

Grass Pollen Allergens

II. ANTIGEN-ANTIBODY PRECIPITATION PATTERNS IN GEL: THEIR INTERPRETATION AS A SEROLOGICAL PROBLEM AND IN RELATION TO SKIN REACTIVITY

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Summary. The Ouchterlony patterns of fifteen grass pollen extracts (against rabbit antisera to the pollens of Cocksfoot—*Dactylis glomerata* and Timothy—*Phleum pratense*) are compared with skin reactivities in allergic subjects of this country and with quantitative assays in terms of a heat labile component of Cocksfoot referred to as antigen A. Problems of standardization in general and of the Noon unit in particular are discussed.

Ten indigenous pollens are shown to give positive skin responses in all the pollen sensitive subjects tested, except in one with a most unusual selective specificity for Timothy alone. All indigenous pollens examined contain substances cross-reacting with antigen A, while the four 'foreign' pollens, which give small or no skin responses, do not share the A-group specificity. Nevertheless A-content cannot be equated with skin reactivity. British pollens have numerous antigenic determinants in common other than the A-group specificity, although the occurrence of completely identical antigens in different pollens is unlikely.

Complications in the interpretation of Ouchterlony patterns of related natural products are discussed from a purely serological point of view with the help of azoprotein models. Evidence is presented for the occurrence of what may be called *bi-specific antibodies*. It is suggested that antibodies specific purely for small haptenic groups such as arsanilic acid are probably never produced.

From gel diffusion tests on numerous bleedings from rabbits it seems that prolonged immunization does not lead to the formation of less specific sera as is usually suggested. On the contrary, more and more precisely fitting antibodies appear to be produced to an ever-increasing number of related antigens and minor impurities, probably to more determinants on one and the same molecule or even to new permutations of adjacent determinants on the same molecule.

The need is stressed for more precise definitions of the terms *bi-specific antibody* and *major and minor antigens*.

INTRODUCTION

GRASS pollen extracts have been shown to contain common components that are antigens for rabbits and guinea pigs by precipitin (Augustin, 1953) and gel diffusion tests (Wodehouse, 1955; Augustin, 1955).

It is the purpose of the present investigation to report a more detailed, and in part quantitative, investigation of a larger number of grass pollens than examined before and to attempt to correlate the findings with skin tests in allergic subjects.

At the European Congress of Allergy in 1956 Taylor and Augustin used azo-protein models to illustrate the type of Ouchterlony patterns obtained with pollen extracts (and other related natural products). The details were not published at the time, and as they can serve to clarify the questions raised by the experiments with pollen extracts they are presented here.

MATERIALS AND METHODS

ANTIGENS

Crude extracts were prepared by shaking intermittently 1 g. samples of the pollens (*Phleum pratense* or Timothy, =T., *Alopecurus pratensis* or Meadow Foxtail, =M.Fox; *Dactylis glomerata* or Cocksfoot, =C., *Festuca pratensis* or Meadow fescue, =M.F., *Festuca rubra* or Red Fescue, =R.F., *Festuca arundinaceae* or Tall Fescue, =T.F., *Lolium perenne* or Italian Rye grass, =I.R., *Cynosurus cristatus* or Dog's Tail, =D.T.; *Arrhenatherum elatius* or Tall Oat, =T.O., *Anthoxanthum odoratum* or Sweet Vernal, =S.V.; *Bromus mollis* or Soft Brome, =B.R.) with 10 ml. of a modified saline solution containing 0.5 per cent phenol (Coca, 1922) at room temperature for 48 hr. One g. samples of *Zea mays* (or Maize, =M.), *Cynodon dactylon* (or Bermuda, =B.E.), *Spartina townsendii* (or Cord grass, =S.) and *Bambusa sp.* (or Bamboo, =B.A.) were extracted with 2 ml. only.

As before (Augustin, 1959a), a carefully stored (0–4°) preparation of the current 'Pollaccine' (a commercially available mixed pollen extract of T. and C.) served as the reference standard. It was assumed to contain 100,000 Noon units (skin test) and 100,000 A-units (gradient diffusion method) per ml.

Azo-proteins were prepared by coupling arsanilic acid (Baker, Campbell, Epstein and Singer, 1956) to crystalline bovine serum albumin (Armour and Co. Ltd.), horse serum globulin (Burroughs Wellcome and Co. Ltd.), rabbit serum albumin (prepared by ammonium sulphate fractionation), proteins of zebra serum and crystalline lysozyme (Armour and Co. Ltd.) (referred to as BSaA*, Hsg*, RSa*, Ze* and LyA* respectively), using 50–100 molecules of diazotized arsanilic acid per molecule of protein. More heavily loaded lysozyme and BSa conjugates were also prepared with double the amounts of arsanilic acid (LyB* and BSaB*); the conjugates were purified by ammonium sulphate precipitation followed by dialysis against physiological saline; most of the conjugates were prepared during 1955 and 1956 in collaboration with Dr. A. B. Taylor (Augustin, 1956; Taylor and Augustin, 1956). All the conjugates except the heavily loaded LyB* cross-reacted with antisera to the uncoupled carriers BSa, HSg, RSa and Ly, and with antisera to HSg*.

ANTISERA

Rabbits were immunized with T. and C. preparations by the multiple depôt method, used in previous investigations (Hayward and Augustin, 1957).

SKIN TESTS

These were carried out by a modification of the prick method as described in the preceding paper of this series (Augustin, 1959a).

ANTIGEN A CONTENT

Assays were carried out by the gradient gel diffusion method in tubes as already described (Augustin and Hayward, 1955) using pooled antisera to C. containing mainly antibodies to the A-antigen. Results were expressed in A-units, in terms of the standard 'Pollaccine'.

GEL PLATE TESTS

The qualitative comparisons of antigen-antibody precipitation patterns were carried out essentially according to Ouchterlony (1953). One per cent solutions of agar, prepared as reported previously (Augustin, Hayward and Spiers, 1958), were poured into Petri dishes to a depth of about 3 mm. Suitable patterns of circular antigen and antibody wells were then punched by means of cork borers. After the wells had been charged, the plates were placed in moist chambers and allowed to develop at room temperature. The plates were examined daily, the development of patterns followed by hand drawings and finally by photographing and/or preservation by staining (Hayward and Augustin, 1957).

The antisera used for the gel plates were specially selected to contain antibodies to as many different determinants as possible. The sera were chosen by following the progress of immunization in each rabbit by Ouchterlony tests. Suitable sera were pooled.

RESULTS

A. POLLENS

Skin Tests and Antigen A Content

In Table 1 the results of skin testing the extracts of ten British and four 'foreign' pollens on eleven allergic subjects are summarized together with the results of quantitative assays of these extracts in terms of antigen A.

Of the British pollens B.R. appears to give often weaker reactions than all the others and T. often stronger ones, but in general similar responses were evoked in all the subjects by all the extracts. There were, however, striking exceptions in some subjects, the most notable being 'Jennifer', who was sensitive to T. alone. It must, however, be emphasized that during six years she has been the only subject found in the clinic or the laboratory of this Institute with a specificity of such high degree (cf. also Frankland, 1955b); she did not suffer clinically from hay fever. Except for the pollen B.R. the subjects B.J.H. and J.H. were almost equally sensitive to all the English grass pollens, and so was A.W.F.—with the inclusion of B.R.—except that his response to S.V. was a 100 to a 1000 fold weaker than that to the other pollens. Brog. also was not very sensitive to S.V., and, in addition, was not sensitive to T.O. or R.F.

While most of the pollens gave similar skin reactions in all the subjects, some striking exceptions occurred; in terms of Pollaccine (1 ml. = 100,000 Noon units) M. Fox scored 50,000 throughout, while the score of S.V. varied from 1000 to 100,000 in different subjects; that of M.F. from 50,000 to 500,000; that of T. from 50,000 to 1,000,000; that of R.F. from less than 10,000 to 200,000 and that of B.R. from 1000 to 100,000 Noon units/ml. Variations in the scores of I.R., C. and T.F. were hardly outside the limits of error of the test.

