

32029

CYTOCHROME *c* ALLERGENS ISOLATED FROM THE POLLENS OF THE DICOTYLEDONS ENGLISH PLANTAIN (*PLANTAGO LANCEOLATA*) AND PATERSON'S CURSE (*ECHIMUM PLANTAGINEUM*)

PETER A. MATTHEWS,* BRIAN A. BALDO† and MERLIN E. H. HOWDEN*‡

*School of Chemistry, Macquarie University; and †Kolling Institute of Medical Research,
Royal North Shore Hospital, Sydney, Australia

(First received 12 June 1987; accepted 28 July 1987)

Abstract—Two cytochrome *c* allergens were isolated from extracts of the pollens of the dicotyledons English plantain (*Plantago lanceolata*) and Paterson's Curse (*Echium plantagineum*) by ion exchange chromatography, gel filtration and preparative isoelectric focusing. They were characterized by their absorption spectra, mol. wt. pI and amino acid composition. The cytochromes *c* bound specific IgE in the sera of hypersensitive patients by RAST. Preliminary evidence for allergenic cross-reactivity between them was obtained by RAST inhibition.

INTRODUCTION

Apart from ragweed (*Ambrosia elatior*) pollen, there has been general neglect of dicotyledon (weed) pollens in research aimed at isolating and characterizing allergens. This is unjustified since such pollens are widely spread and have frequently been implicated in seasonal allergy (Marsh, 1975; King, 1977). Pollens from the dicotyledons English plantain (*P. lanceolata*) and Paterson's Curse or Salvation Jane (*E. plantagineum*) are anemophilous and entomophilous respectively. They have been shown by us to be important in provoking allergy (Baldo *et al.*, 1982; Katelaris *et al.*, 1982).

Recently, cytochrome *c* allergens have been investigated in extracts of *A. elatior* (Goodfriend *et al.*, 1979), Kentucky blue grass (*Poa pratensis*) and perennial ryegrass (*Lolium perenne*) (Ekramoddoullah *et al.*, 1981, 1982), and Bermuda grass (*Cynodon dactylon*) pollens (Schumacher *et al.*, 1982). Ekramoddoullah *et al.* (1982) reported the allergenic cross-reactivity of cytochromes *c* from *P. pratensis* and *L. perenne* pollens. In the present work two cytochromes *c* from the pollens of *P. lanceolata* and *E. plantagineum* have been isolated and partially characterized. They were shown by RAST test to bind specific IgE in the sera of patients who exhibited immediate type I hypersensitivity to extracts of the respective pollens by the skin prick test. Results of preliminary RAST inhibition experiments were consistent with cross-reactivity between these cytochromes *c* allergens.

MATERIALS AND METHODS

Pollen extracts

Echium plantagineum pollen (Commonwealth Serum Laboratories, Parkville, Victoria, Australia) was defatted by diethyl ether and then extracted in 0.125 M NH₄HCO₃ solution, pH 8.2, as described by Katelaris *et al.* (1982). Fifty grams of *P. lanceolata* pollen (Greer Laboratories, Lenoir, U.S.A.) were stirred for 8 hr with successive 600 and 400 ml vols of 0.1 M NH₄HCO₃ solution, pH 8.0, at 4°C. The mixture was centrifuged for 15 min at 4000 *g* at the end of each extraction period. The combined centrifugates were filtered through a sintered glass funnel, and then ultrafiltered through a 15 cm 1000 *M*, retention membrane (Millipore, Bedford, MA, U.S.A.) to a vol of about 160 ml. The retentate was lyophilized.

