

REFERENCES

- ¹ JOHNSON P. and MARSH D. G., *Europ. Polym. J.* **1**, 63 (1965).
- ² JOHNSON P. and MARSH D. G., *Nature, Lond.* **206**, 935 (1965).
- ³ JOHNSON P. and THORNE H. V., *Int. Archs. Allergy* **13**, 257, 276 and 291 (1958).
- ⁴ FEINSTEIN A., Ph.D. Thesis, Cambridge University (1961).
- ⁵ AUGUSTIN R. and HAYWARD B., *Immunol.* **5**, 424 (1962).
- ⁶ MALLEY A., REED C. E. and LIETZE A., *J. Allergy* **33**, 84 (1962).
- ⁷ CALLAGHAN O. H. and GOLDFARB A. R., *J. Immunol.* **89**, 612 (1962).
- ⁸ KING T. P. and NORMAN P. S., *Biochemistry* **1**, 709 (1962).
- ⁹ KING T. P., NORMAN P. S. and CONNELL J. T., *Biochemistry* **3**, 458 (1964).
- ¹⁰ KING T. P. and NORMAN P. S., *Int. Congr. Biochem.* **6**, Abstr. II-95 (1964).
- ¹¹ ROBBINS K. C., WU H., BARAM H. and MOSKO M. M., *J. Immunol.* **91**, 354 (1963).
- ¹² *Bull. Wld. Hlth. Org.* **30**, 447 (1964); *Immunochem.* **1**, 145 (1964). Also reported by COHEN S., *Immunology* **8**, 1 (1965).
- ¹³ WODEHOUSE R. P., *Int. Archs. Allergy* **6**, 65 (1955).
- ¹⁴ MALLEY A., Personal communication (1962).
- ¹⁵ FRAENKEL-CONRAT H., HARRIS J. I. and LEVY A. L., *Meth. Biochem. Anal.* **2**, 359 (1955).
- ¹⁶ GRAY W. R. and HARTLEY B. S., *Biochem. J.* **89**, 59P (1963).
- ¹⁷ PREGL F., *Quantitative Organic Microanalysis* (Edited by GRANT J.), (4th Ed.) p. 78. Churchill, London (1945).
- ¹⁸ MA T. S. and ZUAZAGA G., *J. Indian Eng. Chem. (Anal. Section)* **14**, 280 (1942).
- ¹⁹ HIRS C. H. W., *J. Biol. Chem.* **219**, 611 (1956).
- ²⁰ GOODWIN T. W. and MORTON R. A., *Biochem. J.* **40**, 628 (1946).
- ²¹ TRISTRAM G. R. and SMITH R. H., *Adv. Protein Chem.* **18**, 227 (1963).
- ²² REINDEL F. and HOPPE W., *Chem. Ber.* **87**, 1103 (1954).
- ²³ MICHL H., *Monatsh. Chem.* **82**, 489 (1951).
- ²⁴ RYLE A. P., SANGER F., SMITH L. F. and KITAI R., *Biochem. J.* **60**, 555 (1955).
- ²⁵ WALEY S. G. and WATSON J., *Biochem. J.* **55**, 328 (1953).
- ²⁶ BARROILLIER H., *Naturwiss.* **42**, 46 (1955).
- ²⁷ DAWSON R. M. C., ELLIOT D. C., ELLIOTT W. H. and JONES K. M., *Data for Biochemical Research*, p. 210. Oxford University Press (1949).
- ²⁸ BROWN J. R. and HARTLEY B. S., *Biochem. J.* **89**, 59P (1963).
- ²⁹ GOTTSCHALK A., MURPHY W. H. and GRAHAM E. R. B., *Nature* **194**, 1051 (1962).
- ³⁰ JOHNSON P. and MARSH D. G., *Immunochem.* **3**, 101 (1966).
- ³¹ TREVELYAN W. E., PROCTER D. P. and HARRISON J. S., *Nature, Lond.* **161**, 444 (1950).
- ³² COHN E. J. and EDSALL J. T., *Proteins, Amino Acids and Peptides*, p. 157. Reinhold, New York (1943).
- ³³ KING T. P., Personal communication (1964).
- ³⁴ BOYD W. C., *Fundamentals of Immunology* (3rd Ed.) p. 414. Interscience, New York (1956).

ALLERGENS FROM COMMON RYE GRASS POLLEN (*LOLIUM PERENNE*)—II

THE ALLERGENIC DETERMINANTS AND CARBOHYDRATE MOIETY

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Abstract—The chemical structures of the carbohydrate moieties of both prominent group I rye pollen allergens appeared to be identical; however, it seems unlikely that these moieties are essential for allergenic activity. From investigations of both chemically-modified and enzymically-digested allergen molecules, it is concluded that an intact, or partially intact, tertiary structure is required for full allergenic activity. The demonstration that the group I allergens dissociated into smaller fragments under suitable conditions (e.g. in solutions containing sodium dodecyl sulphate) suggests a possible mechanism whereby these molecules permeate the mucous membranes, causing allergic response in susceptible individuals.

INTRODUCTION

IN PREVIOUS papers,⁽¹⁻³⁾ many close similarities between the two prominent major rye allergens, I-B (α) and I-C (β), were demonstrated by a comparison of immunological, physical and chemical properties. Thus, by spectrophotometric estimation, they were found to contain similar amounts of pentose, hexose and heptose: 6-deoxyhexose, hexuronic acid and hexosamine were not detectable by spectrophotometric analysis.⁽²⁾ Allergenic fraction II-B (γ) also possessed the same classes of monosaccharide, although in smaller proportion than the major allergens. This paper is concerned, in greater detail than hitherto,^(1,4) with the compositions of the carbohydrate moieties, and with the significance of these groupings in allergenicity.

