Immunochemistry. Pergamon Press 1967. Vol. 4, pp. 37–47. Printed in Great Britain

A PROPOSED MECHANISM FOR THE INACTIVATION OF ATOPIC ALLERGENS IN DILUTE SOLUTION

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(*Received* 23 *September* 1966)

Abstract—A study has been made of the ultra-violet absorption spectra of purified atopic allergens at pH 2 and pH 12. The colour change of atopen solutions from deep brown in alkali to light brown or yellow in acid is reflected in the spectra by considerably higher extinction coefficients in alkali. By including native proteins and synthetic protein-sugar model compounds it proved possible to locate the specific differences between the spectra of the allergens in acid and alkali at wavelengths over 325 mμ, i.e. in the near ultra-violet and in the visible region. It is suggested that the chemical shifts in the system: N^{ϵ}-[1-deoxyglycosulosyl-(1)]-lysine residue and its 1·2- and 2·3-enolic tautomers are responsible for the colour-change phenomenon.

The incorporation of these 1-amino-1-deoxy-2-ketose derivatives, especially of the 2·3-enediol form, together with their pathways of degradation in dilute solution and at extreme pH-values, provides a plausible explanation for the inactivation of solutions of atopic allergens.

1. INTRODUCTION

IN A previous study[1] the ultra-violet absorption spectra of a number of purified atopic allergens were compared with those of crystalline proteins and of synthetic model compounds, prepared by subjecting native proteins to Maillard reactions with aldose-sugars. It was found that new chromophores are introduced into the protein molecule during this reaction, giving rise to low-intensity absorption maxima at 305 mμ and at wavelengths over 325 mμ. Further observations on the optical properties of systems containing ε-aminocaproic acid/aldose, n-amylamine/aldose and primary amine/α-hydroxy-aldehydes subsequently led to the conclusion that the chromophore is located at the site of a protein-sugar linkage and encompasses the nitrogen atom of the ε-amino group of lysine residues in the protein and the first two (or three) carbon atoms of the aldose reactant.[2,3]

Since similar structural sites were detected optically and chemically in atopic allergens, it was concluded that these antigens characteristically contain N-substituted 1-amino-1-deoxy-2-ketoses, N^{ϵ}-[1-deoxyglycosulosyl-(1)-]-lysine residues, the absorption peak at 305 mμ being attributable to the 1·2-enol form. The significance of these sites of N-glycosidic coupling in human atopic allergy became especially evident when it could be demonstrated that the specific skin-reactivity of bovine β-lactoglobulin in atopic individuals hypersensitive to cow's milk could be increased a hundredfold by reacting the protein with lactose.[4] It is the purpose of the present article to extend the observations on the u.v. spectra of atopic allergens by differential spectroscopy at extreme pH-values and to identify derivatives of 1-amino-1-deoxy-2-ketoses incorporated in the molecular structure.

2. MATERIALS AND METHODS

The source of most of the allergens used in this work, and their isolation and properties have been given in a previous publication.[1] Four other allergens were

included, viz. purified kapok allergen, feather allergen and allergenic fractions from timothy-grass pollen and from hay; the preparation and properties of these substances will be reported separately. Briefly, the kapok allergen was obtained from a dialyzed aqueous extract of old (yellow) kapok linters, by precipitation with $(NH_4)_2SO_4$ between 35–85% saturation (4°C); the precipitate was then dissolved in water, dialyzed, and titrated with dilute HCl to pH 2·5 to remove precipitable substances from the allergen in the supernatant (kapok fraction E, Ref. 5). The allergenic substance from hay was obtained similarly except for the titration step; 20 μg was needed for positive intracutaneous reactions in persons sensitive to hay (negative in controls) and analysis gave 3·8% N, 14·6% hexoses and 5·4% uronic acid.

