Partial Purification and some Properties of a Cellulase from *Helix pomatia*

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The properties of cellulases from *Helix pomatia* have been studied by many workers, including Seilliere (1906), Karrer, Schubert & Wehrli (1925), Karrer & Schubert (1926, 1928) and de Stevens (1955). Cellulolytic enzymes have also been found in other invertebrates, such as *Teredo* (Greenfield, 1955) and *Ctenolepsima lineata* (Lasker & Giese, 1956), and in a wide range of micro-organisms which include some rumen Protozoa (Hungate, 1942) and bacteria (Halliwell, 1957*a, b*). Many fungi, for example *Aspergillus oryzae* (Gillespie & Woods, 1953) and *Myrothecium verrucaria* (Saunders, Siu & Genest, 1948; Whitaker, 1953; Miller & Blum, 1956; Grimes, Duncan & Hoppert, 1957), have also been shown to produce cellulases.

Although twofold purifications of *H. pomatia* (de Stevens, 1955) and *Teredo* (Greenfield, 1955) cellulases have been reported, only the cellulase from *M. verrucaria* has been purified to give an electrophoretically homogeneous protein (Whitaker, 1953). The properties of this enzyme have been extensively studied; its molecular weight is $63\,000 \pm 15\,000$ (Whitaker, 1952; Basu & Whitaker, 1953; Whitaker, Colvin & Cook, 1954). The enzyme attacks many β -1:4-linked glucosans in an essentially random manner (Whitaker, 1954*a, b*, 1956*a, b*, 1957; Whitaker & Merler, 1956), although there are deviations from this mode of attack with soluble oligosaccharides. The purified enzyme also attacks wheat-straw xylan (Adams, 1952).

The present work was undertaken to purify cellulase from *H. pomatia*, often a more readily available source than a fungal-culture filtrate, and to compare its properties with those of the enzyme from *M. verrucaria*.

METHODS AND MATERIALS

Preparation of crude extract. Hibernating specimens of *H. pomatia* were dissected by the method of Keilin (1956). The digestive tracts and contents were put into a beaker surrounded by ice. They were cut into 1–2 cm. lengths and the mixture was homogenized with an equal volume of ice-cold water in a cooled homogenizer (Potter & Elvehjem, 1936) fitted with a Perspex pestle and surrounded by ice. The tissue was ground at 2000 rev./min. for periods of 30 sec., at 2 min. intervals, until no large particles could be seen. Usually five such treatments were necessary. The

homogenate was centrifuged at 19 000 *g* at 4° for 30 min. and the clear-brown supernatant was dialysed for 16 hr. in cellophan dialysis tubing against a large volume of ice-cold water. Any precipitate which formed was removed by centrifuging at 4° and the resulting solution was used as the starting material from which to purify cellulase. A solution (approx. 100 ml.) containing about 10 mg. of protein N/ml. was obtained from 200 snails.

Preparation of substrates for enzyme assays. At each stage of the purification procedure, the enzymic activity was measured towards cotton linters and Cellofas B.

Bleached cotton linters, kindly given by Lansil Ltd., Shipley, Yorks, were swollen with phosphoric acid for 10 min. at 0° (Walseth, 1952) and the degree of polymerization was estimated by the method of Atchison (1943). The average value was 500.

The degree of substitution of Cellofas B, a soluble carboxymethylcellulose derivative, supplied by Imperial Chemical Industries Ltd. and labelled 'low viscosity grade', was found to be 0.46 by the colorimetric method of Eyler, Klug & Diephuis (1947) and a 0.5% (w/v) solution was prepared. This was stored at 2°.

Methods of cellulase assay. (a) Cellofas B as substrate. Cellofas B (5 ml. of 0.5% solution) was mixed with 0.5 ml. of *m*-buffer (see below) at the required pH and 4 ml. of water in a test tube. The mixture was shaken (50 horizontal strokes of 9.5 cm./min.) in a water bath at 37°. This speed of shaking did not cause any significant loss of enzymic activity (Table 1). Enzyme solution (0.5 ml.) was added and the mixture incubated for 20 min. A sample (2 ml.) was then withdrawn, added to 2 ml. of alkaline copper reagent (Somogyi, 1952) and the mixture heated for 15 min. on a boiling-water bath. It was then cooled rapidly to room temperature, 2 ml. of arsenomolybdate reagent (Nelson, 1944) added, the volume made up to 20 ml. and the colour intensity at 620 μ read on a Unicam colorimeter (model SP. 350) against a blank with boiled enzyme incubated in the same way. The amount of reducing sugar in the sample

Table 1. *Effect of shaking on cellulase activities*

Crude digestive-tract extract was used as the source of cellulase and the incubation periods were 20 min. for soluble-substrate assays and 17 hr. when cotton linters were used.

