

Preliminary Studies on the Mucus Secretion of the Wood Wasp, *Sirex noctilio* F. I. Physicochemical and Biochemical Properties

L. K. Wong and R. K. Crowden

Department of Botany, University of Tasmania,
G.P.O. Box 252 C, Hobart, Tas. 7001.

Abstract

The major component of *S. noctilio* mucus appears to be a protein-polysaccharide complex with probable molecular weight in the range 60 000–100 000. In aqueous solution the macromolecule undergoes slow spontaneous disaggregation to yield dialysable subunits of molecular weight 2000–6000 which retain all the physiological activity of the whole mucus. More rapid disaggregation is brought about by treatment of solutions with moderate concentrations of NaCl, KCl or CaCl₂, or by heating. The native mucus contains amylase, esterase, phenoloxidase and proteolytic enzyme activities, and it is suggested that these enzymes may be responsible, at least in part, for the 'natural' disaggregation process.

Introduction

The reproductive system of the female wood wasp *Sirex noctilio* F. (Hymenoptera: Siricidae) consists of paired ovaries with three pairs of accessory glands, namely the mucus glands and their reservoirs, the oil sacs, and the lateral pouches, and an ovipositor. By virtue of their similar anatomical arrangements, the accessory glands of *S. noctilio* have been likened to the poison glands and sacs, the alkaline glands, and the lubricating glands, respectively, of the honey bee (Boros 1968).

Secretions from the mucus glands of *S. noctilio* have been shown as the causative agent in the primary syndrome of effects which follow the *Sirex* attack on pine trees, *Pinus radiata* D. Don. These effects are described by Coutts (1969*a*, 1969*b*) and Fong and Crowden (1973). All of the 'natural' tree responses can be induced by artificial injection of the tree with a weak aqueous solution of mucus. Coutts (1969*b*) also reported on the heat stability of mucus. Our own data (Fong and Crowden 1973) showed that an autoclaved mucus solution had even greater potency than raw mucus in the earlier induction of senescence responses, for example increase in respiration rate, and loss of chlorophyll.

These present experiments have attempted to define characteristic physicochemical and biochemical properties of the mucus, as a preliminary to the possible identification of the active principle.

Methods

(a) Preparation of Mucus Solutions

Mucus glands were dissected whole from mature female wasps which were captured as they emerged from pine logs held in store at the Forest Research Institute's Field Station (Forest Research Institute, Department of National Development, Hobart, Tas.). The glands were stored deep-frozen

until required. The active agent is stable under these conditions and its activity may be demonstrated after 3 years. The mucus was brought into solution in deionized water by continuous stirring overnight at 2°C to yield a viscous preparation from which tissue debris was removed by filtration through glass wool.

(b) *Chemical Tests*

A range of simple chemical tests for the detection of specific functional groupings in the mucus were carried out as described in the standard reference books (Hawk *et al.* 1947; Dawson *et al.* 1959). In addition, specific tests were made for the following substances:

- (1) Hexosamines were assayed by the methods of Boas (1953) following hydrolysis of the mucus with 8M HCl for 2 h at 100°C.
- (2) Hexuronic acids were assayed by the modified carbazole reaction of Bitter and Muir (1962).
- (3) Sialic acids were assayed by the direct Ehrlich method as adopted by Werner and Odin (1952), and the thiobarbituric acid methods of Warren (1959). To release possible bound sialic acids, the mucus was hydrolysed in 5 mM H₂SO₄ for 1 h at 80°C.
- (4) Sulphate was assayed by the method of Iwasaka *et al.* (1957), following hydrolysis of the mucus in 6M HCl for 18 h at 100°C.

(c) *Viscometry*

Viscosity of mucus solutions was measured in a Cannon-Fenske type glass viscometer (4-ml bulb capacity, 0.6-mm bore capillary), using 9 ml of 0.5% mucus solution at 25°C.

(d) *Dialysis*

Mucus solutions held in cellulose dialysis tubing (Visking Co., U.S.A.) were dialysed against four changes of deionized water for 25 h at 2°C.

(e) *Gel Filtration*

A series of Biogels (Bio-Rad Laboratories, California) having different nominal molecular weight exclusion limits (in parentheses) were used: P2 (1600), P6 (4600), P10 (10 000), P30 (30 000), P60 (60 000) and P100 (100 000). The gels were swollen overnight in water and packed into a precision-bore borosilicate column of 48-ml capacity (Pharmacia, Uppsala, Sweden). The void volume of the gel column was determined using dextran blue (molecular weight 1 000 000). A 4% mucus solution (2 or 4 ml) was admitted from the base of the column and eluted by an upward flow procedure using deionized water. The fractionation was conducted at 2°C. Fractions (1.5 ml) were collected and assayed for carbohydrate, protein, amylase activity and phenoloxidase activity, by the methods described below. Certain fractions were also examined by electrophoresis on polyacrylamide gel.