The grass pollen allergen was the skin-reactive fraction precipitated from a dialyzed aqueous extract of timothy pollen (Sharp and Sharp, U.S.A.) with $(NH_4)_2SO_4$ in the range 35–85% saturation; 11·9% N, 10·8% hexoses and 0·9% uronic acids were detected.*

The feather allergen was a dialyzed and freeze dried extract of aged chicken feathers from an old mattress. Since the substance proved to be extremely polydisperse, without distinct separate compounds being detectable, and because chemical separations turned out unsuccessful, no further purification was attempted. It was active in amounts of 0·2 μg intracutaneously in the majority of atopic patients hypersensitive to house dust and, analytically, gave 4·5% N, 2·7% hexoses and 4·7% hexuronic acid.

A solution of the horse dandruff allergen used previously[1] (35–80% ammonium sulphate precipitate from a solution of the non-dialyzable constituents of horse dandruff) was treated with HCl to pH 4·2 for the removal of precipitable material. The dialyzed and freeze-dried supernatant was used for the studies described here; the preparation of this fraction thus followed exactly the same procedure as applied in the isolation of human dandruff allergen fraction E.[6] The active allergen fraction F from liquorice[7] was used instead of fraction H in the previous study.[1]

Crystalline proteins, human serum albumin (HSA) and β-lactoglobulin (BLG) were obtained as before[1,2] and checked for purity by agar gel electrophoresis. Poly-L-lysine was purchased from Mann Research Laboratories (lot No. ML 804, mean mol. wt. 75·000, degree of polymerization 586).

Synthetic model compounds, 1-(N)-deoxy-ribulose-proteins or 1-(N)-deoxy-ribulose-polylysine, were prepared by keeping 0·5% solutions of the crystalline proteins or the polymer at 50° for 96–144 hr in sterile phosphate buffer pH 7·0 (0·01 M) containing 1% D-ribose. The (pale brown) solutions were then dialyzed exhaustively against running tap water and dried by lyophilization. Three models were used, viz: (deoxyribulose)-human serum albumin (HSAR)†, (deoxyribulose)-β-lactoglobulin (BLGR) and (deoxyribulose)-poly-L-lysine (PLR).

* Clinically, the fraction precipitating at 35% $(NH_4)_2SO_4$-saturation proved more active, while it also gave an absorption spectrum more characteristic of atopic allergens. The yield of this fraction, however, was too low for further studies.

† Analytically, HSAR gave 14·5% protein N as compared to 15·5% for the control (HSAC); lowering of Kjeldahl-nitrogen is a curious phenomenon accompanying browning reactions; the effect may account for the low N-content of some atopic allergens (compare Ref. 3).

The (hydroxymethyl) furfural test with 2-thiobarbituric acid (TBA) was adopted from the method of Keeney and Bassette[8]: 5 mg of the allergen in 2 ml 0·1 N oxalic acid was heated at 100° for 2 hr; after cooling 2 ml of trichloroacetic acid (20%) was added and the solution was centrifuged after mixing. The supernatant was carefully decanted, 1 ml of 0·05 M 2-thiobarbituric acid was added to the solution and the mixture was shaken at 40° for 40 min. Extinction was read at 405 mμ (for pentose-derivatives) and at 443 mμ (for hexose-derivatives) against a blank. The TBA-value was expressed as the difference between this reading and the extinction of a control treated similarly except for the heating step with oxalic acid.

For the oxidation of allergens a modification of the procedure of Heyns and Paulsen[9] was adopted: 10–50 mg of the allergen was dissolved in 10 ml phosphate buffer pH 7·0 (0·01 M), 10 ml $K_3Fe(CN)_6$-solution (0·1% in 0·1 N NaOH) was added and the mixture was shaken at room temperature for 30 min. After thorough dialysis the oxidized product was dried by lyophilization.

Ultra-violet absorption spectra were observed of 0·05% solutions of allergens and model compounds in 0·01 N HCl, 0·01 N NaOH and 0·01 M phosphate buffer pH 7·0 against the corresponding blanks; care was taken to dissolve the dry substance just prior to measurements. A Zeiss u.v.-spectrophotometer Type PMQ II, equipped with 1 cm quartz cells was employed.