M., B.E., B.A. and S. extracts, although five times as concentrated as those of the other pollens, gave very much smaller responses in all the subjects on whom they were tested.

TABLE I
RESULTS OF SKIN TESTS (IN NOON UNITS) AND DIFFUSION TESTS (IN A-UNITS) OF FOURTEEN GRASS POLLEN EXTRACTS
Abbreviations for pollens as given in 'Materials and Methods' under 'Antigens'.

Pollen extract of	Subjects tested											A units per ml. extract
	BjH	AWF	Alderm	Brog.	Noon units per ml. extract						Jennifer	
					J.H.	Barl	CRO	Snalll	Snellm	Collins		
T.O.: Tall Oat ..	150,000	100,000	150,000	1,000	150,000	x	x	x	x	x	x	500,000
I.R.: It. Rye grass	150,000	100,000	150,000	50,000	100,000	x	x	x	x	x	—	150,000
C.: Cockfoot ..	100,000	100,000	100,000	50,000	50,000	x	x	x	x	x	—	150,000
T.F.: Tall Fescue	100,000	100,000	100,000	50,000	150,000	x	x	x	x	x	—	100,000
S.V.: Sweet Vernal	100,000	<1,000	100,000	<1,000	50,000	x	x	x	x	x	—	64,000
M.F.: Meadow Fescue ..	50,000	50,000	500,000	50,000	100,000	x	x	x	x	x	—	220,000
T.: Timothy ..	150,000	70,000	100,000	50,000	100,000	200,000	300,000	200,000	150,000	1,000,000	100,000	150,000
M.Fox.: Meadow Foxtail	50,000	50,000	50,000	50,000	50,000	x	x	x	x	x	x	64,000
R.F.: Red Fescue ..	150,000	100,000	200,000	<10,000	<10,000	x	x	x	x	x	x	50,000
B.R.: Soft Brome	1,000- 10,000	50,000	x	x	1,000	100,000	1,000- 10,000	1,000- 10,000	100,000	10,000	x	25,000
M.: Maize ..	10?	1,000	5,000	—	—	x	x	x	x	x	—	<100
S.: Cord grass ..	100- 1,000	10	5,000	—	—	x	x	x	x	x	x	<100
B.E.: Bermuda ..	10?	—	5,000	100- 1,000	10	x	x	x	x	x	—	<100
B.A.: Bamboo ..	—	100	1,000	—	—	x	x	x	x	x	x	<100
P ₅₆ (Standard) units by definition	100,000	100,000	100,000	100,000	100,000	100,000	100,000	100,000	100,000	100,000	100,000	100,000

— = no reaction x = not tested

Table 1 thus underlines some quite striking specificities of pollens and test subjects. In spite of many similarities not one subject was equally sensitive to all the pollens, but, also,—apart from Jennifer—all the subjects reacted at least to some extent to all the British pollens with which they were tested.

The last column of Table 1 gives the results of the quantitative diffusion tests, i.e. titres in terms of antigen A which will be shown to comprise really a group of antigens. Again differences emerge, but these are not as striking as the differences in the skin tests; also, the foreign pollens, M., B.E., B.A. and S., did not contain any A-antigens. B.R. appeared to contain fewer components of A and less of those it did contain than the other grasses.

Ouchterlony Plates

Comparison of fifteen grass pollens by means of antisera to T. and C. Figs. 1A, 1B and 1C show the antigenic make-up of nine of the British pollens in terms of 'multivalent' antisera to C. (1A), to T. (1B) and to a mixture of the two antisera (1C). Numerous common components (fused lines of coalescence) can be distinguished in terms of both the antisera; however, as would be expected, more lines are obtained with the homologous than with the heterologous antisera, and, for all the pollens, the greatest number of lines with the mixed antisera. All the pollens can be seen to share a composite 'inner' ring of antigens, and all but S.V. (and possibly M.Fox) a similarly composite 'outer' ring. More detailed testing (Figs. 2A, 2B and 2C) showed that S.V. and M.Fox share the outer ring antigens and that the apparent difference in the previous tests was of a quantitative rather than a qualitative nature. R.F. also contained rather less of the outer ring antigens than the other pollens (Figs. 1). As these outer ring antigens are in fact the heat labile A-antigens (Augustin, to be published), it is not surprising that these differences are reflected in the results of the quantitative diffusion tests given in Table 1. Fig. 3 shows in detail how very complicated the make-up of the A-line in fact is. For T.O. at least seven components are clearly separated, all shared with the A-line of C., but possibly only two completely so. These two—comparatively weak—lines are most probably responsible for the 'spur' phenomenon in Figs. 1A and 1C (outer ring).

At least some components of the inner ring antigens are clearly shared by all the nine pollens, but once more there is a spur-like phenomenon, this time for M. Fox with

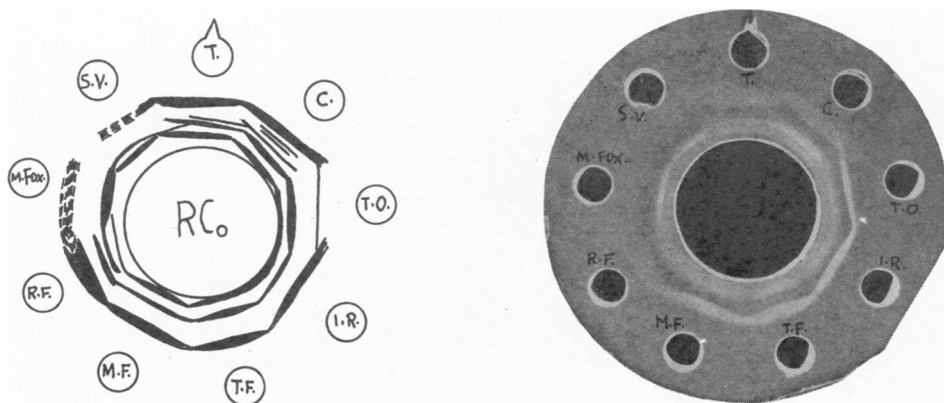


FIG. 1A. Comparison of nine British grass pollens (key to abbreviations in text under 'antigens'). Antigen cups arranged in a circle around a central cup containing antiserum to C.

T.-antiserum (Fig. 1B), and M. Fox and S.V. (Fig. 1A with C.-antiserum). In fact, M.Fox and S.V. can be seen to share fewer antigens with T. and C. than the other pollens. This does not reflect botanical relationships since M.Fox is the only pollen studied that belongs to the same group as T.; S.V. is closely related to T.O., while all the other pollens belong to the same group as C.

T. has two strong individual 'inner' lines not shared with any of the other pollens (1B and 1C) and C. several lines specific to itself between the two main rings (Figs. 1A and 1C).

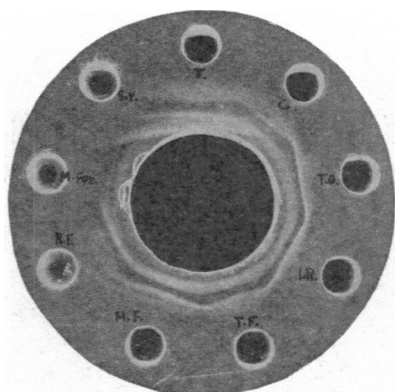


FIG. 1B. Comparison of nine British grass pollens (key to abbreviations in text under 'antigens'). Cups arranged in a circle around a central cup containing antiserum to T.

