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Acid-producing Mechanisms in Minced Leaves

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Bawden & Pirie (1944) observed that the pH of a suspension of washed minced tomato leaves, neutralized with NaOH, drifted back towards the original value of pH 5·8–6·2. By making further additions of alkali during 24 hr. the pH could be finally stabilized at 7·6–8·0. A similar drift has been found in neutralized tobacco and bean-leaf suspensions. The present investigation was undertaken to find the cause of this pH drift. There appeared to be three possibilities: the alkaline hydrolysis of an ester giving rise to an acid, the enzymic hydrolysis of an ester taking place when the pH was raised, or the metabolic production of carbon dioxide. Neuberg & Ottenstein (1928) described the enzymic demethylation of pectin in minced tobacco leaves in the presence of sap. They worked at the normal pH of the mince, whereas the pH drift with which this paper is concerned was only observed on raising the pH. Evidence is, however, presented that the principal mechanism causing the pH drift is the enzymic demethylation of pectin. The question has been investigated in some detail because pH adjustment is an essential preliminary to most work on the liberation of viruses and normal proteins from leaves, and it is an advantage to know what changes the pH adjustment itself brings about.

MATERIAL AND TECHNIQUE

For most of the work leaves of *Nicotiana tabacum* var. White Burley were used, as they were available during the whole year from plants grown in the glasshouse. Other plants, listed below, were also used.

The leaves were minced with a domestic meat mincer, the sap squeezed out through madapollam, and the residue washed by suspending in about five times its own weight of water and squeezing out. After three washes the minced material was squeezed as dry as possible by hand and weighed portions taken. The material thus obtained is called *fibre* and contains about 25% dry matter. Fibre (10 g.) suspended in water (100 ml.) gives a mixture which can be stirred easily; vigorous stirring is necessary during the addition of alkali to prevent local raising of the pH much above 8. Chloroform was used as an antiseptic in fibre suspensions. For some of the work the minced fibre was ground finely in a triple-roller mill as described by Bawden & Pirie (1944). pH measurements were made with a glass electrode. Unless otherwise stated, experiments were carried out at room temperature. The analytical methods which were used are described in the last section (see Methods).

RESULTS

When minced fibre was suspended in water at its own pH (5·8–6·2) the pH remained constant within 0·4 unit for several days. The marked drift of pH towards the acid side took place only after raising of the pH by addition of alkali, and the pH never drifted to below the original value.

The amount of alkali needed to stabilize the pH at 8 varied with different batches of fibre, the average amount for minced fibre being 38 ml. 0·2N-KOH/100 g. wet wt. of fibre. Minced fibre of the leaves of *Nicotiana glutinosa*, wild beaked parsley (*Chaerophyllum sylvestre*), beech (*Fagus sylvatica*), groundsel (*Senecio vulgaris*) and dock (*Rumex obtusifolius*) showed the drift and took up a similar amount of KOH. At the beginning of neutralization larger amounts of alkali were required to raise the pH of the suspension to 8 than were necessary towards the end. As the fibre is neutralized it swells and its water-holding power increases. When fibre that has been finely ground in the triple-roller mill is washed by suspension in water and squeezing out, much of the leaf protein is extracted, and the residue has a lower nitrogen content than the original fibre. Washed milled fibre needs about twice as much alkali to stabilize its pH as an equal weight minced. About one-third of the total alkali taken up is

needed for the initial neutralization of milled fibre and the pH is stabilized more rapidly than with minced material. As the substance responsible for the drift was not extracted by washing the fibre, a cell-wall constituent was indicated.

Location of the acid formed

To determine whether the acid formed was extracted from the fibre, *potassium* was estimated both in the neutral extract obtained by squeezing out the fluid from the fibre after the pH had been stabilized, and in the fibre itself. The potassium content of the original fibre, and of fibre after neutralization, was determined by the method given later (see Methods).