During preliminary stages in the chemical investigation of rye pollen allergens, it had been surmised that the carbohydrate moieties of these allergens might be important in determining allergenic activity. This assumption had been largely derived from many experiments demonstrating the importance of carbohydrate haptens in both natural and synthetic antigens (e.g. Haurowitz⁽⁵⁾). However, an investigation of the immunological significance of a glycopeptide fragment, isolated from allergen I-B after enzymic digestion, revealed that it was non-allergenic and that it failed to inhibit the antigen-antibody reaction between I-B (or I-C) and rabbit anti-rye pollen serum.⁽²⁾ Taken together with independent observations on other pollen allergens by Herbertson *et al.*⁽⁶⁾ (alder), Augustin⁽⁷⁾ (Timothy and cocksfoot) and King *et al.*⁽⁸⁾ (short ragweed), the above findings suggested that a carbohydrate moiety was not essential for the skin activity of pollen allergens.⁽³⁾ This conclusion, was, however, in opposition to the views of Stanworth⁽⁹⁾ who had based his arguments mainly on investigations of horse dandruff allergens. A

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† Much of the work presented here was carried out while one of us (D. G. M.) was a visitor at the Laboratory of Molecular Biology, Cambridge, England.

further enquiry into the nature of rye pollen allergenic determinant groups, using both direct skin testing and immunodiffusion techniques, therefore seemed desirable.

Several workers^(2,4,10) have also questioned the mechanism whereby the major pollen allergens, of molecular weight 30–40,000, succeed in permeating the mucous membranes to induce allergic response. An investigation of the dissociation of major rye allergen I-B in sodium dodecyl sulphate solutions sought to provide an answer to this question.

EXPERIMENTAL

Materials

All chemicals were of 'Analar' grade unless otherwise stated. 2-mercaptoethanol, obtained from Lights Ltd., was redistilled and stored at -20° before use. Iodoacetamide and iodoacetic acid, supplied by B.D.H., were recrystallized from water. Highly purified, crystalline sodium dodecyl sulphate (SDS) was obtained from I.C.I. Ltd. Agar was obtained from Difco Ltd., and Sephadex G-25 from Pharmacia. The enzymes pepsin, pronase and carboxypeptidase were supplied by Worthington, Calbiochem. and CIBA Ltd., respectively. Other chemicals and enzymes were obtained and prepared for use as described in the preceding paper.⁽¹⁾

Method

1. *Qualitative determination of individual monosaccharides in group I rye allergens.* Allergenic samples (5 mg) were hydrolysed with saturated aqueous $\text{Ba}(\text{OH})_2$ (0.15 N; pH 13.4) for 15 hr at 60° , or with 2 N HCl for 2 hr at 100° (Tsugita and Akabori⁽¹¹⁾). Ba^{2+} ions in the alkaline hydrolysates were removed by careful precipitation with 2 N H_2SO_4 , and the hydrolysates were fractionated on a Sephadex G-25 column (74×1.2 cm), equilibrated with 0.05 M $(\text{NH}_4)_2\text{CO}_3$. These Sephadex fractions, or the total acidic hydrolysates, were subjected to high voltage paper electrophoresis at pH 2 (1.4 M HAc; 0.53 M HCOOH), according to the method described previously.⁽¹²⁾ Peptide and carbohydrate fragments were located by staining narrow strips of paper cut in the field direction.

The portions of paper containing carbohydrate material were cut out and eluted with 30 per cent ethanol-water. The carbohydrate fractions from the alkaline hydrolysates were hydrolysed more extensively with 2 N HCl or 2 N H_2SO_4 for 3 hr at 105° to yield the free monosaccharides. The latter were examined by high voltage electrophoresis in 0.2 M borate buffer at pH 10.0 (Foster⁽¹³⁾) and by paper chromatography in $n\text{-BuOH}:\text{CH}_3\text{COOH}:\text{H}_2\text{O}$ (4:1:5) alongside marker sugars. Whatman 3 MM paper was used throughout; in electrophoresis, correction for electro-osmosis was performed using 2,3,4,6-tetra-*O*-methyl-D-glucose as a non-migrating marker. The periodate-starch stain of Metzenberg and Mitchell⁽¹⁴⁾ was employed where great sensitivity was required, and a $\text{AgNO}_3\text{-NaOH}$ stain of Trevelyan *et al.*⁽¹⁵⁾ was used in other cases. Greater sensitivity was obtained if the borate buffer, used in electrophoresis, was neutralized with formic-acetic buffer at pH 2.0 before staining. Peptides were located using the ninhydrin-cadmium acetate stain of Barollier.⁽¹⁶⁾

2. *Further investigations of the monosaccharides and the glycopeptide linkage in group I allergens.* Glycopeptide fragments of allergens I-B and I-C were obtained

by extensive enzymic hydrolysis with trypsin, chymotrypsin, pronase and carboxypeptidase in the manner previously described.⁽²⁾ These fragments could be separated from contaminating peptides by paper electrophoresis at pH 2.0 due to their relatively slow migration rate compared with normal peptides. The glycopeptides were eluted as above. N-terminal amino acids and amino-acid compositions, were determined qualitatively on a micro scale by 'dansylation'* of small fractions of the glycopeptides before and after hydrolysis with 6 N HCl (18 hr at 105°), respectively.