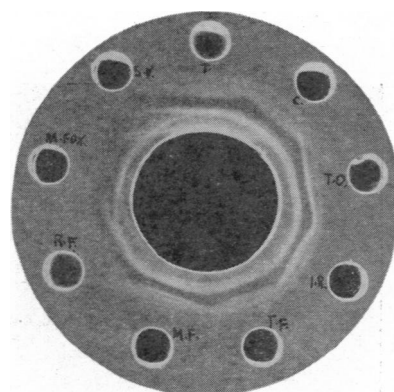


FIG. 1C. Comparison of nine British grass pollens (key to abbreviations in text under 'antigens'). Cups arranged in a circle around a central cup containing equal portions of antiserum to C. and T.

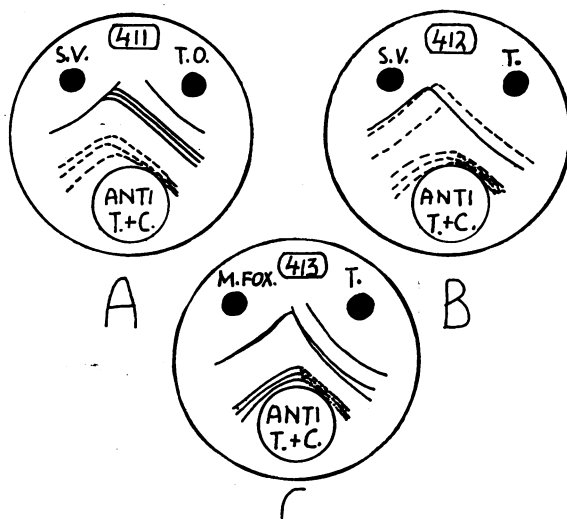


FIG. 2. Comparison of A-antigens in pairs, against mixed antisera against C. and T.

- (A) S.V. and T.O. compared;
- (B) S.V. and T. compared;
- (C) M.Fox. and T. compared.

For abbreviations see text.

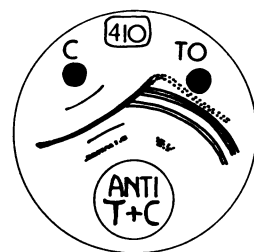


FIG. 3. Comparison of C. and T.O. in terms of a mixture of antisera to T. and C. Density and sharpness of precipitation lines indicated by thickness and sharpness of black lines. Fuzzy weak lines represented by dots. For abbreviations see text.

Grosser botanical differences were reflected in the Ouchterlony patterns. B.R., belonging botanically to yet a fourth group of grasses, appeared to differ from the other British pollens, since the inner and the outer antigens are shared only in part (Fig. 4A and B). The four 'foreign' pollens shared neither the A determinants common to the British pollens, nor those of the inner ring. Some faint precipitin lines could, however, be obtained with the mixed C.- and T.-antisera after concentrating the four extracts but, where these lines extended far enough, they could be seen to cross the lines formed by the neighbouring pollens (reaction of non-identity, Fig. 5); i.e. the indigenous pollens did not contain sufficient amounts of those particular antigens to form these lines even with their own antisera.

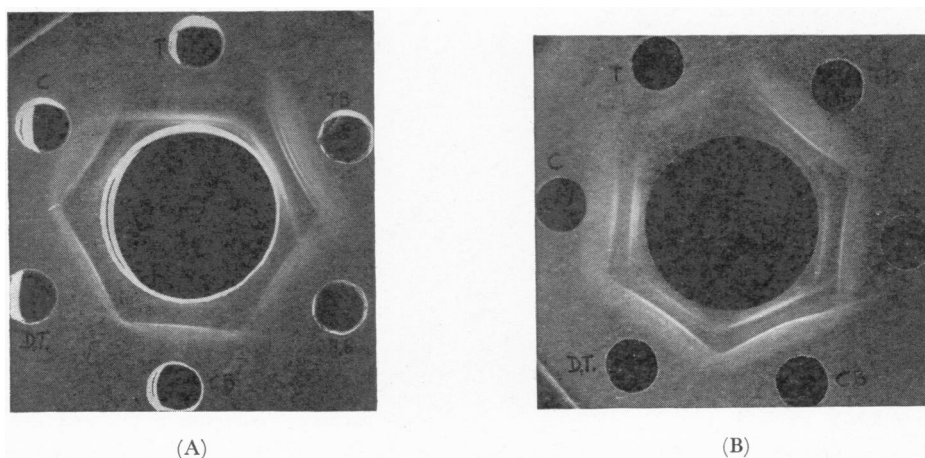


FIG. 4. Comparison of two extracts of T. (T and TB), two extracts of C. (C and CB), B.R. and D.T. in terms of antiserum to T. (Fig. 4A) and antiserum to C. (Fig. 4B). For abbreviations see text.

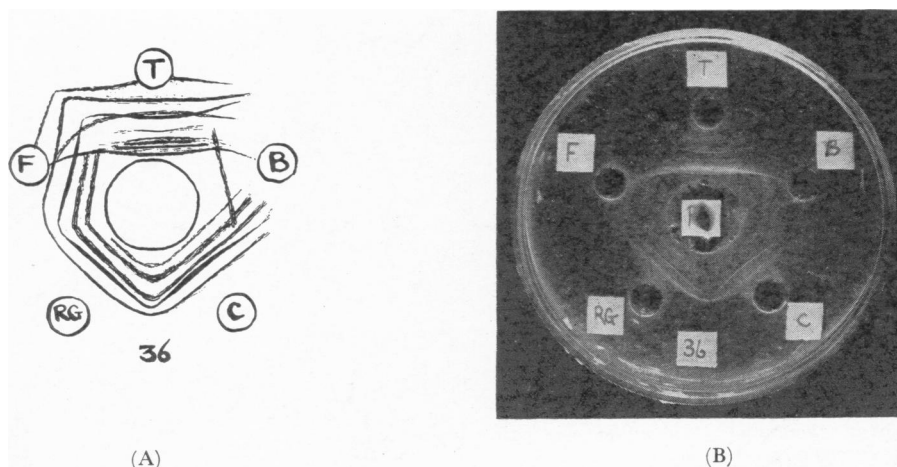


FIG. 5. Comparison of T., C., I.R.G. (=RG), a weak extract of a species of fescue (F) and B.E. (=B) in terms of an antiserum to T. An antibody gradient was established (24 hr.) before adding the antigens. A faint line crossing the T. and C. line has formed with B.E. which has been greatly concentrated. (A) Drawing. (B) Photograph.

This type of comparison in terms of antibodies produced by only two of the pollens is bound to emphasize the similarities between the other pollens. Individual antigens which have no counterpart in T. and C., and to which there are consequently no antibodies available in the antisera used, cannot be discovered in this way. The antigenic make-up of representatives of two of the major hay-fever pollens belonging botanically to different families have therefore been compared in greater detail in terms of both their antisera.

Detailed Comparison of T. and C. In Fig. 6 various extracts of T. and C. pollens have been compared with unheated and heated Pollaccine (1 min. at 100°) in terms of an antiserum to C. There is a composite outer ring due to heat labile antigens, with the majority of the components shared. All the heat resistant antigens forming the inner ring are shared. CJ is a 48 hr. extract of C. (1 g. of pollen per 10 ml. Coca's fluid—Coca, 1922), CW a 1 min. aqueous extract (1 g. per 10 ml.) which was followed by a 48 hr. extraction of the residual pollen (5 ml. of Coca's fluid per g. original pollen) yielding extract CC. TC is the T. equivalent of CJ. Many of the common components of the A-antigens can be seen to occur in variable amounts in the different extracts. It is not possible to tell from this figure whether spur formation between components of CC and CW, and between others of P57 and CJ, represent reactions of partial identity or whether one is dealing with lines due to unrelated antigens superimposed in the reaction with one extract and separated in the reaction with the neighbouring extract.

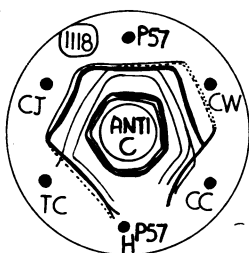


FIG. 6. Comparison of Pollaccine, unheated (P57) and heated for 1 min. at 100° (HP57), variously prepared extracts of C. (CJ, CW and CC) and T. (TC) in terms of antiserum to C. For abbreviations see text.