Electrophoresis using 8% cyanogum and 0.6% starch, and detection of protein, amylase and esterase were carried out according to Mills and Crowden (1968). In addition certain electrophoretograms were stained for the following substances:

- (1) phenol oxidase, using *o*-dianisidine (1.25 mM) in 0.1M acetate buffer, pH 4.5 as substrate;
- (2) glycoprotein, using the periodic acid-Schiff reaction (P.A.S.) as described by Keyser (1964);
- (3) acid mucopolysaccharides, using toluidine blue (Flatters and Garnet Ltd) after Chayen *et al.* (1969), alcian blue (Gurr) after Steedman (1950) and acridine orange (Gurr) after Saunders (1964); the absorption spectrum of the mucus complex with toluidine blue (over the pH range 1-7) was also determined.

(f) *Assay Procedures*

Protein. The method of Lowry *et al.* (1951) was used. Protein concentrations are given as milligrams per millilitre of bovine serum albumin determined by the same procedure.

Amylase. The method of Bernfield (1955) was used.

Esterase. A spectrophotometric method adapted from the procedure used for staining electrophoretograms was used as follows: 0.2 ml of mucus solution was incubated with 2 ml of α -naphthyl acetate (0.002 g in 10 ml of 0.1M phosphate buffer, pH 6.4) for 30 min at 25°C. Then 0.5 ml of a freshly prepared solution of fast blue BB (Gurr) (0.002 g fast blue in 5 ml of deionized water) was

added, the mixture was incubated for 1 h at 25°C, and then immersed for 5 min in a boiling water bath. After cooling, the optical density at 440 nm was determined.

Phenol oxidase. The method used was essentially that of Maehly and Chance (1955) but *o*-dianisidine (1.25×10^{-3} M in 0.1 M acetate buffer, pH 4.5) was used as substrate and H₂O₂ omitted.

Proteolytic enzyme. The method used was that of Bosmann and Pike (1970) in which 'Azocoll' (Calbiochem), a dye-incorporated general proteolytic substrate, was used.

Bioassay. Excised twigs or individual fascicles from *P. radiata* trees previously typed for susceptibility to *Sirex* mucus were used. The plant material was allowed to take up the test solution and degree of senescence was determined visually (Fong and Crowden 1973). A scale of senescence from slight (+) to severe, including foliage death (5+), was adopted.

Results

Raw mucus is a colourless gelatinous material of which 68% is dry weight. A single large female wasp may yield as much as 25 mg of mucus. The mucus dissolved in deionized water to give a viscous solution of pH 6.5 (2% w/v solution, at 20°C),

Table 1. Reactions of *S. noctilio* mucus with some diagnostic chemical reagents

Reagent	Groups tested for	Fresh mucus	Autoclaved or aged mucus
I ₂ /KI	Starch/glycogen	- ve	- ve
Anthrone	Carbohydrate	+ ve	+ ve
Benedicts	Reducing sugar	Weak, + ve	+ ve
Excess ethanol	Precipitation of protein and polysaccharide	+ ve	+ ve
Biuret	Protein	+ ve	+ ve
Ninhydrin	Amino acids	- ve	+ ve
Xanthoproteic reaction	Aromatic nucleus in protein	- ve	+ ve
Ehrlich	Indoles	- ve	+ ve
Folin-Ciocalteu (alkaline)	Phenols and amines	+ ve	+ ve
CuSO ₄ .5H ₂ O	Amines	- ve	- ve
Millon	Aromatic amines	- ve	+ ve
FeCl ₃	Phenols (-SH group)	- ve	- ve
Basic lead acetate	-SH groups	- ve	- ve
Hopkins-Cole	Tryptophan	Weak, + ve	+ ve
Alkaline acetylacetone and <i>p</i> -dimethylaminobenzaldehyde	Hexosamines	+ ve	+ ve
H ₂ SO ₄ /carbazole	Hexuronic acids	+ ve	+ ve
Ehrlich	Sialic acids	- ve	- ve
Thiobarbituric acid	Sialic acids	- ve	- ve
BaCrO ₄ /NH ₃ /Ca ²⁺	Sulphate	- ve	- ve

but did not dissolve in acetate buffer at pH 5.0. It was not coagulated by heat, but was precipitated by cold acetone or ethanol. Spectroscopic examination of the fresh aqueous solution showed a single weak absorbance peak at 290 nm. This absorption peak persisted without increase after autoclaving the mucus. There was no distinguishing chromophoric grouping. Material extracted with acetone or ethanol did not show any significant absorption in either u.v. or visible light.

Some typical diagnostic reactions are given in Table 1. These tests show that raw mucus contains carbohydrate and protein components probably combined in a complex. After electrophoresis, autoclaved mucus gave a magenta-coloured product

with the P.A.S. reagent in regions coinciding with protein and this is consistent with the view that the material is glycoprotein in nature. Further, the positive-staining reactions of electrophoretograms (at pH 4) with toluidine blue, alcian blue and acridine orange and the presence of hexosamines and hexuronic acids suggest that mucus is probably an acid mucopolysaccharide.

