

Clinical, immunologic, and physiologic observations in factory workers exposed to *B. subtilis* enzyme dust

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Approximately one year after enzymes were incorporated into the manufacturing process of one detergent plant in this country, some employees who were exposed to high concentrations of enzyme dust developed both upper and lower respiratory symptoms. These symptoms ranged from nasal stuffiness, rhinorrhea, lacrimation, and throat irritation to chest tightness, cough, and breathlessness. The chest symptoms characteristically occurred 3 to 8 hours after leaving work. Chest x-rays revealed no abnormalities. The prevalence of atopy as determined by personal history was approximately equal to the prevalence in the general population. Intracutaneous skin tests with appropriate enzyme antigens were positive in the majority of affected individuals. Passive-transfer studies with sera from 5 symptomatic employees were positive. Serum precipitins were demonstrated in skin test-negative and skin test-positive employees as well as in persons who had never been exposed to enzyme-containing dust. Complete pulmonary function studies in skin test-negative workers revealed an occasional abnormality. Seventy per cent of the skin test-positive group initially showed disturbances in ventilatory function and/or reduction in the carbon monoxide gas-transfer test. Bronchial challenge tests were positive in 9 of 10 employees with preceding symptoms and positive skin tests. A typical biphasic response pattern was observed in all cases. As stringent dust control measures have been instituted and employees have been instructed in protective measures, the number of respiratory complaints has fallen drastically in this plant.

In the last decade biochemical technologic advances in the large-scale production of microorganisms and their products have resulted in increasing industrial applications of the metabolites, particularly proteolytic enzymes. Proteases obtained from strains of *B. subtilis* and *B. licheniformis*, referred to as subtilisins or subtilopeptidases, exhibit enzymatic activity over wide pH and temperature ranges and are therefore ideally suited for incorporation into household cleaning agents. In 1967, following four years of successful use in Europe, enzyme-containing detergents were introduced in this country and have gained widespread consumer acceptance.

In the latter part of 1967, some employees of an English enzyme detergent producing plant were exposed to high concentrations of enzyme dust and developed both upper and lower respiratory symptoms which suggested some type of sensitization process. In June, 1969, Flindt¹ and Pepys and associates² first

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reported some clinical and immunologic aspects of this problem. Since their initial reports, Wüthrich and Ott,³ Newhouse and associates,⁴ and Greenberg and associates⁵ have also published on this subject. In 1968, before any information about the European experience was available and approximately one year after enzymes were incorporated into the manufacturing process of one detergent plant in this country, we also observed similar symptoms in affected workers. These symptoms ranged from nasal stuffiness, rhinorrhea, lacrimation, throat irritation, and cough to chest tightness, wheezing, and breathlessness. It was especially noteworthy that the upper respiratory symptoms occurred on the job, while chest symptoms characteristically occurred some 3 to 8 hours after the employees left work. The symptoms would generally subside after several hours, and the men would return to work the next day feeling completely normal. Indeed, because of this, physical findings were absent at the time they were seen by the plant physician. Chest x-rays have not revealed any abnormalities either during the stage of acute symptoms or in follow-up periods as long as 36 months after exposure. Since the essential features of this syndrome appeared to be equatable with hypersensitivity responses, we undertook clinical investigations involving direct skin testing, passive-transfer studies, serum precipitin analysis, bronchial challenge tests, and serial pulmonary function testing.

METHODS

The test group of subjects consisted of thirty-eight employees whose symptoms were thought to be related to enzyme exposure. Two of these employees had dermatologic findings only, secondary to the primary irritant nature of the enzyme when handled in high concentrations. Eleven had symptoms limited to the upper respiratory tract. The remaining twenty-five employees exhibited the characteristic complex of chest symptoms which usually occurred after they left the plant. Their ages ranged from 21 to 58, and all had enjoyed reasonably good health. A personal allergy history was taken from each individual to estimate the prevalence of atopy in the group.

All testing utilized the same commercial enzyme preparation being used in the plant at the time symptoms originally developed. It was ascertained that 0.1 mg. per milliliter (1:10,000) solutions of this enzyme material in phosphate-buffered saline (pH 7.2) resulted in only a low incidence of positive reactions (5 in 227) in nonexposed individuals in one series of tests. A sizable incidence of false positive reactions due to primary irritation was obtained in normal subjects if higher concentrations were used. The test solutions were injected intracutaneously in 0.02 ml. quantities on the volar aspect of the forearm, and results were interpreted in 15 to 20 minutes. Buffered saline (pH 7.2), the diluent employed for the enzyme solutions, was injected in adjacent sites as a control. Skin tests with 1:10,000 dilutions were performed only if skin tests were negative at the 1:100,000 strength.