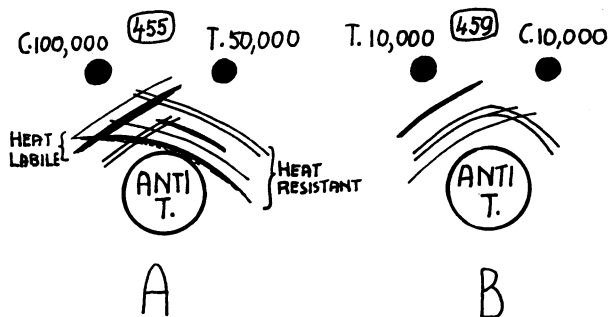


FIG. 7. Comparison of C. and T. antigens in terms of antiserum to T. Concentrations, indicated in Noon units/ml., chosen to demonstrate reactions of non-identity. For abbreviations see text.

In Fig. 7 the antigen concentrations are arranged so that the lines due to the 'inner' and the 'outer antigens' come into positions where interference reactions can take place. In the resultant pattern, lines formed by the two outermost of the heat resistant ('inner') antigens of T. can be seen to cross without deviation the two outer heat labile (A-), lines of C.; and the two innermost T.-lines cross, without deviation, all the lines (heat labile and heat stable) of C.; the two innermost T.-lines (and possibly also the middle T.-line) are, therefore, peculiar to T. (cf. also Figs. 1B, 1C, 4A and 5). Since we know already that some of the heat labile lines of T. are related to the heat labile lines of C., and, similarly, some of the heat stable lines of T. to some of the heat stable lines of C., it follows that the inner and outer antigens of T. are not related to each other nor the inner and outer antigens of C. In Fig. 7B both C. and T. are too dilute to give A-lines. In Fig. 8A and 8B A-lines have formed with anti T. and anti C. sera with both T. and C., and part of the

inner bundle of lines (the composite bundle nearest the anti T. cup) can be seen to be shared between T. and C.

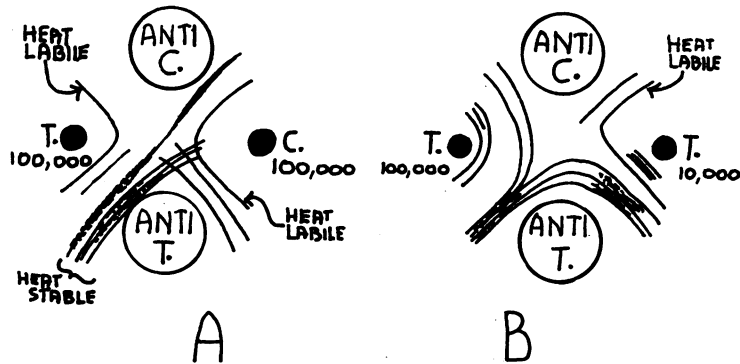


FIG. 8. Comparison of C. and T. Figures indicate Noon units/ml. For abbreviations see text.

A particularly useful arrangement for distinguishing between major related and unrelated components is the cross-diffusion pattern according to Wodehouse (1955) in Fig. 9. The A-line shows up prominently as a component common to T. and C.; and one unshared bundle of T. components (heat stable) can be seen to cross without deviation the shared A-line and another inner component, also shared by T. and C.

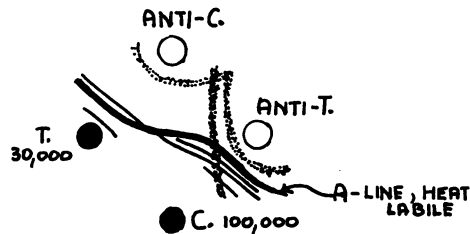


FIG. 9. Comparison of C. and T. at the concentration of 100,000 and 30,000 Noon units/ml. respectively in terms of the corresponding antisera. For abbreviations see text.

The absorption experiments depicted in Fig. 10 demonstrate clearly that practically all the components of T. and C. are either identical or have common determinants or are closely related and that there is qualitatively no difference in the inhibitory power of 1 min. water extracts and 48 hr. coca extracts. When incubated with antiserum to T., CW and CC inhibit, in addition to their own lines, also those belonging to P56, P57, TC and TW, except for the special T-lines (Fig. 10B) already discussed. Similarly TW and TC, when incubated with antiserum to C., inhibit all but one component line of A which is common to CJ, CW and CC (Fig. 10C). Both TC and TW inhibit all the lines obtainable from T. antiserum with either extract, and CC and CW inhibit all the lines that can be formed with either C.-extract.

It follows that T. contains all but one of the minor heat labile determinants of C., but has an additional major heat stable antigen group while C. has only one heat labile antigen in addition to the antigens present in T. and lacks one set of the principal components of T.

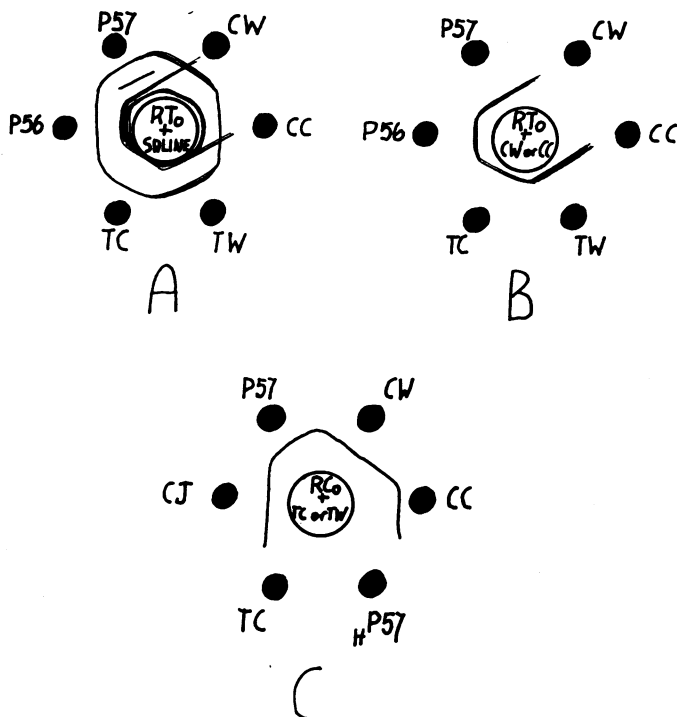


FIG. 10. Absorption experiments. (A) Original pattern obtained with anti T-serum (RTo) (mixed with an equal quantity of saline) for Pollaccines P56 and P57, aqueous 1 min. extracts of C. (=CW) and T. (=TW) and Coca extracts of C. and T. (TC andCC). (B) Saline replaced by freeze dried C.-extracts; similar results were found with CW and CC. (C) Reagents and arrangement as in Fig. 6, but using anti-C. (RCo) incubated with TC or TW in place of anti-C. For abbreviations see text. After incubation of RTo with TC or TW, and similarly, after incubation of RCo with CC or CW, no lines formed with any of the extracts.

Changes of antibody patterns during continued immunization. In Figs. 11A and 11B the antibody patterns of successive bleedings of one rabbit immunized with C. and another immunized with T. are compared in terms of two different concentrations of C. and T., and of Pollaccine.

A single multiple depot injection is equivalent to multiple injection courses; antibody production continues to increase for many weeks without any further injections and falls off very slowly even after several months (Hayward and Augustin, 1957).

Fig. 11 indicates a sharpening of specificities during progressive immunization; while the early bleedings gave nearly equally good patterns with both C. and T., later bleedings

often gave many more and much sharper lines with the homologous than with the heterologous antigen mixture.