3. *Chemical modifications.* Certain chemical modifications of the rye allergens, chosen as involving specifically selected amino acids, were used to investigate the possible significance of these groups in allergenicity (Table 3).

(a) *Reduction.* Disulphide bonds of allergen I-B were reduced with 2-mercaptoethanol and stabilized by alkylation with iodoacetamide using an analogous method to that described for ribonuclease by Anfinsen and Haber.⁽¹⁸⁾ Allergen I-B (15 mg) was dissolved in 0.4 ml of 1 M methylamine-HCl buffer, 7 M with respect to urea, at pH 8.6. To this solution, 20 μl . of mercaptoethanol were added and the mixture was allowed to stand at room temperature under nitrogen for 4½ hr. Recrystallized iodoacetamide (450 mg—i.e. a 10-fold molal excess) was added and the solution left for 15 min at room temperature. A 10-fold molal excess of mercaptoethanol (2 ml) was then added and the solution allowed to stand for a further 45 min. Excess reagents were removed by overnight dialysis against 0.1 M NH_4HCO_3 , followed by passage down a Sephadex G-75 column equilibrated with this buffer.

In another experiment, the extent of reformation of the original disulphide bonds of the allergen, by slow aerial oxidation of the reduced form, was investigated. The reduced form of the allergen, without stabilization by iodoacetamide, was allowed to stand in 0.1 M NH_4HCO_3 , pH 8.2, for 24 hr at room temperature. Very dilute solutions of the allergen (about 0.1 mg/ml) were used for this experiment to minimize the formation of non-specific inter-chain disulphide linkages, in accordance with the 'High Dilution Principle' of Ruggli.⁽¹⁹⁾

(b) *Oxidation.* Allergenic samples were oxidized with performic acid for 2½ hr at -10° using the method of Hirs.⁽²⁰⁾

(c) *Alkylation.* Crestfield *et al.*⁽²¹⁾ have described a method for the selective alkylation of histidine residues in ribonuclease with iodoacetic acid at pH 5.5. This method was applied in the present case to investigate the possible importance of such residues for allergenic activity in allergen I-B, although groups other than histidine could well be involved.

(d) *'Dansylation.'* Derivatives of the allergens containing, on average, 1, 2 and 5 DNS residues per molecule were prepared by mixing the appropriate molar proportions of DNS-Cl, dissolved in acetone (1 mg/ml), with 0.5 ml of a solution of the allergen in 0.1 M NaHCO_3 (4 mg/ml). The mixture was allowed to stand for ½–1 hr at room temperature, and the dansylated allergen was separated from traces of DNS-OH and DNS-NH₂ by passage through a Sephadex G-25

* Reaction with the fluorescent dye, 1-dimethylamino naphthalene 5-sulphonyl chloride (DNS-Cl).⁽¹⁷⁾

column.⁽¹⁷⁾ By spectrofluorometric analysis of the column effluent, virtually all the DNS-Cl was shown to have combined with the allergens.

4. *Immunological investigations.* The absence of a simple assay for allergenic activity *in vitro* has been noted by several authors (cf. Stanworth⁽⁹⁾). The 'prick-test' method of Squire,⁽²²⁾ in which a drop of test solution is pricked directly into the skin of an allergic individual, was used throughout as a direct assessment of allergenic potency. The limitations of this method have already been noted.⁽²⁾ The skin activities of enzymically hydrolysed samples⁽¹⁾ were examined after the digests had been passed down a Sephadex G-25 column to eliminate any possible traces of undigested material. Immunodiffusion experiments were carried out as previously described.⁽²⁾

5. *Sedimentation studies in solutions containing SDS.* Sodium dodecyl sulphate (SDS) has often been used as a disaggregating agent for protein complexes (e.g. Brand and Johnson,⁽²³⁾ Steiner and Edelhoch⁽²⁴⁾). Since it produces disaggregation at a much lower concentration than either urea or guanidine, SDS possesses certain practical advantages in studies using the ultracentrifuge (e.g. it does not produce a markedly curved 'base line'). Neutral or acidic buffer solutions of native allergen, I-B (or a stabilized, reduced form of this allergen, I-B_{red}) containing 0.5 g of SDS per 100 ml of buffer, were examined by ultracentrifugation at 59,780 rev/min. Schlieren optics and a synthetic boundary cell were used for all experiments. The difference in sedimentation patterns between dialysed and non-dialysed solutions was investigated, and also the loss of protein occurring on dialysis against buffer containing SDS (Table 4).

RESULTS

1. Monosaccharide composition

Alkaline and acidic hydrolysates. The main carbohydrate-containing fraction from the Ba(OH)₂ hydrolysate of allergen I-B was eluted from the Sephadex G-25 column soon after the void volume. High voltage paper electrophoresis at pH 2.0 of this fraction and of a sample which had been subjected to partial HCl hydrolysis, each produced major carbohydrate-staining bands of very low positive mobility (0.14–0.18 times the mobility of aspartic acid). These carbohydrate bands were well separated from contaminating peptide material of considerably higher mobility, and did not, themselves, stain with ninhydrin. In certain hydrolysates, faster-migrating fractions which stained very weakly for carbohydrate were observed; these have not been studied.