Duration of shaking	Specific activity	
	Cellofas B	Cotton linters
None	260	210
First half of incubation period	300	220
Second half of incubation period	276	190
Whole incubation period	300	200

was then calculated from a calibration curve prepared from glucose (50–200 $\mu\text{g.}$). Under these conditions the amount of hydrolysis is proportional to the enzyme concentration (Fig 1).

(b) Cotton linters as substrate. Oven-dried-bleached and phosphoric acid-swollen cotton linters (0.2 g.) were weighed into a 20 ml. screw-capped bottle. M-Buffer (1 ml.) of the required pH was added, followed by water to give a final volume of 20 ml. The mixture was incubated at 25° with shaking (60 horizontal strokes of 4 cm./min.) for 3 hr. and 1 ml. of enzyme added. The shaking did not cause any fall in the activity of the enzyme (Table 1). After 17 hr., 2 ml. of the solution was withdrawn and the assay completed in the way already described for Cellofas B.

Units of activity. (a) For the soluble-substrate assays, one cellulase unit was defined as the quantity of enzyme which gave 1 $\mu\text{g.}$ of glucose when incubated for 20 min. at 37° in a reaction medium (pH 5.6, 10 ml.) containing 0.025 g. of Cellofas B.

(b) For the insoluble-substrate assays, one cellulase unit was defined as the quantity of enzyme which gave 1 $\mu\text{g.}$ of glucose when incubated for 17 hr. at 25° in a reaction medium (pH 5.6, 20 ml.) containing 0.2 g. of swollen bleached cotton linters.

(c) Specific activity was defined as the units of activity per ml. of the cellulase preparation divided by the extinction of the solution at 280 $m\mu$, measured in a light path of 1 cm.

Fig. 1. Variation of cellulase activity with enzyme concentration. Activities were measured towards Cellofas B as described in the Methods section.

Ultraviolet-absorption measurements. These were made in a Beckman DU spectrophotometer or a Unicam SP. 500 spectrophotometer.

Nitrogen. Total nitrogen was estimated by micro-Kjeldahl digestion as described by Chibnall, Rees & Williams (1943), followed by distillation and titration, and ammonia nitrogen was estimated by the method of Conway & Byrne (1937).

Buffer solutions. These were prepared from AnalaR reagents. Except where otherwise stated, the buffers used were phosphate from pH 8.0 to 5.6, acetate from pH 5.6 to 3.5 and glycine-HCl at lower pH values (Gomori, 1955).

pH Measurements. These were made with a Pye pH meter and a glass electrode at room temperature, and by standardizing the apparatus with standard buffer tablets, pH values 4.01 and 6.99 (Burroughs, Wellcome and Co.).

Dialysis. Cellophan dialysis tubing (Visking seamless cellulose tubing, Hudes Merchandizing Co. Ltd., London, W. 1) was used wherever possible. When it was necessary to dialyse cellulase preparations for long periods, enzyme preparations in cellophan dialysis tubing 2 cm. in diameter were enclosed in tubing 4 cm. in diameter and then dialysed against a large volume of water. On other occasions, collodion membranes, kindly prepared by Dr G. S. Adair, or pieces of goldbeater's skin (supplied by Frederick Puckridge and Nephew Ltd., London, E. 17), stretched over inverted thistle funnels and attached to them with collodion were used.

Purification of cellulase

H. pomatia cellulase was among the most heat- and acid-labile proteins in the digestive-tract extract; it was not adsorbed on to calcium phosphate gel or aluminium meta-hydroxide under a variety of conditions; it denatured readily in the presence of ethanol or acetone; it precipitated, with other proteins, over a wide range of ammonium sulphate concentrations.

The first stage of the purification procedure (Table 2) was adapted from that used by Keilin (1956) in the purification of helicorubin from *H. pomatia*.

