THE PROTEINACEOUS, GUM POLYSACCHARIDE FROM *Azadirachta indica* A. JUSS*.

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(Received April 5th, 1971; accepted for publication, April 22nd, 1971)

ABSTRACT

The gum exuded by *Azadirachta indica* trees contains 35% of proteinaceous material. The most abundant amino acid is aspartic acid; there are also considerable proportions of serine and threonine, and at least 2% of amino sugars. Attempts to deproteinise the polysaccharide material were unsuccessful. Fractionation of the gum exudate on agarose columns gave a protein-depleted fraction of high molecular weight and a protein-enriched fraction of lower molecular weight. Studies involving molecular-sieve chromatography showed a close correspondence in protein and carbohydrate content, and it appears that the proportion of proteinaceous component that is free or only loosely associated with carbohydrate is small. The carbohydrate component is much more complex than was indicated by earlier investigators. In addition to galactose and arabinose (major components), mannose, xylose, fucose, and rhamnose are also present. The uronic acid content (28%) is higher than previously believed, and a relatively high methoxyl content has been found. The major aldobiouronic acid, previously undetected, is 4-*O*-(4-*O*-methyl-α-D-glucopyranosyluronic acid)-D-galactose, with 4-*O*-(α-D-glucopyranosyluronic acid)-D-galactose also present. The exudate from *Azadirachta indica* is therefore a complex material having several features that are unusual in gum chemistry.

INTRODUCTION

The tree *Azadirachta indica* A. Juss. (Order, *Geraniales*; family, *Meliaceae*), which occurs throughout India, Ceylon, and Burma, has a beneficial effect upon soils[2] and has been introduced extensively in other tropical regions. *Azadirachta indica* (syn. *Antelaea indica*, *Melia indica*) has the native, vernacular names Nim, Neem, and Margosa[3]; it has also been identified in error with the Persian lilac, *Melia azedarach*. Some chemical components of the leaves[4] and wood[5] of *A. indica* have been studied recently.

Previous workers reported that the gum exudate from *A. indica* was a polyelectrolyte[6] containing *ca.* 20% of proteinaceous material[7], with an equivalent

*This is Part 36 of the series "Studies of Uronic Acid Materials". Part 35: Ref. 1.

weight[8] of 1080 and $[\alpha]_D$ −71° (Ref. 9). Electrochemical[10] and thermogravimetric[11] studies were also published. Hydrolysis studies[8] showed that the gum contained galactose and arabinose in the ratio 3:2, together with fucose, a trace of xylose, and the aldobiouronic acid 4-*O*-(α-D-glucopyranosyluronic acid)-D-galactose.

The studies reported here were undertaken in view of our recent interest[12] in the composition and properties of the proteinaceous matter found in association with gum polysaccharides, and because *Azadirachta indica* gum appeared to offer a useful substrate for further studies of the nature of the association between carbohydrate and protein in plant gums.

EXPERIMENTAL AND RESULTS

Origin and purification of samples of Azadirachta indica *gum.* — Gum sample *A* was obtained in October 1967 from Mr. L. Wijesinghe, Research Laboratory of the Conservator of Forests, Kew Road, Colombo, Ceylon. Gum sample *B* was obtained in May 1968 from Dr. D. B. Deb, Regional Botanist, Botanical Survey of India, Coimbatore, India.

Samples *A* and *B* dissolved readily in cold water. After filtration, the solutions were dialysed for 2 days, and the purified gum exudates were recovered as the freeze-dried products. The recoveries, on a dry-weight basis, were 80% and 76% for samples *A* and *B*, respectively.

Results of analyses. — Samples *A* and *B* were studied by the standard, analytical methods described in earlier parts of this series; the results are shown in Table I. Portions (20 mg) of samples *A* and *B* were hydrolysed (0.5M sulphuric acid, 7 h, 98°), and paper chromatography of both hydrolysates in solvents (*a*) benzene–butyl alcohol–pyridine–water (1:5:3:3, upper layer); (*b*) ethyl acetate–pyridine–water (10:4:3); and (*c*) ethyl acetate–acetic acid–formic acid–water (18:3:1:4) indicated the presence, in each, of major amounts of galactose and arabinose, lesser amounts of mannose, small amounts of xylose and fucose, and trace amounts of rhamnose. Chromatography in solvent (*c*) also indicated the presence, in each sample, of two aldobiouronic acids having the mobilities of 4-*O*-(α-D-glucopyranosyluronic acid)-D-galactose (R_{Gal} 0.28, minor component) and 4-*O*-(4-*O*-methyl-α-D-glucopyranosyluronic acid)-D-galactose (R_{Gal} 0.66, major component). Further portions (20 mg) of each polysaccharide were hydrolysed more extensively (M sulphuric acid, 7 h, 98°). Paper chromatography of these hydrolysates in solvents (*a*), (*b*), and (*d*) [butyl alcohol–ethanol–0.1M hydrochloric acid (1:10:5)] indicated the presence, in each, of galactose, arabinose, mannose, xylose, fucose, and rhamnose, together with 4-*O*-methylglucuronic acid and smaller amounts of glucuronic acid and glucurono-6,3-lactone.

