sulphate and 50–72 % as ether glucuronide. 1–3 % is excreted unconjugated.

3. Small amounts of the xylenols have been isolated by direct extraction of the metabolic urines and characterized as carbanilates.

4. Crystalline glucuronides have been isolated as metabolites of all six isomers.

5. Evidence has been obtained by means of paper chromatography of the excretion of small amounts of phenolic and reducing metabolites of the xylenols. 2:5-Dihydroxy-1:3-dimethylbenzene has been identified as a metabolite of both 2:6- and 3:5-xylen-1-ols.

6. The xylenes have been shown to give rise to non-acidic phenol, 0·1–0·3 % being excreted free and 2–4 % conjugated. 2:5-Xylen-1-ol was isolated from *p*-xylene urine and characterized as its carbanilate.

We have to express our thanks to the Royal Society for a Parliamentary Grant which defrayed part of the expenses of this investigation. The micro-analyses were carried out by Drs Weiler and Strauss, Oxford.

REFERENCES

Bray, H. G., Humphris, B. G. & Thorpe, W. V. (1949). *Biochem. J.* **45**, 241.
Bray, H. G., Ryman, B. E. & Thorpe, W. V. (1947). *Biochem. J.* **41**, 212.
Bray, H. G., Ryman, B. E. & Thorpe, W. V. (1948). *Biochem. J.* **43**, 561.
Bray, H. G., Thorpe, W. V. & White, K. (1950*a*). *Biochem. J.* **46**, 271.
Bray, H. G., Thorpe, W. V. & White, K. (1950*b*). *Biochem. J.* **46**, 275.
Bray, H. G., Thorpe, W. V. & Wood, P. B. (1949). *Biochem. J.* **45**, 45.
Johnson, W. C., Shennan, R. J. & Reed, R. A. (1944). *Organic Reagents for Organic Analysis.* London: Hopkin and Williams Ltd.
Nölting, E. & Forel, S. (1885). *Ber. dtsch. chem. Ges.* **18**, 2668.
Thorpe, W. V. (1950). *Brit. Sci. News*, **3**, 78.

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

1. DIGESTIVE JUICE OF *HELIX* ON LEAF FIBRE

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(*Received* 20 *January* 1950)

Knowledge of the detailed composition of the leaf, and more especially of its enzymes and other macromolecular components, has lagged behind the corresponding knowledge of animal tissues and bacteria. There are many reasons for this, but an important one is the mechanical strength of parts of the leaf structure. Furthermore, polysaccharides are important structural elements and leaves have little polysaccharidase other than amylase; they are not therefore weakened by autolytic processes as many other tissues are. Treatments that have been found effective in making extracts from seeds and from most animal tissues are not generally applicable to leaves, and satisfactory alternatives have not yet been developed.

Much work has been done on constituents extracted from leaves that have been dried or frozen before grinding, but many of the materials in which we are interested are not stable in the dry or frozen state. For many years the standard technique with fresh leaves has been to grind by hand in a mortar, or by passage through a domestic meat mincer. This was improved by Osborne & Wakeman (1920), Chibnall (1939) and Lugg (1939), who used mechanical mortars and domestic grinders in which rubbing is to some extent substituted for the cutting action of the meat mincer. This development was extended by Bawden & Pirie (1944) and Crook (1946), who used a power-driven triple-roller mill, and achieved even finer subdivision, and by Wildman & Bonner (1947), who used a colloid mill. These processes increased the extraction of protein, but Bawden & Pirie (1944) pointed out that fine grinding cannot be looked on simply as a subdivision of the leaf with concomitant release of imprisoned substances; the process brings about both combination between substances originally separate and dissociation of complexes originally present. They suggested that the intense local heating, which is a probable result of any grinding process, is responsible for the observed formation of artifacts. Crook (1946) was able to disperse 95 % of the tobacco-leaf protein, but he confirmed that an alteration in the properties of some of the protein is brought about by the grinding. Grinding, by any method involving rubbing, is not therefore a wholly satisfactory pre-

liminary stage in the investigation of labile molecules. The Waring blender and related high-speed machines have been used to subdivide leaves by a more purely cutting action. They depend on the sudden impact of a cutter on leaf particles in dilute suspension. This dilution is a disadvantage, but can be overcome by using one extract as suspending fluid for a further lot of material (cf. Wildman & Bonner, 1947). Local heating is probably avoided, but the action causes the formation of a very large air:liquid interface which is often a disadvantage. The microtome is an ideal extension of this type of action, but technical difficulties limit it to work on a very small scale. For some purposes, chemical methods of dispersing the leaf structure could be used. But all the agents that hold out any promise of success (e.g. urea, phenol, formamide, formic acid, calcium thiocyanate) are well known as protein disintegrators (cf. Bawden & Pirie, 1940*a*, *b*). This direction of approach has not therefore been investigated, because we are primarily interested in the proteins of the leaf and in getting them in as nearly the original state as possible.