The absorption spectrum of an aqueous solution of mucus in complex with toluidine blue shows peaks at 585 nm (max.) and 620 nm. This differs significantly from the spectrum of chondroitin sulphate C (from shark cartilage), which has a peak at 550 nm. The failure to detect sulphate in *Sirex* mucus suggests that it is not a sulphated acid mucopolysaccharide (e.g. chondroitin sulphate or heparin type). Starch, glycogen or sialic acids are not represented in the carbohydrate fraction.

Mucus solutions gave positive assays for amylase, esterase, phenoloxidase and proteolytic enzymes. Peroxidase was absent. Assay of the phenoloxidase with various substrates indicated that it is of the 'laccase' rather than the 'tyrosinase' type (Lindenburg and Holm 1952). Thus it gave a strong reaction with *o*-dianisidine, catechol, and *p*-phenylenediamine, a weak reaction with hydroquinone but no reaction with *p*-cresol or tyrosine as substrate. It was inhibited by 5×10^{-5} M azide (100% inhibition), and (in decreasing order of effectiveness) by H₂O₂, diethyldithiocarbamate, and semicarbazide HCl.

Interpretative drawings of electrophoretograms of mucus, stained for protein, glycoprotein, amylase, esterase and phenol oxidase are given in Fig. 1. There are eight protein bands, four of which have high electrophoretic mobility. Band 1 is a weakly staining zone near the origin. Attempted fractionation of the mucus showed that band 2 is the major component of mucus, though it gives relatively weak staining on the electrophoretograms, possibly due to masking of dye-binding groups on the molecule. Band 2 was eliminated by autoclaving. The P.A.S. stain located glycoprotein bands in positions corresponding to the 'general' protein, though with a change in relative staining intensities. There appear to be two amylase bands, and one each of esterase and phenoloxidase. These enzymes are all associated with the major protein component band 2. Treatment of the mucus with salt solutions had little effect on the electrophoretic patterns.

Dialysis of an aqueous mucus solution yielded a diffusate with weak physiological activity in the senescence test. However, most activity remained with the non-diffusible fraction. The carbohydrate and protein contents, and amylase activity were each reduced slightly during the dialysis, but this was thought to be a normal 'aging' phenomenon of the mucus. The effects of dialysis are shown in the following tabulation.

	Whole mucus (2% solution)	Mucus (2% solution) dialysed for 52 h	
		Residue	Diffusate
Protein (mg/ml)	2.42	2.22	0.01
Carbohydrate (mg/ml)	1.31	1.23	0.01
Amylase activity	2.36	2.05	0.01
Bioassay: score at day 14	5+	5+	+

Effects of 'Aging' on a Mucus Solution

Weak aqueous solutions, sterilized by radiation treatment (1 h exposure to short wave u.v. light of 256 nm), undergo significant changes in physical and chemical properties when left to stand for varying periods of time. Unsterilized solutions become obviously contaminated within 2-3 days at 25°C.

Changes in viscosity. Within 50 h, the relative viscosity of an aqueous solution was reduced to approximately that of deionized water. The viscosity was also lowered by autoclaving, and by moderate concentrations of NaCl, KCl or CaCl₂. Concentrations of CaCl₂ greater than 0.06% caused precipitation of a gelatinous material. Physiological activity of aged mucus of low viscosity was retained in full, as was that of autoclaved mucus and of salt-treated solutions (after dialysis to remove the inorganic ions).

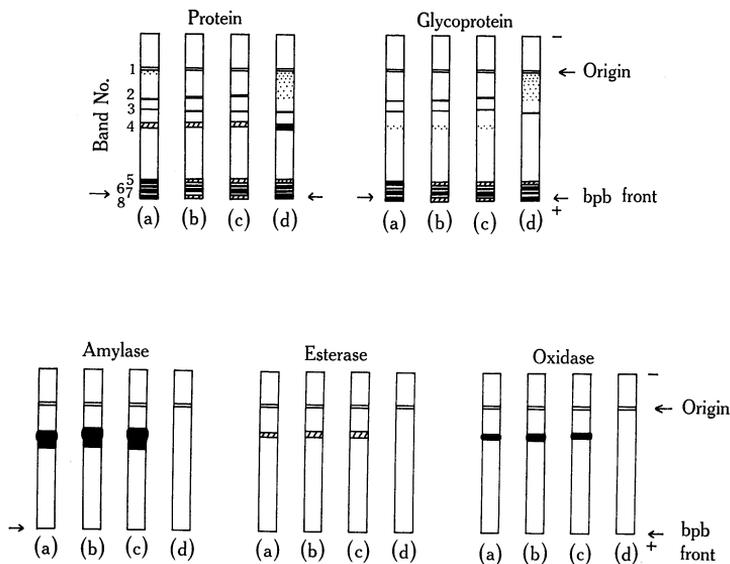


Fig. 1. Interpretative drawings of polyacrylamide gel electrophoretograms of *S. noctilio* mucus. (a) 1% fresh aqueous solution. (b) 1% mucus plus 0.5% NaCl solution. (c) 1% mucus plus 0.5% CaCl₂ solution. (d) 1% autoclaved mucus. bpb front, bromophenol blue front.

