

Studies on 'Allergoids' Prepared from Naturally Occurring Allergens

I. ASSAY OF ALLERGENICITY AND ANTIGENICITY OF FORMALINIZED RYE GROUP I COMPONENT*

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Summary. The highly purified major allergenic component of rye grass pollen (Group I) was used to investigate the possibility of destroying selectively the allergenic properties of an antigen, while largely retaining its original immunizing capacities. The allergen was treated under mild conditions with formalin alone or formalin plus a reactive low molecular weight additive. Certain derivatives (*allergoids*) showed well over 99 per cent reduction in allergenicity, determined by the histamine released from allergic human leucocytes *in vitro*, but were still able to combine with rabbit antibody against native antigen. Furthermore, the allergoids stimulated production (in guinea-pigs) of appreciable amounts of antibody able to inhibit native allergen-mediated human allergic histamine release *in vitro* and to cross-react with native antigen by PCA tests in normal guinea-pigs.

Residual allergenicity and cross-immunogenicity (by the inhibition assay) of the different formalinized derivatives varied appreciably according to the additive used in formalinization, but the cross-reactivities of the different preparations in quantitative precipitin analysis against rabbit anti-native antigen serum were similar. The residual allergenicities of individual derivatives varied by up to 1000-fold in different cell preparations, suggesting a heterogeneity of allergenic determinants. Allergoid derivatives showed no hapten-like activity in that they were unable to inhibit allergen-mediated histamine release from leucocytes.

The theoretical and practical application of allergoids is discussed, including their potential usefulness in improving the immunotherapy of atopic humans.

INTRODUCTION

This paper explores quantitatively the relative stabilities to formalin treatment of the overall *antigenic* and the specifically *allergenic* properties of the highly purified Group I component of perennial rye grass pollen.

Immunochemically closely related proteinaceous fractions, collectively termed Group I

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(Johnson and Marsh, 1965a), are known to be the principal allergens for man in perennial rye grass pollen and a number of other grass pollens (Marsh, Milner and Johnson, 1966; Marsh, Haddad and Campbell, 1968). Group I components have been detected in pollens of many grass genera (Marsh *et al.*, 1968). About 90–95 per cent of grass pollen sensitive individuals show significant skin reactivity to Group I fractions.

Previous experiments by one of us (D. G. Marsh, unpublished) had shown that preparations derived from rye Group I by mild treatment with dilute formaldehyde solution (formalinized derivatives), with or without the chemical incorporation of L-lysine, were significantly less allergenic (over twenty times) than the native allergen by skin tests in allergic individuals. However, the original antigenic properties had apparently been largely retained after modification, since there was a high degree of cross-reactivity between native and formalinized Group I by gel diffusion analysis against anti-native Group I serum.

In view of the considerable therapeutic potential and theoretical interest in allergen derivatives in which the original allergenic properties have been drastically reduced, while the general antigenic properties have been largely maintained, a more thorough and quantitative study of these properties was undertaken using rye Group I as a model system. Particular importance was attached to finding derivatives of low allergenicity which stimulated high levels of antibody in animals which were able to cross-protect against allergic reactions in humans. With this aim in view, formalinized allergen derivatives incorporating several chemically different additives were investigated.

Definitions

In the context of this paper, the term '*allergenic*' will be used only with reference to the properties of a substance associated with induction of specific reaginic antibody synthesis in humans, or with the ability of a substance to combine with homologous reaginic antibody resulting in the manifestation of allergic reactions of the immediate type. '*Antigenic*' will be used, somewhat restrictively, to denote properties of an antigen other than those covered by the term allergenic as defined above.

Both antigenic and allergenic properties may be subdivided into two aspects,* namely: (i) *immunogenic*—the capacity of an antigen to stimulate immunospecific antibody synthesis; and (ii) *reactogenic*—the ability of an antigen to combine with presynthesized antibody.

The term '*allergoid*' will be used to denote an allergen derivative for which the residual allergenic reactogenicity relative to the native allergen has been significantly reduced, while the original antigenic properties (both immunogenic and reactogenic) have been retained to a high degree. The term 'formalinized derivative' will be used where antigenic and allergenic properties had not been fully investigated, or were not as defined by the term '*allergoid*'. (Although all allergoids described in this paper were produced by formalin treatment, we do not wish to restrict the term '*allergoid*' to only formalinized products.)

Any chemically reactive allergoid additive used in the formalinization process in addition to formaldehyde will be denoted by a prefix (e.g. 'lysine-allergoid'). The term 'normal-allergoid' will denote an allergoid prepared using only the allergen and formalin.

* These two aspects are clearly interrelated to some degree. By definition, an antigen is immunogenic. Reactogenicity, a term which has also been used by Farr (unpublished) is a property of both antigens and haptens. It seems probable that both immunogenic and reactogenic aspects should be considered separately for individual immunoglobulin classes, which would imply a further subdivision of the term antigenic as used here.

MATERIALS AND METHODS

Group I allergen

The allergen was prepared as a lyophilized powder from perennial rye grass pollen, according to the procedure of Johnson and Marsh (1965b). A high degree of purity was confirmed by physical, chemical and immunological criteria (Johnson and Marsh, 1965b, 1966a, b).

Rye Group I allergen consists of two major and two minor electrophoretically distinct, but immunochemically closely related, *isoallergens* of indistinguishable molecular weight about 27,000 (Johnson and Marsh, 1965a). The purified preparation is predominantly protein, but contains a small amount of carbohydrate (<5 per cent by weight), considered to be inessential for the allergenic activity of the molecule (Marsh *et al.*, 1966).

Rabbit antisera

Antisera to native rye Group I or fescue Group I were prepared using as adjuvants either Freund's complete or alum, Type C γ (Al₂O₃ · 3H₂O), made according to the Willstaetter procedure (Chase, 1967). Groups of two rabbits received nine fortnightly intramuscular injections of 2 mg of either rye Group I or fescue Group I, emulsified in Freund's adjuvant; bleedings were taken periodically after the sixth, eighth and ninth immunizations. Two other rabbits received four fortnightly injections of 2 mg rye Group I, completely absorbed on alum, administered in the footpads; bleedings were taken two weeks after the final immunization.