Leaf structure can be destroyed by enzymes; this is demonstrated by the symptomatology of bacterial and fungal leaf infections, and by the very existence of herbivorous animals. The digestive fluid from the crop of the snail has long been known to contain carbohydrases, and its action on sugar-beet roots (Colin & Lemoyne, 1936; Colin & Chaudun, 1936), sunflower pith (Lemoyne, 1943) and wood (Ploetz, 1939, 1940) has been intensively investigated. Fabergé (1945) has also used it for making chromosome preparations from root tips. Bawden & Pirie (1946) found that it liberated larger amounts of tobacco mosaic virus from tobacco leaves than could be liberated by grinding and that, after digestion with snail enzyme, no further tobacco mosaic virus could be liberated by the other methods used. From many points of view the crude digestive fluid from snails (*Helix aspersa*) is satisfactory; it contains several carbohydrases but very little protease. It is, however, not conveniently available in quantity at all times of the year, and it contains a large amount of slime which may contaminate the final preparation. We have therefore sought both to fractionate the crude juice and to find alternative sources of enzymes able to destroy leaves. Fractionation has not, so far, given any useful results, but various fungal extracts have given satisfactory digestion of the tobacco leaf (Holden, 1950*b*).

The action of added enzymes on the leaf residue can only be studied in isolation if the leaf enzymes are not active. Since comprehensive inactivation by such methods as heating may alter the state and availability of substrates, and selective inhibition may affect subsequently added enzymes, it was necessary to use leaf tissue in which some or all of the enzymes present in the intact leaf might be active. Their presence was allowed for by the use of controls. The presence of a large number of enzymes has been demonstrated in tobacco leaves (cf. Frankenburg, 1946); some of these are of particular relevance to the present work. There is relatively strong amylase and pectin-esterase activity. Protease is present but not very active, particularly in the absence of reducing agents (Tracey, 1948*a*). Polygalacturonase activity has not been observed and cellulase activity is extremely low (Tracey, 1950*b*).

MATERIALS AND GENERAL PROCEDURES

Leaves between 10 and 20 cm. long from tobacco plants (*Nicotiana tabacum*, var. White Burley) grown in a heated glasshouse were used. They were taken at all periods of the year so that different batches probably differed considerably in age, but we have no reason to think that this has affected our results. Green undamaged leaves were used; the midribs were removed and the laminae minced by two passages through a domestic meat mincer. The fibrous product was then washed well with distilled water and squeezed dry by hand; it was stored at 5° with $CHCl_3$ if not used at once. Material of this nature was used in all experiments, and is referred to as fibre. Where comparisons between minced and milled fibre were to be made, a sample of minced fibre was passed through the triple-roller mill (Bawden & Pirie, 1944), and then used with no removal of soluble fractions. Any differences are therefore due to changes in physical state and in the state of combination, and not to changes in gross composition. Incubations of fibre with snail digestive juice were done at 35–40° in the presence of $CHCl_3$. Soluble carbohydrate is defined as material not sedimented on centrifuging at 3000 rev./min. (1400 *g*) for 15 min., and all fluids received this treatment before analysis. When fibre was incubated once only, it was centrifuged down at the end of the incubation, and the extract was filtered if floating particles were present in it. When the enzyme treatment used caused softening and disintegration of the fibre, and the fibre was reincubated, the same technique was used. When the fibre was not disintegrated and was to be reincubated, the fluid was pressed out through cloth and centrifuged. The volume of the supernatant was measured; any sediment was returned to the fibre, which was reincubated with fresh solutions. In calculating the results of experiments when the fibre was incubated once only, the volume of the fluid was taken to be the volume of the extract plus the amount of fluid still present in the wet fibre. However, with successive incubations no allowance was made for the volume of fluid retained by the residue. Part of the material that was in fact liberated by the first incubation is therefore carried over into the second extract, and estimated in that. This does not affect the figures for the total amount extracted, but tends to minimize the efficiency of the earlier extractions.

The snail enzyme preparation was obtained from *Helix aspersa*, as described by Bawden & Pirie (1946). It was diluted so that 1 ml. contained the contents of one crop. *H. pomatia* digestive juice was used in some experiments, but unless otherwise stated 'snail digestive juice' refers to the diluted juice of *H. aspersa*.

Analytical methods

Dry matter. Weighed portions of fibre were dried at 100–105° overnight and reweighed.

Nitrogen. Total N was determined by a micro-Kjeldahl method using SeO_2 : $CuSO_4$: K_2SO_4 (1 : 1 : 8) catalyst.

Carbohydrate. Total soluble carbohydrate was determined by the orcin method of Pirie (1936). In this method glucose is used as a standard and equal weights of hexoses give values of 100%, of polyhexoses 110%, of pentoses 120%, of polypentoses 136%, and of uronic acids 56%. Though weights calculated for total carbohydrate in solution will only be approximate if sugars of many classes are present, they will be satisfactory for studies where only one class of sugar is liberated.

Reducing sugars were estimated by a modification of the Hagedorn and Jensen method (Hanes, 1929), and are all expressed in terms of glucose, although a mixture of reducing substances will generally be present. This method has the advantage that both ketoses and aldoses are determined, but it was found unsatisfactory for some purposes. With cellobiose, for example, the reducing power was found to be 86% of that of an equal weight of glucose instead of the theoretical value of 53%. In some instances, therefore, a modification of the Willstätter and Schudel alkaline hypoiodite method (Jansen & MacDonnell, 1945) was used. Although this does not estimate ketoses it gives theoretical results for cellobiose and galacturonic acid, and has been used for determining the reducing value of polysaccharides (Bergman & Machemar, 1930).

Uronic acid was determined by a manometric method (Tracey, 1948*b*).

