2. When, however, one-quarter of the nitrogen in the diet was derived from the yeast and three-quarters from whole-wheat flour, the rate of weight increase and utilization of nitrogen was slightly greater than when all the nitrogen was obtained from the whole-wheat flour.

3. This complementary action is presumably due to the relatively high proportion in yeast protein of lysine, an amino-acid essential for growth, which is present in cereal proteins in relatively low amount.

We wish to thank Sir Charles Martin, who kindly examined the histological specimens, and Mr T. C. Fletcher, who kindly prepared them. Our thanks are also due to Mr G. W. Flynn for his help in the preparation of the diets and in the care of the experimental animals.

REFERENCES

Daft, F. S., Sebrell, W. H. & Lillie, R. D. (1941). *Proc. Soc. exp. Biol., N.Y.*, **48**, 228.
Himsworth, H. P. & Glynn, L. E. (1944*a*). *Clin. Sci.* **5**, 93.
Himsworth, H. P. & Glynn, L. E. (1944*b*). *Clin. Sci.* **5**, 133.
Kleinzeller, A. (1944). *Biochem. J.* **38**, 480.
Klose, A. A. & Tevold, M. L. (1944). *Proc. Soc. exp. Biol., N.Y.*, **56**, 98.
Kon, S. K. & Markuse, Z. (1931). *Biochem. J.* **25**, 1476.
Light, R. F. & Frey, C. N. (1943). *Cereal Chem.* **20**, 645.
Macrae, T. F., El Sadr, M. M. & Sellers, K. C. (1942). *Biochem. J.* **36**, 4604.
Osborne, T. B., Mendel, L. B. & Ferry, E. L. (1919). *J. biol. Chem.* **37**, 223.
Still, E. U. & Koch, F. C. (1928). *Amer. J. Physiol.* **87**, 225.

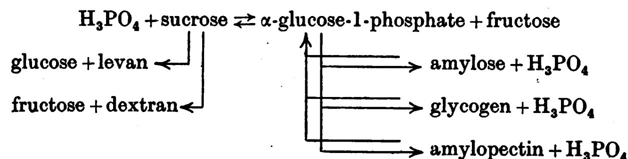
The Mechanism of Polysaccharide Production from Sucrose. 2

BY S. AVINERI-SHAPIRO AND S. HESTRIN, *Chemistry Department, Cancer Laboratories, Hebrew University, Jerusalem*

(*Received* 24 *January* 1945)

Different polysaccharides can be produced from either α-glucose-1-phosphate or sucrose by the action of cell-free enzymes. From α-glucose-1-phosphate there have been obtained amylose, amylopectin, and glycogen (Hanes, 1940; Hassid, Cori & McReady, 1943; Haworth, Peat & Browne, 1944; Cori & Cori, 1943), whereas from sucrose the formation of a glucosan, dextran (Hehre & Sugg, 1942; Hehre, 1943) and a fructosan, levan (Hestrin, Avineri-Shapiro & Aschner, 1943), have been reported.

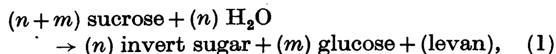
There is a structural similarity between the two substrates which are employed for these polymerizations. The relation of the substrates has been emphasized further by the recent demonstration that α-glucose-1-phosphate and fructose enter into dynamic equilibrium with sucrose and phosphoric acid in the presence of an enzyme which has been isolated from a suitable bacterial species (Doudoroff, 1943; Hassid, Doudoroff & Barker, 1944; Doudoroff, Hassid & Barker, 1944). The known syntheses of different polysaccharides can, in the light of these observations, be fitted into a single scheme:



The group name *polymero-synthease* provides us with a generic description of the enzyme entities which mediate the polymeric steps of the scheme presented above. The elements of water are not included in the balance sheet of the listed reactions. The action of the *polymero-syntheases* on their substrates can therefore be described as a *direct polymeric degradation* (Leibowitz & Hestrin, 1945). *Levansucrase* is the name which has been given to the polymero-synthease which degrades suitable aldosido-fructosides (sucrose, raffinose) polymerically into aldose (glucose, melibiose) and levan.

