SUMMARY

1. It has been found that a prostigmin analogue, the dimethylcarbamate of (2-hydroxy-5-phenylbenzyl)-trimethylammonium bromide (Hoffmann-LaRoche Nu-683) is capable of inhibiting selectively the activity of the non-specific or pseudo-cholinesterase.

2. Injections of this compound have demonstrated that an almost complete inhibition of pseudo-cholinesterase in the blood and tissues of dogs can be effected without eliciting symptoms indicative of the accumulation of acetylcholine.

3. Symptoms indicative of the accumulation of acetylcholine appear only when the activity of the specific or true cholinesterase is significantly depressed as well.

4. These results show that pseudo-cholinesterase plays no essential part in the hydrolysis of acetylcholine *in vivo*.

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The Extraction of Nitrogenous Materials from Green Leaves

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Our knowledge of plant proteins has been reviewed by Vickery (1945). This shows how meagre is the information on the distribution of proteins in green leaves compared with that on proteins in seeds and grains. This difference can partly be explained by the dietetic value of the latter, but it is also a reflexion of the difficulty attending the extraction of the nitrogenous compounds from leaves in forms in which they can be handled conveniently. A systematic study of grinding methods and extracting reagents has therefore been made, to find a short, simple and efficient process for dispersing the nitrogenous compounds of the leaf.

Rouelle (1773) first showed that protein occurs in parts of the plant other than the seed, and by heat coagulation separated the juice of hemlock leaves into a green part and one almost free from pigment. The aim of most subsequent work has been the analysis of the product rather than the preparation of the proteins in a form in which their physico-chemical properties are as little changed as possible. In consequence, many of the extraction procedures were rather drastic, e.g. the use of 60 % ethanol containing 0·3 % NaOH (Osborne, 1924).

Modern work on the extraction of leaf proteins begins with Osborne & Wakeman (1920) who used spinach and Chibnall & Schryver (1921) who used cabbage. By grinding with water or ether-water they obtained about 60 % of the leaf nitrogen in solution. By grinding runner-bean leaves in an end-runner mill with six separate lots of water, Chibnall (1922) was able to separate 80–90 % of the total leaf N from the rest of the cell debris. Noack (1927) and Menke (1938) also separated a portion of the N by simple grinding with water. The 'protein' prepared by heat coagulation of such extracts contains only 11–12 % N and much ash (10–12 %). In an endeavour to obtain purer preparations, Chibnall (1923, 1924) and his co-workers (Chibnall & Grover, 1926; Miller & Chibnall, 1932; Chibnall, Miller, Hall & Westall, 1933) elaborated the well-known method of cytolyzing the leaf with ether or ether-water, pressing out the 'vacuolar' fluid containing a small amount of 'vacuolar' protein and dispersing the remaining 'cytoplasmic' protein by grinding and water extraction. By acid treatment of the extracts they obtained products containing 16·25 % N on an ash-free basis, but in yields of only 40 % or less of the nitrogen of the leaf.

The highest extractions reported to date are those of Lugg (1939). By fine grinding in an end-runner mill in the presence of a large volume of borate buffer, pH 9·2, followed by gentle centrifugation to separate the cell debris, he claims extractions on a small scale of 92–95 % of the total leaf N.

EXPERIMENTAL

Leaves. Tobacco (*Nicotiana tabacum* var. White Burley) has been used because of its availability throughout the year and because of its importance in virus studies. Experiments have also been done with *N. glutinosa*, *Phaseolus vulgaris* var. Canadian Wonder and with *Datura stramonium*. The plants were grown in glasshouses with heat but no extra illumination. The potting compost contained small amounts of added N, P and K and a few batches received top dressings of $(NH_4)_2SO_4$. As far as possible plants about 6 in. high were used. Senescent and very young leaves were discarded. The leaves were cut immediately before beginning extractions, the delay always being less than 20 min. Variations in dry matter and nitrogen content occurred with the size and age of the plants, weather conditions, manurial treatment and the time of day and the season at which they were picked. Although they affect the extraction of nitrogen to some degree, no attempt has been made to control or allow for these variations. In the tables, average figures for extraction are given except in three instances (Tables 3, 10 and 14).

Cutting and grinding machinery. The machines used were a domestic meat mincer, a Latapie mincer, a commercial food cutter, a triple-roller ointment mill, an end-runner mill, a Torrance 'N.T.' conical edge-runner mill and a power-driven laboratory agate mortar. The triple-roller mill has already been described by Bawden & Pirie (1944) and the end-runner is of the conventional laboratory pattern. The food cutter is made by the Hobart Manufacturing Co. of U.S.A. and consists of a rotating bowl carrying the leaves under a set of sharp knives rotating at 1450 r.p.m. The cutting is done with a slicing action and a minimum of incidental cutting and bruising. The N.T. mill is made by Torrance and Sons Ltd., of Bristol, and consists of a cast iron conical bowl of 13 in. diameter, of semiapical angle approx. 60°, rotating under a cast iron conical edge runner with the wide end orientated towards the centre. The inner and outer edges of the runner therefore move considerably faster and slower respectively than the corresponding portions of the bowl. A shearing action is thus introduced into the grinding. A high-speed shaking machine running at 225 strokes/min. was also available. In this small quantities of leaf pulp suspensions could be shaken with sand according to the technique of Curran & Evans (1942).

Analyses. Nitrogen was determined by a micro-Kjeldahl procedure using 1 : 1 : 8 $SeO_2 : CuSO_4 : K_2SO_4$ catalyst. Markham's (1942) still was used, ammonia being collected in 2% boric acid and titrated directly with N/28-HCl. Dry matter was obtained after drying overnight in an electric oven at 95–100°. To obtain an approximation to the amount of protein in extracts, precipitation by 4% trichloroacetic acid has been used. Included in the precipitate will be porphyrin nitrogen and that of N-containing lipids, both of which are included in the plastid fragments which precipitate under these conditions, as well as possible unknown N-containing compounds. For this reason it has seemed desirable not to prejudice the issue by referring to the extraction of 'protein' solely on the basis of total and trichloroacetic-precipitable N estimations, but to leave the matter open for the present and refer only to the extraction of *nitrogen*.

General notes on procedure. Distilled water was used throughout. As the aim has been to prepare the proteins of the leaf with a minimum amount of alteration, all extraction procedures were as mild as possible. Thus, cytolysis with ether or ether-water was provisionally ruled out as being too drastic, because Chibnall and his co-workers had often found difficulty in dispersing the 'cytoplasmic' material after such treatment. The pH of extracting fluids was never allowed to exceed 8·0 since many proteins are altered above this value, e.g. potato virus *Y* and Hyoscyamus virus 3 lose their infectivity at pH 9 (Bawden & Pirie 1939) and many enzymes, e.g. trypsin, are inactivated rapidly above pH 8 (Northrop, 1939).

