phosphorylated or acylated chymotrypsin of peptides in which the phosphoryl or acyl group is attached to a serine hydroxyl group, (b) the increase in the rate of hydrolysis of acylchymotrypsin as the pH is raised, and (c) the increase in the rate of hydrolysis of phosphorylchymotrypsin as the pH is lowered.

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Substrate Specificity of Rumen Cellulolytic Enzymes

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Cell-free preparations of rumen micro-organisms prepared by butanol extraction have been previously shown to contain at least two types of enzyme involved in the hydrolysis of carboxymethylcellulose, one of these, akin to cellobiase, being selectively inhibited by D-glucono-1 → 4-lactone (Festenstein, 1958a). It has also been shown (Halliwell, 1957a, b) that the type of extract studied is without appreciable action on filter paper or cotton fibres. In the present work, specificity studies have been extended to cellophan and cellulose dextrin and the inhibitory effect of gluconolactone has been examined with reference to specific glucose production from cellulosic substrates, as well as from cellosaccharides up to cellopentaose.

EXPERIMENTAL

Substrates. Soluble cellulose dextrin was prepared from absorbent cotton wool and also from filter-paper powder (Whatman, standard grade) by solution in 72% H₂SO₄, as

described by Kooiman, Roelofsen & Sweeris (1953); small amounts of insoluble cellulose dextrin were removed along with the excess of BaCO₃ added for neutralization of acid and the filtrates were clarified by centrifuging at 10 000 g. The final products, twice precipitated by 80% ethanol, gave values of 20 (filter paper) and 30 (cotton wool) for the degree of polymerization, as determined by the cuprimetric and hypiodite methods used for determination of reducing sugar (Festenstein, 1958a); they were soluble in the buffered solutions used in the enzyme-assay procedure, but small amounts of dilute H₂SO₄ gave precipitates which did not dissolve on subsequent neutralization.

Cellobiose, cellotetraose and cellopentaose were gifts from the British Rayon Research Association; they were chromatographically pure and gave reducing values of 1.0, 1.18 and 1.18 respectively, expressed in terms of reduction of the Nelson-Somogyi reagent (Somogyi, 1952) by equimolecular quantities of glucose; the value obtained for cellobiose (L. Light and Co. Ltd.) was 1.12.

Sodium carboxymethylcellulose containing an average of 0.5 carboxymethyl unit per glucose residue (Cellofas B, Imperial Chemical Industries Ltd.), cellobiose (L. Light and Co. Ltd.) and cellophan (PT 300, British Cellophane Ltd.) were commercial preparations. Cellophan strips weighing 25 mg. were cut from the sheets supplied and soaked in water for at least 48 hr. before use, in order to

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remove soluble plasticizers; specimens which were subsequently dried at 105° showed an overall weight loss of 20%.

Enzyme preparations. These were produced by butanol-extraction of sheep-rumen micro-organisms centrifuged at 1500 g, as described previously (Festenstien, 1958*a*). It was established that three extractions recovered about 50% of the carboxymethylcellulase activity of the intact rumen micro-organisms, measured by incubation in the presence of toluene.

Enzyme assay. Substrates were incubated in 0.02M-citric acid-0.04M- Na_2HPO_4 buffer, pH 6.1 (occasionally pH 5.0), at 37° as described previously (Festenstien, 1958*a, b*). Solutions were deproteinized before estimation of reducing sugar (by the cuprimetric method) and also before the specific determination of glucose by glucose oxidase (Huggett & Nixon, 1957), where high enzyme concentrations interfered with colour development; 1 ml. each of 0.3N- $\text{Ba}(\text{OH})_2$ and 5% (w/v) ZnSO_4 were found sufficient to precipitate the protein in 0.8 ml. of enzyme. Toluene (0.1 ml./ml. of aqueous incubation mixture) was added for assay in the presence of micro-organisms and also for assay of cell-free preparations incubated for longer than 24 hr. (0.2 ml./ml.).

Chromatography. The procedures used were as described before (Festenstien, 1958*a*).