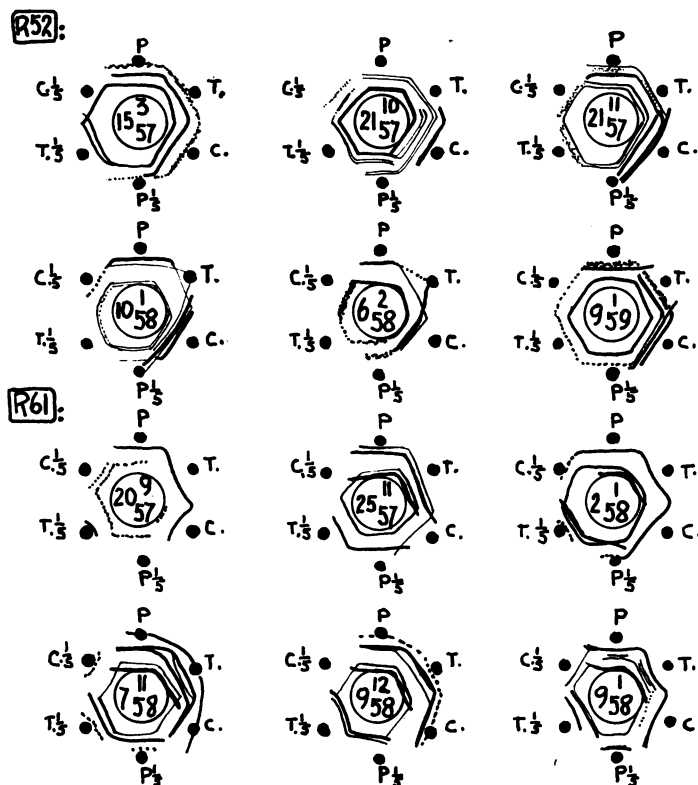


FIG. 11. Changes in Ouchterlony patterns during prolonged immunization. Rabbit R52 received multiple injections of C-preparations on 12.2., 10.7. and 6.9.1957 and on 15.10.1958. Rabbit R61 received multiple injections of T.-preparations on 3.9 and 13.11.1957 and on 15.10.1958. The central cups contain the antisera, with the dates of bleedings indicated thus 15.3.57 = 15.2.1957. The peripheral cups contain Pollaccine, T. and C.-extracts, neat and in dilutions 1/5.

B. ILLUSTRATIONS BY EXPERIMENTS WITH ARTIFICIAL ANTIGENS

Ouchterlony (1953) and Elek (1949) distinguish between three fundamental antigen-antibody interaction patterns in gel; i.e. between the freely intersecting lines obtained with unrelated antigens which do not interfere with each other (reaction of non-identity), the fused arc of coalescence given by identical antigens (reaction of identity) and the spurred arc of partially related antigens (reaction of partial identity).

The main assumption underlying the interpretation of these three reaction patterns is that antigen-antibody precipitation lines are impermeable by the reactants while remaining completely permeable by unrelated compounds. Impermeability by the antigen has been demonstrated by Elek (1949); he found that the edge of a visible indicator effect (such as haemolysis due to antigen and inhibition due to antibody) and the antigen-antibody precipitation line coincide.

Similarities and dissimilarities of antigens can be determined only in terms of the antisera employed. Care must be taken to avoid the escape of reactants behind their barriers for reasons unconnected with serological specificity; for example, the establishment of gradients of one reactant before adding the other is sometimes a useful device, but encourages the escape of antibodies behind the reaction front which may lead to false reactions of non-identity (Elek, 1949); it is necessary to compare reactants under 'balanced' conditions (not too far removed from equivalence conditions); it is further important to avoid unequal exhaustion and repeated refilling of reservoirs. These and other artefacts due to faulty technique of this kind will not be considered here.

However, even purely for reasons of serological specificity it is often difficult to disentangle the various reactions when complicated natural systems, such as grass pollens, are examined. The theoretical possibilities have therefore been investigated by means of azo-protein models.

Reaction of Non-identity. Whenever independently crossing lines are formed, the two antigens under investigation can certainly be regarded as unrelated—provided they have been compared in terms of their mixed antisera which have been prepared by prolonged immunization. Partially related systems may give patterns of non-identity as shown in Fig. 12A. Care must be taken to allow sufficient time for development so that the precipitation lines actually meet and cross or fuse, as the case may be. Further, reactions of non-identity must be distinguished from the phenomenon of double spur formation (Fig. 12B).

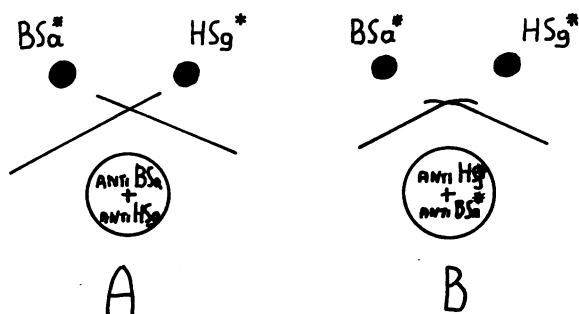


FIG. 12. A. Partially related antigens giving the patterns of non-identity because antisera are employed which do not contain antibodies to the common determinant. B. Double spur formation which may invite misinterpretation as a reaction of non-identity. For abbreviations see text.

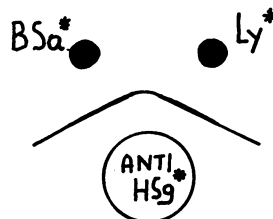


FIG. 13. Partially related antigens masquerading as identical since the reference antiserum contains antibodies for the common determinants only.

Reaction of Identity. Partially related antigens may masquerade as identical if the reference antiserum lacks the antibody for the differentiating component (Fig. 13).

The latter possibility must especially be borne in mind when comparing related natural systems in terms of an antiserum to one or two of them only—as has been done in the present investigation.

Reaction of Partial Identity. First of all a spur phenomenon (Fig. 14A) must be distinguished from what may be called a pseudo-spur phenomenon (Fig. 14B) which is encoun-

tered quite frequently when natural products are compared (cf. Fig. 6). In 14B we have two unrelated antigens HSg and HSa. A little HSa was added to the HSg cup on the left and the third cup contained antibodies to both the antigens. Increasing the HSa in the cup on the left revealed two independent lines on that side (Fig. 14C). Increasing the distance from the antibody reservoir or decreasing the HSg concentration on that side could also have resulted in a separation of the superimposed lines.

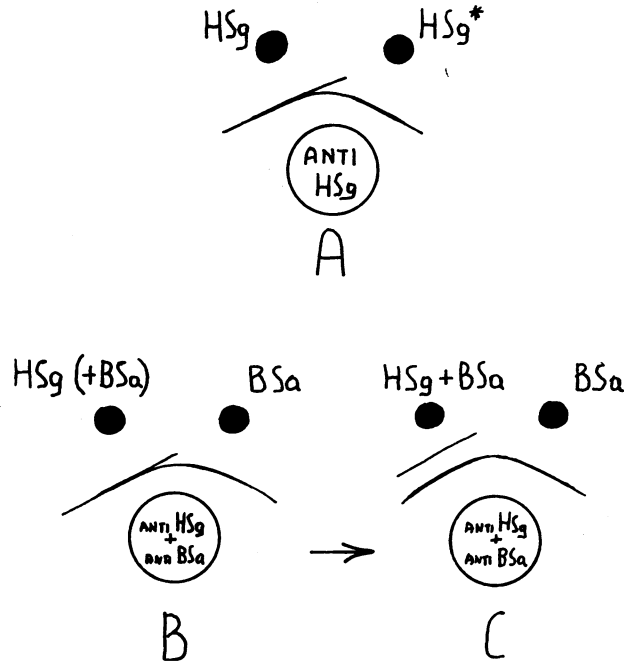


FIG. 14. A. Spur phenomenon due to two related antigens.
 B. Pseudo spur phenomenon; HSg and BSa are not related and the spur is due to superimposition of the HSg and BSa antigen-antibody lines on the L.H.S.
 C. Increasing the BSa concentrations on L.H.S. revealed two distinct lines, one due to an antigen identical with BSa, and the other due to a completely non-identical antigen (HSg).