Changes in enzyme levels. The activities of proteolytic enzyme and amylase fell sharply during 50 h of aging. Phenoloxidase activity remained essentially constant over a 250-h interval, whilst esterase activity appeared to rise slightly. The increased esterase activity may be due to a progressive unmasking of active sites, as no new bands appeared on electrophoretograms. These changes are shown in Fig. 2. Whereas the total protein content of aging mucus remained constant (as measured by the Lowry method), electrophoretograms showed a marked change in the banding pattern (Fig. 3). In particular, bands 5–7 of high electrophoretic mobility became pronounced after 22 h.

Gel Filtration Studies

Specimen profiles for raw mucus and aged mucus from Biogel P60 are shown in Fig. 4. In each case the eluant fractions have been monitored for protein and carbohydrate, and for phenoloxidase and amylase activities. In general protein and carbohydrate eluted in parallel. Raw mucus was eluted predominantly in the void volume (Fig. 4a) indicating the presence of material of molecular weight > 60 000. The elution profile of aged mucus is attenuated suggesting that some molecules of molecular weight < 60 000 are being retained in the gel column (Fig. 4c). An attempted elution of

mucus through Biogel P100 was unsuccessful due to gumming-up of the gel column by adsorption of the mucus. Neither protein nor carbohydrate was detected in the void volume eluate. Thus it appears that no mucus component is of molecular weight $>100\,000$.

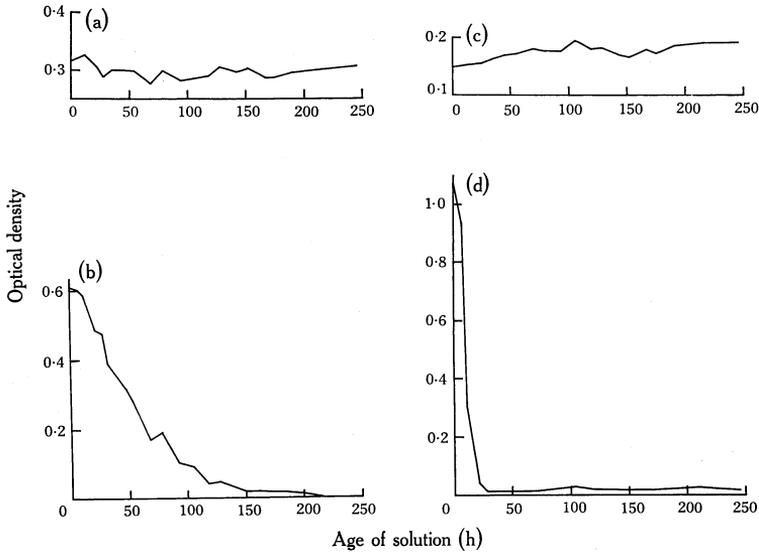


Fig. 2. Changes in enzyme levels of stored aqueous solution of mucus at 37°C. (a) Phenoloxidase, (b) amylase, (c) esterase, (d) proteolytic enzyme.

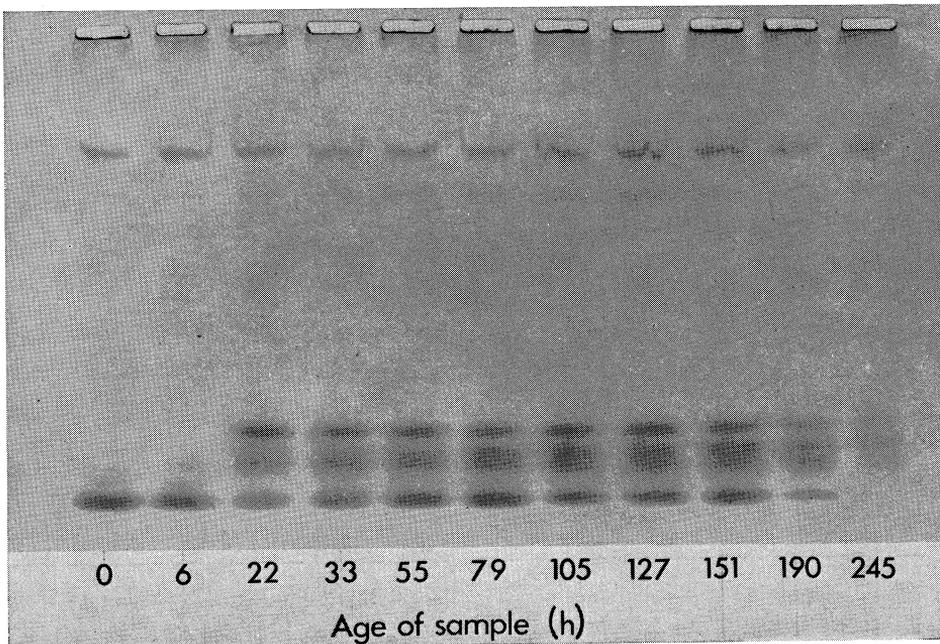


Fig. 3. Electrophoretograms showing protein changes during aging of a mucus solution.

