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Grass Pollen Allergens

IV. THE ISOLATION OF SOME OF THE PRINCIPAL ALLERGENS OF *PHLEUM PRATENSE* AND *DACTYLIS GLOMERATA* AND THEIR SENSITIVITY SPECTRA IN PATIENTS

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Summary. Cocksfoot and Timothy pollen extracts are each found to contain at least fifteen components antigenic in rabbits. Most of these can also be allergens for man, but only a few are regularly so. These 'principal' allergens have now been isolated in highly purified form.

Procedures are given for a simple method of preparing extracts for clinical purposes and for the partial separation, concentration and purification of the allergens by means of differential extractions of the pollens and by means of ultrafiltration, isoelectric precipitation and salt fractionations (at acid and neutral pH) of the extracts. Isoelectric precipitations gave highly pigmented acid complexes, two of which moved as single sharp peaks at pH 7.4 in free electrophoresis, but proved to be hardly active by skin tests. Acid NaCl fractionation of the remainder resulted for Cocksfoot and Timothy in the isolation of a nearly white powder (T21.111121112 = T21B) which was weight for weight 1000–10,000 times as active as the pollen from which it had been derived. The powders have retained their activity for 7 years. By gel diffusion tests, they were found to contain two antigens (one in each preparation) which were immunologically partially related, but the Timothy preparation contained in addition the 'innermost' 'twin' antigens specific for Timothy that we had discovered previously in the crude extracts by gel diffusion methods. Skin reactions could be elicited in hay-fever subjects by prick tests with concentrations of 10^{-9}–10^{-8} g./ml., which is equivalent to intradermal injections of 10^{-11}–10^{-10} mg. and represents a 300-fold purification with respect to the concentrates of crude pollen extracts prepared by ultrafiltration and dialysis.

Fractionation on DEAE-cellulose of one of the highly purified Timothy preparations (T21.11112112 = T21A) and other, crude Timothy and Cocksfoot extracts resulted in considerable and reproducible separation of the various antigens, with no indication of the continuous dissociation into smaller fragments reported in recent publications. The immunologically related antigens of Cocksfoot and Timothy were among a number not retained by the DEAE-cellulose at pH 7.4–8.5. These proved to be skin reactive in all the patients tested. When this fraction was prepared from T21.11112112(= T21A) only one main line (plus a faint subsidiary line) was found in the gel diffusion tests. At pH 6.4 the important dissimilar Cocksfoot and Timothy allergens were eluted; the fraction derived from T21A contained only the special 'innermost' twin antigens in roughly a further ten-fold purification over and above that achieved in the acid NaCl fractionation.

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INTRODUCTION

With the help of preparative immunoelectrophoretic procedures and other gel diffusion methods it has been possible to characterize the individual pollen antigens without having to isolate them. Most of them proved to be very stable low molecular weight proteins with an electrophoretic mobility spectrum wider than that of serum proteins; the skin reactivities for hay-fever subjects could be destroyed by proteolytic digestion (Augustin, 1959b, 1959c) and the antigens associated with skin reactivity were found to have the mobilities of α and β serum globulins. The most electropositive heat and acid labile pollen proteins, the 'A-antigens', present in most English grass pollens, were found not to be allergens for man although they induce precipitin formation in rabbits more readily than the other antigens and have been used for assaying pollen extracts by immunological tests (Wodehouse, 1955; Augustin and Hayward, 1955a; Hayward and Augustin, 1957).

Clinical trials (Frankland and Augustin, 1954) have shown that a purified pollen protein concentrate (now known to have lacked the A-antigens) was as effective in the hyposensitization of hay-fever subjects as the pollen extracts from which it had been derived. There was no difference in the clinical efficacy (Frankland and Augustin, 1962) of heated (4 minutes at 100°) and unheated pollen extracts (only the latter containing intact antigen A), whereas the ultrafiltrates of these extracts were clinically inactive (Frankland and Augustin, 1954) and protein digests (pepsin) hardly active in patients (Frankland and Augustin, 1962).

Meanwhile progress had been made with the immunochemical characterization of reagins and blocking antibodies (Stanworth, 1959; Augustin and Hayward, 1960a, 1960b; Augustin 1960a). Antibodies to pollen constituents in man have been measured by means of passive haemagglutination methods (Orlans, Rubinstein and Marrack, 1953; Feinberg and Flick, 1954; Sehon, 1958, 1960; Augustin, 1960a), but it is by no means clear whether these methods measure blocking antibodies and/or reagins (Augustin, 1960a, 1961; Augustin and Hayward, 1960b). In fact, the follow-up of the recent repository injection treatments by immunological tests has shown that there is no correlation of the passive haemagglutination titres with titres for blocking antibodies, the reaginic titres or with clinical improvement (Arbesman and Reisman, 1961), thus confirming the observations of Orlans *et al.* (1953); i.e. the agglutination methods measure most likely antibodies for pollen constituents other than the allergens (in addition to the blocking antibodies which may form a variable and perhaps small portion of the total sum of the agglutinating antibodies) whereas reagins may escape measurement altogether since their concentration in the serum seems to be very low at all times (Augustin and Hayward, 1960b).

The mechanism of the usual hyposensitization treatment of allergic subjects (Noon, 1911) by a course of injections with crude allergen extracts is incompletely understood (Augustin, 1961) and there is a danger that reagin formation may be induced to constitutents of a crude extract to which the patient has not been previously sensitive. Indeed, it has recently been found that reagin formation may be induced even in supposedly 'normal' subjects during injection treatment with ragweed pollen antigens (Richter, Sehon, Gordon, Gregoire and Rose, 1958; Becker, Sparks, Feinberg, Patterson, Pruzansky and Feinberg, 1961). We do not know whether such newly formed reagins are or can become clinically important, but under the circumstances it seems desirable to prevent their formation by avoiding injecting, inadvertedly, antigens which, in particular patients, are not yet allergens. This would require determining the individual hypersensitivity spectra of patients in terms of the isolated known pollen antigens and may not usually be a practical proposition. It should, however, be undertaken at least in a limited number of patients to gain a better understanding of the methods of hyposensitization generally used.

For all these reasons — and not only to enable us to standardize allergen preparations for clinical use — the isolation of pollen allergens in pure form has become once more one of the most pressing problems facing immunochemists working in this field.

The present work is a contribution to the purification of grass pollen allergens. It includes details of the preparation of the protein concentrates used in our early clinical trial (Frankland and Augustin, 1954) which have so far been published only in abstract form (Augustin, 1953b), the differential extraction of grass pollens by aqueous solvents, the isolation of highly purified allergens from these preparations by means of fractionations on diethylamino-ethyl cellulose and also the sensitivity spectra of individual hay-fever subjects in terms of the purified antigens.

MATERIALS AND METHODS

GRASS POLLENS. Pollens of Timothy (*Phleum pratense*) and Cocksfoot (*Dactylis glomerata*), freshly collected and dry, were available from the 'pollen farm' at Weybridge (which has been attached to the Wright-Fleming Institute since before 1911) where the grasses are grown and the pollen collected under the supervision of a resident botanist.

STANDARD 'POLLACCINE'. As before (Augustin, 1959a, 1959b, 1959c), a carefully stored (0–4°) preparation of the current year's commercially available mixed pollen extract of Timothy and Cocksfoot, prepared at the Wright-Fleming Institute under standard conditions, served as the reference standard. These standards were prepared by using 10 ml. of extracting fluid per g. of pollen (see later) and were sterilized by Seitz filtration. Prepared in the same way each year from pollens grown and collected under standard conditions, the analytical figures for these extracts have remained fairly constant. Over a period of 7 years figures for total nitrogen varied from 1.18–1.43 mg./ml. and for phosphotungstic acid precipitable nitrogen from 0.25–0.32 mg./ml.

As before, these standard extracts were assumed to contain 100,000 Noon units (skin tests on the current and previous year's standards were each year performed on about 100 patients to assure proper continuity) and 100,000 A-units (gradient diffusion method) per ml.

EVANS' FLUID. This buffer contained 0.36 g. KH_2PO_4, 1.431 g. Na_2HPO_4, 5 g. NaCl and 4 g. phenol in 1,000 ml. of distilled water (Evans, 1922).

COCA'S SOLUTION. 1,000 ml. contained 5 g. NaCl, 2.75 g. Na_2HCO_3 and 4 g. phenol in distilled water (Coca, 1922).

STABILIZING BUFFER. This buffer was used routinely for the dilution of pollen extracts which had to be stored for clinical use. It contained, in 1000 ml., 5 g. of glucose, 5 g. of glycine, 0.363 g. KH_2PO_4, 1.43 g. $Na_2HPO_4.12H_2O$ and 2.5 g. NaCl. The preservative was either 0.5 per cent phenol or 1/10,000 merthiolate.

ANTISERA. Rabbits were immunized with Timothy and Cocksfoot preparations by the multiple depot method used in previous investigations (Hayward and Augustin, 1957). This method ensures that antibodies in high titres are produced against the largest possible number of determinants for periods of up to 4 months before reinjections become necessary.

CUTANEOUS TESTS. Prick tests of serial dilutions of the materials under test in comparison with serial dilutions of the standard were done as described previously (Augustin, 1959a). The method has an error of $\pm$ 50 per cent.

ANTIGEN-A ASSAY. This was performed by the gradient gel diffusion methods with antisera which contained mainly antibodies for the A-antigens (Augustin and Hayward, 1955a; Hayward and Augustin, 1957).

GEL PLATE TESTS. These were mostly done as micro modifications of the original Ouchterlony method (Ouchterlony, 1953). One per cent solutions of agar (Augustin, Hayward and Spiers, 1958) were poured on to microscope slides to a depth of 1.5 mm. and the holes cut as required with cork borers and Pasteur pipettes. After the wells had been charged, the slides were allowed to develop in Perspex boxes lined with moist paper and covered with tightly fitting lids. They were read in the usual manner after 24 and 48 hours.

DIALYSES, ULTRAFILTRATIONS AND PRESSURE DIALYSES. Dialyses were performed in Visking seamless sausage skins, 24/32, and in skins of narrower diameter (Hudes Merchandising Corp., Gloucester Place, London) against running tap water or distilled water or by means of the rotating-membrane technique (McMeekin, 1939).

Ultrafiltrations were done through collodion-covered porcelain filters as described in a previous paper of this series (Augustin, 1959a) and pressure dialyses in Visking seamless sausage skins encased in tightly fitting nylon bags left open at the bottom end. Pressures of 10 lb. were applied through glass bulbs communicating with the insides of the Visking cases, the necks of the bulbs being pushed through the rubber stopper around which the two casings were tightly fixed with twine or hose clips.

IMMUNOELECTROPHORESES. These were done as slight modifications of Scheidegger's (1955) micromethod on microscope slides in batches of fifteen (thirty analyses) as described in previous publications (Augustin and Hayward, 1960b, 1961).

ANION EXCHANGE CHROMATOGRAPHY. Peterson and Sober's method (1956) was used with the buffers described in a previous publication (Augustin and Hayward, 1960b). They were 0.01 M Na-phosphate, pH 7.5 (buffer I), 0.02 M Na-phosphate, pH 6.4 (buffer II), 0.05 M Na-phosphate (buffer III), 0.3 M Na-phosphate (buffer IV) and 0.4 M Na-phosphate containing 2 M NaCl (buffer V). In addition buffer O was used (before buffer I) which was 0.005 M Na-phosphate of pH 8.0.

PROTEIN CONCENTRATIONS. These were estimated by their absorption at 280 mμ in a Beckman or in a Unicam SP600 spectrophotometer.

MICRO-KJELDAHL ESTIMATIONS. These were done according to Markham (1942).

CARBOHYDRATE ESTIMATIONS. These were done by a modification of the Orcinol method using galactose as the standard (Friedmann, 1949).

PH MEASUREMENT. Standard glass electrode pH meters (Marconi) or, for rough adjustments, indicator papers were used.

FREE ELECTROPHORESES. These were carried out in a Perkin-Elmer electrophoresis apparatus.

RESULTS

EXTRACTION PROCEDURES

Noon (1911) originally prepared his solutions by extracting a given weight of pollen in fifty times its weight of water, with freezing and thawing, 1 Noon unit being the activity extractable in this way from 10^{-6} g. of Timothy grass pollen.

We accordingly extracted 0.5 g. of fresh Timothy pollens with 25 ml. of distilled water, freezing and thawing five times (over a period of 24 hours) and then filtering over a small pad of Super-Cel. The solutions so obtained were acid and less strong than our routine pollen extracts ('Pollaccine'); the latter were finally prepared by extracting mixtures of acetone-defatted Cocksfoot and Timothy pollens with ten times their weight of Coca's or

Evans' fluid over periods of about 72 hours, with occasional shaking, but without freezing or thawing. As extraction procedures appear to vary from laboratory to laboratory and no systematic extraction studies of grass pollens appear to have been published, some of our own developments are presented here.

Repeated Extractions with Coca's Solution

700 g. of fresh Cocksfoot pollen were first defatted by extraction with 7000 ml. of acetone in a mechanical shaker (three times for 30 minutes, in 1 litre lots, filtrations being quickly achieved on large Buchner funnels over Whatman No. 54 filter paper). The residue (defatted pollen) was then similarly extracted with 7000 ml. of Coca's fluid, a trace of silicon antifoam (MS antifoam A, Hopkins & Williams Ltd.) being added to prevent foaming; the antifoam preparation had no effect on activity. The pollen grains swelled under this treatment and were filtered off over a pad of Hyflo Super-Cel (Johns Manville Co., London, S.W.1), the filtrate being named extract A. The compressed pollen residue was easily lifted off the Super-Cel pad and was extracted, as before, with, this time, 3500 ml. of Coca's solution; this gave extract B. Two further similar extractions (3500 ml. each) gave extracts C and D.

Each of the extracts were then concentrated by ultrafiltration through collodion-coated porcelain filter candles (Augustin, 1959a). The ultrafiltered concentrates were then dialysed exhaustively against distilled water and freeze dried. The non-hygroscopic solids were then weighed and assayed by skin tests for biological activity and by gel diffusion tests for antigen-A content.

