Phytochemistry, 1968, Vol. 7, pp. 2037 to 2044. Pergamon Press. Printed in England

THE DISTRIBUTION OF GALACTOSE AND MANNOSE IN THE CELL-WALL POLYSACCHARIDES OF RED CLOVER (*TRIFOLIUM PRATENSE*) LEAVES AND STEMS

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(*Received* 25 *May* 1968)

Abstract—A study has been made of the quantitative distribution of galactose, mannose and total sugars in various polysaccharide fractions from cell walls of red clover leaves, red clover stems and ryegrass leaves. Only about half of the polymer galactose in the undelignified clover cell walls was extracted by 10% alkali. Although some of the galactose left in the dilute alkali-residue together with about half of the cell-wall mannose was extracted with 24% alkali–4% boric acid, approximately half of the galactose and mannose remained associated with the insoluble cellulose. Mild delignification did not improve the extraction of this alkali-resistant fraction. In contrast with delignification, all of the polymer galactose present in ryegrass cell walls was extracted by the alkali. The ryegrass contained no mannose. Clover cell-walls appear to contain at least two major types of galactose-containing polysaccharide. One type is extracted in 10% alkali, contains no mannose and is not precipitated by iodine from strong calcium chloride solution. The second polysaccharide is extracted by 24% alkali and is precipitated by the iodine. The latter polysaccharide fraction contains mannose possbily present as a galacto- or galactogluco-mannan. The dilute alkali extracts from both red clover and ryegrass cell walls gave appreciable amounts of arabinose-rich arabino-xylan ("linear B"). The yields of this fraction were equal to (clover leaves) and greater than (ryegrass) the yields of the conventional, acid-insoluble hemicellulose-A xylan.

INTRODUCTION

IN A PREVIOUS[1] comparison of the hemicelluloses from Gramineae and Leguminosae the polysaccharides were extracted from the plant holocelluloses with 10% potassium hydroxide and separated into three fractions. These were "linear A" (hemicellulose-A xylan), "linear B" (hemicellulose B arabino-xylan) and "branched B" (hemicellulose B water-soluble hetero-polysaccharide). In this work combined leaves and stems of most of the leguminous plants were used and, as purity of the fractions was the main concern, no attempt was made to obtain an exact quantitative measure of the amounts of the three fractions extracted from the plants. It was shown for both groups of plants that galactose was present only in the "branched B" fractions. Other earlier work[2] in which constituent monosaccharides were measured in both alkali-soluble and alkali-insoluble plant cell-wall polysaccharides, indicated for legumes in particular that not all of the galactose could be extracted by 10% alkali.

Although galactose-free pentosans make up the main portion of the non-cellulose polysaccharides of pasture plant cell-walls, up to 1%, on a dry weight basis, of polymer galactose is also present and the nature of the polysaccharides containing it is of interest. We have, therefore, carried out a more detailed fractionation of the cell-wall polysaccharides of red clover (*Trifolium pratense*) leaves and stems respectively in order to obtain a measure of the distribution of polymer galactose among the various fractions.

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[1] B. D. E. GAILLARD, *Phytochem.* **4**, 631 (1965).
[2] B. D. E. GAILLARD, *J. Agric. Sci.* **59**, 369 (1962).

During the present work it became evident that mannose was associated with the galactose in some of the polymers. Attention was, therefore, also paid to the distribution of mannose. Although the low levels of lignin usually present in the plant material investigated suggested that delignification was unnecessary, the effect of mild delignification on the fractionation procedure was examined. For a comparison, perennial ryegrass (*Lolium perenne*) leaves were also submitted to a similar, but simpler quantitative fractionation.

RESULTS

Galactose and Mannose Levels in Red Clover Cell-Wall Polysaccharides

The amounts of galactose, mannose and total sugars in the polysaccharide fractions obtained, as described in Fig. 1, from ethanol and ammonium oxalate extracted red clover