Pentose was estimated by a colorimetric method using an acetic acid-aniline reagent (Tracey, 1950*a*).

Calcium. Dry fibre (10–20 mg.) was ashed with a few drops of conc. HNO_3, the ash dissolved in N-HCl, Ca precipitated as oxalate in neutral solution, the precipitate dissolved with dilute H_2SO_4 and titrated hot with 0·01 N-$KMnO_4$.

EXPERIMENTS AND RESULTS

Characterization of action of snail digestive juice on fibre

Incubation of snail digestive juice may result in the precipitation of material insoluble at the pH used. This material contains both N and carbohydrate and, if the proportion of snail enzyme to substrate is high, falsely low results for carbohydrate liberation and apparent gains in the total N of the insoluble residue may result. At pH 6–7 the effect due to precipitated carbohydrate is not of importance with the enzyme : substrate ratio used in the experiments subsequently described. At pH 5, however, the precipitation is greatly increased, and the effect of precipitated carbohydrate may have to be allowed for.

Incubation of tobacco-leaf fibre with snail digestive juice results primarily in the removal of the bulk of the polysaccharides present, which may account for 70% of the dry matter (Table 1). Starch is usually completely removed, partly by the leaf amylase and partly by the *Helix* amylase; cellulose is removed by *Helix* cellulase unless heavily lignified, and up to 90% of the original polyuronide content is brought into solution. The pentose content of the fibre is low (about 2–3% of dry matter), and it is all lost from fibre under the action of snail enzymes. The rates of attack on the different components are materially affected by the conditions used and by the pretreatments of the fibre. Some of these effects have been investigated.

Table 1. *Composition of dry tobacco-leaf fibre*

	Weight (% of dry matter)
Starch	10–40
Polyuronide	15–20
Pectin methoxyl	Approx. 1·5
Cellulose	15–25
Pentosan	2–3
Protein	15–30
Extractable with $CHCl_3$	Approx. 8
Cuticle	Approx. 5
Ash	Approx. 7

Effect of pretreatments of fibre on subsequent action of snail digestive juice

Neutralization of fibre to bring about pectin esterase action (Holden, 1945) is an essential preliminary to incubation with polygalacturonases, for Jansen & MacDonnell (1945) have found that removal of methoxyl groups from pectin is necessary for their action. However, if fibre is incubated

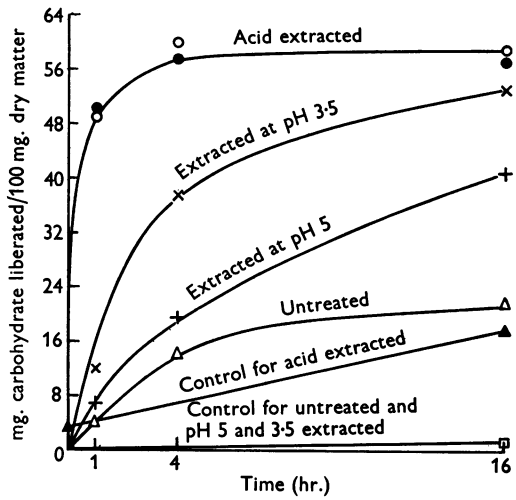

Fig. 1. Effect of decalcification on liberation of carbohydrate from tobacco-leaf fibre by snail digestive juice. After treatment, portions of fibre (dry matter (known) approx. 50 mg.) were incubated for 1, 4 and 16 hr. at 38° with 0·5 ml. 0·2 M-sodium phosphate buffer (pH 7), 0·5 ml. snail digestive juice and 2 ml. water. Controls contained fibre and buffer but no enzyme. For details of extraction procedures see Holden (1950*a*).

with snail digestive juice at pH 6–7 the pectin esterase of the leaf is able to act at the same time, and previous neutralization does not increase the rate of carbohydrate liberation with the snail enzyme. Nevertheless, preliminary neutralization is advisable for otherwise the acid groups liberated by pectin esterase action necessitate frequent adjustment of pH during incubation with snail digestive juice.

Decalcification. Removal of Ca from fibre before incubation with snail digestive juice has a marked effect on the liberation of carbohydrate. Up to 80% of the Ca can be removed by extraction with various salt solutions between pH 5 and 3·5 (Holden, 1950*a*), and substantially complete removal of the remaining Ca can be achieved by subsequent extraction with 0·05N-HCl. The rate of liberation of carbohydrate during a single incubation with snail digestive juice was followed with untreated fibre, and with fibre partly and completely decalcified. The results are given in Fig. 1, which shows that the partly decalcified fibre is attacked more rapidly than the untreated, and that completely decalcified fibre is attacked at an even greater rate. Incubation of acid-extracted fibre without added enzyme led to the release in 16 hr. of about 30% of the carbohydrate liberated by snail digestive juice. The carbohydrate material released without enzyme action is precipitable with ethanol and has a high uronic acid content. It is discussed in more detail in a later paper in this series (Holden, 1950*a*). Decalcification affects the rate of liberation of carbohydrate, but not the total amount liberated. This is illustrated in Table 2. It is clear that the result of three or four extractions is substantially the same whatever the pretreatment.

Milling. Grinding the fibre in a triple-roller mill increases the rate of liberation of carbohydrate on subsequent incubation with snail digestive juice, but does not affect the total amount liberated. The residue from the digestions was assayed for polyuronide and from each 96% was found to have been removed. The results are illustrated in Table 3. Milling does not liberate carbohydrate from minced fibre which has been exhaustively treated with snail enzyme. Furthermore, this milling does not make more substrate accessible to a subsequent incubation with snail enzyme.

