

**The Allergenic Activity and Stability
of Purified Allergens
from the Pollen of Common Rye Grass
(*Lolium perenne*)**

D. G. MARSH, F. H. MILNER and P. JOHNSON

Wellcome Research Laboratories, Beckenham, Kent; Bencard Allergy Unit, Brentford,
and Dept. of Colloid Science, Cambridge

Introduction

Highly purified rye grass pollen allergens have been isolated and extensively characterised by physical and chemical methods (JOHNSON and MARSH, 1965a, b, c, d). The present article reports the detailed immunological behaviour and stability of the allergens and of the crude fractions from which they were derived.

An outline of the purification procedure (JOHNSON and MARSH, 1965a) is presented in Fig. 1. Chromatographic fractions D. 6, 7 and 9, from a DEAE*-cellulose fractionation of the dialysed whole pollen extract, were found to contain the bulk of the allergenic material; further fractionation on 'Sephadex G75' yielded the purified allergens. The allergenic activity of the fractions, all at the same concentration by weight, was shown to increase after each purification step (MARSH, 1964). Allergens I-B (originally termed 'a') and I-C (β) were each shown to be homogeneous by the following criteria: starch gel electrophoresis at pH's 8.5 and 3.5 (without urea), ultracentrifugation, rechromatography on 'Sephadex G 75', N-terminal amino acid analysis and immunodiffusion against rabbit anti-rye pollen sera. Allergenic fraction II-B (γ) appeared to be homogeneous according to the foregoing criteria with the exception of N-terminal amino acid and immunodiffusion

* DEAE will be used as an abbreviation for 'diethylaminoethyl'.

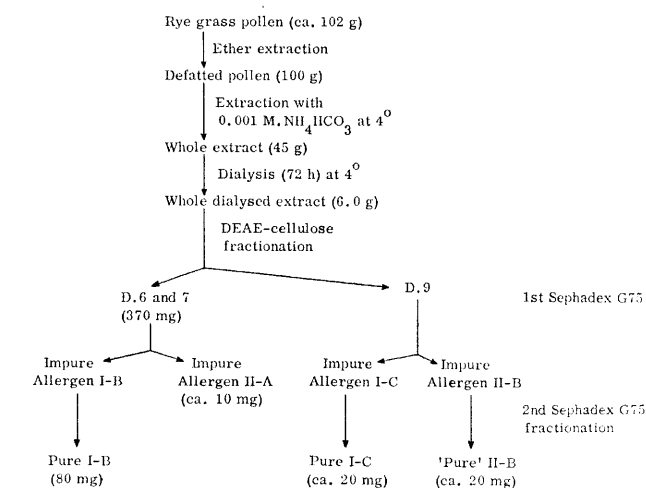


Fig. 1. Scheme for extraction and fractionation of rye grass pollen allergens. Weights given in brackets are of dried solids (containing 12-15% of water) obtained from a 1963 rye pollen. Total amount of allergen I-B in 100 g of defatted pollen $\approx$ 300 mg.

$$\text{Recovery of I-B} = \left(\frac{\text{Pure I-B obtained}}{\text{Total I-B content}} \right) \approx 27\%$$

Yield of I-B (expressed as amount obtained from 100 g of defatted pollen) $\approx$ 0.03%.

analysis: in the latter case, no immunodiffusion lines were obtained, probably due to the low molecular weight of this fraction (10,000). Examination of allergen II-B by starch gel electrophoresis in borate-6 M. urea at pH 8.5 revealed that several discrete components were present, confirming the heterogeneity suggested by the N-terminal amino acid analysis. Preliminary results showed that allergenic fraction II-A (δ) behaved similarly to II-B (MARSH, 1964; JOHNSON and MARSH, 1965b).

Allergens I-B and I-C were both shown to possess mol. wts. of about 31,500. They had slightly differing charges at alkaline pH, manifested by their different behaviour on DEAE-cellulose chromatography and starch gel electrophoresis: in all other respects, including allergenic and antigenic activities, they were experimentally almost indistinguishable. Indeed, there is experimental evidence to suggest that I-B may be converted into I-C by loss of amide group(s) (JOHNSON and MARSH, 1965a). Fractions II-A and

II-B, both of mol. wt. about 10,000, were also related to each other. They were not related immunologically to the allergens of higher mol. wt., since they failed to inhibit the allergenic and antigenic reactions of I-B and I-C. The aforementioned pollen allergens were therefore placed into *isoallergenic* groups I and II and sub-classified according to their apparent mobilities on starch gel electrophoresis at alkaline pH. For example, the so-called major allergenic group I was shown to be composed of four *isoallergens*, I-A, -B, -C and -D, of which I-B and I-C were the most prominent (JOHNSON and MARSH, 1965b).