Freshly minced leaves were squeezed without dilution. For making extracts from fibres from which sap had already been removed, the leaf material was suspended in such a volume of extraction fluid that the final dry-matter content of the mixture was between 5 and 10%.

Squeezing was done by hand through a fine cotton cloth (madapollam), the residue washed twice or thrice with a volume of water equal to one-half that of the extracting fluid (except where noted) and the combined extracts centrifuged for 15 min. at approximately 1500 × gravity. In the earlier experiments, the precipitate thus obtained was returned to the residue for analysis or further extraction.

RESULTS

Grinding methods

Preliminary grinding. In comparing the efficiency of the various methods of grinding, no attempt was made to control the pH. Extractions were done with distilled water and hence at the natural pH of the leaf.

The mincer and food cutter being best suited to the preliminary reduction of large amounts of fresh leaves, comparisons of their efficiency at this stage were made. Table 1 shows the extractions thus obtained from tobacco and beans. Mincing is seen to extract more nitrogen from both species, the nitrogen/dry-matter ratio being in favour of the mincer with tobacco and in favour of the cutter with beans. The additional efficiency of the mincer is shown by mincing the residue obtained after separating the juice from the leaves cut in the food cutter. With tobacco, the extra extraction thus obtained amounts to 12 % of the leaf nitrogen and 10 % of the leaf dry matter, i.e. extractions with mincer alone are very similar to those with cutter + mincer. The mincer has the additional advantage of being able to handle small as well as large quantities of leaves and has therefore been used in almost all experiments as a preliminary cutter. A second mincing brings out a small extra amount of juice, after which the material is washed as above.

Table 1. *Comparative efficiency of food cutter and mincer for preliminary grinding of leaves*

	% extraction of			
	Soluble N		Soluble dry matter	
Species	Food cutter	Mincer	Food cutter	Mincer
Tobacco (*Nicotiana tabacum*)	35	48	35	45
Bean (*Phaseolus vulgaris*)	37	42	31	37

Following Bawden & Pirie (1944), the material obtained after mincing and washing will be referred to as 'fibre'. It consists mainly of leaf fragments in which the cells, although apparently whole when examined microscopically, have been so damaged as to upset their osmotic equilibrium. Precipitates obtained after centrifuging the juice of the leaves plus washings or from any other extracts will be called, for convenience, 'chloroplasts'. They consist mainly of fine cell debris, plastids and plastid fragments and varying amounts of starch. The solution from the centrifugation of leaf juice plus washings will be called 'sap'. Any material remaining after extractions involving operations other than mincing and washing will be referred to as 'fibre residue' or simply 'residue'.

50 % of the nitrogen in the sap of tobacco is, on the average, precipitable by trichloroacetic acid. There are considerable variations around this average depending on the state and age of the leaf, the season, manuring, etc. The extreme values found were 30 and 68 %, but two-thirds of the results fell within the 40–60 % range. The average extractions of nitrogen and dry matter by mincing and washing are 45 and 43½ % respectively of the amounts of these constituents in the intact leaf.

Fine grinding. For finer grinding a comparison was made of the efficiencies of the Latapie mincer, triple-roller mill, Torrance N.T. mill, end-runner mill and the agate mortar. The latter was found wholly unsuitable; the particles of fibre bunch together at the corners of the pestle, which is of the large rectangular type, and are not carried under and crushed. The edge- and end-runner mills had the apparent advantage that the material could be more easily subjected to a larger number of grinding cycles than is possible with the roller mill. Each machine requires fibre of a certain minimum water content before grinding is satisfactory. The Latapie and roller mill will handle fibre as it is obtained after squeezing, i.e. containing 20–25 % dry matter. It is necessary to add an equal volume of water before grinding in the edge-runner and no less than 8 vol. in the end-runner. With the latter, the residue was washed only once. For comparative experiments fibre was put through the Latapie once, the roller mill twice, and ground in the edge- and end-runners for 5 min. These were the treatments after which no further perceptible change occurred.

Table 2. *Comparative efficiency of machines for fine grinding of tobacco fibre*

	% of component in fibre extracted			
	Nitrogen		Dry matter	
Mill	Soluble	'Chloroplastic'	Soluble	'Chloroplastic'
T.R.M.	15	42	8	31
N.T.	11½	30	8	24½
E.R.	10	19	4½	16½
Latapie	14	—	9½	—

T.R.M., Triple-roller mill; N.T., Torrance, N.T. conical edge-runner mill; E.R., End-runner mill.

It can be seen from Table 2 that under these conditions the roller mill was more efficient than any of the others. 'Chloroplast' figures are not given for the Latapie because this machine so chops up the fibre that considerable amounts of cell debris come through the cloth and are included with this fraction. For this reason, and because its use is so laborious for weights of fibre above 10 g., the Latapie was not investigated further. Not only are the extractions with the edge- and end-runners less, but they have the added disadvantage of requiring more time to grind and clean, and of contaminating the extracts with iron.

The triple-roller mill. This was investigated further to find the optimum conditions of grinding (cf. Bawden & Pirie, 1944). Washed fibre was pressed in a hydraulic press to 4000 lb./sq.in., thereby raising the dry-matter content to about 55 % in a typical experiment. Portions of this fibre were mixed with water to give the dry-matter contents shown in Table 3 and each was passed through the mill twice,

Table 3. *Extractions after milling fibre at different water contents*

	% of component in fibre extracted					
	Nitrogen			Dry matter		
Dry matter content (%)	Soluble	'Chloroplastic'	Total	Soluble	'Chloroplastic'	Total
53·1	17	26	43	21	15½	36½
31·9	13	33	46	15½	23½	39
25·8	15	31	46	11½	28½	40
21·2	13	36	49	12½	29	41½
10·6	13	27	40	9	26½	35½
5·3	11	25	36	7½	25½	33

and extracted with water in the standard manner. Preliminary experiments showed that the fibre reaches equilibrium with the added water almost immediately and that pressing has little or no effect

on the degree of extraction. Thus the variations in extraction shown in Table 3 are due to the different moisture contents during milling.

There is a pronounced decrease in the amount of soluble dry matter extracted with decrease in dry-matter content of the fibre milled. When plotted, these figures fall almost on a straight line. The trend in soluble nitrogen is not nearly so pronounced, although, on the whole, it is in the same direction. The effect of the faster falling off in dry-matter extraction is that extracts from wetter fibre are richer in nitrogen. In the chloroplastic fractions, both nitrogen and dry matter show a maximum in the neighbourhood of 20 % dry-matter content, rather more pronounced in the case of dry matter. Throughout the range, the ratio of nitrogen/dry matter in this fraction increases with increasing dry-matter content during milling.