Levansucrase has been obtained in cell-free solution in concentrations suitable for kinetic studies from an organism which has been designated as *Aerobacter levanicum* (Hestrin *et al.* 1943).

Our levansucrase preparation catalyzes the following transformation (Hestrin & Avineri-Shapiro, 1943, 1944):



$$(n+m)\ \text{sucrose} + (n)\ H_2O \rightarrow (n)\ \text{invert sugar} + (m)\ \text{glucose} + (\text{levan}), \quad (1)$$

m and n being molar quantities. The action of the preparation is thus two-fold, hydrolytic and polymeric. Elsewhere evidence has been advanced which shows that the polymeric reaction (m-term of equation (1)) is not a sequence of the hydrolytic reaction (n-term of equation (1)) (Hestrin, 1944). The present paper defines the conditions of pH, substrate concentration, enzyme concentration and reaction time in which levansucrase acts with maximum efficiency in terms of production of polymer.

It is to be assumed that information concerning

the activity of levansucrase with respect to environmental influences will help to shed light on many unknown features of the action of this enzyme, including the following: (*a*) the possible interconnexion of the polymeric and hydrolytic activities of the levansucrase preparation, (*b*) the possibility of obtaining a reversal of the reaction, (*c*) the possibility that the polymeric degradation is effected by a single enzyme entity, and (*d*) the question raised in a general form by Stacey (1943) of the possible participation of polymero-synthease constituents in the lattice of the formed polymer.

An added interest was further given to the study of details of the process of levan production by the fact that levan, alone among the known enzyme-synthesized polysaccharides, consists of fructofuranosidic rather than glucopyranosidic residues. Moreover, the particle weight of levan, as measured by Ingelman & Siegbahn (1944), seems to be unusually high, approaching and even exceeding in magnitude such biological units as viruses and genes.

METHODS

The preparation of the enzyme and the analytical methods have been described (Hestrin *et al.* 1943; Hestrin & Avineri-Shapiro, 1944). The source of the enzyme is the organism *Aerobacter levanicum* which is obtainable from the Department of Hygiene and Bacteriology of the Hebrew University, Jerusalem. The reaction mixtures were maintained at 37°. Determinations of levan were carried out on volumes of reaction mixture sufficient to permit analytical accuracy within a range of error of 3% of the reading obtained. Sterility was maintained by the addition of a drop of chloroform containing thymol to the test mixtures. Free fructose was estimated by subtracting from the total of the reducing sugar the hexose equivalent of the amount of sugar bound as levan, and dividing the result by two. The validity of this method of computation has been demonstrated (Hestrin & Avineri-Shapiro, 1944).

RESULTS

Effect of pH

Solutions of 3 ml. contained 1 ml. of McIlvaine phosphate-citrate mixture of designated pH, 1 ml. of 15% sucrose in water, and 1 ml. of enzyme. Reaction was interrupted after 5 hr. At the time of analysis levan was still being actively formed in all mixtures whose pH permitted levan production.

A typical experiment, set forth in Fig. 1, shows that the pH-activity curve of levansucrase is regular and symmetrical and that there is a well-marked optimum in the region of pH 5·0–5·8. Fructose production from sucrose by the same levansucrase preparation was also optimal in the same region. The pH-activity curve of dextransucrase as established by Hehre & Sugg (1942) shows remarkable similarity to the pH-activity curve of levansucrase. The pH-activity curve of levansucrase is definitely monophasic. Hence it presents no special support for the view that more than one enzyme system in the preparation effects a production of levan from sucrose.

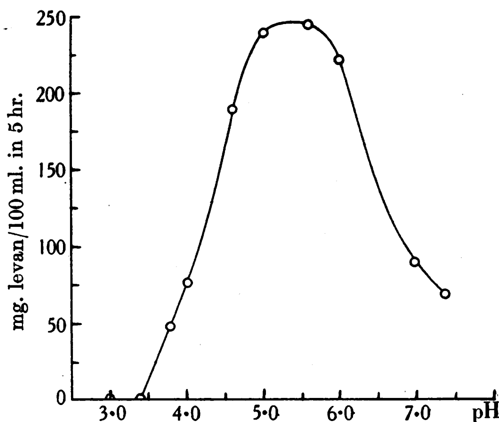


Fig. 1. Effect of pH.

