Carbohydrate Research, 42 (1975) 135–145

PRESENCE OF SOLUBLE LIGNIN–CARBOHYDRATE COMPLEXES IN THE BOVINE RUMEN*

BLANCHE D. E. GAILLARD** AND GEOFFREY N. RICHARDS

Department of Chemistry and Biochemistry, James Cook University of North Queensland, Townsville (Australia)

(Received December 6th, 1974; accepted for publication, December 16th, 1974)

ABSTRACT

The cell-free rumen liquor of a steer on a diet of spear grass has been shown to contain macromolecular substances in which carbohydrates and lignin-derived compounds are covalently bound to each other. The lignin–carbohydrate complexes are soluble at pH 7 or higher, but precipitate at pH 3. At the latter pH, small amounts of a polymer, assumed to be glycoprotein, remain in solution. Some of the lignin–carbohydrate linkages are broken by treatment with alkali. Treatment with 50mM sulphuric acid for a few minutes at room temperature converts part of the complex into an acetone-soluble product, which still contains both carbohydrate and lignin-derived compounds. The formation of soluble lignin–carbohydrate complexes by the action of rumen micro-organisms on the grass may account for the dissolution (and hence the apparent digestion) of about half of the total lignin-intake.

INTRODUCTION

In earlier work, Gaillard and Van't Klooster[1,2] have shown that, in ruminants, the available carbohydrate components of foods are mainly fermented in the rumen, but that in the process, polysaccharides and glycoproteins were added to the digesta and subsequently digested elsewhere in the digestive tract. In order to investigate digestion of the latter polymers, it is necessary to separate the digesta into discrete fractions, *e.g.*, by centrifugation, and in this process we discovered that the cell-free rumen liquor had a quite significant content of soluble carbohydrates, which evidently resisted fermentation or digestion in the rumen. We now describe an investigation of these materials.

RESULTS AND DISCUSSION

The cell-free rumen liquors from a steer fed with spear grass and from a steer fed with Townsville stylo contained only small amounts of total carbohydrate (29.8

*Digestion of Polysaccharide Constituents of Tropical Pasture Herbage in the Bovine Rumen: Part V. Part IV: R. J. Beveridge and G. N. Richards, *Carbohyd. Res.*, 29 (1973) 79.

**Department of Animal Physiology, Agricultural University, Wageningen, The Netherlands.

and 7.0 mg/100 ml, respectively), in agreement with the findings of Bailey[3], and since the spear-grass liquor appeared more promising in this respect, subsequent work was confined to that material.

During dialysis, there was no significant loss of carbohydrate, therefore this method was used to remove the large amounts of material of low molecular weight (mainly salts). The dialysed liquor still had a dark-brown colour. Table I gives the amounts of dry matter, ash, carbohydrate, and protein in the original and in the dialysed rumen-liquor, and also the absorbances at 455 and 280 nm after 10× and 100× dilution, respectively. The former wavelength was chosen arbitrarily as a measure of "colour", and the latter wavelength represents an absorption maximum in these solutions (see below).

TABLE I

COMPOSITION[a] OF RUMEN LIQUOR, DIALYSED LIQUOR, AND PEAKS I AND II OF FIG. 1

	Dry matter	*Ash*	*Carbohydrate*	*Protein*[b]	A_{280}[c]	A_{455}[d]
Rumen liquor	1420	750	29.8	50	0.515	0.458
Dialysed liquor	300	35	29.3	37.5	0.470	0.438
Peak I	100		13.5	15.6		
Peak II	200		16.7	20.0		

[a]All values correspond to mg/100 ml of rumen fluid. [b]N × 6.25. [c]100 × diluted. [d]10 × diluted.

