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A Study of Enzymes that can Break Down Tobacco-Leaf Components

2. DIGESTIVE JUICE OF *HELIX* ON DEFINED SUBSTRATES

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The crop and gut of a starved snail (*Helix aspersa* or *H. pomatia*) contain a reddish-brown liquid (called snail digestive juice in the rest of this paper) that has an acid reaction (Schlemm, 1844). Its pH is in the range of 5·5–6·0 (Kruger, 1925; Duval & Fischer, 1927), and it contains 14–24 % of dry matter (Duval & Fischer, 1927; Režek, 1943). The juice contains protein (Biedermann & Moritz, 1898), and a yellow pigment that is an indicator (Režek, 1943). Values for the surface tension, density, and buffering power are given by Duval & Fischer (1927). Snail digestive juice has been regarded as a secretion of the hepatopancreas, for many of its enzymic properties are shown by hepatopancreas extracts (Krukenberg, 1882), while extracts of the intestine wall do not contain a number of the enzymes (Yung, 1888). Many of the enzymes present in the fresh juice are very stable and are little affected by prolonged storage at 5°. In fact, Voss & Butter (1938) found that the xylanase activity of a preparation after 5 years at room temperature under toluene was nearly as great as that of a fresh preparation.

The juice contains a remarkable number of enzymes; 30 or more have been investigated, and of these about 20 are carbohydrases. When such a complex mixture of enzymes is used on a complex substrate, as it has been in the work described in the previous paper (Holden, Pirie & Tracey, 1950), it is necessary to know what enzymes may be active in producing a particular result. Table 1 is a list of those enzymes that have so far been reported. In this paper the properties and activities of a few enzymes that were of direct interest in our work on the enzymic decomposition of tobacco-leaf fibre (i.e. the portion remaining after mincing leaves and squeezing out the sap) are described. Among these the most prominent was cellulase, which is also the enzyme to which most attention has been given in the past.

Much of the 'cytase' activity observed by early workers was probably due to cellulase. It was found that sections of plant material disintegrated on treatment with snail digestive juice, and that eventually the cell walls could no longer be distinguished. Müller (1901) treated sections of potato tuber with crude trypsin and Merck's 'Ptyalinum siccum'; he regarded the residue as 'pure cellulose'. This material was dissolved by the action of snail digestive juice with the production of sugar. Seillière (1906) found that pure cotton cellulose was not attacked, but after dissolution in Schweitzer's solution and reprecipitation it was hydrolysed to glucose. This work was confirmed by Alexandrowicz (1913), and expanded by Karrer and his school. Untreated cotton cellulose was found to be very resistant to enzymic hydrolysis, although it was readily attacked at an optimum pH of about 5·3 after solution and reprecipitation (Karrer & Illing, 1925). Inactivation of cellulase began at 45–50°, and was complete at 60° (Karrer, Schubert & Wehrli, 1925). If wood was used as a substrate, β-cellulose was attacked more rapidly than α-cellulose; filter paper could be decomposed to the extent of 93 % by prolonged treatment (Karrer & Schubert, 1927). There is an indication that the rate of attack by cellulase is connected with the degree of orientation of the cellulose chains, for the stretching of viscose threads during coagulation increased their resistance to cellulase (Faust, Karrer & Schubert, 1927). Zeise (1931) showed that hydroxyethylcellulose, which is soluble in water, can be used to estimate small quantities of cellulase by observing the reduction in viscosity of the derivative under the action of the enzyme. In a series of papers Ploetz (1939; 1940*a*, *b*, *c*, *d*) found that wood is attacked by snail digestive juice with the liberation of glucose and pentoses. The residues obtained after prolonged enzymic attack contained lignin and carbohydrate apparently in the ratio 1:1. Ploetz suggested that there is a lignin-sugar compound present that is not split by the enzymes of snail digestive juice.