For most pollens so far studied, *isoallergenic* groups can be demonstrated which are analogous with those of the rye allergens, but are not necessarily related either allergenically or antigenically to them (KING and NORMAN, 1962; KING *et al.*, 1964; JOHNSON and MARSH, 1965b; MARSH, 1965). Classification of all these allergens according to the above nomenclature is convenient.

In previous papers (JOHNSON and MARSH, 1965a, b and d), the significance of the carbohydrate moiety of the group I rye pollen allergens was discussed, and it was concluded that this moiety was probably not important allergenically. In the present paper, by skin testing with carbohydrase-digested allergens, further information on this problem has been obtained.

RICHTER *et al.* (1961) and MALLEY *et al.* (1964a, b) reported allergenic activity in dialysable fractions from ragweed and timothy pollens. Other workers (e.g. HERBERTSON *et al.*, 1958; KING and NORMAN, 1962) found little or no activity in similar low mol. wt. pollen fractions. In our laboratories no significant skin activity has ever been observed in the dialysable fraction from rye grass pollen in concentrations as high as 5.5 mg/ml, (BRITTON *et al.*, 1958; JOHNSON and PADLEY, 1959; FEINSTEIN, 1961; MARSH, 1964; MILNER, unpublished observations).

Possible explanations for these apparent discrepancies are: (a) differences in stability of allergens from grass, ragweed and other pollens (see discussion) and (b) the use of dialysis tubing of different permeabilities (CRAIG and KING, 1962). However, RICHTER *et al.* (1961) found that 'desensitised' allergic patients were more skin reactive than untreated individuals towards the dialysable material of ragweed pollen (Untreated individuals usually showed no reaction). This result might be explained by reversible dissocia-

tion between low molecular weight allergenic 'haptenic material' and the non-dialysable allergenic components*. We have tested this hypothesis for rye grass pollen by examining whether a large excess of the dialysable fraction would inhibit skin reactions produced by the rye allergens in untreated allergic individuals.

Materials and Methods

Defatted pollens were kindly supplied by the Bencard Allergy Unit, Beecham Research Laboratories. The carbohydrases cellulase, β -glucosidase, β -galactosidase, β -amylase and lysozyme were supplied by Koch-Light Ltd.

1. Skin Testing

Allergenic activity was determined by the skin testing on rye pollen sensitive individuals. Sterile solutions of whole pollen or purified allergenic extracts were prepared in buffered physiological saline ('tris' or phosphate at pH 7) either by weight or by 'equivalent concentration'. It was often convenient to be able to compare concentrations of purified fractions which were derived from the same weight of whole pollen (i.e. in the same *equivalent* w/v concentration as in the original pollen extract). All the solutions were prepared using silicone-coated glassware to minimise protein loss by adsorption to glass. Drops of solution were placed on the volar surface of the forearm and small volumes were introduced into the skin by pricking through the drops. After 5 min the drops were removed and the areas of the resultant weals measured 10 min later. Two independent methods were used to measure allergenic activity relative to whole dialysed pollen extracts.

Method (a). Using a series of concentrations for the allergenic fractions and whole dialysed pollen extract, approximate weal areas were calculated from the product of two weal diameters measured at right angles to each other. All measurements were averages of at least two similar tests per individual and comparisons of skin activities of whole pollen extracts with those of allergenic fractions were made using test sites which were as near symmetrical as possible. Specific activities of the allergenic fractions relative to whole pollen were determined from graphical plots of 'mean weal diameter' against 'log. concentration' (SQUIRE, 1950).

A typical series of these plots is given in Fig. 2. Since the curves are not exactly parallel, the ratio of the allergen concentration, C_A , producing the same weal diameter as a given concentration of whole extract, C_w , varies with the value of C_w . Hence estimates of specific activity have been made from the limiting C_w/C_A ratios found by extrapolating the linear portions of the graphs to zero weal diameter. Reasonably consistent results could be obtained using a large number of tests with a relatively small panel of 6 allergic individuals (Table I), providing the limitations of this method, discussed by SQUIRE (1950), were taken into consideration.

Method (b). Weal areas were recorded by outlining the raised weals with a ball-point pen and applying adhesive cellophane tape to the weal. The tape was removed with the weal outline, transferred to graph paper, and the areas measured by counting squares through the tape. Fifty allergic volunteers were tested in this manner using two concentrations of the test solutions and whole dialysed pollen controls (Table II).