The total dry-matter content of the dialysed liquor was 300 mg/100 ml. Deducting carbohydrate, protein, and ash, this leaves 200 mg of dry matter/100ml to be accounted for. It seemed likely that the strong u.v. absorption was linked to this fraction, as the protein content was too low to account for it. In an attempt to separate the coloured materials from the carbohydrate by gel-permeation chromatography, columns of Sephadex G-100 and G-200 were monitored for carbohydrate and for absorbance at 455 and 280 nm, respectively. The chromatogram for G-100 is shown in Fig. 1 (G-200 gave a similar pattern with the first peak still at V_0). In peak II, there was coincidence between the two absorbances, and although there was some apparent fractionation of carbohydrate material, this was not separated from the coloured material. Peak I shows coincidence of both absorbances and of carbohydrate, but since the peak is eluted at V_0, this cannot be regarded as evidence of a single component containing both carbohydrate and colour.

The fractions comprising each major peak (I and II in Fig. 1) were combined, deionised by ultrafiltration, and gave the results shown in Table I, confirming that each peak contained both carbohydrate and protein, and that all solutes present were of high molecular weight (greater than 10,000). Since Fig. 1 shows that all components of peak II were eluted well beyond the volume (~200 ml) when solutes of low molecular weight (*e.g.*, D-glucose) emerge from the column, it is evident that there is interaction between the solutes and the dextran gel. Such interaction would be quite likely with aromatic molecules derived from lignin, but most unlikely for poly-

saccharides. Fig. 1 therefore provides good evidence that the carbohydrate constituents are bound to some other type of material which interacts with the dextran gel.

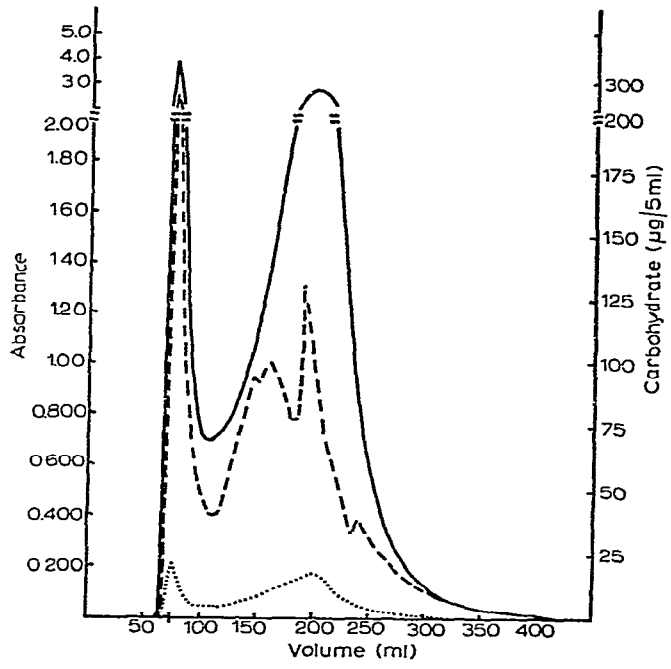


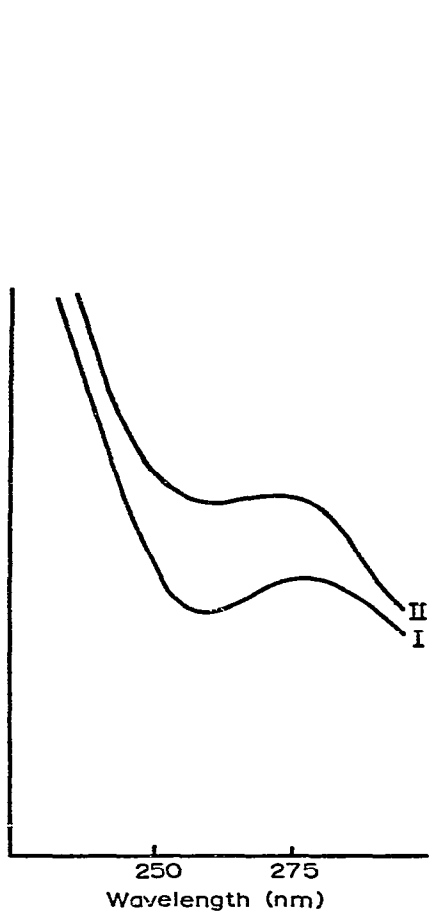
Fig. 1. Fractionation of dialysed rumen-liquor on Sephadex G-100. ———, A_{280}; ·····, A_{455}; – – –, carbohydrate.