Preparation of allergoids

A solution of native Group I allergen (2.00 mg/ml) was prepared in 0.1 M Na₂HPO₄-NaH₂PO₄ buffer at pH 7.50, containing 1 part 'Merthiolate' preservative per 10,000 parts of buffer. In preparing this solution, correction was made for the water content of the lyophilized allergen. The allergen solution (6.0 ml) was mixed with a buffered solution* (3.0 ml) containing 0.2 M of the appropriate additive where applicable (Table 1). To each mixture, freshly-prepared 0.3 M HCHO solution (2.4 ml) in buffer* was slowly added with rapid magnetic stirring. Each reaction mixture was adjusted to pH 7.50 \pm 0.02 by addition of small volumes (about 0.2 ml or less) of 0.5 N HCl or 0.5 N NaOH, with constant stirring of the solution. The solutions were adjusted to 12.0 ml with 0.1 M phosphate at pH 7.50 and their final pHs recorded to an accuracy of \pm 0.01 unit. The solutions were sterilized by cellulose acetate membrane filtration (using 'Millipore' filters, average pore diameter 450 μ) and transferred into sterile serum bottles. Due to the low solubility of adipamide, the reaction mixture containing this additive was prepared by transferring a sterile solution (12.0 ml) containing only allergen and formaldehyde into a sterile serum bottle containing sterile adipamide (86.4 mg, the equivalent of a 0.05 M solution).

The final concentrations of the reactants were as follows: 1.00 mg allergen/ml, containing about 0.001 M available free amino groups, determined from amino acid analysis data (Johnson and Marsh, 1966a); 0.06 M HCHO; and 0.05 M additive, where applicable. All solutions contained 0.1 M phosphate buffer and Merthiolate (1 : 10,000). In addition to these solutions, a sterile native allergen control was prepared containing only the allergen (1.00 mg/ml) in 0.1 M phosphate buffer with Merthiolate at pH 7.50.

* The choice of pH of the 0.1 M phosphate buffer used was governed by the need for easy pH adjustment of the final reaction solutions to pH 7.50 using minimal quantities of acid or alkali.

All solutions were incubated at $32 \pm 1^\circ$ for 32 days, after which their pHs were accurately recorded. The solutions were immediately dialyzed against 0.1 M phosphate at pH 7.5 at room temperature for 3 hours, followed by overnight dialysis at 4° , and stored at -20° . In certain experiments, small aliquots were removed from the solutions after incubation periods of 1, 2, 5, 8 and 18 days and treated in a similar manner.

Allergenicity

The allergenic activities of the formalinized derivatives, relative to the native allergen, were determined quantitatively by the histamine release *in vitro* from grass pollen allergic human leucocyte preparations using the procedure of Lichtenstein and Osler (1964). Allergen control and formalinized preparations were run simultaneously with the same leucocyte preparation. Residual allergenicities (RAI) were calculated from the formula:

$$\text{RAI} = \frac{\text{Concentration of native Group I required for 50 per cent histamine release}}{\text{Concentration of formalinized derivative required for 50 per cent histamine release}} \times 100 \text{ per cent}$$

Inhibition experiments. Experiments were carried out to examine the capacity of the formalinized allergen preparations to inhibit the release of histamine mediated by the native allergen. Leucocytes from the blood of one donor (M. L.—Fig. 1a), for which three formalinized derivatives (the ‘normal-, lysine- and adipamide-allergoids’) showed residual allergenicities of below 0.001 per cent, were incubated at 37° for 60 minutes with allergen and ‘allergoid’ together at various concentrations. In all cases, the ‘allergoids’ were used at concentrations below those necessary for histamine release; allergen was used at concentrations which, without ‘allergoid’, would induce up to 70 per cent histamine release from the cells. The concentration ratios of ‘allergoid’ to allergen were in the range 1000 : 1 to 10,000 : 1. The amounts of histamine released under such conditions were compared with control leucocyte suspensions incubated with the same quantities of allergen or ‘allergoid’ alone.

Antigenicity. Both *reactogenic* and *immunogenic* aspects of the cross-antigenicity between formalinized and native Group I were investigated.

The degree of *cross-reactogenicity* between the antigens was qualitatively determined using double immunodiffusion in agar against the various rabbit anti-native Group I sera.

Quantitative determination was performed by direct comparison of the amounts of anti-native Group I antibody precipitated by the modified and native antigens at equivalence. The nitrogen compositions of antigen-antibody precipitates were determined by the Nessler procedure at a series of antigen concentrations for fixed amounts of antiserum, by a semi-micro modification of the method of Campbell, Garvey, Cremer and Sussdorf (1963). The two rabbit anti-native rye Group I sera (prepared using Freund’s adjuvant) used for this method were previously decomplemented by heating at 56° for 1 hour, followed by extensive adsorption using a non-cross-reacting BSA-anti-BSA immune precipitate (3.5 mg precipitate/ml of serum). All samples, including appropriate solutions of antigen alone, standard $(\text{NH}_4)_2\text{SO}_4$ solutions and saline blanks were run in duplicate. The degree of cross-reactogenicity of modified antigen relative to native antigen was calculated from the formula:

$$\frac{\text{Total N in formalinized Ag-Ab ppt at equivalence} \\ \text{minus N contribution of formalinized Ag in ppt}}{\text{Total N in native Ag-Ab ppt at equivalence} \\ \text{minus N contribution of native Ag in ppt}} \times 100 \text{ per cent}$$

The degree of *cross-immunogenicity* between formalinized and native antigens was determined using antisera raised in guinea-pigs. The antibody content of these antisera was investigated semi-quantitatively by PCA tests in guinea-pigs and quantitatively by the ability of such antisera to inhibit histamine release when allergic human leucocytes were challenged with the native allergen *in vitro* (Lichtenstein, Norman, Winkenwerder and Osler, 1966). The latter test gives a direct comparison of the ability of formalinized and native allergen to induce the formation of antibody capable of inhibiting allergic reaction in a model *in vitro* system.

For quantitative studies, antisera were raised concurrently in groups of ten guinea-pigs, closely matched for weight and sex, of a strain developed by Campbell and later by Trapani for a period of 30 years in closed colony. Each guinea-pig received two immunizations, 28 days apart, of the appropriate antigen completely adsorbed on alum (Type C γ ; Chase, 1967) suspended in physiological borate-saline buffer, pH 8. Both hind footpads were injected on each occasion with 0.3 ml suspension per pad with a total of 150 μ g antigen in the primary and 50 μ g in the secondary immunization. The weights of formalinized derivatives used in such experiments were based on the equivalent weight of the native protein from which they were derived. The animals were bled out 11 days after the secondary immunization. Sera used for the PCA tests were raised similarly in groups of two guinea-pigs using 20 μ g followed by 10 μ g of antigen.

The PCA tests were performed in non-immunized guinea-pigs by intradermal injections (0.1 ml) of eight serial dilutions of an anti-native antigen serum and with eight dilutions of an anti-formalinized antigen serum in each guinea-pig. The animals were later challenged with 500 μ g native antigen or 500 μ g (equivalent weight) homologous formalinized antigen in 0.5 per cent Evan's blue solution (1.0 ml); they were sacrificed and skinned 45 minutes later. Approximate values of cross-immunogenicity were found from the ratio of the dilutions of heterologous and homologous sera which gave equivalent blueing reactions (taking both the size and intensity of blueing into account) on the underside of skin of the same guinea-pig.