The following result is typical: 12 g. minced fibre (dry wt. 2·78 g.) were neutralized by 12·5 ml. 0·091 N-KOH. The potassium content of the original fibre was 3·1 mg., of the neutral extract 19·2 mg. and of the neutralized fibre 24·5 mg. The total potassium accounted for is 40·6 mg., i.e. 92% of the amount added. 43% of the added potassium was in the neutral extract, indicating that the greater part of the acid produced remained on the fibre. The potassium is not simply adsorbed, for when 20 g. fibre at pH 6 were suspended in a potassium sulphate solution containing 100 mg. potassium, after 24 hr. 98·5 mg. were found still in solution.

To test for volatile acid a neutral extract from fibre which had needed 3·3 ml. 0·2N-KOH for neutralization was distilled after acidification with H_2SO_4. Only 0·05 ml. 0·2N-KOH was required to neutralize the acid in the distillate, indicating that there was a negligible amount of volatile acid present.

Carbon-dioxide production in the fibre suspension

That the soluble acid was carbonate, some of the carbon dioxide being formed by metabolic changes in the fibre and some being picked up from the air, seemed probable. Carbonate was determined in the neutral extract, on samples removed at intervals during neutralization, by measurement of the CO_2 liberated on acidification in the Van Slyke manometric gas apparatus (Van Slyke, Page & Kirk, 1933).

Minced fibre (12 g.) was suspended in 150 ml. boiled distilled water in a wide-mouthed bottle, having a stopper holding a burette containing 0·091 N-KOH, and a sampling tube. The latter was a glass tube of 1 cm. diameter reaching nearly to the bottom of the bottle, and having organdie tied over the lower end to prevent fibre being removed when samples were taken. Phenol red was used as an internal indicator in the suspension. Chloroform was omitted as it interfered in the CO_2 determinations.

The results are given in Fig. 1. At the end of the experiment carbonate accounted for 39% of the added alkali, which is in agreement with the value for soluble acid. Making allowance for the carbonate content of the KOH and the CO_2 picked up by a solution at pH 8 from air during sampling, the metabolic production of CO_2 would account for 25% of the added alkali.

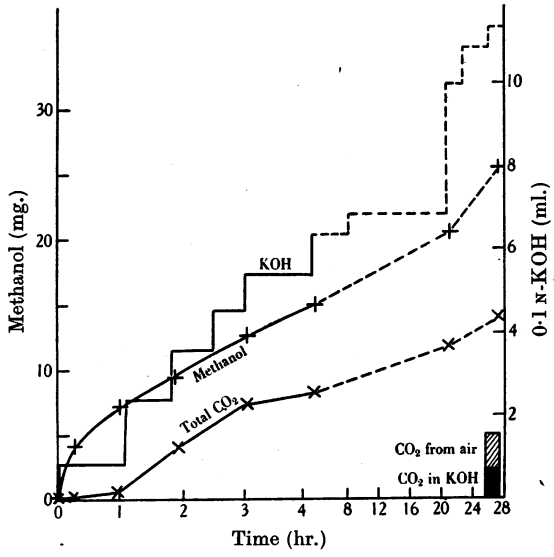

Fig. 1. Relationship between addition of KOH to minced fibre, carbonate content of the solution and methanol liberated. KOH added to 12 g. minced fibre in 150 ml. water to keep the pH at 8. Methanol and CO_2 determined at intervals. Curves are plotted so that ordinates for equimolecular quantities of KOH and methanol are the same, i.e. 1 ml. N-KOH = 32 mg. methanol. CO_2 is plotted in terms of its KOH equivalent. CO_2 from the air and KOH is shown in the bottom right-hand corner.

Titration curves made on a neutral extract showed that it had a low buffering power except in the region of pH 6·4 which is pK_1 for carbon dioxide. This confirmed the finding that the greater part of the acid remained on the fibre and that the soluble acid was carbonate.

Portions of minced and milled fibre were boiled at pH 6 and, when cool, alkali was added to neutralize the suspension. The amount of alkali needed was small and there was little pH drift. The total amount of 0·16N-KOH taken up by 12 g. boiled minced fibre was 1·4 ml., whereas it was 8·0 ml. for unboiled. An enzymic reaction was therefore indicated as the cause of the pH drift.