The u.v. spectra of peaks I and II after ultrafiltration showed (Fig. 2) the maximum at 280 nm and minimum at 258 nm, which are typical for lignin[4]. The i.r. spectra, when compared (Fig. 3) with those of a Klason lignin from bagasse and of a spear-grass hemicellulose B, corresponded well to a combination spectrum of the latter two polymers in the region 1400–1800 cm^{-1}.

The data so far described therefore give a strong indication that both peaks contain carbohydrate–lignin complexes, probably released from the spear grass by the action of the rumen micro-organisms.

A chemical bond between carbohydrate and lignin in the cell-walls of plants is often mentioned[4,5]. Morrison[6] reported the presence of at least 5 complexes in grass. The solubility of these complexes, when isolated, was ascribed to the presence of the carbohydrate. When the complex is hydrolysed, the lignin becomes insoluble in water. Different types of linkage between the carbohydrate and the lignin have been reported. Some are alkali-labile, and some are alkali-stable but can be hydrolysed by acid.

We have tried to obtain some information on the alkali-sensitivity of our



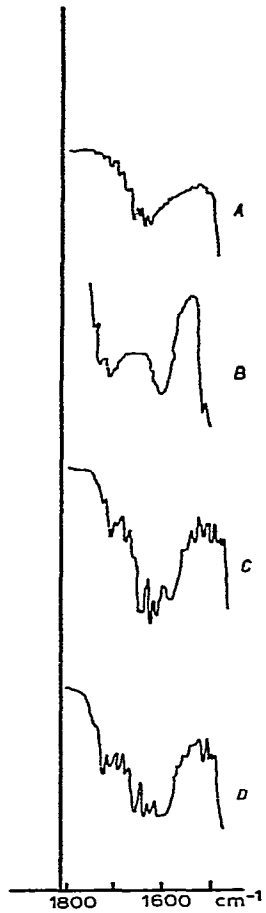


Fig. 2. Ultraviolet spectra of peaks I and II of rumen liquor fractionated on Sephadex G-100.

Fig. 3. Infrared spectra of spear-grass hemicellulose B (*A*), bagasse lignin (*B*), and peak I (*C*) and peak II (*D*) of rumen liquor fractionated on Sephadex G-100.

material by adding sodium hydroxide to the dialysed liquor to a final concentration of 0.1M and keeping the mixture at 37° (see Experimental, alkaline treatment A). Up to 6 h, the total carbohydrate remained constant, as did A_{280}. However, the amount of carbohydrate remaining in solution after acidification of a sample to pH 3 increased in the first 2 h to a value corresponding to 12 mg/100 ml of rumen liquor, and subsequently remained constant up to 6 h, but beyond this time a slow decrease in all carbohydrate values was observed, presumably due to alkaline degradation. We conclude that the alkaline treatment resulted in the scission of some but not all of the carbohydrate material from the lignin, so that it remains unprecipitated on acidification. Fractionation on Sephadex G-100 of the alkali-treated solution after neutralization (Fig. 4) showed that part of the material from the first peak was