The method for determining the blocking activity of an antiserum against allergic histamine release from human leucocytes has been described in detail elsewhere (Lichtenstein *et al.*, 1966). The modification used here employed guinea-pig rather than human antiserum. Individual guinea-pig antisera of appropriate dilutions, between 1 : 30 and 1 : 600, were incubated at room temperature (20–25°) for 30 minutes or more with a concentration of native allergen sufficient for 60–80 per cent histamine release. After this period, suspensions of sensitive leucocytes were added to each tube and the mixtures incubated for 30 minutes at 37°. Percentage inhibition of histamine release by antiserum was calculated relative to release by allergen alone. Another assay of cross-immunogenicity employed pools of equal volumes of each serum within a particular group. Serial dilutions of such pooled sera were used to determine the variation of percentage inhibition with antiserum concentration. The degree of cross-immunogenicity (CI) between the antigens was calculated from the formula:

$$\text{CI} = \frac{\text{Anti-native antigen serum concentration required for 50 per cent inhibition of histamine release}}{\text{Anti-formalinized antigen serum concentration required for 50 per cent inhibition of histamine release}} \times 100 \text{ per cent}$$

RESULTS

Nitrogen analysis of the various formalinized allergen derivatives suggested that high molal ratios of additive had been incorporated into the allergen in Preparations 3–6 (Table 1). The ratios obtained were higher than expected from the numbers of available reactive groupings on the allergen molecule;* this may have resulted from weakly-bound additive not removed by dialysis and, in certain cases, from chains of several molecules of additive linked to single reactive groupings on the protein molecule. There were small, but significant and reproducible, decreases in the pH of buffered reaction solutions containing basic additives (Preparations 4–6), due to the loss of large numbers of positively charged groups on reaction of such additives with formaldehyde and allergen (see 'Appendix', p. 720).

TABLE 1
DESCRIPTION OF 'ALLERGOIDS' PREPARED BY INCUBATION OF NATIVE GROUP I ALLERGEN WITH 0.06 M FORMALDEHYDE AT $32 \pm 1^\circ$ FOR 32 DAYS

Preparation	Additive (0.05 M)	Fall in pH of reaction mixture after incubation*	N composition (g/100g original allergen)†	Approximate moles additive mole allergen‡
1 (Allergen control)§	None	0.01	13.6 ± 0.7 ¶	0
2	None	0.01	14.1 ± 0.5	0
3	Adipamide	0.01	18.1 ± 0.3	43
4	L-Ornithine	0.11	18.0 ± 0.5	42
5	L-Lysine	0.35	24.2 ± 0.6	102
6	L-Arginine	0.35	37.5 ± 0.08	154

* Averages of results from three experiments are given: differences were not greater than ± 0.01 except Preparation 6 (± 0.04).

† Determined from difference in N contents (Nessler procedure) of dialysed allergen and allergoid solutions equivalent to 1.00 mg original allergen/ml.

‡ Molecular weight of allergen assumed to be 27,000 (Johnson and Marsh, 1965b). Values include both reversibly and irreversibly bound additive.

§ No formaldehyde added.

¶ N content by Dumas and Kjeldahl procedures was 13.3 ± 0.3 per cent (Johnson and Marsh, 1965b).

|| Initial pH assumed to be that of the solution added to solid adipamide.

ALLERGENICITY

The residual allergenicity (RAI) of antigen preparations treated with 0.06 M formaldehyde solution at 32° was dependent on the period of incubation, the type of additive (if present) and the leucocyte cell donor (Fig. 1 and Table 2). For a given type of derivative and cell donor, allergenicity generally decreased with increasing incubation time for periods of 1, 2, 5, 8, 18 and 32 days. Normal-, adipamide- and lysine-derivatives were the

* Potential reactive groupings include twenty-seven amines, six guanidines, about four acid amides, nine tyrosines, six tryptophans and three histidines (Johnson and Marsh, 1966a). Formaldehyde reactions with proteins are discussed in the 'Appendix'. (p. 720).

least allergenic (usually ≤ 0.3 per cent RAI to as little as 0.001 per cent RAI with different cells); the arginine-derivative possessed, on average, about 2 per cent RAI, but the ornithine-derivative was still highly allergenic for most cells tested (mean: 38 per cent RAI). Allergic histamine release for the two cell preparations shown in Fig. 1 illustrates the extreme variability in the RAI values which has been observed for each formalinized derivative depending on the cell donor. Residual allergenicities of each of the least allergenic derivatives (32-day preparations) varied by as much as 1000-fold between different cell donors.

No significant difference in allergenicity was detectable between 0- and 32-day allergen controls (Fig. 1a), confirming the considerable heat stability of the rye Group I allergen reported previously (Marsh, *et al.*, 1966; Johnson and Marsh, 1965b).

