

Au antigen, which is a product of an infectious agent. If so, this seems to represent the first recognition in man of a systemic vasculitis mediated by an immunological reaction to a virus or virus-like particle. We do not intend to imply that all cases of polyarteritis are due to this particular antigen. However, the possibility that similar disorders may be caused by other agents operating by a similar mechanism is intriguing.

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ENZYME SENSITISATION IN CONSUMERS OF ENZYME-CONTAINING WASHING POWDER

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Summary Adverse reactions in the eyes and/or airways in connection with the use of enzyme-containing washing powder are described in three female consumers. Prick tests in the patients resulted in immediate skin reactions to test enzyme solutions in concentrations which do not give non-specific weal reactions. Circulating IgE antibodies to the enzyme were also demonstrated. Information about the sensitising allergen in the enzyme preparation was obtained by skin testing with fractions of the gel-filtered and electrophoretically separated enzyme preparation ('Alcalase'). The antigenicity of the enzyme preparation was tested by parenteral immunisation of a rabbit. The results of these analyses indicated that the allergen was immunogenic in the rabbit and, furthermore, that it was identical with the main enzymatically active fractions obtained by gel filtration and electrophoresis. The observations indicate that the danger of sensitisation is not restricted to employees manufacturing enzyme-containing washing powder, but may arise in every situation where these enzymes may be present as dust particles.

Introduction

LABORATORY workers repeatedly exposed to trypsin and chymotrypsin powder can become sensitised to the enzyme particles.¹⁻³ Rhinitis and conjunctivitis suggest a type-I atopic reaction in these cases.

The immunogenic potency of inhaled enzyme powders, however, has not been widely apparent until washing powders containing proteolytic enzyme obtained by submerged cultivation of *Bacillus subtilis* were marketed. A rather high frequency of respiratory disturbances has been noted in workers producing these washing powders.^{4,5} The symptoms and the results of skin and provocation tests indicated involvement of immunological mechanisms. The reactions may be atopic (type I), or possibly type-III reactions may be involved as in some other occupational hypersensitivity reactions of the lung such as farmer's lung.⁶

The *Lancet* suggested that the appearance of allergic lung disease in workers heavily exposed to enzyme powders could have a bearing on the populations using such products.⁷ We have found evidence to support this suggestion in three domestic users, who all had respiratory complaints after they had used a certain enzyme-containing washing powder for some time. Biochemical separations and immunological analyses were made in order to identify the allergen principle in the crude enzyme preparation.

Materials and Methods

'Alcalase' dry powder (Novo Industri A/S, Copenhagen, Denmark) was kindly supplied by the manufacturer. Alcalase solution was prepared with isotonic saline solution buffered with 0.01 M phosphate pH 7.2 (p.B.S.) and freed from insoluble material, bacteria, and spores by centrifugation or filtration through a Millipore GS filter before prick testing and further separation. The alcalase solution was desalted by gel filtration on 'Sephadex G-25'. The excluded fraction, representing 5% of the separated material on a dry-weight basis, was lyophilised and used for immunisation and electrophoresis.

Protein analysis was performed on trichloroacetic-acid-form precipitates by a modified Folin-Ciocalteu method.⁸ Crystalline lysozyme was used as standard protein. 1 g. dry weight of alcalase contained approximately 50 mg. of protein. This finding was in good agreement with protein analysis by the ultraviolet-absorption method.⁹ We concluded that the lyophilised material was pure protein.

Crystallised subtilisin type VII and subtilopeptidase A type VIII were purchased from Sigma Chemical Company, St. Louis, Missouri, U.S.A.

Antiserum was produced in a rabbit by subcutaneous injections of 0.5 mg. of the lyophilised alcalase in 'Bacto-Adjuvant Complete', Difco 0638. 2 weeks after the initial injection, weekly booster injections were given. 1 week after the seventh injection, the animal was exsanguinated.

Prick testing was made on the forearms. Contours of weals were outlined with a ball-point pen after 15 minutes and transferred to protocols by means of transparent tape strips. The mean weal diameters were calculated by measuring the longest and shortest diameters from the traced outlines.