Table 2. *Liberation of carbohydrate from decalcified fibre by snail digestive juice*

(Portions of fibre (dry matter, known, approx. 50 mg.) were incubated 16 hr. at 38° with 0·5 ml. 0·2M-sodium phosphate buffer (pH 7) + 0·5 ml. snail digestive juice + 2 ml. water. Fibre was reincubated for further 24 hr. periods with fresh enzyme and buffer solution. For details of pretreatment of fibre see Holden (1950*a*). % signifies mg./100 mg. dry matter of fibre as taken for the first incubation.)

	Soluble carbohydrate (%)				
Serial number of incubation	Untreated fibre (Ca, 3·76%; polyuronide, 23·6%)	Fibre extracted with NH_4Cl-acetate (pH 5) (Ca, 0·67%; polyuronide, 25·8%)	Fibre extracted with NH_4Cl-acetate (pH 5) then with 0·05N-HCl (Ca, <0·04%; polyuronide, 26·8%)	Fibre extracted with NH_4Cl-acetate (pH 3·5) (Ca, 0·61%; polyuronide, 25·6%)	Fibre extracted with NH_4Cl-acetate (pH 3·5) then with 0·05N-HCl (Ca, <0·04%; polyuronide, 25%)
1	18·7	35·9	52·7	46·5	49·8
2	19·6	23·8	10·5	15·3	9·1
3	15·2	9·8	7·9	8·1	8·2
4	9·3	—	—	—	—
Totals	62·8	69·5	71·1	69·9	67·1

Table 3. *Liberation of carbohydrate by snail digestive juice from minced and milled fibre*

(1 g. lots of trypsin-treated (Holden & Tracey, 1950*b*) fibre (dry matter 23·4%) + 4 ml. snail digestive juice + 2 ml. 0·2M-sodium phosphate buffer (pH 7·2) + 4 ml. water were incubated for 24 hr. at 38°. The remaining fibre was separated and reincubated with fresh juice.)

	Carbohydrate liberated (mg./100 mg. dry matter)	
	Minced	Milled
First incubation	23·0	34·4
Second incubation	20·0	16·8
Third incubation	8·0	4·0
Totals	51·0	55·2

Other treatments. Boiling, freezing, and extraction of fibre with an ethanol : ether mixture (7 : 3, v/v) did not affect the manner in which carbohydrate was liberated by snail digestive juice.

Enzymic treatment. So as to restrict, as far as possible, the number of snail enzymes that would be acting simultaneously some of the substrates were removed from the fibre. Starch was removed by incubation with salivary amylase or with a commercial trypsin preparation (British Drug Houses Ltd. or Hopkin & Williams) (Holden & Tracey, 1950*b*). Removal of starch is advisable because, although fresh snail digestive juice contains amylase, the activity decreases during storage, and the residue after incubations with an old preparation of snail enzyme may contain intact starch grains.

For some experiments the fibre was also incubated with a purified preparation of fungal polygalacturonase (Holden, 1950*a*) which removed a large proportion of the pectic substances present. As an alternative to incubation with polygalacturonase, pectic substances were removed by extracting the fibre with hot ammonium oxalate solution. Treatments that remove pectic substances from leaf mince destroy the integrity of the fragments and leave them soft and swollen with separated cells and fragments. This is presumably due to attack on the 'middle lamella', and might be expected to affect the subsequent liberation of carbohydrates with snail digestive juice. Although the amount of carbohydrate liberated with snail enzyme was less after incubation with polygalacturonase, the rate of liberation was never reduced, and with some samples of fibre was increased. This result is presumably due to the opposed effects of removal of the substrate for one of the snail enzymes, and the facilitation of the action of others by the increased subdivision of the leaf fragments.

Conditions of incubation affecting the action of snail digestive juice on fibre

pH. Bawden & Pirie (1946) have shown that optimum liberation of virus from tobacco fibre by snail digestive juice occurs between pH 6 and 7. The optimum pH for snail

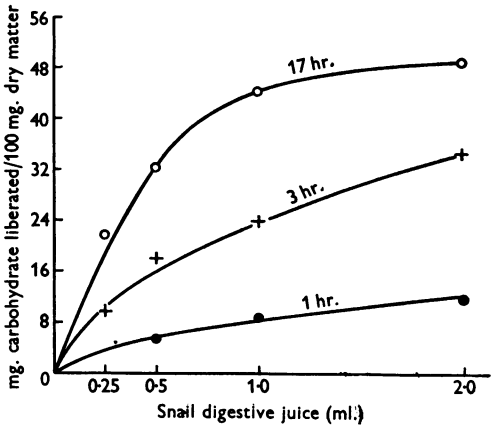

Fig. 2. Effect of enzyme concentration on liberation of carbohydrate by snail digestive juice from fibre. 0·5 g. lots of trypsin-treated fibre (dry matter, 22·7%) + 0·5 ml. sodium phosphate buffer (pH 7) + snail digestive juice (0·25–2·0 ml.) and water to give volume 3 ml. 1, 3 and 17 hr. incubation at 40°.