Stage 1. The crude extract (150 ml.) at 0° was made alkaline to litmus by the addition of aq. 10% (v/v) ammonia solution (sp.gr. 0.88), and ice-cold basic lead acetate solution (25%, w/w) was added to give maximum precipitation, further drops of ammonia being added as required to keep the mixture alkaline. The thick precipitate was removed by centrifuging at 4° and 19 000 g for 30 min. and all subsequent operations were carried out at

Table 2. Purification of *Helix pomatia* cellulase

Activities were measured towards Cellofas B. Overall purification was fivefold. Overall yield, 4%.

Stage past	Method	Volume (ml.)	$10^{-3} \times$ Total activity (cellulase units)	Specific activity
	Initial extract dialysed	150	10 650	445
1	Lead acetate-precipitation and dialysis	90	2 125	995
2	Cellulose column			
	(a) Dark-red fractions	60	180	110
	(b) Pale-brown fractions	8	2 000	2600
3	IRC 50 column	3	420	2350

this temperature. The pH of the solution was adjusted to pH 6.0 by the addition of *m*-acetic acid and cold, saturated sodium sulphate solution was added to precipitate excess of lead. After 1 hr. the mixture was centrifuged and 270 ml. of clear-red solution obtained. Solid ammonium sulphate was added until the solution was 80% saturated with respect to ammonium sulphate. A bright-pink precipitate formed, which was removed by centrifuging at 19 000 *g*. It was dissolved in the minimum amount of water, giving 50 ml. of a dark-brown solution. This was dialysed against four changes of 5 l. of water for 16 hr. and was then free from ammonia. The final volume was 90 ml.

Stage 2. Whatman cellulose powder was packed into a column 49 cm. high $\times$ 5 cm. diameter and equilibrated in *m*-acetate buffer, pH 6.0. Material from stage 1 (90 ml.) was adsorbed on to this column and it was eluted at the rate of 60 ml./hr. with the acetate buffer. Fractions (25 ml.) were collected, and immediately after the hold-up volume of the column had passed through, brown fractions were obtained (225 ml.). A colourless solution (1.2 l.) was then obtained. The protein content of each fraction was estimated from the absorption at 280 *m* μ and the cellulase activities of the fractions were measured. The results (Fig. 2) show that the total and specific activities of the first, coloured fractions were low and that the later fractions contained over 50%

Fig. 2. Cellulose-column fractionation of cellulase (stage 2 of the purification). ●, $E_{280\text{ m}\mu}$; ○, relative activity of cellulase (activity of cellulase adsorbed on to the column was taken as unity). Experimental details are given in the text.

Fig. 3. Fractionation of cellulase on an Amberlite IRC 50 column (stage 3 of the purification of cellulase). ○, $E_{280\text{ m}\mu}$; ●, specific activity of cellulase. Experimental details are given in the text.

of the cellulolytic activity adsorbed on to the cellulose. Fractions 1 to 13 and 14 to 62 were each combined, dialysed against 5 l. of 10% (w/v) ammonium sulphate solution for 16 hr. and any precipitate was removed by centrifuging. The solutions were then dialysed against 10 l. of saturated ammonium sulphate solution for 16 hr. The precipitates which formed were removed by centrifuging at 15 000 *g* for 30 min., redissolved in the minimum volumes of water and dialysed against several changes of water until they were free from ammonia.

A dark-red solution (60 ml.) was obtained from the first fractions and 25 ml. of pale-brown solution from the later ones. The latter solution was frozen at -15° and freeze-dried to a powder, which was redissolved in 8 ml. of water.

Stage 3. Amberlite IRC 50 resin was ground to pass a 200-mesh sieve, washed and converted into the Na^{+} form by the method described by Hirs, Moore & Stein (1953). The resin was stirred in water to which 1 ml. portions of 85% (v/v) phosphoric acid were added until the pH of the solution was about 8. The resin was then poured into a glass tube fitted with a no. 3 sintered-glass disk, to give a column 33 cm. high $\times$ 2.3 cm. diameter, a disk of glass-fibre paper was placed on the column and the resin was washed with 0.05*M*-phosphate buffer, pH 7.05, until the solution eluted was at this pH. The pale-brown solution from the previous stage (8 ml.) was adsorbed on to the column. Fractions (2.2 ml.) were collected and the protein contents and cellulase activities of each are shown in Fig. 3. Fractions 15 to 23 contained the highest total and specific cellulase activity and these fractions were combined and dialysed against saturated ammonium sulphate solution for 16 hr. The precipitate which formed was removed by centrifuging and was dialysed against veronal buffer, pH 7.8, *I* 0.2. It was then examined in a Tiselius electrophoresis apparatus (Perkin-Elmer model 38) against the same buffer. Two boundaries were seen in both the ascending and descending limb of the apparatus.