RESULTS

Effect of pH on enzyme activity. The optimum pH varied with substrate and also with concentration of substrate: thus the pH optimum with 0.2% of carboxymethylcellulose was 6.3, whereas with 0.8% of substrate it was 5.6. As the enzyme was not saturated (see below), the pH optima could be expected to vary with substrate concentration (Dixon & Webb, 1958). Generally the optimum pH was found to lie between pH 5 and 6. Since more than one enzyme is possibly involved, the pH optimum will vary if defined in terms of reducing sugar liberated from a long-chain molecule. The amount of D-glucose produced from cellophan in the initial stages of hydrolysis ($\leq 2\%$) was found to be less than the total reducing material expressed as glucose, the difference varying with pH.

Effect of varying substrate concentration. Whereas cellobiase activity reached a maximum at a substrate concentration of 0.1–0.2% and then showed a fall in activity, the activity of carboxymethylcellulase was still increasing with substrate concentration when the solutions became too viscous to concentrate further (1.5%). Increasing concentration of cellophan up to a 3% (w/v) suspension in the incubation mixture, and cellulose dextrin up to 1%, likewise did not reach the maximum activity of the enzyme.

Relative rates of hydrolysis of substrates. The amounts of reducing sugar (expressed as glucose), produced by 0.2 ml. of enzyme from 0.8% of substrate in 2 hr. at pH 6.1, were 800, 125 and 10 μg . from carboxymethylcellulose, cellulose dextrin and cellophan respectively.

Extent of hydrolysis of substrate by prolonged enzyme action

Incubation mixtures containing 0.08% of carboxymethylcellulose and 0.8 ml. of enzyme were analysed at intervals; the reaction was found to be virtually complete after 3 hr., corresponding to 21% hydrolysis of substrate, assuming 0.5 sodium carboxymethyl unit per glucose residue. With 0.08% of cellulose dextrin, under similar conditions, a figure approaching 10% hydrolysis (5% measured specifically as glucose) was obtained.

Reaction of 0.8 ml. of enzyme with a 0.5% suspension of cellophan produced 16% hydrolysis in 7 days and the washed residue after re-incubation with more enzyme was hydrolysed a further 6%. Substantial inactivation of carboxymethylcellulase activity occurred after incubation for 7 days, amounting to about 80% for enzyme incubated alone and nearly 90% for that incubated in the presence of cellophan.

To establish whether a greater concentration of enzyme produced a substantial increase in hydrolysis of cellophan, a preparation concentrated fivefold by evaporation in a vacuum desiccator at 10° was added, along with buffer, in four daily portions, to 5 mg. of substrate, to constitute finally a volume of 1 ml. After incubation for 4 days total, 25% of the substrate had been hydrolysed. The carboxymethylcellulase activity of enzyme incubated alone was 50% of the original and that incubated with cellophan slightly less.

Dr C. A. Marsh kindly investigated the effect of intact rumen micro-organisms on 20 mg. quantities of cellophan and was able to demonstrate 95% solubilization of substrate, after 4 days. An interesting observation here is that the cellophan lost its glossy appearance and became opaque during digestion by the micro-organisms, but did not disintegrate to any considerable extent, whereas after digestion by cell-free extracts there was no difference in appearance but substantial disintegration into small pieces on gentle shaking.

Effect of gluconolactone on enzymic hydrolysis

It has previously been shown that cellobiase may be completely inhibited by gluconolactone, but that reducing-sugar production from carboxymethylcellulose is only partially inhibited under the same conditions (Festenstien, 1958*a*). Present studies with glucose oxidase to measure glucose specifically have shown that glucose production from both carboxymethylcellulose and cellulose dextrin is very largely inhibited ($\geq 85\%$) by 2.5 mM-lactone, whereas the overall reducing-sugar production is not inhibited to the same extent. The effect of gluconolactone on the enzymic hydrolysis of cellosaccharides is shown in Table 1; similar

Table 1. *Effect of D-glucono-1 → 4-lactone on enzymic hydrolysis of mM-cellosaccharides*

Solutions were incubated with enzyme for 45 min. at pH 6.1; in the range investigated cellobiose and cellotetraose were hydrolysed at a uniform rate.