Preparation of raw mucus in NaCl solution apparently resulted in partial disaggregation of high-molecular-weight components. As with aged mucus a displacement of the elution profile occurred (Fig. 4b), and lower-molecular-weight components were retained on the column. However, there was no comparable displacement of amylase and phenoloxidase activities, which appeared to be associated essentially with the components of high molecular weight eluting in the void volume.

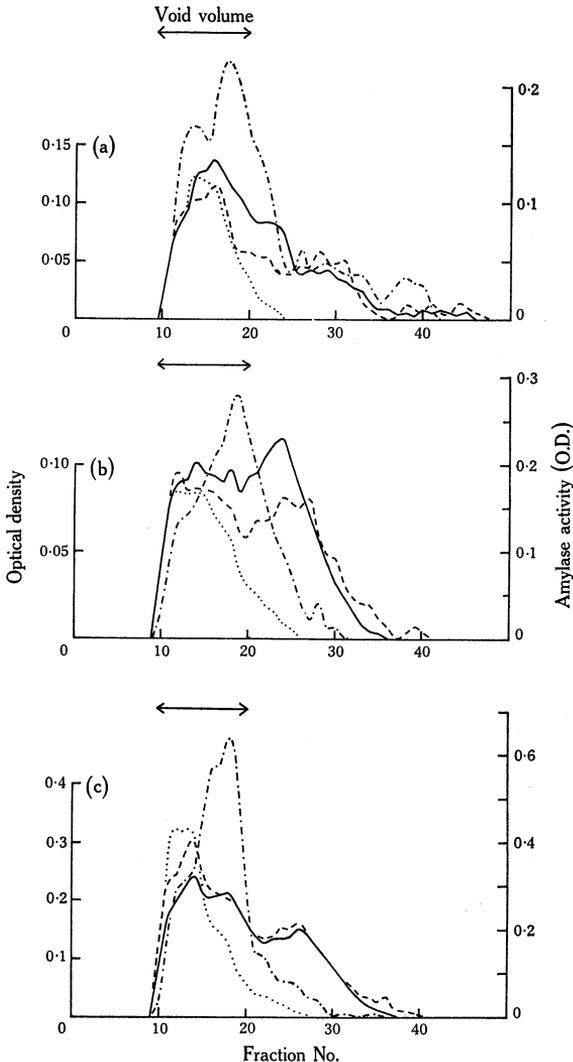


Fig. 4. Specimen elution profiles of mucus components from Biogel P60. (a) 4% fresh mucus solution, 2 ml. (b) 4% fresh mucus solution plus 1% NaCl solution, 2 ml. (c) 4% aged mucus solution, 2 ml, 18 weeks storage at 2°C. — Protein. --- Carbohydrate. -.- Amylase. ··· Phenoloxidase.

Fig. 5 is a series of interpretative drawings of electrophoretograms of eluant fractions from Biogel P60. In each case, only band 2 (protein) appeared in the void volume. Amylase, esterase and phenoloxidase were each located at or near the position of band 2 protein. No bands of high electrophoretic mobility were detected in the column eluates.

The precipitate obtained by treating an aqueous solution of mucus with excess acetone was redissolved in water and passed through Biogel P60. The elution profile,

Fig. 6a, shows appreciable retention of both protein and carbohydrate components beyond the void volume. This material, of molecular weight <60 000 (fractions 21–33), retains most of the physiological activity of the mucus. Fig. 6b shows the elution profile of aged mucus from Biogel P30, with the correlated bioassay of fractions.