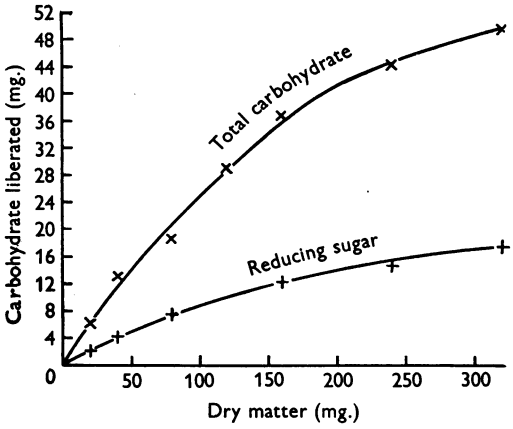

Fig. 3. Effect of amount of substrate on liberation of carbohydrate from fibre by snail digestive juice. Trypsin-treated fibre (100–1600 mg. wet wt.) + 1 ml. 0·2 M-sodium phosphate buffer (pH 7) + 8 ml. water + 1 ml. snail digestive juice. Incubated 5·5 hr. at 38°.

cellulase acting on cellulose lies between pH 5 and 6, but the activity is still considerable at pH 6–7. The action on healthy fibre does not show a marked optimum, but proceeds at about the same rate between pH 5 and 7·5, whether the rate is measured by loss in weight, or loss in polyuronide from the fibre, or appearance of soluble reducing sugar, or of total soluble carbohydrate in the extracts. Difficulty was found in keeping the pH constant during the reaction without the use of strong buffer solutions, which have to be avoided as they depress the enzyme activity. To allow for the acidwards drift of pH, which occurs even when previously neutralized fibre is used, experiments were usually begun at pH 6·8–7·2.

Mixing. The liberation of carbohydrate from starch-free fibre was not affected by keeping the contents of the tubes continually mixed by slow end-over-end rotation during incubation.

Enzyme concentration. Liberation of carbohydrate was found to be approximately proportional to the volume of snail digestive juice used when this amount was small, and not more than a third of the substrate was brought into solution. Fig. 2 shows the result got with different amounts of snail digestive juice acting on starch-free fibre for different times of incubation.

Amount of substrate. The relationship between the amount of carbohydrate liberated by the same amount of snail digestive juice, and the concentration of the fibre suspension is shown in Fig. 3. With less than 1 g. wet weight (250 mg. dry wt.) in 10 ml. the relationship is approximately linear. At higher concentrations there is proportionately less extraction, presumably because the enzymes are no longer in excess. Reducing sugar is about 35% of the total carbohydrate at each ratio of enzyme to substrate.

Effects of salts

Sodium chloride. Figs. 4 and 5 show the effect of different salt concentrations on the digestion of fibre incubated for different lengths of time without and with previous removal of pectic substances. Pectin was removed by extraction with ammonium oxalate solution, which also causes decalcification. A low concentration of salt has a slight enhancing effect on the enzymic action on both types of fibre, but the salt optimum is not sharp. Throughout the range 0·005–0·02 M there is substantially the same activity. Higher salt concentrations are inhibitory, especially if the pectic substances have not been removed.

Calcium. In an earlier section of this paper it was assumed that the increased rate of enzymic attack on fibre which follows extraction with salt or acid was due to the removal of Ca. Other interpretations of the effect are however possible; for example, treatment with acid may loosen bonds that were preventing ready enzyme attack. In Table 4 the results of experiments with added Ca ions are set out. It is clear that Ca actively inhibits the enzymes responsible for the liberation of uronic acid from the fibre and has relatively little effect on the digestion of fibre from which polyuronide has already been removed. The inhibition of polygalacturonase by Ca ions is treated more fully in another paper (Holden, 1950*a*) as is the effect on cellulase (Holden & Tracey, 1950*a*).

In the experiments set out in Table 2 the reduction of the Ca content of a sample of fibre from 3·76 to 0·67% approximately doubled the rate at which snail enzyme acted on it, and further reduction of Ca increased the rate of action still more. Under the conditions used the molarity of the Ca, had it all been in solution, would have been 0·016 M in the first instance and 0·0028 M in the second. As Table 3 shows, such concentrations of Ca^{++} give approximately this amount of inhibition. Although it is obvious that our ignorance of the precise relative positions of the Ca and polyuronide in fibre makes it impossible to assess the Ca concentration at the

actual site of enzyme action, the agreement makes it unnecessary to postulate any other reason for the activation besides the removal of Ca.

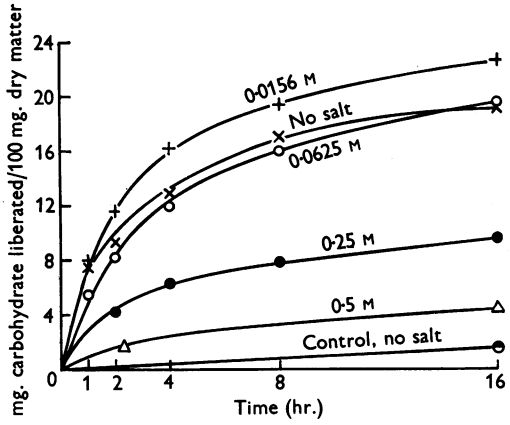

Fig. 4. Effect of NaCl on liberation of carbohydrate from trypsin-treated fibre by snail digestive juice. 0·5 g. lots of fibre (dry matter 19·6%, polyuronide 23·6% of the dry matter) + 2 ml. NaCl solution + 1 ml. water + 1 ml. snail digestive juice. Incubated for different times at 38°. Final concentrations of NaCl are indicated on curves.