Attempted removal of protein from gum sample A. — The following methods, each of which has been effective in removing proteinaceous matter from particular polysaccharide systems, were used in attempts to deproteinise portions (1 g) of gum sample *A* dissolved in water (20 ml). To provide a reference standard, a portion was treated with acetic acid to give pH 5 and acetone (10 vol.) was added. The precipitate

TABLE I

DATA FOR PURIFIED *Azadirachta indica* SAMPLES *A* AND *B* AND FRACTIONS I, II, AND III

	Gum sample A	*Gum sample* B	*Fractions (Sample* A)		
			I	*II*	*III*
Moisture, %	11.9	13.0	13.9	12.8	12.4
Nitrogen, %[a]	6.0	5.6	3.7	6.7	5.2
Protein, % (N × 6.25)	37.5	35.0	23.2	41.9	32.5
$[\alpha]_D$, degrees[a]	−62	−58	−54	−45	−59
Methoxyl, %[a]	2.05	2.36	n.d.	n.d.	n.d.
Methoxyl, %[b]	3.3	3.6	n.d.	n.d.	n.d.
Equivalent weight[a]	990	957	n.d.	n.d.	835
Equivalent weight[b]	620	620	n.d.	n.d.	560
Uronic anhydride[c]	28.3	28.3	n.d.	n.d.	31.4
Limiting flow-time number, mlg^{-1}[a,d]	10.6	13.3	9.7	7.2	7.9
Refractive index, dn/dc[a,d]	0.166	0.163	n.d.	n.d.	n.d.
Molecular weight, $\overline{M}_w$[a,d]	5.2×10^5	7.1×10^5	1.3×10^6	n.d.	5.0×10^5

[a]Corrected for moisture content. [b]Corrected for moisture and protein content. [c]Calculated from equivalent weight on the basis that all acidity arises from uronic acid groups. [d]In M sodium chloride at 27°.

was removed, washed with acetone and then ether, and dried (Found: N, 5.6%). The same isolation procedure was used as the final stage in each of the following tests. (*a*) Ethanol was added until a precipitate appeared. After centrifugation, the supernatant was dialysed for 2 days, and the residual polymeric material was then precipitated as described above (Found: N, 7.5%). (*b*) Treatment with the Sevag reagents, chloroform (4 ml)–butyl alcohol (0.8 ml) (N, 5.6%). (*c*) Treatment with trichloroacetic acid (1.6 g) (N, 5.8%). (*d*) Treatment with trifluoroacetic acid (2M, 20 ml) (N, 5.1%). (*e*) Treatment with 1,1,2-trichloro-1,2,2-trifluoroethane (15 ml) (N, 5.7%). (*f*) Treatment with aqueous sodium tungstate (10%, 6.7 ml)+sulphuric acid (0.3M, 6.7 ml) (N, 4.4%). (*g*) Treatment overnight with sodium borohydride (0.2 g) (N, 5.5%). (*h*) Treatment with pepsin (50 mg, twice recrystallised) at pH 1.6 at 37° for 48 h, followed by treatment with trypsin (50 mg, twice recrystallised) at pH 7.5 at 37° for 48 h (N, 4.4%). (*i*) Treatment with pronase (6.6 mg) at pH 8.0 (Tris buffer) at 60° for 24 h (N, 2.5%).

Molecular-sieve chromatography. — In studies with *Acacia campylacantha* gum, it was found[13] that the degradation of the associated proteinaceous material could be achieved with mixtures of sodium chloride, urea, and dithiothreitol. Molecular-sieve chromatography (m.s.c.) offers a convenient method of studying the progress of such degradations; a technique employing the use of reactive dyestuffs to facilitate the monitoring of the column effluents has been described[14].

