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An Alkali-producing Mechanism in Macerated Leaves

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While investigating the enzymic demethylation of pectin in macerated leaves, a phenomenon not previously described was observed in some members of the Cucurbitaceae (Holden, 1945). When the fibre residue, obtained by squeezing out the sap from minced leaves, was washed with distilled water, it was found that successive washes had higher pH values until a maximum of pH 9·4–9·6 was reached, and this value was maintained on continued washing. The present paper concerns further investigations of this spontaneous rise of pH. The phenomenon would appear to be of importance in the extraction of normal and virus proteins from green leaves since the infectivity of a number of plant viruses is decreased by pH values greater than 9.

MATERIAL AND METHODS

The plants examined were obtained from various sources. All the Cucurbitaceae (except marrow, cucumber and bryony) were from the University Botanic Garden, Cambridge. *Nicotiana tabacum* and *N. glutinosa* were glasshouse grown and the rest of the plants were from gardens or were found growing wild in the neighbourhood of Rothamsted.

The leaves were minced with a domestic meat mincer and the sap squeezed out by hand through madapollam. The fibre was washed by suspending in distilled water (about 5 ml./g. fibre wet weight) and again squeezing out. Measurements of pH were made with a glass electrode.

Calcium was determined on ashed material. Ashing was done in porcelain crucibles over a Bunsen burner at *c.* 500° on weighed portions of fibre or measured volumes of sap that had been dried at 100°. The dried material was moistened with a few drops of HNO_3 to assist ashing. After weighing, the ash was dissolved in N-HCl, filtered, and calcium precipitated as oxalate; the latter was dissolved in H_2SO_4 and titrated with $KMnO_4$ (0·05 N or 0·01 N depending on the amount of oxalate).

Carbonate determinations were made by the method of Hutchinson & MacLennan as described by Piper (1942) except that $Ba(OH)_2$ was used instead of NaOH to absorb the CO_2 liberated.

Phosphorus was determined colorimetrically by a modification of the method of Kuttner & Lichtenstein (1932). Values for inorganic phosphorus were obtained by developing the colour in samples without previous incineration.

EXPERIMENTAL AND RESULTS

Plants showing alkaline drift

Plants of different families, including ten genera of the Cucurbitaceae, were examined and are listed in Tables 1 and 2. As the pH of the expressed sap from the leaves in which the pH rise was first observed was neutral or slightly alkaline, in contrast to the somewhat acid saps found in most plants, other plants known to have more alkaline sap were examined. Haas (1920) had recorded a pH as high as 8·5 for the expressed sap of sweet clover leaves. These were not available, but the sap of common yellow melilot (*Melilotus altissima*), which is closely related, had a pH of 7·8. On washing the fibre, however, the pH fell to 6·3. The pH of sunflower-leaf sap was given by Gustafson (1924) as between 6·3 and 6·9. This was confirmed and both sunflower (*Helianthus annuus*) and Jerusalem artichoke (*H. tuberosus*) were found to show the pH rise, though other members of the Compositae that were examined did not. All other members of the Cucurbitaceae as well as comfrey (*Symphytum officinale*) of the Boraginaceae and stinging nettle (*Urtica dioica*) of the Urticaceae showed the phenomenon. Although the pH tended to be higher in sap from leaves which showed the drift than from those which did not, this was not always so as can be seen from Tables 1 and 2. Young leaves either did not show the pH rise at all or only to a much less marked extent.

Fig leaves (*Ficus carica*) were anomalous in that the pH rose to 8 but no higher; these cannot therefore be included in either group.