In the descending limb these had the mobilities $0.307 \times 10^{-5} \text{ cm.}^2 \text{ v}^{-1} \text{ sec.}^{-1}$ and $1.078 \times 10^{-5} \text{ cm.}^2 \text{ v}^{-1} \text{ sec.}^{-1}$.

RESULTS

Activity of purified cellulase towards carbohydrates.

The crude digestive-tract extract breaks down a number of carbohydrates (Myers & Northcote, 1958) and the purified material could degrade some of these, but it was inactive towards methyl β -cellobioside and xylans (Table 3).

Variation of activity with pH. The variation of activity with pH in the crude digestive-tract extract and the cellulase purified past stage 2 is shown in Fig. 4. The same pH optimum, pH 5.6, is found for the soluble and the insoluble substrates in both preparations but the optimum is broader in the crude than in the purified cellulase.

Variation of activity with substrate concentration. The Michaelis constant for the cellulase purified past stage 3 (Table 2) was determined with Cellofas B as the substrate and expressing its concentration in terms of the glucose equivalent. It was found to be 80 mM, by the method of Lineweaver & Burk (1934) (Fig. 5). This may be compared with a value

Table 3. *Activity of purified cellulase towards other carbohydrates*

Methods of assay are given by Myers & Northcote (1958). The enzyme used was purified to the end of stage 2. In the crude extract, the specific activity of the enzyme degrading Cellofas B and cotton linters was 350 and in the purified material 2030. The activity of the crude extract on each substrate is taken as 100 and the relative activity in the purified material thus expresses the enrichment towards each substrate during the purification.

Substrate	pH of assay (pH for optimum activity)	Temperature	Relative activity
Cellofas B	5.6	37°	580
Cotton linters	5.6	25	560
Methyl β -cellobioside	4.75	37	9
Laminarin	5.1	37	1300
Yeast glucan	5.5	25	530
Chitin	4.75	25	0
Xylan	4.5	37	0
Ivory-nut mannan A	5.5	37	470
Ivory-nut mannan B	5.5	25	500

Fig. 4. pH-Activity curves for the crude extract of *H. pomatia* and cellulase purified past stage 2. ●, Purified cellulase, cotton linters as substrate and 0.5 mg. of crystalline bovine serum albumin were added in each assay. ○, Crude extract, cotton linters as substrate. No foreign protein was added. ▲, crude extract, Cellofas B as substrate.

Fig. 5. Lineweaver-Burk plot for determination of K_m on Cellofas B. The enzyme preparation was purified past stage 3; 0.25% Cellofas B solution is 80 mM in terms of its glucose equivalent.

of 4.4 mm found by Jermyn (1952) with an impure cellulase preparation from *Aspergillus oryzae*.

Products of enzyme action. The solution purified past stage 3 (0.5 ml.) was incubated for 16 hr. at 37° with 9.5 ml. of 0.25% Cellofas B in 0.05M-acetate buffer, pH 5.6. The solution was then boiled, centrifuged and the supernatant passed through a column of Amberlite IR 120 (8 cm. $\times$ 0.9 cm.) to remove Na^+ ions. The eluate was evaporated under reduced pressure to a thin

syrup, which was examined on a paper chromatogram developed in pyridine-ethyl acetate-water (Jermyn & Isherwood, 1949). A spot corresponding to glucose was seen when the paper was coloured with aniline phthalate (Partridge, 1949). A faintly coloured spot near the origin was also seen; this stained yellow when sprayed with bromophenol blue (0.04%) and was due to a carboxymethyl-glucose derivative.