For abbreviations see text.

However, exactly the same pictures can be obtained with two truly related antigens, according to whether the related or the homologous antigen is in excess (or diffuses more quickly), as can be seen from the experiment depicted in Fig. 15. Adding the 'non-spurring' antigen to the 'spurring' antigen will have exactly the same effect as with the unrelated antigens. The two possibilities (Fig. 14 and 15) can be distinguished by comparing each of the antigens (i.e. BSa versus HSg + BSa — Fig. 14, and HSg* versus HSg + HSg* — Fig. 15) in turn in terms of their respective antisera (Fig. 16). Mixtures containing one related and one unrelated antigen (Fig. 14) cannot be distinguished from mixtures containing related antigens (Fig. 15) by absorbing the respective antisera with one of the corresponding mixtures.

By adjusting the concentrations of related antigens it has been possible to obtain parallel precipitation lines similar to those seen with related pollens: Fig. 17A, B and C.

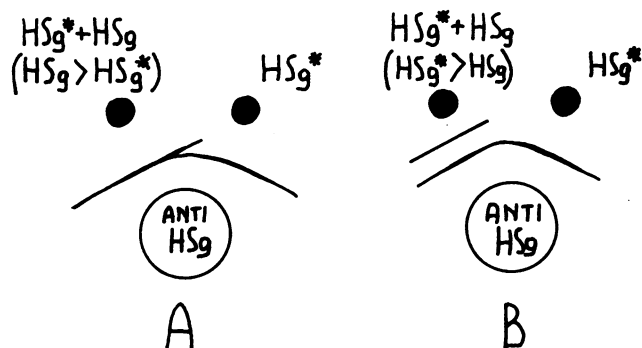


FIG. 15. Comparison of related antigens by means of antibodies to the common component.

A. Homologous antigen in excess on L.H.S.: spur formation.

B. Related antigen in excess on L.H.S.: two lines.

For abbreviations see text.

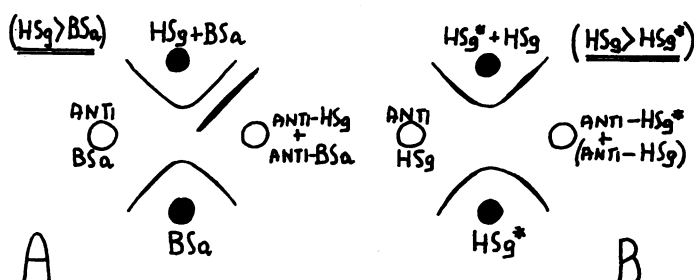


FIG. 16. A. Comparison of a mixture of unrelated antigens with one of the antigens of the mixture in terms of antisera to the mixture and the single antigen.

B and C. The same set-up with related antigens; B, with the 'single' antigen in excess in the mixture, C, with the related antigen in excess.

For abbreviations see text.

Further, it has been possible to obtain a reaction of partial identity when comparing two unrelated antigens by means of an antiserum to an antigen partially related to both (Fig. 18). The fact that the BSa*-anti HSg* line does not extend beyond the HSg-anti HSg* line argues for a bi-specific antibody capable of precipitating with either of the

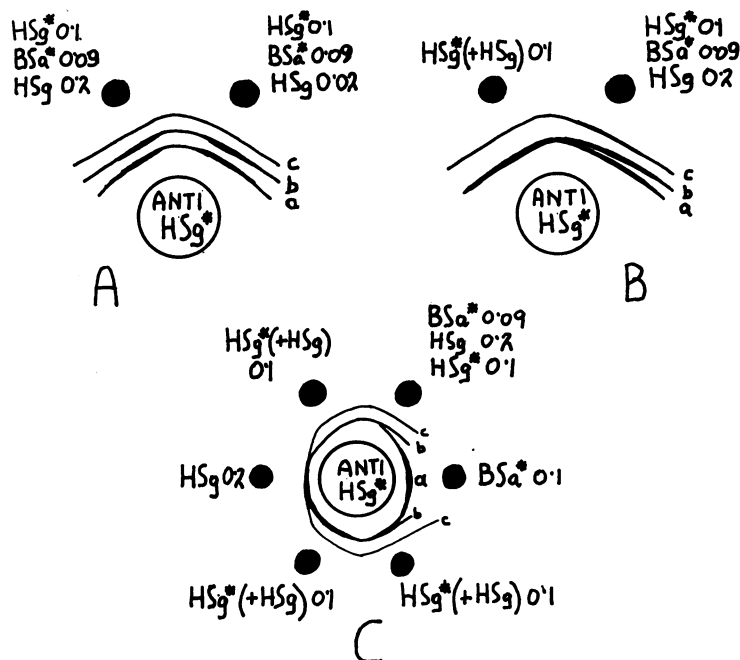


FIG. 17. Patterns obtained with related antigens. Figures besides antigens indicate mg./ml. antigen solution. The HSg* used contained a little uncoupled HSg.

- line due to BSa*-anti HSg* precipitation.
 - line due to HSg*-anti HSg* precipitation.
 - line due to HSg-anti HSg* precipitation.
- For abbreviations see text.

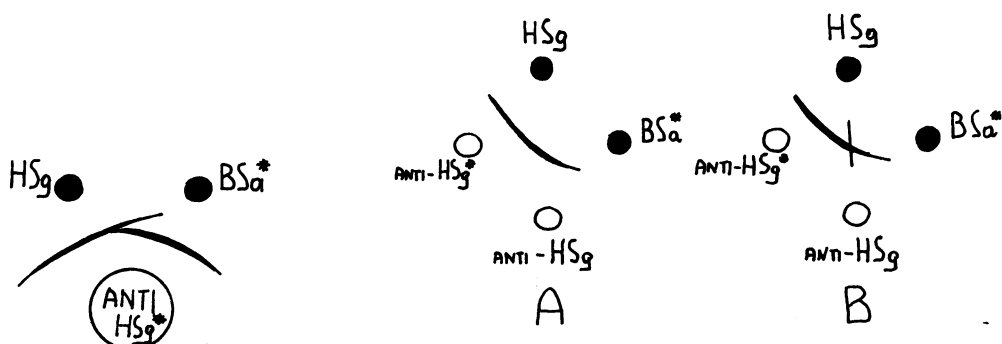


FIG. 18. Comparison of two unrelated antigens by an antiserum to an antigen sharing some of the determinants with both the unrelated antigens.

FIG. 19. Comparison of related and unrelated antigens.

- Pattern obtained experimentally demonstrating bispecific antibodies.
- Theoretical pattern expected in the absence of bispecific antibodies.

For abbreviations see text.

two unrelated antigens HSg and BSa*. It was confirmed experimentally that BSa did not precipitate with the anti-HSg* used. The absence of antibody specific for the arsanilic determinant only was further confirmed with Wodehouse's arrangement (1955) already referred to. The pattern depicted in Fig. 19A was obtained, while that of Fig. 19B should have been obtained in the absence of bi-specific antibodies.

It was also found that azo-proteins with different loads of the identical hapten attached to the identical carrier can give two distinct lines; so far never more than two lines have been obtained (Fig. 20A and 20B).

Sulphanilic acid conjugated with HSg (HSg^s) gave a blurred band (Fig. 21) with an antiserum to arsanilic acid conjugate, while a perfectly satisfactory line was formed when the sulphanilic acid conjugate was allowed to diffuse against antiserum to the unconjugated carrier or its own antiserum. The HSg^s-anti HSg* band took a long time to appear.