3. RESULTS

It is a remarkable property of atopic allergens that the pale brown colour of these antigens in dilute acidic solution deepens considerably upon the addition of alkali. The phenomenon is reversible and is readily detectable with the human eye. Spectroscopically, the colour deepening is manifested in a general rise of the u.v.-absorption spectrum towards increased extinction, with the effect of shifting the curve further into the visible region. The absorption curve at neutral pH occupies a position intermediate between those in acid and alkaline solution; in order to stay outside the range of the iso-electric points (pH 3–7) and to ensure a clear demonstration of colour-deepening the extreme pH-values 2 and 12 were chosen. In Fig. 1 spectra at these pH-values are given of some allergens and in Fig. 2 of a few model compounds. Extinction coefficients at some characteristic wavelengths have been listed in Table 1 for all the substances studied.

The interpretation of the difference in absorption spectra at pH 12 and pH 2 is complicated by a number of factors. Thus, the normal tyrosine/tryptophan ionization shift (275 mμ in acid and neutral, to 295 mμ in alkaline medium) due to the aromatic amino acid residues in the peptide moiety of the allergens causes a shift of the minimum of the absorption curve at 300 mμ to about 320 mμ. Furthermore, alterations due to this effect are not comparable among different atopic allergens because their peptide contents differ considerably.[1] However, although the extinction coefficients increase in the whole region 200–400 mμ with increasing pH, the relative increase is more pronounced at wavelengths over 300 mμ.

Percentage differences between the extinction coefficients at pH 12 and pH 2 (absorbancy difference expressed as % of the extinction at pH 2) have been listed in Table 2; the data reveal significantly higher increments in the region 300–375 mμ for atopic allergens and (deoxyribulose) proteins than for native proteins.

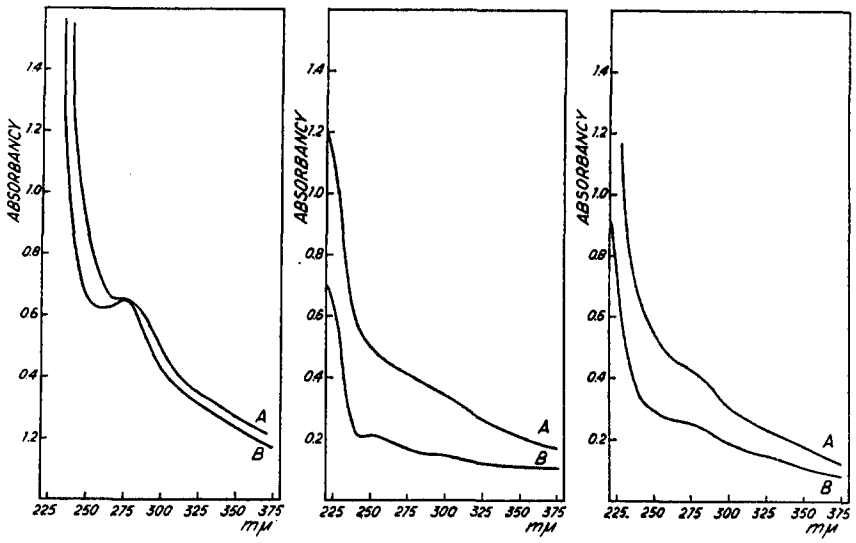


FIG. 1. Ultra-violet absorption spectra in 0·01 N NaOH (A) and 0·01 N HCl (B) of (a) purified house dust allergen 0·05%, (b) feather allergen 0·005%, (c) purified trichophytin 0·05%.