* Evidence that group I allergens dissociate in solutions containing sodium dodecyl sulphate (and possibly urea) has recently been presented (JOHNSON and MARSH, 1965d).

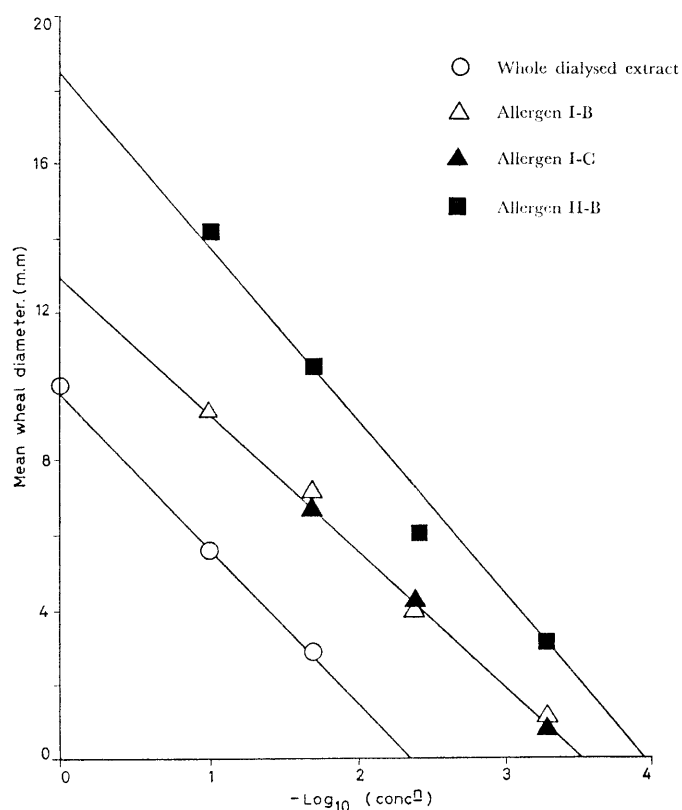


Fig. 2. Graphs used for determination of specific activity by method (a). Concentration expressed in mg of freeze-dried solid/ml. Subject: JPW (Table I).

2. Allergenic Stability

(a) Carbohydrase Digestion

Digestion of allergen I-B with the carbohydrases cellulase, β -glucosidase, β -galactosidase and β -amylase was performed overnight at 37° using an allergen concentration of 4 mg/ml in 0.1 M. pyridine acetate buffer at pH 7.0 and enzyme to allergen ratios of 1:50. Lysozyme digestions were similarly performed, except that a pH of 5.3 was utilised. The allergenic activities of solutions to which enzymes had been added were compared with those of control solutions to which no enzymes had been added.

To determine the extent of carbohydrase digestion of allergen I-B, 1 mg samples of the various digests and the untreated controls were subjected to high voltage paper electrophoresis in 0.1 M. borate buffer at pH 10.0, according to the method of MICHL.

(1951). Marker sugars were also run alongside the digests. The paper was acidified by spraying with pyridine acetate buffer pH 3.5 (JOHNSON and MARSH, 1965d) and stained for carbohydrate using the AgNO_3 -NaOH method of TREVELYAN *et al.* (1950). The enzymes were found to be devoid of any substantial proteolytic activity by staining in parallel with ninhydrin. The presence of free carbohydrate-staining material within the carbohydrases themselves was investigated by allowing solutions of the carbohydrases (in the buffers given above) to stand overnight at 37°. 0.05 mg of each digest (i.e. 2½ times that used for digestion of the allergen) was then subjected to high voltage electrophoresis and stained as above.

(b) *Heating, pH and Ionic Strength Effects*

Solutions of dialysed whole extract (0.24 mg/ml) and of allergen I-B (0.008 mg/ml) in physiological saline (pH 7.3; $\text{I} = 0.15$) were heated in closed siliconed bottles for periods of time ranging from 0–30 min, and then skin tested. The stability of the allergens at a series of pH values was examined by dialysis of whole dialysed extract against the following solutions for 4 h at 4°: 0.1 M. citrate at pH 3.0, 0.1 M. borate at pH 10.0 and 0.2 M. NaOH at pH 11.3. The extracts were then dialysed against distilled water for 3 h before testing. Stability of the allergens towards aqueous solutions of 5 M. NaCl and 2 M. KCNS was similarly tested.

