NON-SOIL CONSTITUENTS OF TERMITE (*COPTOTERMES ACINACIFORMIS*) MOUNDS

By L. Rosemary Gillman*, Marilyn K. Jefferies*, and G. N. Richards*

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Abstract

The exterior wall of the mound of *C. acinaciformis* was extracted with various solvents and the extracts compared with similar products derived from the soil from which the mounds had been constructed. Two types of compound were obtained in greater yield from the mound than from the soil and it was concluded that these compounds had been incorporated into the mound by the termites. The first type of additive was a polysaccharide or mixture of polysaccharides of the hemicellulose group, probably derived from incompletely digested plant particles in the termite faeces. The second type of additive was identified as belonging to the glycoprotein group, is assumed to function as an adhesive in construction of the wall, and may originate in an insect excretion.

I. Introduction

The exterior wall of mounds of the termite species *Coptotermes acinaciformis* (like many other species) is extremely hard and impervious to water, in striking contrast to the surrounding soil which is frequently poorly aggregated. It seemed likely that various types of "additive" responsible for these effects might be incorporated in the mounds by the termites during construction and we have sought to extract and identify such additives. Throughout such work we have compared results with those obtained from the soil surrounding the mound, from which the termites obtain the soil particles used in construction. We have also taken care to use only the "cemented" soil from the mound and to avoid any cemetery or food-storage areas. In earlier studies we attempted to use the mounds of *Amitermes laurensis*, but found it very difficult to avoid such cemetery and food-storage areas in the mound. The species *C. acinaciformis* was much more suitable in this respect since the mound is constructed with an outer envelope (2–3 in. thick) of cemented soil particles which is not used for any other purpose by the insects.

Using a wide range of extractants (including many organic solvents) in preliminary work we were only able to identify two types of non-soil additive in the mounds, viz. polysaccharides and glycoproteins. The most efficient method for extraction of the polysaccharides from the mounds was with 1·0N potassium hydroxide solution at room temperature and this conclusion is in agreement with recent work on extraction of polysaccharides from soils (Swincer, Oades, and Greenland 1968). We found that the acidic extractants favoured by some previous workers (e.g. Barker *et al.* 1965) caused considerable degradation of arabinofuranoside linkages, while organic extractants such as dimethylsulphoxide were very inefficient in solubilizing the polysaccharides. We anticipate that the "hemicellulose-type" polysaccharides were

* Chemistry Department, James Cook University of North Queensland, Townsville, Qld. 4810.

not extensively changed during the alkaline extraction. There is a much greater probability that the glycoproteins which we isolated were degraded during alkaline extraction, but the same type of product (viz. glycoprotein) was also obtained by extraction of the mound with 70% ethanol.

II. Experimental

(a) *General Methods*

All evaporations were carried out at 40°C and 20 mm. For descending paper chromatography on Whatman No. 1 paper the following solvents and sprays were used: solvent A, ethyl acetate–pyridine–water (8 : 2 : 1 v/v); solvent B, ethyl acetate–acetic acid–formic acid–water (18 : 3 : 1 : 4 v/v); solvent C, ethyl acetate–pyridine–water (10 : 4 : 3 v/v); solvent D, butan-1-ol–pyridine–water (6 : 4 : 3 v/v); spray A, alkaline silver nitrate (Trevelyan, Proctor, and Harrison 1950); spray B, *p*-anisidine hydrochloride (Hough, Jones, and Wadman 1950); spray C, ninhydrin (Smith 1960).

Absolute compositional analysis of polysaccharides was carried out by acid hydrolysis and subsequent gas chromatography of alditol acetates as described earlier (Blake and Richards 1970). Uronic acids were separated from the hydrolysis products by ion exchange and tentatively identified by paper chromatography in solvent B, using sprays A and B.

An estimate of the total carbohydrate content of products was obtained by the phenol–sulphuric acid method (Dubois *et al.* 1965). However, instead of measuring absorbance in the usual way at a single wavelength, the complete spectrum was determined from 650 to 400 nm in each analysis. This procedure was adopted because most of the materials analysed in this work produced non-specific background absorption in this region. The background was partly due to colour in the sample being analysed, but mostly due to colour developed by interaction of non-carbohydrate material with the reagents. Obviously the use of absorbance measurements at 488 nm would have given anomalously high results, therefore the height of the inflexion in the absorbance curve at that wavelength was measured and converted to "glucose equivalents" via a calibration with pure glucose. The validity of the method was verified by addition of known amounts of glucose before analysis of several samples.