Liberation of methanol from fibre under various conditions

Ethanol appeared to be absent from a neutral extract of milled fibre, for a distillate from the extract did not give the iodoform reaction. Methanol, on the other hand, was present as shown by (1) forma-

tion of methyl salicylate on warming with sodium salicylate and sulphuric acid; (2) development of purple colour with Schiff's reagent on standing in the presence of conc. H_2SO_4 after oxidation with potassium permanganate. The presence of an appreciable amount of methanol having been established, quantitative determinations were made by a modification of a method given by Schryver & Wood (1920) (see Methods).

The liberation of methanol throughout neutralization was followed in both minced and milled fibre.

The fibre was suspended in water in a bottle with a burette and sampling tube through which samples for methanol determinations were removed at intervals. When the total methanol present at a particular time was calculated, allowance was made for the changes in volume due to removal of samples and the addition of KOH, and for the methanol content of the samples removed previously.

The results (Fig. 1) showed that there was an increase in methanol during neutralization.

Phosphate solution (0·1M-Na_2HPO_4) adjusted to pH 8 had sufficient buffering power to hold the pH of a mixture containing 3·0 g. milled fibre and 60 ml. phosphate solution steady within 0·2 pH unit. Methanol was determined in such a mixture at intervals. Fig. 2 gives the results, which also show

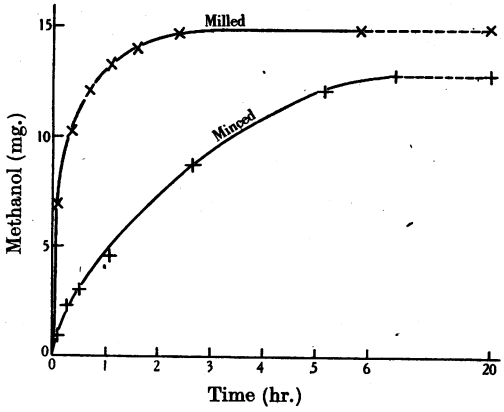

Fig. 2. Comparison of rate of liberation of methanol from minced and milled fibre. Portions (3 g.) of minced or milled fibre in 60 ml. phosphate buffer (0·1M) at pH 8.

that with fibre in buffer solution the liberation of methanol is slower with minced than with milled fibre. Liberation of methanol from buffered fibre is more rapid than from fibre neutralized with alkali.

For the following experiments milled fibre only was used as it provided a more homogeneous substrate than minced fibre. A comparison was made of the rate of liberation of methanol from fibre at pH values from 6·0 to 8·0. The results given in Fig. 3 show that the liberation is most rapid at pH 8 and that there is some demethylation at pH 6, the natural pH of the fibre suspension.

The total methanol liberated from 3 g. fibre boiled at pH 6 in water, cooled, and then suspended in 60 ml. phosphate solution at pH 8 for 20 hr., was 1·74 mg. The distillate from 3 g. fibre boiled at pH 6 contained only 0·3 mg. methanol, showing that the

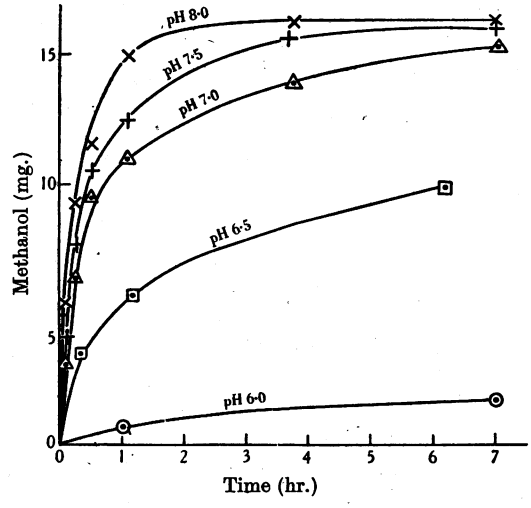

Fig. 3. Comparison of methanol liberation at different pH values. Portions (3 g.) of milled fibre in 60 ml. phosphate buffer solutions (0·1M) at pH values from 6 to 8.

fibre had not been previously demethylated by boiling at pH 6, and therefore that the great decrease in the amount of methanol produced was due to the destruction of the demethylating enzyme.