Gum sample *A* was dyed[14] with Procion Red M-2B, and both this product and the untreated gum were examined by m.s.c. on columns (40 × 1.5 cm) of Bio-Gel A-5M. Two columns were used. With column A, the eluent was M sodium chloride at a flow-rate of 0.3 ml/min; for column B, the eluent (0.1 ml/min) was M with respect

to sodium chloride, 7M with respect to urea, 0.005% (w/v) with respect to dithiothreitol, and 50mM with respect to Tris buffer (pH 9.0). The following experiments were performed. (*a*) Dyed gum (3 mg) was applied to column A, Fig. 1(*a*). (*b*) Undyed gum (6 mg) was applied to column A, Fig. 1(*b*). (*c*) Dyed gum (3 mg) was applied to column B after immersion in the composite dithiothreitol solvent for 0 h and 24 h, Figs. 1(*c*) and 1(*c'*), respectively. (*d*) Undyed gum (6 mg) was applied to column B after storage in the composite solvent for 0, 24, and 114 h, Figs 1(*d*), 1(*d'*) and 1(*d''*), respectively.

The elution patterns for the dyed gum were obtained by the automated colorimeter technique[14]; patterns for the undyed gum were obtained by automatic u.v.-monitoring of the proteinaceous material at 254 nm. The elution patterns for (*d*) each gave a strong absorption, shown to be caused by dithiothreitol, at V_e 80 ml.

To obtain larger peaks in the elution patterns, five portions (10 mg) of *A. indica* gum were dissolved in the composite solvent and applied to column B after various time intervals. The patterns given by the samples, at 0 and 24 h after dissolution, were identical to Figs. 1(*d*) and 1(*d'*), respectively; at 48, 72, and 100 h after dissolution, the patterns were identical to Fig. 1(*d''*).

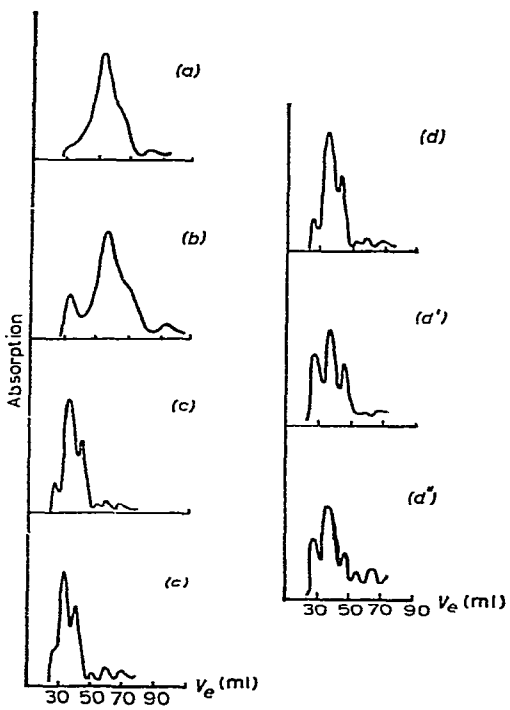

Fig. 1. Elution diagrams (absorption *vs.* elution volume (V_e)) given by *A. indica* gum on Bio-Gel A-5m. (*a*) Dyed gum on column A, colorimetric monitor (c.m.). (*b*) Undyed gum on column A, ultraviolet monitor at 254 nm (u.v.m.). (*c*) Dyed gum on column B, direct (c.m.). (*c'*) Dyed gum on column B after 24 h in eluent (c.m.). (*d*) Undyed gum on column B, direct (u.v.m.). (d')Undyed gum on column B after 24 h in eluent (u.v.m.). (*d''*) Undyed gum on column B after 114 h in eluent (u.v.m.).

To test the effect of the concentration of dithiothreitol, three variations of the composite solvent for column B were prepared in which the dithiothreitol concentration was 0.2, 0.4, and 0.8% (w/v). Two portions of *A. indica* gum (10 mg) were dissolved and kept in the 0.2% dithiothreitol solvent-mixture for 24 and 48 h, respectively, before being applied to column B. Two 10-mg portions were treated similarly with each of the other two dithiothreitol-containing solvents. The elution patterns obtained by u.v.-monitoring at 254 nm are shown in Figs. 2(*a*) and (*b*) (0.2% of dithiothreitol for 24 and 48 h, respectively); Figs. 2(*c*) and (*d*) (0.4% of dithiothreitol for 24 and 48 h, respectively); and Figs 2(*e*) and (*f*) (0.8% of dithiothreitol for 24 and 48 h, respectively). A gum sample dissolved in the 0.8% dithiothreitol solvent for 110 h showed a pattern identical to Fig. 2(*f*). Fig. 2(*g*) shows the pattern given by monitoring with phenol–sulphuric acid[15] for the gum dissolved in the 0.4% dithiothreitol solvent for 48 h; the elution patterns for portions (5 mg) of dyed gum dissolved in this solvent for 24 and 48 h were identical to Figs 1(*c*) and 1(*c'*), respectively. The dyed gum decomposed, giving a brown solution, in the 0.8% dithiothreitol solvent.

