

## Photo-inactivated allergens

### I. Preparation, physicochemical and biological properties

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#### Summary

A method is described for the preparation of inactive atopic allergens by irradiation with ultraviolet light. From an analysis of the absorption and fluorescence emission spectra, and with the aid of synthetic model compounds, it is concluded that the allergenic determinant of lysine-sugar conjugation is extremely sensitive to ultraviolet light. Considerable reduction of allergenicity *in vitro* (diminished capacity to inactivate human complement in fluid phase) and *in vivo* (impressive reduction of skin-reactivity in atopic patients and, by passive transfer, in monkeys) was demonstrated. Only slight modification of the antigenic characteristics was detected.

#### Introduction

It has been proposed that two mechanisms occur in the pathogenesis of atopy, an underlying supersensitivity for activation of mediator systems, and, possibly superimposed, a specific IgE mediated hypersensitivity. It has been postulated that the supersusceptibility may be manifested as overt atopy by the lysine-sugar conjugate common to most atopic allergens; this particular site on allergen molecules has been termed the 'allergenic determinant'. Evidence has been presented that the mediator system primed by the allergenic determinant in solution *in vitro* involves human complement. The IgE reaginic antibody might react with other parts of the molecule, termed the 'antigenic determinants' (Berrens, 1971a, b, 1973; Berrens & Van Rijswijk-Verbeek, 1973).

On the premise that immunotherapy with allergens aims at the induction of high levels of 'blocking' (IgG) antibody by repeated parenteral injections, we considered that this effect might be achieved more readily by eliminating the allergenic determinants from the product used for immunization. This would permit the administration of safe doses of allergens for IgG induction against the antigenic determinants, while at the same time minimizing undesired side-reactions caused by the activation of an effector system by the allergenic determinants.

The lysine-sugar structure has been shown to be sensitive to mild oxidation. A

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decline of allergenicity (by skin tests) has been described for tomato and human dandruff allergens treated with potassium ferric cyanide (Berrens, 1967a; Bleumink, Berrens & Young, 1967); Rockwell (1933) already propagated hydrogen peroxide for inactivating ragweed pollen allergens. In model experiments, diminishing detail was observed in the characteristic absorption and fluorescence spectra of various allergens treated with  $K_3Fe(CN)_6$ ,  $H_2O_2$  or  $O_3$  (Berrens, 1967a, b). Since the powerful oxidizing potential of these agents may cause extensive destruction of those antigenic determinants desired to remain intact for immunotherapy, irradiation with ultraviolet light was preferred as the method of choice for controlled oxidation. This technique had already been applied successfully by Bruchhausen, Geissler & Haschem (1969) in a trial experiment on aqueous crude house dust allergen.

The present reports describe the preparation of photo-inactivated allergens, their characterization and *in vitro* and *in vivo* biological properties.

### Materials and methods

Purified or partially purified allergens included house dust (fractions C and E), human dandruff (fraction E), kapok (fraction E), feather (fraction B), *Lolium perenne* grass pollen (fraction C), ipecac (fraction D), hay (fraction C) and *Alternaria tenuis* (culture filtrate) allergens. Their isolation and codes were in conformity to published isolation schedules (Berrens, 1971a). Synthetic protein-sugar model compounds of increasing lysine-sugar content were prepared and analyzed as described (Berrens, 1971a). An 'allergoid' was prepared from highly purified house dust allergen E by the method of Marsh, Lichtenstein & Campbell (1970), by reacting the allergen in sterile solution with L-lysine and formaldehyde for 32 days at 32°C. The 'house dust allergoid' was recovered by dialysis and lyophilization. UV-absorption spectra were recorded on a Hilger-Watts 'Ultrascan' double-beam spectrophotometer; fluorescence emission spectra were observed in a Zeiss ZFM4 fluorimeter (excitation: 365 nm).

Antiserum to purified house dust allergen was raised in rabbits in a 6-week immunization course, using mixtures of 1.0 mg of allergen in 1 ml of Freund's Complete Adjuvant (Difco). Immunodiffusion and immunoelectrophoresis, intradermal and prick testing in humans and monkeys were performed according to established techniques.