The extractions of total dry matter and total nitrogen show maxima at the same point as the chloroplastic fraction, viz. 20 % dry-matter content. The quantitative picture coincides well with that obtained from experience of the milling process, for, judging from the 'feel', the most satisfactory grinding occurs when the dry-matter content of the fibre is between 15 and 25 %.

Extraction methods

Washing. Serial washing of fibre and milled fibre was investigated to find the most useful degree of washing. Chibnall and his co-workers had used water saturated with ether as a cytolytic agent, and Lugg (1939) showed that the addition of ether to the extracting fluid favours the extraction of nitrogen from the chloroplastic residues. Saturated ether-water was therefore compared with distilled water as a wash fluid.

Table 4. *Effect of serial washing with water and ether-water on the extraction of nitrogen and dry matter from minced and from milled fibre*

	% of original leaf component extracted			
	Soluble nitrogen		Soluble dry matter	
Treatment	Water	Ether-water	Water	Ether-water
Mince and squeeze	39	39	28	28
1st wash	5	4½	4	5½
2nd wash	1½	1	1	1
3rd wash	1½	1	½	½
4th wash	1	½	½	½
5th wash	1	½	½	½
Milled				
6th wash	5	4	3	2½
7th wash	4	3½	2	1½
8th wash	1½	1	1	1
Total extraction	59½	55	40½	41

Portions of unwashed fibre were washed five times with fluid in the proportion of 100 ml. to 50 g. of fibre. They were then milled and the washings repeated three times more. The results are shown in Table 4. Ether-water is seen to be less efficient than distilled water; in every wash it extracts less N, whilst extracting as much or a little more dry matter. The residual fibre from the water washings contains 1·7 % N and that from the ether-water wash 1·9 %. It will be seen that three washes are all that are useful at each stage and this has been made the standard procedure.

Freezing. This is a well-known method of setting free cell contents. Table 5, however, shows that it decreases the extraction from fibre. The decrease is greater for tobacco than for beans and the extraction of nitrogen is more seriously affected than the extraction of dry matter. The effect of freezing is more pronounced than that shown in the table if the whole mince is frozen before squeezing and washing. Freezing the mince denatures protein, which would otherwise pass into the sap, and retains it in the fibre. This denaturation is probably the most important cause of the reduced extractions, and in fact virus workers have used freezing as a means of clarifying the deep green extracts from minced and milled leaves. The present experiments strongly support the statement of Bawden & Pirie (1938) that, '...freezing the tissues may be a dangerous preliminary to work on the labile protein constituents of plants'.

Table 5. *Effect of freezing on extraction of fibre*

	% of component in fibre extracted			
	Soluble nitrogen		Soluble dry matter	
Species	Frozen	Not frozen	Frozen	Not frozen
Bean	8½	10½	10	9½
Tobacco	12	17½	8½	10½

Alkaline extraction. As the isoelectric point of most of the leaf proteins appears to be in the neighbourhood of pH 5, it is to be expected that mild alkaline treatment should increase their solubility. Further, as cellulose and other polysaccharides imbibe more water and become softer as the pH is raised, an increase should also assist extraction by increasing the ease of milling. The upper limit of pH is set only by the susceptibility of the proteins to injury and has been fixed at pH 8·0 for reasons already stated.

Washed fibre was suspended in 6–7 times its own weight of water to give a mixture of convenient consistency. In raising the pH of such a mixture to 8, the acid drift described by Holden (1945) was encountered. However, after 6–7 hr., this had become so slow that the mixture could be squeezed out and the fibre milled without any appreciable

decrease in pH. In this time the alkali taken up was 18–20 ml. of 0·2N-NaOH/100 g. fibre. After milling, the material was again suspended in 6–7 times its weight of distilled water and again adjusted to pH 8·0. Usually only 2–5 ml. of 0·2N-NaOH/100 g. milled fibre were required and very little drift occurred. Squeezing and washing were then carried out as before.

Table 6 shows the increase in extraction obtained by alkali treatment. On the left are the average

Table 6. *Effect of dilute* NaOH *on extraction of tobacco fibre by simple washing and by grinding*

	% of component in material extracted			
	Soluble nitrogen		Soluble dry matter	
Treatment	Washing	Milling after washing	Washing	Milling after washing
Water extract (pH 6·4)	5	21	3	12
NaOH extract (pH 8·0)	18	66	9	24½

extractions with distilled water under the same conditions as with dilute NaOH (below). The average extraction of nitrogen is raised by a factor of 3½ and that of dry matter by a factor of 3. On the right are the extractions from milled fibre expressed as a percentage of the nitrogen or dry matter remaining after washing. Again, the alkali treatment has raised the extraction of nitrogen by a factor of 3 and of dry matter by a factor of 2, and this in spite of the fact that the alkali-washed fibre has already had three times as much nitrogen and dry matter removed. An added advantage is that the nitrogen/dry matter ratio is increased, i.e. proteins are being preferentially extracted.

Since the average extractions of nitrogen and dry matter by mincing and washing are 45 and 43½ %, the average total extractions resulting from these additional processes amount to 58½ and 51½ % nitrogen and dry matter at the natural pH of the leaf fibre, and 85 and 61 % nitrogen and dry matter respectively by extracting at pH 8·0.

The amount of trichloroacetic acid precipitable nitrogen is higher in these extracts than in sap. On an average, 64 % of the nitrogen in the neutral extracts precipitates, the extremes being 41 and 81 %. The precipitable nitrogen in the extracts after milling is 91 % (average), ranging between 88 and 94 %. Thus there is a progressive washing out of the non-precipitable nitrogenous materials in the earlier stages, but this is not complete before the fibre is milled. As the average extractions at the mincing, neutralization and milling stages are approximately 45, 10 and 30 % of the total leaf nitrogen of tobacco, the amounts of 'protein' nitrogen are 23, 6 and 27 % of the total nitrogen. Assuming that the 15 % of the total leaf nitrogen remaining in the milled fibre is 'protein-N', a total of 71 % of the leaf nitrogen is in the form of 'protein'. The average nitrogen content of the leaves used in these experiments was 3·5 %. The 'protein-N' content was therefore 2·5 % which corresponds to a 'protein' content of 15 % of the dry matter. Of this, 85 % is extracted.

Experiments involving extractions of residues from the above treatments at pH 10·2 with dilute NaOH or pH 9·2 with ice-cold 10 % ethanol (cf. Lugg, 1939) are summarized in Table 7. It can be seen that further extractions, even under these rather drastic conditions, result in quite small extra yields, while they probably damage the proteins considerably.