As a result of these experiments we have adopted 24 hr. incubation at about pH 6 with a ratio of 2 ml. digestive juice to 1 g. wet weight of fibre as a standard procedure when normal fibre is being digested. With decalcified fibre less enzyme can be used, and in both methods the salt concentration should be kept low.

Soluble products of fibre digestion

Since snail digestive juice is effectively a mixture of carbohydrases, the soluble end products are carbohydrates, though some may remain polymerized to some extent. The amount of the different end products (hexoses, pentoses and uronic acids) arising from crude fibre will depend on such adventitious circumstances as the initial starch content and the Ca content. The products of digestion of starch-free,

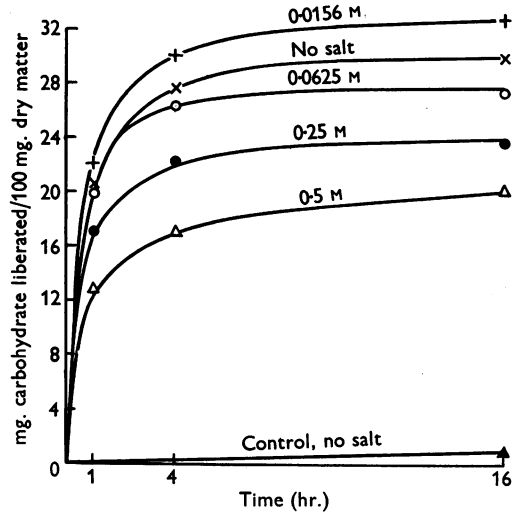

Fig. 5. Effect of NaCl on liberation of carbohydrate from ammonium oxalate-extracted, saliva-treated fibre by snail digestive juice. 0·25 g. lots fibre (dry matter 20·3%, polyuronide 4·5% of the dry matter) + 2 ml. NaCl solution + 1·5 ml. water + 0·5 ml. snail digestive juice. Incubated for different times at 38°. Final concentrations of NaCl are indicated on curves.

partly decalcified fibre were chosen for analysis because they are most nearly derived from leaf fibre alone under optimal conditions of enzyme action. The results are set out in Table 5. The polygalacturonase action takes place more rapidly than the cellulase action. The reducing sugar is about 35–40% of the total carbohydrate in the extracts. The addition of ethanol to snail-enzyme digests gives a

Table 4. *Effect of calcium on carbohydrate liberation by snail enzyme acting on fibre*

	Trypsin-treated, partly decalcified fibre* incubated			Trypsin-treated, partly decalcified fibre that has been incubated with polygalacturonase†	
	1 hr.	21 hr.			
Final concentration $CaCl_2$ (M)	Carbohydrate liberated (mg./100 mg. dry matter)	Carbohydrate liberated (mg./100 mg. dry matter)	Polyuronide loss (mg./100 mg. dry matter)	Incubated 1 hr. Carbohydrate liberated (mg./100 mg. dry matter)	Incubated 19 hr. Carbohydrate liberated (mg./100 mg. dry matter)
0·25	1·4	4·0	0	1·3	5·9
0·1	2·4	7·6	—	5·0	9·4
0·05	3·0	9·3	1·68	5·23	10·5
0·025	4·6	10·6	2·14	5·7	13·2
0·01	6·6	12·8	3·10	6·30	12·8
0·005	7·2	13·8	6·08	6·52	14·1
0·002	11·7	21·9	7·56	7·25	15·8
No Ca added	13·8	20·8	8·45	5·47	15·5

* Portions of fibre (dry matter 79·3 mg., Ca 0·98% of dry matter, polyuronide 25·1% of dry matter) + 7 ml. $CaCl_2$ solution + 1 ml. snail digestive juice. Incubated for 1 and 21 hr. at 38°.

† Portions of fibre (dry matter 51·0 mg. Ca 0·32% of dry matter, polyuronide 2·4% of dry matter) + 5·5 ml. $CaCl_2$ solution + 0·5 ml. snail digestive juice. Incubated for 1 and 19 hr. at 38°.

precipitate, which differs in appearance according to whether the fibre has been decalcified or not. From non-decalcified fibre the precipitate is slightly gelatinous and from decalcified fibre it is finely flocculent. The material contains some uronic acid (approx. 25%) and appears to be similar to that obtained from fibre on incubation with polygalacturonase (Holden, 1950*a*), but is contaminated with ethanol-precipitable material from the snail digestive juice. When pectic substances are removed from fibre before incubation with snail digestive juice, the reducing sugar is over 60% of the total carbohydrate in the snail-enzyme extracts. The addition of ethanol gives only a faint precipitate.

Table 5. *Progressive changes in composition of enzyme digest*

(1 g. lots of trypsin-treated, partly decalcified fibre (dry matter 18·3%, polyuronide 21·4% of dry matter, Ca 0·60% of dry matter) + 2 ml. 0·2M-sodium phosphate buffer (pH 7) + 0·5 ml. snail digestive juice + 7·5 ml. water. Incubated for times given at 35°.)