Chromatography

DEAE-Sephacel anion exchange gel (Pharmacia AB, Uppsala, Sweden) was converted from the sodium to the ammonium form by standard procedures (Pharmacia Fine Chemicals, 1980) and then equilibrated with 0.1 M NH₄OAc, pH 8.0, and used at ambient temp. The gel was packed into a column (2.5 × 90 cm) for initial fractionation of 1–3 g amounts of *P. lanceolata* crude pollen extract. The column was eluted with NH₄OAc solution, pH 8.0, at NH₄OAc ionic strengths of 0.1 M (300 ml), then 0.5–2.0 M (linear gradient, 900 ml), followed by 3.0 M (900 ml). Acetic acid (3% v/v, 900 ml) was the final eluant. *Echium plantagineum* crude pollen extract was initially fractionated in 100 mg amounts using centrifuge tube inserts, each consisting of a plastic tube of about 10 ml capacity with a fine nylon mesh screen across the base, and containing the same

‡Author to whom correspondence should be addressed at:
Division of Biological and Health Sciences, Deakin
University, Victoria 3217, Australia.

gel. Fractionation was achieved by gentle centrifugation (5 min, 200 g) of solutions of the extract through the gel with five 2 ml aliquots each of 0.1 M and 2 M buffer, and 3% v/v acetic acid. Sephadex G-100 superfine gel (Pharmacia AB) was swollen in 0.2 M NH_4OAc , pH 6, and packed into a 1.6×45 cm column at ambient temp. Sephadex G-50 superfine gel (Pharmacia AB) was swollen in 0.1 M NH_4OAc , pH 8, and packed into a 1.6×180 cm column at ambient temp. Sephadex G-25 superfine gel (Pharmacia AB) was swollen in 1.5% v/v acetic acid and packed into a 1.6×40 cm column. All chromatographic eluants were monitored by absorbance at 280 nm.

Preparative isoelectric focusing

Preparative flat-bed isoelectric focusing in Sephadex G-75 superfine gel (Pharmacia AB) was carried out by standard procedures (LKB, 1975) with carrier ampholyte buffer of pH 7–11 (LKB, Bromma, Sweden). Anode and cathode strips were soaked in 1 M phosphoric acid and 1 M NaOH respectively. The gel tray was maintained at 10°C. A constant 7 W power was applied over 16 hr. After isoelectric focusing the pH gradient along the bed was measured by means of a surface pH electrode and a gel divider. Sections at the cathodic end, which were red-brown in colour, were transferred to plastic centrifuge tube inserts of the type described above, and washed with 1.5% v/v acetic acid by centrifugation for 5 min at 200 g. The separated protein-ampholyte solution was lyophilized.

Absorption spectra

Aqueous solutions were scanned from 300 to 650 nm with a Pye-Unicam SP-800 spectrophotometer (Cambridge, U.K.). The difference spectra of 520 nm regions of reduced and oxidized forms of *P. lanceolata* cytochrome *c* and of equine cytochrome *c* were compared. Sodium dithionite and potassium ferricyanide were used to reduce and oxidize the sample respectively.

Molecular wt determination

An estimation of the M_r of proteins was made by the Sephadex G-100 superfine gel filtration method of Andrews (1965). The following proteins with the indicated M_r were used as markers: bovine alkaline phosphatase, 100,000; pepsin, 35,000; equine cytochrome *c*, 12,500 (Sigma Chemical, St. Louis, U.S.A.); bovine serum albumin (fraction V), 67,000; whale myoglobin, 17,800 (Calbiochem, Sydney, Australia).

Chemical analysis

Qualitative spot tests for carbohydrate were performed as described by Vogel (1978). Protein samples were hydrolysed with 6 M HCl at 110°C for 24, 48 and 72 hr. The hydrolysates were analysed with a modified Beckman 120C Amino Acid Analyser

(Beckman, Palo Alto, CA, U.S.A.). Extrapolation of the amounts of amino acid destroyed or liberated with time followed the procedure of Hill (1965). Confirmation of these values was made by applying a factor determined by comparing empirical and actual values obtained for equine cytochrome *c* (Margoliash and Schejter, 1966). The tryptophan contents were determined colorimetrically by the method of Spies and Chambers (1949). Analysis for disulphide bridges was made by the procedure of Cavallini *et al.* (1966). N-Terminal analyses were carried out by attempted dansylation as described by Gray (1967), and by acetyl-end group methylation according to the technique of Ludowieg and Dorfman (1960) as modified by Narita *et al.* (1975).