Substrate	Concn. of lactone (mM)	Inhibition (%)	
		Total reducing substances	Glucose
Cellobiose	0.5	—	99
Cellotriose		74	90
Cellotetraose		71	75
Cellotetraose	2.5	78	92
Cellopentaose		75	82

amounts of hydrolysis products were obtained for the substrates investigated, relative percentage hydrolyses (taking cellobiose as 1.0) for the series from cellobiose to cellopentaose being 1.0:2.4:1.4:1.1.

Effect of other inhibitors on carboxymethylcellulase

Halliwell (1957*a*) has shown that toluene (12%, v/v), NaF (0.02M) and thymol (87.5% saturated solution) cause substantial inhibition of hydrolysis of cellulose powder by rumen micro-organisms; the effect of these substances was tested on reducing-sugar production from carboxymethylcellulose (0.4%). It was found that toluene (0.2 ml./ml. of aqueous incubation mixture) had no effect, NaF (0.05M) inhibited very slightly (6%) and thymol (half-saturated) inhibited slightly (17%); solutions without inhibitor indicated about 4% hydrolysis of substrate.

Chromatographic analysis of hydrolysates

Cellophan. Determination of glucose, as well as total reducing substances, showed that after 0.5% hydrolysis of 10 mg. of cellophan, 50% of the reducing material appeared as glucose, the value rising to 84% for 1% hydrolysis and 96% for 3% hydrolysis, remaining in this region even when the limiting figure had been reached (experiments at pH 5). The products obtained from cellophan incubated with enzyme to give >1% hydrolysis were chromatographed and sprayed with aniline phosphate, which detected principally glucose as expected; there were also substances which gave pink colours with the reagent, R_f 0.11, 0.82 and 1.25, the latter spot coinciding with xylose (solvent butanol-pyridine-water, 6:4:3, by vol.). The AgNO_3 reagent of Trevelyan, Procter & Harrison (1950) detected, in addition, traces of substances

with R_f 0.17 and 0.66 (cellotriose? and cellobiose, possibly) as well as an undifferentiated spot stretching from the origin to include cellotetraose (R_f 0.05).

Cellulose dextrin. Investigations after extended hydrolysis (10%) showed that only half the reducing material consisted of glucose, although this was the major single product even after 2% hydrolysis. Several substances appeared after chromatography, including some undifferentiated spots near the origin; specimen R_f values were 0.13, 0.20, 0.36, 0.48 (solvent butanol-pyridine-water, 6:4:3, by vol.) and 0.06, 0.13, 0.22, 0.34 (solvent butanol-pyridine-water, 6:2:3, by vol.). Corresponding values for cellotetraose, cellotriose and cellobiose were 0.05, 0.25, 0.64 and 0.04, 0.17, 0.47 respectively. The products were detected by both benzidine in trichloroacetic acid and AgNO_3 , though the relative intensities of the spots differed; there was inadequate resolution in acetic acid-propan-2-ol-water (10:58.5:31.5, by vol.) (Hash & King, 1954).

Carboxymethylcellulose. Previous studies of hydrolysates obtained in the presence and absence of gluconolactone and analysed by the acetic acid-containing solvent showed undifferentiated substances up to R_f 0.2 with traces of substances with R_f 0.23, 0.58 and 0.74 in addition to cellobiose (R_f 0.80), the substance with R_f 0.23 appearing only in the presence of lactone (Festenstein, 1958*a*). On the basis of the data of Hash & King (1954), who used this particular solvent system and obtained R_f 0.76 for cellobiose, it was suggested that the substances with R_f 0.23 and 0.58 may have been cellopentaose and cellotriose respectively. However, subsequent studies with authentic cellopentaose and cellotriose gave R_f 0.03 and 0.35 respectively and the value obtained for cellotetraose was 0.09 (Hash & King claimed that a substance with R_f 0.35, obtained from hydrolysis of cellulose by *Myrothecium verrucaria* enzyme, was cellotetraose).