Sucrose concentration. Effect on rate of reaction

The test mixtures contained graded concentrations of sucrose, 1 ml. of enzyme solution, and 1 ml. of Sorensen citrate buffer pH 5·0 in a total volume of 3 ml. Reaction was interrupted after 3 hr. At this time, synthesis was less than 50% complete.

The results, presented in Table 1, show a typical picture: a progressively smaller increase in rate of reaction as the substrate concentration was raised to about 5% sucrose, and constancy or a slow decrease in rate of reaction as sucrose was increased beyond this optimum concentration. The concentration of sucrose which permitted half-optimum reaction velocity varied with the preparation of enzyme. The molar magnitudes were considerably greater than the corresponding value for the hydrolysis of glucose-1-phosphate by muscle phosphorylase, and of the same order as the corresponding values frequently recorded for sucrase of yeast. The data show fair conformity with the Michaelis-Menten theory, but are inadequate as a basis for any definite statement on this point.

Table 1. *Variation of rate of reaction with concentration of sucrose*

Sucrose (%)	Levan (mg./100 ml.) First enzyme sample	Second enzyme sample
0·5	45	20
1·0	64	—
2·0	95	58
3·0	—	84
5·0	117	102
10·0	108	84

Sucrose concentration. Its effect on the final yield of levan

The final yields of levan were determined in test mixtures of similar composition but incubated for 48 hr. Longer incubation of the mixtures failed to bring about any material increase of the levan yield. The results are set forth in Fig. 2.

At substrate concentrations below 3% sucrose there was marked increase in the absolute yield of levan with increasing sucrose concentration. But when the sucrose concentration was increased beyond 3% no further noteworthy increase in the absolute yield of levan was gained. The percentage yield of levan, calculated on total fructose originally present bound in sucrose, shows an opposite trend: it rises with decreasing sucrose concentration, this rise being slow between 18 and 3% sucrose, but increasingly rapid with diminishing substrate concentration below 3% sucrose. Extrapolation of the curve suggests that the levan yield calculated on total fructose residues would be greater than 75% of theory at an infinitely low concentration of sucrose. On 0·5% sucrose, a levan yield amounting to 62% of theory was in fact obtained. We shall later consider some of the factors which shape this curve.

The data presented in Fig. 2 cannot be used for calculating the equilibrium constant of the reaction of levan synthesis even if such a constant exists. Levan yield with high sucrose concentrations (>5%) being independent of the sucrose concentration, it is evident that levan values obtained in this range are not determined by an equilibrium relation between sucrose and levan. Again, levan values on low sucrose concentrations (<3%) are evidently determined by the amount of sucrose which was originally available. However, they cannot be used for the calculation of any equilibrium constant in the absence of parallel data indicating the extent of the hydrolytic breakdown of sucrose effected in the same conditions by the enzyme, and the nature of the relation between the polymerative and the hydrolytic breakdown processes. Nevertheless, the data available are sufficient to establish qualitatively that the ratio of levan to sucrose at the true equilibrium, if there is such a point, is considerably higher than 1/1.

Sucrose concentration. Effect on the levan/fructose ratio

An orienting experiment on the relation between levan and fructose production at varying concentrations of substrate is presented in Fig. 2*a*. Test mixtures were composed in the same manner as for the experiment of Fig. 2*b*. Reaction was interrupted after 5 hr. At this time, synthesis was nearing a standstill in mixtures containing <3% sucrose. Longer incubation was avoided as it is known to favour production of fructose relative to levan (Hestrin *et al.* 1943).

