

Self-contained breathing apparatus, or hoods or suits with an independent air-supply, will be required. It must be appreciated that even weighing out small quantities of the material may be hazardous, and fume-cupboard precautions are recommended for laboratory tasks. Care must be taken in the disposal of the effluent from exhaust ventilation in such a way as not to create an additional hazard.

Those who are already using these enzyme preparations may have to test for evidence of sensitisation in employees and transfer those sensitised to other buildings unless it is found possible to create contamination-free working conditions.

In addition to the specific findings, the outcome of this investigation underlines the need for caution before the handling of any finely particulate organic material.

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ALLERGIC REACTIONS OF THE LUNGS TO ENZYMES OF *BACILLUS SUBTILIS*

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Summary Allergic sensitivity produced by the inhalation of high concentrations of enzyme preparations from *Bacillus subtilis* was found in three workers with mainly bronchial disease. They gave strong immediate reactions to prick tests and immediate, followed by late, mainly asthmatic reactions to inhalation tests. Precipitins were found in the sera of the affected subjects, but even more often in controls.

Introduction

THE development of respiratory disease has been reported by Flindt (1969) in workers producing biological washing preparations, containing proteolytic enzymes and also viable spores of *Bacillus subtilis*. Three affected workers have been investigated in hospital by skin, inhalation, and serological tests.

Clinical Features

Case 1

This man, aged 38 years, who was previously well, developed persistent rhinitis 1-2 days after working with the enzyme material. 4 months later cough and scanty mucoid sputum appeared insidiously, and 2 months after that he had an acute illness starting while at work with sore throat, painful cough, malaise, chest tightness, dyspnoea, and wheezing. Next day his

TABLE 1—PULMONARY-FUNCTION TESTS IN AFFECTED WORKERS

Pulmonary-function tests	Patient no.					
	1		2		3	
	Measured	Predicted	Measured	Predicted	Measured	Predicted
F.E.V. ₁ (l.)	2.3	3.45	1.58	2.6	2.3	3.7
V.C. (l.)	3.86	4.6	2.18	3.71	4.12	4.96
F.E.V. ₁ /V.C. (%)	60	>70	72	>70	56	>70
P.F.R. (l./min.)	360	600	270	525	380	625
Specific conductance (body plethysmograph) (l./cm. H ₂ O. sec.)	0.11	0.23	0.076	0.23	0.077	0.18
F.R.C. (helium-dilution method) (l.)	3.49	3.74	2.26	3.1	4.84	3.74
I.C. (l.)	2.71	2.94	1.58	2.76	2.66	3.09
T.L.C. (l.)	6.2	6.68	3.84	5.86	7.5	6.83
R.V. (l.)	2.34	2.08	1.66	2.15	4.3	1.87
R.V./T.L.C. (%)	37	31	36	30	57	27
CO transfer factor (single breath method) (mm. Hg/ml./min.)	32	30	23	23	29	33
P _v CO ₂ (rebreathing method) (mm. Hg) ..	44	46	..	..	46	46

F.E.V.₁=forced expiratory volume in 1 sec. V.C.=vital capacity. P.F.R.=peak flow-rate. F.R.C.=functional residual capacity. I.C.=inspiratory capacity. T.L.C.=total lung capacity. R.V.=respiratory volume. P_vCO₂=carbon-dioxide tension in mixed venous blood.

scanty sputum became pale-green, and 3 days later he stopped work for 3 weeks, for the first 9 days of which the dyspnoea increased, with five very severe nocturnal attacks. He lost 10 lb. (4.3 kg.) in weight. On return to work he still had mild effort dyspnoea without wheezing. Exposure to the enzyme material provoked immediate chest tightness. On examination rhonchi but no crepitant râles were heard.

Table 1 shows evidence of airways obstruction, the ratio of forced expiratory volume in 1 second to vital capacity (F.E.V.₁/V.C.) being 60%. Isoprenaline inhalation had no effect on the F.E.V.₁ and a subcutaneous injection of adrenaline (0.5 ml. of 0.1% solution) raised the F.E.V.₁ by 0.35 litre. The poor reversibility is supported by the Altounyan test (1966) which compares the rise in F.E.V.₁ after atropine inhalation with the total response produced by an additional inhalation of isoprenaline. The atropine was responsible for 66% of the small total rise of 0.3 litre, a finding thought to indicate that corticosteroid treatment would be unlikely to produce improvement. It was not used in this patient, but was ineffective in the other two patients, who also showed poor reversibility of the airways obstruction. There was no chest radiographic abnormality, leucocytosis, or blood or sputum eosinophilia; and, latex, differential agglutination titre (D.A.T.), and antinuclear factor (A.N.F.) tests were negative.