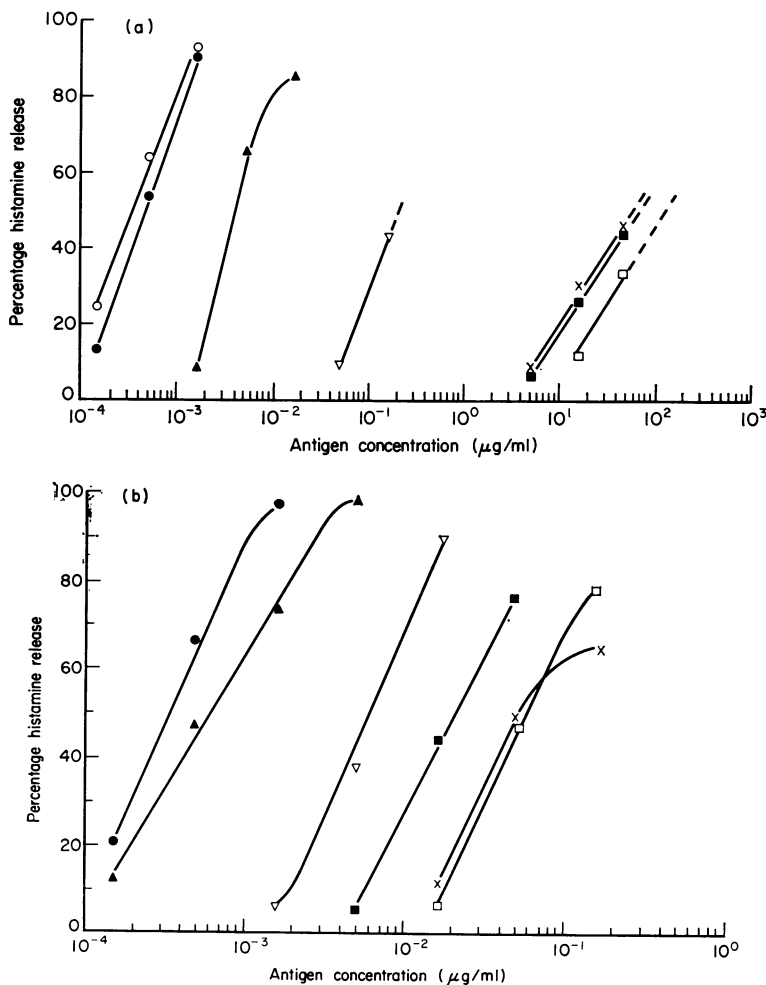


FIG. 1. Histamine release curves for two different human leucocyte preparations challenged with native and formalinized Group I allergen. Curves for the formalinized derivatives are designated by the respective additives: the normal derivative contains no additive other than formaldehyde. (a) Cell donor M.L. (b) Cell donor D.M. \circ , 0-day allergen control; \bullet , 32-day allergen control; \blacktriangle , 'ornithine', ∇ , 'arginine'; \blacksquare , 'lysine'; \square , 'adipamide'; \times , 'normal'.

TABLE 2

PERCENTAGE RESIDUAL ALLERGENICITIES OF FORMALINIZED GROUP I DERIVATIVES RELATIVE TO NATIVE GROUP I RYE ALLERGEN* BY HISTAMINE RELEASE ASSAY *in vitro*

Cell Donor	Native allergen concentration for 50 per cent HR ($\mu\text{g/ml}$)	Days of incubation at 32°	Percentage residual allergenicity of formalinized derivatives				
			Normal	Adipamide	Ornithine	Lysine	Arginine
M.L.	4.6×10^{-4}	18	<0.1	<0.1	1.6	<0.1	0.7
M.L.	4.6×10^{-4}	32	<0.001	<0.001	12	<0.001	0.2
S.Ma.	2.2×10^{-4}	18	3.0	0.7	56	1.4	5.0
S.Ma.	2.2×10^{-4}	32	<0.07	<0.04	62	0.2	0.3
R.K.	2.3×10^{-4}	18	<0.02	<0.02	7.4	<0.02	0.8
T.Do.	5.0×10^{-5}	18	ND	ND	20	ND	3.3
T.De	4.2×10^{-3}	32	<0.2	0.5	67	1.5	3.3
R.W.	2.0×10^{-3}	32	0.03	0.1	40	0.2	1.3
T.M.	3.3×10^{-4}	32	0.7	0.6	55	1.6	5.5
S.Mo.	3.4×10^{-4}	32	0.3	0.09	ND	0.2	ND
A.M.	5.1×10^{-5}	32	0.3	0.3	ND	0.2	ND
L.M.	1.9×10^{-4}	32	0.1	0.06	ND	0.08	ND

ND, Not determined.

* Determined relative to 18- or 32-day controls, as appropriate.

In the series of tests of 'allergoid' inhibition of histamine release from leucocytes by allergen, the demonstration of a positive inhibition would presumably indicate that the formalinized 'allergoid' derivatives were combining with cell bound reaginic antibody without releasing histamine (i.e. acting in a hapten-like manner). However, the normal, adipamide- and lysine-formalinized derivatives produced no inhibition of histamine release by native allergen (indeed, a slight enhancement was noted), using up to 10,000-fold equivalent weight excess of formalinized derivative to native allergen; hapten-like activity of the derivatives can therefore be ruled out.

ANTIGENICITY

Reactogenicity

For immunizations using native Group I antigen emulsified in Freund's complete adjuvant, rabbit and anti-native antigen sera were obtained which showed a high degree of cross-reactivity between native and formalinized antigen preparations by immunodiffusion analysis (Fig. 2a). Zero-day and 32-day native Group I gave similar precipitin lines.

When alum was used as the adjuvant in preparing rabbit anti-native Group I sera, only native Group I (0- or 32-day controls) and the highly allergenic ornithine-derivative showed any significant ability to precipitate with such sera (Fig. 2b). Thus, precipitin analysis in these weaker antisera suggested lack of cross-reaction between most of the formalinized allergen derivatives and the anti-native Group I (alum) sera. However, when dilutions of such sera were used for PCA in guinea-pigs, subsequently challenged with native or formalinized antigens in Evan's blue, some cross-reaction (about 5-20 per cent) was evident.

Subsequent quantitative studies of cross-reactogenicity, by analysis of antigen-antibody precipitates, employed only the stronger antisera prepared by Freund's method. Representative curves from such experiments are shown in Fig. 3. Estimates of cross-reactogenicity between the formalinized and native antigens by this procedure gave values of about 60 per cent cross-reactivity (Table 3).

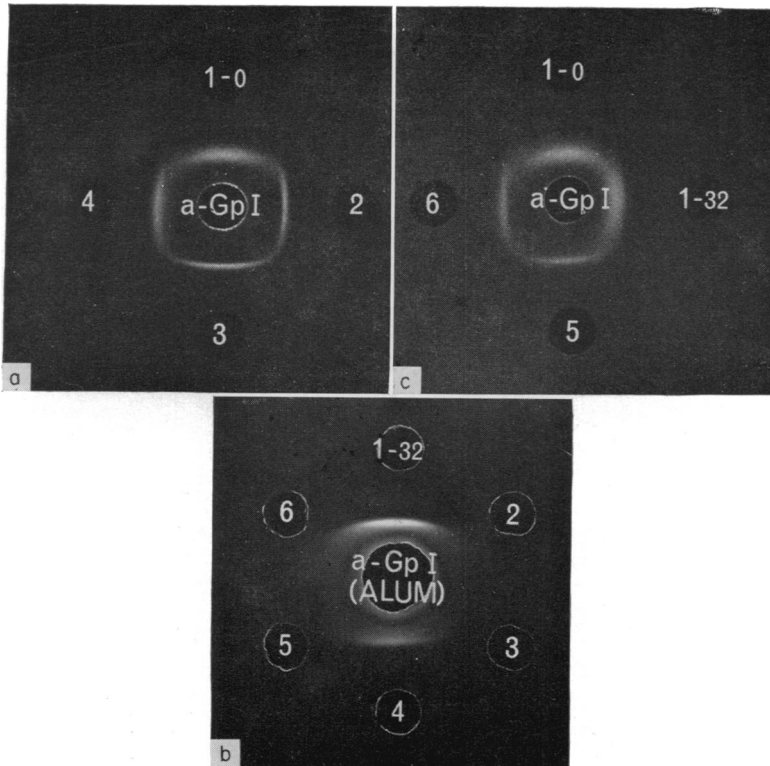


FIG. 2. Immunodiffusion analyses for native and formalized Group I antigen against hyperimmune rabbit antisera to native Group I. The sera were prepared by immunization with antigen in Freund's complete adjuvant or adsorbed on alum. Antigen preparations are numbered as in Table 1; all are '32-day' samples except native antigen Preparation 1-0. Antigen concentrations are 50 $\mu\text{g}/\text{ml}$ in (a) and 500 $\mu\text{g}/\text{ml}$ in (b). [Similar, but less intense, patterns were obtained for antigen concentrations of 50 and 100 $\mu\text{g}/\text{ml}$ in experiments similar to (b).]