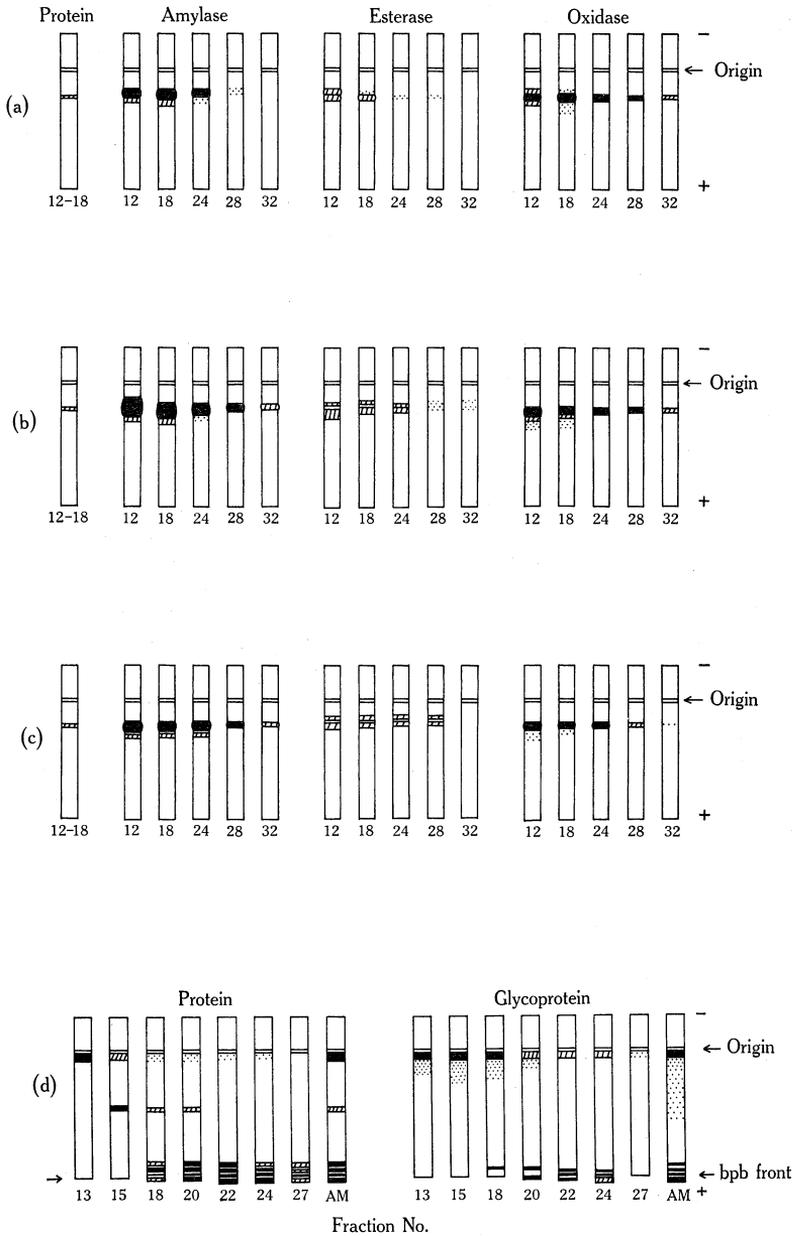


Fig. 5. Interpretative drawings of polyacrylamide gel electrophoretograms of eluant fractions from Biogels P60 (a, b, c) and P10 (d). (a) 4% fresh mucus solution, 2 ml. (b) 4% fresh mucus solution plus 1% NaCl solution, 2 ml. (c) 4% aged mucus solution, 2 ml, 18 weeks storage at 2°C. (d) 1% autoclaved mucus. bpb front, bromophenol blue front.

Attenuation of the elution profile beyond the void volume is evident, and both protein and carbohydrate continue to elute in parallel. It is clear that most of the physiologically active material has been retained to some extent by the P30 gel. Thus prolonged aging of the mucus solution has resulted in the production of physiologically active molecules of molecular weight <30 000 presumably by spontaneous disaggregation of the high-molecular-weight macromolecule contained in raw mucus.

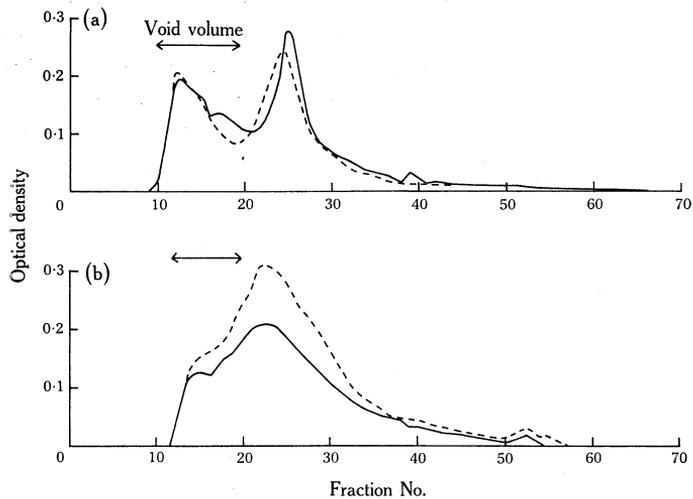


Fig. 6. Elution profiles of mucus preparations from Biogels. (a) Acetone precipitate of fresh mucus, redissolved in water and passed through Biogel P60. (b) Aged mucus solution (18 weeks storage at 2°C) passed through Biogel P30. — Protein. --- Carbohydrate.

	(a)			(b)			
Fractions combined for bioassay	10-20	21-33	34-86	10-18	19-32	33-35	54-68
Protein content (mg)	36	40	17	16	70	15	2
Promotion of senescence (a, twigs; b, fascicles)	3+	5+	+	2+	3+	2+	+

Fig. 7 gives elution profiles for autoclaved mucus on Biogels P2, P6 and P10. It is evident that heating has brought about extensive disaggregation of the raw mucus, such that molecules of molecular weight <10 000 predominate. The P6 profile shows a slight displacement of carbohydrate and protein elution, whilst that from P10 indicates an increased proportion of protein, though the essential parallelism of protein and carbohydrate elution can still be seen. We suggest that the apparent increase in protein is due to an 'unmasking' of more groups on the protein molecules which bind with the Lowry reagent. (Thus, compare Table 1 particularly tests 7, 8 and 11.) All enzymic activity was destroyed by autoclaving but physiological activity was retained by the low-molecular-weight derivatives. Fig. 8 shows comparative bioassays for the eluates from Biogels P6 and P10. Exhaustive washing of the gel columns (beyond fraction 40—Figs 8b and 8d) continued to provide physiologically active material indicating the presence of molecules of molecular weight <5000. Eluates from Biogel P2 were also strongly active in causing twig senescence. However, exhaustive extraction of Biogel P2 beyond fraction 25 failed to yield additional active fractions. Thus the lower limit for active glycopeptide subunits of the mucus appears to be about molecular weight 2000.