Acidic hydrolysis (2 N H₂SO₄ for 3 hr at 105°) of the main carbohydrate fraction from the alkaline hydrolysate yielded the component monosaccharides. Certain of these monosaccharides, or those obtained directly from the carbohydrate band of partial acidic hydrolysates of I-B, were tentatively identified as mannose, galactose and xylose (or glucose) by paper electrophoresis in borate at pH 10. Table 1 gives the migration rates of marker sugars and monosaccharides present in the above hydrolysates, relative to glucose: all values have been corrected for electro-osmosis. Rates for the marker sugars agree well with those already reported.^(13,25,26)

No amino acids were detected by the dansylation procedure in the carbohydrate fraction from the alkaline hydrolysate of allergen I-B.

Monosaccharides, obtained directly from the carbohydrate band of the original partial acidic hydrolysate of I-B, gave spots with the same *R_G* values as galactose and mannose and/or sedoheptulose when examined by paper chromatography in BuOH-HAc-H₂O (Table 2).

Enzymic hydrolysates. Successive hydrolysis of either I-B or I-C with trypsin, chymotrypsin, pronase and, in certain cases, carboxypeptidase gave single slow-migrating, carbohydrate-staining bands on high voltage electrophoresis at pH 2, with migration rates relative to aspartic acid of 0.07–0.09 (cf. ref. 2). These bands stained quite strongly with the chlorine stain,⁽²⁷⁾ but were not detected with

TABLE 1. CORRECTED MOBILITIES, *M_G* (RELATIVE TO GLUCOSE), OF CARBOHYDRATES IN 0.2 M BORATE BUFFER AT pH 10.0

Sugar	<i>M_G</i> *
L-arabinose	0.96
D-xylose	1.02
D-glucose	1.00
D-galactose	0.91
D-mannose	0.70
D-glucoheptulose	0.88
sucrose	0.16
D-glucosamine	0.23
D-galactosamine	0.36
D-glucuronic acid	1.17
Partial HCl hydrolysate of allergen I-B	0.70, 0.90, 1.01
Partial HCl hydrolysate of glycopeptide from I-B	0.70, 0.90

* Average values from 3–5 different experiments. Error = ±1%.

ninhydrin-CdAc. Acidic hydrolysis of these glycopeptide fractions (2 N H₂SO₄ or 2 N HCl for 3 hr at 105°), followed by electrophoresis in borate, indicated a similar monosaccharide composition to that obtained after partial acidic hydrolysis of the whole molecule (Table 1). Dansylation of the glycopeptides before acidic hydrolysis revealed that the only *N*-terminal group was aspartic acid; dansylation after complete acidic hydrolysis showed that only Asp., Glu., Gly., Ala. and Phe. were present in all glycopeptide fractions of I-B and I-C prepared as above.

TABLE 2. RATES OF MIGRATION, *R_G* (RELATIVE TO GLUCOSE), OF CARBOHYDRATES ON PAPER CHROMATOGRAPHY IN *n*-BuOH : HAc : H₂O (4 : 1 : 5)

Sugar	<i>R_G</i>
L-arabinose	1.23
D-xylose	1.35
D-glucose	1.00
D-galactose	0.91
D-mannose	1.17
D-glucoheptulose	0.80
D-sedoheptulose	1.18
D-glucosamine	0.71, 1.17
Partial HCl hydrolysate of allergen I-B	0.90, 1.17

2. The effect of chemical and enzymic modification on allergenic and antigenic activities

Skin-test and immunodiffusion results. Table 3 gives skin-test results for both the chemically-modified and native allergenic samples and Fig. 2 shows corresponding immunodiffusion results. Since these samples were not skin-tested extensively, it is not possible to give an accurate assessment of their activities at this stage.

The introduction of one or two DNS groups produced no marked decrease in skin activity, although a slight decrease was apparent when five DNS groups were introduced: the DNS-labelled molecules were still active antigenically. Reduction with 2-mercaptoethanol, followed by stabilization of the sulphhydryl groups,

TABLE 3. SKIN-TEST RESULTS FOR CHEMICALLY-MODIFIED ALLERGENS

Sample	Concn. mg/ml	Modification	Product of wheal diameters*		
			Modified material	Control	
I-B	0.01	Partial	(1)	20	25
I-B	0.01	dansylation	(2)	25	25
I-B	0.01	No. of DNS	(5)	7	25
I-C	0.01	groups introduced	(2)	20	30
II-B	0.01	in parentheses	(2)	20	35
I-B	0.01	Reduction and	}	5	40
I-B	0.01	stabilization		3	25
I-C	0.01	of -SH groups		1	30
I-B	0.01	Reduction and		10	40
I-B	0.01	reoxidation		0	25
I-B	0.01	Oxidation	}	0	40
I-C	0.01	with		0	30
II-B	0.01	HCOOOH		0	30
I-B	0.01	ICH ₂ COOH at pH 5.5		4	25
I-B	0.1	Trypsin	}	0	25
I-C	0.1	at pH 7.9		0	30
II-B	0.1	Chymotrypsin		0	35
I-B	0.1	at pH 7.9		0	25
I-C	0.1			0	30
I-B	0.5	0.15 N Ba(OH) ₂		0	40
I-B	0.1	100° for 5 min		0	40

* Average for duplicate tests on three individuals, except for results with II-B where only tests on one individual sensitive to this fraction are quoted.