Table 1. *Substrates reported to be attacked by snail digestive juice*

Substrates	Author	Remarks
Starch	Krukenberg (1882)	Amylase action demonstrated has been confirmed by most subsequent workers
	Anker & Vonk (1946)	α- and β-Amylases (pH optima 6·2–6·8 and 4·5) reported
	Karrer, Staub, Weinhagen & Joos (1924)	Activity lost on dialysis at room temperature
Lichenin	Karrer, Joos & Staub (1923)	Both soluble and insoluble forms attacked
	Karrer *et al.* (1924)	Inhibited by glucose and cellobiose
	Karrer & Schubert (1926)	Lichenase activity distinct from cellulase activity
Cellulose	Müller (1901), Seillière (1906)	Discussed in text
Glycogen	Giaja (1914), Gruzewska (1914)	Yeast glycogen rapidly removed. Gruzewska showed starch and glycogen dextrins also split
Yeast 'cellulose'	Giaja (1914)	Yeast cell membrane dissolved
Laminarin	Gruzewska (1920)	Laminarin attacked. Both yeast cellulose and laminarin appear to be 1:3 glucose polymers (Hassid *et al.* (1941) and Barry (1941))
Inulin	Bierry (1910), Karrer *et al.* (1924)	
Laevoglucosans	Karrer & Kamienski (1932), Karrer & Harloff (1933)	A number of laevoglucosans (not attacked by emulsin) were hydrolysed
Mannans, mannogalactans	Bierry & Giaja (1909)	Bierry & Giaja found that mannose was liberated from the mannan of ivory nut and date and that both mannose and galactose were liberated from the mannogalactans of lucerne and fenugreek
Araban	Colin & Lemoyne (1936)	Seillière (1907) was unable to show any action on araban. Colin & Lemoyne (1936) found arabinose was liberated when washed sugar-beet pulp was used as substrate
Xylan	Seillière (1905)	pH optimum 4·65 (citrate buffer), 5·28 (phosphate buffer)
	Ehrenstein (1926)	
Chitin	Karrer & Hofmann (1929)	pH optimum 5·2
	Karrer & White (1930)	*N*-acetyl group essential for hydrolysis
	Neuberger & Pitt-Rivers (1939)	*N*-formyl or *N*-acetyl essential for hydrolysis
Polygalacturonic acid	Ehrlich (1932)	Pectin as substrate
	Colin & Lemoyne (1940)	Pith of *Helianthus annuus* attacked with production of galacturonic acid
Oligosaccharides		
Raffinose	Bierry & Giaja (1906)	
Gentianose and stachyose	Barthet & Bierry (1908)	
Rhamninose	Bierry (1909*a*)	
Manninotriose	Bierry & Barthet (1909)	
Disaccharides		
Lactose, maltose and sucrose	Bierry & Giaja (1906)	
Maltobionic acid, maltosazone, lactobionic acid, and lactosazone	Bierry & Giaja (1908)	
Glycosides		
Amygdalin	Bierry & Giaja (1906)	
α- and β-Methyl-glucosides	Bierry (1909*b*)	
α- and β-Methyl-galactosides	Bierry (1913)	
Arbutin	Graetz (1929)	
Steviosin	Bridel & Lavieille (1931)	
Verbonaloside	Cheymol (1938)	
α- and β-Phenyl-*N*-acetyl-D-glucosaminides	Zechmeister, Tóth & Vajda (1939)	

Table 1 (*cont.*)

Substrates	Author	Remarks
Fats	Yung (1888)	Olive oil emulsified
	Graetz (1929)	Optimum pH 5·7 (acetate buffer), 9 (glycine buffer)
Glycerophosphates	Karrer & Freuler (1926)	Hydrolysis most rapid at strongly acid pH's
Pyrophosphate	Režek (1943)	pH optimum about 2
Acetylcholine	Mentzer, Kaswin, Corteggiani & Gautrelet (1936)	
	Augustinsson (1946)	
Proteins	Rosén (1930, 1933, 1937)	Protease intracellular in wall of mid-gut, none detected in gut contents
	Bawden & Pirie (1946)	Little or no protease detectable
Peptides	Rosén (1933, 1937)	Peptidases were found in gut contents
Lignin	Fernandez & Regueiro (1946)	Possibly demethylated

MATERIALS AND METHODS

Materials. The enzyme preparations used were obtained from the crops and guts of starved *H. aspersa* or *H. pomatia* in the manner described by Bawden & Pirie (1946). A comparison of material obtained in this way from *H. aspersa* and *H. pomatia* is given in Table 2. Preparations were stored at 5° with no added antiseptic and were centrifuged clear before use if necessary.