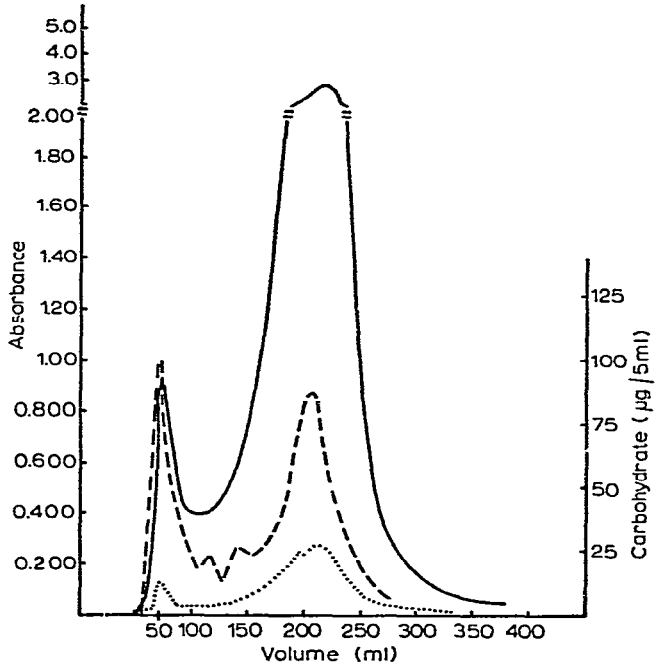


Fig. 4. Fractionation of the alkali-treated rumen liquor on Sephadex G-100. Treatment (*a*) (see Experimental); time, 6 h. ———, A_{280}; ·····, A_{455}; — — —, carbohydrate.

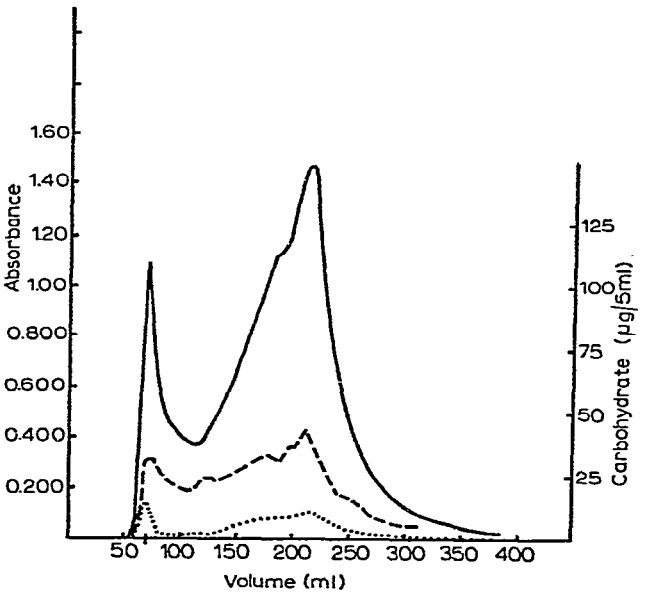


Fig. 5. Fractionation of the acetone extract of the precipitate after treatment of the rumen liquor with 0.5M sulphuric acid for 5 min (Sephadex G-100). ———, A_{280}; ·····. A_{455}; — — — carbohydrate.

converted into material of lower molecular weight, but the A_{280} and carbohydrate profiles still coincide.

In a more extensive fractionation scheme based on the alkali-treatment (Fig. 8), more information was obtained on the reaction of alkali on the complexes. The results of this fractionation are given in Table II.

TABLE II

FRACTIONS OBTAINED BY ACID AND ALKALI TREATMENTS OF THE DIALYSED RUMEN-LIQUOR

	Fractionation as Fig. 8			*Fractionation as Fig. 9*	
	A_{280}[a]	*Carbohydrate*[b]		A_{280}[a]	*Carbohydrate*[b]
Dialysed liquor	0.470	32	Dialysed liquor	0.435	31
Solution I	0.086	5	Solution 1	0.086	6
			Acetone extr. 2	0.222	10
Solution II	0.054	13	Solution 3	0.028	7
Acetone extr. III	0.256	7	Acetone extr. 4	0.049	2
Residue IV	0.075	9	Residue 5	0.030	6

[a]100 × diluted. [b]Mg/100 ml of rumen fluid.

When acid was added to the dialysed rumen-liquor, most of the material was precipitated; 16% of the carbohydrate remained in solution linked to some u.v.-absorbing material, presumably glycoprotein in nature, as 83% of the total protein from the dialysed liquor was also found in this fraction. The precipitate obtained on acidification was treated with alkali and subsequently again acidified (Fig. 8). This procedure converted 41% of the original carbohydrate into the pH 3-soluble form, but only 12% of the original A_{280} component was so converted. Most of the A_{280} material, therefore, remained insoluble at pH 3 after the alkali-treatment, but was now mostly soluble in acetone. The acetone-soluble material (unlike the "glycoprotein" fraction, solution I in Fig. 8) gave a u.v. spectrum in aqueous solution that was typical of lignin. The constituent sugars of all fractions were identified by thin-layer and paper chromatography after hydrolysis. Each contained galactose, glucose, arabinose, and xylose. Solution I also contained mannose, fucose, and rhamnose. During the acid hydrolyses, heavy, dark-brown precipitates were produced from fractions III and IV (Fig. 8).

