The Biochemistry of Rumen Protozoa

4. DECOMPOSITION OF PECTIC SUBSTANCES*

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Among the carbohydrates present in the food eaten by ruminants the pectic substances can form a substantial proportion of the whole, yet their decomposition in the rumen appears not to have been previously investigated. This is in marked contrast to the attention which the other major dietary carbohydrates have received (see, for example, Howard, 1959c). When the present work was commenced, little evidence was available that pectic substances are decomposed in the rumen. Leroy & Michaux (1949) showed, by analysing the food and faeces of sheep, that between 40 and 97 g. of pectic substances were retained by the animal per day. Bryant & Doetsch (1954) observed that some cultures of cellulolytic bacteria isolated from the bovine rumen could ferment pectin. That the decomposition of pectic substances, shown by Leroy & Michaux (1949) to occur in the alimentary tract, could occur in the rumen by the action of the micro-organisms contained there, was shown by our preliminary experiments. Both bacteria and Protozoa were found to be concerned in this decomposition. The present paper describes only the experiments with Protozoa. Three groups of Protozoa were available as fairly pure preparations; *Dasytricha ruminantium*, mixed *Isotricha intestinalis* plus *Isotricha prostoma*, and *Entodinium caudatum*. Their abilities to decompose pectic substances were determined, and a more extended study was made of the most active group, the isotrichs.

MATERIALS AND METHODS

Protozoa. Suspensions of living protozoa and cell-free extracts were obtained as previously described (Howard, 1959a; Abou Akkada & Howard, 1960). *D. ruminantium*

and mixed isotrichs were respectively obtained from two sheep which had been treated as described by Eadie & Oxford (1957) and Howard (1959a). *E. caudatum* was obtained from the same sheep as in previous work (Abou Akkada & Howard, 1960). In some experiments the usual procedure for obtaining a clean suspension of mixed isotrichs (Eadie & Oxford, 1955) was modified by adding xylose (0.5%, w/v) to the rumen liquor instead of glucose. Since xylose is not used by the isotrichs, the protozoa deposited during its fermentation contained much less starch than those in the usual suspensions. Suspensions of isotrichs obtained by adding xylose to the rumen liquor were used immediately. The protozoa were then more active, yet more empty of storage starch, than those prepared by the normal procedure. It was thought that, by using such organisms, the decomposition of substrates that are metabolised with difficulty would be more readily detected.

Substrates. Three polysaccharides were used: (a) pectin (British Drug Houses Ltd., '250 grade apple pectin'); (b) polygalacturonic acid (Nutritional Biochemicals Corp., Cleveland 28, Ohio, U.S.A.); (c) sodium polypectate (Sunkist Growers Inc., Ontario, Calif., U.S.A.). The galacturonic acid content and the yield of methanol on alkaline hydrolysis of these materials were determined by the methods described below. The results, shown in Table 1, indicate that the polygalacturonic acid and the sodium polypectate are almost completely demethylated, and that in the pectin about three-fifths of the galacturonic acid groups are esterified. Samples of the materials were hydrolysed ($\text{N-H}_2\text{SO}_4$ for 2 hr. at 100°), and the sugars in the neutralized hydrolysates were examined by paper chromatography (Howard, 1957). The results are shown in Table 1. None of the sugars was present in the hydrolysates in more than traces, and it was estimated that none of the substrates contained more than approx. 2% of other polysaccharides. Further purification was considered unnecessary.

Solutions (1%, w/v) of the substrates were made as follows. Pectin was added to water or buffer solution in small portions with shaking. A homogeneous, slightly viscous solution was obtained after the mixture had been stored in the refrigerator overnight. Sodium polypectate readily dissolved to give a viscous solution when mixed

* Part 3: Abou Akkada & Howard (1960).

Table 1. *Analysis of pectic materials*

Material	Anhydro-galacturonic acid (% w/w)	Methoxyl ($-\text{O}\cdot\text{CH}_3$) (% w/w)	Neutral sugars in acid hydrolysate
Pectin	67	7.0	Galactose, glucose, arabinose
Polygalacturonic acid	90	0.1	None
Sodium polypectate	62	0.1	Galactose, arabinose

with water or buffer solution. Polygalacturonic acid (1 g.) was suspended in water (50 ml.) containing a little bromothymol blue. 0.5 N-NaOH was added slowly until the solid was all dissolved and the solution was a green-blue colour. About 8.7 ml. of alkali was needed. The solution was finally diluted to 100 ml.