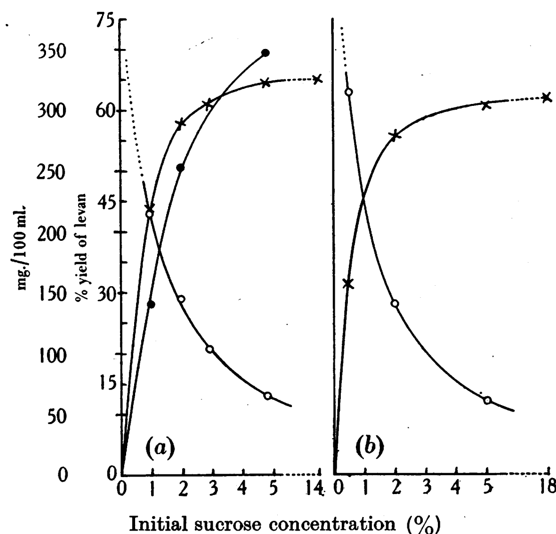


Fig. 2. Enzyme activity in prolonged incubation times as a function of sucrose concentration. o, percentage levan yield calculated on total fructoside supply; ×, levan in mg./100 ml.; •, free fructose in mg./100 ml. Incubation time: (*a*) 48 hr.; (*b*) 5 hr.

It may be seen that low sucrose concentrations tend to favour levan synthesis at the expense of fructose production. A similar conclusion also follows from comparison of the present figure with an earlier balance sheet analysis which showed the reaction undergone by 2% sucrose (Hestrin & Avineri-Shapiro, 1944). The observation suggests that relative diminution of fructose production is a partial explanation of the accelerated rise in the percentage yield of levan observed as the sucrose concentration is diminished.

Enzyme concentration. Effect on specific activity

Experiments showing the variation of specific enzyme activity with the enzyme concentration are presented in Table 2. The data reveal a sharp drop in specific activity as the enzyme concentration is raised, independently of whether activity is measured in terms of levan production or in terms of the total sucrose amounts decomposed. This is a property which, if it is a general one, differentiates levansucrase from yeast sucrase or phosphorylase, both of which show nearly constant specific activity throughout a wide range of concentration.

Considerable variation in activity between different lots of enzyme has been noted. The variation is due largely to the fact that in the method used extraction of levansucrase in soluble form from the

Table 2. *Variation of rate of reaction with concentration of enzyme*

(1 ml. of Sorensen citrate buffer pH 5·0, 1 ml. of 15% sucrose solution and varying amounts of enzyme in a total volume of 3 ml.; incubation times: 2 hr. for enzyme A, and 3 hr. for enzyme B.)

Enzyme preparation	Vol. of enzyme (ml.) (x)	Levan (mg./100 ml.) (m)	Fructose (mg./100 ml.) (n)	Specific enzyme activity (m/x)	Specific enzyme activity ($(m-n)/(x)$)
A	0·2	97	—	485	—
	0·4	138	—	345	—
	0·6	142	—	237	—
	0·8	145	—	181	—
	1·0	156	—	156	—
B	0·3	68	—	227	—
	0·5	112	76	224	376
	0·8	144	94	180	297
	1·0	143	101	143	244

Aerobacter material is never complete. Under the conditions of the preparation, young cells autolyzed in thick suspension consistently yield an active solution. But old cells (48 hr. culture) retain the enzyme more tenaciously; and even young cells fail to release the enzyme if their autolysis is carried out in a dilute suspension.

In comparing the activity of different samples of the bacterial enzyme, the following unit has been found useful. A Levansucrase Unit (L.U.) is defined as that minimum amount of enzyme which induces production of 100 mg. of levan/100 ml. in reaction mixtures of 3 ml. containing 5% of sucrose at pH 5·0 after incubation for 2 hr. at 37°. This definition obviates the complication introduced by the variation of the specific activity of levansucrase with the enzyme concentration and gives the enzyme activity in terms which correspond to measurements carried out in conditions optimal for synthesis. As levan production initially follows a practically linear course under the conditions selected, resort to linear extrapolation in estimating L.U. is permissible so long as the levan concentration actually measured does not deviate from 100 mg./100 ml. by more than about 50%. Using a turbidity equivalent of this amount of levan for reference, observation of this proviso offers no difficulty in practice. The most active of our cell extracts (Preparation A, Table 2) rated 5 L.U./ml.

Enzyme concentration. Effect on the final yield of levan

Mixtures contained graded concentrations of enzyme in a total of 3 ml. fluid containing 5% of sucrose buffered at pH 5·0 with 1 ml. of Sorensen sodium citrate solution.