Table 2. *Properties of digestive juices of* Helix aspersa *and* H. pomatia

	H. aspersa	*H. pomatia*
Average vol. of undiluted digestive juice/snail	0·1 ml.	0·5 ml.
Dilution of juice, used in the experiments reported	1/10	1/3
	Diluted juices	
Dry matter (g./l.) (after centrifuging at 1400 *g* for 15 min.)	30–35	90–100
Total N (g./l.)	3–4	9–13
Trichloroacetic acid-precipitable N (g./l.)	2–3	6–10
Carbohydrate (g./l.) (orcin method)	6–10	20–30
Relative viscosity	1·7*	2·6*
Percentage dry matter sedimented at 90,000 *g*	4·5*	4·0*
Ash (g./l.)	4–6	6·4*
Calcium (g./l.)	0·15–0·25	Approx. 1·0

* Results of single determinations.

Snail digestive juice has a reducing value which, measured by a modification (Hanes, 1929) of the Hagedorn and Jensen method, is about 80% of the total carbohydrate equivalent as determined by the orcin method, and may be higher when determined by the hypoiodite method. The reducing value is only partly due to free sugars, since the precipitate obtained on addition of two volumes of ethanol to the fluid contains about 70% of the original reducing value. Precipitation with tungstic acid and with $ZnSO_4 + NaOH$ removes about 65% of the reducing value from solution. During storage a precipitate separates out and the carbohydrate and reducing values of the centrifuged juice fall. This sediment has a carbohydrate content equivalent to about 15% of its dry matter and contains 12–13% N. After 6 months about half the total dry matter may be precipitated in this way. The material has no cellulase activity using cellophan as substrate.

Dialysis of snail digestive juice. Vellum, nitrocellulose, and rabbit bladder were tested as dialysing membranes, as cellophan is rapidly digested by snail digestive juice. Nitrocellulose, even in the form of fine particles, is not attacked by snail digestive juice. The composition and cellulase activity of the fluid was little altered by dialysis in vellum or bladder for 48 hr. Dialysis in a nitrocellulose membrane resulted in the loss of about 25% of the dry matter and some cellulase activity was lost. Fåhraeus (1947) has also found that cellulase activity is lost when a cell-free extract of *Cytophaga globulosa* (*Sporocytophaga myxococcoides*) is dialysed in a nitrocellulose membrane. This loss of activity could be due to adsorption on the nitrocellulose, as nitrocellulose is sufficiently similar to the normal substrate for specific adsorption to occur. The possibility was tested by adding nitrocellulose particles, which might with their large surface area give a marked reduction in enzyme activity. In some experiments a reduction in enzyme activity was obtained both when the enzyme was treated with nitrocellulose particles which were then removed, and when nitrocellulose particles were present during the action of the enzyme on cellophan. The results were very erratic and large reductions in activity were never found. For all the work reported, snail digestive juice was used without dialysis.

Since many samples of snail digestive juice obviously contain bacteria, it was necessary to examine the possibility that some of the enzymes present were of bacterial origin. This was done with special reference to cellulase. It is impossible to exclude the possibility that cellulase, or other enzymic activity, is due to the presence of stable extracellular enzymes produced by bacteria in the gut of the living snail. If this were so, it would also be necessary to suppose that the production of the enzyme ceased after the removal of the liquid from the gut, for cellulase levels do not rise or fluctuate on storage. The possibility that cellulase activity is due to intact bacteria was excluded by centrifuging a sample of snail digestive juice at 95,000 *g* for 30 min., which would remove any bacteria that it is reasonable to postulate. There was no loss of cellulase activity per

unit dry matter in the supernatant liquid. These experiments were not repeated for other enzyme activities since, like cellulase, they did not fluctuate during storage.

Analytical methods. General analytical methods were those described in the previous paper (Holden *et al.* 1950). Methods developed for the estimation of particular enzymes are described in the following sections.

EXPERIMENTS, RESULTS AND DISCUSSION

Properties of snail cellulase

Cellophan, in the form of tubing for dialysis, was used as substrate in most of the experiments described. Equal lengths of tubing, weighing about 100 mg. air dry, were cut, numbered by scratching and weighed. All were then soaked in water for several hours to remove soluble plasticizers.