The influence of active and inactivated allergens on human complement *in vitro* was assessed by incubating quantities up to 2 µg with 15 µl of serum from a dust-sensitive individual for 30 min at 37°C as described elsewhere (Berrens, 1973); total incubation volume was 0.3 ml of  $Ca^{++}$ - $Mg^{++}$ -containing veronal-saline-buffer ( $VSB^{++}$ ). Reagents for immune haemolysis were made up as prescribed by Kabat & Mayer (1964). After the incubation period, residual human complement was determined by adding optimally sensitized sheep red cells ( $2.5 \times 10^8$  cells EA); haemolysis was read at 541 nm after 60 min at 37°C and removal of the cells by centrifugation. Complement consumption was expressed as decrease of site-forming units per cell (Z-values) according to Rapp & Borsos (1970).

### Results

#### *Preparation of inactivated allergens*

Aqueous solutions of several allergens, even at high dilution, exhibit a brown colour, which hinders calculations of the quantum yield of fluorescent radiation, due to self-absorption. In the present experiments it was therefore decided not to define the exact

energies involved, but instead to irradiate allergen solutions under controlled external conditions. In practice, 0.05% (w/v) solutions in phosphate buffer, 0.01 M, pH 7.0, were irradiated at room temperature at a fixed distance of 10 cm from the centre of the Hg-pressure lamp of the Zeiss ZFM4 fluorimeter. The (unfiltered) emitted UV-light irradiated the whole surface area of a 1 cm quartz cuvette containing 4 ml of allergen solution. Optimal conditions as regards duration of radiation exposure had to be established experimentally for each individual allergen. Initially, this was achieved by observing the drop of fluorescence emission at 445–460 nm of aliquots excited with 365 nm primary energy (Berrens, 1967b). This method was later replaced by measuring the decline of complement-consuming power (see below).

Ultraviolet irradiation of proteins is known to have profound influence on the tertiary and secondary structure, due to loss of three dimensional features, unfolding, and disruption of intramolecular bonds (McLaren & Shugar, 1964). In aqueous solution, this is manifested by increased turbidity, augmented light scattering and lowered stability. The absorption spectra become less prominent and, eventually, the sensitive amino acid residues histidine, tryptophan, tyrosine and cysteine are destroyed.

Atopic allergens, several of which must be regarded as intrinsically denatured glycoproteins (Berrens, 1971a), display an even more sensitive determinant, which, in the conditions of assay, was destroyed even before modification of amino acid residues became noticeable. The absorption spectra of some of the deepbrown allergens (kapok, feathers) were decreased over the entire wavelength range very shortly after starting UV-irradiation; visually, this effect resulted in the gradual disappearance of the brown colour. The spectra of some of the less degraded, more proteinaceous allergens (grass pollen, ipecac), by contrast, increased in absorption due to progressive denaturation and increased light scattering. Fig. 1(a) shows an example of an ovalbumin-glucose model conjugate, containing thirteen sugar-blocked lysine side-chains per mole, and Fig. 1(b) shows some spectra of irradiated purified house dust allergen.

As a more suitable, though not ideal, parameter for observing UV-induced molecular alterations, fluorescence emission of allergen solutions was initially observed, despite the errors inherent to self-absorption (Berrens, 1967b). As shown by the examples of Figs. 2(a) and 2(b), fluorescence emission sharply declines during the first 20 min of irradiation, followed by a gradual increase of emitted fluorescent radiation of longer wavelength. With some allergens of low degree of lysine–sugar substitution, this sequence could barely be observed, because of a low initial 440 nm fluorescence output and a rapid onset of molecular modification generating the 465 nm fluorescent band. Some of the heavily degraded and poorly skin-reactive allergens (kapok, hay, feathers)—which feature a 470–490 nm wave even without irradiation (Berrens, 1967b)—showed a gradual rise of fluorescence intensity with a shift of the maximum to 465 nm. Fluorescence modulation as a function of time for some of the common allergens has been plotted in Fig. 3.