Other materials. Galacturonic acid was supplied by L. Light and Co. Ltd. Di- and tri-galacturonic acid were the gift of Dr W. W. Reid. Tetragalacturonic acid was obtained from the mixture of oligo-uronides resulting from the action of *Isotricha* extract on pectin. The separation was by paper chromatography in the solvent system described below. The portion of the paper bearing the tetragalacturonic acid ($R_{\text{galacturonic acid}}$ 0.12) was cut out and extracted with water. The solution was dried in a vacuum desiccator to yield tetragalacturonic acid as a colourless syrup. Solutions (1%) were made in water and preserved under toluene for use as chromatographic standards. Solutions (1%), for use as enzyme substrates, were made in 0.2 M-NaHCO₃, their pH values were approx. 7.5. Carbazole (British Drug Houses Ltd.) was sublimed before use. Chromotropic acid was used as the 'sodium salt for formaldehyde determination' (British Drug Houses Ltd.).

Standard solutions. (a) Methanol (A.R.) was diluted to 1% (v/v) in water. This stock solution was standardized against a solution prepared from a weighed quantity of a crystalline methyl ester. Methyl *p*-nitrobenzoate was prepared from A.R. *p*-nitrobenzoyl chloride and methanol, and after crystallizing from ethanol had m.p. 96°. About 20 mg. of vacuum-dried substance, accurately weighed, was sealed in a small tube with N-NaOH (2 ml.). The tube was immersed in boiling water for 30 min., cooled, opened, and the contents were diluted to 100 ml. Methanol in this solution was determined after oxidation to formaldehyde by the method described below. A control experiment with the stock solution showed that 1.5% of the methanol was lost during the heating with alkali, and the calculated methanol content of the ester hydrolysate was adjusted accordingly. In this way, the stock 1% (v/v) solution was found to contain 8.0 mg./ml. of methanol. (b) Galacturonic acid was purified with charcoal and crystallized from aqueous ethanol. The standard solution was prepared by dissolving the vacuum-dried substance (0.500 g.) in saturated benzoic acid (50 ml.).

Buffer solutions. In experiments with cell-free extracts of the protozoa, the following buffer solutions were used: (a) phosphate-citrate (McIlvaine, 1921), pH 3-8; (b) KH₂PO₄ + Na₂HPO₄, 0.067 M, pH 6.4-8.4; (c) veronal (Michaelis, 1930), pH 6.8-9.2; (d) Na₂CO₃ + NaHCO₃, 0.2 M (Delory & King, 1945), pH 9.2-10.5.

Enzyme assays

Polygalacturonase. Polygalacturonic acid solution (0.1 ml.), buffer and enzyme solution, in a total volume of 1 ml., were mixed in tubes graduated at 25 ml. The tubes were immersed in a water bath at 38° for 4 hr., and the reducing sugar present was then determined by Nelson's (1944) method. The reducing power was expressed in terms of galacturonic acid, and from this the apparent degree of hydrolysis of the substrate was calculated.

Pectin esterase. The enzyme and substrate were incubated together in small test tubes, capacity about 3 ml., closed by rubber stoppers. In each of four such tubes was placed