(b) *Soil and Mound Samples*

The outer wall of the mound was readily separated from the inner carton structure. A thin layer of dark brown carton material adhering to the inner surface of the wall was chipped away and the wall was ground to a fine powder in a Wiley mill. The control soil was collected as a fine powder to a depth of 8 cm from the A horizon around the base of the mound and was passed through a 30-mesh sieve to remove roots, leaves, etc.

III. Results

(a) *Alkaline Extraction*

Mound and soil samples (1 kg) were rolled for 20 hr with oxygen-free 1·0N potassium hydroxide (1·5 litres) at 25°C. After centrifuging, the volume of the dark brown extract was measured and accepted as an aliquot portion of the total solution in order to avoid extensive washing of the solids. The extract was acidified to pH 3·9 with glacial acetic acid and the resultant precipitate (humic acids) removed by centrifugation. Ethanol was next added to the solution in 50-ml portions with efficient stirring and when a precipitate became evident the mixture was left at room temperature for 2 hr and then centrifuged. The composition of fractions (designated "B fractions") thus obtained are shown in Table 1 and are calculated for complete recovery of extracts. After removal of the B_5 fraction at 66% ethanol, the solution

was concentrated to 100 ml and dialysed against water for 2 days. A small amount of precipitate (B_6) was removed and the pale brown solution was freeze-dried to yield fraction B_7.

TABLE 1

COMPARISON OF YIELD AND CARBOHYDRATE CONTENT OF B FRACTIONS FROM MOUND AND CONTROL SOIL

Fraction	Ethanol concn. (% v/v)	Weight (g)		Carbohydrate in fraction (%)		Derived carbohydrate in original soil or mound (p.p.m.)	
		Mound	Control	Mound	Control	Mound	Control
B_1	32	1·856	0·175	16·3	5·1	491·6	11·2
B_2	45	0·979	0·219	33·1	14·8	528·9	40·5
B_3	50	1·578	0·668	34·4	5·2	884·2	43·4
B_4	63	0·279	0·399	34·4	4·4	156·3	22·0
B_5	66	0·432	0·405	23·8	5·8	167·3	29·4
B_6		0·132	0·084	8·1	0·4	17·5	0·4
B_7		1·916	0·653	14·8	9·2	460·8	74·9
Total		7·172	2·603	23·2*	6·8*	2706·6†	221·8†

* Percentage carbohydrate in combined B fractions.

† Parts per million of carbohydrate in original soil which was isolated from B fractions.

Hydrolysis of the B fractions and qualitative analysis for neutral sugars by paper chromatography (solvent A, sprays A and B) indicated, in decreasing order, xylose, galactose, glucose, mannose, rhamnose, and arabinose. The first four sugars were major components and occurred in approximately constant relative proportions in every fraction. Rhamnose and arabinose either were not detected or were present in very low concentration.

Hydrolysis of B_7 and paper chromatography in solvents C and D, sprays A, B, and C, showed (in addition to the neutral sugars described above), glucosamine and a mixture of amino acids. The absorption spectrum showed inflexions at 280 nm and at 340–380 nm.

(*b*) *Fractionation of the Hemicelluloses*

Fraction B_3, which was pale brown in colour, was designated "crude hemicellulose" and was further investigated. Chromatography on columns of Sephadex G200 and Sepharose 6B yielded good recovery of applied carbohydrate and multiple peaks, but the major carbohydrate peaks in both cases were contaminated by coloured material.

In attempts to remove colour from B_3, batch tests were carried out with QAE-Sephadex (acetate form), DEAE-cellulose (free base), and charcoal (200 mg sorbent in 3·3 ml of 0·25% solution). The first two materials were effective in removing all colour from a solution of B_3, but a large part of the carbohydrate was also sorbed, especially by DEAE-cellulose. Charcoal removed only a part of the colour from the solution (33%, measured at 420 nm) and also sorbed 37% of the carbohydrate. QAE-Sephadex was therefore selected for further chromatography.