Table 7. *Extraction of alkali* (pH 8·0)*-treated residues at higher alkalinities*

	% of residual component in treated fibre extracted	
Treatment	Soluble nitrogen	Soluble dry matter
NaOH (pH 10·2)	12	3½
10 % ethanol (pH 9·2 at 0°)	21	4½

Alkaline earth metal hydroxides offer a convenient means of maintaining a high pH. $Mg(OH)_2$ was chosen for experiment, in spite of its equilibrium pH being 9·7, i.e. above the value considered 'safe' in these experiments. Suspensions containing 1 % of $Mg(OH)_2$ were used both for soaking fibre to pH equilibrium and as extracting fluid after milling. No drift was detectable in $Mg(OH)_2$-treated samples but, as can be seen from Table 8, extractions were low and the decrease in nitrogen extraction greater than the decrease in dry-matter extraction. This effect is presumably due to the Mg^{++} ion.

Table 8. *Extraction of washed fibre by* $Mg(OH)_2$ *suspensions* (pH 9·6) *compared with* NaOH (pH 8·0) *solutions*

	% of component in fibre extracted			
	Soluble nitrogen		Soluble dry matter	
Reagent	Washing	Milling after washing	Washing	Milling after washing
NaOH (pH 8·0)	19½	67	10½	26
$Mg(OH)_2$ (pH 9·6)	5	17	6½	13½

Buffers. The chief drawback to the use of NaOH is the time consumed in bringing the fibre sufficiently close to pH equilibrium to ensure the milling being done at pH 8·0. Suitable buffers in this range are few. As nitrogen is to be estimated in the extracts,

the use of nitrogenous buffers, which are the chief members of the mildly alkaline class, was excluded. Phosphate, the most obvious choice, was, on trial, found to cause the precipitation of large amounts of calcium phosphate which entrained nitrogen. The precipitate contained up to 4 % nitrogen on a dry-weight basis. Both borate and carbonate buffers were tried, although pH 8·0 falls at the end of the borate range and between the two pK's of carbonic acid, where the buffering is least. The only substance found, which was sufficiently inert chemically to be used in protein solutions and which had a pK in the correct range, was *o*-chlorophenyl (pK 8·4). An M/15 solution has sufficient buffering power to hold the pH of washed fibre constant up to 24 hr. with the standard ratio of fibre to volume of solution.

The buffering power of carbonate and borate buffers at this pH is too small to prevent serious drift, but it reduces the extent. Thus, in the time necessary for NaOH adjusted fibre to drift back to pH 5·8, carbonate extracted fibre had reached only pH 7·5. Equilibrium is thus established more quickly with the buffers and from this point of view they have an advantage. The drift was corrected by adding the more alkaline component of the buffer and final concentrations when the mixture was squeezed and milled are given in Table 9.

It can be seen from this table that buffers extract nitrogen and dry matter to the same or a smaller extent than NaOH alone. Even when the pH is allowed to rise to 8·6 by the use of sodium borate alone this picture is not altered—little extra extraction results. Even with chlorophenol, where the pH remained constant, yields were considerably depressed.

Salt solutions. That this deleterious effect of buffers is probably explained by the increase in salt concentration follows from experiments with sodium chloride solutions as extractant. The results of a typical experiment are shown in Table 10. Even so small a concentration as M/1000 reduces the amount of soluble nitrogen. The effect of small concentrations is more pronounced when the fibre is not neutralized, although at higher concentrations the percentage decrease is approximately the same (25 %) whether neutralized or not.

Although the reduction of soluble nitrogen is evident from the lowest concentration, the total nitrogen (soluble + chloroplastic) extracted remains approximately constant until the NaCl concentration reaches 0·1 M. Either the chloroplast material is not being divided so finely in the presence of salt, or that already dispersed is being partly flocculated. The extraction of dry matter is affected in the same way. The apparent increase in the amount of material extracted with M-NaCl may be due to the inaccuracy of estimating the distribution between the extracts and the fibre residue of the considerable amounts of sodium chloride involved.

Grinding in buffer. Lugg (1939) had obtained good extractions by grinding leaves without previous mincing in an end-runner mill with large volumes of M/40 Na borate of pH 9·2. His technique has been tried for comparison with the standard mincing and

Table 9. *Comparative efficiencies of buffer solutions and* NaOH *for extraction of fibre*

		% of component in material extracted							
		Soluble nitrogen				Soluble dry matter			
		Washing only		Milling after washing		Washing only		Milling after washing	
Buffer	Final conc. of buffer (M)	Buffer	NaOH	Buffer	NaOH	Buffer	NaOH	Buffer	NaOH
Na_2CO_3-$NaHCO_3$	0·014	14	14	76	76	8½	7	27	23
Na_2CO_3-$NaHCO_3$	0·53	24	24	32	67	15½	11	22	28
Borate	0·022	17½	22	54	51	6½	5	23	23
o-Chlorophenol	0·065	11½	16	37	58	11½	13½	23	23½

Table 10. *Extraction from washed and milled fibre by dilute sodium chloride solutions*

	% of component in washed fibre extracted							
	Nitrogen				Dry matter			
	Not neutralized		Neutralized		Not neutralized		Neutralized	
NaCl (M)	Soluble	'Chloroplastic'	Soluble	'Chloroplastic'	Soluble	'Chloroplastic'	Soluble	'Chloroplastic'
0·000	12	17	24	24	8	13	14½	13½
0·001	9	18½	21	27½	7	10½	13	16
0·01	—	—	20	29	—	—	12	16½
0·1	9	17	17	24	5½	11	12½	13½
1·0	—	—	17	19½	—	—	20½	15

milling in the triple-roller mill at pH 8·0. For extractions at pH 8·0, the chlorophenol buffer described above was used, at a strength of M/20 (ionic strength equivalent to Lugg's borate). To ensure that differences would not be attributable to the difference in buffers, experiments were also done in the end-runner mill with M/20 chlorophenol buffer at pH 9·2. According to Lugg, any attempt to separate the extract from cell debris by squeezing through cloth, as has been done in the present experiments, results in lowered yields because of retention of chloroplastic material by the remainder of the cell debris. Centrifugation and squeezing were therefore compared in all three buffers.