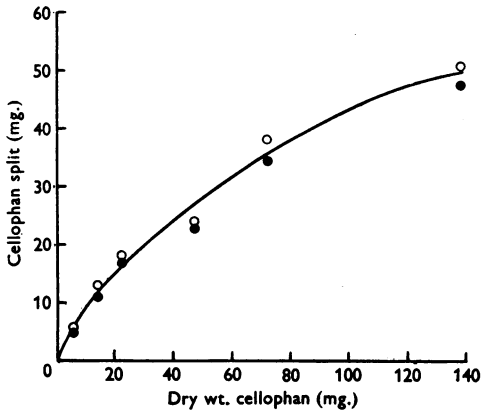

Fig. 1. Variation in cellulase activity with amount of substrate. Weighed pieces of cellophan + 0·5 ml. snail digestive juice + 0·5 ml. water + 2 ml. pH 5 Michaelis veronal-acetate buffer. Incubated 19 hr. at 40°. O——O, Loss in weight of cellophan; ●——●, glucose found calculated as cellulose.

After soaking, the pieces were split open, crumpled, and placed in centrifuge tubes with buffer solution and enzyme, and incubated at 35°. A substrate blank (cellophan + buffer) was set up to indicate any bacterial contamination during incubation. Since material is precipitated from snail digestive juice during incubation, blanks without substrate at all pH's used were set up. In order to calculate the weight of cellulose added to each tube, pieces of soaked tubing were dried at 100° and reweighed. The weights of the dry washed pieces were used to calculate the percentage dry matter of the air-dry cellulose, and hence the weight of cellulose in each tube. At the end of the experiment all tubes were centrifuged, and the total soluble carbohydrate estimated in the supernatant. Cellulose loss was calculated as 90% of the increase in glucose found. In a number of experiments the undigested cellulose was washed free from snail digestive juice and its loss in weight used as a check on the first method. The two methods agreed well.

pH *optimum.* The pH optimum using cellophan as substrate was found to lie between pH 5 and 6 in agreement with the results of previous workers. The optimum pH for cellulase action is discussed further by Holden (1950).

Variation in activity with amount of substrate. Sheet cellophan is insoluble in water, but the effective concentration is proportional to the area exposed to attack, and hence to the weight added. The amount of substrate split in unit time by the same amount of enzyme increases with the amount of substrate, but not linearly (Fig. 1).

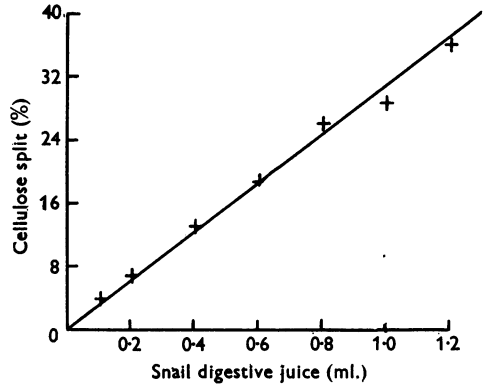

Fig. 2. Variation in cellulase activity with amount of enzyme. Weighed pieces of cellophan (approx. 100 mg. dry weight) + 1 ml. 0·2 M-acetate buffer (pH 5) + water and snail digestive juice to give final volume 4 ml. Incubated 21 hr. at 35°.

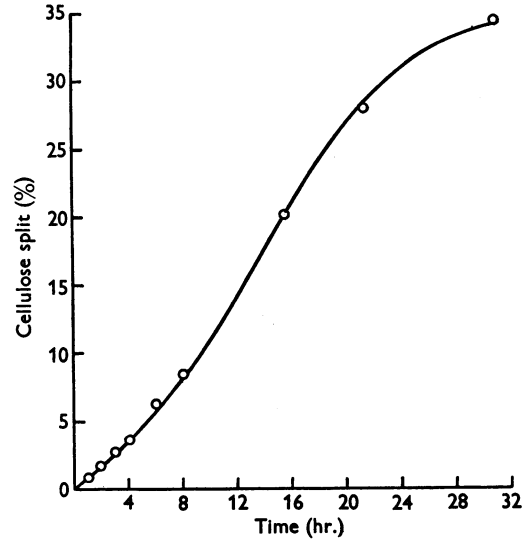

Fig. 3. Variation of cellulase activity with time of action. Weighed pieces of cellophan (approx. 80 mg. dry weight) + 1 ml. snail digestive juice + 1 ml. 0·2 M-sodium phosphate buffer (pH 5·4) + 2 ml. water. Incubated for different lengths of time at 35°.