3. Effect of Dialysable Material on Allergenic Activity

Allergen I-B (0.005 mg/ml) was mixed with several concentrations of dialysable material (up to 20 times its concentration by weight) and skin tested on two untreated allergic individuals. Suitable controls of the pure allergen I-B and of the dialysable material alone were also tested.

Results

1. Skin Activities of Untreated Allergens

In comparing the activities of various fractions it should be noted that 2.5 g of native pollen extracted with 100 ml of water (2.5% equiv. concentration) yielded a solution containing about 82 mg/ml of total solids, 10.8 mg/ml of non-dialysable material and 0.072 mg/ml of allergen I-B.

Fig. 2 illustrates a typical series of graphs used to determine specific activities of the allergens (relative to whole dialysed extract) by method (a) and Table I gives the specific activities for 6 allergic individuals by this procedure. Table II summarises the skin test results using method (b). A valid comparison of the solutions tested at the higher concentrations is not possible since allergenic saturation of the test sites has probably been exceeded in most cases. However, comparison of the results at the lower concentrations indicates that allergens I-B and II-B are about ten times more active than whole *dialysed* extract on a weight basis. The variation in skin reactions between the group I and II allergens for different allergic individuals is illustrated in Table III, which gives the detailed skin test measurements for the first 15 individuals tested by method (b).

Table I

Specific activities of the allergenic fractions. Specific activities given are based on freeze-dried weights and are relative to the whole *dialysed* pollen extract. To obtain specific activities relative to (i) non-dialysed extract, (ii) whole pollen (defatted), or (iii) whole pollen (native) the above values are multiplied by the following factors: (i) 7.5, (ii) 16.7, (iii) 17.0.

Subject	I-B and I-C	Specific activity (C_B/C_A) $lim.$	II-B
JPW	14		37
JCM	9		—
PL	27 (I-B only)*		34
DC	13		—
WS	17		204
MEF	22		50
Average spec. activity	17		81**

* Subject PL was unusual here in that he was apparently less sensitive to I-C than I-B in the above tests. This was not shown in later skin tests on PL. For I-C, $C_w/C_A = 2$.

** Value given is an average for only those subjects shown to be sensitive to II-B.

Table II

Summary of skin test results for 50 pollen-sensitive individuals for whole rye pollen extracts and purified allergens [method (b)]. Values corrected by subtracting control reading.

	-ve control Evan's solution	Native whole extract 2.5% (equiv.)	Native whole extract 0.25% (equiv.)	Dialysed whole extract 10.8 mg/ml	Dialysed whole extract 0.1 mg/ml	Allergen I-B 0.072 mg/ml	Allergen I-B 0.01 mg/ml	Allergen II-B 0.112 mg/ml	Allergen II-B 0.01 mg/ml
Total weal area (mm ²)	362	2051	1456	2005	963	1216	613	1805	807
Mean weal area (mm ²)	—	41.0	29.1	40.1	19.3	24.9	12.3	36.1	16.1
Mean weal diameter (mm)	—	7.2	6.1	7.1	5.0	5.6	4.0	6.8	4.5

2. Allergenic Stability

(a) *Carbohydrase digestion.* Table IV shows a summary of the skin test results for carbohydrase-treated allergen I-B using method (b). High voltage electrophoresis in borate buffer at pH 10.0 revealed that staining for reducing sugar was observed only with cellulase and β -galactosidase digests of the allergen. The control experiments, where either the allergen *alone* or the carbohydrase *alone* was incubated at 37°, showed no material which stained for reducing sugar, except in the case of the cellulase where a weak spot showing approximately zero mobility was observed.

The cellulase digest of the allergen produced two strongly staining regions, one of which corresponded approximately in mobility to mannose and the other of which migrated at about half this rate. A further weak spot with mobility similar to that of xylose was also observed with this digest. The β -galactosidase digest of allergen I-B showed only one spot which stained for reducing sugar: this spot also corresponded in mobility to mannose. The identity of these spots could not be established with any certainty, es-

Table III

Detailed comparison of weal areas (mm^2) produced by dialysed whole extract and purified allergens for the first 15 pollen-sensitive individuals tested by method (b). All samples tested at equivalent concentration of 2.5% in Evan's solution