Batches of 8 g. of tobacco leaves were ground for 5 min. in the end-runner mill with 50 ml. buffer, and washed out with the minimum amount of distilled water. One of each of the buffer extracts was squeezed through cloth and the residue washed three times with 15–20 ml. of water, and one of each was separated according to Lugg with the following modifications. It was found impossible to separate the debris of these leaves by 2 min. centrifugation at 100 × gravity; much material remained suspended and the precipitate was not sufficiently compact for the supernatant fluid to be poured away. It was found necessary to increase the field to 500 × gravity, and this caused the separation of some chloroplastic material. As much as possible of this was removed by washing twice with a volume of water equal to four or five times the volume of the precipitate. All extracts were centrifuged 15 min. at 1500 × gravity to separate chloroplastic material. The results are set out in Table 11.

extract contained a substantial layer of fine cell debris. If the initial separation had been done in lower fields this debris would presumably have been present in larger amounts. The presence of this extra cell debris may account in part for the higher apparent extractions observed by Lugg, although it is possible that the grasses used by Lugg, by reason of their more fibrous leaves, separate more easily at these low fields. The amount of fine debris is a little greater in extracts squeezed through cloth, as might be expected, but the whole chloroplast fraction is greater when prepared thus. It is noticeable, too, that the chloroplastic fraction is greater in extracts at the lower pH. Presumably, one effect of a higher pH is to make more of this fraction soluble. Half the increase in soluble nitrogen between pH 8·0 and 9·2, using chlorophenol buffers, could be accounted for by decrease in the chloroplastic fraction.

Even at pH 9·2, the extractions of soluble material are not as good as those achieved by the mincing, neutralizing and milling at pH 8·0 (80 % at best instead of 85 %). An added disadvantage of the technique is that relatively small amounts of leaves, not more than 50 g., can be handled with apparatus of normal capacity.

Detergents and Ca-*reagents.* As detergents disperse proteins, a mild detergent might be expected to assist extraction of proteins from fibre. Calgon (sodium hexametaphosphate) was chosen as a suitable mild detergent since it has the added advantage of forming non-ionized Ca complexes (Hatch & Rice, 1939), hence preventing the considerable amounts of leaf calcium from forming any insoluble Ca-protein complex. To investigate the importance

Table 11. *Extraction of nitrogen and dry matter by grinding whole tobacco leaves in an end-runner mill in presence of large volumes of buffers*

			% of N and dry matter in leaf extracted			
			Nitrogen		Dry matter	
Method	Buffer	pH	Soluble	'Chloroplastic'	Soluble	'Chloroplastic'
Grind, separate debris by low speed centrifugation	Borate	9·2	73	10	49	10
	Chlorophenol	9·2	76	6½	49	10
	Chlorophenol	8·0	61	11½	54	9
Separate debris by squeezing in cloth	Borate	9·2	77	14	50	30
	Chlorophenol	9·2	80½	8½	47	24
	Chlorophenol	8·0	60	20	54	20

Borate buffer M/40. Chlorophenol buffers M/20.

Lugg's extractions were calculated on the basis of 'soluble' + 'chloroplastic' nitrogen. On this basis, the extractions with tobacco leaves did not reach the values observed by Lugg with certain representatives of the *Graminae*. Even when the separation of cell debris has been done in a centrifugal field five times that used by Lugg, the chloroplastic precipitate from the 1500 × gravity centrifugation of the

of the latter action, experiments were also done with oxalate and citrate solutions as extracting fluids. Oxalate removes Ca in the form of a crystalline precipitate having minimal absorptive powers, and citrate forms non-ionized complexes similar to those with calgon.

All extractions were done at pH 8·0, but the presence of calgon necessitated a different washing

procedure. Its presence made milled fibre slimy and greatly increased its water-holding power. It was impossible to squeeze this in a cloth because the slimy material came through the pores. It was thus necessary to wash by centrifugation, which resulted in less thorough washing. To obtain comparable results, the control experiments with NaOH and experiments with oxalate and citrate were performed in the same way.

Table 12. *Extraction at* pH 8·0 *of* N *and dry matter from washed fibre after soaking and milling in the presence of various reagents*

	% N and dry matter in material extracted			
	Nitrogen		Dry matter	
Reagent	Soak	Mill	Soak	Mill
NaOH	17½	41	10	16
1% 'calgon'	24	53	16	16
1% oxalate	15	49½	8	17½
1% citrate	22	26½	10½	14½

Table 12 sets out the average figures. Calgon increases the extraction of nitrogen both during the adjustment of pH and after milling. At the neutralization stage calgon causes a larger percentage increase in dry matter than in nitrogen extraction, but after milling the opposite is true, i.e. material richer in nitrogen is extracted by calgon after milling but not before. Oxalate depresses the yield of nitrogen at the soaking stage and increases it somewhat after milling, although the total increase is not sufficient to make much difference to the over-all extraction. Citrate has an opposite effect, the reduction of extraction after milling being considerable.

These results indicate that the detergent action of calgon is more important than its Ca-masking action. Calgon is known to be an extremely effective 'anti-Ca' reagent; e.g. Hatch & Rice (1939) found that 1 or 2 parts/million of calgon would prevent the precipitation of 500 parts/million of $CaCO_3$. However, the total amounts of both oxalate and citrate used were two or three times the amount equivalent to the whole leaf Ca, a large part of which is not in solution and probably not involved in this effect.

In spite of its useful nitrogen-extracting powers, calgon has not been generally used in further experiments. As well as making the material slimy and difficult to handle it is difficult to remove.

Extraction sequences

The extractions thus far described are based on the standard operations: mince, wash, soak with dilute reagent at pH 8·0, mill, wash. In most experiments, too, the chloroplastic fraction has been mixed with the remaining fibre to be re-treated in the next extraction. There are several possible variants of this sequence, e.g. the chloroplasts may be regarded at each stage as a separate fraction and not remixed; neutralization of the fibre may be done after milling instead of before; it is possible to carry out more than one milling and washing of the fibre; it would be possible to treat the fibre further in an endeavour to extract residual nitrogen.

Separation of chloroplasts. Although the simplest criterion of extraction was the amount of 'soluble' nitrogen (defined here as material not sedimenting in 15 min. in a field of 1500 × gravity) and dry matter it became desirable at this stage to determine how much of the nitrogen could be separated from the fibre even if it was not all soluble. The observation of Bawden & Pirie (1944) that milling of purified bushy stunt virus with fibre from healthy leaves attaches the virus to chromoproteins made it possible that extraction was being reduced by grinding fibre and previously separated chloroplasts together.

To investigate whether this was the case, chloroplasts were milled with fibre paper pulp. The pulp was prepared by disintegrating filter paper in warm 10 % (w/v) NaOH to hydrate and soften the cellulose so that it would mill more easily. It was then thoroughly washed with water. The damp pulp was mixed with approximately its own weight of chloroplast paste, milled, suspended in about twice its weight of water, and the pH adjusted to 8·0. Controls were done on the unmilled mixture; squeezing, washing and centrifugation was carried out as usual.