Case 2

This man, aged 45 years, was well until 12 months after starting work with the enzyme. Progressive effort dyspnoea with wheezing, chest tightness, and a dry irritating cough developed insidiously and kept him off work for 6 weeks. The symptoms improved after only 3 weeks at home. On return to work they recurred at the end of the 1st day, and later they built up gradually during work, and were worse later at night at home. He improved again on transfer to another department. On examination, basal crepitations and rhonchi were heard.

Table 1 shows slight obstructive and restrictive ventilatory defects. The airways obstruction was not apparent on spirometry but was reflected in a decrease in peak flow-rate (P.F.R.) and specific conductance. Isoprenaline, and corticosteroids, 30 mg. per day for 2 weeks and then gradually reduced, had no effect. There was no chest radiographic abnormality, leucocytosis, or eosinophilia; and latex, D.A.T., and A.N.F. tests were negative. The patient had a budgerigar for 10 years, but skin and precipitin tests to avian antigens were negative.

Case 3

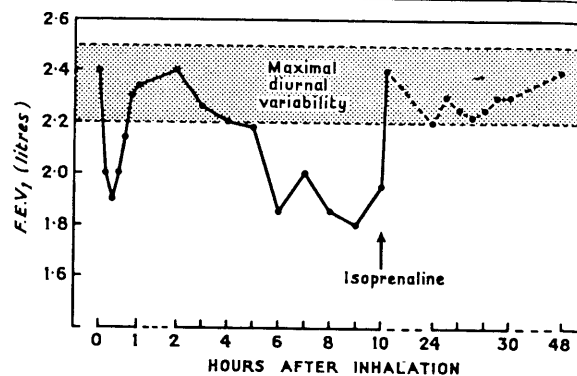
This man, aged 30 years, was successfully treated for

pulmonary tuberculosis in 1953. Two months after starting work in the non-soap detergent section, but not with the enzyme material, he developed, insidiously, a progressive decrease in exercise tolerance, and also had episodes of chest tightness, dyspnoea, and wheezing at rest. These symptoms became much worse after starting work with the enzyme material, when he also developed a persistent painful cough without sputum. The enzyme dust caused immediate acute symptoms, which progressed during the day and were often worse after leaving work. On examination, rhonchi but no crepitant râles were heard.

Table I shows an obstructive ventilatory defect. The F.E.V.₁ varied spontaneously between 1.5 and 2.9 litres, and was raised 0.6 litre by isoprenaline inhalation, 0.7 litre by subcutaneous adrenaline, and 0.75 litre by atropine inhalation, the highest F.E.V.₁ being 2.9 litres. In the Altounyan test, atropine produced 90% of the rise in F.E.V.₁, suggesting poor reversibility by corticosteroids, and, indeed, treatment with 30 mg. per day of prednisolone for 9 days and 60 mg. per day for 6 days, followed by gradual reduction, had no effect. The raised residual volume and functional residual capacity (F.R.C.) suggest air-trapping. The chest radiograph showed pleural thickening and a calcified opacity, presumably related to his previous tuberculosis. There was no leucocytosis, or eosinophilia; and latex, D.A.T., and A.N.F. tests were negative.

Skin Tests

Two enzyme preparations, 'Alcalase' ("A") and 'Maxatase' ("M"), and spores of *B. subtilis* cultured from "A" were defatted in ether overnight, extracted for 7 days in carbol/saline, seitz-filtered, dialysed, and freeze-dried. Prick tests were then made using these extracts and a purified crystalline proteinase from *B. subtilis* (Koch-Light). In control tests in patients attending the Brompton Hospital there were virtually no unequivocally positive reactions to any of the extracts at 10 mg. per ml., and there were no reactions to the 1 mg. per ml. strength (Flindt 1969). All three affected subjects tested at 10 mg. per ml. gave very strong, immediate, whealing reactions, particularly to the enzyme preparations (table II). The prick tests were repeated in patients 2 and 3 with weaker solutions of extract "A" before the inhalation tests. Patient no. 2 gave a 4.5 mm. wheal to the 0.1 mg. per ml. strength and



Dual, immediate, and late asthmatic reactions to inhalation test with "A" enzyme extract.

a 9 mm. wheal to the 1 mg. per ml. strength, and patient no. 3 gave a 7 mm. wheal to the 0.1 mg. per ml. strength, thus showing even further their high degrees of sensitivity. Intracutaneous tests in patients 2 and 3 with 0.01–0.02 ml. of the "A" extract at 0.01 and 0.1 mg. per ml. strength gave strong immediate reactions in both patients, and patient no. 3 also gave a second reaction maximal at 6 hours to both concentrations. There were no reactions to prick tests with extracts of 23 of the commoner inhalant and food allergens.