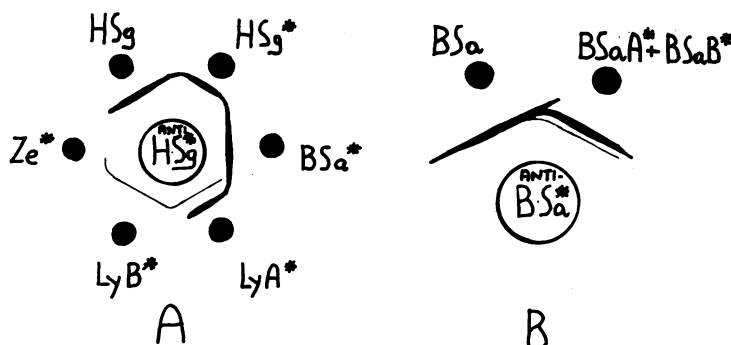


FIG. 20. Comparison of antigens related by carrier or hapten against antiserum to one of the conjugates. For abbreviations see text.



FIG. 21. Comparison of antigens containing chemically similar determinants. For abbreviations see text.

DISCUSSION

A. POLLENS

The grass pollens compared here include nine of the grasses which are quoted by Wodehouse (1935) as the most important hay fever producers (in America), and two, B.R. and D.T., which Wodehouse considers to be of minor importance. These grasses belong botanically to four different families (Table 2) and the four 'foreign' pollens represent a further four families. All the British pollens, whether or no of primary importance as hay fever producers, appear to have numerous common determinants which may belong to unrelated or to related antigens. The fact that there was little spur formation may merely be a consequence of comparing many pollens in terms of antisera to only two of them. Antibodies specific for two pollens only can merely indicate similarities to and differences from these two pollens; differences in determinants lacking in these two pollens remain undetected. However, a more detailed comparison was possible for T. and C., since multivalent antisera were available for both. Yet here too one could not be sure of true spur formation, i.e. of reactions of partial identity. In any case it would be unwise to draw definite conclusions in view of the great complexity of the patterns obtained; the antigenic components will have to be obtained largely free from each other so that they can be compared individually instead of in mixtures.

Nevertheless, the indications are that T. and C. share most of their more important antigenic components except for the 'innermost' antigens of T. and one of the minor A-components of C. M.Fox., S.V. and B.R. appear to share fewer antigens (or determinants) with T. and C. than the nine other pollens, i.e. C. (*fescucae*) appears to have more in common with T.O., belonging to the *avenae* (with S.V.) than T. with M.Fox. although the latter two belong to the same family botanically (the *angrostideae*); i.e. it seems from the experimental patterns that the antigenic make-up cuts across botanical classifications, and only gross botanical differences are reflected, e.g. the botanically very different pollens of B.A., B.E., M. and S. share very little antigenically (for rabbits) with the British grass pollens.

TABLE 2
BOTANICAL RELATIONSHIPS OF THE EXAMINED GRASS POLLENS
ACCORDING TO HUBBARD (1954) AND RENDLE (1925-30).

Gramineae	
Tribe	Genus and Species
Agrostideae	<i>Phleum</i> : <i>Phleum pratense</i> or Cocksfoot: C.
	<i>Alopecurus</i> : <i>Alopecurus pratensis</i> or Meadow Fox-tail: M.Fox.
Festuceae	<i>Festuca</i> : <i>Dactylis glomerata</i> or Cocksfoot: C.
	<i>Festuca rubra</i> or Red Fescue: R.F.
	<i>Festuca pratensis</i> or Meadow Fescue: M.F.
	<i>Festuca arundinacea</i> or Tall Fescue: T.F.
	<i>Lolium perenne</i> or Italian Rye grass I.R.G.
Aveneae	<i>Cynosurus cristatus</i> or Dog's Tail: D.T.
	<i>Arrhenatherum</i> : <i>Arrhenatherum elatius</i> or Tall Oat: T.O.
	<i>Anthoxanthum odoratum</i> or Sweet Vernal: S.V.
Maydeae	<i>Zea</i> : <i>Zea mays</i> or Maize: M.
Bromeae	<i>Bromus</i> : <i>Bromus mollis</i> or Soft Brome: B.R.
Spartineae	<i>Spartina</i> : <i>Spartina townsendii</i> or Cord grass: S.
Chlorideae	<i>Cynodon</i> : <i>Cynodon Dactylon</i> or Bermuda: B.E.
Bambosideae:	<i>Bambusa</i> : <i>Bambusa sp.</i> or Bamboo: B.A.

Individual responses of hay fever sufferers to the pollens were very variable and could not be correlated with any particular antigen. While most hay fever sufferers reacted equally well to a number of pollens, there were always some exceptions. If the small sample presented here (Table 1) can be taken as representative, Noon was correct in stating that T. pollen gives on the whole stronger reactions than pollens of the other grasses, and from the present limited series it would seem that B.R. gives on the whole less strong reactions than the other grasses.

Whether all these skin responses are clinically significant is another question. They must be indicative of the presence of the appropriate reagins in the skin—and possibly in other tissues. However, any of these reagins will become clinically important only if the subject is exposed to a sufficiently large quantity of the corresponding allergen. In the present study it has not been possible to determine which—if any—of the antigens forming lines in the Ouchterlony plates are allergens. It would, however, seem that English grass pollens have not only many antigens (for rabbits) in common, but also many allergens. Not only do all the subjects tested react to at least some extent to all the British grass pollens, but Frankland (1955a) found that it was irrelevant whether preseasonal hypoimmunization treatment was carried out with T. or C. alone, or with a mixture of the two, and that high dosage treatment with Pollaccine was successful in over 90 per

cent of the patients. This would certainly indicate that British hay fever grasses share most of their pollen allergens (or determinants of these) with C. and T., or that those not shared with T. and C. have little clinical importance. Freeman (1955) even found that hyposensitization with Pollaccine reduced skin responses to B.E.

Skin reactions are usually reduced after hyposensitization with pollen extracts in comparison with those recorded before treatment, sometimes very much so, but they do not disappear completely. There is sometimes a comparative enhancement of non-T. and -C. reactions after treatment with Pollaccine; i.e. these patients are specially immunized against C. and T. Minor components of C. and T. or allergens present in other pollens only may have been covered inadequately or not at all. Clinically this does not seem to matter, probably because these minor components will not usually assail the patients in quantities sufficient to sensitize or, if the patient has become sensitized, to provoke an attack.

However, it will be clear from this discussion that it is in general not permissible to assay pollen extracts by skin tests in terms of any individual extract chosen at random. Not only are skin tests on patients very inaccurate, but by its very nature the activity represented by a given number of Noon units, i.e. by a given amount of pollen, must vary from pollen to pollen and patient to patient. Nevertheless skin reactions on subjects whose sensitivity to various pollens is known remain useful for a preliminary assessment of potencies and Noon units still have a meaning when they are applied to a particular pollen product whose method of preparation is maintained constant. Also it is possible to eliminate to some extent variations between individuals by carrying out tests on a sufficiently large number of subjects. Potency figures derived in this way may be used as a guide for comparing the potencies of commercially available extracts, but will give no indication as to how a particular patient will react to any particular extract. In a proper analysis potencies must be given for each extract in terms of the individual allergens. From the figures given in Table 1 it seems unlikely that antigen A is an allergen.

When sera of rabbits are tested in the course of immunization the antigen-antibody lines of the outer ring due to the antigens referred to as the A-antigens are usually the first to appear, suggesting that A-antigens either form a large proportion of the pollen antigens or that they induce antibody formation more readily than the other antigens. For both these reasons they have often been referred to as main or principal pollen antigens (Augustin, 1955; Wodehouse, 1955), although strictly speaking the terms major and minor antigens have not been properly defined. Also it must be borne in mind that the early appearance of the A-lines may merely be an indication that 'balanced' conditions were obtained first for the A-antigens. Since ultrafiltrates of pollen extracts contain a small amount of 'inner ring' antigens and never any A-antigens, it appears that the 'inner ring' antigens diffuse more quickly than the A-antigens. They will therefore meet the advancing antibody front more quickly at higher concentrations than the A-antigens, and a higher antibody concentration may be required to halt them even if their concentration in the pollen is no higher than that of the A-antigens.