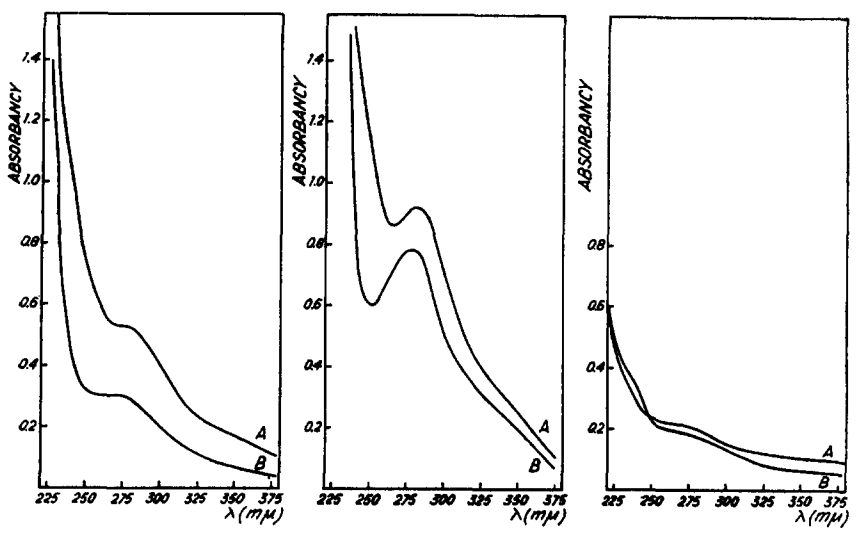


FIG. 2. Ultra-violet absorption spectra of (deoxyribulose)-model compounds in 0·01 N NaOH (A) and 0·01 N HCl (B); (a) HSAR, 0·05%, (b) BLGR, 0·05%, (c) PLR, 0·05%.

TABLE 1. EXTINCTION COEFFICIENTS $E^{1\%}_{1cm}$ OF ATOPIC ALLERGENS AND MODEL SUBSTANCES AT SOME CHARACTERISTIC WAVELENGTHS AND AT TWO DIFFERENT pH-VALUES*

Substance	250 mμ		275 mμ		300 mμ		325 mμ		350 mμ		375 mμ	
	pH 2	pH 12	pH 2	pH 12	pH 2	pH 12	pH 2	pH 12	pH 2	pH 12	pH 2	pH 12
House dust	13·2	17·5	12·8	12·8	8·3	9·8	6·2	7·0	4·7	5·3	3·3	4·1
Horse dandruff	5·2	19·4	6·9	7·2	2·5	5·8	1·7	1·8	1·2	1·4	0·9	1·2
Feathers	68·2	100·8	56·4	82·0	37·4	66·4	28·2	53·0	19·8	43·0	15·0	34·4
Kapok	82·0	208·0	74·0	176·0	60·0	150·0	48·0	124·0	38·0	100·6	28·0	84·0
Liquorice	34·0	94·0	31·0	75·6	26·0	65·2	23·4	60·0	20·0	54·2	16·0	42·0
Ipecac	13·6	35·6	14·0	16·8	5·6	12·3	3·6	5·1	2·6	3·9	1·8	3·1
Grass pollen	7·2	22·8	9·6	10·6	3·8	8·0	3·3	3·3	3·0	3·0	1·8	2·8
Hay	56·2	128·0	50·0	110·0	39·0	91·0	33·6	74·6	27·8	63·0	22·0	56·0
Trichophytin	5·6	10·8	5·0	8·5	3·6	6·3	2·8	4·6	2·2	3·5	1·5	2·7
PLR	4·5	4·6	3·6	4·1	2·5	3·0	1·8	2·4	1·2	2·1	0·9	1·6
BLGR	12·0	23·0	15·5	18·0	10·3	14·2	6·3	8·1	4·0	5·0	2·2	3·1
HSAR	6·4	14·4	6·0	10·6	3·9	7·9	2·3	4·7	1·4	3·4	0·8	2·2
HSAC	2·7	7·0	3·9	3·4	0·8	1·8	0·4	0·6	—	—	—	—
Pepsin	4·9	37·0	14·3	12·0	1·4	8·6	0·1	0·2	—	—	—	—
Trypsin	4·9	32·5	12·0	12·8	2·0	8·0	0·1	0·1	—	—	—	—
Ovalbumin	2·5	6·4	6·0	6·0	1·0	1·8	—	—	—	—	—	—

* The extinction coefficient for allergens and model substances may not be absolute, because the degree of browning may be different for products prepared from different batches of material.