For the production of inactivated allergens for clinical trials, 0.05% allergen solutions were as a rule irradiated for 20–30 min, on occasion for 60 min; whenever feasible, the initial drop of 445 nm fluorescence emission was taken as a guide for optimal allergen inactivation with the least possible damage to the antigenic structure of the carrier molecules.

#### *Antigenicity*

To examine the effect of UV-radiation on antigenicity, i.e. on the capacity of allergens

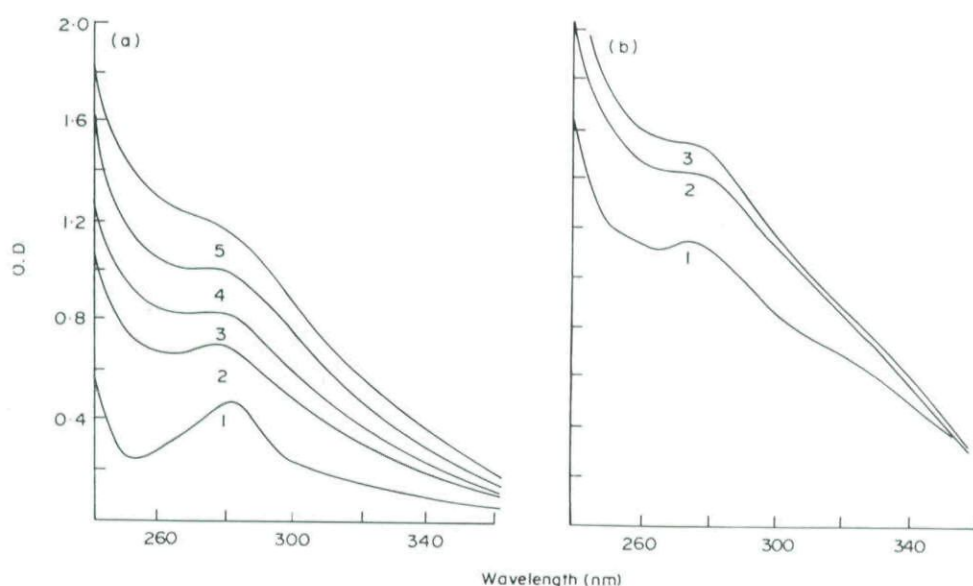


**Fig. 1(a)** Ultraviolet absorption spectra of 0.05% solution (0.01 M phosphate buffer, pH 7.0) of ovalbumin-glucose conjugate, containing thirteen sugar-blocked lysine residues per mole, before and after UV-irradiation for various periods of time. (1) Before irradiation; (2) after 30 min of irradiation; (3) after 60 min; (4) 120 min; (5) 240 min. **(b)** UV-absorption spectra of purified house dust allergen, fraction E, 0.05% in neutral phosphate buffer after: (1) 0 min; (2) 30 min, and (3) 60 min of irradiation with unfiltered (Hg) UV-energy.