In another series of studies using the method of radial immunodiffusion after Mancini, Carbonara and Heremans (1964), values of cross-reactogenicity were about 30 per cent—roughly half those found by quantitative precipitation. Subsequent molecular weight studies on the formalized derivatives (unpublished) revealed that partial polymerization of the protein had taken place, accounting for these lower results. The radial immunodiffusion method showed no detectable antigenic difference between 0- and 32-day native antigen controls. This result, together with the allergenicity studies, demonstrated that no significant denaturation of the native antigen had occurred during a 32-day incubation at 32°.

Immunogenicity

All formalized antigens induced in guinea-pigs the formation of antibody which cross-reacted with native Group I antigen by PCA tests in non-immunized guinea-pigs; conversely, antibody raised against the native antigen, when injected into guinea-pigs, gave positive PCA reactions upon subsequent challenge with any formalized antigen. In either case, challenge with the homologous antigen produced significant reactions at anti-serum dilutions substantially greater than when heterologous antigens were used (Table 4),

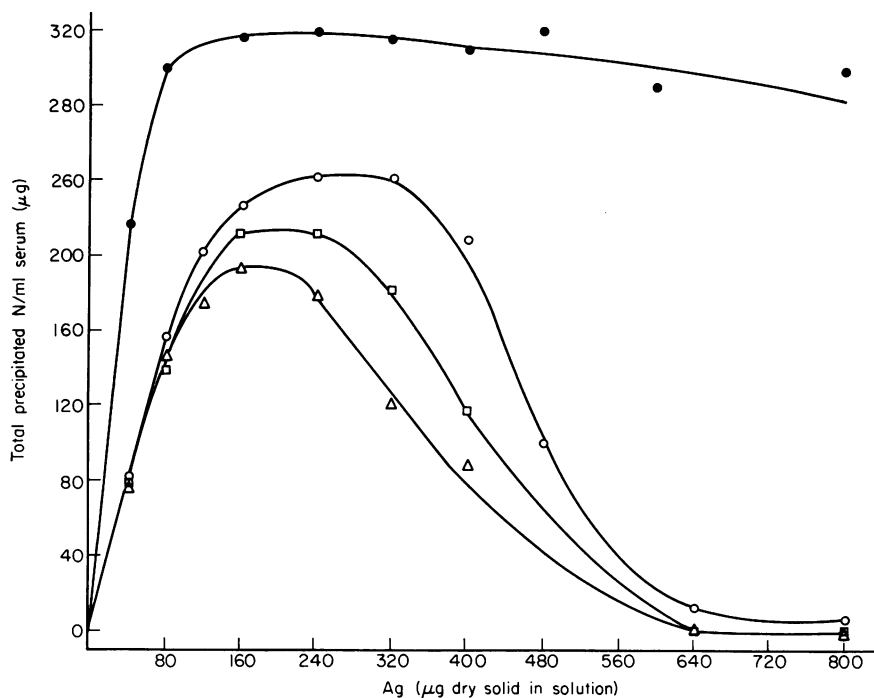


FIG. 3. Quantitative precipitin analyses for native and formalized antigens against decompemented rabbit anti-native Group I Serum A (Freund's complete adjuvant). ●, Native antigen (0-day) control; ○, 'lysine'; □, 'adipamide'; △, 'normal'.

excluding the case of the ornithine derivative. Within the limitations of the PCA test, these semi-quantitative results suggest that the degree of cross-reaction between native antigen and anti-formalized antigen sera is greater than between formalized antigens and anti-native antigen sera.

For the leucocyte allergen neutralization assay, in order to make quantitation of the results significant, sera were produced in larger matched groups of ten guinea-pigs under more carefully controlled conditions, using immunizing doses of antigens sufficient to give consistently high blocking antibody titres. Scattergrams showing two separate studies of the comparative abilities of individual antisera to block histamine release from allergic human leucocytes mediated by native Group I allergen are shown in Fig. 4. Antisera

TABLE 3
PERCENTAGE CROSS-REACTOGENICITY OF FORMALIZED RYE GROUP I WITH NATIVE GROUP I RYE ALLERGEN (0-DAY CONTROL) BY QUANTITATIVE PRECIPITATION

Anti-native Group I rye serum*	Formalized antigen derivative				
	Normal	Adipamide	Ornithine	Lysine	Arginine
A	57	61	ND	64	ca 62
B	52	61	ca 69	71	ND

Estimated maximum error = ± 4 per cent of quoted percentages.

* Sera prepared by hyperimmunizing rabbits with antigen emulsified in Freund's complete adjuvant. Serum A contained 293 μg Ab nitrogen/ml and Serum B 230 μg /Ab N/ml homologous for the native Group I antigen.

TABLE 4
APPROXIMATE PERCENTAGE ANTIGENIC CROSS-IMMUNOGENICITY BETWEEN
NATIVE GROUP I ANTIGEN AND FORMALINIZED ANTIGENS DETERMINED
BY PCA TESTS OF GUINEA-PIG ANTISERA IN GUINEA-PIGS

Formalinized derivative	Per cent cross-immunogenicity with native allergen*	
	1	2
Normal	40	10
Adipamide	50	10
Ornithine	> 80	100
Lysine	30	20
Arginine	70	10

All percentages are quoted relative to the homologous Ag-Ab cross-reaction on the same animal.

* Column 1: Guinea-pigs used for PCA were injected with dilutions of both anti-allergen and anti-allergoid sera on the same animal; they were all challenged later with the native allergen (500 μ g). Percentages quoted are average results from four animals.