The action of acid on the complexes is not easy to understand. The freeze-dried, dialysed liquor was completely insoluble in acetone. However, when it was dissolved in water to reproduce the dialysed liquor, the complexes precipitated with 50mM sulphuric acid (leaving the glycoprotein in the solution), and the precipitate then directly extracted with acetone (fractionation scheme, Fig. 9), a considerable part of the complex became soluble in acetone. The residue was then treated with alkali. In all fractions from this scheme, total carbohydrate and A_{280} were measured (Table II). It seems that, although the freeze-dried material is not soluble in acetone, 32% of the original carbohydrate and 51% of the u.v.-absorbing material became soluble in the

acetone after the very brief and mild treatment with acid (~5 min in 50mM sulphuric acid at room temperature). More drastic treatments with sulphuric acid had little further effect on acetone-solubilisation of carbohydrate or A_{280} fractions (Table III).

TABLE III

RECOVERY OF CARBOHYDRATE AND A_{280} AS WATER- AND ACETONE-SOLUBLE PRODUCTS AFTER VARIOUS TREATMENTS WITH SULPHURIC ACID

		A_{280}[a]	Carbohydrate[b]
Dialysed liquor		0.435	31
50mM Acid, 5 min,	supernatant	0.086	6
	acetone extract	0.222	10
50mM Acid, 20 h,	supernatant	0.086	7
	acetone extract	0.260	11
0.5M Acid, 5 min,	supernatant	0.055	7
	acetone extract	0.223	13
0.5M Acid, 20 h,	supernatant	0.048	6
	acetone extract	0.248	14

[a]100 × diluted. [b]Mg/100 ml of rumen fluid.

The acetone extracts, after removal of acetone, were applied to a column of Sephadex G-100. The very brief treatments with 50mM and 0.5M H_2SO_4 again gave two peaks. A_{280} in the first peak at the void volume was slightly lower after treatment with 0.5M acid (Fig. 5) than with 50mM acid, but the second peak was wider for both treatments, indicating some increase in heterogeneity. The carbohydrate in the first peak again corresponded sharply with A_{280}. The subsequent carbohydrate peaks were less sharp than with the original, dialysed liquor. The precipitate resulting from prolonged treatment with 0.5M acid gave an acetone extract in which the A_{280} maximum in the first peak was markedly decreased, and that in the second peak was increased (Fig. 6). There was also a marked increase in carbohydrate in both peaks. When the total precipitate resulting from the prolonged treatment with 0.5M sulphuric acid was redissolved in alkali, neutralised, and then fractionated on Sephadex G-100 (Fig. 7), two major peaks were again observed, with general correspondence of A_{280}, A_{455}, and carbohydrate. The general conclusion from Figs. 4–7 is that all of the types of polymer present appear to be subject to some action by both acid and alkali, but that no new type of polymer is produced; *e.g.*, there is no evidence for production of lignin-free polysaccharide or carbohydrate-free lignin. It is possible, however, that some free carbohydrate of low molecular weight may be produced on prolonged treatment with acid.

Our results are, in general, compatible with the conclusions of Stewart[5] that alkali-labile bonds, such as esters, function as linkages between lignin and polysaccharides. The production of an acetone-soluble product on very mild treatment with acid (Table III) could arise either by scission of an extremely acid-labile linkage