Passive-transfer studies were performed on the backs of 2 normal volunteer subjects who had no history of prior exposure to enzyme dust. Skin sites were prepared by intracutaneous injections of varying dilutions of fresh unheated sera. One site was prepared with undiluted serum heated at 56° C. for 30 minutes. Forty-eight hours later these sites were challenged with 0.02 ml. quantities of 1.0 mg. per milliliter enzyme solution (1:1,000). Appropriate controls included phosphate-buffered saline (pH 7.2) and 1:1,000 dilutions of unrelated enzymes, papain, and bovine ribonuclease. Additional controls consisted of skin tests with all the above substances in areas of skin not previously injected with test sera.

Serum precipitins were assayed by the standard Ouchterlony double-gel diffusion method using agarose (Seakem, Bausch and Lomb, Rochester, New York) as the gel medium and a citric acid-phosphate buffer, pH 7.0. Sera were placed in the central wells. Varying concentrations of enzyme solution and a buffer control were placed in the peripheral wells

located radially 11 mm. from the central well. The plates were incubated for seven days at 4° C.

Bronchial challenge tests were performed with enzyme solutions in serial concentrations nebulized through a No. 40 DeVilbiss nebulizer by compressed air. Each subject was instructed to inhale 0.5 ml. of the nebulized solution within a 5 minute period. On each test day, the first exposure was to buffered saline which served for both control and practice. With one exception all subjects had initial normal peak expiratory flow rates. Responses to the various challenges were evaluated by chest auscultation and measurements of the peak expiratory flow rate utilizing a Wright peak flowmeter. In 4 subjects, diffusion capacity was measured by the single-breath carbon monoxide method before and after provocation.

During the provocative experiments several other laboratory assessments were also made. These included peripheral leukocyte counts and quantitative assay of β_2C levels.

RESULTS

Table I summarizes results of direct intradermal tests in this group of workers. As expected, individuals with only the irritant-type dermatitis did not show positive skin tests. Only 3 of 11 patients with upper respiratory symptoms were positive, whereas 22 of 25 with characteristic chest symptoms were positive. It should be noted that the prevalence of atopy, as determined by personal questionnaire in both the skin test-positive and skin test-negative groups, was similar to that present in the general population, approximately 20 per cent.

Passive transfer studies were limited to 5 of the above patients who exhibited strongly positive skin test reactions. Results of these tests are shown in Table II. Positive results were obtained with test sera from all 5 subjects, but the reactions were abolished by heating the sera to 56° C. for 30 minutes. All control sites were negative.

Precipitins were found in 3 of 13 skin test-positive individuals and in 9 of 12 skin test-negative subjects. The sera of a separate group of 41 normal individuals who had never been exposed to enzyme-containing dust were also investigated. Twelve of this group were found to have precipitating antibody directed against subtilisin.

Complete pulmonary function tests were performed on 28 subjects at a time when they were entirely asymptomatic. The data obtained in the skin test-negative group are presented in Table III. Subjects 6 and 7 originally presented with lower respiratory symptoms, Subject 8 with dermatitis, and the remainder of the group with upper respiratory symptoms. Normal pulmonary function results were observed in all subjects with the exception of Subjects 3 and 8. Both demonstrated a slight restrictive abnormality in lung volumes and a slightly reduced single-breath diffusing capacity of lungs for carbon monoxide (DLCO). Isolated abnormalities in ventilatory indices were observed in Subjects 9 and 10 but are not interpreted as significant.

Table IV lists similar pulmonary function data in the skin test-positive group. Subjects 1, 2, and 3 originally presented upper respiratory symptoms, whereas all others in the group presented lower respiratory symptoms. Over-all interpretation of these results at the onset of the study reveals that some pulmonary function abnormality was present in 13. Subjects 1, 2, 9, 12, and 15 were

TABLE I. Comparison of skin tests with symptoms

Skin tests	Total	Atopic	Symptoms		
			Dermatologic	Upper respiratory	Lower respiratory
Number of subjects	38	8	2	11	25
Positive	25	5	0	3	22
Negative	13	3	2	8	3

TABLE II. Results of passive transfer study

Serum dilution	Skin test grade*							
	Test sites, challenged with subtilisin					Test sites, Subject 1, challenged with		
	Subject 1	Subject 2	Subject 3	Subject 4	Subject 5	Saline	Papain	Ribonuclease
Undiluted	4+	4+	4+	4+	4+	0	0	0
1-10	2+	—	—	—	—	0	0	0
1-100	1+	—	—	—	—	0	0	0
1-1,000	0	—	—	—	—	0	0	0
Undiluted (heated at 56° C.)	0	—	—	—	—	—	—	—

Additional control sites in all subjects (without prior injection of serum) were tested with all the above antigens. These were all negative. —, Not performed; 0, negative.