Time (hr.)	Carbohydrate liberated (mg./100 mg. dry fibre taken)		
	Total	Reducing sugar	Uronic acid
Initial control*	3·0	About 0·5	—
0·5	7·1	2·7	4·3
1	12·8	4·5	5·2
2	22·4	7·3	5·9
4	27·8	10·5	7·1
8	31·4	11·1	7·8
16	41·5	16·5	9·6
16 hr. control*	9·5	1·2	5·7

* Incubated without enzyme.

Leaf fractions resistant to snail enzymes

After a sample of tobacco-leaf fibre has been repeatedly digested, a residue amounting to about 30% of the initial dry matter remains. These residues have a high N content (9–10% of the dry matter) because little or no N has been removed, and some may have been added by precipitation from snail digestive juice. The greater part of this N can be brought into solution by subsequent treatment with trypsin. The polyuronide content of the residue is about 5% of the dry matter. The residue is a fine sludge which may contain fibrous knots. Microscopic examination shows these knots to be interwoven masses of secondarily thickened vessels. Four components of the residue can be identified microscopically. These are fibres showing secondary thickening, fragments of cuticle still showing the outlines of epidermal cells and intact guard cells, minute intensely birefringent particles of calcium oxalate, and protoplasts and their fragments. About one-fifth of the residue dry matter is cuticle, another fifth fibres, and the remainder protoplasts, their fragments and calcium oxalate.

Residues after digestion of minced fibre contain few intact protoplasts and most of the fragments appear to be as small as chloroplasts. Intact protoplasts can be got by infiltrating snail digestive juice into intact pieces of leaf by the continued application and removal of reduced air pressure until all the intercellular spaces appear to be filled. After a few days' incubation, the top and bottom layers of cuticle may be removed by the aid of a fine brush. There remains a suspension of intact protoplasts containing calcium oxalate particles and a web of secondarily thickened vessels. The results obtained appear similar to those obtained by Whittenberger & Naghski (1948) using *Clostridium roseum*.

About 25% of the initial dry matter remains sedimentable after repeated digestion, i.e. almost as much as when minced fibre is used. Apparently loss of soluble proteins, sugars and salts is counterbalanced by the retention of chloroplasts normally removed in the sap and by precipitation of unstable sap proteins.

Superficially similar fractions are got by the action of boiling 0·5% (w/v) ammonium oxalate solution on the intact leaf. This treatment removes pectic substances from the leaf, with the result that the cells separate and the leaf disintegrates into four fractions similar to those described above. The protoplasts are, however, still enclosed in cellulose walls and the epidermal cells remain attached to the cuticle. The vascular elements remaining after the action of snail digestive juice are degraded to some extent, for many are evident only in the form of the partially unwound threads of what was originally the spiral thickening. The resistant components may be separated by hand if intact leaf is used, or chemically if fibre is used. Protoplasts are soluble in dilute hypochlorite solution, protoplasts and fibres in cold 70% aqueous H_2SO_4, while the cuticle is insoluble in both reagents. The cuticle fraction is contaminated with lignin, but may be obtained free from it by mechanical separation from the residues got from whole leaf.

Effect of snail digestive juice on chloroplast fraction

When crude sap is centrifuged, a deposit is obtained. The upper layer of this is composed of cell fragments rich in chloroplasts. This material is also present in fibre, and the use of the sap fraction permitted study of the action on it of the snail enzymes in the absence of fibrous constituents. Incubation of the chloroplast fraction from sap with snail enzymes at pH 5 has no obvious effect on the microscopic appearance of the fraction, and probably little on its composition. There is a loss of N from solution due to precipitation from the snail digestive juice, and an increase in soluble carbohydrate. This latter increase is less than that found in the autolysis of chloroplast fractions (Holden & Tracey, 1950*b*), and after prolonged incubation net decreases of soluble carbohydrate occur as a result of precipitation from the snail-enzyme solution. Since the fraction appears to be composed mainly of protein, starch, inorganic salts and a small amount of lipids (Holden & Tracey, 1950*b*), it is not surprising that snail digestive juice has little action on it, for it is almost devoid of protease activity and its amylase activity is weak.

DISCUSSION

Snail digestive juice, acting on tobacco-leaf fibre under suitable conditions, brings the greater part of the fibre into solution, and leaves a residue of indigestible or only slowly digestible materials. A similar effect was noticed by Naghski, White, Hoover & Willaman (1945) in the fermentation of *Cryptostegia grandiflora* leaves by *Clostridium roseum*. After fermentation and screening, a sludge of coagulated contents of chlorenchyma cells settled

out from the fluid, while the screenings contained cuticle and veins. Baker & Harriss (1947), examining the contents of sheep rumen and intestine, found that lignified vessels and cuticle were more resistant to digestion by rumen microflora than other leaf components. Consequently spiral, annular and other lignified vessels and sheets of cuticle carrying intact guard cells accumulate in the faeces. In addition to the range of microscopically visible particles set free by the dispersal of the main leaf structure by the snail digestive juice, heterogeneous materials are liberated in solution. There is little protein, because most of the leaf protein coagulates on incubation and so appears in the residue, but other macromolecules (e.g. polysaccharides) accompany the monosaccharides that have been made by the snail carbohydrases. These resistant macromolecules probably arise in different ways. Some will be held mechanically and will diffuse out as the leaf structure is digested; some are probably attached by a linkage that is susceptible to one of the enzymes in the digestive mixture used. This distinction is in part arbitrary; a succession of steps can be envisaged forming a smooth gradation without rigid division into categories. The mode of linkage which Powell (1948) has called clathrate, and in which a molecule is imprisoned in a cage made of other materials to which it is not attached by any normal chemical valencies, has not yet been recognized in biological structures. Nevertheless, it probably does occur and if the imprisoning material were damaged the two components would separate. Furthermore, substances that are held together by weak links, e.g. hydrogen bonds or adsorption, may well separate if one of the substances is altered by partial hydrolysis, even although the action is not taking place at a bond adjacent to the link in question. These considerations would complicate the interpretation of the results of enzymic digestion of a biological structure even if only one enzyme were acting; in a system where several must be assumed to be acting the situation is even more confused.