Electrophoresis

Vertical slab polyacrylamide gel electrophoresis in the presence of SDS with discontinuous buffers was conducted according to standard methods (LKB) using the Laemmli Tris-glycine buffer system (Laemmli, 1979). A 3% w/v stacking gel overlaid a 12.5% w/v resolving gel ($12 \text{ cm} \times 1.5 \text{ mm}$). Electrophoresis was carried out at 15°C with a constant 15 mA current applied over 15 hr. The gels were then fixed in trichloroacetic acid, stained overnight in 0.025% w/v Coomassie brilliant blue R-250 in ethanol/water (95:5, v/v) and destained in ethanol/acetic acid/glycerol/water (10:3:2:27, v/v).

Analytical isoelectric focusing

Rod gel polyacrylamide gel isoelectric focusing was performed according to the technique of Wrigley (1971) in 11×0.6 cm gels containing carrier ampholytes of pH 8–10.5 (LKB). Electrofocusing was carried out by the application of an initial current of 2 mA per gel to a maximum of 0.5 kV over approx. 4 hr at 6°C. The gels were then fixed in 12% v/v trichloroacetic acid and stained with Reissner stain (Baldo and Uhlenbruck, 1977).

RAST tests

The method of Ceska *et al.* (1972) was employed. Sample solutions were incubated with cyanogen bromide-activated paper discs at concns of 20–100 $\mu\text{g}/\text{disc}$. Sera (50 μl) were from hypersensitive patients whose clinical diagnoses had been confirmed by case history and skin prick tests (Baldo *et al.*, 1982; Katelaris *et al.*, 1982). Cord serum was used as an 'IgE-free' control. Measurement of the uptake of ^{125}I -labelled anti-IgE (Pharmacia AB, Uppsala, Sweden; 40,000 disintegrations/min) was made by a Packard Auto Gamma Counter (Packard Instrument Company, Sydney, Australia).

RAST inhibition

The procedure followed was essentially that of Gleich *et al.* (1974). *Plantago lanceolata* cytochrome *c* was coupled to discs, as above, at 50 $\mu\text{g}/\text{disc}$. Sera from 10 patients who were hypersensitive to *P.*

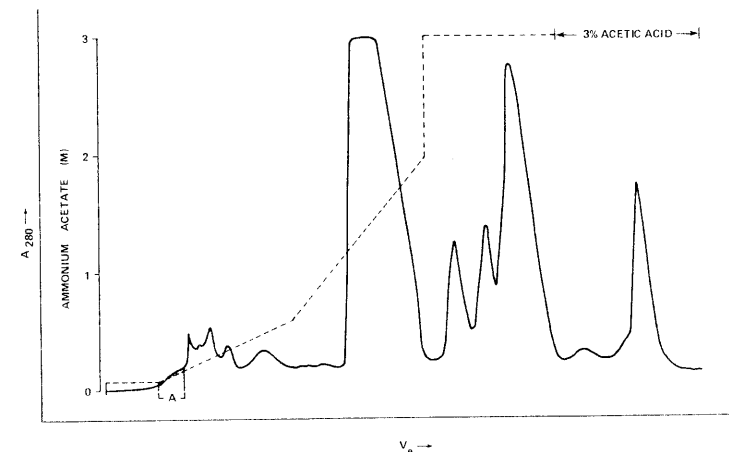


Fig. 1. Elution profile (solid line) of crude *P. lanceolata* pollen extract chromatographed on an anion exchange column. The pollen extract was chromatographed on a 2.5×90 cm column of DEAE-Sephacel equilibrated with 0.1 M NH_4OAc solution, pH 8.0. The NH_4OAc solution (pH 8.0) gradient used for elution is shown by the dashed line. Elution was completed with 3% (v/v) acetic acid/water as indicated between the arrows. Material eluted in the area marked A was pink-brown in colour and was labelled fraction 1A. It contained the cytochrome *c* allergen. The full scale of the abscissa corresponded to 2.0 absorbance units, and of the ordinate 41.