*Each serum was placed on two recipients and the results were averaged.

TABLE III. Pulmonary function results in the skin test-negative group

Subject	Age	FEV ₁ / FVC (%)	FEV ₃ / FVC (%)	MVV*	MEFR*	MMF*	VC*	FRC*	RV*	TLC*	DLCO*
1	25	81	97	106	117	95	113	103	99	109	100
2	31	84	96	116	96	105	112	96	102	109	94
3	38	85	98	116	90	92	83	51	70	79	67
4	30	80	96	131	111	94	127	81	56	107	103
5	50	78	92	103	93	86	98	95	116	104	81
6†	32	82	98	84	100	91	106	53	74	97	76
7†	41	79	93	127	101	91	103	78	77	95	95
8‡	25	77	96	75	68	65	81	72	80	81	72
9	28	75	91	93	88	59	89	90	134	101	113
10	26	83	98	90	49	75	92	76	74	87	83

FEV₁ = forced expiratory volume in one second; FVC = forced vital capacity; FEV₃ = forced expiratory volume in 3 seconds; MVV = maximal voluntary ventilation; MEFR = maximal expiratory flow rate; MMF = maximal midexpiratory flow rate; VC = vital capacity; FRC = functional residual capacity; RV = residual volume; TLC = total lung capacity; DLCO = diffusing capacity of the lungs for carbon monoxide.

*Expressed as per cent of predicted.

†Lower respiratory symptoms.

‡Dermatitis.

classified as having normal pulmonary functions. Some measure of ventilatory disturbances was noted in 10 individuals (Subjects 3 to 8, 10, 11, 13, and 17) with Subjects 8, 11, and 13 showing evidence of hyperinflation. The single-breath diffusion study was borderline or reduced in 8 persons (Subjects 3, 5 to 7, 10, 14, 16, and 18), and in 5 (Subjects 3, 5 to 7, and 10) this finding was

TABLE IV. Pulmonary function results in the skin test-positive group

Subject	Age	FEV ₁ / FVC (%)	FEV ₃ / FVC (%)	MVV*	MEFR*	MMF*	VC*	FRC*	RV*	TLC*	DLCO*
1†	48	84	96	125	120	93	106	80	104	106	113
2†	21	77	94	97	99	59	98	57	74	92	93
3†	42	79	93	83	62	77	91	78	69	84	56
4	35	78	95	141	53	66	151	118	111	139	101
5	22	63	88	60	44	25	81	66	109	89	66
6	34	70	93	74	65	45	104	98	113	106	68
7	36	72	90	107	71	63	106	73	59	92	65
8	43	67	91	114	†	†	127	93	152	119	90
9	22	78	97	113	99	77	116	94	69	103	122
10	29	74	94	68	†	†	71	76	93	84	62
11	55	65	84	116	†	†	128	146	221	154	130
12	36	76	95	88	78	80	120	112	86	110	126
13	58	77	90	88	62	56	98	172	264	158	86
14	26	81	100	80	88	99	95	97	91	94	66
15	21	94	100	93	108	74	94	78	81	91	79
16	22	82	98	88	†	†	93	65	61	85	74
17	29	73	93	99	70	46	128	95	65	111	89
18	29	90	98	85	84	80	68	39	55	65	74

See legend to Table III for meanings of abbreviations.

*Expressed as per cent of predicted.

†Upper respiratory symptoms only.

‡Unable to calculate flow rates because of the slow chart speed used in these subjects.