Double diffusion-in-gel analyses were performed according to Ouchterlony in the micromodification of Wadsworth.¹⁰

Immuno-electrophoresis and agar electrophoresis was performed according to the technique described by Wadsworth and Hanson.¹¹

Passive haemagglutination tests were performed according to Stavitsky¹² using tanned red blood-cells coated by incubation with 1 mg. desalted alcalase protein per ml. of

P.B.S. pH 6.4. The rabbit hyperimmune serum agglutinated these cells in a titre of 1/5160.

Gel filtration was performed by upward flow on a sephadex G-100 column (2.5 by 90 cm.) using P.B.S. pH 7.2 as the eluting fluid.

Assay for protease activity. Hagihara's method, modified by Matsubara et al.¹³ was used. The enzyme activity of fractions was expressed in percentage of the activity of the starting material.

Case-reports

Case A

This woman, aged 44, has no family history of allergies and she has had no allergic symptoms herself. She is a non-smoker and lives with her fiancé. For many years, but especially during the past 6, when she has been working as a controller in a metal factory, she has had recurring respiratory complaints. Thus there have been periods of coughing and expectoration each spring and autumn after common colds. In this connection she has also felt a tightness in her chest, especially on physical effort and in the mornings.

She started to use an enzyme-containing washing powder in 1967 without noticing any associated adverse reactions. In August, 1969, she changed to another enzyme-containing washing powder, said to be more effective. During the next 5 months she often got violent attacks of coughing with slight wheezing and tightness in the chest in the daytime and at night. There was a considerable mucous expectoration but no indication of infection either in the appearance of the sputum or in the body temperature. She always got worse the 2-3 times in the week when she did the laundry for herself and her fiancé in the bathroom. The following nights were especially troublesome because of the coughing and shortness of breath. There were no symptoms from the upper respiratory tract. Realising the connection between her discomfort and the laundry, she first attributed it to the physical effort, but then she changed washing powder and the respiratory symptoms subsided. Using simple detergent washing powder she has been healthy and completely free from the described symptoms now for more than half a year. She has recalled a violent attack preceding the described troublesome period once during the summer, 1969. This happened a night after she had visited some friends living in a trailer where the enzyme-containing washing powder, which she later employed, had just been used.

Examined at the allergy outpatient clinic in December, 1969, she seemed quite healthy. Spirometry normal. The erythrocyte-sedimentation rate (E.S.R.) was 6 mm. in the 1st hour (Westergren) and circulating eosinophils were 4% of a normal number of circulating white blood-cells. Intradermal skin tests with common standard allergens were all negative. Prick tests with enzyme preparations

TABLE II—RESULTS OF PRICK TESTING WITH ALCALASE PREPARATIONS AFTER ABSORPTION WITH HYPERIMMUNE RABBIT SERUM

Preparation	Mean weal diameter (mm.) in		
	A	B	C
Unabsorbed (1 mg./ml.)	7	5	5
Absorbed*:			
Supernatant	2.5	1.5	1
Precipitate in 0.1 N sodium hydroxide	5.5	5	4
0.1 N sodium hydroxide	1.5	1	2
Histamine (1%)	N.D.	6.5	7

* Equal parts of alkalase solution (1 mg./ml.) and hyperimmune rabbit serum.

were performed in May, 1970. The results are presented in detail in figs. 2 and 4, tables I and II.

Case B

A woman aged 68 years keeping house only for her husband. She had some family history of atopic disease. For several years she has had a mild hypertension, and for the past year she has had diabetes mellitus controlled by oral medication and diet. During the past few years she has had intermittent periods of purulent bronchitis and slight wheezing mainly in the mornings, when coughing and exposed to cold air. She has never had genuine asthmatic dyspnoea. She also intermittently had slight palpebral oedema without any subjective complaints until the autumn of 1968, when she noted irritation and itching in her eyes when she did the weekly laundry in her bathroom or the monthly machine washing. She had used an enzyme-containing washing powder since it was introduced in 1967, and she had found it unpleasantly dusty but very effective. When she abandoned this washing powder the eye symptoms vanished, but they returned when she tried the same washing powder once again. She has been able to use two other, less dusty, washing powders containing granular enzyme with only slight discomfort.