The same cellulase sample (1 ml.) was incubated for 24 hr. at 25° with 19 ml. of a 1% suspension of

cotton linters, the mixture was filtered and the experiment described above repeated. Glucose only was detected chromatographically.

Temperature stability of cellulase. The crude extract was unstable when heated or cooled to very low temperatures (Table 4).

Ammonium sulphate inhibition of cellulase. The results of activity determinations carried out when different concentrations of ammonium sulphate were present in the assay system are given in Table 5. The amount of reducing sugar was calculated in each case from a calibration curve prepared from glucose (25–200 μ g.) in the presence of the same concentration of ammonium sulphate as that used in the enzyme assay.

The inhibition was shown to be reversible. The initial extract (2 ml.) was dialysed against 2 l. of 10% (w/v) ammonium sulphate solution for 24 hr. at 4° and a portion of the solution shown to have no activity when assayed in the presence of ammonium sulphate. The remaining solution was then dialysed in a collodion sac against several changes of water until it was salt-free. The specific activity of this solution towards Cellofas B was the same as that of the starting material.

Effect of protein on cellulase activity. The activity of the enzyme purified past stage 2 (Table 2) was greater in the presence than in the absence of

Table 4. *Temperature stability of cellulase*

The initial extract (2 ml. samples) was used. Any precipitate formed during the heating or cooling was removed by centrifuging and the supernatants were diluted for assay.

Treatment	Activity (units/ml.)
Crude digestive-tract extract	7500
Heated at 30° for 5 min.	4000
Heated at 50° for 5 min.	940
Heated at 70° for 5 min.	0
Cooled to -15° and thawed	7500
Cooled to -72° and thawed	0

Table 5. *Inhibition of the initial extract by ammonium sulphate*

The specific activity in the absence of ammonium sulphate was 300.

Concn. of ammonium sulphate (g./l. of assay medium)	Relative cellulase activity
0	[100]
5.5	50
31.2	45
76	40
87.5	0
113	0

Table 6. *Effect of added protein on cellulase activities*

Activities were measured towards bleached, phosphoric acid-swollen cotton linters. The activity of both crude and purified materials in the absence of foreign proteins is taken as 100.

Enzyme preparation	Protein added (mg./20 ml. of assay medium)	Relative activity
Initial extract	Lysozyme (50)	100
	β -Lactoglobulin (50)	100
Cellulase purified past stage 2	Insulin (0.5)	150
	β -Lactoglobulin (0.5)	150
	Crystalline bovine serum albumin (0.5)	150
	Lysozyme (0.5)	145
	Lysozyme (10)	150
	Lysozyme (50)	155

Table 7. *Effect of protein on cellulase activity*

Incubations were carried out at 25°. The added protein was 0.25 mg. of crystalline bovine serum albumin/ml. of incubation mixture.

Duration of incubation (hr.)	Reducing sugar formed during incubation (mg.)			
	Cotton linters (1.0% suspension)		Cellofas B (0.025% solution)	
	Protein absent	Protein present	Protein absent	Protein present
1	0.175	0.18	0.16	0.16
3	0.5	0.54	0.28	0.35
5	1.05	1.22	0.31	0.42
8	1.25	1.6	0.40	0.51
12	2.13	3.04	—	—

protein (Table 6) but the activity of the impure enzyme preparation was not affected by the protein. The extents of the hydrolyses of both soluble and insoluble substrates in the presence of added protein were measured over periods of several hours (Table 7). The effect of pre-incubation on the activity of the enzyme in the absence of its substrate is shown in Table 8.

When the initial extract was used, ivory-nut mannan B, lobster chitin and yeast glucan were degraded more rapidly in the presence than in the absence of added protein (Table 9).

DISCUSSION

M. verrucaria cellulase, the only one purified to electrophoretic homogeneity, has a specific activity only twice as great in the pure solution as in the initial extract. The present work has resulted in the separation of a cellulase with five times the specific activity of the starting material. The purified enzyme is not able to attack xylan or methyl β -cellobioside. It is not electrophoretically homogeneous, however, and is able to degrade a number of carbohydrates other than cellulose. In the purification a cellulose column was particularly useful since it was the only way found to separate the enzyme from contaminating pigments; its success was probably due to interaction between the enzyme and its substrate.