A graphic presentation of the results is given in Fig. 3. It is seen that enzyme concentration under the conditions of the experiment determines levan yield where small amounts of enzyme are employed. When a sufficiency of enzyme is initially added, however, further increase in the enzyme concentration does not bring about any further increase in the yield.

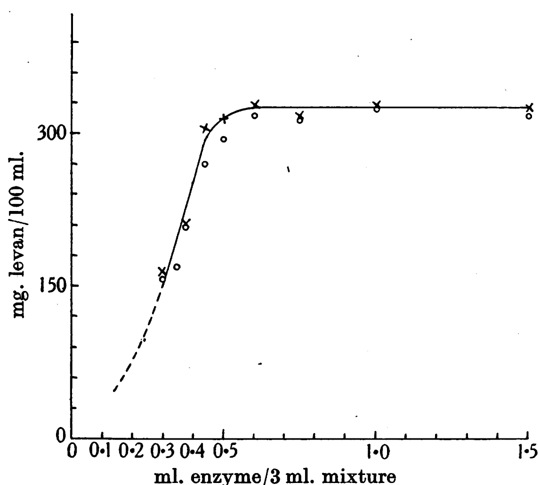


Fig. 3. Effect of enzyme concentration on levan yield. o, levan after 48 hr. incubation; ×, levan after 72 hr. incubation.

Two explanations of the rising sector of the curve may be mentioned: (1) in low concentration the enzyme is relatively unstable and limits the levan yield because it is thermally inactivated in the course of the incubation; (2) polymer production involves a stoichiometric inclusion of some enzyme element within the molecule of the polymer, and levan yield becomes dependent on this factor when the concentration of the latter becomes a limiting one.

The flat sector of the curve in conjunction with the data presented in Fig. 2*b* shows that conditions can be realized experimentally in which the final absolute yield of levan stays constant despite large variation in the enzyme and sucrose supply. Under these conditions some unidentified factor, which is neither the initial sucrose nor enzyme supply but may possibly be the accumulation of a reaction product, puts a stop to levan synthesis long before the supply of sucrose has been exhausted.

Time relations

The time course of the production of levan by the action of levansucrase has been investigated for the condition of non-limiting sucrose concentrations. The test mixtures contained 5% of sucrose and 1 ml. of enzyme liquid (or suspension) in a total volume of 3 ml. and were buffered at pH 5·0.

Results pertaining to several lots of soluble enzyme and one sample of desmo-enzyme (cell sediment of an autolysate) are assembled in Fig. 4 and its inset.

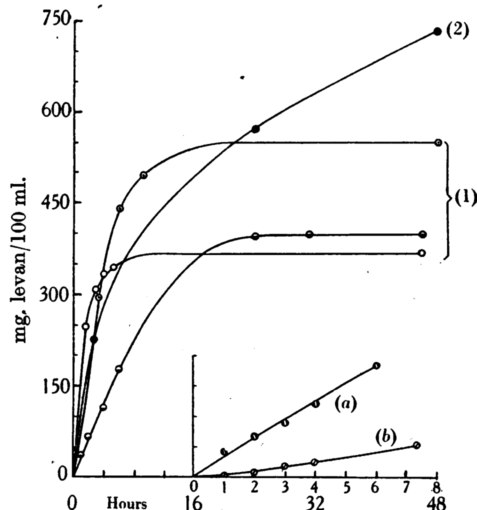


Fig. 4. Kinetics of levan production *in vitro*. Curves, (1) levan production by different soluble levansucrase preparations; (2) by an insoluble levansucrase preparation. Inset, (a) soluble enzyme without added glucose; (b) the same with 4% added glucose.

Levan production in the condition investigated is seen to follow initially a practically linear course. This is maintained both in the absence and presence of added glucose during a period of 3 hr. or until 150 mg. of levan/100 ml. have formed, whichever is the shorter. There is no sign here of an autocatalytic trend such as has been noted in glycogen production *in vitro*. Since no decrease in reaction rate takes place over a considerable interval it has to be concluded that no rate-limiting component is being used up during this time. Neither the amount of glucose, which inhibits markedly when sufficiently concentrated relative to sucrose (see Fig. 4 inset and Hestrin & Avineri-Shapiro, 1944), nor the fructose nor levan formed at this stage, exerts any notable influence on the early course of the synthesis.