lanceolata pollen were pooled and diluted 1:3 v/v with RAST buffer (Ceska *et al.*, 1972). The inhibiting allergen, *E. plantagineum* cytochrome *c*, was dissolved in RAST buffer to a concn of 1 mg/ml, and 200, 100, 50, 25, 10, 1 and 0.1 μl aliquots were micropipetted or syringed into small tubes. Fifty μl of the serum solution were added to each tube, and the tube stood for 1 or 2 min. One coupled disc was then placed in each tube, and the tube stood overnight. The discs were washed, labelled and counted by the method of Ceska *et al.* (1972). Percentage inhibition was calculated with reference to those discs to which only sera which had not been exposed to inhibitor were added.

RESULTS

Isolation of cytochromes *c*

Ion exchange chromatography of *P. lanceolata* crude pollen extract on DEAE-Sephacel gave the elution profile shown in Fig. 1. An initial pale pink-brown band followed by seven or more yellow bands could be seen in the column as elution proceeded. The 0.1 M NH_4OAc fraction eluted during the small-scale chromatography of *E. plantagineum* crude pollen extract was also pink-brown in colour. These initial fractions were then each gel filtered on Sephadex G-50 superfine and red-brown fractions 2A and 2B were recovered (Fig. 2). It was found that these fractions were similar in colour, approximate elution position and chromatographic behaviour on the anion exchange column as expected of cytochrome *c*-type proteins. They both gave absorption

maxima at 550, 521 and 415 nm, typical of those for cytochrome *c* haeme groups. The carbohydrate spot tests gave negative colour reactions, indicating that no carbohydrate residues were present. SDS-polyacrylamide gel electrophoresis of fractions 2A and 2B showed two to four major bands of similar mol. wt (Fig. 3).

Distinct red-brown bands were visible during the preparative isoelectric focusing of fractions 2A and

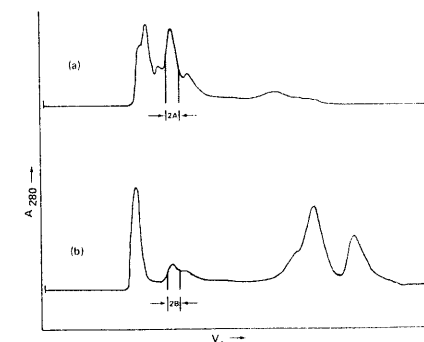


Fig. 2. Gel filtration of (a) *P. lanceolata* fraction 1A (Fig. 1) and (b) a similar fraction from *E. plantagineum* pollen extract. The elution profiles are shown for chromatography on 1.6×180 cm columns packed with Sephadex G-50 superfine gels swollen in 0.1 M NH_4OAc , pH 8.0. The columns were eluted with the same buffer. The areas marked 2A (*P. lanceolata*) and 2B (*E. plantagineum*) correspond to red-brown coloured fractions which contained the cytochromes *c* and were recovered. The full scale of the abscissa corresponded to 0.2 absorbance units, and of the ordinate 390 ml.