B. COMPARISON WITH RESULTS OBTAINED WITH ARTIFICIAL ANTIGENS

The study of the patterns given by artificial antigens leads to some conclusions about the information that can be derived from Ouchterlony tests with natural systems.

In Fig. 17 we see that two dissimilar antigens that do not contain a determinant in

common may give a reaction of partial identity. This indicates that antibodies must be present in the antiserum that will combine with two distinct determinants. This inference conflicts with the commonly accepted view that bi-specific antibodies are not formed—although there are occasional claims for the existence of bi-specific antibodies in the literature. The matter depends on the meaning of the word bi-specific. In his original discussion of the formation of antibodies Pauling (1940) suggested that antibodies might be formed with two receptors adapted to entirely distinct antigens. Attempts to make such antibodies have failed; the matter is discussed by Kabat (1956) and by Kabat and others (1957). However, it is possible that the determinant with which a receptor of an antibody combines can be divided into two distinct parts. The extent of antigen with which an antibody receptor combines is not limited to one small group. Thus Kabat (1956b, 1957) considers that some antibodies against (1→6) dextran are adapted to five or six glucose residues of the antigen, since the most efficient inhibiting haptens are isomaltopentaose and isomaltohexaose. When an artificial antigen P_1D is used as immunizing antigen some of the antibodies are adapted both to the determinant D and to the adjacent part of the protein P_1 . This was shown by Haurowitz and Schwerin (1942) who found that some of the antibody in their antisera was not precipitated by P_1 nor by the 'test' antigen P_2D (where P_2 is an unrelated protein), but were precipitated by P_1D . More recently Francis, Mulligan and Wormall (1955) have come to similar conclusions.

If two receptors of antibodies are thus adapted to D and adjacent parts of P_1 , the parts of the unrelated protein P_2 adjacent to D in the test antigen P_2D may obstruct the approach of the receptor to D . This may be one explanation for the association constant of the combination of P_2D with antibody being less than that of D with antibody (Epstein, Doty and Boyd, 1956).

It appears from Fig. 17 that the antiserum used in this experiment contained no antibody against the determinant D (in this case *p*-phenyl arsonic acid) that could not also combine with the protein carrier P_1 ; if there had been such an antibody a spur would have formed on the left owing to the reaction of these anti-arsonic antibodies with the arsonic acid in BSaA*. The anti serum therefore contained anti- P_1D which may be called bi-specific, since it formed a precipitate with P_1 and with P_2D —two unrelated antigens.

One theory of cross-reactions between two natural antigens such as hen and duck ovalbumin is that the determinants of the two antigens are similar but not identical; that some of the receptors of the anti-hen antibodies are less specific than others and capable of combining with the determinant of duck ovalbumin. Another theory is that hen and duck ovalbumin both have several determinants per molecule and that some of these determinants are common to both; this is the theory held by Hooker and Boyd (1934) and has been much supported and strengthened in recent years by Lapresle and co-workers (1955, 1958) who actually isolated three determinants from the digest of a single molecule such as serum albumin.

We may suppose that pollen antigens also have several determinants per molecule. Some of these may be common to one pair of pollens of different species and others common to another pair. We may conclude from the experiment with artificial antigens, that demonstrates the formation of a kind of bi-specific antibody, that if antigens A and B from two pollens give a reaction of identity with antiserum against a third pollen C , they do not necessarily have a determinant in common; one may suppose that A and C share a determinant and B and C share another determinant. A study of the reaction of A and B with antiserum against C tells us only about the resemblances and differences between

A and C, and between B and C, but not about the resemblances and differences between A and B.

According to the first theory of cross-reactions, the increase of cross-reactivity with prolonged immunization is due to the increased formation of antibodies that can accept determinants similar to but not identical with that of the immunizing antigen. This theory is supported by the results of experiments with artificial antigens (Hooker and Boyd, 1941). But when natural antigens with several potential determinant groups per molecule are involved antibodies may be formed to more and more determinants of the molecule; the increase of the range of cross-reaction may be due to formation of antibodies against determinants shared with other antigens. From Fig. 21 it can be seen that reactions with related determinants may be expected to give diffuse bands rather than sharp lines. In experiments with pollens one finds that prolonged immunization gives rise to sera that form, in Ouchterlony plates, more and more sharp lines, and that the lines become sharper as immunization continues. Although thus all the evidence from Ouchterlony plates goes to show that increasing amounts of strictly specific antibodies are formed, it must be borne in mind that less specific antibodies could easily be missed in this type of test, since they would form diffuse bands which may take many days to develop.

If a pollen extract contains a group of similar antigens such as the outer group of A-antigens, the homologous pollen extract may form a group of fine sharp lines in gel diffusion plates when relatively concentrated reagents are used. A heterologous extract with one antigen in common with the homologous extract may form only one line. When the reagents are much more dilute, as in the quantitative gradient gel diffusion method used by us (Augustin and Hayward, 1955), the homologous extract may form a narrow diffuse band instead of a group of lines, whereas a heterologous extract forms a sharp line. This is what was occasionally found experimentally. Some antisera to Timothy gave diffuse lines with Timothy extracts at the low concentrations used in our quantitative tests, while perfectly sharp lines were obtained with antisera to Cocksfoot.

Whenever a determinant is artificially introduced into a protein, this protein conjugate has the specificity of this new determinant, D (e.g. phenylarsonic acid), but one of the original determinants, O, of the protein may be blocked in the process. Antibodies to O can therefore pass precipitation lines due to D and combine behind it—owing to O-specificity—with the original carrier; i.e. in Fig. 17 the *a*-line is due to antibodies with D-specificity, while *b* is due to antibodies specific for the carrier but lacking in O-specificity. Antibodies with O-specificity can therefore pass lines *a* and *b* and form behind them a line with the original carrier—owing to a determinant obliterated in the coupling process. It is difficult to explain the three lines, formed in these experiments, in any other way.

The formation of two lines due to heavily and lightly loaded protein can be similarly explained. During conjugation with diazonium salts azo-linkages are first formed with tyrosine groups, but at a later stage also with histidine groups. Both conjugates have the same artificial hapten specificity, but the heavily coupled conjugate may lack two carrier determinants while the lightly coupled conjugate may lack only one. This can lead to the formation of two lines despite identical carriers and identical haptens. Different antibodies, one corresponding more closely to tyrosine-D, the other to histidine-D, are formed, according to Hooker and Boyd (1933).

Ouchterlony patterns with azo-proteins have been introduced here in an attempt to find out how much can justifiably be deduced from the coalescent, spurring, parallel and independently crossing lines obtained in gel diffusion plates when comparing natural

antigen mixtures such as pollen proteins. Summarizing, coalescent lines of identity may be due to identical antigens or to partially related antigens if the reference serum lacks the antibody for the differentiating component. Reactions of non-identity may also be given through the reference antiserum lacking antibody to the common determinant and it may be confused with the phenomenon of double spur formation. Greatest difficulties may be experienced in the attempt to interpret reactions of partial identity. Pseudo spur formation is very difficult to distinguish from true spur formation and bi-specific antibodies may simulate reactions of partial identity between molecules with nothing in common. Incidentally, antibodies bi-specific in the sense used by Pauling (1940) would not form precipitation lines with an antigen possessing only one of the specificities, since such antibodies would be univalent for it. At the most the formation of a precipitation dot may be envisaged where the two antigens—each with a different determinant—and the bi-specific antibodies meet. It has also been demonstrated that parallel noncrossing lines in pollen plates could be due to related antigens or even to antigens with identical carriers and identical haptens, one of them much more heavily loaded with determinant than the other. The formation of two lines with heavily and lightly halogenated proteins has been reported by Wilson and Pringle (1956). Fuzzy bands instead of lines indicate related but not identical determinants, provided that broad bands owing to unbalanced conditions are excluded.

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