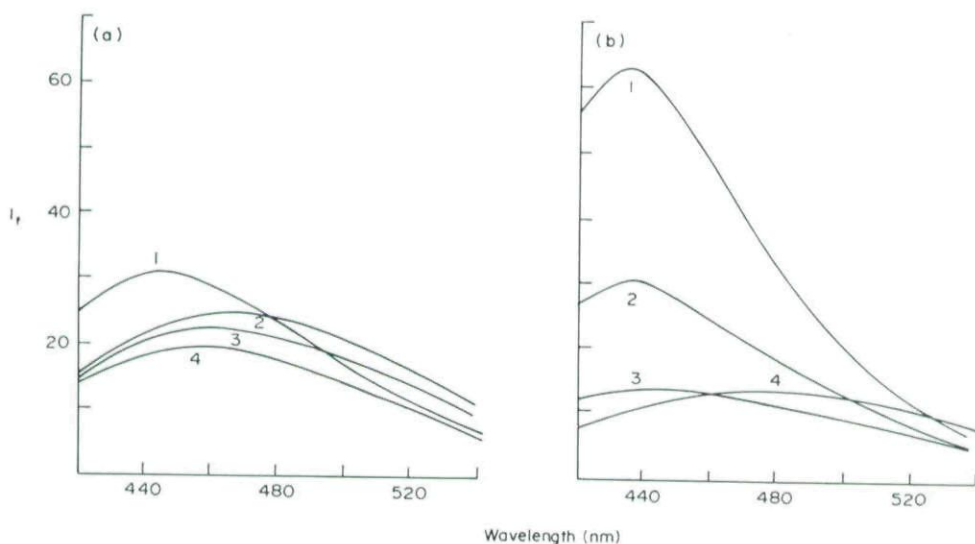


**Fig. 2(a).** Fluorescence emission spectra ( $\lambda_{prim.} = 365$  nm Hg) of 0.05% solutions of model compound  $\beta$ -lactoglobulin-lactose, containing twelve sugar-blocked lysines per mole, after (1) 0 min of UV-irradiation (Hg, unfiltered); (2) 20 min; (3) 60 min; (4) 120 min. Fluorescence intensity in arbitrary units. **(b)** Fluorescence emission spectra of purified human dandruff allergen, fraction E, 0.05% in neutral buffer, after irradiation with UV-energy for (1) 0 min; (2) 20 min; (3) 40 min; (4) 240 min.

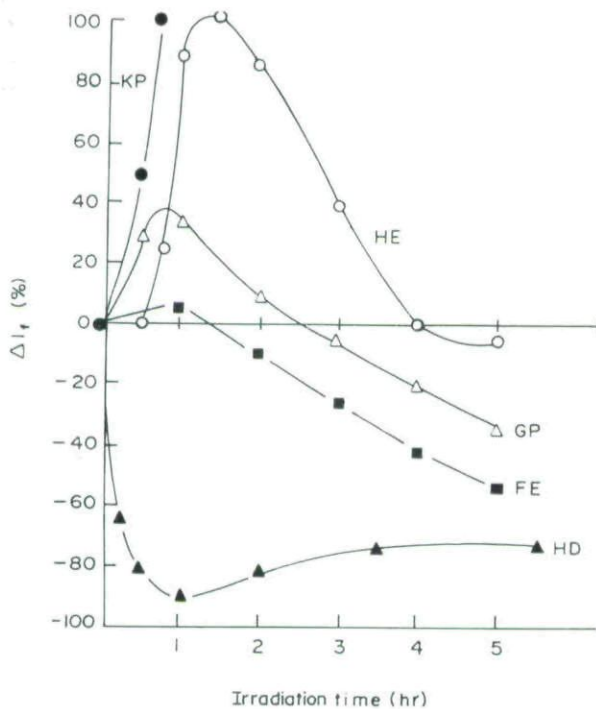


**Fig. 3.** Variation of fluorescence output, measured at 460 nm ( $\lambda_{prim.} = 365$  nm), of 0.05% allergen solutions in 0.01 M phosphate buffer, pH 7.0, with time of irradiation with unfiltered UV-light (Hg). KP = kapok KPE; HE = house dust fraction E; GP = rye grass pollen, fraction C; FE = feathers, fraction B; HD = human dandruff allergen, fraction E.

to react with precipitating antibody raised in laboratory animals, rabbits were immunized with a potent, partially purified house dust preparation, fraction C (Berrens, 1971a). Although the homologous photo-inactivated product appeared to have lost some of its precipitating potential, the immunoelectrophoretogram of Fig. 4 demonstrates that, despite the one hundred times lowered allergenic potential (Fig. 6), the loss of antigenic characteristics was not extensive. To date, no experiments have been performed to check on the immunogenicity of UV-inactivated allergens, i.e. on their capacity to induce antibody capable of reacting with the active parent compound.