She attended the allergy outpatient clinic in April, 1970. No pathological findings appeared at the physical examination. Intradermal skin testing with some 40 standard allergen extracts was negative. Spirometry normal. E.S.R. was 7 mm. in 1st hour. Differential count of a normal number of white blood-cells revealed 1.5% eosinophils. Prick tests with enzyme preparations were performed in May, 1970 (figs. 2 and 4, tables I and II).

Case C

A woman, aged 35, managing a three-person household. She and her family have no history of allergies. Through the years she has had rather frequent upper-respiratory-tract infections.

She started to use an enzyme-containing washing powder in late 1967 both for regular weekly machine-washing and also for occasional hand laundry in her bathroom. During the autumn and winter she had daily attacks of sneezing and watery nasal secretion and a slight secretion from her eyes. There was no coughing or wheezing. The eye and nose symptoms presented mainly in the mornings. They were increased in immediate connection with the use of the enzyme-containing washing powder, and the morning symptoms were more pronounced on the day after washing than on subsequent days. She stopped using this washing powder in spring 1969 with immediate relief of the symptoms. For soaking only, however, she now uses a washing powder with granular enzyme without discomfort.

This woman never sought medical consultation but her reactions were known by case B, who told us about her. Asked to cooperate she has permitted us to do prick testing with enzyme preparations in May, 1970 (figs. 2 and 4,

TABLE I—RESULTS OF PRICK TESTING AND PROTEASE ASSAY WITH HEATED AND UNHEATED ALCALASE PREPARATIONS

Concn. (mg./ml.)	Min. at 100°C	Mean diameter of weal (mm.) in			Protease activity (%)
		A	B	C	
10	0	8	6	10	100
5	0	9	6	8	100
5	1	N.D.	5	N.D.	0
5	5	N.D.	5	N.D.	0
5	15	N.D.	5	N.D.	0
5	60	N.D.	4	N.D.	0
1	0	6.5	4.5	5.5	100
1	1	5.5	N.D.	5.5	0
1	5	5	N.D.	4	0
1	15	5	N.D.	4.5	0
1	60	4	N.D.	5.5	0

N.D. = Not done.

tables I and II, but no other tests have been performed. She seems to be quite healthy.

Results

Serological Findings

Circulating specific IgE antibodies, kindly analysed by Dr. L. Wide, Uppsala, were demonstrated in all three cases with a radioallergosorbent test (R.A.S.T.) using sephadex particles coupled with both crude and purified alcalase preparations.¹⁴ The three cases had serum-IgE levels determined by a radioimmunosorbent technique (R.I.S.T.) ranging between 50 and 90 ng. per ml.¹⁵

Double diffusion-in-gel analyses revealed fuzzy, indistinct precipitation lines when the sera of the three cases as well as of twenty healthy blood-donors were

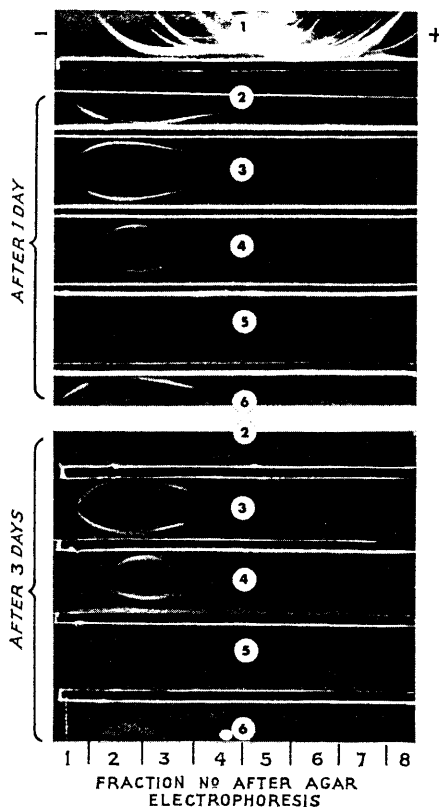


Fig. 1—Immunoelectrophoretic analysis by rabbit hyperimmune serum to alcalase of desalted protein fractions of alcalase and of alkaline peptidases.