buffered pectin solution (1 ml.), freshly made by mixing the stock (1%) pectin solution (1 ml.) with the appropriate buffer (4 ml.). Enzyme solution (1 ml.) was added to each of two of the tubes, and water (1 ml.) to the others. The tubes were then stoppered, shaken and immersed in the water bath at 38° for a period of 1-4 hr., depending on the potency of the enzyme solution and the pH of the mixture. A 'blank' tube containing water (2 ml.), and two standard tubes containing water (1.5 ml.) and methanol [0.5 ml. of 0.01% (v/v) solution, i.e. 40 µg. of methanol], were incubated at the same time. At the end of the incubation period the enzyme activity was stopped by the addition of 2N-H₂SO₄ (0.1 ml.) to each tube. The determination of the methanol content of the solutions was then carried out by a modification of the method of Feldstein & Klendshoj (1957), as follows. One ml. of the acidified mixture from each tube was placed in the outer compartment of a Conway unit, the inner compartment of which contained 10% (v/v) H₂SO₄ (1 ml.). Liquid paraffin was used to seal the lids of the units, which were left overnight at room temperature. One ml. of liquid from the inner compartment was then transferred to a tube, and one drop of 0.3 M-KMnO₄ was added. After 10 min. at room temperature, excess of permanganate was destroyed with one drop of saturated NaHSO₃. The formaldehyde resulting from oxidation of the methanol was determined by heating with the chromotropic acid-H₂SO₄ reagent (Lambert & Neish, 1950) and measuring the colour intensity with the use of an Ilford no. 626 filter. The reading obtained with the solution containing the methanol standard was used to calculate the quantity of methanol in the other tubes. A small proportion of free methanol was always found in the pectin solutions, and, at pH values above 8, some non-enzymic hydrolysis occurred.

Other analytical methods

Galacturonic acid was determined by the H₂SO₄-carbazole method of Dische (1947). Nitrogen analyses were carried out as described by Howard (1959*a, b*). Solutions to be examined chromatographically for galacturonic acid or oligo-uronides were first diluted with an equal volume of ethanol to precipitate polysaccharide (Smith, 1958*b*). The precipitate was removed by centrifuging, and cations were removed from the supernatant solution by treatment with Amberlite IR-120 (H⁺) resin. The resulting solution of acids was evaporated to dryness in a vacuum desiccator at room temperature. Chromatography of the residue was carried out on Whatman no. 541 paper in ethyl acetate-acetic acid-water (3:1:1, by vol.) during 8-12 hr. Solvent was removed from the paper first by air-drying and finally by steaming. The acidic spots were then revealed by dipping in an ethanolic solution of bromocresol green (0.04%) adjusted to pH 7.

Manometric methods

Experimental conditions were, in general, similar to those previously used (Abou Akkada & Howard, 1960). Antibiotics and Na₂S were omitted from the buffer solutions, which contained bicarbonate but no acetate. The main chambers of the Warburg flasks contained protozoal suspension (5 ml., containing about 2 mg. of total protozoal N), and the side arms contained 1% solutions in the buffer of pectin or sodium polypectate (1 ml.). The experimental period was 4 hr.; at the end of this time the flasks were

disconnected from the manometers, and the protozoa were killed by adding 2N-H₂SO₄ (0.1 ml.). The suspensions were centrifuged, and portions of the supernatants were removed for the determination of methanol, reducing sugar and total galacturonic acid, and also for chromatographic examination.

When methanol was to be added to protozoa, the main chambers of the flasks contained protozoal suspension (2 ml.), and the side arms buffer solution (0.5 ml.) containing methanol (250 µg. approx.). Duplicate flasks were set up containing buffer solution without protozoa in the main chambers. The methanol contents of the flasks were determined after incubation for 4 hr.

RESULTS

Properties of cell-free extracts of protozoa

A quantitative comparison of the pectin esterase and polygalacturonase activities of extracts of the three groups of Protozoa is shown in Table 2. Preliminary experiments with the *Isotricha* extract had suggested that both enzymes were most active at a pH of about 8; the comparative experiments were therefore carried out at this pH. Qualitative experiments in the phosphate buffer, pH 6, gave similar results. Incubation of the extracts from *D. ruminantium* or the isotrichs with solutions of pectin or polygalacturonic acid at pH 7.5 or 8 caused the disappearance of ethanol-precipitable material. At the same time, oligo-uronides and galacturonic acid appeared in the solution. Chromatographic examination of samples of the mixtures taken at different times during incubation showed that during the greater part of the period there was very little galacturonic acid present. Moderate amounts of di-, tri- and tetra-galacturonic acid were formed but the greater part of the material occurred as higher uronides was not separable on the paper. The yields of hydrolysis products appeared from the chromatograms to be the same, whether pectin or polygalacturonic acid was the substrate.