Column 2: Guinea-pigs were injected as in Column 1; they were each challenged later with the allergoid (500 μ g) homologous to the anti-allergoid serum previously injected. Percentages quoted were each determined from results in two animals.

against native Group I, normal-derivative and lysine-derivative all showed considerable blocking activity, although the average blocking activities of both groups of anti-derivative sera were significantly lower than the anti-native antigen control. Statistically, the two sets of inhibition data for anti-native Group I and anti-lysine-derivative sera (diluted 525 times), for which all points lie on expected near-parallel linear regions of the inhibition

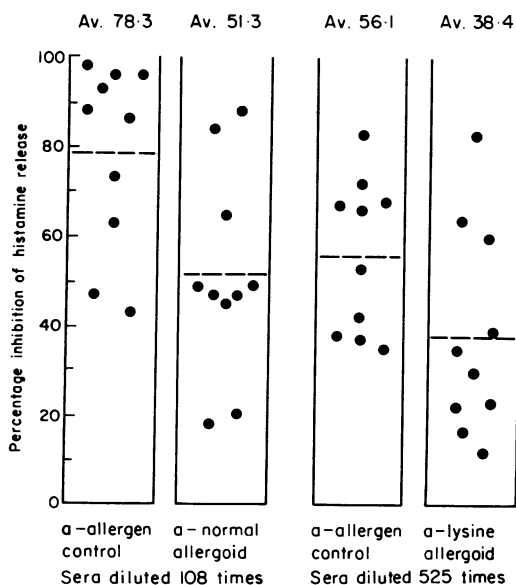


FIG. 4. Scattergrams showing percentage inhibition of native Group I allergen-mediated histamine release from allergic human leucocytes, using individual guinea-pig antisera against native Group I and normal- and lysine-allergoids (at dilutions indicated).

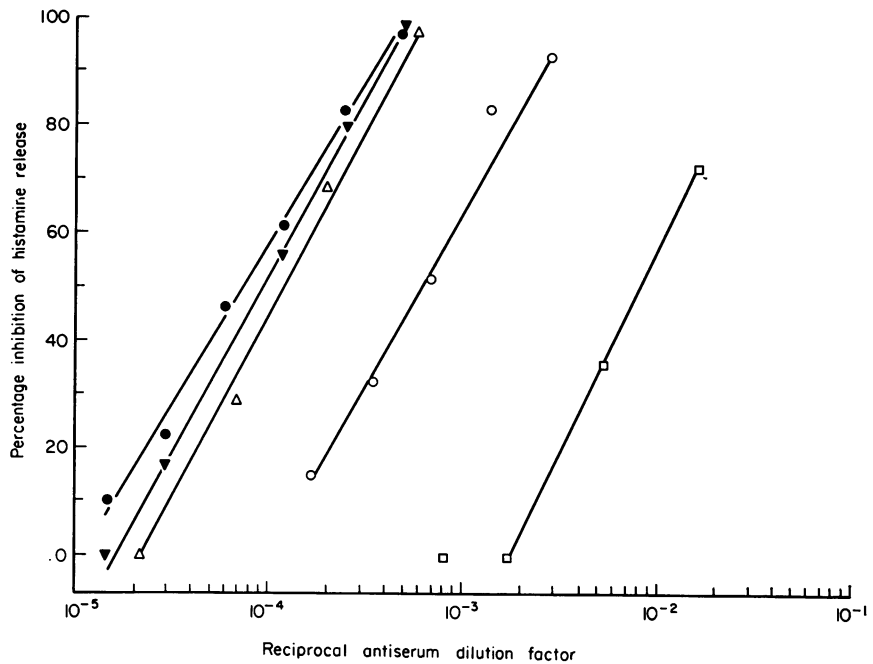


Fig. 5. Inhibition curves for native Group I allergen-mediated histamine release from allergic human leucocytes, using pooled guinea-pig antisera against native Group I and formalized derivatives. ●, Anti-native antigen (32-day) control; ▼, anti-'arginine'; Δ, anti-'lysine'; ○, anti-'normal'; □, anti-'adipamide'. Cell donor S.Ma.

curves (cf. Fig. 5), were significantly different ($0.05 > P > 0.025$). In similar assays, individual anti-adipamide-derivative sera were found to possess very low or undetectable blocking activities, whereas the anti-arginine-derivative sera all had high blocking activities approaching those of the anti-native antigen controls.

Equal volumes of the ten individual sera within a group were pooled in order to obtain a quantitative comparison of the cross-immunogenicities of the formalized derivatives

TABLE 5

PERCENTAGE CROSS-IMMUNOGENICITY OF FORMALIZED GROUP I DERIVATIVES RELATIVE TO NATIVE GROUP I RYE ANTIGEN, BY THE COMPARATIVE ABILITIES OF GUINEA-PIG ANTISERA AGAINST SUCH ANTIGENS TO INHIBIT ALLERGEN-MEDIATED ALLERGIC HISTAMINE RELEASE *in vitro*

Cell donor	Anti-normal derivative*		Anti-adipamide derivative	Anti-lysine derivative	Anti-arginine derivative
	A	B			
M.L.	15	ND	<0.1	60	ND
S.Ma.	23	12	1	62	80
N.H.	12	ND	<2	53	ND
L.M.	ND	5	ND	ND	64

Estimated error = ± 5 per cent of quoted percentages. ND, Not determined.

Antisera were raised simultaneously in matched groups of 10 guinea-pigs for the '32-day' native antigen and '32-day' allergoids: pools were prepared from equal volumes of each serum within a group.

Two less precise analyses using other individuals' cells gave qualitatively similar cross-immunogenicity values to those quoted above.

* Two separate experiments (A and B) were performed at different times with different matched pairs of groups of guinea-pigs immunized with native antigen or normal-derivative.

relative to native antigen, in terms of their respective capacities to induce synthesis of antibody capable of inhibiting allergic histamine release by native allergen. An example of a series of inhibition curves is given in Fig. 5; quantitative cross-immunogenicity data using four different allergic human leucocyte preparations are summarized in Table 5. By this assay, the cross-immunogenicities between the lysine- and arginine-derivatives and the native antigen were always very appreciable (over 50 per cent); the values for the normal-derivative were somewhat lower (over 10 per cent except in one case); for the adipamide-derivative, cross-immunogenicity with the native antigen was always very low (about 1 per cent or less). Small variations in the cross-immunogenicity figures for each formalinized derivative were found from donor to donor; these variations were considerably less than the comparable variations in residual allergenicity (Table 2). A further variation was noted in the cross-immunogenicity figures for the normal-derivative when comparing, with the same cell donor (S.Ma.—Table 5), antisera raised in different matched paired groups of guinea-pigs immunized with test and control antigens.

We were unable to detect precipitating antibody by ring test or immunodiffusion analysis in several of the guinea-pig sera which possessed high titres of antibody capable of blocking histamine release from allergic human leucocytes.

DISCUSSION

Two rye Group I allergen derivatives, formed by treatment with formalin alone or formalin plus L-lysine, may be classified as *allergoids* by previous definition. These 'normal'- and 'lysine-' allergoids possessed low residual *allergenic reactogenicity* and high *antigenic reactogenicity and immunogenicity* relative to the native allergen. The lysine-allergoid appears to be the most promising immunotherapeutic preparation based on: (a) its residual allergenicity of 0.001–0.3 per cent, determined by assay of histamine released by allergen and allergoid from different human leucocyte preparations; (b) its previously observed low skin reactivity (Marsh, unpublished); and (c) its ability to induce in guinea-pigs the biosynthesis of antibody possessing 60 per cent of the blocking capacity of anti-native Group I antibody, determined by inhibition of allergen-mediated allergic histamine release from isolated human leucocytes.