Variation in activity with amount of enzyme. With the amount of substrate used (approx. 100 mg. dry matter) the activity was proportional to the amount of enzyme used for amounts up to 1 ml. of snail digestive juice (Fig. 2). The method was therefore suitable for comparing the activities of different preparations.

Variation of activity with time of action. Fig. 3 shows that the rate of action at 35° increased initially and then diminished after 16 hr. This effect is presumably due to an increase in the surface of the substrate during the early stages of enzyme action. It was observed that the cellophan became fragile early in the action, and was readily broken into small pieces.

Effect of salt on action of snail cellulase. The inhibitory effect of high concentrations of NaCl and $CaCl_2$ is shown in Table 3. The enzyme action is inhibited at a lower concentration of $CaCl_2$ than of NaCl. Similar salt effects were observed when leaf fibre was the substrate (Holden *et al.* 1950).

Table 3. *Effect of salt on action of snail cellulase*

(Weighed pieces of cellophan (approx. 80 mg. dry matter) + 0·5 ml. snail digestive juice (pH 5·3) + 3·5 ml. water containing the amount of NaCl or $CaCl_2$ needed to give the required final concentration. Incubated 21 hr. at 38°. The final concentration of Ca derived from the snail digestive juice was 0·0006 M and that of NaCl was <0·006 M.)

Final concentration of added salt (M)	Percentage of cellulose split	
	NaCl	$CaCl_2$
1	5·9	—
0·5	5·8	—
0·25	7·8	1·6
0·1	12·6	4·8
0·05	13·5	6·9
0·025	14·6	10·2
0·01	15·0	12·5
0·005	—	15·0
0·002	—	15·1
No salt added	15·2	14·6

Effect of temperature in the absence of substrate. (*a*) Short periods at high temperatures: 2 ml. of snail digestive juice were kept at the temperatures shown in Table 4 for 10 min. After cooling, the activities of the heated portions of juice were compared with that of an unheated control. There was no detectable activity in the sample heated to 86° when tested on cellophan, but some activity could be detected even in a sample heated to 100° when tested by the much more sensitive method (see p. 412) using carboxymethylcellulose as substrate.

Table 4. *Effect of temperature on stability of snail cellulase*

(2 ml. portions of snail digestive juice kept for 10 min. at temperatures given. When cool, set up with weighed pieces of cellophan (approx. 80 mg. dry matter) + 2 ml. water + 1 ml. 0·2 M sodium phosphate buffer (pH 5·4). Incubated for 24 hr. at 35°.)

Temperature (°)	Percentage of cellulose split
(Control) 5	72·3
54	44·3
65	7·3
75	2·9
86	0

(*b*) Long periods at 35°: samples of snail digestive juice were kept at 35° for 1 and 3 days in the absence of substrate. The activities of the incubated samples were then compared with that of a control sample kept at 5°. The control sample split 72% of the substrate, that incubated for 1 day split 63%, and that incubated for 3 days split 36%, all being tested under similar conditions.

Effect of exposure to extremes of pH. The preparations used were stored without adjustment of pH and were usually at a pH of 4·5–5·5 when used. Fresh preparations were usually at the higher pH's of the range and a slow acid drift occurred with ageing. Samples of juice were brought to various pH's and allowed to stand 3 hr. at room temperature. Their activities were then compared at pH 5·4 after allowing for any dilution during pH adjustment. The results are shown in Table 5. Inactivation was rapid below pH 4 and above pH 9.

Table 5. *Effect of* pH *on stability of snail cellulase*

(Portions of snail digestive juice adjusted to pH values shown then all brought to the same volume. Activity measured after standing 3 hr. at room temperature. Weighed pieces of cellophan (approx. 80 mg. dry matter) + 1 ml. snail digestive juice + 3 ml. 0·2 M-sodium phosphate buffer (pH 5·4) + 1 ml. water. Incubated 17 hr. at 35°.)

pH	Percentage of cellulose split
2·4	2·1
3·7*	11·0
5·0	23·6
6·8	21·1
8·6	19·3
10·9	3·2

* Precipitate formed which had negligible activity.