A 3% solution (5 ml) of B_3 from the mound in 0·01M sodium acetate was loaded onto a column of QAE-Sephadex (100 by 2·5 cm, acetate form) and eluted with 0·01M sodium acetate (450 ml). Neither carbohydrate nor colour were detected in the effluent. A gradient of 0–0·75M potassium chloride (2 litres) was next applied to the column and analysis of the effluent fractions for carbohydrate gave the results shown in Figure 1. The fractions containing carbohydrate were opalescent. Those emerging

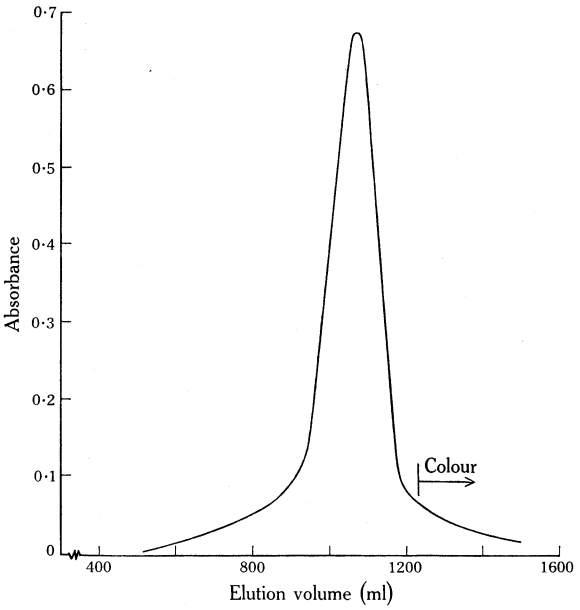

Fig. 1.—Fractionation of mound hemicellulose on QAE-Sephadex (0–0·75M KCl gradient).

prior to the appearance of colour were combined, concentrated, dialysed, and freeze-dried to a white solid which represented 69% of the total carbohydrate added to the column and 24% by weight of the original B_3 sample.

A similar fractionation of B_3 from control soil on QAE-Sephadex yielded a colourless product which represented 61% of the total carbohydrate and 3% by weight of the original B_3 sample.

Hydrolysis of the colourless hemicellulose product from mound fraction B_3 and gas-chromatographic analysis of the derived alditol acetates gave the results shown in the following tabulation:

Sugar	Molar ratio	Anhydro sugar in fraction B_3 (%)
Rhamnose	0·05	3·5
Arabinose	0·04	3·1
Xylose	1·00	72·2
Mannose	0·05	3·5
Galactose	0·18	13·3
Glucose	0·07	4·7
Total	—	100·3

Qualitative paper chromatography of the uronic acids derived from the hydrolysis indicated the presence of 4-*O*-methyl-D-glucuronic acid, $R_{GU} = 1·29$ in solvent B, where $R_{GU} = 1·00$ for D-galacturonic acid.

(c) *Extraction of Glycoprotein*

Mound and soil samples (1·5 kg) were rolled for 20 hr at room temperature with 7% EDTA disodium salt solution (2 litres), which had been adjusted to pH 4 with acetic acid before adding the soil. After removal of the extract by centrifugation the soil was further extracted with 7% EDTA in 0·03M sodium phosphate buffer at pH 7·5. No evidence for glycoprotein was found in either extract. The washed soil was next extracted with 70% aqueous ethanol (2 portions of 2 litres each for 20 hr each), the extracts were concentrated, dialysed against water, and freeze-dried to a pale yellow powder, yielding 1·92 g (0·13%) (found: N, 1·87%, Kjeldahl) from the mound and 0·69 g (0·05%) (found: N, 1·03%) from the control soil.

(d) *Hydrolysis of Crude Glycoproteins*

The above products (0·025 g) were dissolved in 4·0N hydrochloric acid (5 ml) and hydrolysed at 100°C for 4 hr in sealed tubes under nitrogen. The filtered solutions were then evaporated to dryness several times with intermediate additions of water to remove hydrochloric acid. The residues were dissolved in water, passed through columns of Amberlite resin IR-120 (H^+), and concentrated for analysis of the neutral sugars by gas chromatography after conversion to alditol acetates. Results are shown in the following tabulation:

Sugar	Ratio by weight (mound)	Ratio by weight (control soil)
Rhamnose	0·18	0·19
Arabinose	0·48	0·20
Xylose	0·13	0·41
Mannose	0·88	0·99
Galactose	0·54	0·75
Glucose	1·00	1·00

The IR-120 resin columns were eluted with 0·1N hydrochloric acid and the eluates examined by paper chromatography in solvents C and D, sprays B and C. Only one amino sugar spot was detected, corresponding to authentic D-glucosamine. An Elson–Morgan analysis (Gottschalk 1966, p. 226) of the column eluate indicated an amino sugar content of 5·2% in the crude glycoprotein from both mound and control soil.