A suspension of 3 g. milled fibre in 100 ml. water to which 1 ml. 5N-NaOH had been added was boiled and 50 ml. distillate collected. The methanol content of the distillate was 14·4 mg., which corresponded with the amount produced enzymically at pH 8. von Fellenberg (1918) found that ester-combined pectin methyl groups were split off by dilute alkali, whereas the ether-linked lignin methyl groups remained attached.

Non-enzymic demethylation. Fibre (3 g.) that had been boiled at pH 6 was boiled in 100 ml. 0·05N-NaOH; the methanol found in the distillate was 15·6 mg., showing that fibre in which enzyme had been destroyed could be demethylated by boiling with dilute alkali.

The non-enzymic demethylation of boiled fibre at different pH values at room temperature was investigated. Portions (0·5 g.) of boiled fibre were suspended in 10 ml. solution for 20 hr. and methanol determined. Table 1 shows that although there is some non-enzymic demethylation at pH 8 it is more extensive in solutions of higher pH.

Heat stability of enzyme. Portions of fibre were suspended in water and kept at different temperatures for 15 min. and cooled; each portion was then

Table 1. *The non-enzymic liberation of methanol from boiled fibre*

(0·50 g. boiled fibre in 10 ml. solution at different pH values for 20 hr. Room temperature.)

pH	Medium	Methanol (mg.)
6·05	0·1 M-phosphate solution	0
6·88	,,	0
7·95	,,	0·31
9·40	,,	0·75
12·3	0·02 N-NaOH	1·44
Boiled in 0·02 N-NaOH		2·34

squeezed out and suspended in 0·1 M-phosphate solution at pH 8 and methanol was determined at intervals. The results are given in Table 2.

Table 2. *Methanol liberation in heated fibre*

(Suspensions of 3 g. milled fibre in 50 ml. water, at pH 6, kept at the temperatures given in the first column for 15 min. before suspending in 50 ml. 0·1 M-phosphate at pH 8.)

	Methanol (mg.) liberated in		
°C.	2 hr.	17 hr.	41 hr.
18	12·3	13·5	13·8
60	7·3	11·5	12·0
70	6·1	10·7	11·2
80	4·7	9·6	10·0
90	1·8	3·1	3·5
100	1·3	2·7	2·7

Demethylation in the presence of sap

Neuberg & Kobel (1927) obtained pectase from the expressed sap of tobacco leaves which could demethylate extracted citrus pectin. Neuberg & Ottenstein (1928) showed that enzymic demethylation of pectin took place in a mince of tobacco leaves at the normal pH of the minced leaves. As demethylation is slow in well-washed minced or milled fibre at pH 6, an experiment was made with fibre in the presence of sap to obtain conditions comparable with those used by Neuberg & Ottenstein.

An examination was made of methanol liberation in fibre + sap under various conditions, and also from boiled fibre. The details of this experiment are given later (see Methods). The results are given in Table 3. This shows that demethylation of the fibre has taken place to the extent of 28 % at 16° and of 48 % at 40°, in 24 hr. at pH 5·9. At pH 8 in 24 hr. demethylation was practically complete and was more rapid at 40° than at 16°. There was little increase in the amount of methanol from boiled fibre at pH 5·9 and a small increase at pH 8 due to non-enzymic demethylation. These results are somewhat different from those of Neuberg & Ottenstein (1928), who found that 50 % of the methanol present in the form of esters was liberated in 2 hr. at pH 6.

Table 3. *The liberation of methanol from fibre + sap under various conditions*

(Values at pH 5·9 obtained by adding 40 ml. water to 10 g. fibre + sap. Values at pH 8·1 obtained by neutralizing the mince with alkali and then adding 40 ml. 0·1 M-Na_2HPO_4 solution at pH 8·1.)