The combined ultrafiltrates were ultrafiltered once more, giving again a strongly pigmented concentrate which also was freeze dried and assayed.

The results of this and a similar extraction of Timothy pollen are given in Table 1.

TABLE 1

REPEATED EXTRACTIONS OF TIMOTHY AND COCKSFOOT GRASS POLLENS WITH COCA'S SOLUTION

The C109 extracts were derived from 700 g. Cocksfoot pollen and the T112 extracts from 400 g. Timothy pollen. FD = freeze dried.

Sample	*Freeze-dried weight (g.)*	*Noon units/mg.*		*Total activity of extract (in 10^6 Noon units) of A-antigen*
		Skin test	*Diffusion test (A-antigen)*	
C109A	11.0	30,000	30,000	330
C109B	7.5	100,000	50,000	375
C109C	5.0	20,000	25,000	153
C109D	1.4	10,000	20,000	28
C109UF	1.1	1,000	4,800	5
				Total 863
T112A	7.53	50,000	40,000	300
T112B	1.9	50,000	20,000	40
T112C	0.8	20,000	20,000	16
T112D	0.8	10,000	25,000	20
				Total 376

From this it appears that Timothy pollen is more readily extracted than Cocksfoot, but that the total amounts of activity (and A-antigens) extracted are roughly the same for both pollens. Somewhat more solids (and activity) were, however, extracted from the Cocksfoot pollens. The ultrafiltrates of Cocksfoot always contained less than 1 per cent of the starting activity while those of Timothy varied and sometimes contained as much as 5 or 6 per cent in these large-scale preparations from fresh pollens. It seems that Timothy pollens contain larger quantities of dialysable allergens than Cocksfoot pollens. This had not been observed in our early investigations which were concerned with the ultrafiltration of 'Pollaccine' solutions (i.e. mixtures of extracts of Timothy and Cocksfoot) which had been preserved in the cold (4°) for periods of a year or more.

TABLE 2

EXTRACTION SCHEME

(EXTRACTS I)

Code letter	*Time of extraction*			*Total time of extraction*
	Stirring	*Shaking*	*Standing* (0–4°)	
G	—	1 min.	—	1 min.
H	1 min.	—	—	1 min.
J	30 min.	—	1 hr.	1½ hr.
K	—	1 min.	1 hr.	1 hr.
L = R	30 min.	—	5 hr.	5½ hr.
M = S	30 min.	—	24 hr.	24 hr.
P	—	1 min.	—	1 min.
Q	—	1 min.	1 hr.	1 hr.

All the pollens were extracted twice; first extractions are indicated as 'I' and second extractions as 'II'. All the second extractions were allowed to proceed overnight and the code letters of 'II' extracts do not refer to times of extractions, but merely serve to relate the 'II' extracts to the appropriate 'I' extracts.

The Differential Extractions of Pollens and the large-scale Preparation of Extracts

While it is quite easy to filter pollen extracts over Super-Cel immediately they have been prepared, the further preparation of sterile solutions for clinical purposes is made difficult by crystallization of 'Dactylen' (Augustin, 1959a) which continues for prolonged periods and makes large-scale Seitz filtration (and clarification) through a small filter press very slow; the filter pores become clogged through incipient crystallization. Attempts have therefore been made to dissociate the extraction of these crystalline glucosides and pigments from that of the active materials and prepare at the same time more highly purified starting materials.

One lot each of defatted Timothy and Cocksfoot pollens were treated for varying lengths of time in various ways (Table 2) with 10 ml. quantities of Coca's solution (C123CI and T123CI), Evans' solution (C124EI and T124EI) or with plain distilled water (C125WI and T125WI) respectively. The mixtures were then immediately filtered over a small pad of Super-Cel which took less than 30 seconds. The filtrates (extracts I) were then allowed to stand overnight in the cold (4°). Next day the amount of crystallized 'Dactylen'

(Augustin, 1959a) was noted and the filtrates (CI, EI and WI, Table 3) were analysed for skin activity, antigen-A content, total nitrogen, carbohydrate (as galactose), and their pH was noted also. The pollen residues (plus Super-Cel) were extracted overnight with a further 10 ml. of the same extraction fluid (extracts II). As before, the mixtures were shaken for 1 minute by hand and filtered once more. The second filtrates (CII, EII and WII) were analysed in the same way as the first filtrates. The extraction scheme is given in Table 2 and the results in Tables 3–6.

TABLE 3

EXTRACTION OF COCKSFOOT (C123) AND TIMOTHY (T123) POLLENS WITH COCA'S SOLUTION; EXTRACTION CODE GIVEN IN TABLE 2

Code No. (*Table 2*)	*T.N.* *mg./ml.*	*C.H.* (*G.A.*) *mg./ml.*	*Noon units/ml.*		*Dactylen*
			Skin test	*Diffusion test* (*antigen A*)	
C123					
GCI	0.54	19.0	1,000	30,000	++++
HCI	0.58	20.0	100	30,000	++++
JCI	0.85	16.5	10,000	30,000	+++++
KCI	0.57	15.0	50,000	30,000	++++
LCI	1.12	19.0	10,000	50,000	Trace
MCI	1.03	27.0	100,000	70,000	Trace
GCII	0.52	5.0	100,000	30,000	Nil
HCII	0.61	3.2	100,000	40,000	Nil
JCII	0.34	6.0	100,000	25,000	Trace
KCII	0.37	4.5	100,000	25,000	Trace
LCII	0.55	4.5	50,000	30,000	Trace
MCII	0.34	2.8	20,000	40,000	Trace
T123					
GCI	0.49	19	20,000	30,000	+++
HCI	0.53	14	20,000	30,000	+++
JCI	1.00	17	20,000	35,000	++++
KCI	0.70	(9)	10,000	32,000	++++
LCI	1.30	18	100,000	70,000	+++++
MCI	1.24	17	100,000	80,000	Trace
GCII	0.62	3.2	100,000	40,000	Trace
HCII	0.74	2.5	100,000	50,000	+
JCII	0.39	2.5	100,000	32,000	Trace
KCII	0.46	1.5	100,000	50,000	Trace
LCII	0.39	2.2	100,000	50,000	Trace
MCII	0.48	2.2	100,000	50,000	Trace

pH of all extracts = 7.5. T.N. = Total nitrogen. C.H. = Carbohydrate. G.A. = Galactose.

To start with it should be noted that Coca's solution is a more efficient extractant for the skin-reactive allergens than Evans' fluid or than plain distilled water, most probably because the allergens either are themselves acid or are associated with acidic proteins (Augustin, 1959a). Evans' fluid is not an efficient enough buffer to keep the pH of the extracts at neutrality and the aqueous extracts are even more acid. Surprisingly enough the total amounts of nitrogen extracted by the three solvents do not differ very much from each other (Table 6).

It can further be seen that the carbohydrate content (the figures have relative value only since there was interference by pigments and probably other substances and no attempt was made to assess the exact nature of the carbohydrate; see, however, Augustin 1959a) of the Cocksfoot extracts was much higher than that of the Timothy extracts. There is also a distinct lack of correlation between skin tests and diffusion tests which is not surprising since the latter were used to measure only one particular pollen antigen now known *not* to be an allergen (Augustin, 1957, 1959b, 1959c).

TABLE 4

EXTRACTION OF COCKSFOOT (C124) AND TIMOTHY (T124) POLLENS WITH EVANS' FLUID; EXTRACTION CODE GIVEN IN TABLE 2

Code No.	*T.N.* *mg./ml.*	*C.H. (GA)* *mg./ml.*	*Noon units/ml.*		*Dactylen*
			Skin tests	*Diffusion tests (antigen A)*	
C124					
PEI	0.57	30	<1,000	20,000	+++++
QEI	0.87	32	50,000	32,000	+++++
REI	0.91	25	50,000	40,000	++
SEI	1.2	32	100,000	64,000	+
PEII	Accident	4	50,000	16.000	Trace
QEII	0.25	7	50,000	16,000	Trace
REII	0.31	7	20,000	20,000	+
SEII	0.25	6	50,000	16,000	+
T124					
PEI	0.67	9	20,000	16,000	+++++
QEI	0.91	16.5	100,000	32,000	++++
REI	1.1	6	100,000	40,000	++++
SEI	1.3	6	100,000	64,000	+
PEII	0.48	0.5	80,000	20,000	Trace
QEII	0.30	1.5	10,000	16,000	Trace
REII	0.36	1.0	10,000	20,000	Trace
SEII	Accident	1.0	5,000	12,000	Trace

pH of EI extracts = 5–5.5.
pH of EII extracts = 5.2.
T.N. = Total nitrogen. C.H. = Carbohydrate. G.A. = Galactose.

The lack of 'Dactylen' in the second extracts is merely a reflection of the ease with which Dactylen can be filtered off in small-scale operations.

We concluded that particularly with Cocksfoot a 1-minute extraction removed most of the Dactylen and also much pigment without extracting much activity; in fact, it was for this reason possible to omit the defatting of pollen (which also removes much pigment but no activity) when 1-minute aqueous extractions preceded the extractions with Coca's solution. In this way much purified, low nitrogen, lightly coloured, very active Coca extracts were obtained. Although with Timothy as much as 20 per cent of the skin-active substances may be removed even during a 1-minute extraction, we found that this step was still worth while in order to obtain the second, still very active, low nitrogen and low carbohydrate extract, particularly since the first Timothy extracts were always especially highly pigmented. Also, the first extracts could easily be worked up separately since the

TABLE 5

EXTRACTION OF COCKSFOOT (C125) AND TIMOTHY (T125) POLLENS WITH UNBUFFERED DISTILLED WATER

Extraction code in Table 2.

Code No.	*T.N.* mg./ml.	*C.H. (GA)* mg./ml.	*Noon units/ml.*		*Dactylen*
			Skin tests	*Diffusion tests (antigen A)*	
C125					
PWI	0.65	9	5,000	32,000	+++++
QWI	1.02	30	50,000	64,000	++++
RWI	1.35	14	50,000	80,000	+
TWI	1.34	14	100,000	100,000	Trace
PWII	0.51	1.2	10,000	2,000	—
QWII	0.44	3.5	10,000	<1,000	Trace
RWII	0.24	0.9	5,000	<1,000	+
TWII	0.25	0.5	5,000	<1,000	++
T125					
PWI	0.69	9	10,000	25,000	+++++
QWI	1.08	20	100,000	40,000	+++++
RWI	1.22	20	100,000	80,000	++++
TWI	1.41	18	100,000	100,000	Trace
PWII	0.62	1.5	100,000	<1,000	Trace
QWII	0.39	2.5	5,000	<1,000	Trace
RWII	0.20	3.0	5,000	<1,000	+
TWII	0.30	1.0	1,000	<1,000	++

pH of WI extracts = 5.0
pH of WII extracts = 4.8

TABLE 6

SUMMARY OF NITROGEN (mg./ml.) EXTRACTED BY THE VARIOUS PROCEDURES

CI+CII	*T123*	*C123*
G	1.11	1.06
H	1.27	1.19
J	1.39	1.19
K	1.16	0.94
L	1.69	1.67
M	1.78	1.37
First 24 hr. extract	1.24	1.03
EI+EII	*T124*	*C124*
P	1.15	0.57 (some spilled)
Q	1.21	1.12
R	1.46	1.22
S	1.30	1.45
First 24 hr. extract	1.30	1.20
WI+WII	*T125*	*C125*
P	1.31	1.16
Q	1.47	1.46
R	1.42	1.79
T	1.71	1.59
First 24 hr. extract	1.41	1.34

Dactylen appeared to crystallize out in a more easily filterable form than from the long time extracts.

The findings reported here have been incorporated in our routine large-scale preparations of Pollaccine over the past 3 years and have given satisfactory results in the treatment of patients. The extracts thus obtained (by extracting with Coca's solution undefatted pollen

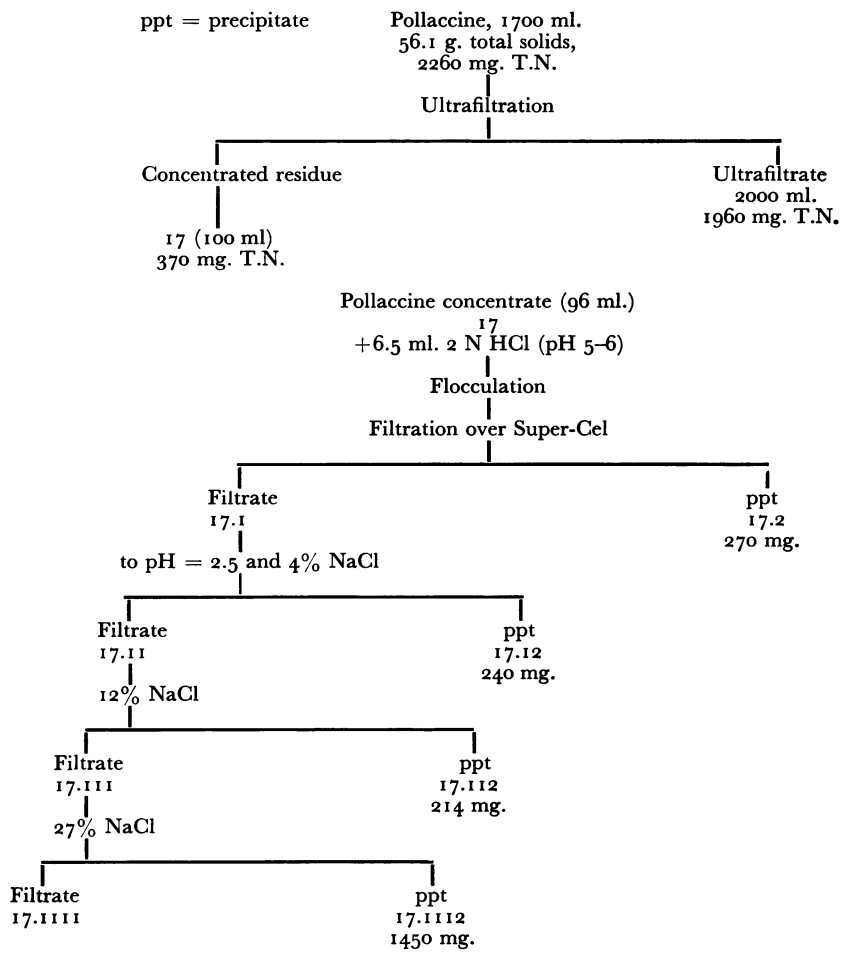

FIG. 1. Ultrafiltration of 'Pollaccine' and NaCl fractionation of the ultrafiltered residue. T.N. = total nitrogen (micro-Kjeldahl).

previously extracted for 1 minute with water), were however much stronger (in terms of skin reactivity) than the 'Pollaccine' solutions prepared previously (1 g. of pollen per 10 ml. of Evans' fluid) and had to be diluted for clinical use with the indicated stabilizing buffers containing glycine and glucose. The nitrogen content of these solutions therefore bears no relation whatever to their activity. The keeping properties of the extracts were in no way affected by these alterations in procedure.