Patient	-ve control Evan's solution	Dialysed whole extract, W	Purified Allergens		Ratios of areas of weals*		
			I-B	II-B	I-B/W	II-B/W	II-B/I-B
1	9	68	56	40	0.79	0.53	0.66
2	4	37	15	12	0.33	0.24	0.73
3	0	30	3	0	0.10	0.00	0.00
4	0	6	5	4	0.83	0.67	0.80
5	10	16	15	8	0.83	0.00	0.00
6	0	12	0	4	0.00	0.33	∞
7	3	16	36	8	2.53	0.38	0.15
8	7	29	22	10	0.68	0.14	0.20
9	0	26	22	3	0.85	0.12	0.14
10	0	29	2	21	0.07	0.72	10.5
11	12	102	23	82	0.12	0.78	6.37
12	0	34	26	4	0.76	0.12	0.15
13	0	12	3	0	0.25	0.00	0.00
14	7	209	87	25	0.40	0.09	0.22
15	4	83	80	98	0.96	1.19	1.24
Averages	3.7	47.3	26.3	21.3	0.63	0.38	—

* Corrected by subtracting negative controls.

Table IV

Summary of corrected skin test results for 50 pollen-sensitive individuals for native and carbohydrase-digested allergen I-B [method (b)] at 2.5% equivalent concentration (Results: Section 1).

	-ve control Evan's solution	Dialysed whole extract	Carbohydrase digests of allergen I-B					Controls (I-B alone)	
			Cellulase	β -Glucosidase	β -Galactosidase	β -Amylase	Lysozyme	pH. 7.0	pH 5.3
Total weal area (mm^2)	528	2005	705	652	670	472	576	608	683
Mean weal area (mm^2)	—	40.1	14.1	13.0	13.4	9.4	11.5	12.2	13.7
Mean weal diameter (mm)	—	7.1	4.2	4.1	4.1	3.5	3.8	3.9	4.2

Table V

Effect of heating (100°C at pH 7.3) on the allergenicity of dialysed rye extract (0.24 mg/ml) and allergen I-B (0.008 mg/ml). A negative control solution (saline) gave no reaction in any of the above individuals.

Fraction	Time of heating (s)	Product of weal diameters (mm ²)		
		JPW	WS	MEF
Whole dialysed extract	0	135	100	60
	10	70	300	50
	30	40	100	10
	120	70	100	40
	480	0	25	50
	1800	28	60	32
I-B	0	9	16	35
	10	4	16	25
	30	25	4	10
	120	0	0	6
	480	8	4	16
	1800	12	20	4

Table VI

Effect of extreme pH conditions and high salt concentrations on allergenicity of whole dialysed rye extract (0.12 mg/ml)

Conditions	Product of weal diameters (mm ²)		
	JCM	DC	PL
pH 3	36	36	30
pH 10	40	84	36
pH 11.3	0	1	10
5 M. NaCl	0	12	9
2 M. KCNS	4	2	48
+ve Control	42	40	42
- ve Control (saline)	0	0	0

Table VII

Skin test results for mixtures of the dialysable material and allergen I-B from rye grass pollen [method (a)]. Negative control (saline) gave no reaction in either subject. The values are averages of 2-4 tests per allergic individual.

Concentration of dialysate mg/ml	Concentration allergen I-B mg/ml	Product of weal diameters (mm ²)	
		WS	JPW
0.10	0	2	0
0	0.005	12	17
0.0001	0.005	30	20
0.001	0.005	29	18
0.01	0.005	13	24
0.10	0.005	24	30

pecially in view of the probable presence of oligosaccharide material in the digests.

(b) *Other stability tests.* Tables V and VI summarise results of tests investigating the effect of heat, pH and high ionic strength on allergenic stability. It has not yet been possible to perform the large numbers of tests necessary to observe whether any statistical significance may be attached to the variation of the skin test results after different periods of heating. However, the preliminary results suggested, that boiling either purified I-B or a whole dialysed extract for up to 30 min caused no detectable loss in allergenicity. Other preliminary data for the whole dialysed extract (Table VI) suggested that allergenicity was retained at pHs 3.0 and 10.0, but not at 11.3. Exposure to 5 M. NaCl apparently caused reduction in skin activity in all cases. The effect of 2 M. KCNS was uncertain, although dialysis against 2 M. KCNS, followed by dialysis against distilled water, resulted in precipitation of a large proportion of the solids present in the whole extract. In this case, the difference in behaviour between subject PL on the one hand, and JCM and DC on the other, may be due to the partial or complete destruction of the group I, but not the group II, allergens. Of the three sensitive individuals tested, only PL was shown to be sensitive to the group II allergens (Table I).

3. Effect of Dialysable Material on Allergenic Activity

Table VII summarises preliminary results examining the possible blocking of skin activity of allergen I-B by the dialysable fraction from the whole rye pollen extract. These results confirmed previous results in our laboratories which showed that no appreciable skin activity could be found in the dialysable fractions, and strongly suggested that dialysable material does not inhibit the normal allergic reaction produced by allergen I-B.