TABLE 2. Percentage differences: $\frac{E_{NaOH}-E_{HCl}}{E_{HCl}} \times 100\%$

Substance	250 mμ	275 mμ	300 mμ	325 mμ	350 mμ	375 mμ
House dust	35	1	20	11	14	17
Horse dandruff	288	6	58	12	17	33
Feathers	46	65	77	86	113	148
Kapok	150	136	158	158	168	190
Liquorice	180	141	141	158	168	162
Ipecac	161	20	119	39	49	70
Grass pollen	245	10	105	0	0	50
Hay	124	120	133	124	132	175
Trichophytin	92	80	63	64	63	71
PLR	7	5	17	44	66	54
BLGR	91	15	38	28	25	34
HSAR	131	71	105	109	143	158
HSAC	157	−10	100	50	0	0
Cryst. pepsin	640	−17	543	125	0	0
Cryst. trypsin	557	−2	300	200	0	0
Cryst. ovalbumin	170	+1	104	20	0	0

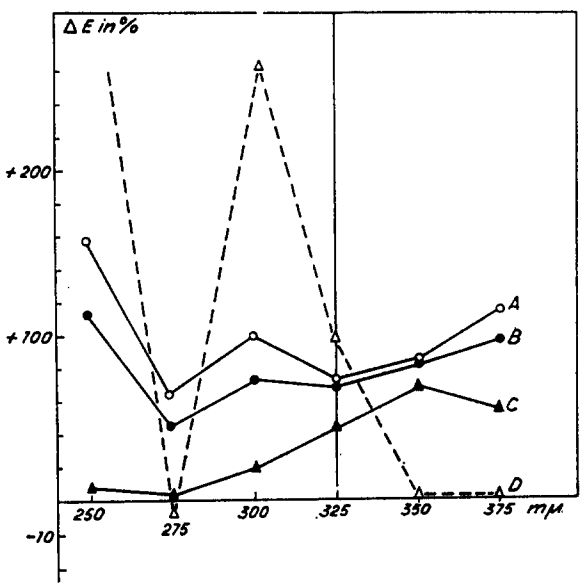


FIG. 3. Percentage difference curves; absorbancy difference ΔE (extinction at pH 12 minus extinction at pH 2) expressed as % of absorbancy at pH 2. Compare Table 2. A: average of 8 allergens, not including trichophytin; B: average of 2 model compounds (HSAR and BLGR); C: PLR; D: average of 4 native proteins, including HSAC.

To illustrate this further, percentage differences have been averaged for the allergens, for the synthetic models, and for the crystalline proteins used here. Although this is an arbitrary procedure, the resulting 'percentage difference curves' (Fig. 3) indicate the *specific* alterations for the allergens and models, as compared to the crystalline protein controls, to be at wavelengths over 325 mμ,

the differences below this point being largely attributable to the tyrosine/tryptophan shift in the peptide moieties; the difference curve for the (deoxyribulose)-polylysine compound is in accordance with this conclusion (Fig. 3).

The chromophores in atopic allergens responsible for absorption at 305 mμ were previously shown to be N-substituted 1-amino-1-deoxy-2-ketoses in the 1·2-enol form.[1,2] These are the only extra structures incorporated in the model compounds used in this study. Chemically, such residues may be demonstrated by taking advantage of their easy cleavage from the carrier protein under mild hydrolytic conditions in 0·1 N oxalic acid. Gottschalk[10] has shown that the ease of formation of (hydroxymethyl)-furfural under relatively mild conditions is a specific chemical test for 1-amino-1-deoxy-2-ketoses. The application of this method to some atopic allergens according to the experimental procedure developed by Keeney and Bassette[8] indeed gave significant values for 1-amino-1-deoxy-2-ketoses in these antigens. Although only relative values are obtained with the method as applied here, the data (in ΔE/5 mg) in Table 3 show the 'TBA-index' to be considerable for atopic allergens and model substances; crystalline proteins do not react in this test.