Other formalinized allergen derivatives described can probably not be classified strictly as allergoids.* The adipamide-derivative, although possessing low residual allergenicity and high antigenic reactogenicity relative to the native allergen, showed very low cross-immunogenicity in the blocking assay. The arginine-derivative showed a somewhat greater allergenicity (RAI = about 2 per cent) than the aforementioned derivatives, but possessed good antigenic properties. The ornithine-derivative was too allergenic to be a desirable immunizing agent.

Formaldehyde reactions

Formaldehyde has long been used to convert bacterial and other proteinaceous toxins into virtually non-toxic toxoid derivatives, capable of immunizing protectively against the corresponding native toxins (e.g. Linggood, Stevens, Fulthorpe, Woiwod and Pope, 1963). Carter (1935) claimed to have 'detoxified' pollen extracts in a similar manner, but

* We are provisionally considering an allergoid to be a derivative possessing less than 1 per cent residual allergenicity, by direct histamine release assay using a number of allergic human leucocyte preparations, and a minimum of 10 per cent cross-immunogenicity in our blocking assay.

presented no experimental data. Stull, Cooke, Sherman, Hebdal and Hampton (1940), probably following a similar methodology to Carter, treated crude aqueous ragweed and timothy grass pollen extracts with dilute formalin, but were unable to produce satisfactory derivatives. More recently, Naterman (1957, 1965) formalinized precipitated pollen materials, apparently with some success, although the clinical experiments were not controlled and no immunological analyses were performed.

The immunochemical complexity in preparing allergoids is considerably greater than for toxoids, since in the former case it is necessary to modify significantly a number of different allergenic determinants (see 'Allergenicity' section) without destroying all the original antigenic determinants; for toxins, the destruction of only one type of toxic centre is necessary.

The chemistry of formaldehyde reactions with proteins and reactive additives is treated in detail in the 'Appendix' (p. 720). Rye Group I allergen contains amino groups only in its protein moiety; no amino sugars or other carbohydrate groups able to react with formaldehyde under the reaction conditions described have been detected (Johnson and Marsh, 1966b). Therefore, the extensive destruction of allergenicity for most formalinized Group I preparations further strengthens previous evidence (Marsh *et al.*, 1966), strongly suggesting that only the protein moiety is involved significantly in allergenic determinants. The special case of the ornithine-derivative is treated in the 'Appendix' (p. 721).

Antigenicity

The antigenic cross-reactogenicity of the allergoids with native Group I antigen, determined by quantitative precipitation, did not vary from allergoid to allergoid very appreciably (range: about 70–50 per cent) compared with the large variations in antigenic cross-immunogenicity determined by the inhibition assay (range: about 80 to <0.1 per cent). Three of the possible explanations for the wide variations in the immunogenicity results are: (i) a large differential in the rates of metabolism of different allergoids, leading to a longer availability of antigen for initiation of antibody synthesis of the less readily metabolized derivatives,* (ii) variations in the ability of animals to process and/or to recognize the different allergoid antigens; and (iii) variations in the average ratios of the probable guinea-pig γ_1 and γ_2 'blocking antibodies' produced against the different antigens. The latter factor would certainly influence cross-immunogenicity results obtained by PCA, due to the different tissue-fixation characteristics of these classes of antibody (Ovary, Benacerraf and Bloch, 1963; Strejan and Campbell, 1968): its possible influence on the results obtained by the inhibition assay remains to be determined.

There is a marked correlation between the cross-immunogenicity values for different allergoids with respect to allergen, and the corresponding figures for diphtheria toxoids relative to diphtheria toxin. Linggood *et al.* (1963) found that both lysine- and arginine-diphtheria toxoids were more cross-immunogenic with the native toxin than was the normal-toxoid. In the present assay of the inhibition of allergic histamine release from leucocytes, higher cross-immunogenicity was found between lysine- and arginine-allergoids and native antigen than between normal-allergoid and native antigen. It seems possible that the incorporation of lysine or arginine into a formalinized allergoid or toxoid confers on such a substance an intrinsic adjuvant-like activity, possibly due to the factors stated in the previous paragraph.

* In support of this view, normal- and lysine-allergoids were shown to be much less susceptible than the native allergen to proteolytic enzymatic digestion by trypsin, chymotrypsin and pepsin (Marsh, unpublished).

Precipitin analysis revealed extensive cross-reactogenicity between formalinized antigens and anti-native Group I sera when the sera were prepared using antigen in Freund's complete adjuvant, but not when alum was the adjuvant (Fig. 2). The highly allergenic ornithine-derivative was an exception to this general rule (see 'Appendix'). Varying the dilutions of the antigens and of both types of antisera did not change the relative appearance of the precipitin lines against the native antigen and the formalinized antigens. Thus, we feel that the antisera raised using Freund's adjuvant is able to recognize more antigenic determinants on the native molecule than 'alum-antisera'. The inability of *allergoids* to precipitate with antisera raised against native antigen using alum as the adjuvant shows that lack of precipitation is not necessarily a good indication of whether a modified antigen is capable of immunizing protectively against the native antigen.

Allergenicity

Some pronounced variations in the residual allergenicity of identical formalinized derivatives with different cell preparations were observed (Fig. 1 and Table 2). These differences showed no correspondence with the donor's cell sensitivity and thus did not apparently reflect differences in the levels of cell-bound anti-Group I reaginic antibody (IgE). The most plausible explanation seems to be that different allergic individuals' IgE-producing systems vary in their capacity to recognize different determinants on the native allergen molecule. The binding affinities of different individuals' IgE antibody molecules to the various allergenic determinants are probably the important factors to be considered in this respect.

The failure of allergoids, in 10,000-fold excess, to inhibit allergen-mediated histamine release from leucocytes suggests that the allergoids did not combine with cell-bound IgE in a hapten-like fashion.

Therapeutic potential of allergoids

Conventional immunotherapy of human allergic (atopic) disorders involves injection with gradually increasing doses of crude extracts of the materials toward which the individual is sensitive. Immunotherapy with crude aqueous ragweed pollen extract or the purified ragweed allergen, Antigen E, was shown to decrease the degree of allergic symptoms in patients with ragweed hay fever (Lowell and Franklin, 1965; Lichtenstein, Norman and Winkenwerder, 1968; Norman, Winkenwerder and Lichtenstein, 1968). The extent of relief appeared to increase by increasing the total dosage of antigen administered and was usually accompanied by an increase in specific blocking antibody level in the serum (Lichtenstein *et al.*, 1968).