SALT FRACTIONATIONS

Acid NaCl Fractionation of 'Pollaccine'

All operations were done in the cold (0–4°). 1700 ml. of 'Pollaccine' were ultrafiltered through collodion-covered porcelain filter candles (Augustin, 1959a) and the residue exhaustively dialysed against distilled water and clarified over Super-Cel. This yielded 100 ml. of a dark brown liquid. The light-coloured ultrafiltrate was set aside for further investigations. Pilot experiments indicated that fractionation with ammonium sulphate did not bring about much separation from the pigments and that sodium chloride fractionation at neutral pH was not possible. Fractionation at acid pH proved more hopeful and brought about the separation of a lightly coloured active precipitate from a deeply pigmented solution. The final scheme adopted is briefly outlined in Fig. 1. It consisted of a preliminary iso-electric precipitation at pH 5–6, followed after acidification to pH 2.5 by addition of increasing amounts of a saturated solution of sodium chloride with stirring. Definite precipitation steps could readily be distinguished. The precipitates were allowed to settle in the cold and were then filtered off over Super-Cel. After washing with the appropriately adjusted wash liquids (pH and NaCl concentration corresponding to those of the mother liquor), the precipitates were dissolved with the help of a little alkali and immediately neutralized (universal indicator paper). The dissolved and clarified solutions of the precipitates were then stirred into 10 volumes of acetone, which was found to precipitate all the activity (skin tests) and dissolved out some of the pigments. Sedimentation always took place quite readily and the precipitates could be collected by centrifugation (refrigerated centrifuge) and were dried by repeated re-suspension in dry acetone, ethanol and ether. The final drying took place in the filter-paper-covered centrifuge tubes in a vacuum desiccator.

The preliminary iso-electric precipitation was difficult because of the strong pigmentation,

TABLE 7

NaCl FRACTIONATION OF 'POLLACCINE'

Sample	*NaCl concentration % w/v*	*Dry weight mg.*	*T.N.*	*C.H.*	*Molisch reaction*	*Skin activity Noon units*	*% Recovery (Noon units)*
17 (original, undialysed, 1,700 ml.)	—	33/ml.	1.33 mg./ml.	Not done	+ + + + + +	100,000/ml.	—
UF 2,000 ml.	—	—	0.93 mg./ml.	Not done	+ + + + + +	5,000/ml.	—
UF concentrate 100 ml.	—	—	3.7 mg./ml.	Not done	+ + + + + +	200,000,000/ml.	100
17.2	—	270	9.8%	12	+ + + +	50,000/mg.	7
17.12	0–4	240	11.7%	5	+	50,000/mg.	6
17.112	4–12	214	10.1%	10	+	100,000/mg.	11
17.1112	12–27	1,450	6.0%	2	Trace	125,000/mg.	91

UF = ultrafiltrate, UF concentrate = bag contents after ultrafiltration, T.N. = total nitrogen (micro-Kjeldahl), C.H. = carbohydrate, estimated as galactose by the orcinol method (much interference by pigments of solutions, i.e. figures only approximate).

and optimum conditions varied a little with different pollen extracts. They had to be determined afresh each time; i.e., in a series of small test tubes, aliquots of the particular batch in hand were diluted with equal volumes of distilled water; acetic acid was then added to give optimum flocculation and the main batch treated accordingly. The precipitate removed in this way was never very large but contained much pigment.

The iso-electric and the 0–4 per cent NaCl precipitates were difficult to dissolve and some insoluble residue had usually to be discarded, i.e. some denaturation must have occurred at this stage, but possibly not of the active material, since recovery figures were always very good. All the later NaCl fractions dissolved with great ease in water or saline.

The final precipitation was done by adding solid NaCl to the last filtrate; however, only very little more precipitate formed and the final filtrate still contained about 1 per cent of the starting activity.

Table 7 gives some of the analytical data relating to this fractionation. Ultrafiltration alone achieved already a six-fold purification in terms of total nitrogen (T.N.). The bulk of the activity was precipitated between the NaCl concentrations of 12 and 27 per cent, which, in terms of T.N., achieved a further four- to five-fold purification, giving roughly a thirty-fold overall purification in terms of T.N. The purification figure in terms of total solids must have been higher.

The fractions differed in pigment and carbohydrate content (Table 7) and had very different electrophoretic mobilities (Fig. 2).

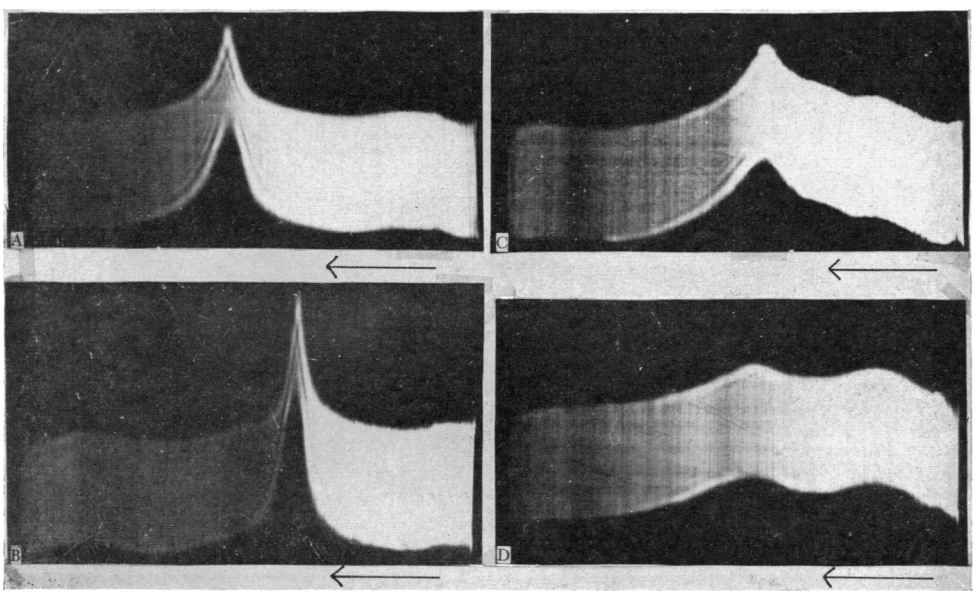

FIG. 2. Descending electrophoretic patterns of 'Pollaccine' fractions (Fig. 1) at concentrations of 10 mg./ml. in phosphate buffer of pH 7.4 and I = 0.1. The first pattern (A = 17.2) was taken after a run of 100 minutes and the remainder (B = 17.12, C = 17.112, D = 17.1112) after runs of 120 minutes.

Acid Sodium Chloride Fractionation of a Timothy Pollen Extract

500 g. of the fresh pollens were extracted with ether, acetone and petrol ether at room temperature until the extracts were colourless and gave no residue on evaporation. The defatted pollens were then extracted twice with 4 l. followed by 2 l. of Coca's solution

(mechanical shaking for 30 minutes in a stoppered glass container). The combined extracts were then ultrafiltered through collodion-covered filter candles, and the ultrafiltered residue exhaustively dialysed against tap water and finally against distilled water in Visking membranes 24/32, care being taken that the membrane bags were not taut at any time during the dialysis. This yielded finally 420 ml. of a dark brown solution which was fractionated essentially according to the scheme outlined for 'Pollaccine' (Fig. 1), except that each NaCl-fraction was always redissolved and re-fractionated according to the steps that preceded its isolation. On acidification two precipitations occurred before pH 3.0 was reached, one at pH 5.4 which gave an easily soluble dark brown precipitate (T21·12) soluble at acid pH, but only partially soluble at neutral pH; after drying, the residue insoluble at neutral pH weighed 112 mg. and the soluble part 132 mg. All the precipitates were dissolved in the minimum of distilled water, neutralized, dialysed free from salt and finally isolated as dry powders by precipitation with acetone, etc., as described in the fractionation of 'Pollaccine'.

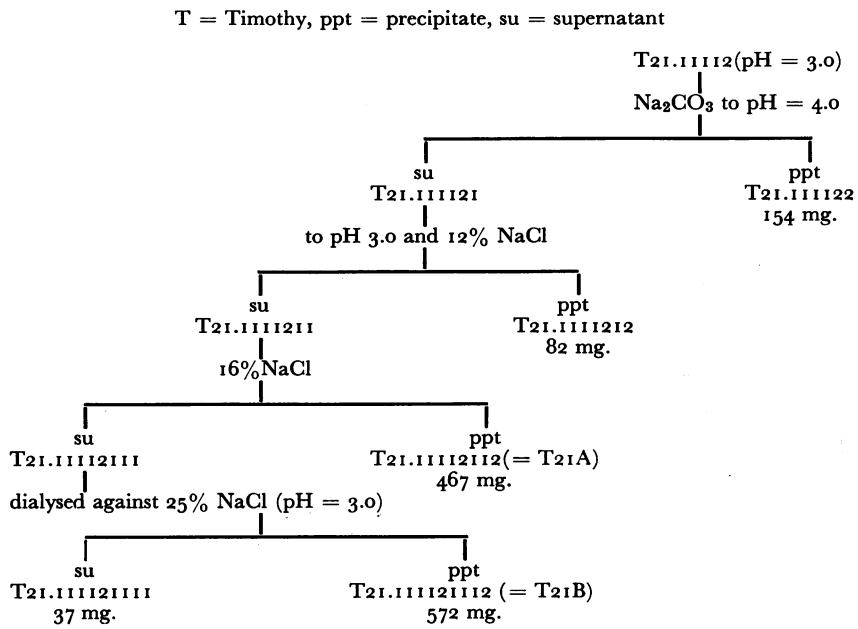

FIG. 3. Fractionation of the 12–15 per cent NaCl precipitate (pH = 3.0) from Timothy pollen extract.

In Fig. 3 the re-fractionation of the 12–25 per cent NaCl precipitate is outlined. This precipitate dissolved very readily, giving a solution of 50 ml. During drop-by-drop neutralization with a sodium carbonate solution a precipitate separated out at pH 4.0. It was removed by centrifugation and yielded 154 mg. (T21.111122) of a very easily soluble brown powder. The supernatant (T21.111121, 56 ml.) was adjusted to pH 3.0. The careful and slow addition of sodium chloride did not produce any turbidity until the concentration was 7 per cent. This turbidity did not increase on standing until the NaCl concentration was increased from 11 to 12 per cent, and this precipitate was centrifuged off (T21.1111212), yielding 82 mg. of a dry powder. The addition of 3 g. of NaCl to the supernatant produced copious precipitate formation, giving 467 mg. of a light brown

powder. The supernatant (T21.11112111) from this was only very slightly coloured and had a sodium chloride concentration of 16 per cent. Further precipitation was produced by dialysing this solution (rotating membrane technique) against a 25 per cent NaCl solution of pH 3.0. The precipitate so formed [T21.111121112 (= T21B), 573 mg. dry weight] was almost colourless. It was found to contain 5–10 million Noon units per mg. when skin tested repeatedly in the usual manner in comparison with the current 'Pollaccine' standard. The fraction T21.11112112 (= T21A) contained 1–5,000,000 Noon units per mg. while the activities of the other fractions were negligible in comparison.

In this fractionation a total of 2.4 g. of non-dialysable material has been isolated from 500 g. Timothy grass pollens; of this more than 50 per cent had an activity of 1–10 million Noon units (skin activity) per mg., none of which was antigen A since this had been destroyed during the acidification. As freeze drying of the ultra-filtered and exhaustively dialysed starting solution yielded a material of 30,000 Noon units per mg., a 30 to 300-fold purification has been achieved in comparison with that already very much purified material. According to Noon 1 g. of pollen is equivalent to 1,000,000 units, i.e. our most active preparation was weight for weight 10,000 times as active as the pollen from which it had been derived. At the same time the total activity isolated exceeded by any standards that of the starting material. Even if we consider that we isolated by our extraction procedures more nearly 2 million than 1 million Noon units per g. of pollen, 500 g. of pollens are the equivalent of 1000 million Noon units while 1.3 g. of a material assaying 1–10 million units per mg. gives us 1300 to 13,000 million Noon units, i.e. even the most conservative estimate gives a yield of over 100 per cent. The assays on the dry powder, which were stored in wax-sealed glass containers at 0–4°, were repeated at yearly intervals and have remained constant (5,000,000 ±50 per cent) for 7 years.

Gel diffusion tests showed that even the most active preparation still contained at least three components.

The NaCl preparation T21.111121112 (= T21A) was found to be fully antigenic in rabbits, the antisera thus produced giving precipitation lines identical with those produced by the antisera for crude extracts.

Since the concentrated mixed antisera precipitated almost all the activity (skin test) from the NaCl-preparations (and from crude pollen extracts) it seems justifiable to conclude that probably all the allergens of these pollens produce precipitating antibodies in rabbits although not all rabbit antisera can be expected to contain antibodies for all the pollen components; i.e. particular antisera will not be able to precipitate all the skin reactive substances of pollen extracts.