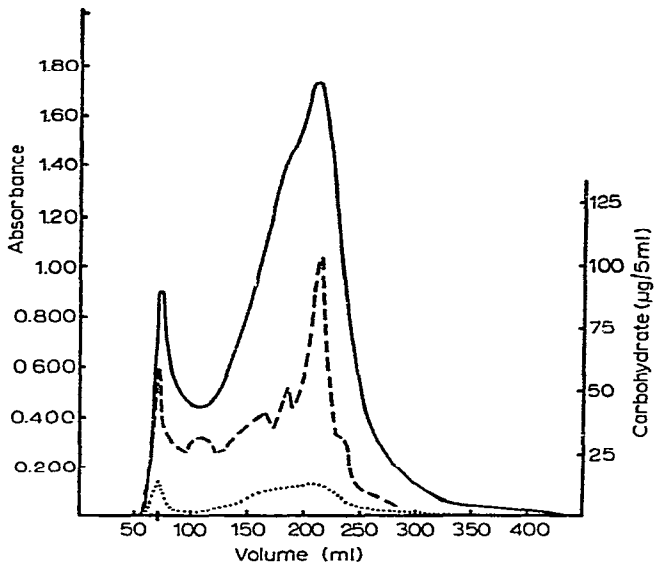


Fig. 6. Fractionation of the acetone extract of the precipitate after treatment of the rumen liquor with 0.5M sulphuric acid for 20 h (Sephadex G-100). ———, A_{280}; ·····, A_{455}; — — —, carbohydrate.

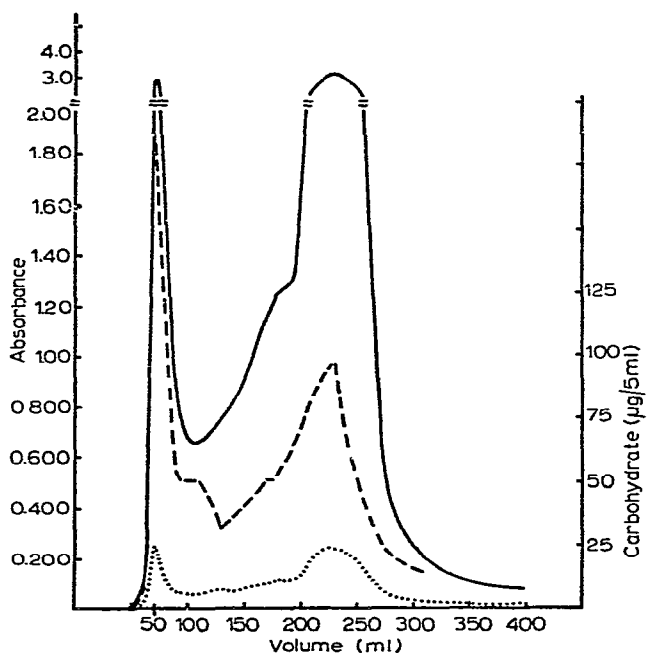


Fig. 7. Fractionation of the redissolved precipitate from the rumen liquor after treatment with 0.5M sulphuric acid for 20 h (Sephadex G-100). ———, A_{280}; ·····, A_{455}; — — —, carbohydrate.

such as an ortho-ester, or by conversion of an acetone-insoluble metal salt of the lignin–carbohydrate complex into an acetone-soluble, free-acid form. At present, the former explanation seems the less likely because it would be expected to lead to greater changes in the gel-chromatography profiles, *e.g.*, producing lignin fragments considerably depleted in carbohydrate.

The observation that soluble lignin–carbohydrate complexes are produced from fodder in the rumen has significant implication in studies of the ruminant digestion process. There have been reports (*e.g.*, refs. 1 and 7) that lignin is partly digested. Such reports are based on the disappearance of lignin from the fibrous digesta, and it now seems certain that at least part of this "digestion" is in fact due to the dissolution of the lignin–carbohydrate complex, which passes from the rumen as a polymer in solution and quite possibly evades further degradation and emerges in the faeces.

The liberation of the soluble complexes probably follows upon the enzymic scission of both cellulose and hemicellulose chains of the grass cell-wall in the rumen, as there is no suggestion that any lignin-containing material is extractable by water from the grass without involvement of rumen micro-organisms. If we assume that the volume of fluid leaving the rumen and entering the abomasum is ~150 litres/day and that, under the conditions of our experiment, the animal consumes ~7 kg dry-weight of spear grass containing 10% of lignin, the presence of 200 mg of lignin in the lignin–carbohydrate complex per 100 ml of rumen fluid corresponds to ~43% of the total lignin-intake. It is probable that most of the dissolved lignin–carbohydrate complexes are precipitated in the low pH of the abomasum, and there may be some redissolution at the later stages in the gastro-intestinal tract. The possibility of digestion of the complexes after leaving the rumen should be further investigated.