Table 1. *Calcium distribution in plants not showing alkaline drift*

Family	Common name	Latin name	pH of sap	Dry wt. as % wet wt.	Ca as % dry wt. of sap	Ca as % dry wt. of fibre	% total Ca in fibre
Solanaceae	Tobacco	*Nicotiana tabacum*	6·05	8·7	1·5	1·6	52·5
		Nicotiana glutinosa	5·28	8·9	8·2	1·8	16·8
	Tomato	*Lycopersicum esculentum*	6·20	10·6	3·7	1·9	35·0
Leguminosae	Melilot	*Melilotus altissima*	7·79	20·2	1·9	2·3	61·2
Cruciferae	Broccoli	*Brassica oleracea*	5·80	17·3	3·9	1·7	44·3
Labiatae	Dead-nettle	*Lamium purpureum*	6·11	16·3	1·4	0·9	48·7
Rosaceae	Strawberry	*Fragaria vesca*	—	32·9	0·5	1·3	82·9
Compositae	Marigold	*Calendula officinalis*	5·93	7·3	0·9	0·9	19·6
	Groundsel	*Senecio vulgaris*	5·86	8·0	1·4	0·8	38·6
	Chrysanthemum	*Chrys. hortorum*	5·80	19·6	2·5	1·4	47·3
Labiatae	Peppermint	*Mentha piperata*	6·30	14·5	5·3	1·0	44·5
Caprifoliaceae	Elder	*Sambucus nigra*	5·85	19·7	0·9	2·3	72·0

Table 2. *Calcium distribution in plants showing alkaline drift*

Family	Common name	Latin name	pH of sap	Dry wt. as % wet wt.	Ca as % dry wt. of sap	Ca as % dry wt. of fibre	% total Ca in fibre
Cucurbitaceae	Marrow	*Cucurbita ovifera*	7·00	16·1	1·7	10·2	90·3
	Cucumber	*Cucumis sativus*	7·43	11·7	4·7	11·3	85·2
		Sicyos angulata	6·29	7·6	4·2	5·3	50·0
	Calabash	*Lagenaria leucantha* v. *longissima*	6·15	11·8	6·3	4·2	38·0
		Momordica charantica	6·61	24·1	0·7	7·5	90·5
	Squirting cucumber	*Ecballium elaterium*	6·80	11·1	2·5	6·9	66·1
	Loofah	*Luffa cylindrica*	5·50	19·7	1·8	6·1	77·8
	Bryony	*Bryonia dioica*	6·98	11·1	1·1	5·9	91·3
		Thladiantha dubia	—	13·9	—	6·1	—
		Cyclanthera explodens	—	12·0	—	5·9	—
Compositae	Sunflower	*Helianthus annuus*	6·60	*c.* 15·0	2·3	7·3	85·3
	Jerusalem artichoke	*Helianthus tuberosus*	6·11	20·3	1·5	6·2	85·5
Urticaceae	Nettle	*Urtica dioica*	—	21·8	3·0	6·5	90·0
Boraginaceae	Comfrey	*Symphytum officinale*	7·00	13·2	0·5	3·6	96·0

The leaves of all species showing the alkaline pH drift have a characteristic dry and rough feel due to the covering of large hairs which are a conspicuous feature of all of them. A microscopical examination of sections stained in ethanolic purpurin to distinguish calcified areas (Baecker, 1930) showed that both uni- and multicellular hairs and the walls of cells in the region of the hairs were calcified. In addition cystoliths occurred beneath the upper epidermis in *Urtica dioica*.

General observations on the pH *drift*

Water volume. When the maximum pH value is reached, the volume of water used for washing does not influence the pH to any great extent. In one experiment, a wash with 10 ml. water/g. wet weight nettle fibre had a pH of 9·5 while a wash with 200 ml./g. had a pH of 9·6. No volatile alkali was found in alkaline washes, but an appreciable amount of calcium (0·15–0·35 mg./ml.), traces of phosphorus (2–4 μg.) and magnesium were present.

Milling. When minced fibre is finely ground in a triple-roller mill (Bawden & Pirie, 1944) the pH is raised even without previous washing. In some species, a pH of 9 or higher is not reached by washing minced fibre but only after grinding. The pH of fibre of leaves which do not show the high pH is raised by about 0·2 unit on milling.

Heating. The pH rise is not prevented by boiling a suspension of fibre; it may actually be speeded up and the maximum pH may be higher. Whole leaves dipped into boiling water gave an extract with a pH between 8 and 9. The action appears therefore not to be enzymic.

Effect of KCl *and* $CaCl_2$. Minced fibre washed with N-KCl solution instead of water gave the alkaline pH drift, but the soaking of fibre in 0·2M-$CaCl_2$ solution tended to prevent the pH rise.