	Methanol (mg.) liberated in				
Treatment	10 min.	30 min.	2·25 hr.	6 hr.	24 hr.
pH 5·9, 16°, boiled	—	1·37	—	1·51	1·70
pH 5·9, 16°	0·28	1·31	2·25	2·86	4·95
pH 5·9, 40°	—	2·32	4·13	6·76	8·43
pH 8·1, 16°, boiled	—	—	1·93	2·32	3·27
pH 8·1, 16°	—	10·00	13·56	16·05	15·94
pH 8·1, 40°	—	13·05	15·01	16·51	16·34
Boiled with NaOH ...		16·63			

The total amount of methanol liberated in the absence of sap

Minced fibre (10 g.) was washed with three 50 ml. portions of water and methanol determined in the washes. The fibre was then suspended in 100 ml. water and methanol determinations made during 5 days; in this time the pH fell from 6·34 to 5·63. The fibre was squeezed out and suspended in 80 ml. 0·1 M-phosphate solution at pH 8; methanol was again determined at intervals. Finally, the fibre was boiled with alkali and methanol determined in the distillate.

Table 4 shows that at pH 6 demethylation is at first fairly rapid, even in the absence of sap, but slows off considerably. Then at pH 8 the fibre is rapidly and completely demethylated.

Table 4. *The total liberation of methanol from minced fibre in the absence of sap*

(10 g. fibre were washed 3 times, then suspended in water for 5 days and afterwards in phosphate buffer (0·1 M) at pH 8 for 2 days. The suspension was finally boiled with alkali. Samples were taken at intervals, as shown, and methanol determined.)

Treatment		Methanol (mg.)
Three washes, 1 hr.		5·4
Soaking at own pH:	1 day	4·8
	2 days	6·2
	5 days	7·4
Soaking at pH 8:	2 hr.	12·8
	20 hr.	19·1
	2 days	20·5
Boiling with NaOH		0·4
	Total	33·7 mg.

Examination of various enzyme preparations for demethylating activity

Experiments were made to find means of demethylating boiled fibre enzymically. Some commercial enzyme preparations were tested for demethylating activity. Portions (2 g.) of boiled

fibre were suspended in 40 ml. 0·1M-phosphate solution at pH 8 with, respectively, 80 mg. trypsin (British Drug Houses Ltd.), 80 mg. taka-diastase (Parke Davis and Co.), 170 mg. 'Luizym' (Luitpold-Werk, Munich). Another 2 g. portion was tested with snails' stomach juice, the quantity used being equivalent to the juice from one stomach. None of these enzymes was effective in demethylating boiled fibre.

Boiled fibre (3 g.) was suspended in 88 ml. centrifuged sap of *Nicotiana tabacum* that had been adjusted to pH 8. The high buffering capacity of the sap was sufficient to hold the pH at the initial value. The methanol content of the sap was 4·26 mg., and after 48 hr. there was an increase of 11·43 mg. It is clear, as Neuberg & Kobel (1927) found, that the sap contains a demethylating enzyme. It might be thought that the methanol found in the sap was produced only on mincing, as the result of the enzyme and substrate being brought together. However, methanol was found in the juice squeezed out from frozen whole leaves, which suggests that the methanol was preformed.

Extraction of enzyme from fibre. Preliminary experiments showed that water extracts of milled fibre at pH 6 had no demethylating activity on boiled fibre, but that extracts at pH 8 made either with alkali or phosphate had strong activity. Extracts at pH 8 from bean and tomato fibre also could demethylate tobacco fibre. By distilling *in vacuo* below 30° the extracts could be concentrated and methanol removed. The active portion of the extracts could be precipitated by half-saturation with ammonium sulphate.

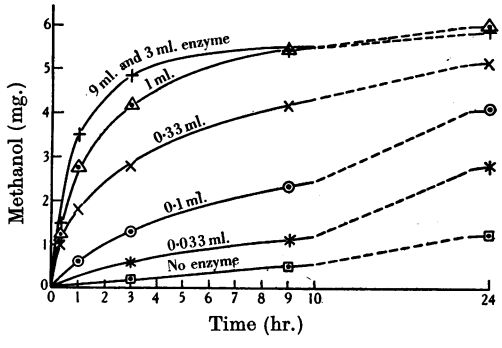

Fig. 4. Dependence of methanol liberation on enzyme concentration. Portions (2 g.) of boiled milled fibre with amounts of enzyme concentrate 9·0–0·033 ml. in phosphate buffer (0·1M) at pH 8.