The crude glycoproteins and also the alkali-extraction product B_7 were also hydrolysed with constant-boiling hydrochloric acid at 100°C for 24 hr and analysed for amino acids using a Paton–Simmonds automatic amino acid analyser. Columns of Aminex resin were used at 50°C, and the initial eluting buffer was pH 3·23 with a change to pH 4·26 after 200 ml of eluent. Results are shown in Table 2. The "unknown" was ninhydrin-positive and eluted approximately in the position of phosphoserine or phosphothreonine, but no authentic samples of these compounds were available. It was not cysteic acid. The unidentified compounds eluted as follows:

(1) preceding aspartic acid;
(2) between serine and glutamic acid;
(3) between glutamic acid and glycine;
(4) between leucine and phenylalanine.

There was insufficient sample to analyse for basic amino acids, but proline was detected qualitatively.

(e) *Fractionation of the Crude Glycoprotein*

The crude glycoprotein from the mound (2·5% solution, 1 ml) was chromatographed with 0·005M ammonium acetate on a column of Sephadex G75. Fractions

TABLE 2

AMINO ACID ANALYSIS OF GLYCOPROTEINS

n.d., not detected

Amino acid residue	Relative molar yield			Amino Acid Residue	Relative molar yield		
	Fraction B_7	Mound	Soil		Fraction B_7	Mound	Soil
Aspartic acid	3·5	1·2	2·2	Phenylalanine	0·3	0·6	n.d.
Threonine	1·0	1·0	1·0	Lysine	n.d.	—	0·9
Serine	0·9	1·1	1·2	Histidine	n.d.	0·6	0·5
Glutamic acid	2·8	1·0	1·7	Unknown	n.d.	2·9	1·4
Glycine	5·0	1·6	4·1	Unidentified (*a*)	2·0	n.d.	n.d.
Alanine	6·8	1·9	2·3	(*b*)	1·8		
Valine	3·0	1·6	3·1	(*c*)	4·5		
Isoleucine	1·3	0·9	1·4	(*d*)	1·8		
Leucine	1·1	n.d.	2·2				

were analysed by the phenol–sulphuric acid method for carbohydrate and by optical density at 280 nm for protein. The results for the product from the mound are shown

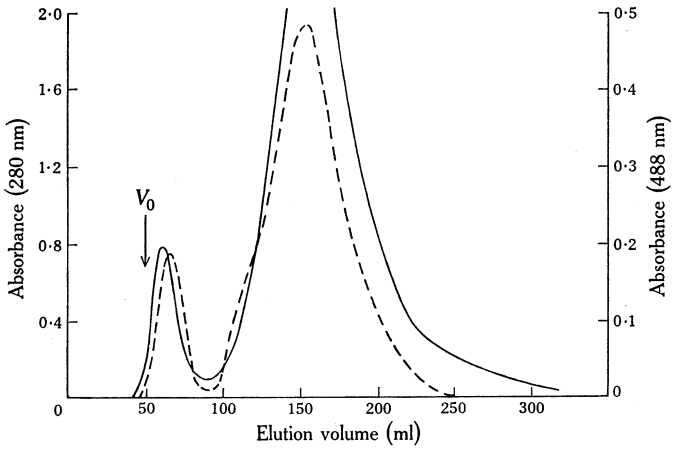

Fig. 2.—Gel chromatography of mound glycoprotein on Sephadex G75.
—— Absorbance at 280 nm. – – – Phenol–sulphuric acid reaction.

in Figure 2. Some coloured material remained sorbed on the top of the column, but all of the fractions were also slightly coloured. The two peaks were combined, concentrated, and finally freeze-dried. Hydrolysis and qualitative paper chromato-

graphic analysis gave results from each peak which were very similar to those described above for the crude glycoprotein.

A similar fractionation of fraction B_7 on Sephadex G75 yielded two peaks showing absorption at 280 nm at approximately the same elution volumes as those in Figure 2. Only the second peak, however, contained carbohydrate.