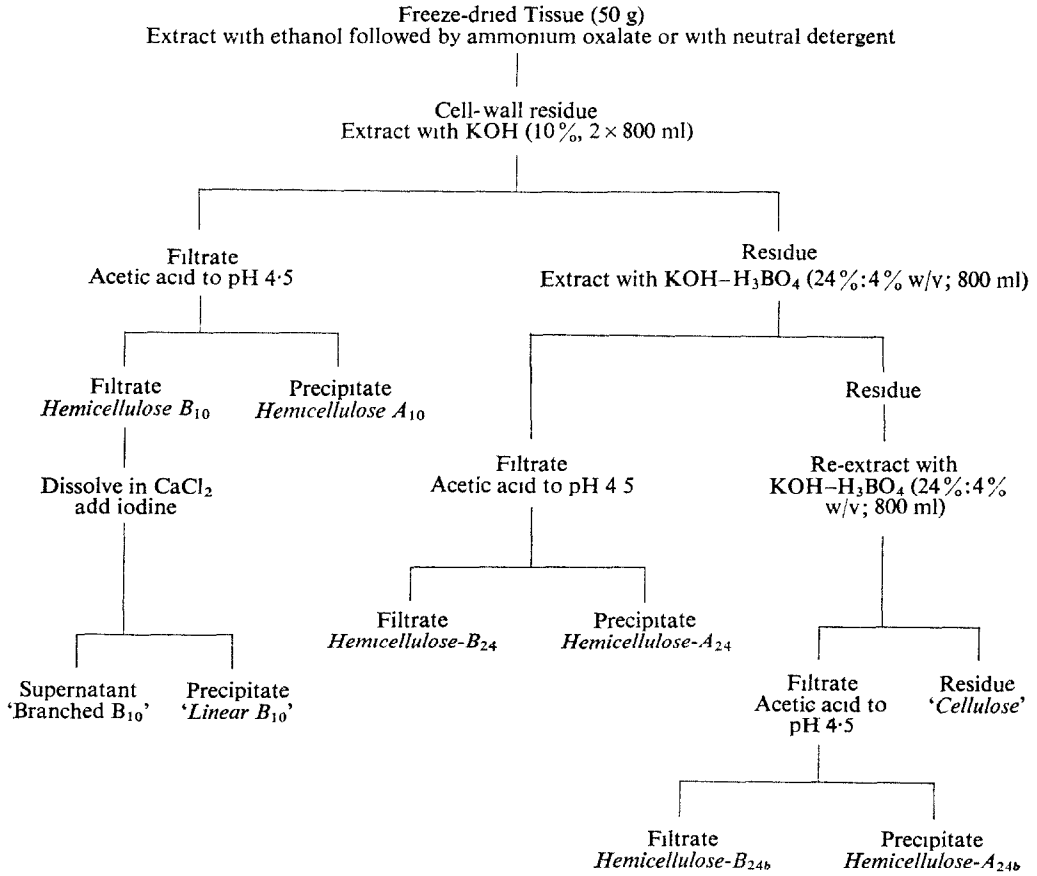

FIG. 1. EXTRACTION AND FRACTIONATION OF PLANT CELL-WALL POLYSACCHARIDES

(*Trifolium pratense*) leaves and stems are given in Tables 1 and 2. All results are calculated as a percentage of moisture-free (oven dry) original freeze-dried plant tissue. An indication of the recoveries of polymer galactose and mannose is given by comparing the totals of each sugar in the fractions with the amount present in the original cell-wall material. Recoveries

of galactose appeared to be better than recoveries of mannose. Overall accuracy of the whole procedure was estimated to be between ±90 and 110 per cent. In Tables 1 and 2, and throughout the text, the subscript numeral 10 (e.g. hemicellulose B_{10}, "Branched B_{10}") refers to fractions extracted by 10% alkali and the subscript 24 refers to fractions extracted by 24% alkali–4% boric acid.

TABLE 1. GALACTOSE AND MANNOSE CONTENTS OF CELL-WALL POLYSACCHARIDE FRACTIONS OF RED CLOVER STEMS

Fraction†	Total sugars (g-anhydro)	Galactose (mg-anhydro)	Mannose (mg-anhydro)
	(Amounts per 100 g dry weight of original stems)		
Hemicellulose-A_{10}	5·90	—*	—*
"Linear B_{10}"	2·23	135	—
(Corrected)	(1·95)	(—)	
"Branched B_{10}"	0·28	60	—
(Corrected)	(0·56)	(195)	
Hemicellulose A_{24}	0·21	—	—
Hemicellulose B_{24}	1·11	119	192
Hemicellulose B_{24b}	0·28	43	34
"Cellulose" residue	23·6	370	265
Total		727	491
Original oxalate-extracted cell wall		729	720

*— Indicates absent.
† See Fig. 1.