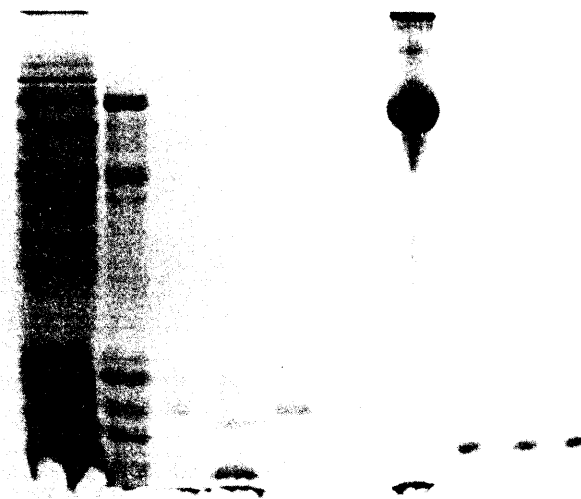


Fig. 3. SDS-polyacrylamide gel electrophoresis of extracts, fractions and standards involved in isolation of pollen cytochromes *c*. See the text for experimental details. The cathode is at the top. From left to right: crude *P. lanceolata* pollen extract; fraction 1A (Fig. 1); next four electrophoretograms—bands of *pI* 8.0-8.5 from preparative isoelectric focusing of fraction 2A (Fig. 2) after removal of ampholytes; from top to bottom (individual components were not correlated with bands), bovine serum albumin (M_r 66,000), human albumin fraction V (65,000), trypsinogen (24,000) and insulin (5734); cytochrome *c*, *pI* 9.85, from *P. lanceolata* pollen; cytochrome *c*, *pI* 9.85, from *E. plantagineum* pollen; and equine cytochrome *c* (Sigma Chemical).

2B. They both focused at *pI* 9.85 at 10°C. Ampholytes were removed by gel filtration on Sephadex G-25 superfine. The first eluted peak in each of these chromatograms was lyophilized and examined by SDS-polyacrylamide gel electrophoresis, and each gave a single band (Fig. 3). Confirmation of homogeneity was achieved by analytical isoelectric focusing which also revealed a single sharp band for each compound.

Properties of cytochromes *c*

An M_r of 12,800 was deduced by gel filtration on Sephadex G-100 superfine for both of the homogeneous proteins isolated as above. Their absorption spectra gave maxima typical of the α , β and γ bands of cytochrome *c* haeme groups (Fig. 4). Disulphide bridge and N-terminal analyses were consistent with those expected of single polypeptide chains having acetyl-masked N-terminal groups, but no disulphide bridges nor free sulphhydryl groups. Amino acid analyses of the cytochromes *c* from *P. lanceolata* and *E. plantagineum* pollens are given in Table 1. Both of them contained 111 residues. This number and the relative proportions of the amino acids are similar to those of most plant-derived cytochromes *c* (Table 1) (Dayhoff and Eck, 1972, 1973, 1976).

Allergenic properties of the cytochromes *c*

Plantago lanceolata and *E. plantagineum* cytochromes *c* were found by RAST to bind specific IgE

in the sera of hypersensitive patients, each giving mean radioactive uptakes of approx. 10% (Fig. 5). In order to investigate possible allergenic cross-reactivity between these cytochromes *c*, RAST inhibition tests were performed. Varying amounts of *E. plantagineum* cytochrome *c* were used to inhibit the binding of specific IgE in the sera of *P. lanceolata*-sensitive patients to *P. lanceolata* cytochrome *c*. The results, based on discs exposed to 50 μ g of *P. lanceolata* cytochrome *c*, indicated that 50% inhibition occurred with approx. 50 μ g of *E. plantagineum* cytochrome *c* (Fig. 6).

DISCUSSION

Plantago lanceolata and *E. plantagineum* are not closely related taxonomically (Watson and Wrigley, 1982). However, Katelaris *et al.* (1982) found that the sera of 28 patients shown by RAST to react with *E. plantagineum* pollen extracts also reacted by RAST with pollen extracts from two to eight different dicotyledon and three monocot species, including *A. elatior* and *C. dactylon*. All of these sera also reacted with discs coupled to *P. lanceolata* pollen extract. These results suggested that either the patients had been separately sensitized by these pollens or that allergenic cross-reactivity was involved. The isolation in the present work of two cytochrome *c* allergens from *P. lanceolata* and *E. plantagineum* pollens brings the number of such known allergens to six, three from