Milled tobacco fibre (15 g.) was demethylated in phosphate solution (pH 8) and the neutral extract concentrated to 50 ml. Portions (2 g.) of boiled fibre were taken and different amounts of the concentrated fibre extract, from 9·0 ml. down to 0·033 ml., were added and the volume made up to 40 ml. with 0·1M-phosphate solution pH 8. Boiled concentrated fibre extract (3 ml.) was added to one portion, and there was a control to which no enzyme was added. Samples were removed at intervals. The results given in Fig. 4 show that the amount of methanol liberated was dependent on the enzyme concentration. The curve for 9 ml. of enzyme solution was the same as that for 3 ml. except that the 20 min. value was slightly higher.

Demethylation of pectin. Enzyme concentrates were tested for their ability to demethylate pectin extracted from tobacco leaves by ammonium oxalate solution (see Methods). The ammonium oxalate-extracted fibre (3 g.), when boiled with 0·05N-NaOH, gave 2·1 mg. methanol, which showed that most of the methanol-producing substance had been removed. When neutralized fibre was extracted with ammonium oxalate solution the precipitated pectic material had no methoxyl content.

Pectin was dissolved in water; to one portion, phosphate buffer at pH 6 was added + 15 ml. enzyme solution (= amount of enzyme extracted from 1·5 g. fibre); to two other portions, phosphate buffer at pH 8 was added, and 15 ml. enzyme to one of them. Samples were removed at intervals and methanol estimations made. As soon as the sample was withdrawn acid was added to it to lower the pH to 4 in order to stop any further action and to prevent demethylation when the sample was distilled. On acidification of the samples from the preparations with enzyme a gelatinous precipitate was obtained.

The results given in Table 5 show that the extracted enzyme is effective in demethylating extracted tobacco pectin. There was some demethylation at pH 8 without enzyme. At pH 8 with enzyme demethylation is more rapid than at pH 6.

Table 5. *Liberation of methanol from extracted tobacco-leaf pectin*

(200 mg. portions of pectin in 40 ml. water + 25 ml. 0·1M-phosphate solution (at pH 6 or at pH 8) + 15 ml. enzyme solution.)

	Methanol (mg.) liberated in			
	5 min.	45 min.	3 hr.	17 hr.
pH 6 + enzyme	1·15	2·40	3·79	7·05
pH 8, no enzyme	0	Trace	—	1·57
pH 8 + enzyme	2·46	4·45	·6·67	8·96

100% liberation = 9·9 mg.

Demethylation in minced leaves of members of the Cucurbitaceae

In the plants so far considered the pH of the expressed sap was 6 or less. It was thought that an investigation of the methanol liberation in plants with more alkaline sap might be of interest. Bawden & Pirie (1937) recorded the pH of the expressed sap of cucumber leaves as between 7 and 8. Cucumber leaves from glasshouse plants gave on mincing a

sap with a pH of 7·2. After three washes the pH of the fibre suspension (prepared as previously described) had risen to 9·6. Other members of the Cucurbitaceae were also found to have relatively alkaline saps: white bryony (*Bryonia dioica*) pH 6·6–7·2, marrow (*Cucurbita pepo*) 6·9–7·4 and melon (*Cucumis melo*) 7·4. On washing the fibre of these leaves the pH increased; the value of pH 9·4–9·6 was reached, which was maintained but not exceeded when washing was continued. In these plants the methanol content of the sap was from 0·25 to 0·5 mg./ml., i.e. from five to ten times as high as in tobacco. The freeze-juice from whole leaves had also a higher methanol content. The addition of alkali to bring about demethylation in bryony, etc., is not necessary; methanol is liberated spontaneously owing to the high pH. When the fibre of these plants is boiled, demethylation takes place rapidly although the enzyme is destroyed. A concentrate of the extract of bryony fibre soaked at the pH reached on washing, i.e. 9·4, was effective in demethylating boiled tobacco fibre. The phenomenon of rise of pH on washing is at present under investigation.