All the three fractions which were separated by

the third stage of the purification procedure showed the same order of cellulase activity (Fig. 3). None of these fractions had higher activity than the material adsorbed on to the column, but since the proteins moved at different rates it seems likely that there are at least three cellulolytic enzymes in the digestive tract of *H. pomatia*, possibly differing in their specificities towards the length of glucose chain attacked and the mechanism of the degradation.

Florkin & Lozet (1949) showed that the snail digestive tract harbours cellulose-decomposing bacteria and stated that the cellulase of *H. pomatia* is of bacterial origin: earlier workers (Biedermann & Moritz, 1898; Karrer *et al.* 1925) had assumed that the enzyme was produced by the animal. It is suggested from the present work that since a number of cellulases occur in the digestive tract of *H. pomatia* it is possible that one or more of these are produced by the intestinal flora and others by the animal itself. Tracey (1951) concluded that the cellulase and chitinase of earthworms are of both bacterial and animal origin.

Insoluble substrates are degraded much more slowly than soluble ones, and cellulose is especially inconvenient as a substrate for enzyme assays, since the material is heterogeneous in both its physical and chemical structure and may differ from sample to sample. It was often more convenient therefore to use Cellofas B as the substrate for assays. Only glucose and carboxymethyl-glucose were found from an enzymic hydrolysate of this substrate, and previous work has shown that carboxymethylcelluloses are degraded in the same way as cellulose (Siu, 1951). Further, the same pH optimum, pH 5.6, was found for cellulase acting on both soluble and insoluble substrates in the present case. However, wherever possible, the results obtained with Cellofas B were confirmed with the insoluble substrate.

Xylans are usually found associated with cellulose preparations and the measured activities of the enzyme could have been partly due to decomposition of these polysaccharides (Halliwell, 1957*a, b*). However, during the process of purification the xylanase activity of the original extract (Myers & Northcote, 1958) has been lost and in addition a chromatographic examination of the enzymic hydrolysate of both soluble and insoluble substrates showed the presence of glucose or glucose derivatives only. Preparations of α -cellulose from cotton linters have a very low pentosan content of approximately 0.07% (Adams & Bishop, 1955), and although the original polymer chains have undoubtedly been shortened, especially by the phosphoric acid treatment, the enzymes purified do produce free glucose from these derived glucosans of cellulose and therefore can be defined as cellulases.

Table 8. *Effect of pre-incubation on enzymic activity*

Samples of cellulase (1 ml.) purified past stage 2 and diluted as for assay were pre-incubated as indicated for 17 hr. at 25°. Crystalline bovine serum albumin (2 mg.) was added, as shown in the Table, and activity determinations were made with Cellofas B as the substrate.

Enzyme treatment	Relative activity
Not pre-incubated; no protein added at assay	[100]
Not pre-incubated; protein added at assay	200
Pre-incubated without protein; none added at assay	55
Pre-incubated with protein; none added at assay	190

Table 9. *Effect of added protein on carbohydrase activities towards insoluble substrates*

Assays were carried out at 25° by the methods described by Myers & Northcote (1958). Crystalline bovine serum albumin (5 μ g.) was added to each assay mixture.

Substrate	pH for optimum activity	Relative activity (100 in the absence of protein)
Lobster chitin	4.75	215
Ivory-nut mannan B	5.5	350
Yeast glucan	5.5	195

Basu & Pal (1956) reported that a fungal-cellulase preparation was denatured when shaken during assay. However, it can be seen from Table 1 that there was no loss of activity at the speeds of shaking used during assay in the present work. In fact, the tubes were always shaken to ensure both constant-temperature conditions throughout the liquid and thorough mixing of all the components of the system, a particularly important factor when insoluble substrates were used.

Whitaker (1952) and Basu & Whitaker (1953) found that the cellulase activity of a cellulolytic-enzyme preparation from the culture medium of *M. verrucaria*, 40% of the protein of which was cellulase, was increased by the addition of enzymically inactive protein. They presented evidence that the enhancement of activity was due to some effect of the protein on the cellulose substrate, possibly lowering the surface potential. Tracey (1955), working with a highly active chitinase preparation from *Lycoperdon*, found a similar effect which he attributed to the same cause. He suggested that the effect, if confirmed, should be termed an opsonic effect, in contradistinction to the more usual one which is due to the added foreign protein preventing enzyme denaturation. When, therefore, it was found that the activity of a partially purified cellulase preparation from *H. pomatia* was increased by the addition of a number of proteins the possible mechanism of this was further investigated. The evidence reported suggests that the higher activity in the presence of foreign protein is due to the protection of the enzyme from denaturation. This conclusion is based on the following observations:

(a) The activity is increased with both soluble and insoluble substrates.