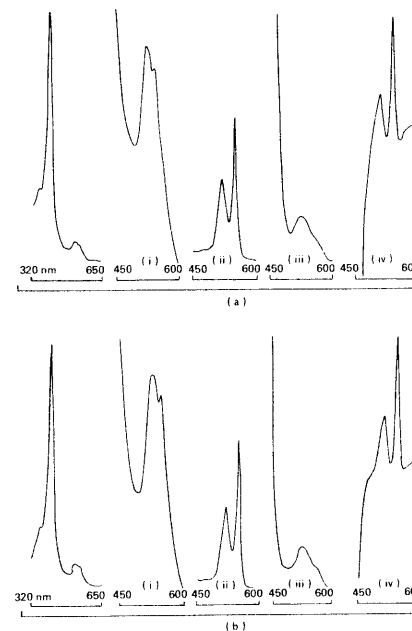


Fig. 4. Comparison of the absorption spectra of (a) cytochrome *c* from *P. lanceolata* pollen and (b) equine cytochrome *c* in their (i) native, (ii) oxidized and (iii) reduced forms. The concns of the cytochromes *c* were 0.5 mg/ml in water in each case. The vertical scale is absorbance. Similar spectra were obtained for the cytochrome *c* from *E. plantagineum* pollen. Traces (iv) are the difference spectra, for reduced minus oxidized forms. Oxidized and reduced forms were obtained by addition of excess sodium dithionite and potassium ferricyanide respectively. The full scale of the abscissae corresponded to (left to right) 1.0, 0.1 and [ii-iv] 0.2 absorbance units respectively.

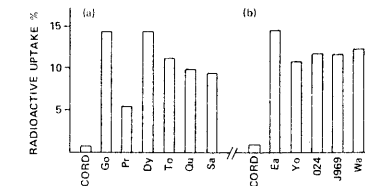


Fig. 5. RAST results for cytochromes *c* from (a) *P. lanceolata* and (b) *E. plantagineum* pollens. The cyanogen bromide-activated discs were exposed to 50 μ g/disc of cytochrome *c*. Patient identification for sera is given along the horizontal axis. Sera were obtained from patients found (Katelaris *et al.*, 1982; Baldo *et al.*, 1982) to be hypersensitive to the respective pollens by RAST and skin prick test.

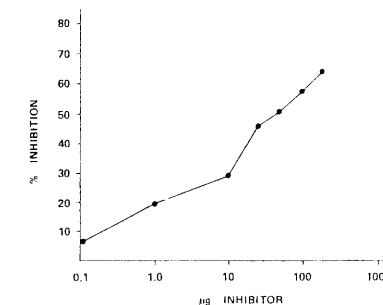


Fig. 6. RAST inhibition of *P. lanceolata* pollen cytochrome *c* by *E. plantagineum* pollen cytochrome *c*. The procedure followed that of Gleich *et al.* (1974). Cyanogen bromide-activated discs were coupled to *P. lanceolata* pollen cytochrome *c* at 50 μ g/disc. Sera from 10 patients hypersensitive to *P. lanceolata* pollen extract were pooled. Pooled serum was mixed with varying amounts of the inhibitor, *E. plantagineum* pollen cytochrome *c*. Coupled discs were then incubated with inhibited and uninhibited serum. The percentage inhibition of radioactive uptake for specific IgE was measured with reference to uninhibited serum.

Table 1. Amino acid compositions of cytochrome *c* allergens from *P. lanceolata* and *E. plantagineum* pollens^a

Amino acid	<i>P. lanceolata</i>				<i>E. plantagineum</i>			
	Hydrolysis time (hr)			Integral value	Hydrolysis time (hr)			Integral value
	24	48	72		24	48	72	
Lys	14.0	13.9	14.0	14	12.2	12.3	12.0	12
His	2.7	2.9	2.5	3	2.0	2.6	2.2	2
Arg	1.8	1.9	2.1	2	1.7	1.9	1.8	2
Asx	9.9	9.8	9.7	10	10.1	9.8	9.8	10
Thr	8.0	6.9	5.5	9	7.9	7.0	6.2	9
Ser	4.0	3.1	2.2	5	6.4	5.8	5.3	7
Glx	10.2	9.9	9.9	10	8.8	9.0	8.6	9
Pro	7.1	7.0	6.8	7	7.8	7.6	7.4	8
Gly	12.0	12.3	12.3	12	13.3	13.2	13.2	13
Ala	11.2	11.5	11.3	11	9.8	9.6	9.6	10
Cys	1.8	1.4	1.3	2	1.7	1.5	1.3	2
Val	3.0	3.5	4.0	4	3.0	3.6	4.2	4
Met	1.8	1.6	1.2	2	1.8	1.4	1.3	2
Ile	2.8	3.0	3.2	3	3.5	4.0	4.0	4
Leu	7.3	7.8	8.0	8	6.8	6.8	6.9	7
Tyr	3.7	3.7	3.4	4	4.8	4.6	4.4	5
Phe	4.1	3.8	3.7	4	4.0	3.7	3.5	4
Trp	1.0			1	1.0			1
Total residues	111				111			