Discussion

1. Activity of Purified Allergens

Skin activity has been conclusively demonstrated in two discrete groups of rye pollen allergens in a large number of individuals allergic to grass pollens. The results obtained with the graphical method (a), suggested that the group I allergens were active in all rye pollen allergic individuals, but that the group II allergens were only active in certain cases. As a result of more extensive skin tes-

ting using method (b) (Tables II and III), it is clear that allergy towards the group II fraction is much more widespread than had previously been assumed (JOHNSON and MARSH, 1965a). The mean skin reactions for both the whole dialysed extracts (W) and the purified allergens using method (b) were all slightly, but not significantly, below those calculated from method (a) for the same solute concentrations (Fig. 2 and Table II). However, in view of the different procedures involved in methods (a) and (b), particularly the numbers of patients tested and the concentration ranges utilised, the results obtained show an encouraging measure of agreement.

The pronounced variation in the ratio of weal areas obtained for groups I and II in different allergic individuals (Table III) is well outside that to be expected from the errors inherent in the tests. There was no experimentally detectable difference between the skin activities of the group I isoallergens (Fig. 2), except in one individual; their specific activities relative to whole dialysed extract were also similar from individual to individual (Table I).

Preliminary skin tests on several other crude rye pollen fractions (MARSH, 1964) have suggested that further minor allergens in the whole dialysed extract show pronounced skin reactions only in certain people. This would probably account for most of the relatively high reactions observed for the whole dialysed extract in certain individuals (Table III). These further allergens occur in predominantly polysaccharide fractions of low or negative apparent mobility on starch gel electrophoresis at pH 8.5.

Detailed analysis by microdensitometry of starch gel patterns obtained using known amounts of the group I allergens from a 1963 rye pollen and the corresponding whole dialysed extract (JOHNSON and MARSH, 1965a) indicated that allergen I-B constituted about 1/20th of the extract and allergen I-C about 1/80th (although these relative proportions varied from extract to extract); other group I allergens were present to a much lesser degree. On this basis, one would expect a specific activity for group I (total) to be about 16* relative to the *dialysed* extract, neglecting the contributions of other allergenic groups. The value of 17* found by quantitative skin testing using the graphical method (a) shows good

* These values correspond to specific activities relative to the *native* rye pollen of about 270 and 280 respectively and relative to *non-dialysed* extract of about 120 and 130 respectively (Table I).

agreement with this figure, but is possible slightly high considering the contribution of other allergenic groups. There is therefore no support in this work for the occurrence of major rye pollen allergens of such high specific activity (300 times those of dialysed extracts) as previously quoted for other grass pollens by AUGUSTIN and HAYWARD (1962), unless their fractionation procedures led to the isolation of minor allergenic fractions. Our figures agree much more closely with those of 100–1000 for purified group I ragweed allergens, relative to *non-dialysed* whole ragweed extracts (see preceding footnote), determined after extensive skin testing by KING *et al.* (1964), LEA *et al.* (1962) and ARBESMAN *et al.* (1962); although ROBBINS *et al.* (1963) found 10-fold higher specific activities not only for his ragweed allergenic fraction but for those of KING and also GOLDFARB (ARBESMAN *et al.*, 1962). Probably errors have arisen mainly due to the inaccuracies of 'end-point' methods of skin testing.

2. Significance of the Carbohydrate Moiety of Group I Allergens

Earlier precipitin inhibition results of JOHNSON and MARSH (1965a), which suggested that the carbohydrate moiety of group I allergens was probably unimportant allergenically, have been considerably substantiated by the finding that after positive digestion with crude cellulase or β -galactosidase, no measurable loss in allergenicity relative to the control was apparent (Table IV). After treating whole grass pollen extracts with the carbohydrases α -amylase, emulsin or takadiastase, AUGUSTIN (1959) reported no loss in allergenic activity, although in her experiments it was not feasible to check if any digestion of the allergen molecules had taken place; KING *et al.* (1964) believed that the low carbohydrate content (0.5–0.6%) of their group I ragweed allergens was due to an impurity, and HERBERTSON *et al.* (1958) showed that their alder allergenic fraction contained no carbohydrate. The bulk of the available evidence would therefore support the view that a carbohydrate moiety is not responsible for allergenic activity in the major allergens of pollens (see also JOHNSON and MARSH, 1965d).