Alkali content of fibre. It was found that addition of dilute acid to fibre caused effervescence and evolution of CO_2. When 0·2N-HCl was added to a minced fibre suspension to lower the pH to 3·5, the pH rose after a short time to between 7 and 8 and it

only remained stable at 3·5 after several additions of acid. The pH did not rise above 7 between additions of acid unless the solution was squeezed out from the fibre and the fibre resuspended in water, when the pH rose to above 9. Milled fibre took up a large amount of acid in one addition and no pH rise occurred.

Calcium was found in the acid extract and quantitative determinations showed that it was present in an amount sufficient to account for most of the acid added. The calcium content of the sap washes and of the fibre was determined in the various species and the results expressed on a dry matter basis. The results given in Tables 1 and 2 show that the 'alkaline drift' plants have a much higher calcium content than those of the other group. The calcium content of the sap as a percentage of dry matter is similar in both groups, but the fibre calcium is considerably higher in plants giving the pH rise; the proportion of the total calcium which is insoluble is thus greatly increased.

The calcium content of the fibre of young leaves of 'alkaline drift' plants is lower than that of fully grown leaves. Young bryony leaves (picked in June) had a fibre calcium of only 2·2 % dry matter whereas the larger older leaves from the same plants had a value of 6·2 % dry matter.

The carbonate content of milled marrow fibre was found to account for 80 % of the total calcium content. The greater part of the calcium is thus present as $CaCO_3$.

Phosphorus content of fibre. Phosphorus determinations were made on fibres of a number of species. The P content of 'alkaline drift' fibres was considerably higher than that of fibres which do not show the drift. Table 3 gives a comparison of the P distribution in fibre of nettle and tobacco, as examples of both types of fibre.

Portions of fibre (0·5 g.) were extracted with 10 ml. 0·2 N-HCl, centrifuged after 2 hr. and a further 5 ml. acid added. After the second portion of acid had been decanted, 10 ml. ethanol-ether (7 : 3) were added to remove lipid P. After a further 2 hr., a second extraction with a 5 ml. portion of ethanol-ether followed and the fibre was then dried. (HCl (0·2 N) was used for acid extraction in the earlier experiments, but later cold 10 % trichloroacetic acid was used as recommended by Schneider (1945) for fractionation of P in animal tissue.) Total and inorganic P were determined on the acid extract, total P on the ethanol-ether extract and on the fibre residue.

The amount of inorganic P in nettle fibre is remarkably high but even higher values of up to 1·2 % of the dry matter have been found. The highest value for tobacco fibre was less than 0·4 % and these plants had been given a large amount of phosphate fertilizer. The P content of the fibre of young leaves is higher than that of old even in 'alkaline drift' plants, but the amount of inorganic P is less. Table 4 compares the P distribution in the fibre of old and young nettle leaves picked in June.

Thoday & Evans (1932) stated that the phosphate in *Helianthus tuberosus* was localized in the multi-

Table 3. *Comparison of fractionation of fibre* P *in tobacco and nettle*

	Tobacco			Nettle		
Fraction	P as % total dry wt.	Total P (%)	Acid-extractable P (%)	P as % total dry wt.	Total P (%)	Acid-extractable P (%)
Total	0·240	100	—	0·720	100	—
Extracted by 0·2 N-HCl	0·103	43	100	0·634	88	100
Inorganic P in acid extract	0·072	17	70	0·570	79	90
Ethanol-ether (7 : 3) extractable	0·024	10	—	0·043	6	—
Residue	0·113	47	—	0·043	6	—

Table 4. *Comparison of* P *fractionation in young and old bryony leaves*

	Young leaves			Old leaves		
Fraction	P as % total dry wt.	Total P (%)	Acid-extractable P (%)	P as % total dry wt.	Total P (%)	Acid-extractable P (%)
Total	0·650	100	—	0·420	100	—
Extracted by 10 % trichloroacetic acid	0·514	79	100	0·294	70	100
Inorganic P	0·108	16·6	21	0·265	63	90
Ethanol-ether (7 : 3) extractable	0·059	9	—	0·063	15	—
Residue	0·078	12	—	0·063	15	—