The present model study of allergoids has employed derivatives of the highly purified potent Group I allergen of rye grass pollen. Provided this model can be extended to other allergenic extracts of practical clinical value, and the cross-immunogenicity between allergoid and native antigen can be confirmed in man, allergoids are potentially of considerable therapeutic interest. They would allow the administration of much higher doses of antigen than currently possible with the highly allergenic native extracts. Thus, high immunizing doses of allergoid could be given on a restricted immunizing schedule, leading probably to the development of high blocking antibody levels (and, hopefully, to greatly improved clinical relief). High dosage allergoid immunotherapy could well assist in determining the significance of blocking antibody and other factors involved in symptomatic relief of human atopy.

In treating allergic individuals with allergoids, the question arises of both reagenic antibody (IgE) and blocking antibody (IgG and IgA) systems competing for antigenic stimulation by allergoid. That is to say, the allergoid *per se* may be potentially immunogenic in a specifically allergenic as well as a generally antigenic sense. However, we do not anticipate that this factor will present a problem, since it has been shown that following repeated parenteral immunization there is little or no overall increase in the level of specific IgE antibody in individuals who were already producing such antibody to ragweed allergens (Sadan, Rhyne, Mellits, Goldstein, Levy and Lichtenstein, 1969). Should any allergoid-specific IgE be produced *de novo*, it would be directed against a substance which does not exist in nature and, as our experiments reveal, there is virtually no allergenic cross-reactivity between allergoid and allergen.

The use of formalinization as a chemical method for the preparation of allergoid derivatives is probably restricted mainly to allergenic materials which are essentially protein in nature. This is considered to be the case for principal grass and ragweed pollen allergens (Marsh *et al.*, 1966; King, Norman and Lichtenstein, 1967), and also allergenic components of cod fish (Aas and Jebsen, 1967). Allergenic components of some other biological materials either do or may contain important glycosidic determinants (Kabat, Turino, Tarrow and Maurer, 1959; Stanworth, 1963; Berrens, 1968).

We should stress that the Group I allergoids are basically prototypes for modified allergens suitable for immunotherapy of atopic humans. Some modifications in experimental procedure may be necessary to produce the best allergoids in the cases of complex allergenic mixtures and other purified allergens.

APPENDIX

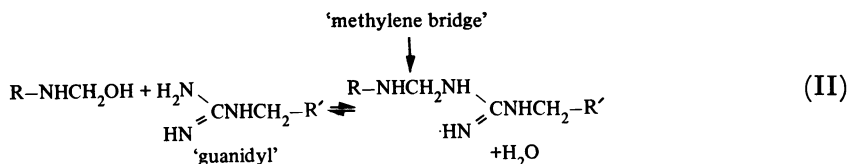
Chemistry of formaldehyde reactions

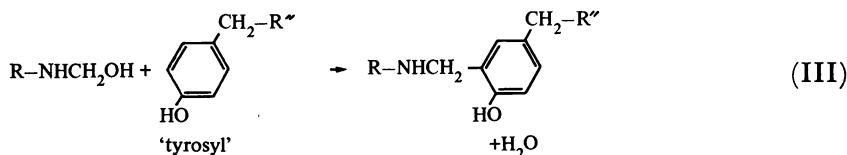
Under the reaction conditions described, formaldehyde is known to react rapidly and reversibly with amino groups (of the allergen or additive) to give aminomethylol derivatives (I):



Reactions of this type would have reached equilibrium prior to the pH adjustment of the reaction mixtures.

Subsequent reactions occur much more slowly and involve either reversible combination of the aminomethylol derivatives with protein guanidyl and acid amido groupings or irreversible Mannich substitution reactions of the aminomethylene radical at reactive positions on certain aromatic amino acids (tyrosine, histidine and tryptophan) present in proteins: for example,

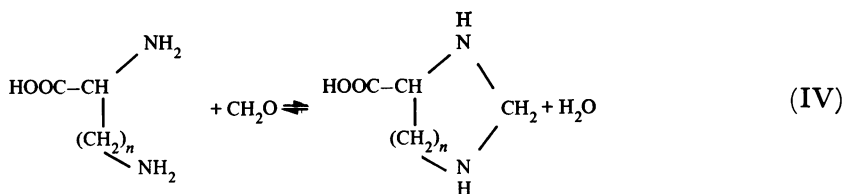




With the relatively low concentrations of allergen (1.00 mg/ml) and formaldehyde (0.06 M) used, the chemical reactions should principally involve intra- rather than inter-allergenic molecular cross-linking. This conclusion has been substantiated by molecular weight studies on the normal- and lysine-derivatives (unpublished results). The reactions should take place only between reactive groupings which are stereochemically relatively close together. Where a large excess of reactive groupings is contributed by an additive present in the incubation mixture, it is expected that the principal reactions involve extensive inter-molecular methylene bridge linking between additive and allergen. This conclusion is substantiated for the basic lysine- and arginine-derivatives by the relatively large drop in pH (0.35 unit) of the strongly buffered reaction solutions during 32 days of incubation and by the substantial incorporation of adipamide, lysine and arginine in Preparations 3-6 (Table 1). In a chemically analogous preparation of a lysine-diphtheria toxoid, the substitution of radioactive lysine into protein tyrosyl groups was demonstrated (Blass, Bizzini and Raynaud, 1968).

Compounds of the type $\text{R-NH-CH}_2\text{-NH-R}'''$, formed by the linkage of two amino groups by formaldehyde, are known to be generally unstable except where stabilizing factors are operating, such as in certain ring structures. The work of Ruzicka (1935) and others has clearly shown that five-, six-, and to a lesser degree, seven-membered alicyclic ring structures are relatively stable and easily formed. Ring sizes larger than seven atoms become increasingly difficult to produce. Thus, the probability of formation of relatively stable ring structures of the type IV shown below, from the homologous basic amino acid series ($n \geq 1$), is likely to be in the order:

diaminopropionic acid \approx diaminobutyric acid $>$ ornithine $>$ lysine $>$ higher members of the series (assuming other factors such as the increasing insolubility of the derivatives for $n > 5$ are ignored).



The relative ease of ring formation with ornithine as compared with lysine would effectively incapacitate considerably more formaldehyde in the former case. This explains the relative lack of chemical alteration in the ornithine-derivative, reflected particularly in its high residual allergenicity (Fig. 1 and Table 2) and antigenicity (Fig. 2b and Table 4) compared to the lysine-allergoid. Further unpublished results using diaminopropionic acid (Dpr) as the additive, fit in well with this analysis. The Dpr-formalinized derivative was not detectably altered allergenically, antigenically, nor in its electrophoretic mobility in starch gel at pH 8.5, compared with the native allergen control.

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