Variation of activity with age and between preparations. A large number of preparations of digestive juice from *H. aspersa* and two from *H. pomatia*, after being stored for varying periods, were tested at the same time. The activities of *H. aspersa* preparations from 1 to 22 weeks old were found to be of the same order. Preparations 15 months to 3 years old had activities slightly less than half the mean for the others. The two *H. pomatia* preparations had activities on a dry-matter basis similar to that of *H. aspersa* preparations of the same age.

The action of cellulase on cellulose derivatives. Zeise (1931) showed that hydroxyethylcellulose, which is soluble in water, can be used to estimate small quantities of cellulase by observing the reduction in viscosity of the derivative under the action of the enzyme. Three cellulose derivatives soluble in water were tested as substrates for snail digestive juice. Sodium carboxymethylcellulose (Cellofas WFZ) in 0·5% (w/v) solution, ethylmethylcellulose (Cellofas WLD) in 1·2% solution (both from Imperial Chemical Industries Ltd., Nobel House, Stevenston, Ayrshire) and methylcellulose in 0·5% solution all showed a marked reduction in viscosity and an increase in reducing value under the action of snail digestive juice at pH 5. The increase in reducing value was measured by both the Hagedorn and Jensen method, and by the modified Willstätter and Schudel hypoiodite method. The first method gave higher results than the second. It was found that it also gave anomalously high results with cellobiose, whereas the hypoiodite method gave results in accordance with theory. The hypoiodite method was therefore regarded as the more

reliable. Results of exhaustive hydrolysis by snail digestive juice are given in Table 6. Initial hydrolysis is rapid, reaching values over half the final values in an hour.

Table 6. *Hydrolysis of substituted celluloses by snail cellulase*

(100 ml. of solution contained 8 ml. 0·2 M-sodium acetate-acetic acid buffer, 2 ml. snail digestive juice, and either 811 mg. sodium carboxymethylcellulose or 1275 mg. ethylmethylcellulose. The pH of both solutions was 5·0, and had not changed at the end of the experiment. The solutions were incubated at 35° for the times given. At 160 hr. a further 2 ml. of snail digestive juice were added. Blank values for snail digestive juice and substrate at each time were determined and have been allowed for. Analyses were made by the modified Willstätter and Schudel hypoiodite method (Holden *et al.* 1950) using glucose as a standard. For ethylmethylcellulose the basis of calculation used is equivalent to following the loss by the substrate of residues substituted by 1·3 methyl substituents, this residue weight being the same as the mol. wt. of free glucose. The ratio of methyl to ethyl substituents in the substrate is not, however, known.)

Time (hr.)	Sodium carboxymethylcellulose. Reducing sugar formed (calculated as singly substituted residues) as percentage substrate weight	Ethylmethylcellulose. Reducing sugar formed (calculated as glucose) as percentage substrate weight
18	35	12
42	37	13
90	38	14
160	39	16
232	43	17
310	41	17

The increase in reducing value with sodium carboxymethylcellulose was equivalent to the splitting of 40% of the substrate; for ethylmethylcellulose the figure was 17% (Table 6). The degree of substitution of the sodium carboxymethylcellulose used was stated by the manufacturers to be 0·45, i.e. on an average, 1 residue in 2·2 has a substituent group. On the assumption that the ratio of the reactivities of the 2-, 3- and 6-hydroxyl groups is independent of the degree of substitution of the cellulose chain as a whole or of the state of substitution of neighbouring hydroxyl groups, Spurlin (1939) calculated the proportions of residues of different degrees of substitution to be expected in cellulose derivatives of different degrees of substitution. The results obtained agreed well with the few published figures. Using Spurlin's data, about 60% of the residues in the sample of carboxymethylcellulose used would be expected to be unsubstituted, or about 36% of the residues would be both unsubstituted and next to other unsubstituted residues. Thus if only those glycoside links are split that lie between two unsubstituted residues, it would be expected that about 36% of the substrate would appear as reducing sugar after exhaustive enzyme action. The amount found, 40%, is in fair agreement with this expectation. The degree of substitution of the ethylmethylcellulose used is stated to be 1·3. Making the same assumptions as before, this corresponds to 18% of unsubstituted residues and 3·3% of unsubstituted contiguous residues. The figures in Table 6 show that 17% of the links are attacked, so it would appear that in this instance only one of a pair of contiguous residues need be unsubstituted for the link to be attacked. It is assumed that in neither case is the enzyme system capable of removing single unsubstituted residues from the ends of the chains formed when the first bond is attacked.