TABLE V. Results of bronchial challenge studies

Subject	Dose (0.5 ml.)	Percentage decrease in the peak flow	Wheezing	Bronchodilators
1	1/1,000,000	35	Equivocal	Reversed
2	1/100,000	15	+	Reversed
3	1/100,000	21	+	Reversed
4	1/100,000	15	Equivocal	—
5	1/100,000	35	+	—
6	1/10,000	33	+	Reversed
7	1/10,000	20	+	Reversed
8	1/1,000	24	+	Reversed
9	1/1,000	45	+	Reversed
10	1/1,000	None	None	—
11 (Control)	1/1,000	None	None	—
12 (Control)	1/1,000	None	None	—
13 (Control)	1/1,000	None	None	—
14 (Control)	1/1,000	None	None	—
15 (Control)	1/100	None	None	—

associated with reduced indices of obstructive ventilatory functions. A reduced diffusion capacity in Subjects 14 and 16 was not accompanied by any other physiologic abnormality. In one subject (Subject 18) a deficit in DLCO was associated with evidence of mild restriction in the lung volume but without evidence of obstruction. Subsequent tests, however, did not produce any evidence of restrictive lung disease in any of these subjects.

An over-all comparison of the pulmonary function data between the two groups shown in Tables III and IV indicates an obvious difference. Whereas

TABLE VI. Effect of enzyme inhalation on DLCO (milliliters per minute per millimeters of mercury) before and after bronchial challenge tests

Subject	Predicted	Provocation			
		Before	First week after	3 mo. after	5 mo. after
1	32.8	31.0	29.2 (day 1)	23.6	31.0
2	30.2	34.2	27.3 (day 1)	27.2	30.3
			24.3 (day 6)		
3	29.4	19.2	11.2 (day 4)	20.5	23.0
4	31.6	28.6	20.0 (day 5)	22.0	29.0

70 per cent of the skin test-negative group was found to have normal pulmonary function measurements, only 22 per cent of the skin test-positive group had all normal pulmonary function measurements. Furthermore, a notable feature of this study was the number of individuals demonstrating an impairment of the single-breath diffusion.

Enzyme inhalant provocative studies were performed on 10 employees who had reported pulmonary symptoms and on 5 normal subjects. Table V lists these results. A positive immediate response was obtained in 9 of 10 subjects in the test group, while all control individuals were negative. In general, decreases in the peak expiratory flow rate correlated well with auscultatory evidence of wheezing and symptoms of chest tightness and cough.

Five subjects who exhibited immediate symptoms and signs after bronchial challenge were observed closely for an additional 10 hours. All of these men developed a delayed type of bronchospasm, the onset of which varied from between 4 to 6 hours after the initial challenge. Fig. 1 depicts the time course of a typical dual-type response which was allowed to proceed spontaneously. This pattern of reaction was observed in all 5 subjects. Fig. 2 depicts a case in which a bronchodilator was used to reverse the bronchospasm observed in both the immediate and late reactions. Its effectiveness in both phases of the reaction is clearly demonstrated. In addition to recurrent wheezing and a decrease in peak expiratory flow in the late reaction, crepitant rales were heard in 2 subjects. There was no indication of any febrile episodes. Two individuals exhibited flushing of the face. All commented that the symptoms induced by provocation were similar to those which they had previously experienced following exposure in the plant.

In addition to the ventilatory measurements described above, DLCO studies were performed in 4 of these subjects before and after provocative exposure. Table VI presents the results of these studies. In all cases there was a slight to moderate drop in DLCO. This decrease in DLCO persisted for 3 months. However, continued follow-up with complete pulmonary function testing, including the DLCO, showed that all subjects have returned to normal.

In all test subjects, blood was drawn immediately before and after provocation for a leukocyte count and β_1C analysis. There was only one individual who demonstrated an elevation of the leukocyte count from a basal level of 6,400 per cubic millimeter to 12,400 per cubic millimeter following the test. In none of the group was there a change in the level of β_1C .

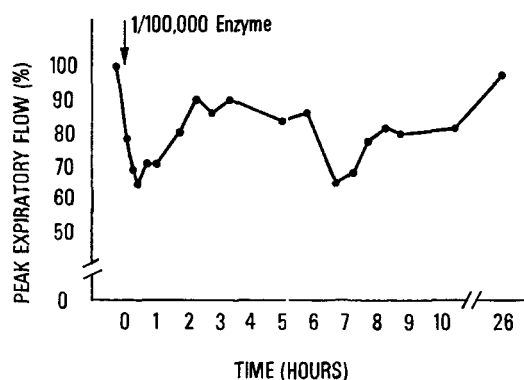


FIG. 1. Time course of a typical bronchial challenge test allowed to proceed spontaneously. Peak expiratory flow rates were measured with a Wright peak flowmeter.