Table 13. *Effect of milling chloroplasts with filter paper pulp*

	% of component in mixture extracted			
	Nitrogen		Dry matter	
Fraction	Milled	Not milled	Milled	Not milled
Soluble	35½	52	16	19½
Chloroplast	3½	46	2	22
Residue on filter paper pulp	61	1	82	59

Average results are shown in Table 13. Milling chloroplasts with cellulose has caused more than half of the nitrogen present to adhere to the fibres, whereas simple mixing has resulted in a loss on the fibres of 1 % only. The nitrogen content of the milled residue is 2·5 % compared with 0·1 % in the unmilled residue. The chloroplast fraction is reduced by the milling to a very small proportion and the amount of soluble material resulting from the action of the dilute alkali on the chloroplasts much decreased. Thus the material attached to the fibres is less susceptible to the action of dilute alkali. The loss of

material on the fibre was so large that considerable increases of extraction were to be expected by not remixing chloroplasts for further treatment.

Table 14 sets out the results of two series of experiments to show the effect with leaves. In those marked (*a*) the chloroplast fraction has been mixed with the residue at each step and the whole subjected soaking at pH 8·0. Together with the fact that yields of soluble fraction after milling (columns 6 and 13) seem to be little affected by the presence or absence of chloroplasts, this means that the over-all yield of soluble material is a little reduced by not including the chloroplasts from mincing in the preliminary soak at pH 8·0.

Table 14. *Effect of re-extracting chloroplasts with the fibre and of keeping them separate*

	% extraction of component in leaf													
	Nitrogen							Dry matter						
	Mince		Neutralize		Milled			Mince		Neutralize		Milled		
Species	Soluble	Clp.	Soluble	Clp.	Soluble	Clp.	Residue	Soluble	Clp.	Soluble	Clp.	Soluble	Clp.	Residue
(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)	(9)	(10)	(11)	(12)	(13)	(14)	(15)
(*a*) Chloroplasts returned to fibre for extraction														
Tobacco	44	—	7	—	29	—	20	39	—	3	—	9½	—	48½
Tobacco	37	—	10	—	31	—	22	45½	—	7	—	11	—	36½
Nicotiana glutinosa	36	—	9½	—	12½	15½	26½	51	—	4½	—	5	9	30½
(*b*) Chloroplasts kept separate														
Tobacco	33	18	5	1½	30	6	6½	37	13	3	1	12	8	26
Tobacco	34	20	4	1½	21½	6	13	40	15	2½	1	9½	4½	27½
Nicotiana glutinosa	36	22	4	3	9	8	18	51	10½	2	2	4	4½	26

Clp. = 'chloroplastic'.

to the next extraction. In those marked (*b*) the chloroplasts were kept separate. As was to be expected from the above results, a considerable increase in total extraction over series (*a*) can be observed in series (*b*) experiments. An identical picture is shown in Table 15 from which it can be

Table 15. *Extraction (soluble + chloroplast) when milling is done after or before neutralizing*

% extraction of component in material milled			
Nitrogen		Dry matter	
Milled after neutralizing	Milled before neutralizing	Milled after neutralizing	Milled before neutralizing
Chloroplasts returned to sequence			
63	49	25½	24½
Chloroplasts kept separate			
78	57	42½	35½

seen that whether milling is done before or after neutralization, separating the chloroplasts at each stage increases the total extraction from the fibre. The residue (columns 8 and 15, Table 14) from series (*b*) experiments contains only about half of the nitrogen and dry matter found in the others. The values in columns 2 and 9 will not, of course, be affected, while values only appear in columns 3 and 10 for those experiments in which chloroplasts were separated. It can be seen that failure to replace chloroplasts reduces the yield of soluble fraction by

That the extra amounts of nitrogen and dry matter in the residue from series (*a*) (columns 8 and 15) is not wholly due to the inclusion of the chloroplasts from the milling stage is shown in the *Nicotiana glutinosa* experiments, where mill chloroplasts and residue were estimated separately. The series (*a*) residue is still higher in both nitrogen and dry matter, showing that the effect observed with cellulose fibres also holds good for leaf fibres. The difference would not be expected to be so great for leaf fibres, for filter paper mills with great difficulty and any deleterious effect on the chloroplastic material would be expected to be greatly intensified.

Neutralization after milling. By far the most time-consuming step in the sequence so far described is the adjustment of the fibre to pH 8·0 (6–7 hr.). If the fibre is first milled, pH equilibrium is reached very much more rapidly and the greater part of the soda can be added within 2 hr. (cf. Holden, 1945). The effect on the extraction of this reversal of the order of neutralization is shown in Table 15 where the figures are for total extraction at each step (soluble + chloroplast). From this it can be seen that the order of neutralization has a considerable effect, the extractions being appreciably higher when the fibre is neutralized before milling. This is true whether the chloroplasts are kept separate or returned to the extraction sequence. More serious, the reduction of nitrogen extraction is a good deal more than of dry matter when the fibre is milled before neutralization. It seems unlikely that the

alkaline wash would promote extraction by removing salts not already extracted by the water washing to which the fibre has already been subjected. It is possible that some other inhibitory substance has been removed. However, the most plausible explanation is that increase of pH has lead to extra imbibition of water by the polysaccharide components of the leaf with consequent swelling and softening. Considerable swelling occurs as is shown by settling experiments with washed fibre suspended in a large volume of water. At pH 8·0 the volume occupied by the settled fibre is three or four times that occupied at pH 6·5. Neale (1929, 1930) has shown under more extreme conditions that cellulose in the form of cotton, cellophan and rayon swells considerably in 0·1 M- and perceptibly in 0·01 M-NaOH. Further, Cross & Bevan (1885) observed that cellulose in the green leaf was much less resistant to reagents before it had been dried, which would indicate that swelling of undried leaf fibre would be considerable even when the NaOH concentration was below 0·01 M. The behaviour of the leaf fibre is thus consistent with what might have been expected in mildly alkaline fluids although leaf fibre is a good deal more sensitive than prepared cellulose. The resulting softening of the fibre might well lead to more efficient milling and hence to a higher extraction.

Serial milling. The average extractions of nitrogen and dry matter after more than one milling are shown in Table 16. After each milling, the fibre was adjusted to pH 8·0 and washed twice in the usual way. Series in which neutralization was done before milling and in others in which it was done afterwards are included.

Table 16. *Serial milling and its effect on extraction*

	% extraction of component in fibre			
	Nitrogen		Dry matter	
Extraction process	Milled after neutralizing	Milled before neutralizing	Milled after neutralizing	Milled before neutralizing
Neutralization	15½	—	11½	—
1st milling	63	61	34½	36
2nd milling	8½	19	5½	10½
3rd milling	—	6	—	1½

It can be seen that a second milling is as effective as a preliminary adjustment of pH. Similarly, the over-all extraction for neutralization plus two millings is approximately the same as for three millings. However, the extraction of nitrogen becomes progressively more difficult. Thus the second milling removes 40–50 % of the nitrogen remaining after the first milling, but the third removes only 30 % of that left after the second milling. At this stage, the residue contains only 0·5 % nitrogen on a dry-weight basis and usually represents only 5 % of the nitrogen of the intact leaf.