Table 2. *Hydrolysis of pectic substances by cell-free extracts of rumen protozoa*

Incubation of substrate+enzyme was in phosphate-citrate buffer, pH 8 (McIlvaine, 1921). Other conditions and methods of analysis were as described in the text. Pectin and polygalacturonic acid were substrates for the determination of pectin esterase and polygalacturonase activity respectively. The figures in the Table are the weights of product (µg. of methanol or reducing sugar as galacturonic acid respectively) produced per hr. per mg. of protein N in the enzyme solution. Two separate preparations each of *Isotricha* spp. and *D. ruminantium*, and one of *E. caudatum*, were tested.

Protozoa	Pectin esterase activity	Polygalacturonase activity
<i>Isotricha</i> spp.	164, 130	505, 1350
<i>D. ruminantium</i>	33, 22	62, 101
<i>E. caudatum</i>	< 1	< 3

Di-, tri- and tetra-galacturonic acid were themselves hydrolysed by the *Isotricha* extract; hydrolysis of di- and tri-galacturonic acid was slow, and yielded, respectively, galacturonic acid and a mixture of galacturonic acid and digalacturonic

Fig. 1. Variation of activity of *Isotricha* polygalacturonase with pH. Conditions of assay were as described in the text. ●, 0.067 M-Phosphate buffer; ○, veronal buffer; △, carbonate buffer.

Fig. 2. Variation of activity of *Isotricha* pectin esterase with pH. Conditions of assay were as described in the text. ●, Phosphate-citrate buffer; ○, veronal buffer.

Table 3. *Effects of suspensions of isotrichs on pectic substances*

Incubation was for 4 hr. in Warburg flasks. Concentrations of substrates were equivalent to 1 mg. of galacturonic acid per ml. of suspension. Pectin esterase activity is given as the percentage of total methoxyl liberated as methanol; polygalacturonase activity as the apparent percentage hydrolysis of substrate into galacturonic acid, and 'fermentation' as the percentage decrease in total galacturonic acid during the incubation; (a), (b) and (c) refer to three separate suspensions of protozoa. X, Test not applicable; —, not determined.

Activity of Protozoa	Substrates				
	Galacturonic acid	Pectin			Sodium polypectate
		(a)	(b)	(c)	
Pectin esterase	X	50	66	67	X
Polygalacturonase	X	36	—	—	42
'Fermentation'	10	16	11	—	8

Fig. 3. Gas production by *Isotricha* during incubation with pectic substances. The buffer solution contained NaHCO_3 (0.1%). The gas phase was $\text{N}_2 + \text{CO}_2$ (95:5). Other conditions were as described in the text. O, Endogenous fermentation; Δ, sodium polypectate; ▲ pectin; ●, methanol.

acid. Hydrolysis of tetragalacturonic acid proceeded more rapidly and yielded chiefly digalacturonic acid with a little galacturonic acid, but no trigalacturonic acid.

The *Isotricha* extract was incubated with polygalacturonic acid at various pH values, and the formation of reducing sugar was measured. A number of different buffer solutions was required to cover the range of activity. Fig. 1 shows the results obtained in such experiments, in which a single batch of enzyme extract was used. The activity was greatest between pH 8.7 and 9.0. Similarly, the *Isotricha* pectin esterase was found to have maximum activity at pH 8.6–8.8 in veronal buffer (Fig. 2).

Table 4. *Pectic enzymes in buffer solution in which isotrichs have been incubated*

Concentrations of substrates and measurement of activities were as described in Table 3. X, Test not applicable.

Enzyme activity	Substrates	
	Pectin	Sodium polypectate
Pectin esterase	72	X
Polygalacturonase	40	43

Effect of Isotricha suspensions on pectic substances

The amounts of gas produced in manometric experiments in which pectin or sodium polypectate was added to *Isotricha* suspensions are shown in Fig. 3. At the end of the experiments the total galacturonic acid, reducing sugar and methanol contents of the solutions were determined. The results are summarized in Table 3, which also includes for comparison the results of a similar experiment in which galacturonic acid was used instead of the polysaccharides. Paper-chromatographic examination showed that the considerable amount of reducing sugar present in solution after incubating protozoa with pectin or sodium polypectate consisted almost wholly of galacturonic acid. To estimate roughly the extent to which the enzymes diffused into the medium from the protozoal cells, a suspension of *Isotricha* was shaken for 4 hr. in buffer solution alone. The protozoa were then removed by centrifuging, and the supernatant was tested for enzyme activity against pectin and sodium polypectate, under the same conditions as would have been used had the solution been a suspension of protozoa. The enzymic activities of this protozoa-free solution are shown in Table 4.