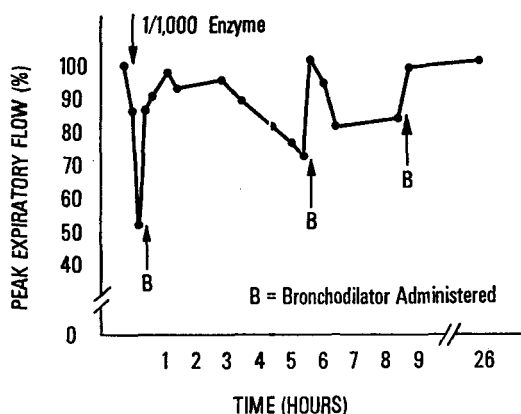


FIG. 2. Time course of a bronchial challenge test in which a nebulized bronchodilator (isoproterenol 1 to 200) was administered promptly after objective evidence of airways obstruction. In this case, a brisk bronchodilator response was obtained in both the early and delayed phases of the reaction.

DISCUSSION

These studies confirm published reports that subtilisin is capable of inducing anaphylactic sensitization in exposed detergent plant workers. The incidence of sensitization was greater among employees exposed to higher levels of enzyme. Although several reports^{4, 5} have stressed the increased incidence of positive skin tests to the enzyme among atopic workers, atopy was distributed evenly among skin test-positive and skin test-negative individuals in this study. Our results could be due to the small size of our sampling population or to the fact that the identification of atopic persons in this study was based only on history and not on skin testing with common allergens. Intracutaneous skin testing is a reliable method of corroborating clinical allergy in these workers. At least part of this sensitization is mediated by reaginic antibodies as demonstrated by the

passive-transfer data and by the immediate response observed in bronchial challenge testing.

We also observed the unusual delayed bronchial reactions reported by Pepys and associates.² Although late responses are occasionally encountered after bronchial challenge with pollen and dust atopens, they appear more frequently after provocation with crude enzyme antigens. Pepys and co-workers suggest that these findings are similar to those seen in other organic dust syndromes. Serum precipitins are usually found in organic dust syndromes, and considerable attention has been drawn to their possible role in the pathogenesis of bronchial and alveolar inflammatory lesions. However, the importance of precipitins in respiratory disease caused by subtilisin is much less clear. Wüthrich and Ott³ did not find precipitating antibodies to subtilisin in the serum of their subjects. Pepys and associates observed precipitins in both exposed and nonexposed subjects and in fact reported that the incidence of positive precipitins appeared to be greater in unexposed as compared to exposed individuals. Our results in this regard are similar to those of the latter group of investigators, and at the present time we are unable to explain the significance of precipitins in subtilisin allergy.

The physiologic response to bronchial provocation merits further discussion. It is noteworthy that both the early and late episodes of bronchospasm were readily reversible by inhaled bronchodilators. Response to these agents has also been observed clinically in workers with mild to moderate obstructive symptoms occurring after exposure to dust in the plant environment. The reductions in diffusion capacity observed in the 4 subjects followed serially before and after provocation are significant, as is the fact that the carbon monoxide transfer defect was not entirely reversed during the 3 month follow-up period in 3 of the 4 subjects. However, all of those with reduced values have returned to normal within 6 months. Our observations of decreased diffusion values following provocation are in contrast to those reported by Pepys and associates in that abnormalities in DLCO were not demonstrated in the 3 subjects they provoked.

These physiologic observations in gas transfer are subject to several interpretations. Although the carbon monoxide transfer factor is usually normal during periods of remission,⁷ it has been reported to be reduced during acute asthmatic episodes.⁶ The majority of patients tested in the present investigation demonstrated evidence of airways obstruction. Even in those patients with normal pulmonary function tests, it is conceivable that considerable small airways obstruction could have been present, thereby leading to an unequal distribution of ventilation.⁸ In support of this, it has been demonstrated that abnormalities in regional alveolar ventilation-perfusion ratios, detected by the radioactive xenon-133 technique, are present in asymptomatic subjects with reversible airways obstructive disease.⁹ Alternatively, a reduced gas-transfer factor may be the result of direct alveolar membrane and/or interstitial tissue involvement. In any event, these results indicate that bronchial challenge tests of this nature are not to be taken lightly, and, if performed, long-term observation including complete pulmonary function evaluations are in order.