cellular epidermal hairs and in *H. annuus* in the basal cells of the hairs and the cortical cells below. The method they used involved treatment of sections with a solution of ammonium molybdate in HNO_3 which produced a yellow crystalline precipitate with the phosphate in the tissues; this was then reduced to a bluish-black oxide of molybdenum with phenylhydrazine hydrochloride. A microscopical examination of minced fibre of 'alkaline drift' plants showed that there were large numbers of undamaged epidermal hairs. When marrow leaf fibre was treated by the above method it gave a large quantity of yellow crystals which were concentrated to some extent in and around the hairs, but owing to the acidity of the reagent, which must be maintained, much phosphate was extracted from the fibre into solution. It seems probable that much of the calcium and phosphate are localized in the same cells and that in addition to calcium carbonate some of the calcium is present as a calcium phosphate.

Conditions under which a pH *rise takes place with 'non-drifter' fibre*

A pH rise could be obtained with 'non-drifter' fibre by adding $CaCO_3$ and phosphate and, when the necessary conditions had been established, the investigation was continued to find which constituent

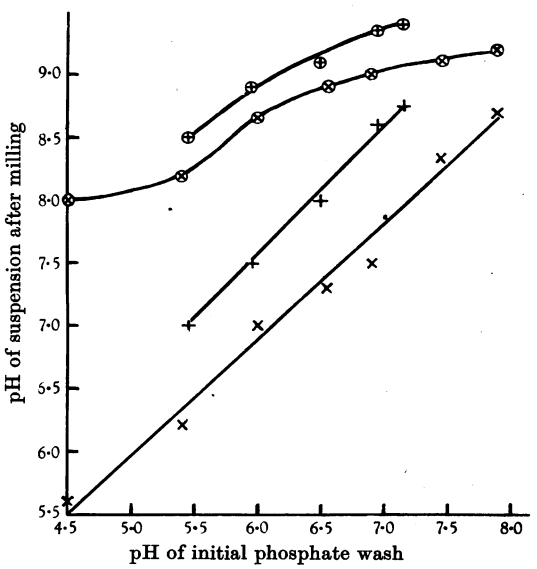

Fig. 1. The effect of milling fibre 'phosphated' at different pH values. ×—× nightshade fibre; ⊗—⊗ nightshade fibre + $CaCO_3$; +—+ tomato fibre; ⊕—⊕ tomato fibre + $CaCO_3$.

of fibre, in addition to $CaCO_3$ and phosphate, could be responsible. When tobacco leaf fibre was milled with $CaCO_3$ the pH did not rise above that of the $CaCO_3$ itself (i.e. pH 8). When, however, it was neutralized by soaking in 0·2 M-Na_2HPO_4 adjusted to pH 7·9, washed with water and then milled with $CaCO_3$, a pH of 9·3 was obtained. Unless the fibre was thoroughly washed after 'phosphating', the pH rise did not occur, due undoubtedly to the buffering action of the phosphate. 'Phosphated' washed fibre had a P content about twice the original value. Fibre which had been soaked in phosphate solution and milled without $CaCO_3$ showed a rise but the pH was not as high as with the $CaCO_3$. The actual pH reached on milling, with or without $CaCO_3$, was found to depend on the pH of the phosphate solution in which the fibre had previously been soaked. The results of an experiment using nightshade (*Solanum dulcamara*) and tomato fibre are shown in Fig. 1.

A similar effect could be obtained by replacing sodium phosphate as a soaking fluid by sodium citrate or potassium oxalate but not by sodium acetate, sodium sulphate or sodium chloride. The pH rise was also obtained with acid-extracted nettle fibre under the same treatment. Table 5 gives the results of an experiment using minced nettle fibre.

Table 5. *Reconstitution of* pH *rise in acid-extracted nettle fibre*

(15 g. minced nettle fibre soaked in 100 ml. N-HCl overnight, squeezed out and washed twice with 100 ml. water. pH of fibre suspension 2·7.)