(*f*) *Evidence for Sialic Acid*

An aliquot portion from each glycoprotein peak (Fig. 2) was hydrolysed in 0·1N sulphuric acid at 80°C for 1 hr, neutralized with barium carbonate, filtered, concentrated, and passed through a column of Sephadex G25. The later fractions from the column were concentrated and heated in 0·1N sodium hydroxide for 30 min at 100°C. The spectrum of the resultant solution from each of the samples showed a marked inflexion at 256 nm superimposed on a general background absorption which vitiated the use of this method for quantitative analysis of sialic acid (Gottschalk 1966, p. 216).

IV. Discussion

Lee and Wood (1971), in a book published after our work had been completed, state that the soil used in the capping of *C. acinaciformis* mounds is generally derived from the subsoil (B horizon), but in some instances is derived from topsoil (A horizon) when this layer contains a high proportion of clay. Certainly the soil in our experiments contained a high proportion of clay, and also it is most probable that the subsoil would contain less carbohydrate material than the topsoil. As a control to provide evidence of addition of carbohydrate materials by the termites during mound construction therefore, our use of topsoil still appears valid.

The alkaline extracts of both mound and soil yielded large quantities of precipitated humic acids on neutralization and these were not investigated further. The fractional ethanol precipitation of the neutralized extracts yielded, first of all, dark brown precipitates (fractions B_1 and B_2, Table 1) which we classify as fulvic acids (Greenland 1965) and have not investigated further at this stage. The fraction B_1, however, might merit some further investigation since it is present in much larger amount in the mound than in the soil and, like all of the fractions obtained from the mound (Table 1), has a higher carbohydrate content.

Further precipitation with ethanol yielded fractions B_3–B_5 which were a pale brown in colour and we have carried out a preliminary investigation on B_3 since this fraction is present in larger amount and has a much higher carbohydrate content in the mound than in the soil. The separation of the coloured components of B_3 from polysaccharide proved rather difficult but was achieved on QAE-Sephadex. The polysaccharide component was eluted mainly as a single peak (Fig. 1) and comprised about a quarter of the total B_3 in the mound, whereas in soil the polysaccharide comprised only 3% of the total B_3. We conclude therefore that this polysaccharide (which we will term "hemicellulose") was incorporated into the mound by the termites. The tabulation in Section III(*b*) shows that the hemicellulose is predominantly composed of xylose and galactose and we have tentatively detected the presence of 4-*O*-methyl-D-glucuronic acid. It seems reasonable to assume therefore that this hemicellulose may originate in the major food materials of the termite, e.g.

spear grass and various woods, which have arabino-4-*O*-methylglucuronoxylans as major constituents (Timell 1965; Blake and Richards 1970). The relatively high proportion of galactan is rather surprising. It probably does not originate from plant pectic substances since no galacturonic acid was detected and the galactan is not necessarily part of the same polysaccharide molecule as the xylan.

After removal of fulvic acids and hemicelluloses from the neutralized alkaline extract, a polymeric substance remaining in the solution was found to yield several neutral sugars, glucosamine, and amino acids on hydrolysis. Since this substance was also known to be soluble in 70% ethanol the presence of a glycoprotein was suspected and the probability of alkaline degradation (Gottschalk 1966, p. 284) during extraction was therefore of concern. An extraction of the mound with 70% ethanol was therefore carried out, after a preliminary washing with EDTA at two different pH values. The yields of polymeric material from this extraction were 0·13% and 0·05% from mound and soil respectively. Since the nitrogen content of the mound extract was also significantly higher (1·87%, cf. 1·03%) we regard these results as very tentative evidence that this type of product has been added to the soil by the termites. Both products yielded a considerable range of neutral sugars on hydrolysis [tabulation, Section III(*d*)], the major differences between the products from mound and soil being in the reduced amount of xylose and galactose and increased arabinose in the mound product. The high xylan content of the product from soil may indicate its contamination by some hemicelluloses or their degradation products, presumably of relatively low molecular weight.