In the over-all group of enzyme-sensitive employees, the physiologic measure-

ments in part confirm the data obtained in the provocative experiments, but they also provide additional evidence that alterations in pulmonary function may occur after prolonged exposure to large amounts of enzyme-containing dust. Nearly 70 per cent of the allergic group demonstrated some type of pulmonary function impairment. Many of these men also had decreased or borderline gas-transfer factor abnormalities. Subsequent studies in this group showed that the diffusion capacity returned to normal, although some evidence of ventilatory disturbance was still present. However, all pulmonary functions returned to normal within 5 months. One subject with nasal symptoms only also showed a reduction in pulmonary function. Thus, these data support the concept that a thorough evaluation of pulmonary function should be undertaken on symptomatic workers who develop sensitivity. Data in the skin test-negative symptomatic group of workers would also indicate that pulmonary physiologic evaluations should be performed if their symptoms persist or worsen. Workers demonstrating a diffusion abnormality should be removed from exposure to subtilisin if dust control measures are not adequate.

Although the immediate part of the biphasic reaction can be clearly assigned to a reagin-mediated mechanism, an explanation of the delayed response requires further investigation. As Pepys and co-workers have suggested, it is possible that the delayed clinical manifestations could be the result of an Arthus reaction. The delay of the reaction, occurring 4 to 6 hours after inhalation, and the reduction in diffusion capacity are compatible with this hypothesis, while the absence of precipitins in most and the favorable response to the bronchodilator in the acute and late phases are not. This explanation could be accepted only if the deposition of antigen-antibody complexes could be demonstrated at the appropriate tissue sites. Delayed bronchospasm conceivably could also be elicited by another population of reagins analogous to different types of homocytotropic antibodies encountered in other animal species.^{10, 11} Such antibodies could then mediate the release of histamine, slow-reacting substance of anaphylaxis, or both with attendant effects on bronchial smooth muscle. However, since the effects of these mediators would be reversible, they would not be expected to result in persistent diffusion abnormalities.

Certain nonimmunologic phenomena must also be considered since subtilisins are potent proteolytic enzymes. Direct proteolysis of bronchial mucosa and/or alveolar membranes is one possibility. However, previously unexposed human volunteers experienced neither subjective nor objective abnormalities following inhalation of an enzyme solution containing 1 mg. per milliliter and, in one case, 10 mg. per milliliter. Another possibility based on the proteolytic nature of these enzymes is the formation of kinins. Henningsen and Zachariae¹² have shown that subtilisin is capable of causing the formation of kinins from plasma substrates. In this connection, it is interesting that 2 subjects experienced facial flushing during their delayed episodes of bronchospasm. Thus, liberation of a potent vasoactive substance could occur independently of an antigen-antibody reaction. Metabolites derived from *B. subtilis* are also capable of inactivating complement in vitro.¹³ If similar effects took place in vivo, it could be postulated that anaphylatoxin might be liberated by cleavage fragments of complement

(C3a, C5a). Finally, another interesting in vitro effect of subtilisin is its inhibition of the lipolysis mediated by catecholamines in isolated rat adipose tissue.¹⁴ This, of course, suggests that these enzymes may block beta adrenergic receptors.

The nature and composition of the antigenic substances in commercial subtilisins merits special consideration. The industrial enzyme preparation contains a small number of spores, small amounts of ingredients from the grain culture media, and various inorganic salts. It is generally agreed that the low level of spores in these preparations is insignificant in terms of human pathogenicity. It has been speculated by Pepys that spore cell walls may be capable of producing an antigenic response, but his results did not show this to be of any clinical significance. Nevertheless, the possibility exists that prior exposures to *B. subtilis* may account for the incidence of precipitating antibodies in persons not exposed to enzyme dust. The allergenic potential of cell wall material as well as trace grain proteins utilized in the fermentation process have not yet been investigated. However, the combined clinical and immunologic data derived from these and other studies indicate that the main allergenic principle of the commercial concentrate appears to reside in the proteinase component.

Prior to the introduction of proteolytic enzymes into detergents, the dust which existed in these plants had not been the cause of any industrial health problem. Dust is a natural byproduct in plants in which very large quantities of detergents are produced monthly, and its control is a complex and costly procedure due to the enormous size and mechanical sophistication of the facilities. As greatly improved dust-control measures have been instituted and employees have been instructed in protective measures which reduce undue exposures, the number of respiratory complaints has fallen drastically.

In addition to the engineering dust-control systems which have been installed, manufacturing process modifications have been designed and implemented to produce a coated nonrespirable enzyme particle. All of these changes have contributed greatly to the reduction of airborne respirable enzyme dusts and the number of the adverse respiratory complaints and findings. As the etiology of this industrial problem is immediately evident, namely, the inhalation of enzyme dust, so is its elimination: stringent control of respirable airborne dust in the plant environment.

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