The time-action curves clearly reveal the intervention of a sharply inhibiting influence in a late phase of the synthetic process. As a limiting concentration of levan is approached, the curves for soluble levansucrase bend rapidly and flatten. The limiting levan concentration, variable to some extent with the particular preparation of enzyme, is quite low (< 500 mg./100 ml.). It has been shown to be independent of the sucrose and enzyme concentrations (cf. Figs. 2*b*, 3). No similar limit point is discernible when the synthesis is mediated by desmo-levansucrase (cf. Fig. 4).

When a large excess of yeast invertase is incorporated in the same test mixtures together with levansucrase, the final amount of levan formed is smaller than in mixtures without added yeast sucrase. This effect is certainly due to the removal of sucrose from the system at an early stage by the vigorous action of yeast invertase. It is significant that the removal of sucrose by this agent does not lead to a reversal of the direction of the levansucrase action which would express itself in a removal of already formed levan.

SUMMARY

1. The activity of the levansucrase of *Aerobacter levanicum* has been investigated as to dependence on pH, sucrose concentration and enzyme concentration. Levan synthesis was found to proceed at an optimum rate in mixtures containing 5% of sucrose buffered at pH 5·0–5·8. A levansucrase unit which expresses the enzyme activity in optimum reaction conditions has been defined on this basis.

In estimating levansucrase activity, consideration must be given to the factor of the specific enzyme activity. This decreases sharply as the enzyme concentration is raised.

2. The time-action curve of soluble levansucrase in the presence of a non-limiting concentration of sucrose and a sufficiency of enzyme follows initially a practically linear course, but later bends sharply and flattens as an unidentified inhibiting influence comes into play.

3. The final absolute levan yield is a function of the initial sucrose and enzyme supply when low concentrations of substrate or enzyme are employed, but becomes independent of either of these factors when the latter are increased sufficiently.

On a suitably low sucrose concentration, levan production accounts finally for 62% of the total of the fructoside residues. The levan/sucrose ratio at the true equilibrium point is therefore certainly greater than 1.

The authors wish to acknowledge their indebtedness to Dr M. Aschner and Dr J. Leibowitz for their interest in this work.

REFERENCES

Cori, G. T. & Cori, C. F. (1943). *J. biol. Chem.* **151**, 57.

Doudoroff, M. (1943). *J. biol. Chem.* **151**, 351.

Doudoroff, M., Hassid, W. Z. & Barker, H. A. (1944). *Science*, **100**, 315.

Hanes, C. S. (1940). *Proc. roy. Soc.* B, **129**, 174.

Hassid, W. Z., Cori, G. & McReady, R. (1943). *J. biol. Chem.* **148**, 89.

Hassid, W. Z., Doudoroff, M. & Barker, H. A. (1944). *J. Amer. chem. Soc.* **66**, 1416.

Haworth, W. N., Peat, S. & Browne, E. (1944). *Nature, Lond.*, **154**, 236.
Hehre, E. J. (1943). *Proc. Soc. exp. Biol., N.Y.*, **54**, 240.
Hehre, E. J. & Sugg, W. (1942). *J. exp. Med.* **75**, 339.
Hestrin, S. (1944). *Nature, Lond.*, **154**, 581.
Hestrin, S. & Avineri-Shapiro, S. (1943). *Nature, Lond.*, **152**, 49.
Hestrin, S. & Avineri-Shapiro, S. (1944). *Biochem. J.* **38**, 2.
Hestrin, S., Avineri-Shapiro, S. & Aschner, M. (1943). *Biochem. J.* **37**, 450.
Ingelman, B. & Siegbahn, K. (1944). *Nature, Lond.*, **154**, 237.
Leibowitz, J. & Hestrin, S. (1945). *Advances in Enzymology*, vol. **5** (in the Press). New York: Interscience Press.
Stacey, M. (1943). *Chem. Ind.* **62**, 110.

Acid-producing Mechanisms in Minced Leaves

By MARGARET HOLDEN, *Rothamsted Experimental Station, Harpenden, Herts*

(*Received* 5 *February* 1945)

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