TABLE 2. GALACTOSE AND MANNOSE CONTENTS OF CELL-WALL POLYSACCHARIDE FRACTIONS OF RED CLOVER LEAVES

Fraction†	Total sugars (g-anhydro)	Galactose (mg-anhydro)	Mannose (mg-anhydro)
	(Amounts per 100 g dry weight of original leaves)		
Hemicellulose A_{10}	0·65	—*	—*
"Linear B_{10}"	0·56	43	—
(Corrected)	(0·47)	(—)	
"Branched B_{10}"	0·41	111	—
(Corrected)	(0·48)	(155)	
Hemicellulose A_{24}	0·26	—	—
Hemicellulose B_{24}	0·55	60	51·0
Hemicellulose A_{24b}	0·04	—	—
Hemicellulose B_{24b}	0·10	33	—
Cellulose residue	9·4	117	149
Total	—	367	200
Original oxalate-extracted cell walls		449	255

*— Indicates absent.
† See Fig. 1.

The pectic substances extracted in the ammonium oxalate during the preparation of the stem cell-walls were analysed and found to contain, on the same quantitative basis, 940 mg of anhydro-galactose but no mannose.

In previous work[1] repeated iodine precipitation was used to give a "linear B" fraction entirely free of galactose, as some of the "branched B" polymer is carried down with "linear B" during the first precipitation. In order to avoid losses which are likely during extensive purification, only a single iodine precipitation was used in the present work. With clover stems in particular (Table 1) it can be seen that much galactose is present in the "linear B_{10}" fraction. Reprecipitation of this "linear B_{10}" with iodine gave a galactose-free precipitate with the soluble polysaccharide in the supernatant having galactose, arabinose and xylose in the correct ratio (3:2:1) for the "branched B" fraction. A corrected "branched B_{10}" galactose was therefore calculated and, from the galactose:pentose ratios, amended "linear B_{10}" and "branched B_{10}" total sugar values were calculated. These amended results are given in brackets in Tables 1 and 2.

Clover stem cell-walls were also prepared by extraction with the neutral detergent solution of Van Soest.[3] This method has the advantage of eliminating protein along with sugars and the pectic substances, and hence avoids foaming during alkali extraction. The resulting preparation had the same galactose and mannose content as the ethanol and oxalate extracted residue. Extraction of the neutral detergent residue with ammonium oxalate dissolved only a trace of polymer galactose confirming that the detergent had removed all but an insignificant amount of the pectin galactan. On fractionation, the detergent residue gave the same quantitative distribution of galactose and mannose with similar amounts of the two sugars remaining unextracted in the final cellulose residue.

To check on the effect of delignification, neutral detergent cell-wall residue was treated with chloramine-T and ethanolamine.[4] The final residue was extracted with 10% followed by 24% alkali, and galactose and mannose remaining in the cellulose residue were determined. Residual galactose was 91% and residual mannose 85% of that present in the cellulose residue from undelignified material. This method of delignification did not, therefore, markedly improve the extraction procedure.

Extraction of Polysaccharides from Ryegrass Leaves

Neutral detergent extracted cell-wall residue from perennial ryegrass leaves contained, per 100 g dry weight of original leaf, 0·47 g of anhydro-galactose, but no mannose. Separate portions of the cell-wall preparation, with and without delignification respectively, were submitted to the fractionation procedure in order to see how delignification affected the extraction of the grass polysaccharides. In this case all but 10–15% of the polysaccharide galactose was extracted in the 10% alkali but the unextracted polysaccharide galactose was only dissolved by the stronger (24%) alkali when the tissue had been delignified.

The total anhydro-sugar contents of the polysaccharide fractions from the delignified ryegrass cell-wall residue were, per 100 g dry weight, hemicellulose A_{10}, 0·37 g; "linear B_{10}", 3·96 g; "branched B_{10}", 1·67 g; hemicellulose A_{24}, 0·0; hemicellulose B_{24}, 0·47 g and cellulose, 14 g.