DISCUSSION

All the observations make it clear that the pH drift is mainly due to the enzymic breakdown of pectin to methanol and pectic acid.

The slower liberation of methanol from fibre neutralized with alkali than from fibre in buffer solution is due to the pH falling between each addition to a value where demethylation is slower. Minced fibre contains a large proportion of intact cells, whereas in milled fibre all the cells have been ruptured and torn apart. There is therefore a larger surface area of pectin exposed to the enzyme and the solution of higher pH can permeate more easily. Because of this, demethylation in milled fibre is more rapid than in minced.

Methanol liberation is considerably affected by keeping fibre at different temperatures before neutralization (Table 2). The values in Table 2 and Fig. 4 show that the relationship between enzyme and substrate, in the form in which it is present in boiled fibre, is complex, and that with small amounts of enzyme the rate of liberation of methanol is such that the total possible methanol would not be produced.

Neuberg & Kobel (1927) gave the pH optimum for the action of tobacco pectase on extracted citrus pectin as 5·6, while Kertesz (1936, 1937) stated that pectin-methoxylase (pectase) had no pH optimum, the action increasing with decreasing hydrogen-ion concentration until the effect of the alkalinity of the medium superseded the enzymic action. In the present investigation, although the enzymic demethylation of extracted pectin was fairly rapid at pH 6, it was very slow at this pH when the pectin was on the fibre. As the pH was raised the action of the enzyme increased, but even at pH 8 the non-enzymic demethylation was appreciable. Owens, McCready & Maclay (1944) gave pH 9 as the maximum value for the *in situ* enzymic demethylation of citrus-peel pectin; at higher pH values the alkaline de-esterification was considerable.

The presence of pectase in the leaf-sap of many plants is well known, but it is of interest that a pectin-demethylating enzyme should remain on the fibre and only be extracted when the pH is raised.

METHODS

Potassium determinations. The potassium was precipitated as potassium-silver-cobaltinitrite with 25% w/v Analar sodium cobaltinitrite in the presence of $AgNO_3$. The precipitate was washed with aqueous acetone, acetone and finally ether and allowed to dry. It was then dissolved in dilute HNO_3 and ethanolic ammonium thiocyanate added. This gave a blue colour (Breh & Gaebler, 1930), which was compared in a visual colorimeter with standards from a range containing 0·1–0·5 mg. of potassium. Weighed amounts of fibre which had been dried at 100° were ashed in a porcelain crucible with H_2SO_4 at 500°. The hot-water extract of the ash was filtered and potassium determined in the filtrate. To the neutral extract a few drops of glacial acetic acid were added and the solution filtered to remove precipitated protein. A sample was taken containing less than 0·5 mg. potassium.

Methanol determinations. A method for estimating methanol described by Schryver & Wood (1920) was based on the colour reaction between phenylhydrazine hydrochloride, potassium ferricyanide, hydrochloric acid and formaldehyde. The methanol was converted to formaldehyde by ammonium persulphate. This method is laborious and not suitable for routine estimations, as it is necessary to determine the amount of persulphate required for the oxidation of each sample. Snell & Snell (1937) suggest that the potassium permanganate and oxalic acid reagents used in the modified Denigès method for methanol estimation could be used instead of ammonium persulphate. Solutions of $KMnO_4$ (3 g.) in 85 ml. water + 15 ml. 85% (w/w) H_3PO_4 and oxalic acid 5% (w/v) in 49% (w/w) H_2SO_4 were tested. The presence of conc. H_2SO_4 reduced the sensitivity of the test so much that it was useless. With lower concentrations of H_2SO_4 the permanganate solution did not clear. However, when a more concentrated solution of oxalic acid was used, and the solution warmed, it became clear and colourless and the H_2SO_4 could be omitted.