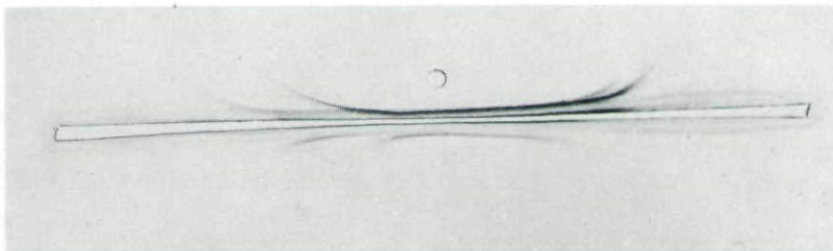


**Fig. 4.** Immunoelectrophoresis of partially purified active house dust allergen, fraction C, 3% solution (top well) and the same product inactivated by 30 min UV-irradiation (bottom well) against rabbit antiserum to the active preparation. Anode to the right. Stain: Coomassie blue.

*Allergenicity (in vitro)*

The recently described complement-binding properties of active allergens have been shown to be related to the estimated mean number of reactive (i.e. reducing) lysine-sugar determinants per mole of allergen. A similar relationship has been found to exist with skin reactivity as a parameter of biological activity (Berrens, 1971a, b, 1973; Berrens & Van Rijswijk-Verbeek, 1973). Because the irradiation experiments pointed towards marked sensitivity of these lysine-sugar sites to ultraviolet light, the interaction of photo-inactivated allergens with human complement was examined.

Whereas most of the common allergens—with the exception of ovomucoid and grass pollen allergens—readily inactivate human C in fluid phase, photo-inactivated house dust, dander, kapok, feather and other allergens lose this property. In a typical

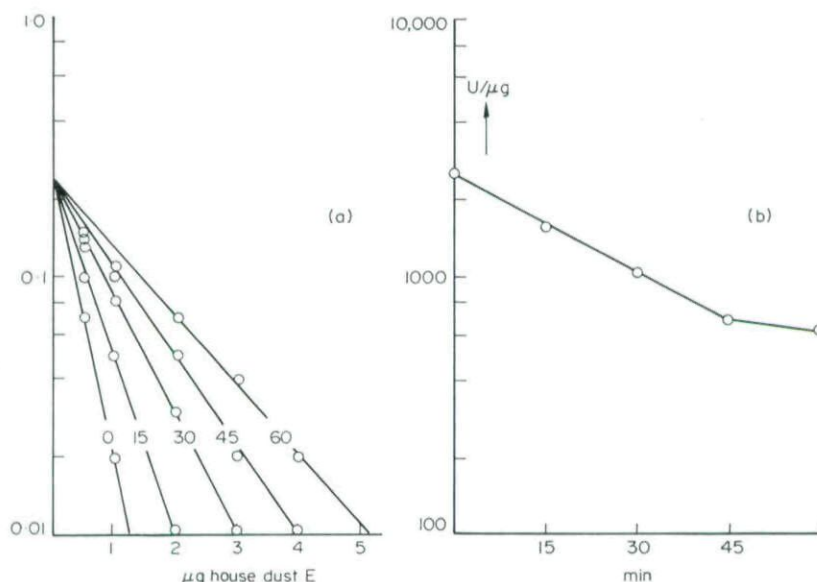


Fig. 5(a). Inactivation of human serum complement (15  $\mu$ l 1:20) by purified house dust allergen, fraction E, irradiated for periods up to 60 min. A single serum from an atopic individual was used in all the tests. Conditions: see text. (b) Plot of the slopes ( $K$ ) of the dose-response curves of Fig. 5(a) against time of irradiation. Slopes  $K$  were multiplied by 1000 and arbitrarily plotted as 1000  $K$ , or 'units/ $\mu$ g allergen'.