TABLE 3. EXAMPLES OF THIOBARBITURIC ACID TESTS FOR 1-AMINO-1-DEOXY-2-KETOSES (TBA-INDEX, IN ΔE/5 mg) IN SOME ALLERGENS AND CONTROL SUBSTANCES

Substance	ΔE/5 mg 405 mμ	ΔE/5 mg 443 mμ
House dust	*	0·128
Feathers	*	0·033
Kapok	0·052	0·144
Liquorice	*	0·340
Ipecac	*	0·060
Grass pollen	—	0·070
Tomato	*	0·177
HSAR	0·041	0·026
HSAC	0·007	0·010
PLR	0·459	0·177
Polylysine	—	—
Cryst. ovalbumin	—	—
β-lactoglobulin	0·010	0·010

* Not determined.

Because N-substituted 1-amino-1-deoxy-2-ketoses possess the properties of reductones,[9,11] it was reasoned that oxidation of these derivatives in atopic allergens would result in a decrease of colour intensity in NaOH. In a few experiments atopic allergens were accordingly treated with $K_3Fe(CN)_6$ in NaOH[9] (see Methods section) at room temperature, and were recovered by dialysis and lyophilization; this is illustrated by spectra of the ipecac allergen and of the human dandruff allergen in 0·01 N NaOH before and after oxidation (Fig. 4). A considerable decrease in absorbancy of the oxidized allergens was indeed observed, percentage decrease being most pronounced at wavelengths over 350 mμ.

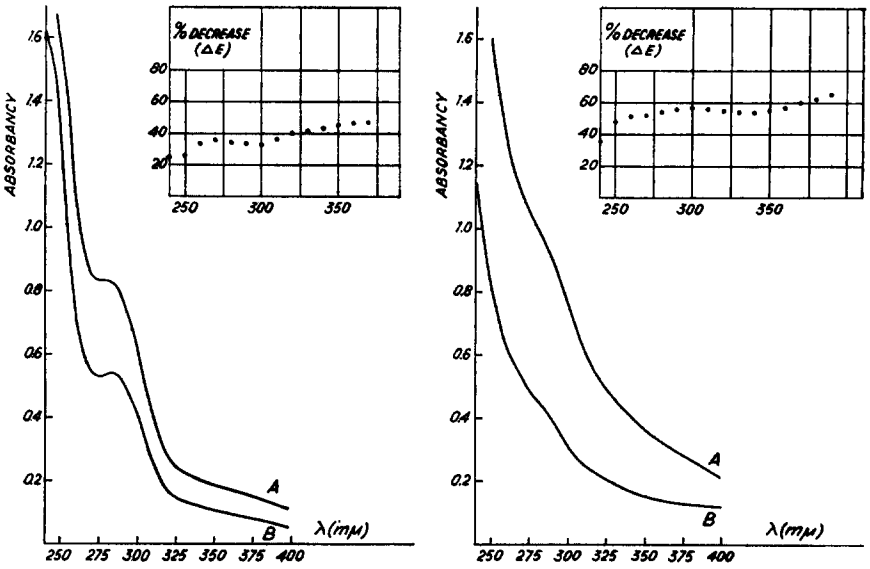


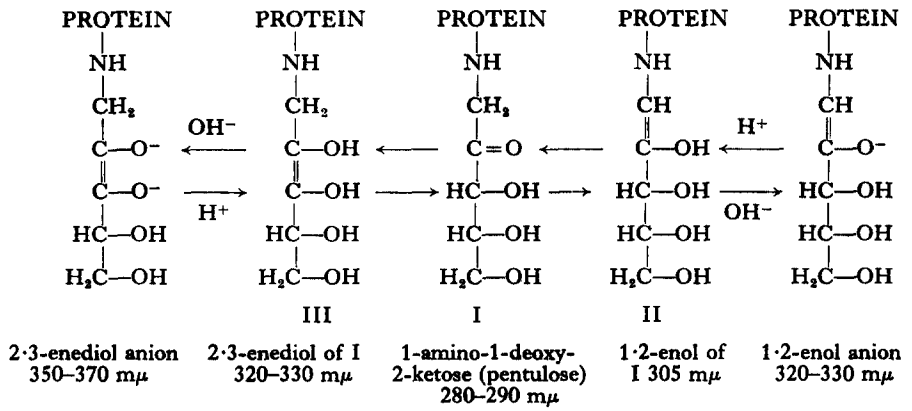
FIG. 4. Ultra-violet absorption spectra of two atopic allergens before and after oxidation; all solutions 0·05% allergen in 0·01 N NaOH; A: before oxidation; B: after oxidation (a) purified ipecac allergen (b) purified human dandruff allergen. Percentage decrease (difference A–B expressed as % of A) has also been indicated.