Electrophoretograms of eluant fractions from Biogel P10 are shown diagrammatically in Fig. 5*d*. Differences in staining patterns compared to non-autoclaved mucus are immediately apparent and the following points are noted.

- (1) There is intense staining of band 1 (fraction 13, void volume) and band 4 (fraction 15).
- (2) Band 2 has totally disappeared.
- (3) There is elution in strength of bands of high electrophoretic mobility (low molecular weight) in fractions 18 onwards. These bands appeared in the void volume of P6 and may therefore be assumed to be of molecular weight 5000–10000. For comparison P.A.S. staining for glycoprotein is also shown, and positive stains were obtained with toluidine blue, alcian blue and acridine orange, confirming that they are glycopeptide in nature.

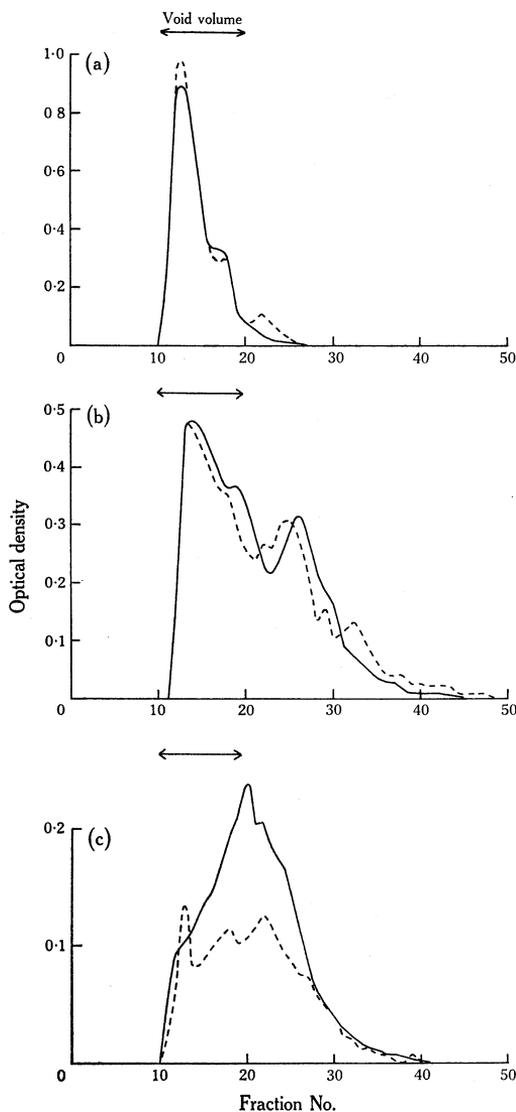


Fig. 7. Elution profiles of autoclaved mucus (4%, 4 ml) from Biogels P2 (a), P6 (b), and P10 (c). — Protein. --- Carbohydrate.

Discussion

The mucus secretion of *S. noctilio* appears to be a fairly homogeneous protein-carbohydrate macromolecule with properties of a non-sulphated acid mucopolysaccharide. Whereas our techniques do not rigorously exclude the possibility that contaminant, high-molecular-weight protein-free carbohydrate is present in raw mucus, our data suggest that the material is glycoprotein in nature. This is supported by the similar elution profiles of both protein and carbohydrate after a variety of treatments, and the reactivity of all electrophoretically separated protein bands towards the P.A.S. reagent.

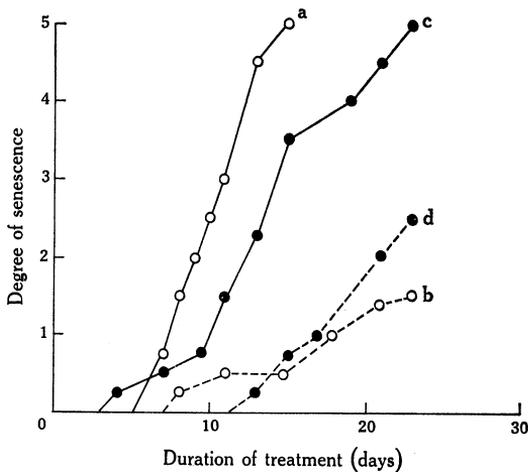


Fig. 8. Bioassays of Biogel eluates and residues. (a) Eluant fractions of autoclaved mucus from Biogel P6. (b) Column residue of (a). (c) Eluant fractions of autoclaved mucus from Biogel P10. (d) Column residue of (c).

The molecular weight of *Sirex* mucus is estimated to lie between 60 000 and 1000 000, from a series of Biogel (P series) fractionations. This is only an approximate value, as other more precise methods of molecular weight determination have not been employed. Results presented in this paper are consistent with the view that this macromolecule, in dilute aqueous solution, may undergo spontaneous disaggregation into smaller subunits of molecular weight less than 10 000, still glycoprotein in nature and still retaining the physiological activity of the whole mucus. Disaggregation of the macromolecule may also be effected by heating, or by treatment with weak salt solutions.