^aValues given were determined for the products of hydrolysis of the cytochromes *c* by 6 M HCl, except in the case of tryptophan which was determined spectrophotometrically. Integral values were obtained by extrapolation to zero time.

dicot and three from monocot pollens. The observation in preliminary RAST inhibition experiments of significant allergenic cross-reactivity between the two cytochrome *c* allergens isolated in this work is not surprising considering the relative uniformity, especially in respect of conformation, in the structures of cytochromes *c* (see Table I and Dayhoff and Eck, 1972, 1973, 1976). Interestingly, equine cytochrome *c*-coupled discs incubated with sera from patients (Fig. 5) sensitive to either of the dicot pollens gave radioactive uptakes in RAST that were low, but significantly higher than the cord serum control (Matthews, Baldo and Howden, unpublished results). Cross-reactivity between cytochrome *c* allergens could explain, at least in part, apparent cross-reactivity between dicot pollens, and even between monocot and dicot pollens. The allergenicity of dicot whole plant fragments (Baldo *et al.*, 1982) may also to some extent be due to the presence of cytochrome *c* allergens.

The probable ubiquity of cytochromes *c* allergens in pollens make them prime candidates for application in the standardization of extracts of allergenic pollens for use in diagnosis and immunotherapy. Such standardization is sorely needed, but is at an early stage of development (Baldo and Howden, 1983). Pollen cytochrome *c* allergens are also potentially of value since their well-defined structures mean that they are particularly amenable to elucidation of their allergenic determinants, and to correlation of these with allergenicity and cross-reactivity. Such studies are currently being undertaken by the authors. We assume that the allergenic determinants reside in the protein portion of the cytochromes *c*.

Acknowledgements—The authors thank the Asthma Foundation of New South Wales and the National Health and Medical Research Council of Australia for support, Drs A. Cripps, R. Baker, C. Katelaris, J. Skalla and R. S. Walls for sera, and Drs J. Dopheide, D. D. Sheumack, Messrs D. Barnett, P. L. Macoun, P. J. Sharp and Mrs D. L. Matthews for special assistance.