Inhalation Tests (table III)

Aerosols of the appropriate concentrations of solutions of enzyme "A" extract (as judged by the prick-test reactions) were produced by passing oxygen at 8 litres per minute through a Wright's nebuliser (Pepys, Hargreave, Chan, and McCarthy 1968). No reactions were given by a healthy volunteer (F. E. H.) to the inhalation of a 10 mg. per ml. concentration for 5 minutes. The effects on the reactions of inhalation of disodium cromoglycate were also tested. Control tests with a carbol/saline challenge showed variations in the F.E.V.₁ during the day in patient no. 1 between 2.2 and 2.5 litres, in patient no. 2 between 1.54 and 1.7 litres, and in patient no. 3 between 1.6 and 2.25 litres.

Case 1

In test 1 (1 mg. per ml.) (see accompanying figure) there was an immediate asthmatic reaction, with a fall in F.E.V.₁, which reversed spontaneously in 50 minutes. A second asthmatic reaction followed, beginning at 5 hours and reversed by isoprenaline. The F.E.V.₁ returned to pre-test levels by 24 hours. In test 2 (1 mg. per ml.) the inhalation of disodium cromoglycate before the challenge inhibited the immediate reaction; no observations were made for late reactions. In test 3 disodium cromoglycate was given 12 minutes before the challenge with

TABLE II—PRICK TESTS (WHEAL DIAMETER IN mm.)

	Patient no.		
	1	2	3
<i>Test extracts at 10 mg./ml.:</i>			
Product "A" (alcalase)	5	7.5	12
<i>B. subtilis</i> from "A"	4	1	6
Product "M" (maxatase)	5	6	10
<i>B. subtilis</i> from "M"	0	3	3
<i>B. subtilis</i> , crystalline proteinase (Koch-light)	..	10	11

TABLE III—INHALATION TESTS WITH EXTRACT OF *B. SUBTILIS* ENZYME PREPARATION "A"

Test no.	Date (May, 1968)	Concentration (mg./ml.)	Time (min.)	Volume (l.)	Asthmatic reaction (% fall)				White-blood-cell count (per c.mm.)		Eosinophil count (per c.mm.)	
					Immediate		Late		Before test	After 7 hr.	Before test	Maximum
					F.E.V. ₁	V.C.	F.E.V. ₁	V.C.				
Patient no. 1												
1	7th	1	3	0.8	21	7	25	13	6800	11,400	120	220
2	9th	1*	5.5	1.5	4	2	..	..	..	..	..	..
3	11th	10*	5	1.0	38	24	25	16	5800	11,200	200	210
4	13th	1	5	1.3	14	5	24	13	..	..	310	280
Patient no. 2												
1	14th	0.1	5	1.0	8	0	21	11	..	..	120	430
2	16th	1	5	1.1	6	7	44	32	9100	12,200	430	600
3	20th	1	5	0.8	26	24	51	47	9700	16,500	600	770
4	24th	1†	5	0.9	34	24	34	22	10,000	14,500	770	1180
5	27th	1‡	5	1.0	18	17	17	9	11,200	14,700	1180	1380
Patient no. 3												
1	22nd	0.1	1	0.4	59	63	59	66	6100	8600	160	110

* Disodium cromoglycate, 20 mg., administered as a powder through a 'Spinhaler' before challenge.

† Disodium cromoglycate given 1, 4, 7, and 10 hours after challenge.

‡ Disodium cromoglycate given before challenge, and 3, 6, and 10 hours afterwards.

a higher concentration (10 mg. per ml.); this was followed by an immediate asthmatic reaction and then a late asthmatic reaction which was weaker than might have been expected judging by tests 1 and 4 in which the late reaction was greater than the immediate reaction. In test 4 (1 mg. per ml.) immediate and late asthmatic reactions comparable with those in test 1 (1 mg. per ml.) were produced. The tests were followed by leucocytosis without fever; no crepitations appeared, and there was no decrease in total lung capacity (T.L.C.) or CO transfer factor to suggest a peripheral, "alveolar" reaction.