Acid Sodium Chloride Fractionation of Cocksfoot Pollen Extract

10 g. of C 109 A (Fig. 5) were dissolved in 330 ml. of distilled water and the solution clarified by filtration through a clarifying Ford FCB pad. At pH 5.4 the solution became turbid, but no precipitate settled even after 'maturing' in the cold room for several hours; some turbidity could be removed by centrifugation (ppt. C109.2); the supernatant (su C109.1) was not completely clear even after filtration over Super-Cel, but clarified completely on adjusting the pH to 3.0.

In a preliminary study an attempt was made to establish more accurately than in the previous experiments the correct precipitation steps in the NaCl precipitation. Increasing amounts of NaCl were weighed into calibrated 2 ml. bottles to cover a range of NaCl concentrations (in 2 ml.) from 2–33 per cent. 1.5 ml. of C109.1 (pH = 3.0) were then

added to each bottle and the volume made up to the 2 ml. mark with distilled water slightly acidified so that the final pH of the solutions remained 3.0. Rubber stoppers were pressed well home and the small air bubble at the top of the liquids removed by means of a syringe needle which was pushed through the rubber stopper. The mixtures were rotated at 4° overnight, spun clear at the same temperature, the supernatants pipetted off and their absorption at 280 mμ measured in a UV spectrophotometer. A calibration curve relating optical density to weight of 'protein' in C109.1 was established from dilutions of the 2 per cent NaCl bottle in which no precipitate had formed and the absorption figures of the supernatants were by this means converted to 'protein' figures. They were used to construct a precipitation curve (Fig. 4). Only four precipitation steps could be distinguished,

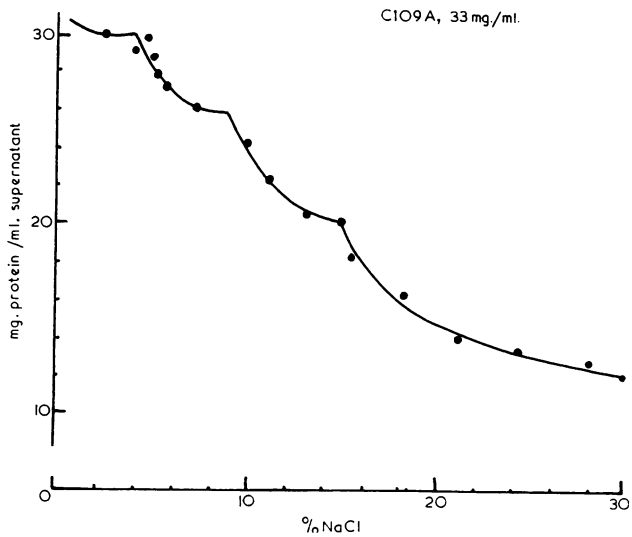

FIG. 4. Solubility curve of dialysed freeze-dried Cocksfoot pollen extract C109A in NaCl (pH 3.0) at 2°. The supernatants were diluted from 1/20–1/100 to give convenient spectrophotometric readings at 280 mμ. These were converted to 'protein' figures by means of a standard curve constructed from a 2 per cent NaCl solution (pH = 3.0) of C109A.

the largest amount being precipitated between 15 and 30 per cent. Incipient precipitation started at 4 per cent and the precipitation steps appeared to be 4–6, 6–10, 10–15 and 15–30 per cent.

The bulk of C109.1 was fractionated accordingly (Fig. 5). As before, the centrifugally separated fractions were dialysed free from salt and precipitated with acetone, etc. The balance sheet of the fractionation is given in Table 8. There were three principal active fractions, C109A.111212, C109A.111122 and C109A.11112112, all of which were in some manner derived from the 15–30 per cent NaCl precipitate (Fig. 5), i.e. within the same range of NaCl concentration in which the main activity of Timothy was precipitated. The supernatant from the 30 per cent NaCl precipitation retained only 3 per cent of the starting activity, but in association with at least 25 per cent of the starting material by weight.

The most active preparations were not quite as active as those obtained from Timothy pollen, but the percentage yield (in terms of skin units) was again far too high. As with Timothy, the activity of the Cocksfoot preparations has remained constant for 7 years.

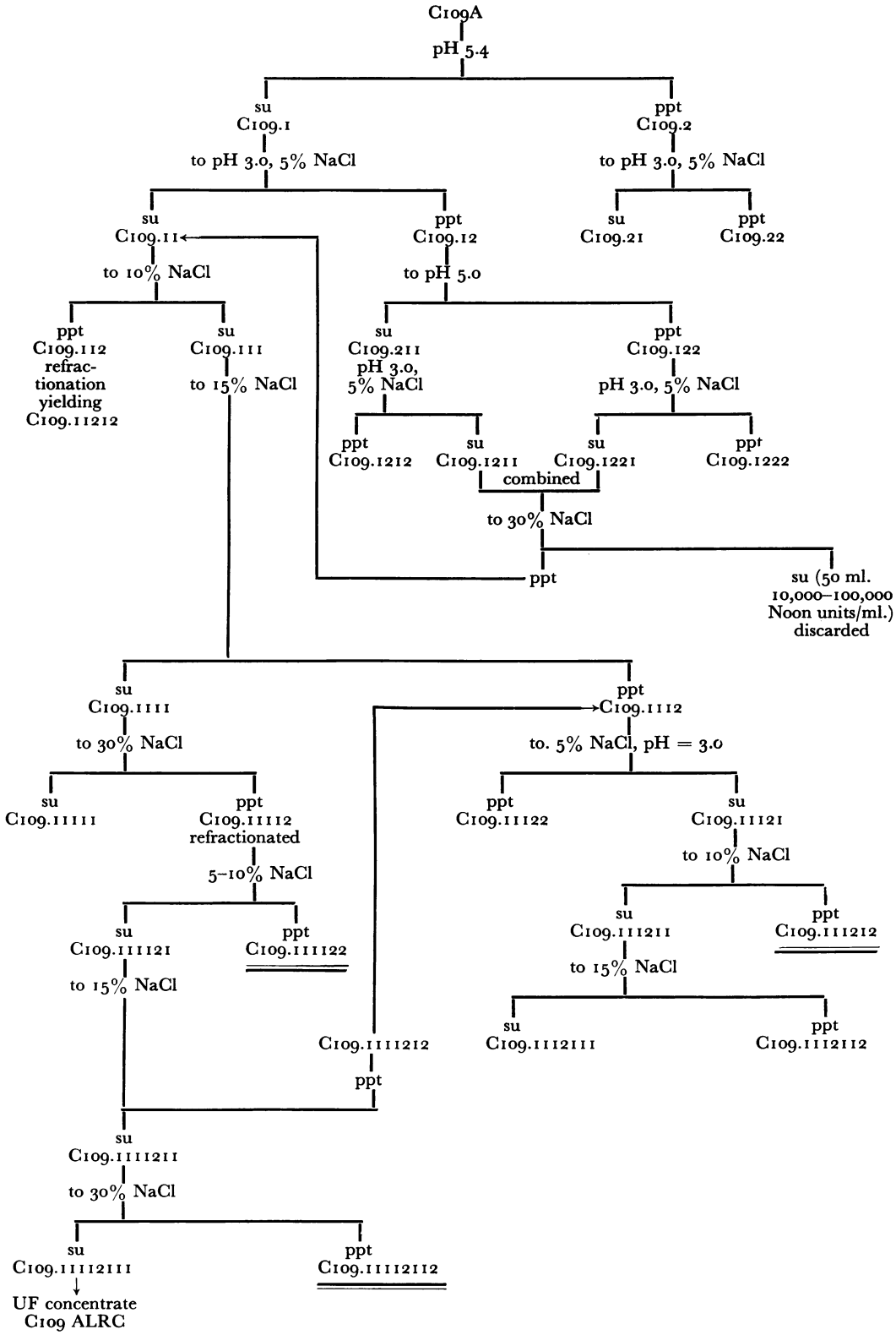

FIG. 5. NaCl fractionation of dialysed freeze-dried Cocksfoot pollen extract C109A.

Electrophoresis in free solution gave a simple fast-moving peak for both the iso-electric precipitates (C109A.22 and C109A.122) while all the other fractions gave composite pictures.

TABLE 8

NaCl FRACTIONATION OF COCKSFOOT POLLEN EXTRACT. BALANCE SHEET

Sample	*NaCl concentration w/v*	*Dry weight mg.*	*Skin activity Noon units per mg.*	*Skin activity Noon units Total × 10⁻⁶*	*% Recovery (skin reactivity)*
C109A (starting material)	—	10,000	30,000	300	
C109A.22	Iso-electric ppt.	109	<100,000		
C109A.1212	0–5% of 0–5% ppt.	164	<160,000		
C109A.1222	Iso-electric of 0–5% ppt.	345	<1,000		5–10%
C109A.11212	5–10%	158	20,000	3.1	
C109A.11122	0–5% of (10–15% + 15–30% ppt.)	139	100,000	13.9	
C109A.1112112	10–15% of 5–10% ppt.	62	100,000	6.2	
C109A.111212	5–10% of (10–15% + 15–30% ppt.)	250	1,000,000	250	
C109A.111122	5–10% of 15–30% ppt.	398	500,000	200	>200
C109A.11112112	15–30% of 15–30% ppt.	225	1,000,000	225	
C109AL.CR	supernatant from 30% ppt, ultrafiltered, dialysed	2,640	5,000	13.2	3

Immunological comparison of the most active Timothy and Cocksfoot preparations. Fig. 6A shows that at levels of 1 mg./ml. the Timothy preparation contained in terms of a rabbit anti-Timothy serum three components, one of these giving a reaction of identity with an antigen

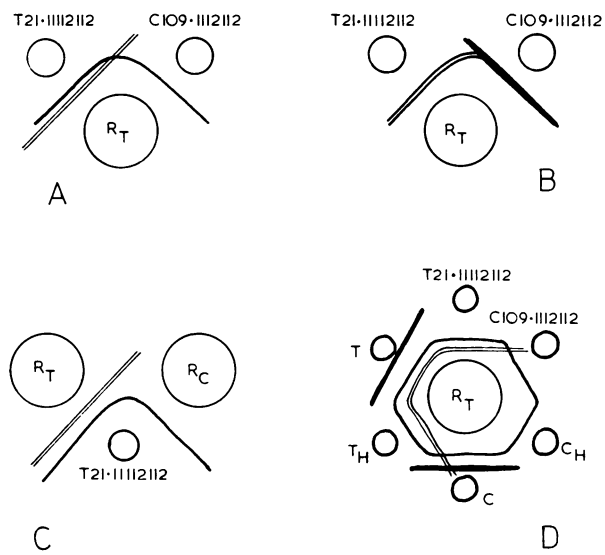

FIG. 6. Immunological comparison of Cocksfoot and Timothy pollen preparations, similarly purified by acid NaCl fractionations (Figs. 3 and 5) and of similar potency (by skin tests 1–10,000,000 Noon units/mg. with crude Cocksfoot (C) and crude Timothy (T) pollen extracts, in terms of antisera R_C and R_T. T21.11112112 = T21A. Suffix H indicates heating at 100° for 1 minute.

of the Cocksfoot preparation (at 1 mg./ml.). The two antigens specific for Timothy appear to be identical with the two antigens of crude pollen extracts which give the 'innermost' 'twin' precipitation lines in Ouchterlony tests (Fig. 6D) and had previously been recognized as specific for Timothy alone amongst fifteen different pollens (Augustin, 1959b). Fig. 6D further shows that these Timothy specific twin antigens are not in any way related to the heat labile A-antigens of Timothy and Cocksfoot and confirms that the crude and purified Timothy and Cocksfoot preparations share the other 'inner' antigen in terms of the anti-Timothy serum R_T.

In Fig. 6B the purified Cocksfoot and Timothy preparations are compared in terms of an anti-Cocksfoot pollen serum (R_C). It can be seen that R_C had no antibodies for the specific twin antigens of Timothy and that the antigen 'shared' by the two preparations consisted actually of two Timothy antigens which were only partially identical with the Cocksfoot antigen. Another interpretation of the B-pattern would be that the Cocksfoot pollen preparation contained three antigens, two of them shared with T21B and the third specific for Cocksfoot.

Fig. 6C confirms that T21B has (at least) one antigen for which R_T and R_C have the same antibodies and in addition the twin antigens for which R_C has not got any antibodies.

Ammonium Sulphate Fractionation of 'Pollaccine' and Cocksfoot Extracts at Neutral pH.

In the hope of separating the (acid-labile) A-antigens from the skin-reactive allergens, fractionations were done at neutral pH on preparations which were already known to contain disproportionate amounts of A-antigen and skin reactivity, i.e. we used a 1-minute water extract of Cocksfoot pollen (C59W) containing 20,000 Noon units of A-antigen, but only 2000 skin-reactive units and the subsequent Coca extract (C59CI) (30 minutes) of the same pollen preparation which assayed 30,000 A-units and about 10,000 skin-reactive units.

TABLE 9

AMMONIUM SULPHATE FRACTIONATION OF A COCKSFOOT POLLEN EXTRACT. BALANCE SHEET

Fraction	*% saturation* $(NH_4)_2SO_4$	*Total Noon units* $\times 10^{-6}$		*% Recovery (skin reactivity)*
		Skin test	*Antigen A*	
C127, 50 mg./ml. (starting material)	—	90	135	
C127.2	0–30	5	5	About 5
C127.12	30–45	45	40	About 50
C127.112	45–58	45	40	About 50
C127.1112	58–80	4·5	<5	About 5

Preliminary investigations indicated that, at concentrations of 10 mg./ml., 50 per cent saturation with ammonium sulphate produced only a faint cloudiness in the W-preparation, while 'Pollaccine' gave a somewhat stronger cloudiness and the Coca extract definite precipitation. As even at 75 per cent saturation with ammonium sulphate only a very slight precipitation could be produced in the W-preparation, the possibilities of the method could only be pursued with Coca extract C126CI.