(b) The extent of the increase in activity increases with the length of the incubation period (Table 7).

(c) A number of proteins with different properties and isoelectric points give the effect (Table 6).

(d) Pre-incubation of cellulase with protein in the absence of substrate prevents loss of activity (Table 8).

(e) Chitinase, yeast glucanase and ivory-nut mannanase activity in a crude digestive-tract extract from *H. pomatia* were stimulated by the addition of protein with no carbohydrate activity (Table 8). The structures of these polysaccharides differ markedly, as they are made up from different monosaccharide units with different linkages between the units in the molecule, so it is improbable that added protein could have the same effect on each. It should, however, be noted that in these cases the stimulation was observed when the enzymically active solution already contained much inactive protein.

SUMMARY

1. A five-fold purification of a cellulolytic enzyme has been obtained from the digestive tract of *Helix pomatia*. The yield of the enzyme was 4%.

2. Although not specific to cellulose, the purified enzyme did not degrade methyl β -cellobioside or xylans.

3. The purified enzyme showed optimum activity at pH 5.6, was inactivated reversibly by ammonium sulphate and was denatured by heating or cooling.

4. The Michaelis constant of the purified enzyme was 80 mM.

5. The addition of protein to the purified preparation increased its enzymic activity and evidence is presented to indicate that this added protein does not affect the substrate.

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The Transport of Carotenoids, Vitamin A and Cholesterol Across the Intestines of Rats and Chickens

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During his classical studies on the isolation and characterization of carotenoids from the tissues of animals, Zechmeister (1937) noticed that different species of animals are able to store different carotenoids selectively. The animals were broadly classified into four groups according to the nature of the carotenoids in their tissues: thus cows have mostly β -carotene, birds mostly hydroxy carotenoids, man and frogs all carotenoids, while most mammals have none. This has subsequently been confirmed by Goodwin (1950) and by Ganguly, Mehl & Deuel (1953). Ganguly, Krinsky, Mehl & Deuel (1952) and Ganguly *et al.* (1953) have postulated that the presence or absence in the tissues of animals of receptor proteins capable of specifically combining with the carotenoid concerned might be the reason for the wide variation in the distribution of carotenoids as related to species. Although Zechmeister & Tuzson (1934, 1935) and Ganguly *et al.* (1953) were able to conclude from the available indirect evidence that this is due to selective absorption in the intestine itself, very little direct evidence was available. Moreover, the exact mechanism and the exact site of selection in the intestine remained obscure.

The selectivity in the absorption of lipids is not confined to carotenoids alone. It has long been

known that a similar situation is encountered in the absorption of sterols, since animals do not absorb plant sterols efficiently. Recently Glover & Green (1957) have suggested the possible presence in the intestinal cells of lipoproteins capable of combining stereo-specifically with the particular sterol to facilitate its absorption. By the use of techniques similar to those of Glover & Green (1957) it is demonstrated in the present communication that highly specialized mechanisms exist in the mucosal cells of animal intestines for selective admission of the carotenoids into their tissues.

MATERIALS AND METHODS

Light petroleum (b.p. 40–60°; Burmah-Shell) was allowed to stand over an aqueous solution of potassium permanganate for a few days, washed with water, dried over calcium chloride and double-distilled. A.R. acetone was obtained from British Drug Houses Ltd. and A.R. chloroform from Merck. Ethanol was kept over solid potassium hydroxide for 7 days and double-distilled. Antimony trichloride was obtained from Thomas Tyrer Co. Ltd., London; alumina from Merck; Celite 535 from the Johns-Manville Co. Ltd., London; magnesium oxide from British Drug Houses Ltd.; synthetic vitamin A from Hoffman-La-Roche, Basle; and crystalline cholesterol from British Drug Houses Ltd.

β -Carotene. Fresh carrots were minced and saponified at room temperature in brown bottles and exhaustively extracted with light petroleum. The concentrated extracts

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