Case 2

Test 1 (0.1 mg. per ml.) caused a late pulmonary reaction, and test 2 (1 mg. per ml.) a stronger one. The late reaction seemed to consist of both bronchial asthmatic and peripheral "alveolar" components. Chest tightness, high-pitched expiratory rhonchi, and crepitations developed, and only partial improvement in ventilation followed isoprenaline and adrenaline. The CO gas-transfer factor was not reduced. Test 3 (1 mg. per ml.) showed that the sensitivity had increased, for on this occasion there was, in addition, an immediate reaction, followed by a more vigorous late reaction with a temperature of 38.1°C. In test 4 (1 mg. per ml.) the challenge was followed after 1, 4, and 10 hours, and after the onset of the immediate reaction, by the inhalation of disodium cromoglycate. The immediate asthmatic reaction was stronger than in the previous test, and the patient was flushed, felt nauseated, and had tachycardia. The late pulmonary reaction, however, was reduced. In test 5 (1 mg. per ml.) the disodium cromoglycate was given 12 minutes before the challenge, and 3, 6, and 10 hours afterwards on this occasion. The immediate asthmatic reaction was reduced, there were no systemic manifestations, and there was an even greater reduction of the late reaction than in test 4.

The tests were followed by a leucocytosis maximal at 7 hours, when the eosinophil count was low. As the leucocytosis decreased, the numbers of eosinophils rose progressively 24–92 hours after each test, reaching a maximum of 1380 per c.mm. The increase in eosinophils seems compatible with the increasing immediate reactions to successive tests. The leucocytosis and eosinophilia did not seem to be modified by the administration of disodium cromoglycate.

Case 3

This patient was highly sensitive to the enzyme preparation. He gave a vigorous immediate skin-test reaction and a vigorous immediate asthmatic reaction to the inhalation test (0.1 mg. per ml.), followed by a late asthmatic reaction appearing after about 4 hours. He maintained that the asthmatic reactions he had had at work were more severe than this. Because of the extent of the immediate reaction he was given an injection of adrenaline, and there was a return of the F.E.V.₁ to pre-test levels by 2 hours. Later inhalations of isoprenaline and injections of adrenaline and aminophylline did not prevent the appearance of the late reaction. No crepitations appeared, and there was no decrease in the T.L.C. and CO transfer factor to suggest a more peripheral reaction.

Serological Tests

Agar-gel double-diffusion precipitin tests were made at 25°C and 4°C with the crystalline proteinase of *B. subtilis*, with carbol/saline freeze-dried extracts of preparations "A" and "M", and with extracts of spores of *B. subtilis* cultured from preparation "A". Table IV shows that more reactions were obtained, particularly to the crystalline proteinase, in asthmatic and healthy subjects not working with the enzyme material than in the exposed subjects. Reactions were given only to the "A" extract by two of the group of exposed workers and one control, and only to the "M" extract by one asthmatic patient. All three of the workers who were tested by inhalation gave positive precipitin reactions to the crystalline proteinase. Patient no. 3 also reacted to the "A" and "M" extracts. Of the sera giving positive reactions at 4°C, only

TABLE IV—NUMBERS OF SUBJECTS WITH AGAR-GEL PRECIPITIN REACTIONS (4°C)

Subjects	No.	Crystalline proteinase		Extracts (30 mg./ml.)		Extract of <i>B. subtilis</i> ("A", 10 mg./ml.)
		1 mg./ml.	10 mg./ml.	"A"	"M"	
Exposed workers with symptoms	18	4	5	3/13	3	0
Exposed symptom-free workers	4	1	0	1	0	0
Asthma patients	20	15	11	12	7	0
Healthy subjects	20	10	9	8	8	0

two-thirds reacted at 25°C, and then far more weakly, presumably because the proteolytic enzymes had digested the immunoglobulins or the formed complexes. Hydrolysis of precipitin complexes produced by a similar proteolytic enzyme from *B. licheniformis* in tests against rabbit antisera has been reported by Bernlohr (1964). We observed similar effects of the *B. subtilis* enzyme preparations on immunoglobulins and complexes produced by human precipitins with antigens of *Aspergillus fumigatus*. The precipitin reaction was shown to occur between the γ -globulin, separated from, the sera of patient no. 3, one of the asthma patients, and one of the normal subjects by the method of Levy and Sober (1960), and the "A" extract.

Discussion

Respiratory symptoms developed after starting work with high concentrations of enzyme preparations from *B. subtilis* in two subjects (nos. 1 and 2). In a third (no. 3) symptoms appeared after working with other non-soap detergents, but there was evidence from skin and inhalation tests that he later developed strong allergic sensitivity to the enzyme preparations. These materials are irritant to the skin in areas of high perspiration. An allergic mechanism for their effect on the respiratory tract is, however, suggested by the inhalation-test reactions, and by the strong immediate prick-test reaction to low concentrations, particularly of the crystalline proteinase, in the three affected workers studied here and in the other exposed workers reported by Flindt (1969). No reactions were obtained with much higher doses to an inhalation test in a healthy subject not working with this material, and virtually none to skin tests in control subjects.