In a preliminary experiment the oxidized human dandruff allergen was finally screened for skin-reactivity in a number of atopic subjects giving positive intracutaneous reactions to 0·2 μg of the untreated purified allergen. The oxidized compound proved about 10 times less active, but since structures other than the aminodeoxyketoses may have been involved, some caution should as yet be exercised before the exclusive implication of the N-glycosidic structural unit as a biologically active determinant.

4. DISCUSSION

4.1 *Explanation of colour deepening*

The incorporation of N-substituted 1-amino-1-deoxy-2-ketoses within the molecular structure of atopic allergens now seems to be sufficiently established to warrant the suggestion that these particular N-glycosides are responsible for changes over 300 mμ in the allergen spectra as a function of pH. The hydroxyl at C-2 of these Amadori products in the 1·2-enol form (II) is slightly acidic; the formation of anions of acidic hydroxyls is known to induce a bathochromic shift of the absorption peak (compare ascorbic acid: 240 mμ at pH 2 to 265 mμ at pH 12, Ref. 12). Hence, the loss of a proton from the C-2 hydroxyl of II already accounts in part for the observed deepening of colour:



The 2-ketose I may enolize in two ways, viz. by the loss of a proton from either C-1 or C-3; in the latter case the 2·3-enediol is formed. According to Anet,[13] Amadori compounds derived from strong bases (in this case the primary ϵ-amino group of lysine residues) undergo 2·3-enolization under alkaline conditions. The specific tautomer III may be responsible for the weak absorption band at about 325 mμ often observed in the spectra of atopic allergens and model compounds;[1,2] this assumption is sustained by the maximum at 320–330 mμ in the u.v.-spectra of enolized 3-deoxy-glycosuloses.[14] Thus, while alkali would promote 2·3-enolization accompanied by increased absorption at 320–330 mμ, the dissociation of the

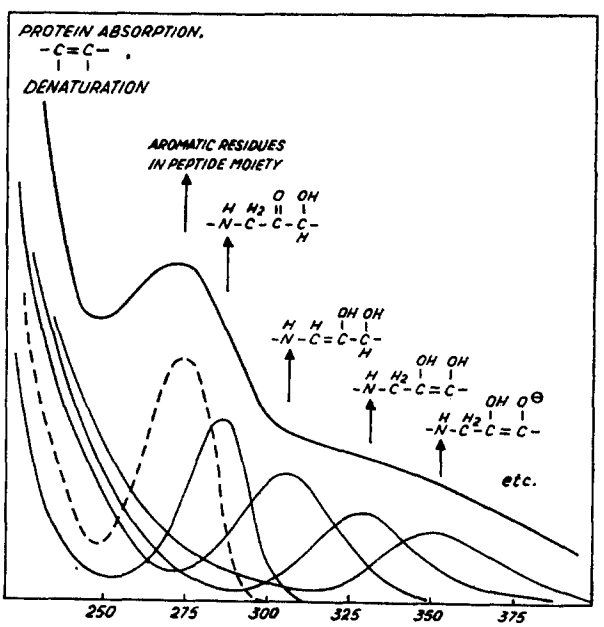


FIG. 5. Schematic representation of structural units in atopic allergens together with the approximate position of their absorption maxima contributing to the overall allergen spectrum. The dotted curve represents the normal contribution to absorption by the peptide moiety of the allergen.

resulting tautomer III would, moreover, shift the absorption peak still further to about 350–370 mμ.