At 50 mg./ml. precipitation started at 30 per cent saturation with ammonium sulphate. In free electrophoresis this fraction gave a fast-moving single sharp peak, but contained only about 5 per cent of the starting activity (skin test). Further fractions were collected at 30–45, 45–58 and 58–80 per cent saturation of ammonium sulphate and proved all inhomogeneous by free electrophoresis and by gel diffusion tests. At 80 per cent saturation the supernatant was practically inactive by skin test, the bulk of the activity being recovered between 30 and 58 per cent saturation. The balance sheet is given in Table 9. There was no separation of skin reactivity and antigen A. The percentage recovery (about 110 per cent) was not beyond what is reasonable if it is accepted that none of the skin reactivity was destroyed during the fractionation.

THE ULTRAFILTRATES

By freeze drying or by precipitation with phosphotungstic acid it was possible to concentrate the antigens of the ultrafiltrates. They were found to contain two dialysable (unstretched Visking membranes 24/32) non-skin-reactive antigens (precipitable by the appropriate rabbit antisera) and one non-precipitable (at any rate by our rabbit antisera) non-dialysable allergen; in other words, in the final analysis even the allergens of the ultrafiltrates (i.e. the antigens that can just about pass the membranes we use) remain inside the membrane sacks unless the membranes are stretched or dialysis is carried on for prolonged periods; either prolonged ultrafiltration or prolonged dialysis did in fact cause leakage once more. The colourless relatively non-dialysable allergen was precipitable by trichloracetic acid and contained eight amino acids.

ANION EXCHANGE FRACTIONATIONS

Since diethyldiaminoethyl (= DEAE) cellulose (Peterson and Sober, 1956) had proved so successful for the fractionation of serum proteins in most peoples' hands, including our own (Augustin and Hayward, 1959, 1960a, 1960b), we decided to attempt the fractionation of crude and purified pollen extracts in the same manner. The same buffers (I to V) as in our serum fractionations (Augustin and Hayward, 1960b) were used for the elution of the pollen proteins from the DEAE-columns, with the addition of a 0.001 M phosphate buffer of pH 8.0 (buffer O).

Fig. 7 gives the fractionation of four pollen preparations, three of which (T112A, T21.11112112 and C59CI) have been discussed in previous sections of this paper. T59W was a 1 minute aqueous extract of Timothy pollen, prepared as described for C59W. Thus three Timothy preparations in greatly varying states of purification and one rather impure Cocksfoot preparation were fractionated. The elution patterns in Fig. 7 are arranged to make comparisons easy; i.e., regardless of the number of tubes over which elution took place with a particular buffer, the same space on the abscissa was allotted for the equivalent buffers whatever the pollen preparation; the elution patterns were compressed or stretched proportionately, although in actual fact little adjustment was necessary. Buffer O was introduced for the fractionation of T112A when it was noted that many components were crowded together in the first eluate (buffer I, see T21.11112112 = T21A in Fig. 7) which corresponds to the slow γ-globulin position in the corresponding fractionation of serum proteins. This crowding together in the unabsorbed part was not surprising since we knew already from previous immunoelectrophoretic studies that the range of charges of the pollen proteins was wider than that of the serum proteins (Augustin, 1959c). The more

alkaline lower ionic strength buffer O was introduced to fractionate this part of the protein spectrum and in the hope of isolating antigen A, whose electrophoretic mobility lies within this range.

As would be expected, the elution patterns are the more complicated the less pure were

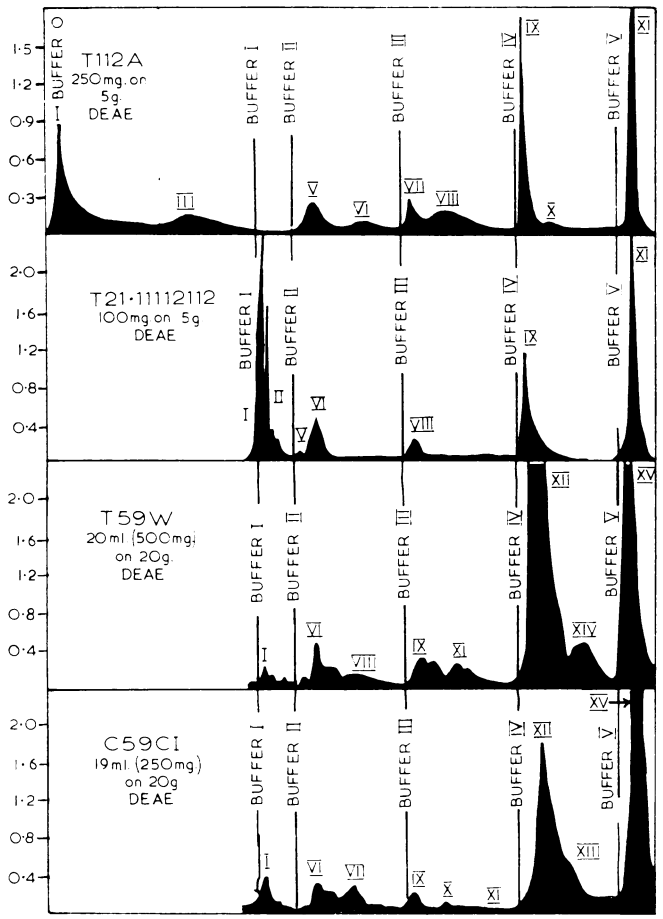

FIG. 7. Fractionation on DEAE-cellulose of three Timothy grass pollen preparations (T112A, a dialysed freeze-dried Timothy pollen extract in Coca's solution of a skin potency of 30,000 Noon units/mg.; T59W, a freeze-dried 1 minute water extract of Timothy pollen of a skin potency of 5,000 Noon units/mg. and T21.11112112 (= T21A), a NaCl fraction of Timothy pollen extract of a potency of 1,000,000 Noon units/mg.) and one dialysed freeze-dried Cocksfoot pollen extract C59CI assaying 10,000 Noon units/mg. by skin test and 30,000 A-units/mg. The composition of the buffers used in the elution are given under 'Materials and Methods'.

Figures along the ordinates indicate absorptions at 280 mμ of the consecutive column eluates (5 ml. quantities), the latter being plotted along the abscissa. The Roman figures indicate how eluates forming one (or sometimes several) absorption peaks were bulked.

the starting materials. Even in the compressed pattern of T59W sixteen peaks can be distinguished compared with only nine for T21.11112112 (= T21A).

The eluted fractions were bulked and pressure dialysed as usual in Visking membrane cases surrounded by tightly fitting nylon bags. This led to our surprise to some leakage of activity which sometimes amounted to as much as 20 per cent of the starting material;

with the help of gel diffusion analyses (Figs. 8 and 9) we were able to ascertain that this leakage was selective and not a general leakage of all the components in the dialysing bag. The pressure dialysates could be dialysed without or with very little loss if care was taken not to stretch the pores of the membranes.

All the bulked eluates were reduced to volumes of 2 ml. for the analyses.

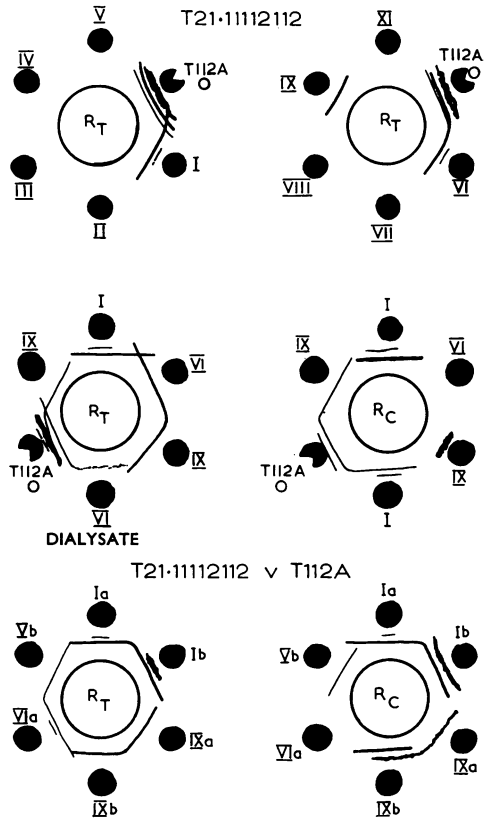

FIG. 8. Immunological comparison of DEAE fractions of Timothy pollen preparations by double gel diffusion on microscope slides. Reservoirs containing the bulked pressure dialysed concentrates (or where indicated their dialysates) of the DEAE fractions were arranged as indicated (by Roman numerals) around central reservoirs containing rabbit antisera for unfractionated crude pollen extracts of Timothy (R_T) and Cocksfoot (R_C) respectively. The Roman numerals correspond to the Roman numerals of Fig. 7 and 'O' designates the unfractionated starting material.
Upper part: DEAE fractions of T21.11112112 (= T21A) in terms of R_T and R_C.
Lower part: DEAE fractions of T21.11112112 (= T21A) and T112A (which have been labelled 'A' and 'B' respectively) in terms of R_T and R_C.

Fig. 8 gives comparative gel diffusion analyses of the DEAE-Timothy fractions in terms of a rabbit antiserum against whole Timothy extract (R_T) and another against whole Cocksfoot extract (R_C). Not all the bulked concentrated fractions of T21A gave diffusion lines, but all the lines given by the crude starting material T112A (O) (except the heat and acid labile A-lines) appear to be accounted for by some DEAE fraction; this includes one thin line of T112A which can be seen (left middle pattern of Fig. 8) to have given a reaction of identity with the (pressure) dialysate of fraction VI derived from T21A. Since

this thin line can be seen to have given (on the left) a reaction of non-identity with fraction IX of T21A and since fractions IX and VI of T21A gave a reaction of identity (on the right), it follows that fraction VI of T21 contained at least two types of molecules, one more readily dialysable than the other; indeed, the pressure dialysed residue of fraction VI (simply designated 'VI' as were all the other pressure dialysed

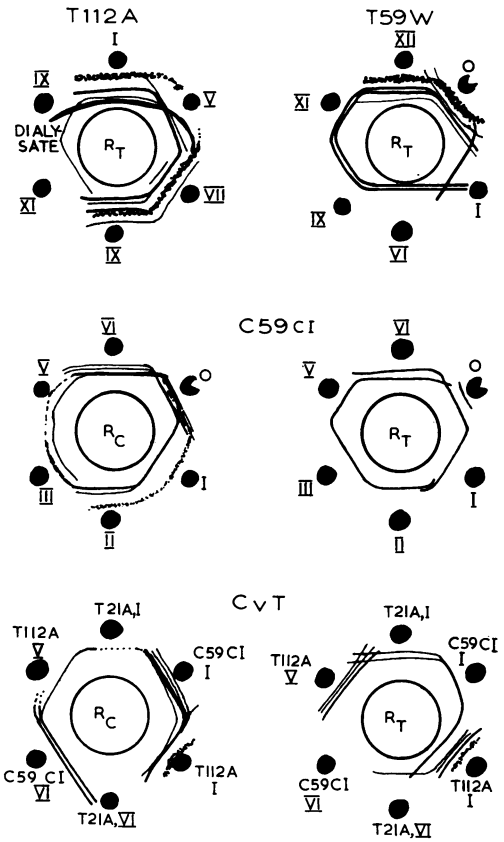

FIG. 9. Immunological comparison of DEAE fractions of Timothy and Cocksfoot pollen preparations (cf. Fig. 7) by double gel diffusion in terms of concentrated rabbit antisera for crude extracts of Timothy (R_T) and Cocksfoot (R_C) pollen extracts. In the upper part of the figure different fractions of particular extracts are compared amongst themselves as indicated, while the corresponding fractions of these Timothy (T) and Cocksfoot (C) preparations are compared with each other in the lower part ('C v. T'). T21A = T21.11112112 (Figs. 3 and 7). Key, otherwise, as for Fig. 8.

residues in Figs. 8 and 9) can be seen to give a second fine line (in terms of R_T, bottom row of Fig. 8) in addition to the main line of this fraction. This second fine line is most probably due to the dialysable component although this was not especially tested for.

It should further be noted that the identical components are eluted with particular buffers, regardless of the state of the purity of the starting material. Further, when we refractionated (not shown) some of the (purer) eluted bulked fractions, identical peaks were eluted in the same positions as before. These two facts, together with the fact pointed out before — namely that the components of the isolated fractions gave reactions of

identity with particular components of the mixed starting materials — rather conflict with the widely accepted idea (Loveless, Wright and Ryan, 1951; Loveless and Timasheff, 1955; Sehon and Richter, 1959; Johnson, Thorne, Britton and Coombs, 1958) that there is a continuous breakdown of easily dissociable pollen allergens.

Antigen A from T112A was not isolated in pure form, but can be seen from the position and shape of the lines that it forms to occur most probably in fraction I of T112A (Ib, bottom row of Fig. 8).

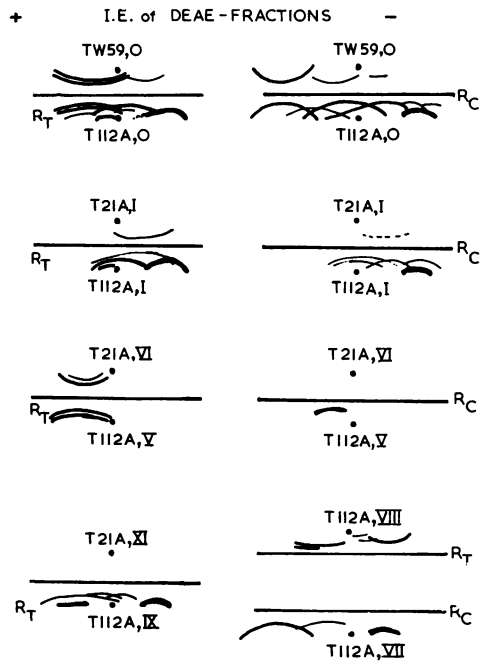

FIG. 10. Immunoelectrophoretic (= I.E.) comparisons of DEAE fractions of grass pollen preparations (cf. Fig. 7) in terms of rabbit antisera to the crude pollen preparations as indicated. The antiserum troughs are indicated by horizontal lines and the antigen reservoirs on either side of the troughs by black dots. The + and − signs indicate the polarities of the slides during the electrophoreses. Key otherwise as for Figs. 7 and 8.