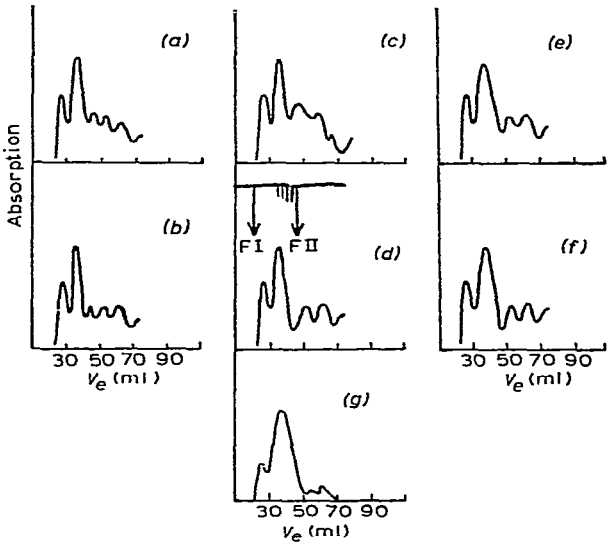

Fig. 2. The effect of varying the dithiothreitol concentration and the reaction time on the elution diagrams given by *A. indica* gum on Bio-Gel A-5m (u.v. monitor at 254 nm): (*a*) 0.2% dithiothreitol for 24 h, (*b*) 0.2% dithiothreitol for 48 h, (*c*) 0.4% dithiothreitol for 24 h, (*d*) 0.4% dithiothreitol for 48 h, (*e*) 0.8% dithiothreitol for 24 h, (*f*) 0.8% dithiothreitol for 48 h, (*g*) 0.8% dithiothreitol for 48 h, phenol–sulphuric acid detection.

Fractionation of A. indica *gum.* — Fig. 2(*d*) indicated that dissolution of the gum for 48 h in the composite solvent containing 0.4% of dithiothreitol was the least-drastic treatment that removed the component eluting at V_e 45 ml and resulted in considerable simplification of the elution pattern of the gum as monitored at 254 nm. A column (31 × 3.7 cm) of Bio-Gel A-5m was prepared, using the M sodium

chloride–7M urea–0.005%(w/v) of dithiothreitol–50mM Tris buffer(pH 9.0) composite solvent as eluent at a flow-rate of 0.5 ml/min. *A. indica* gum (100 mg) was dissolved in the solvent mixture (8 ml) corresponding to 2M sodium chloride–7M urea–0.4%(w/v) of dithiothreitol–50mM Tris buffer(pH 9.0), and was applied to the column after 48 h. The elution pattern was monitored at 254 nm and was identical to that shown in Fig. 2(*d*). The material eluted over the ranges V_e 20–45 ml and 45–70 ml was collected as fractions I and II, respectively. A total of 20 × 100 mg portions of *A. indica* gum was fractionated in this way; fractions I and II from each run were combined. After exhaustive dialysis and centrifugation to remove a slight precipitate arising from "column-bleed", the total yields of freeze-dried products from 2 g of gum were: fraction I, 1.18 g; fraction II, 0.52 g.

To allow further studies of the effect of dithiothreitol on *A. indica* gum, a further portion (2 g) of the gum was dissolved and allowed to stand for 48 h in the solvent (160 ml) containing 0.4% of dithiothreitol. The solution was dialysed exhaustively and filtered, and the product (fraction III) was isolated by freeze-drying: yield, 1.62 g.

Analysis of fractions I, II, and III. — Analytical data for fractions I, II, and III are shown in Table I. Hydrolyses were carried out in the same manner as already described for gum samples *A* and *B*; each of the fractions contained the same neutral and acidic sugars, in essentially the same proportions, as the whole gum.