It would appear from the fact that the ratio % nitrogen extracted/% dry matter extracted is higher in the later millings, that the nitrogen/dry matter ratio in the later millings might be higher than in the earlier ones. That the opposite is true indicates that only a proportion of the residue is affected by the grinding process.

Three millings or two millings subsequent to neutralization are all that are worth while in most cases. The extracts have by this stage become very dilute—only 0·25 g./l., and progressively larger amounts of the structural material of the leaf are broken down with each milling.

Shaking with sand. To investigate the possibility of removing some of the nitrogen remaining in the residues after milling, they were shaken with sand according to the technique of Curran & Evans (1942). Suspensions of fibre residue, 10 mg. dry matter/ml., were shaken for 5 hr. with an equal weight of graded sand on the high-speed shaking machine (225 strokes/min.). The results were disappointing. Approximately 10 % of the nitrogen in the fibre residue was brought off a fibre containing 0·6–0·7 % N. With fibres of higher N content, more success was attained, but these are more easily dealt with by the triple-roller mill. The two techniques, therefore, appear to have approximately the same limits of extraction.

DISCUSSION

The final sequence of extraction processes elaborated during this investigation is: mincing and washing, milling twice or thrice in the triple-roller mill and extraction of this material with dilute NaOH at pH 8·0. By this means it is possible consistently to separate 90–95 % of the nitrogen of tobacco leaves from the insoluble residue. On occasions the extraction rises to 99 %. At the same time, approximately 80 % of the total dry matter is also dispersed.

So far as can be judged, the most important factors giving maximum extraction are: the introduction of as much shearing stress into the grinding as possible, the use of extractions at a pH as high as possible without damaging the proteins, keeping the salt concentration low and separating the chloroplast fraction from the residue at each stage. The material must not be frozen at any stage. Any departure from these conditions lowers the yield from tobacco leaves. Thus, freezing leads to considerable denaturation when losses of 30 % may occur (Table 5).

Increasing the pH of extracting fluids from 6 to 8 has been shown to increase the extraction of nitrogen

and dry matter by a factor of three (Table 6). The work of Lugg (1939) indicated that even better results could be obtained by increasing the pH still further and this has been confirmed here (Table 11). The problem of setting an upper limit to the pH of extracting fluids is a difficult one. If the properties of a particular protein are known the fixing is more or less automatic, but when, as here, an attempt is made to extract all proteins with a minimum of damage some more or less arbitrary general level must be fixed. This will be determined by the more sensitive components of the leaf proteins and a consideration of the properties of certain viruses and enzymes already mentioned has led to the fixing of the pH at 8·0.

Many extracting solutions such as buffers and calcium reagents, which would appear to offer definite advantages, all suffer from the defect that they increase the salt concentration. The degree of dispersion and the ease of extraction of the leaf proteins are extraordinarily sensitive to small concentrations of salts (Table 10). Most satisfactory results have therefore been obtained by avoiding salts, even in the form of buffers, and using only dilute solutions of sodium hydroxide to adjust the pH of extraction fluids. This experience is in direct contradiction to the suggestion of Vickery (1945). He suggests, presumably from analogy with the behaviour of seed proteins, that the difficulty experienced by Chibnall in dispersing the 'cytoplasmic' protein after ether cytolysis was due to removal of the proteins from a medium of high to one of low ionic strength when the cell was broken up. He suggests, too, that the extra efficiency of 'used' ether-water was due to small amounts of salts introduced during its previous history. In the present experiments it has been shown that small quantities of salts, either at the natural pH of the leaves or at pH 8·0, decrease the amount of nitrogen and dry matter extracted. The properties of the green leaf proteins of tobacco are therefore considerably different from those of many seed proteins whose solubility is greatly increased by small concentrations of salts.

The most important factor in the grinding process is the amount of shearing stress to which it subjects the leaves. Thus, in the comparison of the mincer and the food cutter, it was found that the size of the leaf fragments was very similar, yet the extraction from minced leaves was higher (Table 1). The higher efficiency of the mincer appears to be due to the crushing and bruising to which it subjects the leaves. Among the machines used for the reduction of minced fibre, the order of increasing amounts of shearing during grinding is approximately: Latapie, end-runner, edge-runner and triple-roller mills, and this again is the order of increasing extraction from the ground products. In the Latapie, the emphasis is upon a cutting action with incidental shearing occurring at the places where the cut occurs. Microscopic examination shows fragments having many cells with apparently undisturbed contents. At the other end of the scale, shearing and pressure in the roller mill tears apart the tough cell walls, flattens out the fragments and scours off the cell contents. This picture is supported by the results of several millings, when it is still possible to separate 30 % of the nitrogen from fibre containing only 0·7 % N on a dry-weight basis, together with very small amounts (approx. 4 %) of the dry matter. The shearing action of the mill must be 'peeling off' from each particle, a layer comparatively rich in nitrogen rather than breaking up the particles and dispersing the fragments. Leaves, in general, appear to be very susceptible to shearing stresses. Thus, Phillis & Mason (1937) found that such a small amount of shearing as that induced by 'gently rubbing between finger and thumb' the leaves of the cotton plant, was sufficient to increase the amount of sap, which could be pressed out in a hydraulic press, by a factor of four and to reduce the pressure necessary to express it from 4000 lb./sq.in. to something too small to show on the gauge.

Increasing the alkalinity of extracting fluids, besides promoting solution of the proteins, increases the efficacy of the shearing action. This probably occurs through increased imbibition of water by cell-wall materials which leads to swelling and softening. Another possible promoting action of a high pH is its liberation of pectase (Holden, 1945) which attacks the leaf pectin and would be expected to weaken the middle lamella and make grinding easier. The opposite effect is seen when the moisture content of fibre is reduced preparatory to milling. Then, the extraction of dry matter tends to decrease more rapidly than the extraction of nitrogen (Table 3); the proteinaceous parts of the leaf have a greater water-holding power than the 'structural' parts.

As has been noted by many workers, leaf proteins are difficult to disperse and bring into solution. Thus from Table 14 it can be seen that approximately 30 % of the total nitrogen separated from the tobacco leaf remains in the chloroplast fraction. By regrinding this with fibre, nearly one-third of it can be dispersed so that it does not sediment in a field of 1500 × gravity applied for 15 min. The shearing action of the mill is thus having an effect other than that of scouring cell contents off the wall. It is also breaking up and dispersing those contents after the manner of a colloid mill.