experiment, highly purified house dust allergen, fraction E, was irradiated with unfiltered UV-radiation from a mercury lamp; aliquots were withdrawn from the 0.05% solution every 15 min and were incubated in a dilution series with 15  $\mu$ l of allergic serum diluted 1:20 in VSB<sup>++</sup> buffer (30 min, 37°C). Residual complement was then determined by immune haemolysis, using haemolysin-sensitized sheep red cells (EA). The results of this experiment, shown graphically in Fig. 5(a), clearly demonstrate that the ability to inactivate human C progressively decreases with time of irradiation. Interestingly, the slope ( $K$ ) of  $Z (= -\ln(1-y))$  versus allergen concentration plots, expressed as units/ $\mu$ g allergen and plotted logarithmically in Fig. 5(b), decreases linearly with time up to 45 min of irradiation. This indicates that the photochemical process in the first 45 min, and with haemolytic complement as the detection system, affects one particular chemical grouping which is quantitatively being eliminated from the allergen molecules. This is compatible with the idea that the lysine-sugar determinants (in

reducing, enolic form) are rapidly being inactivated, presumably by photochemical oxidation.

By contrast, it was found that an 'allergoid' (Marsh *et al.*, 1970) prepared from the dust fraction E with lysine and formaldehyde, had fully retained the complement-binding property of the parent product.

#### *Allergenicity (in vivo)*

To test the effect of UV-irradiation of allergens on the *in vivo* allergenicity, pilot studies and clinical trials were performed on two representative pairs of treated and untreated products, viz house dust and grass pollen. In the first series, a group of 102 atopic patients with asthma, rhinitis and/or eczema was injected intradermally with a sterile solution of partially purified house dust allergen (HEC, 2 µg/ml) and its derivative HEC/UV obtained after 30 min of UV-irradiation. The results, collected graphically in Fig. 6, demonstrate that, whereas 70% of this group reacted to the untreated

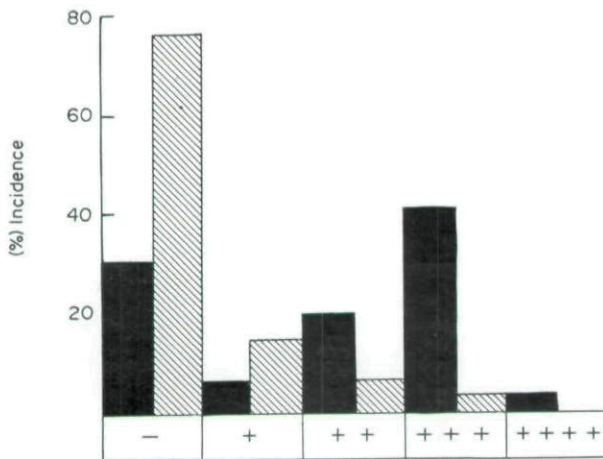


Fig. 6. Combined results of intradermal tests with house dust fraction C (HEC, black columns) and its UV-irradiated derivative (HEC/UV, hatched columns) in a group of 102 dust-allergic patients; the + signs refer to a visual appreciation of the intensity and area of the wheal and erythema reaction.

material with wheal and erythema of 1+ or higher intensity, only a minority of patients continued to respond to an equal dosage of the irradiated product. A conservative estimate indicates that the skin-reactive potential decreased by a factor of one hundred or over. Nevertheless, some individuals continued to react to the photo-inactivated product, and this led us to increase the radiation period to 60 min in later experiments; also, it gradually became clear to us that, in such patients, a different mechanism mediating the allergic response must be involved.

As shown in Table 1, clinical trials of irradiated grass pollen extracts produced less unequivocal results. Although prick tests with a partially purified extract of rye grass pollen (including Group I and II allergens) and its irradiated derivative indicated some loss of skin-reactivity, intradermal tests did not confirm appreciable suppression of the skin test response. These results, obtained at the Pasteur Institute, were confirmed independently in a different group of patients examined in Liège (Table 2).