Fig. 9 gives further gel diffusion comparisons in terms of more concentrated antisera and includes analyses of the Cocksfoot fractions. It can be seen that while one part of the A-antigens of T59W appears in fraction I, another (different) portion of them appears in fraction XII. In fact, from the top left corner picture of Fig. 9 it looks as if fractions VII and IX (which were still very inhomogeneous) of T112A also contained some A-antigens. The same Ouchterlony plate shows also that the (pressure) dialysate of fraction IX forms a single line giving a reaction of identity with a thin line of fraction XI which can be seen also in fraction I of T112A. This situation is thus reminiscent of that discussed regarding fraction VI of the T21A preparation (Fig. 8); for, once more, the pressure dialysis resulted in the selective dialysis of one particular component. Fraction IX of T112A can be seen to have many other constituents, all of which remained inside the dialysing sac.

All the fractions of the crude preparations (T112A, T59W and C59CI) were found to give lines in terms of R_C and R_T; in fact in most instances several lines. Part of the

A. antigens of Cocksfoot appeared in Fractions I and II of C59CI (middle row of Fig. 9). From the figures it is almost impossible to decide how many of the A-antigens Cocksfoot and Timothy have in common (bottom row of Fig. 9); in fact Figs. 8 and 9 demonstrate clearly that to make full use of the DEAE fractionations it is necessary to do preliminary fractionation on the crude pollen extracts.

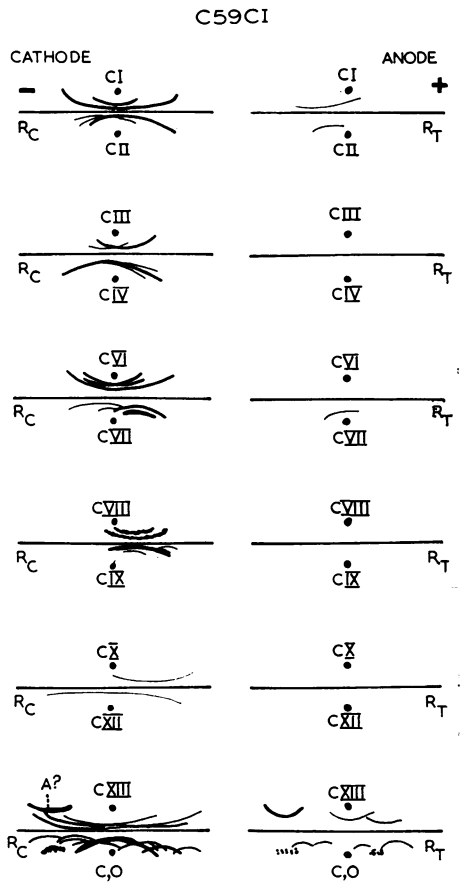

FIG. 11. Immunoelectrophoretic comparisons of the DEAE fractions (cf. Fig. 7) of the Cocksfoot preparation C59CI. Key as for Fig. 10.

Fraction VI of the T21A preparation gave only one line (together with another faint additional line) with R_T and no line at all with R_C (Figs. 8 and 9). Vice versa, the corresponding fraction VI of the Cocksfoot preparation gave two lines with R_C and none with R_T. Nevertheless, Timothy pollens must contain some antigens like the fraction VI Cocksfoot antigens, for the lines for the latter can be seen to bend round towards the cup containing the corresponding fraction (V) of the T112A preparation.

Fig. 10 gives the immunoelectrophoretic pictures of the starting materials and of the various Timothy fractions in terms of R_C and R_T. From Fig. 10 the 1 minute aqueous extract (T59W, O) is immunologically much more simple than the corresponding 24-hour Coca extract (T112A, O). A detailed comparison of the immunoelectrophoretic

pictures for T112A in terms of R_T and R_C (not shown) indicated a minimum of fifteen different antigen lines for this preparation, while T59W formed, by the same criteria, at the most five different lines.

The purest fractions have been isolated from the T21A preparation and are fractions I and VI. Although fraction I gave only one line, and in the identical positions, with R_T and R_C (Fig. 10), two lines have occasionally been obtained in Ouchterlony patterns (Figs. 8 and 9). Fraction VI of T21A gave only a single line in some Ouchterlony plates (Figs. 8 and 9), but the immunoelectrophoretic analyses (Fig. 10) showed clearly at least two components as parallel lines for this fraction in terms of R_T. The corresponding fraction of T112A was fraction V which gave with R_T the same two lines in the identical position. Since T112A had not undergone any acid treatment, it is unlikely that these double lines are due to any splitting of a mother component. Anyhow, we know of twin antigen lines for Timothy from the gel diffusion patterns of crude extracts (Augustin, 1959b and Fig. 6). Fraction V of T112A was less pure than fraction VI of T21A, for it gives a line with R_C, as well as the two lines with R_T. Although this line appears once more immunoelectrophoretically in the same position as that with R_T, it must be due to an additional antigen, since fraction VI of T21A gives no line whatever with R_C. Fig. 10 includes further comparisons of the corresponding factions of T21A and T112A; the latter always formed many more lines than the former; R_T and R_C usually produced very different pictures.

Fig. 11 gives the DEAE fractions of C59CI in terms of R_C and R_T. Most of the fractions gave complicated immunoelectrophoretic patterns (in terms of R_C), except fractions X and XII which gave (different) single lines (in terms of R_C) and no lines at all in terms of R_T.

It was estimated with the help of different antisera (not shown) that the original crude preparation C59CI contained at least seventeen different antigens; in Fig. 11 (bottom row) ten precipitation lines can be seen to have formed with R_C and additional lines with R_T. This means that the DEAE procedure has brought about a considerable fractionation of antigens, in spite of the fact that almost all the fractions still contained several antigens.

Fig. 12 gives the immunoelectrophoretic analyses of what proved to be the most active Timothy and Cocksfoot preparations in patients (see later). They were the unabsorbed proteins (fraction I) and the fractions eluted with buffer II at pH 6.4; i.e. they were fractions eluted under conditions identical with those under which the bulk of the serum γ globulins and reaginic globulin respectively have been eluted by us (Augustin and Hayward, 1960a, 1960b). The most potent pollen allergens are, therefore, at least physically, similar to serum γ globulins. Fraction I of T59W appeared to be as pure as fraction I of T21A; this is interesting in view of the crude starting material which was used. Fraction VI of C59CI still gave three lines in terms of R_C; and the two and three lines respectively for fractions VI of T21A and V of T112A were confirmed. Fraction VI of T59W appeared to be identical with fraction V of T112A.

Fig. 12 confirms that the fractions VI of Timothy and Cocksfoot pollens are immunologically completely unrelated.

SENSITIVITY SPECTRA IN PATIENTS

The DEAE fractionations of T112A, T59W and C59CI were done simultaneously. Two ten-fold dilutions of each fraction were tested in hay-fever subjects. For this purpose an aliquot of each bulked fractions was made up to the equivalent of 500 ml. of the main

Table 10

SENSITIVITY SPECTRA OF HAY-FEVER PATIENTS IN TERMS OF THE ISOLATED DEAE FRACTIONS OF THE TWO CRUDE TIMOTHY POLLEN PREPARATIONS (T59W AND T112A, FIG. 7)

The + signs in each vertical column represent skin reaction to the various preparations (indicated on the left) in a single patient, each preparation being tested in two dilutions, the second being ten times as dilute as the first. The number of + signs indicates the severity of the reactions. Poll. = 'Pollaccine'. − = no reaction.

DEAE fractions		*T59W*								*T112A*			
I	N	++++	++++	+++	−	±	++	+	−	++++ ++++	+	++++	++++
	1/10	+	++	+	−	−	−	−	−	++++	−	+++	++
II	N	−	++	−	−	−	−	−	±	++++ ++	+	+++	+
	1/10	−	+	−	−	−	−	−	−	+++	−	+	−
III	N	−	+++	−	−	−	−	−	−	+++	−	++	−
	1/10	−	+++	−	−	−	−	−	−	−	−	+	−
IV	N	−	−	−	−	−	−	+	−	++	−	−	−
	1/10	−	−	−	−	−	−	+	−	−	−	−	−
V	N	±	++	−	−	−	−	−	−	+++	+	+++	++++
	1/10	−	+	−	−	−	−	−	−	++	−	−	−
VI	N	+++	++++ ++++	++++	++++ ++++	−	+++	+++	+++	++++	−	++	+++
	1/10	+	++++ ++++	+	++	−	+	+	+	+	−	−	+
VII	N	++	++++ ++++	+	++++	+	−	−	+++	++	−	−	−
	1/10	−	++++ +++	−	±	−	−	−	−	−	−	−	−
VIII	N	+	+++	+	++	−	−	++	++	−	−	−	−
	1/10	−	++	−	−	−	−	−	−	−	−	−	−
IX	N	−	+++	−	−	−	−	−	−	++++	++	++	+
	1/10	−	++	−	−	−	−	−	−	++++	−	−	−
X	N	+++	−	+	+	+	++	±	−	−	−	+	−
	1/10	−	−	−	−	−	−	−	−	−	−	−	−
XI	N	+	−	+	+	−	+	+	−	−	−	−	+
	1/10	−	−	−	−	−	−	−	−	−	−	−	−
XV	N	−	−	−	−	−	−	−	−				
	1/10	−	−	−	−	−	−	−	−				
Poll.	1/10,000	+	++	+	+	±	+	+	+	++	±	+	+
	1/100,000	−	+	−	−	−	−	−	−	−	−	−	−

Table 11

SENSITIVITY SPECTRA OF HAY-FEVER PATIENTS IN TERMS OF SOME OF THE DEAE FRACTIONS OF TIMOTHY AND COCKSFOOT POLLENS (FIG. 7)

The + signs in each vertical column represent skin reactions to the various preparations (indicated on the left) in a single patient, each preparation being tested in two dilutions, the second being ten times as dilute as the first. The number of + signs indicates the severity of the reactions. Poll. = 'Pollaccine'. C59O = starting material. C59CI as put on the DEAE column. − = no reaction. Other symbols as in Figs. 7–12.

Preparation		*Patients*					
		A	*B*	*C*	*D*	*E*	*F*
Poll.	1/1,000	+++	++	+++	+	+	+
	1/10,000	+	+	+	−	−	−
C59 O	N	+++++++	+++	++++	++++	+++++++	+++++
	1/10	++	+	++	++	+++++	+
C59 I	N	+++++++	+++++	++++++	+++++	+++++++	+++
	1/10	++	+	++	+	++	+
C59 VI	N	+++	+++++++	+++++	+++++	++++++++++++	+++++
	1/10	+	+	++	+	+	++
C59 VII	N	+++	+++	+++	++++	++++	++
	1/10	+	+	+	−	−	−
C59 IX	N	++	+	++	++	++++	+++++
	1/10	+	−	+	−	+	+
C59 XII	N	++	+	++++	+?	+++++	+
	1/10	+	−	+	−	+	+
C59 XV	N	+	+	+++	+++	++++++	+
	1/10	+	−	+	+	+	−
T59W I	N	+++	++	++++	++	+++	++
	1/10	+	+	+	−	−	−
T59W VI	N	++	+++++++	++++++++++	+++++	+	++++++++++
	1/10	+	+	++++	++	−	+
T59W XI	N	++	++	++++++	++	+	+++
	1/10	+	+	+	−	−	−
T59W XII	N	+	+++	++++	+	++++++	++++
	1/10	+	−	++	−	+	+
T59W XIV	N	−	−	++	−	−	−
	1/10	−	−	++	−	−	−
T59 XV	N	−	−	−	−	−	−
	1/10	−	−	−	−	−	−
T112A I	N	++++	++++	+++++	++++	+++++	++++++
	1/10	++	+	++	+	+++	+
T112A V	N	+	++	+++++	++	++++	++++
	1/10	−	±	+	+	−	−
T112A IX	N	−	++	++++	+	+++	++++
	1/10	−	+	+	−	−	−
T112A XI	N	−	−	−	−	−	−
	1/10	−	−	−	−	−	−

fraction and this and a 1/10 dilution of this in physiological saline was used for skin testing in comparison with 1/1000 and 1/10,000 dilutions of standard 'Pollaccine'.

Table 10 gives the results of the prick tests for T59W and T112A. The tests in each vertical column were done on the same patient. It is immediately obvious that there are great individual variations between different patients, but that the majority reacted strongly to fractions I and VI of the two preparations and that no one at all reacted to fraction XV of either preparation although these contained large amounts of protein as judged from the heights of the peaks in Fig. 7.

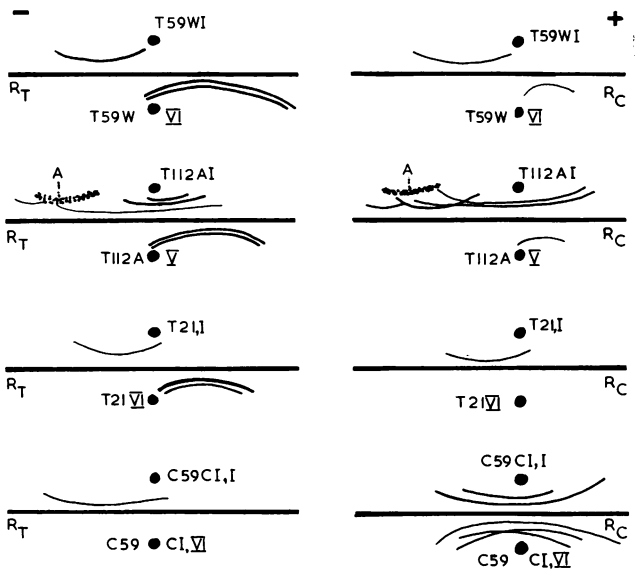

FIG. 12. Immunoelectrophoretic comparisons of the most active (by skin test) DEAE fractions of crude and purified Timothy and Cocksfoot preparations (cf. Fig. 7). Key as for Figs. 10 and 11. Antigen A is indicated by the letter A.