Treatment	pH
Fibre (3 g.) milled	5·4
Fibre (3 g.) milled with 1 g. $CaCO_3$	7·4
Fibre (6 g. in 40 ml. 0·2 M-Na_2HPO_4) squeezed out and washed	7·9
Fibre (3 g.) milled	8·4
Fibre (3 g.) milled with 1 g. $CaCO_3$	9·7

The constituent of the leaf responsible is not protein since incubation with a commercial trypsin preparation removes a large proportion of the nitrogenous constituents from tobacco fibre but does not prevent the pH rise. Cellulose also does not appear to be involved since experiments with filter paper (treated with alkali to render it suitable for milling, and then well washed) do not give the effect. When, however, pectic acid was removed from fibre it was not possible to obtain a pH rise.

Tobacco fibre (10 g., dry wt. 2·6 g.), suspended in water, was incubated at 40° and pH 5 for 48 hr. with 1 mg. of a preparation of polygalacturonase (purified from a commercial pectinase of fungal origin) to convert pectic to galacturonic acid (Jansen & MacDonnell, 1945). The fibre lost 450 mg. of carbohydrate and, when soaked in neutral phosphate solution, washed and milled with $CaCO_3$, did not show a rise above pH 8. The same effect was brought about by incubation with the crop juice of *Helix aspersa* and was due presumably to its polygalacturonase action. As the enzymic removal of pectic acid from fibre thus abolished the effect, experiments were done with pectic

acid. This was prepared by de-esterification of citrus pectin (British Drug Houses Ltd., 100 grade) enzymically with tobacco pectase and also with 0·1 N-NaOH. The pectic acid (0·5 g.) was soaked in 25 ml. 0·2 M-Na_2HPO_4 solution for 2 hr. during which a small amount dissolved. The undissolved portion was centrifuged down and then washed three times with 50 ml. water. $CaCO_3$ (0·5 g.) was then added which caused a pH rise to 9, and when the paste of pectic acid and $CaCO_3$ was milled the pH obtained was 9·8. A similar experiment, in which 0·5 g. pectic acid and 0·5 g. $CaCO_3$ were soaked in phosphate solution and the solid material then washed, showed a pH rise to 9·2 by the fourth wash and to 9·9 when milled.

Pectic acid thus behaves in the same way as whole fibre of 'non-drifter' leaves when soaked in phosphate solution, washed and milled with $CaCO_3$, and the phenomenon is similar to that originally observed with the fibre of leaves rich in calcium carbonate and in phosphate.

DISCUSSION

Wilkins (1917) reported very high values for the calcium content of various members of the Cucurbitaceae, but made no suggestion as to its mode of combination. Smith (1944) stated that 70 % of the calcium of squash leaves was immobilized in cell walls or intracellular substances and was readily extracted with dilute acid. It was also observed that the cell wall residue after various extraction treatments when suspended in water had a pH of 9·0.

Although the presence of calcified areas in plant tissue has been known for a considerable time, it has not been observed that a large proportion of the total calcium and as much as 10 % of the dry weight of the leaves of some species is calcium carbonate. The amount of insoluble inorganic phosphate in 'alkaline drift' plants, presumably as calcium phosphate, is also noteworthy.

Two species which show the pH rise, nettle and sunflower, were those investigated by Spoehr and his coworkers from 1923 to 1940 (see Spoehr, Smith, Strain & Milner, 1940; and Smith, 1940) on account of their ability to absorb carbon dioxide when killed by drying or freezing. Smith (1940) concluded that the absorptive agents were calcium and magnesium carbonates, and possibly phosphates.

SUMMARY

1. A pH rise to *c.* 9·5 on washing the fibre of macerated leaves was observed in all species of the Cucurbitaceae examined and some members of the Urticaceae, Boraginaceae and Compositae.

2. The rise is not prevented by heating and is speeded by fine grinding.

3. 'Alkaline drift' plants have a high calcium content, a large proportion of which is insoluble and occurs as calcium carbonate. The calcium content of the fibre may be as high as 11 % of the dry matter and the inorganic phosphorus content is also high, up to 1 % of the dry matter. The calcium and phosphorus appear to be localized in and near the epidermal hairs.

4. Young leaves which have much lower contents of calcium and inorganic phosphorus do not show much pH rise.

5. A pH rise to >9 can be produced with 'non-drifter' and acid-extracted 'drifter' fibre and with pectic acid by soaking in neutral phosphate solution, washing and milling with calcium carbonate. After incubation of 'non-drifter' fibre with polygalacturonase the pH rise cannot be obtained.

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