In a preliminary experiment, conducted in Liège, the activity of native and irradiated rye grass pollen extract by *in vitro* neutralization and passive transfer to monkey

**Table 1.** Intradermal and prick tests with active *Lolium perenne* grass pollen extract (GPA) and grass pollen extract treated by ultraviolet radiation (GPA/UV) in a group of pollen allergic patients

| Preparation     |        | Prick (100 µg/ml)<br>(fifteen patients) |         |           | Intradermal (10 µg/ml)<br>(sixteen patients) |         |           |
|-----------------|--------|-----------------------------------------|---------|-----------|----------------------------------------------|---------|-----------|
| No. of patients | GPA    | 0*                                      | + / 2 + | 3 + / 4 + | 0                                            | + / 2 + | 3 + / 4 + |
|                 | GPA/UV | 2                                       | 7       | 6         | 2                                            | 3       | 13        |
|                 |        | 12                                      | 3       |           |                                              | 9       | 5         |

\* Score of skin reaction.

**Table 2.** Intradermal and prick tests in a group of pollen-allergic patients (at Liège) with active rye grass pollen preparation GPA and with the same product irradiated with ultraviolet light (GPA/UV). Numbers indicate mean diameter in mm

| Patient | Prick (5 µg/ml) |          |        |          | Intradermal (1 µg/ml) |          |        |          |
|---------|-----------------|----------|--------|----------|-----------------------|----------|--------|----------|
|         | GPA             |          | GPA/UV |          | GPA                   |          | GPA/UV |          |
|         | Oedema          | Erythema | Oedema | Erythema | Oedema                | Erythema | Oedema | Erythema |
| S.A.    | 6               | 10       | 0      | 0        | 8                     | 37       | 0      | 3        |
| C.F.    | 10              | 40       | 0      | 0        | 11                    | 40       | 3      | 10       |
| B.G.    | 6               | 15       | 0      | 0        | 17                    | 40       | 11     | 20       |
| V.L.    | 6               | 20       | 0      | 0        | 16                    | 40       | 10     | 30       |
| J.J.    | 8               | 35       | 4      | 18       | 17                    | 50       | 17     | 40       |
| O.V.    | 4               | 25       | 0      | 0        | 10                    | 40       | 0      | 20       |
| L.J.    | 10              | 32       | 0      | 0        | 22                    | 40       | 10     | 30       |

**Table 3.** *In vitro* neutralization of reagins in the serum of a grass pollen-allergic patient (PK-titre in man 1000, in monkey 50). Serum mixed in a ratio of 1:2 and 1:10 with rye grass pollen extract GPA or with its UV-irradiated derivative GPA/UV (0.5 mg/ml), incubated overnight at 4°C and centrifuged. Passive transfer of 0.1 ml supernatants to monkey skin. Challenge after 24 hr by i.v. injection of 3 ml pollen 'Pasteur' (0.1%) and 3 ml Evans blue (0.5%). Experiments in duplicate. Results in mean diameter (mm) of blueing area

| Serum/allergen ratio |   | GPA |    | GPA/UV |    |
|----------------------|---|-----|----|--------|----|
| 1:2                  | 0 | 0   | 40 |        | 40 |
| 1:10                 | 0 | 0   | 30 |        | 30 |

skin was evaluated. The results of this experiment, shown in Table 3, demonstrate that the irradiated preparation had apparently been deprived considerably of its *in vitro* capacity to react with human reagins.

### Discussion

Our results demonstrate that several of the common allergens are readily inactivated by ultraviolet radiation. Analysis of the absorption and fluorescence spectra shows that the 'allergenic' determinants are highly susceptible and can be eliminated even before radiation-induced modification of essential amino acid residues becomes detectable. However, it is virtually impossible to achieve completely inactive allergens without causing some damage to the 'antigenic' determinants of the carrier molecules. This conclusion is based on the reaction of the photo-inactivated products with precipitating antisera to the parent allergens, and on the appearance of additional fluorescent derivatives masking the decline of lysine-sugar derived fluorescence.