Table 11 confirms this impression. In fact, all the patients can be seen to have reacted very strongly to fraction I of C59CI, T59W and T112A, and many of them if anything still more strongly to fractions VI (and V of T112A). Although patients reacted to the last eluate from the Cocksfoot preparation C59, all the last eluates from the Timothy preparations were inactive.

The fractions of T21.11112112 (= T21A) were tested on ten patients. All ten reacted strongly to fraction I, residue and dialysate; nine of them reacted strongly to fraction VI, residue *or* dialysate, but the reactions to the residue were stronger (some of them stronger than to fraction I) than those to the dialysate. Only six reacted (slightly) to fraction IX, either to residue *or* to the dialysate. Five patients reacted to fraction VIII which did not appear to be dialysable, four to fraction X (not the dialysate) and only one (slightly) to fraction XI.

Since the area of fraction VI of T21A (Fig. 7) comprises less than 10 per cent of the total fractionation area and most of the activity appeared to be concentrated in fractions VI and I, it is probably correct to assume that the DEAE fractionation has brought about a further ten-fold purification. It certainly has brought about the separation of the antigen

common to Cocksfoot and Timothy (Fig. 6) from the two 'innermost' antigens specific for Timothy; in fact fraction VI of T21A consists of these two innermost specific Timothy antigens, one or both of which appear to be very active indeed. Since T21A assayed about 5,000,000 Noon units per mg. (skin test) this would make 50,000,000 Noon units per mg. for fraction VI (and/or I). Since the original ultrafiltered, dialysed and freeze-dried Timothy extract T21 assayed 30,000 Noon units per mg. it appears that something like a 1000-fold purification has been achieved. Weight for weight fraction VI of T21 may be expected to be up to 50,000 times as active as the pollen from which it has been derived.

However, accurate data must await the isolation of pure fractions in larger quantities, particularly in view of the inaccuracy of the skin tests.

DISCUSSION

In allergy to-day probably two of the most important — and most controversial — questions for immunologists and clinicians alike are

(*a*) whether all or any of the constituents of pollen (and of other allergenic materials) that are antigenic for rabbits can be identified with the allergens for man in these materials, and

(*b*) whether pollen allergens are stable substances — of which eventually stable standardized preparations will be available for clinical purposes — or whether they are the intrinsically unstable, easily dissociable substances they have generally been reported to be (Loveless *et al.*, 1951, 1955; Sehon and Richter, 1959; Johnson *et al.*, 1958).

Further, it is still being disputed whether pollen allergens are dialysable or non-dialysable (Loveless *et al.*, 1951, 1955; Sehon and Richter, 1959; Goldfarb, Moore, Rapaport, Sklarofsky, Gettner and Abramson, 1954; Johnson *et al.*, 1958) and we can still not be sure of the chemical nature of allergens. One would also very much like to know whether allergens can be distinguished, chemically or physico-chemically, as a class from non-allergens or whether any substance can become an allergen under certain conditions; and if that were so, what these conditions are.

ALLERGENS VERSUS OTHER ANTIGENS

In the present investigation it is shown that extracts of Cocksfoot and Timothy pollens are not only chemically (Augustin, 1959a) but also antigenically even more complex than was suspected hitherto (Augustin, 1959b, 1959c). We have now demonstrated a minimum of fifteen antigens in each of the crude pollen extracts by means of rabbit antisera to crude Cocksfoot and Timothy pollen extracts. Some of these could only be revealed by cross precipitation with the heterologous antisera which sometimes have larger amounts of antibodies for what must be antigens, immunologically at least partially identical in the two pollens, but present in very different amounts.

We have been able to separate several of these antigens from the majority of the remainder and could show that the activity of these purified fractions varied independently of each other in different patients.

Early on in this series of investigations (Augustin, 1953a, 1957) we found that thoroughly washed precipitates of pollen extracts with their rabbit antisera were highly skin reactive (for pollen-sensitive subjects) when dissolved in dilute alkali and neutralized; however, in spite of the careful washing, this activity could have been due to allergens being carried

down non-specifically with non-allergen antigen-antibody precipitates; more recently (Augustin, 1960b) we were able to report that most of the skin reactivity of pollen extracts can be precipitated by rabbit antisera for pollen extracts. The antisera used for this purpose were concentrated mixed high titre antisera for crude Cocksfoot and Timothy pollen extracts, to ensure the presence of antibodies for as many different determinants as possible. We were never able to precipitate all the skin reactivity, probably because antibodies for particular allergens were lacking or in too low a concentration for precipitation; and one could again argue that skin reactivity was carried down non-specifically with non-allergen antigen-antibody precipitates; it is, however, unlikely that most of the skin reactivity should be carried down non-specifically.

Also, in further confirmation that allergens are antigens for rabbits, the purification of allergens could be followed by gel diffusion tests and the most pure allergens could be identified by precipitation lines found (amongst others) in the crude starting preparations. Further, a highly active allergen concentrate (T21.111121112 = T21B) was found to induce, in rabbits, formation of antibodies which gave, with this preparation, gel diffusion patterns identical with those given by antisera produced with the crude pollen extracts. At the same time, this is also evidence that the acid NaCl fractionation used in the isolation of this allergen concentrate had not harmed (at least not in any detectable way) the allergens. In further confirmation that the acid fractionations are in no way harmful, all the antigens isolated from the 'acid' concentrate could also be isolated under the identical experimental conditions from unfractionated crude preparations and preparations fractionated at neutral pH. Antigens isolated by a particular set of conditions proved to be immunologically identical (in terms of the antisera used by us), regardless of the past history of the starting materials.

The immunoelectrophoretic behaviour of the three most active allergen fractions agreed with what had been deduced from the (preparative) immunoelectrophoretic analyses of the unfractionated extracts (Augustin 1959c); they were found to have the electrophoretic mobilities of serum α and β globulins.

Timothy and Cocksfoot pollen preparations yielded one fraction (fraction I) which contained an antigen identical for the two pollens and this fraction was highly active in all the patients tested. The other most active fraction which was in most, but not in all patients, even more active than fraction I, was fraction VI (fractions V of T112A) (Tables 10 and 11). However, although this fraction was isolated under the identical experimental conditions from both pollens, there was no immunological cross reaction whatever between these two fractions in terms of our rabbit antisera; also, although most patients reacted strongly to the Timothy as well as to the Cocksfoot pollen fraction, some reacted more strongly to the Timothy and others more strongly to the Cocksfoot preparation. DEAE-fractions VI of Timothy and Cocksfoot pollens were associated with a very small protein peak (Fig. 7) although they had, of all the fractions, the highest skin reactivity in patients.

These two most active fractions of Timothy and Cocksfoot pollens were found to belong to what has been called the 'innermost' group of antigens (Augustin, 1959b, 1959c) forming the diffusion lines nearest the antibody reservoirs in gel diffusion plates (Fig. 6). This again confirms previous experiments in which it was shown that destruction of the outermost heat labile pollen antigens (A-antigens) had no effect on skin reactivity (Augustin and Hayward, 1955b; Augustin, 1955, 1959b, 1959c) or clinical efficacy (Frankland and Augustin, 1962). Moreover, crude pollen extracts were inactivated (skin

test) by digestion with a mould protease which left the A-antigens intact while destroying the heat stable antigens (Augustin, 1959c). We can therefore be reasonably sure that the A-antigens are not allergens although one would like to confirm this indirect evidence by direct testing with the isolated A-antigens free from other antigens. We have not yet succeeded in doing this; although we were able to concentrate the A-antigens in one particular DEAE-fraction, this fraction still contained at least six other antigens (Fig. 12) including the very active allergen common to Cocksfoot and Timothy.

The purest fraction I preparation was derived from an already highly purified Timothy preparation, T21.11112112 (= T21A), and formed two precipitation lines with antisera capable of forming fifteen lines with the crude preparations. T21A also yielded the purest fraction VI preparation which was found to be responsible for the innermost 'twin precipitation lines' which are prominent in the Ouchterlony patterns of the crude Timothy extract with the homologous antisera (Augustin, 1959b and Fig. 6). The corresponding Cocksfoot preparation formed three lines and neither preparation reacted at all with antisera for the heterologous pollen, again confirming earlier tests with crude preparations (Augustin, 1959b).

Thus all our evidence goes to show that allergens are indeed antigens for rabbits. It is unlikely that our most potent allergen preparations (which remained associated with antigen-rabbit-antibody gel diffusion lines) should have consisted largely of non-allergenic material, for on a weight for weight basis these allergen preparations appear to rank amongst the biologically most potent substances known (see later).

It has been suggested (Raynaud, Relyveld, Couzinet and Bepoldin, 1959) that diphtheria toxin does not precipitate with antitoxin specific for the toxic group of the molecule because only one such group is carried by each toxin molecule. Similarly it is possible that allergens do not form precipitates with reagins because these are antibodies for a particular allergenic group of which only one is carried by each allergen molecule.

ARE ALLERGENS REALLY UNSTABLE EASILY DISSOCIABLE SUBSTANCES?

While it has been very difficult to come to quantitative conclusions as regards yields and activities of these preparations because of the notorious inaccuracy of the skin tests, the activities are certainly very high (see later) and we have never been able to discover any losses during our fractionations. This suggests either the production of active molecules by breakdown from inactive material or from other active molecules or the removal of inhibitors. We have not had any evidence for either possibility; an increase of the activity of Rye grass extracts during storage in the cold has been reported by Kammann (1912), but we have not been able to discover such an increase with any of our own pollen extracts.

The spontaneous crystallization of glucosides ('Dactylen') from crude pollen extracts (Augustin, 1959a and this work), the fact that during storage insoluble pigmented precipitates form in crude extracts which are inactive and the production of partially insoluble (and inactive) precipitates at pH 4.0 and 5.0 during the fractionation of crude extracts, all point to some denaturation and dissociation. However, we have no indication that these reactions are in any way concerned with the active molecules. On the contrary, the antigens of the most pure preparations could be identified with the antigens from the impure preparations, giving reactions of identity with them. One might argue that the haptenic groups could have remained the same and that an inactive part of the molecules

had been split off. While such a split may occur immediately after or even during the extraction of the pollens, all our evidence goes to show that there is no further splitting later on; the most pure fractions were no more dialysable than the crude starting materials, even though the crude and the purified materials contained some molecules small enough to diffuse through stretched Visking membranes, which held them back in the unstretched state. The crude and purified preparations contained a small percentage of molecules which were a little more easily dialysable than the rest and some of these were active by skin tests. These molecules were immunologically (and mostly also by skin test) distinct from the less readily dialysable antigens. Moreover, the two highly active Cocksfoot and Timothy preparations prepared by acid salt fractionation retained their activity for 7 years during storage at 4°. Crude pollen extracts have been shown to withstand heating at 100° and a large range of acid and alkaline pH changes (Augustin and Hayward, 1955b; Augustin, 1959b, 1959c).

Thus all this suggests that pollen allergens are very stable. The deterioration of crude extracts after storage of 1 year or longer (Augustin and Hayward, 1955b; Augustin, 1955) and the fact that highly dilute pollen extracts can lose activity within 30 minutes of their preparation (Hjorth, 1957, 1958) probably means that crude extracts contain highly reactive substances that are injurious to allergens; possibly the various flavonoid impurities are involved; also it should be appreciated that pollen solutions containing 100 Noon units or less per ml. are very dilute allergen solutions indeed and may contain no more than 10^{-5} mg. of allergen protein per ml. Under these conditions deterioration may be expected to occur with all proteins and may be in no way peculiar to pollen allergens.

COMPARISON WITH OTHER WORK ON GRASS POLLENS

It is difficult to compare the results of the extensive electrophoretic centrifugal and salt fractionation studies of a preliminary nature by Johnson and collaborators (Johnson *et al.*, 1958) with our own. However, their molecular weight estimations are of the same order of magnitude as has been reported for Timothy pollen allergens (Augustin, 1953b), i.e. 17,000–19,000 compared with our 14,000, although Johnson *et al.* seem to have incurred rather higher losses than ourselves during dialysis; but this may possibly be attributable to their using rather less fresh pollens than we have done and it is also possible that they have increased the pore size of their membranes by stretching, as in the membrane pressure dialyses reported here. It seems to us that fractionation by electrophoretic convection is not a very good method for separating structurally similar, relatively fast diffusing molecules. Johnson *et al.* report their inability to fractionate by means of NaCl at acid pH although this has proved a most useful method in our hands (Augustin, 1953b and the present work). Like ourselves (Augustin, 1953b and here) they were able to isolate a relatively pure component by precipitation at pH 5.0. However, the present work shows clearly that free electrophoresis as used by Johnson *et al.* (1958) and earlier on also by ourselves (Augustin, 1953b, 1956) is not a sufficiently discriminating analytical tool; in fact, in our hands progress was only possible after we had developed gel diffusion and immunoelectrophoretic techniques (Augustin and Hayward, 1955a, 1957; Augustin 1959b, 1959c). In spite of their intensive investigations, which did, however, not include immunochemical analyses, they were led to believe that Rye and Timothy grass pollen allergens were very smiliar. Previous investigations (Augustin, 1959b, 1959c) and the present work have shown that grass pollens contain individual as well as shared allergens.