EXPERIMENTAL

Materials. — Klason lignin from bagasse and spear-grass hemicellulose were kindly supplied by Mr. R. F. H. Dekker.

Methods. — Total carbohydrate was determined by the phenol–sulphuric acid method[8] and expressed as "glucose equivalent" by reference to a D-glucose calibration curve. Gel-permeation chromatography was carried out on 50 × 2.5-cm columns unless otherwise stated, by upward flow using 25mM ammonium acetate. Where necessary, polymer solutions were concentrated and/or deionised by ultrafiltration through a Micron membrane U.M.10 (mol. wt., 10,000). Infrared spectra were determined with mulls of freeze-dried materials in Nujol or hexachlorobutadiene. Thin-layer chromatography was carried out on Baker gel silica 1 B plates with ethyl acetate–pyridine–water (8:2:1), and sugars were detected with the aniline phosphate spray. Instead of using the multiple-development technique, a form of continuous chromatography was achieved by attaching a piece of thick filter-paper to the top of the plate. In this way, a good separation of all sugars was obtained in 6 h. Butan-1-ol–ethanol–water (7:2:2) was used for paper chromatography.

Isolation of soluble polymers from rumen liquor. — A Droughtmaster steer with a rumen fistula was pen-fed for 7 days with spear grass (*Heteropogon contortus*) (7 kg/day). The following morning before feeding, the rumen was bailed out, and the contents were immediately filtered, cooled to ~5°, and transported in a cooled container to the laboratory where the filtrate was immediately centrifuged at 19000 r.p.m. for 20 min. The clear liquor was poured off, leaving a slime layer with a cake of bacteria, protozoa, and small particles of food. In the same way, a clear rumen-liquor was obtained after feeding Townsville stylo (*Stylosanthes humilis*). The rumen liquors were dialysed against running water for 2 days and then freeze-dried. Aliquot portions of the products were redissolved in appropriate volumes of deionised water for use as "dialysed rumen-liquor". The yields are shown in Table I.

Fractionation of constituents of the dialysed rumen-liquor. — (*a*) *Treatment with alkali.* To 10 ml of dialysed rumen-liquor was added 1 ml of M sodium hydroxide, and the mixture was kept at 37°. At intervals, 1-ml samples were taken and neutralized to pH 7, and carbohydrate and A_{280} were determined. The sample was then acidified to pH 3 with sulphuric acid. The colour of the solution changed from dark brown to light brown, and a precipitate was formed. For the resulting supernatant solution, carbohydrate and A_{280} were measured.

(*b*) To 10 ml of dialysed rumen-liquor was added 1 ml of 0.5M sulphuric acid. A brown precipitate formed, which was immediately removed by centrifugation and redissolved in 10 ml of 50mM sodium hydroxide. The remaining fractionation is shown in Fig. 8.

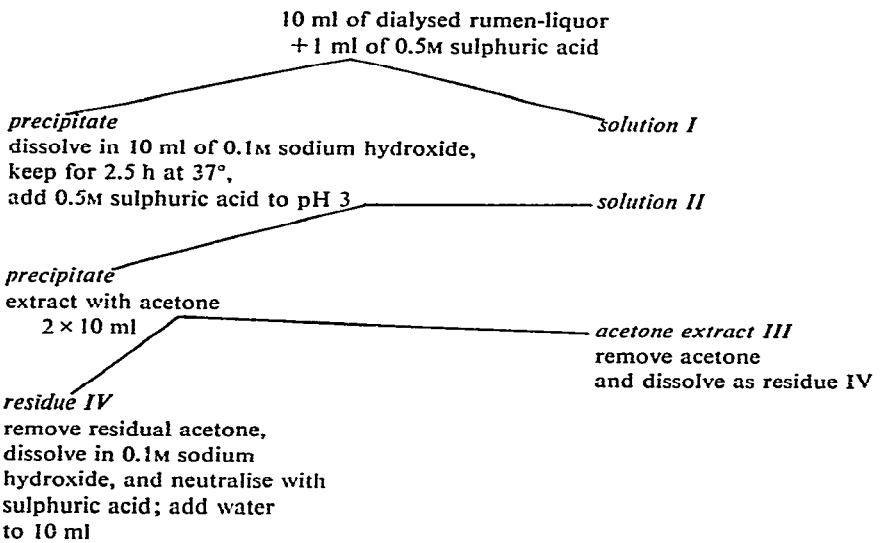


Fig. 8. Fractionation scheme (*b*).