Our analysis of the optical data leads to the conclusion that the most radiation-sensitive structural determinant is the site of lysine-sugar linkage, which is rapidly lost as a result of photochemical oxidation. The decline of 445 nm fluorescence may be due to loss of lysine-sugar determinants in 1,2-enol form (Berrens, 1967b); it is possible that the decline of the 470 nm peak in the spectrum of the more degraded allergens is attributable to loss of the same determinant in the (more stable) 2,3-enediol form. The linearity of the plot of photochemical *K*-values with time and the abrupt shift after 45 min (Fig. 5b) also points towards two structures of different UV-sensitivity. It has been pointed out that the 2,3-enediol tautomer may become stabilized in the form of a pseudo-isoxazoline structure (Berrens, 1971a). The above conclusions may then be linked up with the findings of Lambein *et al.* (1969), who reported UV-sensitivity of 2-alanyl-3-isoxazolin-5-one and derivatives.

The appearance of a new fluorescent maximum at 450 nm on prolonged irradiation may be explained by assuming that tryptophan and histidine residues are being modified; photoconversion of tryptophan into kynurenin and formylkynurenin, which exhibit a fluorescent peak at 450 nm, has been described (Savige, 1971). Since this involves destruction of side-chains probably important as antigenic determinants, photo-inactivation of allergens should be conducted under conditions minimizing this effect. Though, initially, we attempted to do so by discontinuing the irradiation process at the point of minimum fluorescence, the complement-inactivation test developed later probably should be recommended for better control in future experiments.

In contrast to the experience of Bruchhausen *et al.* (1969), it proved possible to photo-inactivate house dust allergen as well as kapok, feather, human dandruff allergen and several others. However, UV-irradiation of the grass pollen allergens only caused a partial reduction of allergenicity. It should be mentioned that, in the group of house dust sensitive patients, individuals were occasionally encountered who showed skin reactions of undiminished intensity (and could not be hyposensitized) to inactivated dust allergen. Taken together with the observation that powerfully skin-active grass pollen allergens exhibit only weak potency in *in vitro* complement consumption (Berrens & Van Rijswijk-Verbeek, 1973), this phenomenon again points at two different mechanisms capable of initiating the allergic response. In this line of thinking, the first pathway, at least *in vitro*, is complement-dependent and is being primed non-specifically by (UV-sensitive) common determinants of lysine-sugar conjugation acting on the first component of human complement (most likely by mediation of an as yet

unidentified serum protein). If extrapolation to the *in vivo* situation is in order, such a mechanism might be prevalent in cases of immediate type I house dust atopy. The second pathway is mediated by specific (IgE) antibody directed against characteristic antigenic determinants of the allergen molecule, and probably is complement-independent (Grant & Liechtenstein, 1973). This mechanism may prevail in grass pollen allergy, at least in pollen rhinitis, though it is conceivable that a combination of both mechanisms may occur in individual patients. If one considers employing photo-inactivated allergens for immunotherapy, optimal results may on this basis be expected with house dust allergen derivatives, not necessarily with those of grass pollen.

Although the photo-inactivation process may cause some damage to the antigenic structure, the disadvantage should not be overrated. The majority of the allergens of the house dust, kapok, feather group have already undergone uncontrollable modification and denaturation in the original substrates prior to their extraction for clinical use (Berrens, 1971a). The alternative of 'allergoids', prepared by Marsh *et al.* (1970) by treatment of pollen allergens with formaldehyde and lysine in a Mannich reaction, may become complicated by chemical side-reactions and crosslinking, thereby creating novel antigenic determinants. In our hands, a 'house dust allergoid' had unimpaired capacity to bind human complement *in vitro*.

As pointed out before (Berrens, Young & Zuidema, 1971), the UV-sensitivity of inhalant allergens may provide an explanation for the lowered level of allergenically active material in mountainous areas, and suggests a means of passively reducing the allergenic potential within indoor dwellings. The alternative of active treatment by immunotherapy with UV-inactivated house dust allergen is discussed in the next paper.

### Acknowledgments

Dr L. Vacková (guest worker from Prague, Czechoslovakia) and Dr E. Young (Clinical Allergy Department, Utrecht) collaborated in the early phases of this study. Mr P. van Liempt and Mr C. L. H. Guikers assisted in the complement assays.

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