- (1) Immunoelectrophoretic pattern of human serum.
- (2) Alcalase 20 mg./ml.
- (3) Alcalase 2 mg./ml.
- (4) Alcalase 0.2 mg./ml.
- (5) Subtilisin 15 mg./ml.
- (6) Subtilopeptidase 15 mg./ml.

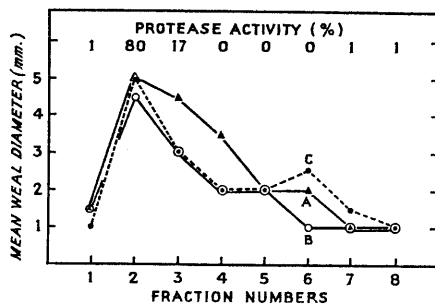


Fig. 2—Results of prick testing in cases A, B, C and protease assay of eluates after electroseparation of alcalase in agar at pH 8.2.

Fraction numbers designate eluates from cathode to anode (see fig. 1).

tested against crude alcalase solution and its desalted protein fraction. After gel filtration on sephadex G-200 of a patient's serum and concentration of the fractions to the serum volume, applied to the column, the precipitating serum factor was found in the 19 S peak. Sera from the three patients were precipitated with 50% ammonium sulphate. The redissolved precipitates did not give rise to any precipitation lines with the alcalase preparations.

Passive haemagglutination tests were negative in all three cases using desalted alcalase protein as antigen.

Diagnostic Prick Testing

The three women were prick tested with alcalase at concentrations of 10, 5, and 1 mg. dry weight per ml. Positive immediate weal reactions were elicited with mean weal diameters ranging between 6 and 10 mm. using the highest concentration and between 4 and 6 mm. using the lowest concentration. The allergenic activity did not notably decrease after heating the solution of 5 or 1 mg. dry weight per ml. to 100°C for 1–60 minutes (table I), although this treatment destroyed the enzyme activity.

Analyses of Alcalase by means of Hyperimmune Serum

Double diffusion-in-gel analyses of alcalase solution by means of a hyperimmune serum produced in a rabbit revealed one main precipitation band and 1 or 2 very faint lines. In an attempt to relate the allergenic activity in alcalase with precipitinogenic antigens absorption experiments were carried out by incubating equal parts of the alcalase solution (1 mg. per ml.) and the hyperimmune serum for 1 hour at 37°C and 4 hours at 4°C. After centrifugation the supernatant as well as the precipitate dissolved in 0.1 N sodium hydroxide were used for prick testing. The supernatant was considerably less allergenic than the dissolved precipitate (table II). In similar absorption experiments we found that the enzyme activity was notably reduced by the hyperimmune serum as compared with the activity after incubation with normal rabbit serum.

Immunoelectrophoresis in agar at pH 8.2 using three concentrations of the desalted protein fractions of alcalase and two crystalline alkaline peptidases from *B. subtilis*, subtilisin, and subtilopeptidase-A showed

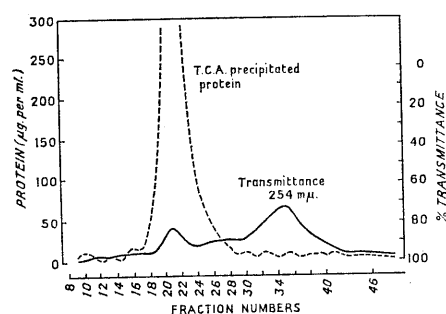


Fig. 3—Elution curves of 1 g. alcalase filtered through sephadex G-100.