3. Allergenic Stability

Rye pollen allergens have been shown to be stable towards heating (100° at pH 7.3) and also within the pH range 3–10: similar findings have been reported by AUGUSTIN (1959) and MALLEY *et al.* (1962) for other grass pollen extracts. Purified grass

pollen allergens were also stable towards freeze-drying and storage at 4° in the dried state (MARSH, 1964), or in sterile concentrated solution (AUGUSTIN and HAYWARD, 1962; MALLEY *et al.*, 1962). On the other hand, the purified ragweed allergens showed maximal stability only between pH 6 and 8.5 and were 'polymerised' by freeze-drying followed by storage at -20° (KING *et al.*, 1964), although they were much less susceptible towards proteolytic digestion than the corresponding rye allergens. These stability differences between purified grass and ragweed allergens are probably a reflection of their widely differing amino acid compositions and sequence (JOHNSON and MARSH, 1965c). These differences would also account for some of the conflicting reports in the literature in relation to allergenic stability.

It is of interest to note here that the group I rye allergens also retained their antigenicity (measured by gel diffusion analysis against rabbit antiserum towards whole pollen) after the heat treatment reported above (JOHNSON and MARSH, 1965a, b). Other rye pollen fractions have been shown to contain chiefly the main thermolabile 'A-antigens' of rye pollen (AUGUSTIN, 1959); but these fractions proved to be non-allergenic (MARSH, 1964). Any quantitative assay of grass pollen extracts based on the proportion of A-antigen is therefore a completely unreliable guide to the allergenic content. This finding confirms previous conclusions of AUGUSTIN (1959) and FEINSTEIN (1961), obtained using whole grass pollen extracts.

4. Dissociation of Allergenic Determinants

Since the dialysable material present in whole rye pollen extract produced no measurable inhibition of the allergic skin reaction of the group I rye pollen allergens and no reaction by itself on untreated allergic subjects, it seems highly improbable that normal non-dialysable allergens dissociated during dialysis at 4° to give fragments possessing their allergenic determinants. It is therefore unlikely that such 'haptenic material' related to the major rye allergens is present in whole rye pollen dialysate. The recent findings of MALLEY *et al.* (1964a, b) for timothy grass pollen demonstrating allergenic activity in dialysable material appear to oppose this conclusion. However, since these workers were using different conditions for extraction and dialysis, the possibility of a physical or proteolytic dissociation of haptenic material cannot be ruled out.

Other factors which could have been responsible for the observed activity in the dialysate are: diffusion of the group II timothy allergens (mol. wt. about 10,000) through the dialysis tubing (CRAIG and KING, 1963), or the use of desensitised individuals for skin tests. It is significant that not all the individuals tested by MALLEY *et al.* (1964b) were skin-active towards the dialysable material; possibly those which reacted had previously been desensitised. RICHTER *et al.* (1961) observed skin activity for the dialysable ragweed pollen fraction in all desensitised subjects, but no reaction in over 80% of the untreated cases. Analogous behaviour might be expected for the corresponding timothy fraction. Further experiments along these lines should provide valuable information regarding the desensitisation procedure.

Summary

Allergenic activity has been found in two discrete groups of isoallergens from rye grass pollen. These two groups have previously been shown to be quite distinct physically, chemically and immunologically. Different allergic individuals showed different degrees of skin reaction towards the two allergenic groups. A few individuals were also allergic towards further allergens which have not yet been characterised.

No detectable loss of skin activity was observed after digestion of a homogeneous group I allergen with the carbohydrases β -galactosidase and cellulase, both of which were shown to release carbohydrate fragments from this allergen. This result strongly suggests that a carbohydrate moiety is inessential for allergenic activity in the group I rye allergens, especially when considered together with earlier experimental evidence supporting this view.

It has been shown that rye allergens are very heat stable and retain full allergenic activity over a wide pH range. Allergenic activity of the major rye allergens could not be inhibited by a large excess of the non-allergenic dialysable material obtained from whole pollen extracts. This result suggests that no 'haptenic material' is removed by dialysis.

Acknowledgements. We are pleased to thank Drs. A. CRONIN and R.R.A. COOMBS for their help with skin testing and for useful discussions. We are also grateful to Messrs. C.A. FRASER and C.G. LEE and Dr. B. DYBAS for their assistance with the large scale skin testing. One of us (DGM) was financially supported by a grant from DSIR during part of this work.