REFERENCES

- Andrews P. (1965) The gel-filtration behaviour of proteins related to their molecular weights over a wide range. *Biochem. J.* **96**, 595–606.
- Baldo B. A., Chensee Q. J., Howden M. E. H. and Sharp P. J. (1982) Allergens from plantain (*Plantago lanceolata*). Studies with pollen and plant extracts. *Int. Archs Allergy appl. Immun.* **68**, 295–304.
- Baldo B. A. and Howden M. E. H. (Editors) (1983) Allergen standardization in Australia and current allergen research. *Proc. Sydney Allergen Group 3*, Macquarie University, Sydney.
- Baldo B. A. and Uhlenbruck G. (1977) Selective isolation of allergens. Reaction of house dust mite extracts with tridacnin and concanavalin A and examination of the allergenicity of the isolated components. *Clin. Allergy* **7**, 429–444.
- Cavallini D., Graziana M. T. and Dupre S. (1966) Determination of disulphide groups in proteins. *Nature* **212**, 294–295.
- Ceska M., Eriksson R. and Varga J. M. (1972) Radio-immunosorbent assay of allergens. *J. Allergy clin. Immun.* **49**, 1–9.
- Dayhoff M. O. and Eck R. V. (1972, 1973, 1976) *Atlas of Protein Sequence and Structure*. National Biomedical Research Foundation, Washington.
- Ekraddoullah A. K. M., Kisil F. T. and Sehon A. H. (1981) Isolation of allergenically active cytochrome *c* from Kentucky blue grass pollen. *Int. Archs Allergy appl. Immun.* **65**, 367–376.
- Ekraddoullah A. K. M., Kisil F. T. and Sehon A. H. (1982) Allergenic cross-reactivity of cytochromes *c* of Kentucky bluegrass and perennial ryegrass pollens. *Molec. Immun.* **19**, 1527–1534.
- Gleich G. J., Larson J. B., Jones R. T. and Baer H. (1974) Measurement of the potency of allergy extracts by their inhibitory capacities in the radio-allergosorbent test. *J. Allergy clin. Immun.* **53**, 158–169.
- Goodfriend L., Choudhury A. M., Carpio J. D. and King T. P. (1979) Cytochrome *c*: new ragweed pollen allergens. *Fedn Proc.* **38**, 1415.
- Gray W. R. (1967) Dansyl chloride procedure. In *Methods in Enzymology* (Edited by Hirs C. H. and Timasheff S. N.) Vol. 11, pp. 139–151.
- Hill R. C. (1965) Hydrolysis of proteins. *Adv. Protein Chem.* **20**, 37–107.
- Katelaris C., Baldo B. A., Howden M. E. H., Matthews P. A. and Walls R. S. (1982) Investigation of the involvement of *Echium plantagineum* (Paterson's Curse) in seasonal allergy. IgE antibodies to *Echium* and other weed pollens. *Allergy* **37**, 21–28.
- King T. P. (1977) Chemical and biological properties of some atopic allergens. *Adv. Immun.* **23**, 77–105.
- Laemmli U. K. (1979) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**, 680–686.
- LKB 2001 *Vertical Electrophoresis Unit Application Notes*. Bromma, Sweden.
- LKB *Application Notes* 198 (1975) Bromma, Sweden.
- Ludowig J. and Dorfman A. S. (1960) Micromethod for the colorimetric determination of *N*-acetyl groups in acid mucopolysaccharides. *Biochim. biophys. Acta* **38**, 212–218.
- Margoliash E. and Schejter A. (1966) Cytochrome *c*. *Adv. Protein Chem.* **21**, 114–287.
- Marsh D. G. (1975) Allergens and the genetics of allergy. In *The Antigens* (Edited by Sela M.), Vol. 3, pp. 271–359. Academic Press, New York.
- Narita K., Matsuo A. and Nakajima T. (1975) End group determination. In *Protein Sequence Determination, A Sourcebook of Methods and Techniques* (Edited by Needleman S. B.), 2nd edn, pp. 30–103. Springer, Berlin.
- Pharmacia Fine Chemicals AB (1980) *Ion Exchange Chromatography: Principles and Methods*, p. 38. Uppsala, Sweden.
- Schumacher M. J., Wagner C. M. and Cusanovich M. A. (1982) Cytochrome *c* as a possible allergen in Bermuda grass pollen extract. *J. Allergy clin. Immun.* **69**, 145.
- Spies J. R. and Chambers D. C. (1949) Chemical determination of tryptophan in proteins. *Analyt. Chem.* **21**, 1249.
- Vogel A. I. (1978) *Textbook of Practical Organic Chemistry*, 3rd edn, p. 1078. Longmans Green, London.
- Watson L. and Wrigley C. W. (1982) The classification and distribution of grain- and pollen-bearing plants relevant to allergy in Australia. In *CSIRO Wheat Research Unit, Technical Publication No. 8*. CSIRO, Sydney.
- Wrigley C. W. (1971) Electrofocussing of proteins. In *New Techniques in Amino Acid, Peptide and Protein Analysis* (Edited by Niederweiser A. and Pataki G.), pp. 291–339. Ann Arbor Science, Ann Arbor, Michigan.