The gas production found when methanol was added to a suspension of *Isotricha* in Warburg flasks is also shown in Fig. 3. During this experiment the methanol concentration in the suspension fell from 90 to 87 $\mu\text{g./ml.}$

DISCUSSION

It is commonly agreed that enzymic decomposition of pectin requires the action of two enzymes or enzyme systems (Deuel & Stutz, 1958). The first of these is pectin esterase, which hydrolyses the ester groups in pectin to yield methanol and polygalacturonic acid (pectic acid), and the second is polygalacturonase, which breaks the polysaccharide chain by rupturing glycosidic linkages. Enzymes of this latter type from different sources vary widely in properties, for example, in type of substrate preferred (i.e. degree of esterification), in pH optimum, in mode of action and in end products. The pectin esterases from different sources have not been subjected to such a detailed comparison; in any case, the possibilities of variation here are much less.

Among micro-organisms which decompose pectin, it would be expected that there would be some correlation between the production of the two enzymes. This was the case, for example, with most, though not all, of the bacteria examined by Smith (1958*a*). In the three protozoal genera which are the subject of the present study, the production of the two enzyme activities falls into the expected pattern, as shown in Table 2. Although there were considerable differences in activity between preparations from the same organism, the differences between organisms were much greater. The *E. caudatum* extract contained neither enzyme, the *D. ruminantium* extract a little of each, and each was most abundant in the *Isotricha* extract. It is clear that *E. caudatum* can play no part in ruminal pectin decomposition; this finding reinforces our previous conclusion (Abou Akkada & Howard, 1960) that this organism uses nothing but granular starch to satisfy its need of carbohydrate. Of the two genera of rumen holotrich protozoa, *Dasytricha* and *Isotricha*, the latter seemed more active in pectin decomposition, and most of our experiments were performed with this organism.

Investigation of the protozoal pectin esterase was made easier by applying a modified assay method, which we believe to be an improvement over those used hitherto. These latter may be divided into two groups, according to whether the hydrolysis of the ester is measured by determining the acidity produced or the methanol liberated. Methods of the former kind usually require that the enzyme acts in a nearly neutral, unbuffered solution (Reid, 1950; Solms & Deuel, 1955), which limits the possible biochemical investigations. The determination of the methanol produced by the action of pectin esterase has hitherto involved distillation (see, for example, Pogell, Moseley, Likes & Koenig, 1957), which can be inconvenient when numerous determinations are to be done. However, a method for determining methanol in

tissues, in which the distillation step is replaced by a diffusion step in a Conway unit has been described (Feldstein & Klendshoj, 1957). The modification of this procedure described in the Materials and Methods section has proved convenient and accurate. Many determinations may be carried out together, and the sensitivity of the chromotropic acid colour reaction permits the experiments to be done on a very economical scale. By means of this technique, and using concentrated enzyme solutions for short incubation periods, the action of *Isotricha* pectin esterase has been investigated over almost the whole pH range of its activity. The enzyme is most active at pH 8.6–8.8, which is well within the region in which pectin undergoes spontaneous chemical hydrolysis.

Since the *Isotricha* extract converts pectic substances chiefly into oligo-uronides, with very little galacturonic acid, the polygalacturonase of *Isotricha* must attack the polysaccharide chains either at random or perhaps preferentially at inner linkages. It should therefore be classified as a liquefying polygalacturonase (Deuel & Stutz, 1958), as distinct from the saccharifying type which remove single galacturonic acid units from the ends of the chains. The splitting of tetragalacturonic acid almost wholly into digalacturonic acid by the *Isotricha* extract supports this classification of the enzyme. As the enzyme has so far not been examined in the absence of pectin esterase, it is not possible to say whether it can attack a highly esterified pectin on its own; the glycosidic hydrolysis of pectin by *Isotricha* extracts might take place after demethylation by the pectin esterase.