The estimations were made as follows: Samples of methanol-containing solutions (0·5–5·0 ml.) with a methanol content of 0·03–0·40 mg. were distilled in the steam distillation apparatus described by Markham (1942). Most of the samples in which methanol was to be determined were coloured and cloudy so that it was necessary to use a distillate. Possible interfering substances were also avoided by this means. To ensure complete recovery of methanol, 5 ml. of distillate were collected. To the distillate 1 ml. $KMnO_4$ (as above) was added, and after standing 10 min. 1 ml. 10% (w/v) aqueous oxalic acid. The time of oxidation is of importance because although on longer standing the

amount of methanol converted to formaldehyde is increased, there is also a great increase in the amount further oxidized to formic acid. Under the conditions used about 26% of the methanol was converted to formaldehyde. After the addition of oxalic acid the solution was warmed. When cool 2 ml. 1% (w/v) phenylhydrazine hydrochloride was added followed by 1 ml. 2% (w/v) potassium ferricyanide and 3 ml. conc. HCl, making a total volume of 13 ml. On the addition of the ferricyanide solution a precipitate formed which disappeared on the addition of the HCl, when the cherry-red colour developed immediately. Freshly prepared reagents must be used or the precipitate does not disappear and the colour developed is paler. The intensity of colour was compared in a visual colorimeter with standards containing 0·04–0·32 mg. methanol prepared from a solution containing 0·08 mg./ml. methanol, in the same way and at the same time. The colour is fairly stable but fades on long standing, so comparisons were made within 15 min. of the colour being developed.

In all experiments when serial estimations of methanol were made samples were kept until there was a convenient number (about 12) to be assayed in a batch. Samples free from suspended fibre gave constant values for methanol, whether assayed immediately or after being kept 24 hr.

Experiment to determine the rate of demethylation in the presence of sap. Leaves were minced and 10 g. portions of fibre + sap (dry wt. 1·15 g.) weighed out as quickly as possible. Three portions were each diluted with 50 ml. water, the pH on dilution being 5·91. One of these was kept at room temperature (16°), another at 40° and the third boiled and then kept at 16°. Two portions were adjusted to pH 8 with 0·2N-NaOH and diluted with 50 ml. 0·1M-phosphate solution (pH 8·1). One of these portions was kept at 16° and the other at 40°. Another portion was diluted with 10 ml. water, boiled, and when cool adjusted to pH 8 and 40 ml. 0·1M-phosphate solution added. A further portion was distilled with 1 ml. 5N-NaOH added to the suspension. The methanol content was determined in the distillate to give the value for the total amount of loosely combined methanol. The first sample was taken 10 min. after mincing, and the methanol concentration found was taken as the initial value for the series.

Extraction of pectin. Well-washed milled fibre was extracted with ethanol to remove pigments, then suspended in water and boiled for 5 min. to destroy the demethylating enzyme. Aqueous ammonium oxalate (0·5% w/v) was used for pectin extraction. This was carried out at pH 4 and 25° for 48 hr., the extraction solution being changed frequently. An equal volume of 95% ethanol was added to the extract in order to precipitate the pectin. The precipitate was dissolved in water and reprecipitated three times. After drying over H_2SO_4 the product had a methoxyl content of 4·8%. When dissolved in water the solution was opalescent, rather viscous, and had a pH of 5·4. The methoxyl content of the pectin was low but it was possible to use the material to demonstrate the demethylating action of the enzyme on pectin.

SUMMARY

1. The drift of pH towards the acid side observed when minced tobacco leaves are neutralized with alkali is mainly due to the enzymic demethylation of pectin when the pH is raised above that of the normal sap. The metabolic production of carbon dioxide is also in part responsible for the drift.

2. The liberation of methanol is more rapid in milled than in minced leaf fibre, and at pH 8 more rapid than at pH 6, whether in the presence or absence of sap.

3. Heating to 100° prevents the pH drift, and the small amount of methanol liberated at pH 8 is due to non-enzymic demethylation.

4. The pectase is extracted from fibre at pH 8 but not at pH 6. The pH 8 extract will demethylate boiled fibre and extracted pectin.

5. Minced leaves of some Cucurbitaceae have a spontaneous alkaline drift when washed, which permits demethylation without the addition of alkali.

6. An improved method for the estimation of methanol is described.

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