Portions (50 mg) of gum samples *A* and *B* and fractions I, II, and III were dyed[14] with Procion Red M-2B. The dyed polysaccharides (3–4 mg) were dissolved in 2M sodium chloride (0.5 ml) and examined by m.s.c. on a column (40 × 1.5 cm) of

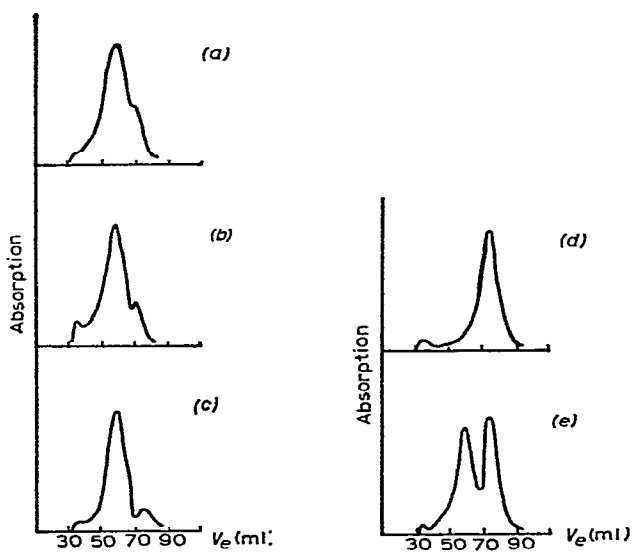

Fig. 3. Elution diagrams given by *A. indica* samples *A* and *B* and fractions I–III on Bio-Gel A-5m (samples dyed; M sodium chloride eluent; colorimetric monitor). (*a*) Gum sample *A*. (*b*) Gum sample *B*. (*c*) Fraction I. (*d*) Fraction II. (*e*) Fraction III.

Bio-Gel A-5m with M sodium chloride as eluent at a flow-rate of 0.5 ml/min. The elution patterns, obtained by the automated colorimetric method[14], are shown in Fig. 3.

The amino acid composition of the proteinaceous components. — Portions (calculated to contain *ca.* 2 mg of protein) of *A. indica* gum samples *A* and *B* and fractions I, II, and III were hydrolysed (6M hydrochloric acid, sealed tube), and analyses of their amino acid compositions were carried out by the methods already described[12]. The results are given in Table II.

TABLE II

THE AMINO ACID COMPOSITION[a] OF *Azadirachta indica* GUM

Amino acid	*Gum sample*	*Gum sample*	*Fractions*		
	A	B	*I*	*II*	*III*
Lysine	44	59	35	30	43
Histidine	17	18	11	26	16
Arginine	27	28	35	25	28
Aspartic acid	138	145	129	169	146
Threonine	66	64	59	62	63
Serine	75	79	66	88	76
Glutamic acid	78	81	84	63	71
Proline	73	57	83	91	68
Glycine	73	71	79	70	70
Cystine	18	14	6	23	15
Alanine	53	55	62	40	54
Valine	75	76	71	80	76
Methionine	3	0	0	6	1
Isoleucine	51	51	64	41	59
Leucine	84	80	87	59	83
Tyrosine	30	31	22	28	24
Phenylalanine	57	57	66	36	57
2-Amino-2-deoxy-D-glucose	38	34	41	63	50
Nitrogen recovery[b]	83	74	75	77	72

[a]Expressed as μmoles of amino acid per 1000 μmoles total. [b]Expressed as % recovery of Kjeldahl nitrogen.

The hexosamine content of A. indica *gum.* — Since the amino acid analyses indicated significant amounts of a component that corresponded chromatographically to 2-amino-2-deoxy-D-glucose, independent analyses were made by two procedures based on the *p*-dimethylaminobenzaldehyde colorimetric method of Elson and Morgan[16].

The first procedure was that described by King[17], in which the method of Belcher *et al.*[18] was modified at the neutralisation stage. Portions (30 and 50 mg) of gum sample *A* were hydrolysed with 2M hydrochloric acid for 6 h on a boiling water-bath, conditions which should result in complete hydrolysis of hexosamine without its degradation[7,19]. In duplicate experiments, 4M acid gave a concordant result, but degradation occurred when 6M acid was used. The calibration curve was prepared

with 2-amino-2-deoxy-D-glucose. (Found: hexosamine in *A. indica* gum sample *A*, 2.1%).