'Control' refers to untreated allergens.

produced a marked drop in skin activity and antigenicity although, in one experiment, allergenicity was recovered partially after aerial reoxidation of the reduced form. Alkylation also produced a decrease in allergenic, but not antigenic activity, and oxidation or alkaline hydrolysis completely destroyed both activities. Small skin reactions were occasionally observed with enzymic hydrolysates of the allergens at high concentration (about 1.0 mg/ml) in cases where non-hydrolysed material had not been removed by Sephadex treatment. However, none of the fractions obtained from Sephadex G-25 chromatography of tryptic or chymotryptic digests (where un-digested material had been eliminated) were found to be allergenic.

Inhibition results. Complete digestion of allergen I-B by either trypsin, chymotrypsin or pepsin gave fragments which showed no tendency to inhibit antigen-

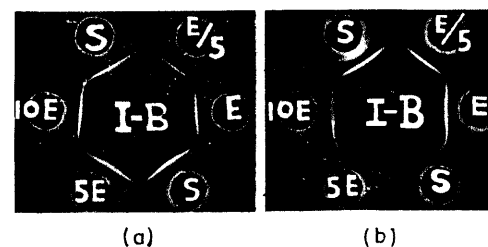


FIG. 1. Gel diffusion patterns showing attempted inhibition of precipitin line formation between allergen I-B (concn. 0.05 per cent in saline: approximately 7.5 γ per well) and rabbit antiserum S.

10E, 5E, etc. refer to the number of equivalents of the chymotryptic (a) or tryptic (b) digest of I-B mixed with antiserum S prior to diffusion.

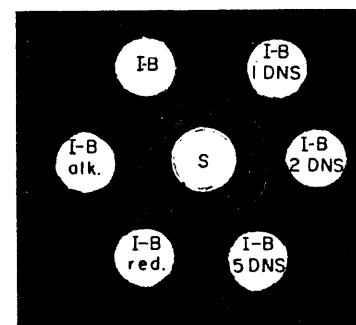


FIG. 2. Immunodiffusion patterns between antiserum S and native or chemically-modified allergen I-B (concn. 0.02 per cent in saline: approximately 3.0 γ protein per well).

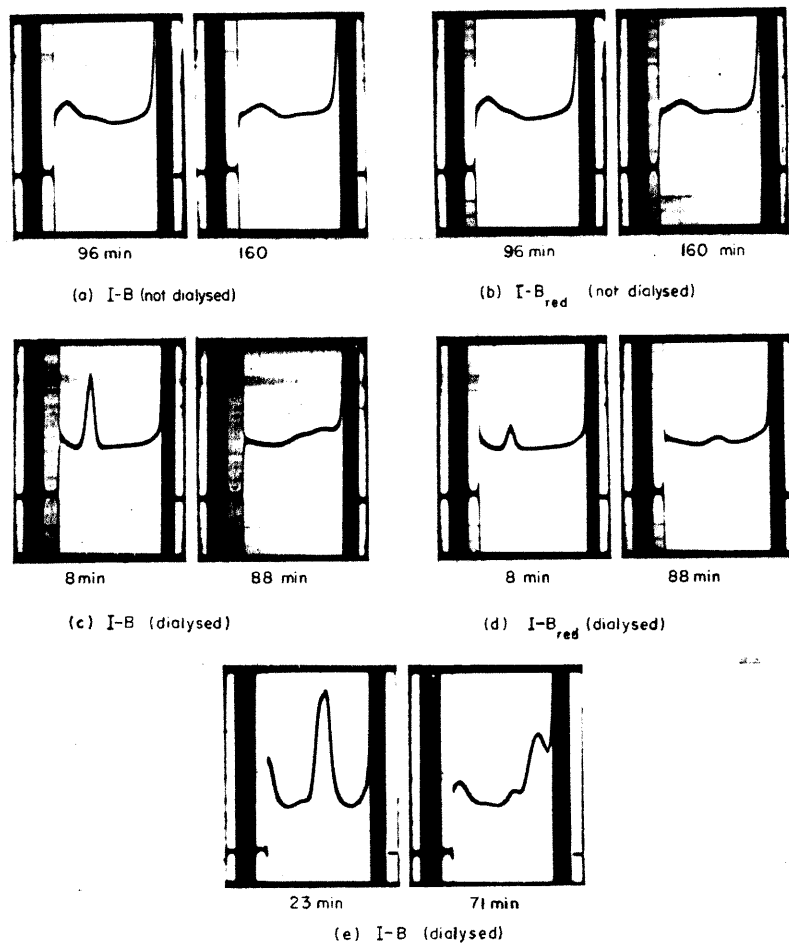


FIG. 3. Sedimentation patterns, at $18 \pm 2^\circ$ for various allergen (I-B and I-B_{red}) solutions containing SDS (0.5 g/100 ml). Speed: 59,780 rev/min. See Table 4 for further details.

antibody precipitin formation between I-B (or I-C) and rabbit anti-rye pollen serum, even where up to twenty equivalents of the enzymic digests were mixed with the antiserum prior to diffusion (Fig. 1). The failure of the glycopeptide fragment from I-B to produce similar inhibition has previously been reported.⁽²⁾ However, inhibition of precipitation was observed where only partial chymotryptic or peptic hydrolysis had occurred, due to the use of enzyme solutions of low activity. It was confirmed that when either I-B or I-C were mixed with the antiserum prior to diffusion, and these mixtures were allowed to diffuse against I-B or I-C, no precipitin lines were observed.