The products from both mound and soil were shown to contain 5·2% amino sugar (tentatively identified by paper chromatography as glucosamine) and their amino acid analysis is shown in Table 2. The mound product was separated into two components by chromatography on Sephadex G75 (Fig. 2). Each component contained both carbohydrate and a 280 nm chromophore (assumed to be associated with polypeptides). Each peak from Figure 2 was subjected to mild acid hydrolysis and the resultant low molecular weight products treated with alkali. The resulting solution showed an absorption peak at 256 nm which is assumed to be due to pyrrole-2-carboxylic acid derived from sialic acid (Gottschalk 1966, p. 216).

The products extracted by 70% ethanol are tentatively classified as glycoproteins on the basis of their amino acid, sialic acid, and carbohydrate content and solubility in aqueous ethanol. The results in Figure 2 suggest that they are not physical mixtures of polysaccharides and proteins.

Any hypothesis on the source and mechanism of incorporation of the hemicellulose and glycoprotein into the mound must remain very speculative. However, it has been observed (Gay, personal communication) that in building the wall of the mound a termite will select a soil particle and carry out actions which appear to result in the application of a mandibular excretion to the particle which is then placed into the wall on a small amount of previously deposited faecal matter. It seems probable that the hemicellulose originates in incompletely digested plant materials from the faecal matter of the insect and we have confirmed microscopically that the lower gut contents contain much incompletely digested plant fibre. It might be postulated that the glycoprotein arose either from an excretion in the mandibular region of the insect, or from microorganisms of the gut, via the faeces. In either case it appears valid to

suggest that the glycoprotein functions as an adhesive in cementing soil particles together in the wall of the mound. The excretion of major amounts of nitrogen by the termite in fact may be regarded as rather surprising, since this insect is known to be a protein economizer (e.g. via controlled cannibalism) (Krishna and Weesner 1969). However, the use of the glycoprotein "adhesive" is justified anthropomorphically since a stable mound construction is essential to the survival of the species. This type of concept of the glycoprotein as a positive evolutionary survival factor tends to favour its origin in a specialized excretion rather than its fortuitous presence in faeces and there would be merit in a search for a gland with such a function in the insect.

The probable "function" of the hemicellulose in the mound is less clear-cut. Polysaccharides, e.g. from microorganisms, have been shown to cause aggregation and stabilization in soils (Clapp, Davis, and Waugaman 1962), by mechanisms which may involve the interaction of both hydroxyl and carboxyl groups with the soil particles. In such studies, however, polysaccharides in solution have normally been utilized, whereas the hemicellulose will presumably be present mainly in more or less intact plant fibres within the faeces. It seems unlikely that any readily water-soluble hemicelluloses will be present in the termite faeces and therefore this type of stabilization of the mound is improbable and the faeces may simply provide the function of a space-filling mortar in the wall structure.

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VI. References

Barker, S. A., Finch, P., Hayes, M. H. B., Simmonds, R. G., and Stacey, M. (1965).—*Nature, Lond.* **205**, 68.

Blake, J. D., and Richards, G. N. (1970).—*Carbohydrate Res.* **14**, 375.

Blake, J. D., and Richards, G. N. (1970).—*Aust. J. Chem.* **23**, 2353.

Clapp, C. E., Davis, R. J., and Waugaman, S. H. (1962).—*Proc. Soil Soc. Amer.* **26**, 466, and references therein.

Dubois, M., Gilles, K. A., Hamilton, J. K., Rebers, P. A., and Smith, F. (1956).—*Analyt. Chem.* **28**, 350.

Gottschalk, A. (1966).—"Glycoproteins." (Elsevier: Amsterdam.)

Greenland, D. J. (1965).—*Soils Fertilisers* **28**(6), 521.

Hough, L., Jones, J. K. N., and Wadman, W. H. (1950).—*J. Chem. Soc.* p. 1702.

Krishna, K., and Weesner, F. M. (Eds.) (1969).—"Biology of Termites." Vol. 1. p. 410. (Academic Press: New York.)

Lee, K. E., and Wood, T. G. (1971).—"Termite and Soils." (Academic Press: New York.)

Smith, I. (1960).—"Chromatographic and Electrophoretic Techniques." Vol. 1. (Heinemann: London.)

Swincer, G. D., Oades, J. M., and Greenland, D. J. (1968).—*Aust. J. Soil Res.* **6**, 211.

Timell, T. E. (1965).—*Adv. Carbohyd. Chem.* **20**, 410.

Trevelyan, W. E., Proctor, D. P., and Harrison, J. S. (1950).—*Nature, Lond.* **166**, 444.