However, the chloroplasts are the most resistant cell constituents, probably because of their high lipid content, and show up most clearly the disadvantages of the triple-roller mill. Once separated from the bulk of the leaf residue, they must be kept separate for maximum extraction. Any small gain

in soluble material obtained by regrinding them with the remaining fibre is more than offset by the loss in extraction caused by the fixation of chloroplasts to the residue (Table 14). This fixation is sufficiently firm to resist elution by NaOH at pH 8·0 and may represent considerable amounts of nitrogen, as in the filter-paper experiments (Table 13). A similar result has been obtained by Bawden & Pirie (1944) with tomato bushy stunt virus. As the chloroplasts are the most difficult to disperse it seems likely that a considerable proportion of the nitrogen remaining on the fibre will be of this origin. However, that virus also may be caused to adhere indicates that other cell constituents are represented among this firmly adhering material. Thus it would appear that some degree of equilibrium between material on the fibre and that dispersed is reached at each grinding.

The major constituent of the residue is carbohydrate. Approximately 75 % of the dry matter of the residue estimates as carbohydrate by the orcin method of Pirie (1936). The nitrogen content is generally of the order of 1 %, so that approximately 6 % of the residue could be accounted for by protein. Approximately 4 % is lipid material (soluble in acid-alcohol-ether) and 15 % is ash. According to Kissling (1926), the cellulose content of the tobacco leaf is 10–15 % of its dry matter, but it is unlikely that all of this is concentrated in the final residue. Appreciable quantities of fine cell-wall debris, often with protein attached, find their way through the cloth during squeezing and contaminate the chloroplast fractions. As well as cell-wall material, the chloroplast fractions contain starch, particularly in summer. There is some evidence that other formed elements of the cell, such as mitochondria are also present.

It has been emphasized that the definition of 'soluble' adopted in this work has been material which does not sediment in 15 min. in a centrifugal field of approximately 1500 × gravity. Most of the solutions thus obtained are deep green and contain particles visible under the microscope. These are largely plastid fragments varying in size from 5 μ diameter down to the limits of resolution, but starch fragments and minute cell-wall debris are also present. Some of this material can be removed by prolonged spinning at 1500 × gravity, and the bulk of it sediments at 8000 × gravity. There is little evidence whether or not the protein fraction of the sedimentable material consists wholly of plastid fragments (chromoprotein) and other similar formed elements. The observation that tomato bushy stunt virus is attached to chromoprotein by milling and only liberated therefrom by the action of trypsin (Bawden & Pirie, 1944), suggests that other soluble cell constituents are attached in a similar manner. This attachment of soluble material to chromoprotein is a phenomenon similar to the attachment of chromoprotein to cell debris. It indicates a certain amount of 'scrambling' of the cell contents and is the greatest disadvantage of the milling process. Bawden & Pirie suggested that it was due to momentary heating as the leaf material passed between the rollers.

However, in spite of these defects, the high extractions here reported have been obtained under conditions less likely to cause grave alteration to the proteins than any so far mentioned in the literature. Much valuable data should result from further study of the products obtained.

It would seem impossible to find any means of grinding which is as efficient in dispersing the leaf proteins and yet at the same time does not cause this local heating with consequent alteration of proteins and their attachment to one another and the cell debris. Further, with 95–98 % extraction, the limit of removal in the form of solutions sufficiently concentrated for easy manipulation is almost reached. The problem then is one of finding a means of reduction of the leaf more mild than grinding and one which will not leave so much nitrogen attached to a residue. The most hopeful solution to the first would appear to be the use of enzymes. Using purified pectinases and cellulases, the cell contents should be altered only to the extent that extra- and intracellular environments are different. The contents would not be dispersed to the same degree as would be obtained from the shearing action of the mill. On the other hand, most of the formed elements of the cell would be intact and an opportunity presented of studying not only the types of protein present, but their spatial distribution in the cell.

SUMMARY

1. A process for extracting 90–95 % of the nitrogenous material from tobacco leaves is described.

2. The steps in this process are: preliminary mincing and washing, grinding in a triple-roller ointment mill, extraction with dilute sodium hydroxide at pH 8·0, repetition of grinding and extraction.

3. The triple-roller mill is effective because of the rubbing to which it subjects the leaves.

4. The salt concentration must be minimal for best extraction; buffers and compounds preventing the precipitation of Ca-complexes reduce extraction.

5. Regrinding of separated chloroplastic material with partially extracted leaf fibre decreases rather than increases the extraction of protein.

6. Grinding under Lugg's (1939) conditions was found to be less effective with tobacco leaves than the process described here.

7. All grinding methods have the disadvantage that they cause some degree of alteration of the

proteins. Nevertheless, the high extractions here reported have been obtained under conditions less likely to cause grave alteration to the proteins than any so far reported in the literature.

8. Approximate calculations based on the extraction give the average protein content of tobacco leaves to be 15 % of the dry matter, of which 95 % is extractable.

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Oxidations in Acetobacter

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The question of the intermediary stages of the bacterial oxidation of acetic acid is one of the major unsolved problems in the intermediary metabolism of bacteria. Quastel & Webley (1941) showed that aneurin and co-carboxylase can increase the rate of oxidation of acetic acid in strain number 4759* of the National Collection of Type Cultures, but other evidence indicated that pyruvate is not an intermediate in acetate oxidation.

In *Acetobacter*, acetic acid is an end-product under some conditions, especially when ethanol is the substrate (Brown, 1886), but it is readily oxidized under other conditions (Tosic, 1942). This observation was studied in detail, as it was thought that an organism in which incomplete oxidations occur might be specially suited for work on intermediary metabolism.

* This organism is listed as a '*Propionibacterium*', but it was shown by Krebs & Eggleston (1941) that the organism does not possess the generic characteristics of *Propionibacterium*.

EXPERIMENTAL

Material and methods

The organism. The original strain of *Acetobacter turbidans* (Cosbie, Tosic & Walker, 1942) was used in most experiments.

The cells were grown on a basal medium to which suitable organic substrates, such as glucose, glycerol, ethanol, and sodium lactate, were added. The basal medium contained: 5·0 g. 'Difco' yeast extract, 2·0 g. $(NH_4)_2SO_4$, 2·0 g. KH_2PO_4, 0·2 g. $Na_2HPO_4.2H_2O$, 2·0 g. $MgCl_2.6H_2O$ in 1000 ml. tap water. Larger quantities of the organism were grown in Roux bottles containing 100 ml. basal medium, substrates and 2% agar, at the optimum temperature for growth (25°). The inoculum per 100 ml. medium was a 2 ml. portion of a 24 hr. old test-tube culture grown in the basal medium. After 40 hr. incubation (unless otherwise stated) cells were washed off the agar with distilled water, centrifuged, resuspended in water, centrifuged again and finally suspended in a small volume of water. The concentration of the 'stock suspension' was determined by drying 1 ml.