3. Disaggregation experiments

Figure 3 shows the sedimentation patterns for solutions of I-B or I-B_{red} in SDS solution (0.5 g/100 ml) and Table 4 gives the sedimentation coefficients of the components present. These 's' values were corrected to water at 20° , assuming the same correction factors (i.e. the same η , ρ and $\bar{v}$) as for buffer solutions without

TABLE 4. SEDIMENTATION COEFFICIENTS OF COMPONENTS PRESENT IN SOLUTIONS CONTAINING SDS (0.5 g/100 ml)

Sample	pH of solution†	Protein concn. g/100 ml* Before dial.	After dial.	<i>s</i> ₂₀ values (Svedberg units)
a I-B	7.8	1.0	Not dialysed	1.7; 3.2
b I-B _{red}	7.8	1.0		1.6; 3.8
c I-B	7.8	2.0	0.7	1.8; 3.4
d I-B _{red}	7.8	1.0	0.15	2.84
e I-B	3.0	2.0	1.33	1.7; 3.8 (1.1)†
f I-B	7.8	No SDS		2.89 (ref. 2)

* Protein concentrations determined on the basis of freeze-dried material before dialysis and using the interferometer after dialysis.

† Sedimentation coefficient for SDS micelles.

‡ Buffer solutions used were as follows: phosphate-NaCl, pH 7.8, I = 0.1 (5.790 g Na₂HPO₄·12H₂O, 0.197 g KH₂PO₄ anhydrous, 2.923 g NaCl); citric-Na citrate, pH 3.0, I = 0.6.

SDS. Synthetic boundaries were formed successfully only where allergen solutions had been previously dialysed: in other cases, complete mixing occurred and boundaries formed at, and moved away from, the meniscus as in experiments utilizing normal cells. Since a considerable proportion of the solute was found to diffuse through the dialysis bags, particularly in alkaline solution (Table 4), it seems probable that the presence of this rapidly-diffusing material was responsible. The formation of a slowly sedimenting boundary at the solvent meniscus in the acidic buffer (Fig. 3e) could almost certainly be attributed to the presence of large SDS micelles at this pH.

DISCUSSION

Unequivocal identification of the monosaccharides present in group I rye allergens has not yet been accomplished, but it seems probable that galactose and mannose are the major hexoses present. The heptose and the pentose, suggested by earlier spectrophotometric determination,⁽²⁾ are possibly sedoheptulose and xylose, respectively.

The nature of the glycopeptide linkage is of considerable interest. A glycosidic ester linkage would account for the presence of the amino acids aspartic acid and glutamic acid in the glycopeptide obtained by enzymic digestion, and also the alkali-lability of the glycopeptide linkage⁽²⁷⁾ (no peptide material was detected in the carbohydrate fraction obtained from the $\text{Ba}(\text{OH})_2$ hydrolysate). Linkage through a hexosamine may be ruled out since this type of sugar was not detected either by spectrophotometric assay or by analysis of a partial acidic hydrolysate of allergen I-B on the Amino Acid Analyser.⁽²⁸⁾ However, the glycopeptide from the trypsin-chymotrypsin-pronase digest was not susceptible to treatment with hydroxylamine,⁽¹⁾ toward which the glycosidic ester linkage is normally labile (Gottschalk *et al.*⁽²⁷⁾). A further possibility might be an N-glycosidyl linkage of the carbohydrate either through glutamine or asparagine. The expected stability of this type of linkage should be in accordance with the observed properties. Further investigation of the chemical nature of the carbohydrate moiety and linkage was prevented by shortage of material and, when it seemed probable that carbohydrate was not essential for allergenic activity,^(2,3) the enquiry was not pursued further.

The general nature of allergenic determinants of pollen allergens may now be considered in the light of current knowledge of rye and other pollen allergens.^(6,7,8,29,30) Studies on the inhibition of immunodiffusion precipitin line formation, between the group I rye allergens and rabbit anti-rye pollen sera, showed that fragments from complete proteolytic digestion of I-B with trypsin, chymotrypsin or pepsin, failed to produce inhibition: it therefore seems highly improbable that such digests contained the main *antigenic* determinant groups. All skin activity, and therefore allergenicity, was destroyed by complete proteolytic digestion. Similar results have been quoted by King⁽³⁰⁾ for a total digest of group I ragweed allergens (his 'fraction IV') with nagarse. A partial digestion of this fraction with pepsin yielded a high molecular weight material (about 30,000) which retained a large proportion of the original skin activity (King and Norman⁽²⁹⁾). Hence, although the major group I rye and ragweed allergens possessed different susceptibilities toward proteolytic enzymes,⁽¹⁾ in each case small fragments from complete hydrolysates seemed to have lost all antigenic and allergenic properties. Certain larger fragments from ragweed (and possibly rye) appear to have retained these properties to a large degree.

A precise interpretation of the inhibition experiments in terms of allergenic, rather than antigenic, activity depends on establishing whether the allergenic determinant groups for man also act as prominent antigenic determinants for the rabbit. Although this has not yet been proved, there are good reasons for selecting it as a useful working hypothesis. Human allergic antibody ('reagin') has been classified as an IgA antibody,^(31,32) and this type of immunoglobulin has been shown to possess a very similar gross structure to the normal IgG group, particularly in the regions attributed to the antigenic combining sites (Cohen⁽³³⁾). Mechanisms of synthesis are thus probably similar in the two cases, and it seems probable that a foreign chemical grouping constituting an *allergenic* determinant in allergic humans would, for normal individuals and probably rabbits, act also as an *antigenic* determinant. Hence, any peptide fragment derived from an allergen and failing to inhibit the homologous antigen-antibody precipitin reaction by

virtue of the absence in it of a prominent antigenic determinant, would seem also not to possess the equivalent allergenic determinant.