Although several other hydrolysates were examined, differentiated spots up to R_f 0.2 were not generally obtained and the substance with R_f 0.23 sometimes extended in position from R_f 0.20 to 0.35. It was possible, however, occasionally to identify substances with R_f 0.01 and 0.07, which were definitely increased in the presence of lactone and migrated with cellopentaose and cellotetraose in butanol-pyridine-water (6:4:3 and 6:2:3, by vol.), though in these solvents cellopentaose hardly moved from the origin; it was not possible to identify the original substance with R_f 0.23 after chromatography with butanol-containing solvents, but it seems likely that it behaved like cellotriose, which appeared in the same area as gluconolactone on the chromatograms.

DISCUSSION

The relative rates of hydrolysis of carboxymethylcellulose and cellophan resemble those obtained by Jermyn (1952) for *Aspergillus oryzae* enzyme and by Holden & Tracey (1950) for snail digestive juice, though in the latter case the enzyme preparation appears to be much more powerful than the rumen extracts, since 70 % hydrolysis of cellophan could be demonstrated in 19 hr.

The lower limiting hydrolysis of cellophan by the rumen preparation, about 25 %, may be due to exposure of resistant crystalline regions in the substrate rather than to the presence of an intrinsically resistant residue requiring a different mechanism for hydrolysis. The physical appearance of the residual cellophan was quite different according as hydrolysis had taken place by the rumen extracts or by the intact micro-organisms. It would seem that the influence of local enzyme concentration and also possibly inhibition by glucose should be further investigated.

Toluene, sodium fluoride and thymol have only small effects on reducing-sugar production from carboxymethylcellulose, yet Halliwell (1957*b*) found substantial inhibition of digestion of filter paper by intact rumen micro-organisms. Since these materials interfere with bacterial metabolism and cause glucose to accumulate, it is possible that continuous removal of glucose and its further metabolism are factors accelerating the initial stages of breakdown of filter paper and also cellophan by intact rumen micro-organisms; also the initial stage of breakdown may require energy, which is provided by the catabolism of glucose.

The effect of gluconolactone on hydrolysis of the lower cellosaccharides shows that inhibition is strongest towards the smallest molecule, cellobiose, and becomes progressively less effective as the chain length increases. However, inhibition of cellopentaose hydrolysis is still substantial, with 2.5 mM-lactone, particularly in respect of glucose production. The general finding of inhibition by lactone, along with the chromatographic studies of the hydrolysis of carboxymethylcellulose in the presence of lactone, suggests that small amounts of cellobiose, cellotriose and higher saccharides accumulate in the presence of lactone, but the dividing line is diffuse rather than sharp and several products accumulate rather than a single one.

The rate of hydrolysis of the cellosaccharides is not significantly increased with chain length, in accord with the finding of Grassmann, Zechmeister, Tóth & Stadler (1933) for *Aspergillus oryzae* enzyme, rather than that of Whitaker (1956) for *Myrothecium verrucaria* culture filtrates. Youatt (1958) has recently shown that a cellobiase preparation from *Stachybotrys atra* hydrolyses cellosac-

charides from cellobiose to cellopentaose, and also a cellulose dextrin of mean chain length 11, at comparable rates, but has no action on carboxymethylcellulose. This particular cellobiase is evidently not inhibited by gluconolactone (M. A. Jermyn, personal communication).

Cellobiose was not detected in cellulose dextrin hydrolysates and only traces appeared in the cellophan hydrolysates; evidence for the higher cellosaccharides was only suggestive, emphasizing the powerful cellobiase activity of the rumen preparations.

SUMMARY

1. Specificity studies on cell-free extracts of cellulolytic enzymes from sheep rumen have been extended to cellophan and cellulose dextrin, which have been found to be hydrolysed at lower rates than carboxymethylcellulose, particularly cellophan.

2. D-Glucono-1 → 4-lactone has been shown to be a specific inhibitor of glucose production from carboxymethylcellulose, cellulose dextrin and also cellosaccharides from cellobiose to cellopentaose, producing at least 80 % inhibition at a concentration of 2.5 mM.

3. Inhibition of hydrolysis of mM-cellosaccharides in the series cellobiose-cellopentaose by 2.5 mM-gluconolactone has shown the hydrolysis of cellobiose to be completely (99 %) inhibited, the inhibition decreasing with chain length to 82 % for cellopentaose. The inhibition measured in terms of reducing sugar is generally less than that in terms of glucose.