Monosaccharide Composition of Clover and Ryegrass Polysaccharide Fractions

The monosaccharides detected on chromatograms of hydrolysates of the various polysaccharide fractions are listed in Table 3. Sugars were classified as major components when,

[3] P. J. VAN SOEST, *J. Ass. Off Agr Chem.* **46**, 825 (1963).
[4] B. D. E. GAILLARD, *J Sci. Food Agric.* **9**, 170, 346 (1958).

on visual assessment of sprayed chromatograms, the intensity of the spots corresponded to 10% or more of the sugar in the polysaccharide, and minor when they corresponded to below 10%.

The composition of the hemicellulose A, "linear B_{10}" and "branched B_{10}" fractions was essentially similar to that already reported for these fractions.[1] Mannose was clearly absent from the "branched B_{10}" fractions. When the clover hemicellulose B_{24} was dissolved in alkali and treated with barium hydroxide[5] a heavy precipitate of a possible heteromannan was obtained. The monosaccharide composition of polysaccharide from this precipitate was galactose, glucose, mannose and xylose in the ratios, by quantitative paper chromatography,

TABLE 3. MONOSACCHARIDES PRESENT IN RED CLOVER AND RYEGRASS CELL-WALL POLYSACCHARIDE FRACTIONS

Fraction†	Major monosaccharides	Minor monosaccharides
(a) Clover leaves and stems		
Hemicellulose A_{10}	Xylose	Uronic acid
"Linear B_{10}"	Xylose, arabinose, glucose	Uronic acid
"Branched B_{10}"	Galactose, arabinose, xylose, uronic acid	Glucose*
Hemicellulose A_{24}	Xylose, glucose	Uronic acid, arabinose
Hemicellulose B_{24}	Galactose, glucose, mannose, xylose	
Cellulose residue	Glucose	Xylose, galactose, mannose
(b) Ryegrass leaves		
Hemicellulose A_{10}	Xylose	Arabinose, uronic acid
"Linear B_{10}"	Xylose, arabinose, glucose	Uronic acid
"Branched B_{10}"	Xylose, arabinose	Galactose, glucose, uronic acid
Hemicellulose A_{24}	—	—
Hemicellulose B_{24}	Xylose, arabinose, glucose	Galactose, uronic acid
Cellulose residue	Glucose	—

* Variable, often absent.
† See Fig. 1.

of 1:4·0:2·0:1·3. The chromatograms (solvent b) showed no sign of arabinose, so that mannose could be clearly detected on chromatograms developed with solvent (a) as well as on those developed with solvent (b). When this possible heteromannan was dissolved in calcium chloride solution and iodine added a heavy blue precipitate formed. Only a negligible amount of polysaccharide remained in the supernatant. The polysaccharide precipitate gave on hydrolysis, after removal of iodine, galactose, glucose, mannose and xylose in the same ratio as in the original heteromannan. It should be noted that the "linear B_{10}" fraction on reprecipitation with the iodine gave polysaccharide containing no galactose or mannose but rich in arabinose and xylose (Table 3).

In general there did not appear to be any marked differences in monosaccharide composition between corresponding polysaccharides from clover leaves and stems.

[5] T. E. TIMMELL, *Methods in Carbohydrate Chem.* (edited by R. L. WHISTLER), vol. 5, p. 134, Academic Press, New York (1965).

DISCUSSION

In cell-wall preparations from both red clover leaves and stems only half of the polymer galactose and none of the mannose was extracted with 10% alkali compared with nearly 90% of the galactose from ryegrass cell-walls. Much of the residual galactose and mannose in red clover cell-walls was resistant to extraction with stronger alkali and mild delignification did not improve its extraction. Results reported for soya bean seed testa or hulls[6] showed a similar pattern with part of the polymer mannose resistant to strong alkali extraction after mild delignification. Delignification by severer methods was avoided in the present work in order to prevent losses of the more soluble fractions. It is possible that such delignification, particularly of the residue from the dilute alkali extraction, might aid the solubilization of the resistant polymer galactose and mannose or break any existing links between it and cellulose.

Although many reports[7-10] have been made of the monosaccharide composition of cell walls of leguminous plants, a detailed examination of the various polysaccharide fractions present in the leaves and stems of a pasture legume such as red clover does not appear to have been made. The present results indicate that at least two types of galactose-containing polysaccharide are present in red clover cell-wells. These are, one which is not precipitated by iodine from calcium chloride solution ("branched B_{10}") and one which is so precipitated (hemicellulose B_{24}). In the latter case the galactose may be present as a galacto-mannan or galactogluco-mannan as the fraction contains mannose and glucose as well as galactose.