It is possible that the endogenous enzyme activities of the mucus may contribute to the break-up of the macromolecule following injection of the mucus into the sap stream of the tree during oviposition. It is also possible that the enzymes of the tree under attack may contribute to the break-up of the molecule. In this context we envisage a situation in which the mucus undergoes progressive disaggregation at the site of oviposition, so providing a continuing supply of physiologically active subunits. Thus the whole syndrome of senescence effects in the host tree may be maintained over an extended period of time. This may explain the 'conditioning' effect of mucus on *P. radiata*, referred to earlier (Coutts 1969*b*; Fong and Crowden 1973), by which means successful establishment of the symbiotic fungus *Amylostereum aerolatum* is promoted, and survival of the wasp's eggs and larvae is enhanced.

Acknowledgment

We are grateful to the Forest Research Institute, Forestry and Timber Bureau, Department of National Development, for making facilities available to us. L. K. Wong also acknowledges receipt of a postgraduate scholarship from the Forest Research Institute.

References

- Bernfield, P. (1955). Amylases α and β . In 'Methods in Enzymology'. (Eds S. P. Colowick and N. O. Kaplan.) Vol. 1, pp. 149–58. (Academic Press: New York.)
- Bitter, T., and Muir, M. H. (1962). A modified uronic acid carbazole reaction. *Anal. Biochem.* **4**, 330–4.
- Boas, N. F. (1953). Method for the determination of hexosamines in tissues. *J. Biol. Chem.* **204**, 553–63.
- Boros, C. G. (1968). The relationship between the woodwasp *Sirex noctilio* F. and the wood-rot fungus *Anylostereum* sp. M.Sc. Thesis, University of Adelaide, South Australia.
- Bosmann, H. B., and Pike, G. Z. (1970). Glycoprotein synthesis and degradation: glycoprotein : N acetyl glucosamine transferase, proteolytic and glycosidase activity in normal and polyoma virus transformed BHK cells. *Life Sci.* **9**, 1433–40.
- Chayen, J., Bitensky, L., Butcher, R. G., and Poulter, L. W. (1969). 'A Guide to Practical Histochemistry.' pp. 68–71. (The University Press: Aberdeen, U.K.)
- Coutts, M. P. (1969a). The mechanism of pathogenicity of *Sirex noctilio* on *Pinus radiata*. I. Effects of the symbiotic fungus, *Amylostereum* sp. (Thelophoraceae). *Aust. J. Biol. Sci.* **22**, 915–24.
- Coutts, M. P. (1969b). The mechanism of pathogenicity of *Sirex noctilio* on *Pinus radiata*. II. Effects of *S. noctilio* mucus. *Aust. J. Biol. Sci.* **22**, 1153–61.
- Dawson, R. M. C., Elliott, D. C., Elliott, W. H., and Jones K. M. (Eds). (1959). 'Data for Biochemical Research.' (Oxford University Press.)
- Fong, L. K., and Crowden, R. K. (1973). Physiological effects of mucus from the wood wasp, *Sirex noctilio* F., on the foliage of *Pinus radiata* D. Don. *Aust. J. Biol. Sci.* **26**, 365–78.
- Hawk, P. B., Oser, B. L., and Summerson, W. H. (1947). 'Practical Physiological Chemistry.' 13th Edn. (McGraw-Hill Book Co. Inc.)
- Iwasaki, I., Utsumi, S., Hagino, K., Tarutani, T., and Ozawa, T. (1957). Spectrophotometric method for determination of small amounts of sulphate ions. *Bull. Chem. Soc. Jpn* **30**, 847–51.
- Keyser, J. W. (1964). Staining of serum glycoproteins after electrophoretic separation in acrylamide gels. *Anal. Biochem.* **9**, 249–52.
- Lindeberg, G., and Holm, G. (1952). Occurrence of tyrosinase and laccase in fruit bodies and mycelia of some *Hymenomycetes*. *Physiol. Plant.* **5**, 100–14.
- Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951). Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **193**, 265–75.
- Maehly, A. C., and Chance, B. (1955). The assay of catalases and peroxidases. In 'Method of Biochemical Analysis'. (Ed. D. Glick.) Vol. 1, pp. 357–408. (Interscience: New York.)
- Mills, A. K., and Crowden, R. K. (1968). Distribution of soluble proteins and enzymes during early development of *Pisum sativum*. *Aust. J. Biol. Sci.* **21**, 1131–41.
- Saunders, A. M. (1964). Histochemical identification of acid mucopolysaccharides with acridine orange. *J. Histochem. Cytochem.* **12**, 164–70.
- Steedman, H. F. (1950). Alcian blue 8GS: a new stain for mucin. *Q. J. Microsc. Sci.* **91**, 477–9.
- Warren, L. (1959). The thiobarbituric acid assay of sialic acids. *J. Biol. Chem.* **234**, 1971–5.
- Werner, I., and Odin, L. (1952). On the presence of sialic acid in certain glycoproteins and in gangliosides. *Acta Soc. Med. Ups.* **57**, 230–41.