(*c*) The fractionation scheme described in Fig. 9 was carried out

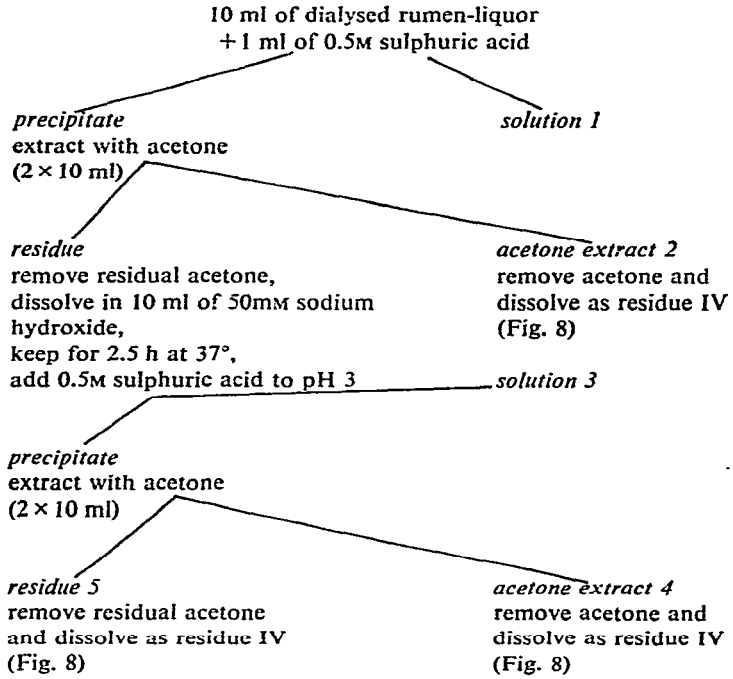


Fig. 9. Fractionation scheme (*c*).

(*d*) In order to determine the effect of the acid treatment on acetone-solubilisation, the first two steps of Fig. 9 were repeated with 0.5M sulphuric acid for 20 h, and with 5M sulphuric acid for ~5 min and for 20 h. The results are shown in Table III.

ACKNOWLEDGMENTS

The authors thank Dr. M. J. Playne for arranging the supply of rumen liquor from the Lansdown station of C.S.I.R.O. Division of Tropical Agronomy, and Mr. R. G. Meggarity of the same Division for assistance with analyses in Table I. Financial assistance was provided by the Rural Credits Development Fund of the Reserve Bank of Australia.

REFERENCES

1 B. D. E. Gaillard and A. Th. van 't Klooster, *Mededel. Landbouwhogeschool Opzoekingsta. Staat Gent*, 69 (1969) 11.
2 B. D. E. Gaillard and A. Th. van 't Klooster, *Neth. J. Agr. Sci.*, 21 (1973) 217.
3 R. W. Bailey, *New Zealand J. Agr. Res.*, 2 (1959) 355.
4 K. V. Sarkanen and Ch. H. Ludwig, *Lignin*, Wiley–Interscience, 1971, p. 218.
5 C. M. Stewart, *Cellulose Chem. Technol.*, 7 (1973) 691.
6 I. M. Morrison, *Phytochemistry*, 12 (1973) 2979.
7 R. F. H. Dekker, G. N. Richards, and M. J. Playne, *Carbohyd. Res.*, 22 (1972) 173.
8 M. Dubois, K. H. Gilles, J. K. Hamilton, P. A. Rebers, and F. Smith, *Anal. Chem.*, 28 (1956) 350.