(See fig. 4.)

two strong precipitation arcs close to each other, located toward the cathode, when analysed with the rabbit antiserum to alcalase (fig. 1). In the highest concentration of the protein preparation of alcalase two or three faint arcs were also seen localised closer to the starting basin and towards the anode. In contrast to these faint precipitates the two main arcs on the cathode side gradually became weaker during the second and third day of diffusion.

Allergen and Enzyme Activity in Fractions after Agar Electrophoresis

2 mg. alcalase were electrophoretically separated from a transverse well in the mid-position of an agar layer on an 8×8 cm. glass plate under conditions identical to the immunoelectrophoretic analysis described above. Eight transverse 1 cm. broad strips were then cut out in the agar as indicated in fig. 1 and each eluted in 2 ml. of saline solution for 24 hours. Subsequent prick testing and enzyme activity determination showed that both allergen and enzyme activities were localised in the same agar sections as the above cathodic precipitates (fig. 2).

Allergen and Enzyme Activity in Fractions after Gel Filtration

Gel filtration on sephadex G-100 of 1 g. of alcalase showed a single protein peak. Non-protein material was eluted after the protein (fig. 3). Based on earlier runs using this column with molecular-weight markers the elution volume of the protein indicated a molecular weight of around 20,000. Enzyme-activity determination and prick testing of the gel-filtered fractions revealed maximum activity for both these properties coinciding with the main protein peak. The results of the prick testing and enzyme determinations are given in fig. 4.

Discussion

The allergenic potency of airborne enzyme particles was clearly demonstrated by the relatively common finding, in workers producing enzyme-containing washing powders, of sensitisation.^{4,5,16,17}

We have studied three female domestic users who had adverse reactions of local, atopic type in connection with the use of enzyme-containing washing powder. Besides the rather suggestive history, assumption of an

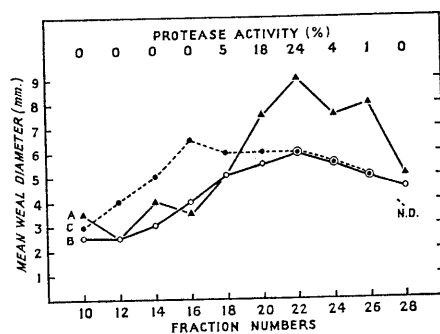


Fig. 4—Results of prick testing in cases A, B, and C and protease assay of gel-filtered alcalase (sephadex G-100).

Same fractions as designated in fig. 3.

immunological type-I mechanism behind these reactions was supported by positive prick-test results with concentrations of the crude enzyme solutions far below the level at which non-specific reaction may be elicited.⁴ Support for a type-I hypersensitivity reaction was also obtained by the demonstration of circulating enzyme-specific IgE antibodies by means of the R.A.S.T. This method has been reported as a sensitive in-vitro method to detect reaginic antibodies to allergens responsible for atopic reactions.¹⁴ In the epidemiological study reported by Newhouse et al.⁵ no circulating IgE antibodies against the enzyme could be detected by the red-blood-cell-linked antigen-antiglobulin test.¹⁸ On the other hand, these authors did find circulating IgG antibodies by this technique. In the first report on enzyme sensitisation in workers, precipitating antibodies were found both in sensitised persons and in controls,⁴ but in later investigations^{14,17} such antibodies could not be demonstrated. In the sera of our cases as well as in control sera presumably non-specifically precipitating components strongly interfered in the immunodiffusion analyses. These components are probably α_2 -M globulins.¹⁹ We did not find any precipitins in the γ -globulin fraction obtained by ammonium-sulphate precipitation against the actual enzyme preparation. We also failed to demonstrate any circulating antibodies by passive haemagglutination tests using cells coated with alcalase protein; nor did Wüthrich and Ott.¹⁶ Thus we found no serological indication that the hypersensitivity reaction in our cases might be based on local type-III Arthus reactions.