While mannans of various kinds are commonly isolated from seeds[11] and woods,[5] their presence in leguminous leaves and stems is to be expected as small amounts of mannose have been reported in the hydrolysates of the cell walls of various legumes such as lucerne,[7] bean[9,10] shoots and clovers.[8]

The main differences between red clover leaves and stems was in the overall higher levels of the various polysaccharide fractions in the stems. While the clover leaves gave approximately equal amounts of hemicellulose A_{10} (xylan) and "linear B_{10}" (arabino-xylan) the stems contained a much higher proportion of the hemicellulose A_{10}. As with the clover leaves, the rye grass leaves also contained a relatively low level of hemicellulose A compared with the "linear B_{10}". The amounts of these fractions present in plant tissues may vary considerably. Thus while Sannella and Whistler[12] obtained a good yield of hemicellulose B containing arabinose from soya bean hulls, Aspinall *et al.*[6] obtained none of this type of polysaccharide from this source. Lucerne[13] stems have also been reported to yield xylan but not arabino-xylan of the linear B type. Such variations in the levels of these fractions may be related to maturity of the plant tissues.

EXPERIMENTAL

Plant Material

Red clover (*Trifolium pratense*) was cut, to within 5 cm of ground level, from a pure stand growing 30 cm high. Samples (1–2 kg wet weight) of leaves and stems (stems plus petioles) respectively were rapidly dissected out and deep frozen. A similar sample of Grasslands Ruanui perennial ryegrass (*Lolium perenne*) leaves

[6] G. O. ASPINALL, K. HUNT and I. M. MORRISON, *J. Chem Soc.* 1945 (1966).
[7] E. L. HIRST, D. J. MACKENZIE and C. B. WYLAM, *J. Sci. Food Agric.* **1**, 19 (1959).
[8] M. SALO, *J. Sci. Agric. Soc. Finland* **37**, 127 (1965).
[9] T. NAGASAKI and S. KAWAMURA, *Bull. Fac. Agric., Kagawa Univ. Japan* **15**, 154 (1967).
[10] D. J. NEVINS, P. D. ENGLISH and P. ALBERSHEIM, *Plant Physiol.* **42**, 900 (1967).
[11] R. L. WHISTLER and C. L. SMART, *Polysaccharide Chemistry*, Academic Press, New York (1953).
[12] J. L. SANNELLA and R. L. WHISTLER, *Arch. Biochem. Biophys* **98**, 116 (1962).
[13] G. O. ASPINALL and D. MCGRATH, *J. Chem. Soc.* 2133 (1966)

15–20 cm long was cut from a stand of single plants growing in full leaf to about 25 cm high. The frozen plant samples were freeze-dried, weighed and finally ground in a Wiley mill (1 mm mesh). The moisture content (9–10%) of the ground material was measured on portions heated overnight at 110°.

Preparation of Cell-Wall Residues

Ethanol–ammonium oxalate extraction. Freeze-dried plant material (50 g) was extracted twice by boiling for 5 min with ethanol (80% v/v, 2 l.) followed by filtration. The residue from this extraction was then extracted by refluxing, in portions, for 2 hr in ammonium oxalate (0·5% w/v, total vol. 1 l.) and filtered. The oxalate filtrate was acidified with conc. HCl (5 ml), dialysed and freeze-dried (pectic substances).

Neutral detergent extraction. Freeze-dried plant material (50 g) was divided into six equal portions which were each extracted by gentle boiling under reflux with the neutral detergent solution (300 ml) of Van Soest.[3] The combined residues were collected by filtering the boiling solutions in a porosity 1 sintered-glass funnel and washed well with hot water.

Delignification. Neutral detergent or oxalate-extracted residue was delignified with chloramine-T and ethanolamine essentially by the method of Gaillard.[4] Water was added to the residue to provide a slurry which was heated in a beaker to 80° in a water bath. Chloramine-T (1 g to each 75 ml water up to a maximum of 3 g) was added followed by acetic acid (1 ml to each 1 g of chloramine-T) and the mixture heated for 2 hr at 80° with occasional stirring. The hot slurry was filtered in a porosity 1 sintered-glass funnel and the residue washed with ethanol followed by boiling ethanolamine (3% w/v in ethanol) and finally ethanol. During the ethanolamine washing the residue was kept covered with the hot ethanolamine for 3 min before applying suction. The entire chloramine-T–ethanolamine treatment was repeated two times to give a final residue of holocellulose, which was washed with ethanol followed by acetone and dried at 40°.