As might be expected, the rate of hydrolysis of cellulose derivatives was found to be much greater than that of cellophan; the derivatives, being soluble in water, present a vastly greater surface to the attack of the enzyme. Very dilute solutions of snail digestive juice were therefore used to follow the change of viscosity of the cellulose derivatives. Another factor also makes the viscosimetric method sensitive to very small amounts of enzyme. If all the chains of carboxymethylcellulose are split in half, the increase in reducing value will only be about 0·3% which is hardly detectable, but the viscosity increment (relative viscosity − 1) will be reduced to a considerable extent. A similar effect has been described with the pectic acid-polygalacturonase system; thus Jansen & MacDonnell (1945) observed a 50% loss of viscosity increment at 2% hydrolysis. In the present work, viscosity measurements were made on solutions of derivatives (0·3–1·2%, w/v) having relative viscosities of about 3·0. All solutions had the same salt concentration (0·05 M). Ostwald viscometers with capillaries of about 20 cm. and flow times of about 90 sec. for 8 ml. water at 20°

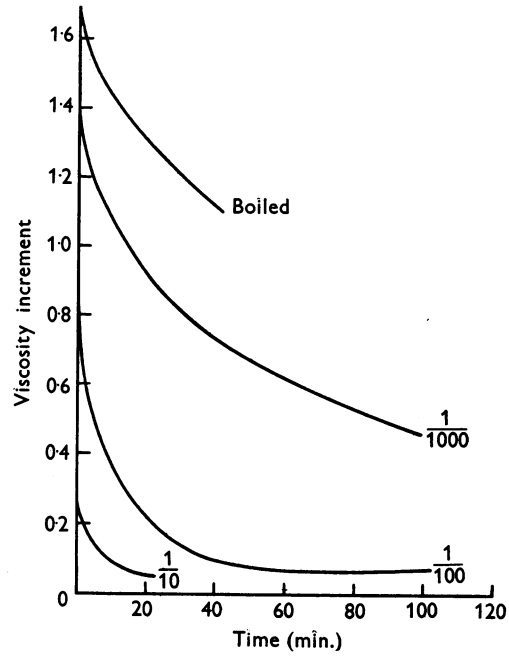

Fig. 4. Viscosity reduction of sodium carboxymethylcellulose with cellulase. 10 ml. of solution contained 2 ml. pH 5 Michaelis veronal-acetate buffer, 1 ml. *H. aspersa* juice of the dilution indicated and 32·5 mg. sodium carboxymethylcellulose; incubated at 20°. 1 ml. of the supernatant from a sample of *H. aspersa* juice that had been heated 10 min. in a boiling-water bath and then cooled and centrifuged was used for the 'boiled' determination.

were used. It was found that 1 ml. of 1:1000 dilution of *H. aspersa* digestive juice (dilution from original crop contents 1:10,000) added to 9 ml. of sodium carboxymethylcellulose (final concentration of sodium carboxymethylcellulose 0·32%, veronal-acetate buffer (pH 5·0) final molarity 0·05 M, Michaelis, 1931) halved its viscosity increment in about 50 min. at 20°. Since the viscosimetric method proved so sensitive it was used to estimate the amount of enzyme destroyed by heating for 10 min. at 100° (Fig. 4). It was found that about 0·03% of the activity remained after this treatment.

Snail polygalacturonase

The polygalacturonase activity of snail digestive juice was compared with that of a purified fungal preparation (from Dr H. Lineweaver) by determining the amount of reducing sugar produced from pectic acid under standard conditions (method based on that of Jansen & MacDonnell, 1945). The pH optimum was found to be in the range pH 4–5 and was not as sharp as that of the fungal preparation. High salt concentrations were found to be inhibitory, inhibition beginning at 0·125 M-NaCl and becoming almost complete at 0·5 M-NaCl. Ca ions, even in very low concentration (0·0005 M), had an inhibitory effect. Ca likewise inhibited the polygalacturonase action of snail digestive juice on leaf fibre (Holden *et al.* 1950). The contents of one crop of *H. pomatia* hydrolysed pectic acid at the same rate as 0·015 mg. purified fungal polygalacturonase. The corresponding figure for *H. aspersa* was 0·003 mg. The crop contents of *H. aspersa* and *H. pomatia* had similar activity on a dry-matter basis.