After gel filtration on sephadex G-100 of crude enzyme solution prick-test reactions were elicited by fractions containing as little as 1 µg. protein per ml., which is similar to the lower limit of the biological allergen activity of purified rye grass²⁰ and birch-pollen allergen.²¹ The allergenic activity coincided with an enzyme-active protein peak and had an elution volume which indicated a molecular-weight class of 20,000. This figure accords well with the findings of Keay et al.²² and is also well within the molecular-weight ranges in which many allergens have been found.^{20,21,23} By more direct methods the molecular weight of pure *B. subtilis* protease has been estimated to be 27,000–30,000.

This protein was immunogenic in the rabbit, and immunoelectrophoretic analysis in agar by means of the rabbit hyperimmune serum revealed two main precipitinogens localised on the cathodic side of the start basin and two or three more rapidly moving antigens forming very faint precipitation arcs. The localisation of the two main precipitates, when compared with a human serum run as reference in the same experiment (fig. 1), shows that these components have a high isoelectric point. Ottesen and Spector²⁴ reported the isoelectric point of two alkaline peptidases from *B. subtilis* to be 9.1 and 9.4, suggesting that the two main components observed by us may also be alkaline peptidases (proteases). This assumption was also supported by the fact that the cathodic dense precipitates, in contrast to the anodic ones, soon showed an appearance suggesting auto-digestion. The enzyme and allergenic activity was also eluted from these parts of the agar after electrophoresis. Thus we assume identity between the enzyme, the two slow-moving precipitating components, and the allergen principle. The relatively low electric charge of this enzyme-allergen is in contrast to the acidic nature usually found in other isolated allergens.^{20,21,23} Knowledge on the relation between molecular structure and allergen activity is limited, but may be increased by further studies of this allergenic enzyme since it has been extensively analysed biochemically, including establishment of its primary structure, composed of 75 aminoacids.

Besides such obvious factors as intensity of exposure and particle size, sensitisation to this allergen may also be favoured by an enzymatically increased penetration through the mucous membrane. As a challenging allergen, however, the enzyme was active even after enzyme inactivation by heat. In our patients we did not consider it necessary further to establish the diagnosis by provocation tests, but in more obscure cases such tests may be indicated and can probably be done safely with heat-inactivated enzyme-allergen.

Even if sensitisation to proteolytic enzyme in washing powder is much less common in domestic users than in factory workers the problem is evidently more than just one of occupational sanitation and elimination of easily sensitised employees. The use of proteolytic enzymes is not restricted to laundering. The manufacturers of alcalase in Denmark describe its use in baking, brewing, fish, leather, and silk industries. We would like to stress that, in every situation where these enzymes are used, it should be certified that they do not occur in a dusty form capable of polluting the air.

In November, 1969, the manufacturers of the enzyme-containing washing powder investigated by us changed the dispersion of the enzyme component from a powder to granules, thereby probably reducing the risk for sensitising considerably. However, one cannot completely exclude the possibility that materials such as clothes and sheets, treated with solutions containing these proteolytic enzymes and dried without sufficient rinsing, may release enough enzyme to elicit unrecognised allergic type-I reactions.

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ANTIBIOTIC RESISTANCE OF SHIGELLA SONNEI

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Summary Examination of 1102 representative strains of *Shigella sonnei* isolated in London in the past two years has shown that 70% of strains are now resistant to three or more antibacterial drugs. Neomycin-resistant strains, though uncommon, are isolated more frequently than in the past. The significance of these findings is discussed in relation to the treatment of acute diarrhoea and of the convalescent excreter.

Introduction

THE antibiotic resistance of *Shigella sonnei* is of interest for a number of reasons. The clinician is concerned with treatment of the acute case and also of the symptomless excreter. Should he use antibiotics at all? If so, which one? The epidemiologist can trace the spread of particular strains in his area, using a combination of drug-resistance pattern and colicine type to characterise a strain. The bacterial geneticist, interested in the problems of acquired antibiotic resistance, has in *Sonne* dysentery a ready-made working model to observe and test his hypotheses.