A surprising finding in this work was that, although *Isotricha* possesses pectic enzymes, it appears to lack almost all capacity to ferment or otherwise utilize the products of hydrolysis. Thus sodium polypectate was hydrolysed to the extent of 42% during incubation for 4 hr. with a protozoal suspension (Table 3), yet during the same period fermentation of the hydrolysis products into acids or gases was negligible (Fig. 3). The loss of total galacturonic acid during this 4 hr. period, 8%, may be compared with unpublished observations by one of us (B.H.H.) that under comparable conditions *Isotricha* suspensions fermented 80–95% of added glucose within 20 min. When protozoal suspensions were incubated with pectin some gas production was observed (Fig. 3). When, however, the methanol in the solution was measured at the end of the experiment, the corresponding acid groups liberated from the pectin accounted for almost all this additional gas, as carbon dioxide from the bicarbonate buffer. As with the sodium polypectate, so the pectin was broken down into reducing sugars to a large extent, and the loss of total galacturonic acid was also small (Table 3).

At the end of the 4 hr. manometric experiments involving pectin, the protozoa were exposed to methanol in a concentration of about 50 $\mu\text{g./ml.}$ The possible effect of methanol on the protozoa was the subject of a separate experiment, in which the substance was added to approximately twice the concentration found in the pectin experiments. The loss of methanol during 4 hr. was negligible, and the fermentation rate of the protozoa was the same as that of a control sample of the suspension (Fig. 3). The protozoa were normally active when examined microscopically at the end of the experiment, and it was concluded that the methanol produced by hydrolysis of pectin is not metabolized further by, and has no harmful effect on, the isotrichs.

The ease with which the pectic enzymes diffuse out of the protozoal cells is demonstrated by the results of the experiment in which isotrichs were incubated in buffer alone, and then removed. The protozoa-free fluid was then shown to be as active in the hydrolysis of pectin and polygalacturonic acid as a suspension of living protozoa (Tables 3 and 4).

While this work was in progress an investigation into the same problem was reported by Wright (1960). The suspensions of mixed protozoa from the bovine rumen, from which Wright prepared his enzyme extracts, contained both oligotrichs (principally *Epidinium* species, which we have not examined) and holotrichs (*Dasytricha* and *Isotricha*). The properties which Wright reported for the pectin esterase in his material agree with our own findings, but the polygalacturonases in the two cases appear to differ, both in pH optimum [Wright (1960), pH 6.5-7.2; present work, pH 8.7-9.0] and in the end products of hydrolysis (Wright, almost wholly galacturonic acid from pectin, and galacturonic acid plus small amounts of oligo-uronides from low-methoxyl pectin; present work, mainly oligo-uronides, with little galacturonic acid, from both pectin and polygalacturonic acid). Perhaps the most probable cause of these discrepancies is the presence in Wright's (1960) protozoal suspensions of pectolytic organisms, the individual properties of which are unknown.

Some features of our results are very puzzling. Why *Isotricha* possesses enzymes for hydrolysing pectin but cannot use the products of hydrolysis, and why these enzymes have, *in vitro*, pH optima approximately two pH units above the pH of the normal milieu of the organism, are questions to which we cannot at present suggest answers.

SUMMARY

1. Extracts of the rumen Protozoa *Entodinium caudatum*, *Dasytricha ruminantium* and mixed *Isotricha* species have been tested for hydrolytic activity against pectic substances.

2. The *E. caudatum* extracts contained no pectin esterase or polygalacturonase activity, the *D. ruminantium* extracts contained both enzymes in low concentration, and the highest concentrations of both enzymes were found in extracts of the isotrichs.

3. An improved method for measuring pectin-esterase activity, which involves the determination of methanol after diffusion in Conway units, is described. By the use of this method the *Isotricha* enzyme was shown to have maximum activity at pH 8.6-8.8.

4. The extracts of *D. ruminantium* or mixed isotrichs decomposed pectin or polygalacturonic acid principally into oligo-uronides; very little galacturonic acid was produced. Polygalacturonase activity of the *Isotricha* extract was greatest at pH 8.7-9.0.

5. Suspensions of living isotrichs readily hydrolysed pectic substances. Galacturonic acid was the main product, and methanol also was produced from pectin. *Isotricha* appears to possess little or no ability to ferment pectic substances or their hydrolysis products.

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