The results of the inhibition experiments and the direct skin tests of chemically-modified rye allergens are seen to be complementary, in agreement with the above hypothesis. Slight distortion of the tertiary structure, such as introduction of one or two DNS groups, failed to destroy the allergenic and antigenic activities, but more extensive disruption completely destroyed them. Similarly, reduction followed by aerial reoxidation, in favourable cases, produced material possessing some allergenic activity. Alkylation of the major rye allergens and acetylation of the major ragweed allergens⁽³⁰⁾ have both been shown to cause a diminution in allergenicity, but apparently not in antigenicity. It is not possible, however, to decide from these results, whether the diminution in skin activity arises from the introduction of the new groups or from a more subtle subsequent rearrangement of the tertiary structure of the molecules. The results with both enzymically-digested and chemically-modified allergens would suggest that the integrity of a large part of the tertiary peptide structure is essential for full allergenic activity. Further, earlier inhibition results suggest that the carbohydrate moiety is probably not implicated in this active centre.^(2,3)

More recent skin-test results⁽³⁴⁾ on twenty-two allergenic individuals, using allergen I-B treated with the carbohydrases cellulase, β -glucosidase, β -galactosidase, β -amylase or lysozyme, showed no loss in activity as compared with control solutions of untreated allergen. These enzyme preparations (from Koch-Light Ltd.) were found to be devoid of proteolytic activity and contained no carbohydrate detectable by paper electrophoresis in borate buffer. Carbohydrate fragments were detected by electrophoresis of the allergenic digests with cellulase and β -galactosidase, but not in other enzymic digests (results to be reported in detail in a later paper). Hence removal of at least part of the carbohydrate by cellulase or β -galactosidase was shown to produce no measurable diminution in allergenicity. These recent results considerably substantiate the suggestion that carbohydrate plays no direct role in pollen allergenic activity.

Sedimentation studies of allergen I-B in SDS solution have shown that the allergen may disaggregate under favourable conditions. A similar mechanism may allow penetration of major allergen molecules through the mucous membranes of allergic individuals to produce initial sensitization and subsequent allergic reaction. The pronounced disaggregation occurring at alkaline pH (Fig. 3) may also account for the blurred starch gel patterns for both I-B and I-C in gels containing urea at pH 8.5.⁽²⁾ Such disaggregation of the major allergens implies the existence of more than one peptide chain, but since a low recovery (10 per cent) of a single N-terminal group (isoleucine) was observed,^(1,2) other N-terminals must be 'blocked'. A thorough reinvestigation of the disaggregation process may well throw considerable light on the mechanism of allergic sensitization and the overall structure of the major rye pollen allergen molecules.

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REFERENCES

- ¹ JOHNSON P. and MARSH D. G., *Immunochem* **3**, 91 (1965).
- ² JOHNSON P. and MARSH D. G., *Europ. Polym. J.* **1**, 63 (1965).
- ³ JOHNSON P. and MARSH D. G., *Nature, Lond.* **206**, 935 (1965).
- ⁴ FEINSTEIN A., Ph.D. Thesis, Cambridge University (1961).
- ⁵ HAUROWITZ F., *Ann. Rev. Biochem.* (Edited by LUCK J. M., ALLEN F. W. and MACKINNEY G.) Vol. 29, p. 609. Annual Reviews Inc., Palo Alto (1960).
- ⁶ HERBERTSON S., PORATH J. and COLLEDAHL H., *Acta Chem. scand.* **12**, 737 (1958).
- ⁷ AUGUSTIN R., *Immun.* **2**, 148 (1959).
- ⁸ KING T. P., NORMAN P. S. and CONNELL J. T., *Biochemistry, N.Y.* **3**, 458 (1964).
- ⁹ KING T. P., NORMAN P. S. and CONNELL J. T., *Biochemistry, N.Y.* **3**, 458 (1964).
- ¹⁰ STANWORTH D. R., *Adv. Immunol.* (Edited by DIXON F. J. and HUMPHREY J. H.) **3**, 181. Academic Press, New York (1963).
- ¹¹ KING T. P. and NORMAN P. S., *Biochemistry* **1**, 709 (1962).
- ¹² TSUGITA A. and AKABORI S., *J. Biochem., Tokyo* **46**, 695 (1959).
- ¹³ MICHL H., *Monatsh. Chem.* **82**, 489 (1951).
- ¹⁴ FOSTER A. B., *J. chem. Soc.*, 982 (1953).
- ¹⁵ METZENBERG R. L. and MITCHELL H. K., *J. Am. chem. Soc.* **76**, 4187 (1954).
- ¹⁶ TREVELYAN W. E., PROCTER D. P. and HARRISON J. S., *Nature, Lond.* **166**, 444 (1950).
- ¹⁷ BARROILLIER H., *Naturwissenschaften* **42**, 46 (1955).
- ¹⁸ GRAY W. R. and HARTLEY B. S., *Biochem. J.* **89**, 59P (1963).
- ¹⁹ ANFINSEN C. B. and HABER E., *J. biol. Chem.* **236**, 1361 (1961).
- ²⁰ RUGGLI P., *Annln. Phys.* **392**, 92 (1912).
- ²¹ HIRS C. H. W., *J. biol. Chem.* **219**, 611 (1956).
- ²² CRESTFIELD A. M., STEIN W. H. and MOORE S., *J. biol. Chem.*, **238**, 2413 (1963).
- ²³ SQUIRE J. R., *Clin. Sci.* **9**, 127 (1950).
- ²⁴ BRAND B. P. and JOHNSON P., *Trans. Faraday Soc.* **52**, 438 (1956).
- ²⁵ STEINER R. F. and EDELHOCH H., *J. Am. chem. Soc.*, **83**, 1435 (1961).
- ²⁶ MICHL H., *J. Chromat.* **1**, 93 (1958).
- ²⁷ WEIGEL H., *Adv. Carbohydr. Chem.* (Edited by WOLFROM M. L. and TIPSON R. S.) **18**, 61. Academic Press, New York (1963).
- ²⁸ GOTTSCHALK A., MURPHY W. H. and GRAHAM E. R. B., *Nature, Lond.* **194**, 1051 (1962).
- ²⁹ MARSH D. G., Ph.D. Thesis, Cambridge University (1964).
- ³⁰ KING T. P. and NORMAN P. S., *Proc. 6th Int. Congr. Biochem., New York*, Abstract II-95 (1964).
- ³¹ KING T. P., Personal communication (1964).
- ³² HEREMANS J. F., HEREMANS M. T. and SCHULTZE H. E., *Clinica. Chim. Acta* **4**, 96 (1959).
- ³³ FIREMAN P., VANNIER W. E. and GOODMAN H. C., *J. exp. Med.* **117**, 603 (1963).
- ³⁴ COHEN S., *Biochem. J.* **89**, 334 (1963).
- ³⁵ MILNER F. H., Personal communication (1965). Results to be reported in detail later.