References

- ARBESMAN, C. A.; REISMAN, R. E.; BONSTEIN, H. S. and ROSE, N.: Allergic and immunologic studies of a 'purified' fraction of ragweed pollen. *J. Immunol.* 90: 612 (1962).
- AUGUSTIN, R.: Grass pollen allergens. III. Their differentiation from other pollen antigens by immuno-electrophoretic studies in relation to skin reactivity, enzymic digestions, heat and pH stabilities. *Immunology* 2: 230 (1959).
- AUGUSTIN, R. and HAYWARD, B. J.: Grass pollen allergens. IV. The isolation of some of the principal allergens of *Phleum pratense* and *Dactylis glomerata* and their sensitivity spectra in patients. *Immunology* 5: 424 (1962).
- BRITTON, C. J. C.; COOMBS, R. R. A.; JOHNSON, P. and THORNE, H. V.: Grass pollen extracts. IV. The biological activity of rye pollen extracts and their fractions. *Int. Arch. Allergy* 13: 305 (1958).
- CRAIG, L. C. and KING, T. P.: 'Dialysis' in GLICK, D.: *Methods for Biochem. Anal.*, vol. X, p. 175 (Wiley, New York 1963).
- FEINSTEIN, A.: Studies of pollen extracts (Ph. D. Thesis, Cambridge University 1961).
- HERBERTSON, S.; PORATH, J. and COLLEDAHL, H.: Studies on allergens from alder pollen (*Alnus glutinosa*). *Acta chem. scand.* 12: 737 (1958).
- JOHNSON, P. and MARSH, D. G.: The isolation and characterisation of allergens from the pollen of rye grass (*Lolium perenne*). *European Polymer J.* 1: 63 (1965a).—'Isoallergens' from rye grass pollen. *Nature* 206: 935 (1965b).—Allergens from common rye grass pollen. I. Chemical composition and structure. *Immunochemistry* (in press, 1965c).—Allergens from common rye grass pollen. II. The allergenic determinants and carbohydrate moiety. *Immunochemistry* (in press, 1965d).
- KING, T. P. and NORMAN, P. S.: Isolation studies of allergens from ragweed pollen. *Biochemistry* 1: 709 (1962).—*Idem* Proc. 6th Int. Congr. Biochem., New York. Abstr. II: 95 (1964).
- KING, T. P.; NORMAN, P. S. and CONNELL, J. T.: Isolation and characterisation of allergens from ragweed pollen. II. *Biochemistry* 4: 458 (1964).
- LEA, D. J.; FREEDMAN, S. O. and SEITON, A. H.: The skin activity of non-dialysable constituents of ragweed pollen. *Int. Arch. Allergy* 20: 220 (1962).
- MALLEY, A.; REED, C. E. and LIETZE, A.: Isolation of allergens from timothy pollen. *J. Allergy* 33: 84 (1962).
- MALLEY, A.; CAMPBELL, D. H. and HEIMLICH, E. M.: Isolation and immunochemical properties of haptenic material from timothy pollen. *J. Immunol.* 93: 420 (1964a).
- MALLEY, A.; SAHA, A. and CAMPBELL, D. H.: Preliminary chemical investigations of a haptenic fraction isolated from timothy grass pollen extracts. *Immunochemistry* 1: 237 (1964b).
- MARSH, D. G.: Biophysical and chemical studies of pollen extracts (Ph. D. Thesis, Cambridge University 1964).
- MARSH, D. G.: unpublished observations (1965).
- MICHL, H.: Hochvoltektrophorese, *Msehr. Chem.* 82: 489 (1951).
- RICHTER, M.; GRIFFITHS, B. W. and ROSE, B.: The allergens in ragweed pollen. Physicochemical and immunologic studies of a purified fraction of the dialysate of ragweed pollen. *J. Immunol.* 87: 439 (1961).
- ROBBINS, K. C.; WU, H.; BARAM, P. and MOSKO, M. M.: Isolation of non-dialysable allergens and antigens from low ragweed pollen (*Ambrosia elatior*). *J. Immunol.* 91: 354 (1963).
- SQUIRE, J. R.: The relationship between horse dandruff and horse serum antigens in asthma. *Clin. Sci.* 9: 127 (1950).
- TREVELYAN, W. E.; PROCTER, D. P. and HARRISON, J. S.: Detection of sugars on paper chromatograms. *Nature* 166: 444 (1950).

Authors' addresses: Dr. D. G. Marsh, Wellcome Research Laboratories, Langley Court, Beckenham, Kent;
Dr. F. H. Milner, Beecham Research Laboratories, Brentford; Dr. P. Johnson, Department of Colloid Science, Cambridge (England).