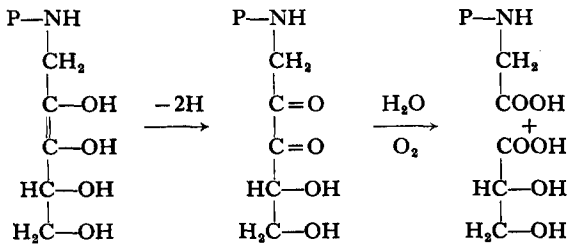
For a better insight into this complex and dynamic system, the approximate position of the absorption maxima of the intermediates proposedly contributing to the allergen spectra have been drawn in idealized curves in Fig. 5.

The chemical shifts induced in this system by alterations of the pH would satisfactorily explain the observed colour change phenomenon.

4.2 *A mechanism for the inactivation of atopic allergens*

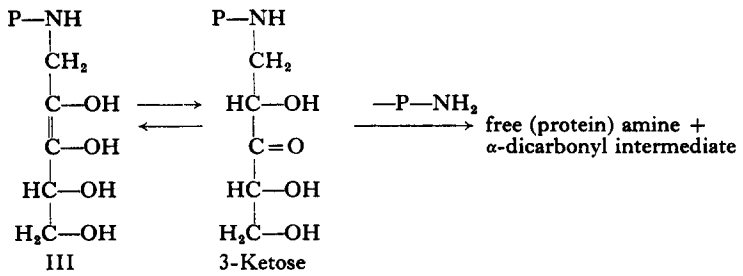
The existence of N^{ϵ}-[1-deoxyglycosulosyl-(1)]-lysine residues and the enolic tautomers thereof in atopic allergens may also serve to explain some curious observations in the clinical literature on the instability of these antigens in dilute aqueous solution. Hjorth[15,16] and Derrick and Trinca[17] have drawn attention to the so-called 'volume effect': the activity of grass pollen extracts used for diagnosis and hyposensitization therapy depends upon the degree of filling of the vials; the activity of extracts in partially filled vials is consistently lower than the activity of identical solutions in completely filled bottles. This difference, moreover, becomes more pronounced with decreasing concentration, i.e. deterioration of the antigen occurs at a faster rate in more dilute solutions. Stull *et al.*[18] reported the loss of neutralizing capacity of a 1 year old pollen extract to be disproportionate to the decrease of protein content. Hjorth attributed the volume effect to adsorption of the antigen onto the glass wall and substantiated this conclusion by counteracting the effect with the surface active detergent Tween 80. Similar results have led Malley *et al.*[19] to recommend the use of silicone coated glassware.

The inactivation of pollen allergens seems to be especially pronounced in dilute extracts made up in the alkaline *Coca* solution often used in allergy practice. A plausible explanation for this phenomenon and for the 'volume effect' may be found in the particular properties of the aminodeoxyketose structures incorporated in the allergen molecules. As discussed above, alkaline conditions promote 2·3-enolization of the aminodeoxyketose I and the resulting tautomer III is liable to undergo further degradation reactions; decomposition of III may follow various pathways among which, by virtue of the reducing power of III, oxidation by molecular oxygen:



Oxidation of this type of diols is catalyzed by traces of heavy metal ions (compare ascorbic acid). The 'volume effect' would therefore find an explanation in the ratio of molecular oxygen dissolved in the solvent to concentration of the allergen, decomposition being fastest in dilute solutions. The adsorption to surfaces (a general phenomenon, which also occurs non-specifically to celluloses and dextrans

during column chromatography of atopic allergens) might possibly assist in providing the required activation energy, while the alkalinity of the glass wall, promoting 2·3-enolization, favours the release of the entire sugar residue by β-elimination at C-1, i.e. by 'amine elimination'.[11,13]



The allergen is consequently deprived of important stabilizing sugar residues and is left as an essentially denatured (glyco) protein.

In acid media the pathway of (slow) degradation largely involves 1·2-enolization with elimination of the functional group at C-3.

Acknowledgements—The author wishes to thank Dr E. Bleumink for preparing the sample of (deoxyribulose)-β-lactoglobulin and for stimulating discussions. The kind gifts of dialyzed horse dandruff extract by Dr D. R. Stanworth, and of purified trichophytin by Dr J. H. Morris are gratefully acknowledged. Thanks are also due to Miss H. de Lange for able technical assistance.

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