breakdown.

Barley β -glucosan

Unheated-culture filtrate attacked barley β -glucosan with the production of cellobiose, glucose and laminaribiose in roughly equal quantities, as estimated chromatographically. Heated filtrate produced roughly the same amount of cellobiose, less glucose and no laminaribiose. These results, together with those with laminarin and laminaribiose, are summarized in Table 2.

DISCUSSION

The variability in enzyme activity between filtrates of cultures, grown under conditions ostensibly the same, is a prominent feature of the above results. The observed differences in cellobiase activity clearly correspond with the various results recorded by Kooiman *et al.* (1953), by Whitaker (1953) and by Levinson, Mandels & Reese (1951). As all attack on cellobiose was prevented when filtrates were heated

Table 2. *Action of M. verrucaria culture filtrate on β -1:3- and β -1:4-linked polyglucoses*

Action of filtrate *D*, unheated (*u*) and heated (*h*) at 60° for 10 min., on laminarin, laminaribiose, cellobiose and barley β -glucosan for 24 hr. at 30°; -, + and ++ represent rough quantitative assessments of the sugars appearing on paper chromatograms prepared as in the Methods section.

Substrate	Filtrate	Sugars detected on chromatograms		
		Glucose	Cellobiose	Laminaribiose
Laminarin	<i>u</i>	++	-	+
	<i>h</i>	+	-	-
		(very faint)		
Laminaribiose	<i>u</i>	++	-	++
	<i>h</i>	++	-	++
Cellobiose	<i>u</i>	+	+	-
	<i>h</i>	-	++	-
Barley β -glucosan	<i>u</i>	++	++	++
	<i>h</i>	+	++	-

for 10 min. at 60°, while that on several forms of cellulose was not, it is plain that the cellobiase is distinct from cellulase.

With heated filtrates, glucose was produced as well as cellobiose from several types of cellulose, which confirms the conclusions of Whitaker and of Kooiman *et al.* that cellulase can produce glucose directly. Nevertheless, in filtrates with an active cellobiase, the ratio glucose:cellobiose was much greater with the unheated than with the heated filtrate, so that, in such filtrates most of the glucose must be produced by cellobiase. The activity towards cellulose was roughly halved by the standard heat treatment, though the exact degree of inactivation varied from filtrate to filtrate, and between substrates.

The broadly similar degrees of inactivation on heating, with insoluble or soluble forms of cellulose as substrate, and in both viscometric and reductometric observations on methylcellulose, are consistent with the view that all forms of cellulose are broken down by a single, chain-splitting β -1:4-glucosidase (C_x), which does not attack cellobiose. But this view is unsatisfying in detail. When two different filtrates acted on several different forms of cellulose, there were quite different proportions between the relative rates at which the several substrates were broken down. Whitaker (1953), working with successively purified fractions of a single filtrate, observed that the proportions between attack on various substrates remained constant, and from this inferred that his preparations contained a single enzyme. On that basis, our different experience suggests that the situation in a less purified preparation may be more complex. Further, in one experiment with cellulose powder (Fig. 4*a*), we observed an inactivation on heating which was so unusually large (95 %) as to hint that some different enzyme might be involved (cf. Saunders, Siu & Genest, 1948). Clearly, however, these experiments give no definite evidence of the existence of a separate enzyme C_1 .

With insoluble cellulose, only a small fraction was broken down at an appreciable rate, and even with soluble-cellulose derivatives breakdown was less than 50 %. With regenerated cellulose this might have been due to an inhibitory effect of cellobiose on cellulase (cf. Reese, Gilligan & Norkrans, 1952), but with cotton wool and filter-paper powder the stasis seemed to be more directly related to the solubility of the substrate than to the small amounts of cellobiose present. This, together with the fact that shaking increases the conversion, suggests that cessation of attack on the cellulose complex is caused by some physical obstacle.

There was some evidence, with filtrates lacking cellobiase, for the existence of another enzyme destroying cellobiose with the production of glucose

and an oligosaccharide higher than cellobiose. This activity, like that of cellobiase, is destroyed on heating at 60° for 10 min. It is not known whether this enzyme is present in all filtrates, since its action would be masked in the presence of an active cellobiase; nor has it been decided what factors in the culture determine whether this enzyme, or cellobiase, is produced in the medium, or both.