Snail pectin esterase

The pectin esterase activity of snail digestive juice is very low, being of the order of 10^{-5} units/ml. when determined by measurement of the methanol liberated from citrus pectin under standard conditions (Holden, 1946). Incubation periods of 24–28 hr. were necessary to obtain amounts of methanol that could be estimated with any accuracy. Holden (1945) stated that snail digestive juice did not demethylate the pectin in tobacco-leaf fibre. As the amount of pectin esterase activity is so low, this result is not unexpected.

Snail amylase

Fresh snail digestive juice preparations have an amylase activity of the order of one-tenth that of saliva. Snail amylase was found to be more active at pH 4·5–5·5 than at pH 7. The amylase activity of one preparation decreased during the first month of storage to about 50% of its initial value. Thereafter there was little change in activity for a period of 3 months. Old preparations have little or no activity.

Action on xylan

The observation of Seillière (1905) that xylan is hydrolysed by snail digestive juice has been confirmed using a preparation of maize xylan. In one experiment 56% of the dry weight of the xylan appeared as xylose after incubation for 5 days at pH 5·1. Hydrolysis also occurred at pH 7, but to a lesser extent.

Fructosans

The weak inulase activity reported by Bierry (1910) was confirmed. After incubation for 10 days at pH 5·3, 25% of inulin (Kahlbaum) was split by a preparation of digestive juice of *H. aspersa* and 66% by one of *H. pomatia*. Under the same conditions a levan from grass and a levan from *Bacillus subtilis* were both split 33% by the digestive juice of *Helix aspersa*, and 50% by that of *H. pomatia*. Irisin was only attacked to the extent of 12% by *H. aspersa* digestive juice and 4% by *H. pomatia* juice under the same conditions.

Action on yeast 'cellulose'

A preparation from baker's yeast was rapidly attacked by snail digestive juice. This glucan has been shown to be a 1:3 β-linked glucose polymer (Hassid, Joslyn & McCready, 1941). This agrees with the observation of Giaja (1914) that the yeast cell membrane was dissolved.

Substrates not attacked

Hyaluronic acid, prepared from umbilical cord (Hadidian & Pirie, 1948), and alginic acid were not attacked by snail digestive juice, and the observation of Bierry & Giaja (1909) that agar was not attacked was confirmed. A dextran from *Betacoccus arabinosaceus* (*Leuconostoc mesenteroides*) (Birmingham) which contains predominantly 1:6 linkages (Stacey & Swift, 1948) underwent some changes when treated with snail digestive juice. There was a slight fall in viscosity, but after prolonged action the preparation had lost only 15% of its viscosity increment. There was production of reducing sugar on prolonged incubation, indicating the appearance of 10% of its weight as reducing sugar. It seems probable that the 1:6 links were not split, and that, while some degradation occurred, long chains of residues still remained intact. Galactan ('galactogen') prepared from the albumen glands of *Helix aspersa* (Baldwin & Bell, 1938) was not attacked after 14 days' incubation at pH 5, by either the digestive juices of *H. aspersa* or of *H. pomatia*.

SUMMARY

1. The action of snail digestive juice on cellulose (cellophan) and on pectic acid has been investigated.

2. A number of other polysaccharides and polysaccharide derivatives have been tested as substrates for snail digestive juice. Hyaluronic acid, alginic acid, a dextran from *Betacoccus arabinosaceus* (*Leuconostoc mesenteroides*), galactogen from *Helix aspersa* and nitrocellulose were not affected, while irisin, yeast glucan, a levan from grass, a bacterial levan, methylcellulose, ethylmethylcellulose, and carboxymethylcellulose were attacked.

3. A sensitive method for the detection of cellulase has been developed. The reduction in viscosity of a water-soluble cellulose derivative is used as an index of cellulase activity.

4. Sodium carboxymethylcellulose is split enzymically to an extent corresponding with the breaking of glycoside links between unsubstituted residues. Ethylmethylcellulose is split to an extent corresponding with the breaking of one of the glycoside links in which each unsubstituted residue is involved.

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