The second procedure used was that of Lakshmi and Pattabiraman[7], based on the method of Levvy and McAllan[20] (Found: hexosamine in gum samples *A* and *B*, 2.0 and 2.2%, respectively).

DISCUSSION

The results of these experiments indicate that the gum exuded by *Azadirachta indica* trees is more complex than was indicated by previous investigators. The two specimens examined in this work are less laevorotatory than was reported previously[9], and, although slightly lower values for the hexosamine content were found than in earlier work[7], the amount of proteinaceous matter found is much greater.

Chromatographic evidence indicated that mannose and trace amounts of rhamnose were present, in addition to the neutral sugars identified by Mukherjee and Srivastava[8]. The acidic sugars are also more complex than was reported previously, and the presence of a high methoxyl content is a hitherto unsuspected feature. The major aldobiouronic acid is 4-*O*-(4-*O*-methyl-α-D-glucopyranosyluronic acid)-D-galactose, and only relatively minor amounts of the aldobiouronic acid previously identified[8], 4-*O*-(α-D-glucopyranosyluronic acid)-D-galactose, are present; calculation shows that these two acids are present in the ratio 3:1 if all the methoxyl content of the gum polysaccharide occurs in the 4-*O*-methyl-D-glucuronic acid residues.

The uronic acid content of the exudate is also higher than was reported previously[8]. Furthermore, a value (1080) reported[8] for the equivalent weight of the acidic polysaccharide was misleading, since it was not subjected to correction for the unusually high proteinaceous content of *A. indica* gum. Our values for the equivalent weight of the purified gum exudate, corrected for protein, indicate a value of 620 for the equivalent weight of the carbohydrate component; this corresponds to a "uronic anhydride" content of 28.4%.

One of the purposes of this study was to examine the analytical differences in the composition of the gum exudate obtained from different geographical locations. The analytical data in Tables I and II show that the composition and properties of gum samples *A* and *B* are strikingly similar. It therefore appears that, in contrast to other genera *e.g. Prunus*[21] and *Combretum*[22], the gums from *Azadirachta* resemble *Araucaria*[23] and *Lannea*[1] in the constancy of composition of their gum polysaccharides. *Azadirachta indica* has the highest protein content of any gum exudate studied to date, and it is interesting that the constancy of composition extends so closely to the amino acid composition of the proteinaceous component.

The physical and chemical methods commonly used to de-proteinise plant polysaccharides were markedly unsuccessful with *A. indica* gum. Precipitation with ethanol gave a product enriched in protein. However, the removal of considerable amounts of protein was achieved by the use of enzymes, particularly pronase.

Molecular-sieve chromatography gave some complex elution patterns, but the

results of monitoring the eluent by different techniques indicating (*a*) the polysaccharide and (*b*) the proteinaceous components reinforced the evidence, from attempted de-proteinisation, that these components are closely, rather than loosely, associated. The controlled fractionations and the degradation of one component caused by the use of the composite sodium chloride–urea–dithiothreitol chromatographic solvent, which should weaken all but covalent bonds between carbohydrate and protein, also supported this evidence: Fraction I corresponded to the material of high molecular weight in the unfractionated gum, and fraction II to the material of lower molecular weight. Although one minor, protein-rich peak was eliminated from the molecular-sieve chromatograms, the similarity of the analytical parameters of gum *A* and fraction III (*i.e.* gum *A* after treatment with the composite hydrogen bond-breaking, disulphide bond-reducing solvent) indicated that the latter did not cause extensive general degradation; in contrast to parallel studies[13] with *Acacia* gums, complete re-aggregation did not occur on removal of the urea and dithiothreitol from the solutions. The amino acid compositions of fractions I–III are little different from that of gum sample *A* from which they were derived. The presence of significant amounts of aspartic acid, serine, threonine, and amino sugar in the hydrolysates examined (Table II) gives additional circumstantial evidence for the presence of covalent linkages between the proteinaceous and polysaccharide components of this gum exudate. Linkages between amino sugars and particular amino acids have been established as the basis of carbohydrate–protein linkages in other polysaccharides[24].

The proteinaceous gum polysaccharide from *Azadirachta indica* therefore has several features that are uncommon in plant gums. Structural studies will be undertaken, together with attempts to isolate the amino sugar–amino acid unit involved in the carbohydrate–protein linkage.

ACKNOWLEDGMENTS

We thank the Science Research Council for a maintenance award (to A. H.), and Rowntree–Mackintosh Ltd. (York) and Laing–National Ltd. (Manchester) for financial support.

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