Work has already been published on the release of viruses from the insoluble parts of the leaf by enzymic digestion (Bawden & Pirie, 1946). Interest then centred on the fact that larger yields of virus could be separated enzymically than by the other methods tried. It is possible that the results presented in the present series of papers could be used to study the location and mode of attachment of viruses and enzymes in the cell. When relatively specific enzymes can be used, the possibility arises that one enzyme may bring about a dispersal of the structure without releasing a component of it into the medium, whereas another enzyme preparation would release that component from the detritus. The search for such a system was one of the objects of this research and work on it is still in progress.

SUMMARY

1. The effect of the polysaccharidases of *Helix* digestive juice on tobacco-leaf fibre has been investigated.

2. Various pretreatments of the fibre modify the action of these enzymes. 90% of the carbohydrate of the fibre can be brought into solution. The nature of the insoluble residue is discussed.

3. Cellulase and polygalacturonase are principally concerned in this digestion. The action of the latter is favoured by preliminary decalcification of the fibre.

4. Snail digestive juice has little action on the green 'chloroplast' fraction of sap sediment.

5. The merits of disintegrating leaves enzymically are considered, but the fact that release by a specific enzyme does not give unequivocal evidence about the original linkage of a substance is recognized.

REFERENCES

Baker, F. & Harriss, S. T. (1947). *Nutr. Abstr. Rev.* **17**, 3.
Bawden, F. C. & Pirie, N. W. (1940*a*). *Biochem. J.* **34**, 1258.
Bawden, F. C. & Pirie, N. W. (1940*b*). *Biochem. J.* **34**, 1278.
Bawden, F. C. & Pirie, N. W. (1944). *Brit. J. exp. Path.* **25**, 68.
Bawden, F. C. & Pirie, N. W. (1946). *Brit. J. exp. Path.* **27**, 81.
Bergman, M. & Machemar, H. (1930). *Ber. dtsch. chem. Ges.* **63** B, 316.
Chibnall, A. C. (1939). *Protein Metabolism in the Plant.* New Haven: Yale University Press.
Colin, H. & Chaudun, A. (1936). *C.R. Acad. Sci., Paris*, **202**, 973.
Colin, H. & Lemoyne, S. (1936). *Bull. Soc. Chim. biol., Paris*, **18**, 1578.
Crook, E. M. (1946). *Biochem. J.* **40**, 197.
Fabergé, A. C. (1945). *Stain Tech.* **20**, 1.
Frankenburg, W. G. (1946). *Advanc. Enzymol.* **6**, 309.
Hanes, C. S. (1929). *Biochem. J.* **23**, 99.
Holden, M. (1945). *Biochem. J.* **39**, 172.
Holden, M. (1950*a*). *Biochem. J.* **47**, 415.
Holden, M. (1950*b*). *Biochem. J.* **47**, 426.
Holden, M. & Tracey, M. V. (1950*a*). *Biochem. J.* **47**, 407.
Holden, M. & Tracey, M. V. (1950*b*). *Biochem. J.* **47**, 421.
Jansen, E. F. & MacDonnell, L. R. (1945). *Arch. Biochem.* **8**, 97.
Lemoyne, S. (1943). *Bull. Ass. Chim. Sucr.* **60**, 12.
Lugg, J. W. H. (1939). *Biochem. J.* **33**, 110.
Naghski, J., White, J. W., Hoover, S. R. & Willaman, J. J. (1945). *J. Bact.* **49**, 563.

Osborne, T. B. & Wakeman, A. J. (1920). *J. biol. Chem.* **42**, 1.
Pirie, N. W. (1936). *Brit. J. exp. Path.* **17**, 269.
Ploetz, T. (1939). *Ber. dtsch. chem. Ges.* **72** B, 1885.
Ploetz, T. (1940). *Ber. dtsch. chem. Ges.* **73** B, 57.
Powell, H. M. (1948). *J. chem. Soc.* p. 61.
Tracey, M. V. (1948*a*). *Biochem. J.* **42**, 281.
Tracey, M. V. (1948*b*). *Biochem. J.* **43**, 185.
Tracey, M. V. (1950*a*). *Biochem. J.* **47**, 431.
Tracey, M. V. (1950*b*). *Biochem. J.* **47**, 433.
Whittenberger, R. T. & Naghski, J. (1948). *Amer. J. Bot.* **35**, 719.
Wildman, S. G. & Bonner, J. (1947). *Arch. Biochem.* **14**, 381.

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

2. DIGESTIVE JUICE OF *HELIX* ON DEFINED SUBSTRATES

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(*Received* 20 *January* 1950)

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.