The ability of our filtrates to degrade laminarin has recently been confirmed by Morris (1955), who records the production of readily fermentable reducing substances, presumably glucose. The action of the filtrate in producing laminaribiose and greater amounts of glucose from laminarin is analogous to the production of cellobiose and glucose from cellulose, and to the production of xylobiose and xylose by *A*- and *B*-xylanases (Sørensen, 1952). However, the behaviour of the 1:3-glucosidases after 10 min. heating at 60° is the opposite of that observed with the corresponding 1:4-ases; i.e. the hydrolysis of laminarin is almost completely prevented by such treatment and that of laminaribiose is practically unaffected.

When the filtrates act on the barley β -glucosan, the products are cellobiose, laminaribiose and glucose. The cellobiose could be produced by the β -1:4-glucosidase (cellulase), the laminaribiose by the corresponding 1:3-ase, and the glucose, if in no other way, by the action of laminaribiase on laminaribiose. When the culture filtrate has been heated at 60° for 10 min. before reacting with the β -glucosan under otherwise identical conditions, at least as much cellobiose is produced, less glucose and no laminaribiose.

This might be expected from the observed behaviour of the enzymes. The chain-splitting β -1:3-ase is inactivated, hence no laminaribiose should be produced, while the activity of the laminaribiase remains unimpaired. On the other hand, there is partial inactivation of the chain-splitting 1:4-ase producing cellobiose, while the systems producing glucose from cellobiose would be totally inactivated. Hence the production of glucose by either the 1:3- or the 1:4-route should be negligible. Nevertheless, there is a residual production of glucose, conceivably due to the ability of cellulase to produce glucose directly. The above argument depends on the assumption that the β -1:3- and β -1:4-linkages occur in separate groups in the β -glucosan molecule. If, on the contrary, the 1:3- and 1:4-linkages were to occur alternately in the glucose chains the action of the 1:3-ase would yield cellobiose and not laminaribiose, its inactivation by heating should result in a decreased production of cellobiose and the 1:4-ase would not produce any cellobiose. If the 1:3- and 1:4-linkages alternated in that way, our results would be incomprehensible, hence the view is

favoured that at least the greater proportion of these linkages occur in separate homologous groups in the β -glucosan of barley.

SUMMARY

1. Cell-free culture filtrates of *Myrothecium verrucaria* contain a cellulase, producing cellobiose and glucose from complex and single-chain cellulose molecules, which is partially inactivated by heating at 60° for 10 min.

2. No evidence has appeared of the existence, in these filtrates, of a further enzyme breaking down complex cellulose into straight-chain fragments.

3. They also contain, though not always, cellobiase, hydrolysing cellobiose to glucose, which is completely inactivated in 10 min. at 60°.

4. When cellobiase is absent there is evidence of transglycosidase activity, producing glucose and cellobiose from cellobiose. This activity is also completely abolished by heating for 10 min. at 60°.

5. In addition to β -1:4-glucosidases, culture filtrates of *M. verrucaria* contain β -1:3-glucosidases, hydrolysing laminarin and laminaribiose.

6. When β -1:3- and β -1:4-ases act together on barley β -glucosan the products are cellobiose, laminaribiose and glucose.

7. The results obtained after the filtrate has been heated at 60° for 10 min. suggest that the β -1:3- and β -1:4-linkages occur separately in distinct groups in the barley β -glucosan.

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Protein of Pasture Plants

CYTOPLASMIC PROTEIN OF WHITE CLOVER AND ITALIAN RYEGRASS

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Studies of the proteins of the leaves of pasture plants have in general been confined to estimation of the amount of crude protein present, and analysis of this crude protein in terms of amino acid content. (For reviews see Chibnall, 1939; Lugg, 1949.) For this type of work, quantitative separation of the total protein of the leaf is desired, and the physical state of the extracted protein is relatively unimportant. The methods used frequently lead to denaturation, and very little has been learned of the properties of the native protein of the leaf cells.

The methods of extraction which are used when the protein is to be recovered without denaturation have recently been reviewed by Wildman & Jagendorf (1952), who discuss at length the work of

the Californian school on the physical chemistry of the leaf proteins of several higher plants. They draw attention to the compromise aimed at in devising methods severe enough to cause rupture of a reasonably large percentage of leaf cells, yet not so severe as to denature the soluble protein within these cells. The essence of the methods used is cell rupture by mechanical stress, the acid of the liberated vacuolar fluid being neutralized by extracting into buffer solution. The protein is thereby subjected to no chemical denaturant in extraction, and is protected from acid precipitation when liberated; but no safeguard can yet be offered against changes in protein structure brought about by the disorganization of cell contents, soluble and in-