Isolation of Polysaccharide Fractions

Extraction of hemicellulose A and B fractions. These fractions were extracted according to the scheme outlined in Fig. 1. All alkaline extractions were carried out at room temperature by continuous stirring overnight under a stream of N_2 and the extracts filtered on a porosity 1 sintered-glass filter with exclusion of air. Residues were washed with dilute KOH (5%) followed by water and the washings added to the alkali extract. After both 10% and 24% KOH extractions the water-washed residues were washed with ethanol followed by acetone then dried at 40° and sampled. Alkaline filtrates were acidified under N_2 while cooled in ice water. Hemicellulose A precipitates were collected by centrifuging (20,000 *g*) after standing the cooled acidified solution for 2–3 hr. After grinding in ethanol followed by ether they were dried *in vacuo* ($CaCl_2$). All acidified hemicellulose-B solutions were dialysed for several days against tap water, concentrated under vacuum on a rotary evaporator and freeze-dried.

Fractionation of hemicellulose-B_{10}. Freeze-dried hemicellulose-B_{10} (1 g) was dissolved in conc. $CaCl_2$ (100 ml s.g. 1·3) by stirring overnight and iodine solution (I_2 3%, KI 4% w/v, 15 ml) added. The blue precipitate was allowed to settle for 1 hr and then collected by centrifuging for 1 hr at 20,000 *g*. I_2 in the supernatant solution of "branched B_{10}" and in an aqueous solution of the blue precipitate of "linear B_{10}" was neutralized by treatment with conc. $Na_2S_2O_3$ solution. The solutions were finally dialysed, concentrated and freeze-dried in the usual way.

Carbohydrate Analyses

Acid hydrolyses. "Branched B" fractions (10 mg) were hydrolysed in *N* H_2SO_4 (25 ml) by heating for 2 hr at 100°. Other polysaccharide fractions (10 mg) were treated with H_2SO_4 (72% w/w, 0·7 ml) at room temperature for 2 hr, diluted with water (23 ml) and refluxed for 2 hr. Cell-wall preparations and residues (100 mg) were hydrolysed either by refluxing for 2 hr in *N* H_2SO_4 (50 ml) for galactose determinations or by treating with the 72% H_2SO_4 (1·4 ml), followed by dilution with water (40 ml) and refluxing for 2 hr for mannose determinations. Hydrolysates were neutralized with alkali for total sugar analysis or with $BaCO_3$ for paper chromatographic analysis when the neutralized hydrolysates were freeze-dried and taken up in water (1 ml). These latter hydrolysates were satisfactory for the chromatographic analysis of all sugars present except mannose. For the analysis of this sugar separate acid hydrolysates were neutralized with $BaCO_3$ and the filtrates treated with a little Biodeminrolit (CO_2 form) before freeze-drying. This was done to remove uronic acids which moved in the same position as mannose in solvent (b).

Paper chromatography. Chromatograms on acid-washed paper were developed with either solvent (a), ethyl acetate–water–pyridine (2:2:1) or (b), ethyl acetate–acetic acid–formic acid–water (9:1·5:0·5:2·0). Sugars were located with aniline hydrogen phosphate.

Total reducing sugars. These were measured by the microcuprimetric procedure of Nelson[14] using a xylose standard.

[14] N. Nelson, *J. Biol. Chem.* **153**, 375 (1944).

Individual monosaccharides. These were measured by the quantitative method of Wilson[15] except that the papers were sprayed with aniline hydrogen phosphate. For the measurement of mannose, papers were developed with solvent (b) and for galactose and other monosaccharides they were developed with solvent (a).

All sugar analyses were calculated, as the appropriate anhydro-sugar, for the total fraction from 100 g of moisture-free, original freeze-dried plant tissue, so that moisture contents of each fraction need not be measured.

Acknowledgements—The technical assistance of Mrs. V. Payne is gratefully acknowledged.

[15] C. M. WILSON, *Analyt. Chem.* **31**, 1199 (1959).