4. Chromatographic analysis of carboxymethylcellulose hydrolysates with cellosaccharides as reference substances suggests that small amounts of cellobiose and possibly higher saccharides accumulate in the presence of gluconolactone; other substances, probably substituted derivatives, complicate the analysis.

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Sterol Metabolism

4. THE ABSORPTION OF 7-DEHYDROCHOLESTEROL IN THE RAT*

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It was shown previously that sterols undergoing absorption in the guinea-pig intestine become distributed uniformly among the cell fractions and are not confined to fatty globules (Glover & Green, 1957). The sterol composition of each cell fraction was found to be the same, indicating that an exchange and transfer of the sterols took place between them. It was suggested that this process played an important part in absorption and indicated that sterols were absorbed at the molecular level.

The present work was undertaken to see if a similar situation obtains in another species, the rat, which has been the main experimental animal used for lipid-absorption studies. A preliminary account of the work has been described (Glover & Stainer, 1957).

7-Dehydrocholesterol was selected for the test because the rat intestine, in contrast with that of the guinea pig, contained little (Glover, Glover & Morton, 1952) and its characteristic spectral absorption serves as an excellent label for tracing its movement across the cell.

EXPERIMENTAL

Materials. 7-Dehydrocholesterol was obtained from Peboc Ltd., Liverpool, and was recrystallized from methanol before use until $E_{281}^{1\%}$, 281.8 m μ was 286–290.

Animals. Male rats in the weight range 140–265 g. were divided into groups of two having approximately the same mean weights. Four days before selection for experiment they were placed on a lipid-free diet of solvent-extracted food cubes (no. 4 diet, British Extracting Co. Ltd., Port Sunlight). Control groups (C1–C7) were fasted for 24 hr. and then killed for examination as described previously (Glover *et al.* 1952). Each animal in the test groups (D1–D8) was given a dose of 150 mg. of 7-dehydrocholesterol

dispersed in 0.5 ml. of arachis oil and mixed into 10 g. of lipid-free diet. The animals were killed 5 hr. later for examination. In four experiments (D5–D8), however, each animal was given a similar dose 24 hr. before the final dose. The additional material was administered to ensure a reasonable level of absorption along the intestine at the time of examination.

Preparation of mucosal cell homogenate. The proximal half of the small intestine was removed from each animal and immediately flushed out several times with ice-cold 0.25 M-sucrose solution. The tissue was then cut into 4 in. lengths, opened to expose the mucosa and laid flat on top of large Petri dishes containing salt-ice mixture. The mucosal layer was scraped off gently with the back of a scalpel and transferred with five times its volume of 0.25 M sucrose solution into a pre-cooled Potter–Elvehjem-type homogenizer having a Teflon (Fluon) pestle. The mucosal cells were comminuted for 2 min. with the tube immersed in salt-ice mixture.

The volume of the homogenate was noted (about 25–30 ml.) and a portion, usually one tenth, taken for sterol analysis. The fractionation of the remainder was carried out in a refrigerated centrifuge at -5 to $+5^{\circ}$. A modification of the procedure used by Glover & Green (1957) was introduced for the control groups C4–C7 and the dosed animals D1–D8. It was desired to obtain more concentrated groundplasm fractions for nitrogen assays and electrophoresis studies (results to be reported in another paper). Consequently, the homogenate in the centrifuge tubes was covered with a layer of light petroleum-ether (50:50, v/v) and subjected to 20 000 g for 90 min. The centripetally moving fatty particles collected in the organic solvent (F layer) and the supernatant groundplasm fraction (I) were then carefully separated; the latter was thus obtained without dilution with washings. The sedimented particulate material was recomminuted in the homogenizer with the same volume of 0.25 M-sucrose as before and then fractionated to yield the fractions: 'cell debris and nuclei', mitochondria, microsomes, groundplasm (II). Although the two supernatant fractions, groundplasm (I) and (II), were analysed separately, the results for sterol analysis have been combined for presentation. The above-mentioned microsome fraction is obtained

* Part 3: Glover & Green (1957).