METHODS FOR THE SEPARATION, PURIFICATION AND MEASUREMENT OF NINE COMPONENTS OF HEMOLYTIC COMPLEMENT IN GUINEA-PIG SERUM

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Abstract—C'1 precipitates from normal guinea-pig serum at ionic strength 0.04 and pH 7.5. It may be purified further by reprecipitation during dialysis against 0.005 M phosphate buffer, pH 7.5. C'3b precipitates at ionic strength 0.04-0.06 and pH 5.0. It is easily purified by elution from DEAE-cellulose at about 0.12 ionic strength. C'3c precipitates at about 0.065 ionic strength. C'2 and C'3d remain soluble when $(\text{NH}_4)_2\text{SO}_4$ is added to about 2.2 M and then can be precipitated at about 3.0 M $(\text{NH}_4)_2\text{SO}_4$. These two components can be separated easily by chromatography on CM-cellulose. The sequence and the approximate ionic strength at which eight components elute from CM-cellulose is: C'2, 0.05; C'3f, 0.12; C'4, 0.13; C'3c, 0.135; C'3b, 0.14; C'3e, 0.145; C'3a, 0.18; and C'3d, 0.20. The sequence and ionic strength of elution from DEAE-cellulose is: C'3e, 0.03; C'3a, 0.035; C'3c and C'3f, 0.065; C'3d, 0.095; C'3b and C'4, 0.12. The yield of C'2 from DEAE-cellulose has been low. A substance in normal guinea-pig serum which inactivates the C'3c site on the intermediate complexes, EAb C'1,4,2,3c, EAb C'1,4,3c or EAb C'4,3c, elutes from DEAE-cellulose at about 0.05 to 0.06 ionic strength. Another substance which inactivates C'1 elutes from DEAE-cellulose at about 0.12 ionic strength.

INTRODUCTION

IN DECEMBER of 1963 a summary⁽¹⁾ was prepared which outlined the general characterization of nine fractions of guinea-pig serum which appeared to be essential for the reactivity of complement (C') in immune hemolysis. The report described the discovery that the hemolytic reactivity of the third component of complement, C'3, which was shown by Rapp⁽²⁾ in 1958 to consist of two distinct fractions, C'a and C'b, actually functioned through the sequential reactivity of six separate fractions of serum. The existence of four components, tentatively named C'3a, C'3b, C'3c and C'3d, was first described by Nishioka and Linscott.^(3,4) Further purification of these four components, as well as confirmation that the order of reaction with sensitized sheep erythrocytes (E) in the stage, EAb C'1,4,2, was C'3c, C'3b, C'3a, C'3d, was performed by Nelson, Oikawa and Rommel.^(5,6) Subsequently Inoue and Nelson^(7,8) obtained evidence that the fraction of serum designated C'3a could be separated into two distinct fractions, C'3a and C'3e, and that another essential factor, C'3f, was present in the preparations of C'2 made by the method of Borsos, *et al.*,⁽⁹⁾ as well as in fraction IV₄ of bovine plasma, which had been used by Nishioka and Linscott as a source of C'3d. Repeated trials have indicated that the order of reaction of guinea-pig components was C'1, C'4, C'2, C'3c, C'3b, C'3e, C'3f, C'3a and C'3d.

At about the same time that these data were emerging from studies on guinea-pig serum, an independent investigation of human C' was proceeding by Muller-Eberhard and collaborators. Their work indicated that at least three fractions of