TABLE 12

TENTATIVE ESTIMATES OF THE SKIN REACTIVITIES AND PURIFICATIONS OBTAINED UP TO DATE FOR THE MOST HIGHLY PURIFIED ALLERGENS

Allergens from	*Purified preparation skin reactive at intradermal injection of* (μg.)	*Purification with respect to dialysed crude starting material*	*Minimum number of precipitation lines with antiserum for starting material*		*Molecular weight*	*Chemical nature*	*Precipitation of allergens between ammonium sulphate concentrations % saturation*	*Authors*
			Starting material	*Purified preparation*				
Grass pollens	10^{-7}–10^{-8}	300-fold	15–17	2 (DEAE fraction I) 2 (Timothy-DEAE fraction VI)		Protein (+carbohydrate?)	30–80 (crude preparation)	Augustin, 1960 and this paper
					14,000 17–20,000 }	crude preparations		Augustin, 1953b Johnson *et al.* 1958
Ragweed Giant	10^{-3}	?	5	1 (splitting into 3 on storage)	Not done	Protein + carbohydrate	?	Goldfarb, 1958
Short ('deproteinized')	10^{-4}	50-fold*	2	?	S = 1.2	Polypeptide	?	Sehon and Richter, 1959
Short (dialysed residue)	10^{-4}–10^{-5}	?	?	?	S = 3.6	Protein + carbohydrate	?	Lea, Meacock, Sehon and Freedman, 1961
			10–12	?				Gerschenfeld *et al.*, 1961
Alder pollen	?	42-fold†	Not done	Not done	Not done	Polypeptide	?	Herbertson *et al.*, 1958
Horse dander	10^{-4}	<2-fold	7	2 (+small quantities of serum albumin and globulin)	34,000	Protein + carbohydrate	55–80	Stanworth, 1957, 1958
Diphtheria toxin	2×10^{-3}	15-fold	15	1	74,000	Protein	45–80	Pope and Stevens, 1958, 1959; Relyveld *et al.*, 1959

* The authors state that the 'deproteinized' solution had been purified 1000 times, but that the purification figure in terms of dialysed crude residue is 50.

† The authors have purified the crude alder pollen extracts 300-fold, but this includes a 7-fold purification by dialysis.

Relative Potencies of the Most Active Allergen Preparations known

In Table 12 a very tentative attempt has been made to compare the activities (and other properties) of our most potent grass pollen preparations (Augustin, 1960b, and the present work) with the most potent allergen preparations from other laboratories. In view of the great inaccuracy of the skin tests and the fact that different test procedures have been employed by different workers, some of the figures in column 2 (Table 12) may possibly be wrong by a factor of about 100. Stanworth (1957, 1958) and ourselves have used skin prick tests to estimate biological activity, Stanworth by comparing the size of skin tests and we by comparing different dilutions, which included one which was too high to give any reaction (Augustin, 1959a); all other workers have tested by means of intradermal injections. Squire (1950) has shown that a prick test is equivalent to an intradermal injection of 3×10^{-6} ml. of the same preparation and we have used a factor of 10^{-5} for converting prick tests into intradermal tests.

The minimum figures for skin reactivity in Table 12 (column 2) apply to intradermal injections of the purified preparations. The figure of 10^{-8}–10^{-6} µg. for grass pollens was arrived at by assuming an activity of 10 million Noon units per mg. for our purest preparations (although some of the DEAE-fractions may have been more active still). Since pollen extracts containing 10-100 Noon units per ml. give positive prick tests in most allergic subjects this means that solutions of 10^{-6}–10^{-5} mg. of the highly purified pollen preparation/ml. give positive skin reactions and, thus, an intradermal injection 10^{-11}–10^{-10} mg. (10^{-8}–10^{-7} µg.) of purified pollen proteins.

In 1958, Goldfarb, Bhattcharya and Koerner reported that solutions of their most pure Giant Ragweed preparation, Trifidin A, gave strong skin reactions when 0.02 ml. of solutions containing 0.033–0.33 µg/ml. were injected intradermally, i.e. 0.0006–0.006 µg. of Trifidin A. It is therefore probable that 10^{-3} µg. (or even less) could still have given positive reactions.

Sehon and Richter (1959) reported a fifty-fold purification of a 'deproteinized' extract of Short Ragweed (the purification involved heating at 60° for 15–20 minutes) which gave positive skin tests on injecting 10^{-7} mg., i.e. 10^{-4} µg. Very recently, Lea, Meacock, Sehon and Freedman (1961) reported that a fraction 'active at 0.001 µg./ml. in PK-tests' had been isolated from a dialysed residue from extract of Short Ragweed. We take this to mean that the intradermal injection of 0.05 ml. of this solution, i.e. 5×10^{-5} µg. gave a positive skin test and a figure of 10^{-5}–10^{-4} µg. has therefore been entered in Table 12.

Stanworth (1957) reported positive skin tests at concentrations of 1 mg./ml. of his horse dander preparation. From our experience with pollen extracts it is likely that solutions giving the size of skin response reported by him will still be positive when diluted one hundred-fold. In this way a figure of $10^{-2} \times 10^{-5}$ mg. ($= 10^{-7}$ mg. or 10^{-4} µg.) was arrived at for a minimal intradermal reaction.

Kuhns and Pappenheimer (Kuhns, 1955, 1959) have advocated the diphtheria system as a model system for immediate type hypersensitivities since, provided the allergen really is the diphtheria toxin, additional independent methods of estimations are available. The toxin preparation used by them (Kuhns and Pappenheimer, 1952) contained 0.46 µg. of specifically precipitable (by antiserum) toxin nitrogen (N) per Lf unit while the purest toxin preparations have been reported to contain 0.33 µg. of toxin per Lf unit. Kuhns used 0.1 Lf units of toxoid and 0.0002–0.0004 µg. of toxin N to produce immediate type wheals; this, converted to protein, gives a figure of about 2×10^{-3} µg. for positive skin reactions.

Table 12 thus shows that several allergen preparations have been purified to the extent that the intradermal injection of 10^{-3} µg. or less gives positive skin responses in the appropriately allergic subjects. The most active preparation appears to be that which we have isolated from grass pollens, although the figure entered in the table is a conservative estimate; it is possible that some of the grass pollen allergens, particularly the 'innermost' Timothy twin antigens are intrinsically more active than other allergens, for our extracts have probably not been much more highly purified than other pollen preparations (300-fold for grass pollens compared with about fifty-fold for Alder pollen and Ragweed pollen). It is to be expected that Stanworth's still heterogeneous horse dander preparation (Stanworth, 1958) can be further purified, particularly in view of the low purification figure (less than two-fold) up to date (Stanworth, 1957).

From this table it appears that diphtheria toxin is weight for weight no more active as an allergen than the other preparations quoted. However, from the recent work of Relyveld, Henocq and Raynaud (1961) it appears that the allergen(s) in Kuhn's toxin preparation(s) were after all not the toxin itself, but an impurity. This impurity was found to differ also from the non-toxic P-proteins which Kuhns had shown not to be the allergens (Kuhns, 1955); i.e. the suspicions of Vaughan and Kabat (1953) have come true.

This means that the diphtheria system can unfortunately not provide the short cut to the solution of the mechanism of immediate type hypersensitivities for which Kuhns (1952, 1955) had hoped.

THE BIOLOGICAL POTENCY OF ALLERGENS IN RELATION TO THAT OF THE MOST POTENT BACTERIAL TOXINS

On intravenous injection the lethal dose of pollen allergens for subjects sensitive to them may be no higher than about 10,000 times the minimum skin-reactive dose; that is, lethal doses may be of the order of 10^{-3} µg. of our purest preparations of grass pollen allergens (Table 12) and 1 µg. of the purest Ragweed pollen allergens. In fact, near-fatal reactions occur occasionally in the clinic when exceptionally highly sensitive hitherto untreated subjects receive their first hyposensitizing dose according to Noon's method (1911). In present-day practice this dose (which is given subcutaneously) consists usually of 0.1 ml. of a solution containing 100 Noon units/ml., i.e. near-fatal reactions can occur with subcutaneous injection of only 0.001–0.01 µg. of pollen allergens. (A solution containing 100 skin-reactive Noon units/ml. contains no more than 10^{-5}–10^{-4} mg. of the allergens described here, i.e. 0.01–0.1 µg./ml.). Death has been reported from the accidental intravenous injection of pollen extracts (Frankland, 1958) two weeks after 20, 40 and 60 Noon units had been tolerated satisfactorily.

If it is remembered that the lethal doses of botulinus, tetanus and dysentery toxins for man are 0.06 µg. and the lethal dose of diphtheria toxin 20 µg. (Van Heyningen, 1955), it can be appreciated how highly potent the isolated allergens are, even though we cannot claim that they have been isolated in pure form. However, the term 'pure' is relative as pointed out by Marrack (1958) for diphtheria toxin. Successful physico-chemical and structural studies have been done on bacterial toxins, myoglobins, etc. (Marrack, 1958) which may have been no more highly purified than the grass pollen allergens described in the present work.

Many fatal and near-fatal reactions have occurred with a chemically pure substance, penicillin (Garrod, 1958; Levine, Perri and Anthony, 1960). Penicillin has been

regarded as a particularly strong sensitizer. However, it should be appreciated that routine therapeutic doses (intramuscular injections) are of the order of 250,000–1,000,000 units (Martindale, 1958); this is equivalent to 150–600 mg. of penicillin G (1,670 units/mg.) — in terms of hypersensitivity reactions a simply enormous dose. Shock has been produced with as little as 10 units (during testing for hypersensitivity (Mayer, Mosko, Schutz, Osterman, Steen and Baker, 1953)) which represent 6 μg. of penicillin. This is still a large dose in respect to hypersensitivity reactions. Penicillin may be a less potent allergen than the allergens of grass pollens, but under present condition much larger quantities are encountered of the former. If testing is attempted at all in allergic subjects, it should certainly be done with much lower concentrations of penicillin than is common practice now.

SPECIAL PROPERTIES OF ALLERGENS IN GENERAL

Can we come to some conclusions as to general features of allergens as a class? Table 12 shows that all the allergens are of relatively low molecular weight and we know that most of the grass pollen allergens are of a size that is only just held back by the Visking membranes we use. Table 12 also shows that all the purest allergen preparations contain proteins but that (whenever tested) they were found to contain also carbohydrates; pollen preparations were generally found to contain arabinose and galactose (Augustin, 1959a; Goldfarb *et al.*, 1958; Lea *et al.*, 1961). All the pollen allergens are relatively fast diffusing molecules, the active molecules always forming the 'inner antigen lines' next to the antiserum reservoirs in gel diffusion tests. All the allergens are substances which are relatively heat stable (Augustin and Hayward, 1955b; Augustin, 1959b, 1959c; Sehon and Richter, 1959). The 'outer' antigens of pollens (and probably those of other crude allergen preparations (Augustin, 1960b)) appear to be heat labile molecules and may not be allergens for man although they are antigenic in rabbits. The most conclusive evidence for this view has perhaps been provided for grass pollens (Augustin and Hayward, 1955b; Augustin, 1959b, 1959c) but seems to hold good also for Ragweed; for Wodehouse has shown that the 'outer antigens' of Ragweed were heat labile and Goldfarb *et al.* found that what he assumed to be the only allergen of Ragweed, was an 'inner' antigen.

Although evidence has accumulated that allergens are digestible by proteolytic enzymes (Augustin, 1955, 1959c, 1960b) this can no longer be taken as evidence that allergens are proteins; for Pusztai and Morgan, (1961) have shown that the specificity of blood-group substances is destroyed by proteolytic digestion although it is well established that their immunological specificity depends on their carbohydrate moiety. Therefore, although we can still be sure that grass pollen allergens (and other allergens, Augustin 1955, 1959c, 1960b) require a structurally intact protein part to remain active, it is possible that their immunological specificity (and allergenic activity) is not dependent upon the protein part.

It has been pointed out in the experimental section that the most active grass pollen allergens (from both Timothy and Cocksfoot) were eluted from the DEAE-cellulose columns under the same conditions under which serum γ globulins and reagins are eluted. One is therefore tempted to speculate whether there are some, possibly not chemical, but physical properties (and therefore behaviour) which highly active allergens share with human serum or tissue proteins; this would provide a ready explanation why reagins (i.e. antibodies for allergens) should have a special affinity for human body tissues. However, it remains to be seen whether potent allergens other than those of grass pollens exhibit the same physico-chemical behaviour.

PRACTICAL ISSUES

A special object of this work was (*a*) the development of a quick and simple method suitable for the large-scale preparation of stable purified extracts for clinical use containing all the allergens of crude extracts but with a minimum of pigments and other low molecular impurities, and (*b*) the development of a method for making a more highly purified stable preparation in solid form containing the minimum number of non-allergenic antigens, but a high yield of the principal allergens, to serve as a standard for testing and clinical purposes as well as to form the starting material for the final isolation of the pure allergens.

The allergen solutions prepared by the extraction with Coca's solution of pollens previously extracted for 1 minute with water, as described here, fulfil the requirements of (*a*) and the highly active powders we obtained by means of the NaCl fractionation of acidified concentrates of the dialysed pollen extracts fulfil the requirements of (*b*).

Extracts prepared according to (*a*) gave gel diffusion patterns identical with those of the crude extracts used in previous years, were as active by skin tests and have for the past 3 years replaced in clinical practice the original 'Pollaccine' solutions that had been prepared essentially according to Noon (1911); the slight reduction of antigens of lowest molecular weight in the recent extracts, as compared with those of the original 'Pollaccine' did not seem to matter clinically and stability was, if anything, improved.

The powders prepared from Cocksfoot and Timothy grass pollens by means of the acid NaCl fractionation were found to retain their high activity for 7 years, whereas it is usually regarded unsafe to use extracts that have been stored (4°) for more than 1 year for clinical purposes (cf. also Augustin and Hayward, 1955b). We show that the most active preparations made by the NaCl fractionation contain only three to four components that give rise to antibody formation in rabbits, that all these antigens are important allergens (although we do not yet know whether both or only one of the 'twin' antigens of the Timothy pollen preparation are allergens since they have not yet been separated from each other) and that the described process of preparation entails no loss of activity; it will be remembered that similar preparations had been found previously to be clinically as effective as crude extracts (Frankland and Augustin, 1954). We also show that these preparations are particularly suitable starting materials for the final separation and isolation of the pure allergens (e.g. by fractionation on DEAE cellulose).

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