Inhalation tests with low concentrations of the enzyme extracts caused immediate and late bronchial reactions, and in one patient a more peripheral reaction. These resembled the dual reactions in patients with allergic bronchopulmonary aspergillosis (Pepys, Hargreave, Chan, and McCarthy 1968), and in some patients with avian allergy (Hargreave 1969), in whom disodium cromoglycate had an inhibitory effect similar to that in the patients reacting to the enzyme material. One of the two affected workers also gave a dual skin-test reaction like that observed in the above diseases. Dual reactions of this type are associated with precipitins and seem to be dependent upon the presence of immediate-type reactions as well (Pepys et al. 1968); both were present in the three patients. It is not clear what may be the significance of the precipitins and of their greater incidence in asthmatic and healthy subjects who gave no skin-test reactions, nor why fewer of the exposed workers gave positive reactions. One possibility is that the exposure of the workers to the dust decreased their precipitin levels, as Massoud and Taylor (1964) observed in patients with precipitins to cotton extracts following inhalation tests with these extracts.

The increasing immediate reactions and eosinophilia

on repeat inhalation tests in one of the patients (no. 2) reflects the possible effects of repeated exposure to high concentrations of this particular enzyme material at work. The persistence of airways obstruction, only partially or not reversible by isoprenaline or corticosteroids after cessation of exposure, is like that seen in patients basically non-atopic in whom the late bronchial reactions seem to be mediated by precipitins. The three affected workers seemed to be non-atopic subjects: none of them gave a history suggestive of atopic sensitivity or reacted to prick tests with a battery of common allergens.

The enzyme materials can be added to the list of organic dusts, many of which are innocuous in themselves, but are capable of provoking complex allergic reactions in the bronchi and in the peripheral lung tissues (Pepys 1969). The part played by their enzymatic effects in themselves is difficult to estimate.

Requests for reprints should be addressed to J. P.

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PHLEBOTOMY AND IRON-OVERLOAD IN PORPHYRIA CUTANEA TARDA

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Summary The part played by hæmosiderosis in porphyria cutanea tarda (P.C.T.) has been investigated in three patients with the syndrome. Iron storage was evaluated before and after remission of the porphyria had been induced by multiple phlebotomies. Liver-biopsy specimens showed residual hepatic siderosis in two patients after remission. The liver of the third had become free from stainable iron, but her serum iron and transferrin levels were normal. In two patients iron excretion was determined after test doses of intramuscular deferoxamine, and in both it fell from a slightly raised level before therapy to a normal level after therapy. A separate study in rats with experimental (hexachlorobenzene-induced) porphyria showed that the superimposition of hepatic siderosis did not increase the severity of the porphyria. It is concluded that hepatic siderosis may not play as crucial a role in the pathogenesis of P.C.T. as has previously been surmised, and that the response to phlebotomy therapy depends probably on a more complex mechanism than simple elimination of iron.

Introduction†

ALTHOUGH it has been nearly a decade since Ippen (1961) first showed that multiple venesections produced clinical improvement and biochemical remission in most patients with porphyria cutanea tarda (P.C.T.), we still do not understand clearly how these occur. The prevailing

opinion is that improvement is related to elimination of the excessive storage-iron found in most, but not all, cases of P.C.T.

The kinetics of iron metabolism have been investigated in a number of these patients (Saunders 1963, Price et al. 1968), often with equivocal results, but only a few workers have re-evaluated the iron status after phlebotomy-induced remission of the P.C.T. (Hickman et al. 1967, Strickland 1968). In any given case it is impossible to predict, from the severity of the clinical manifestations or from the degree of porphyrinuria, whether or not hæmosiderosis is present. In this study we report, firstly, an experimental study on the effect of iron administration on the course of hexachlorobenzene-induced porphyria in rats (admittedly, only a rough counterpart of human hepatic porphyria), and secondly our experience with three patients with P.C.T. and associated hæmosiderosis in whom iron-overload was evaluated before and after phlebotomy therapy.

Methods

Animal Experiments

Female Sprague-Dawley rats, averaging approximately 75 g. body-weight, were divided into five groups according to their diets and whether or not they had had injections.

Group I—standard diet.

Group II—standard diet and injected iron-dextran (100 mg. I.P. at start of experiment).

Group III—0.2% w/w hexachlorobenzene (H.C.B.) diet.

Group IV—0.2% H.C.B. diet and injected iron-dextran (100 mg. I.P. at start of experiment.)

Group V—0.2% H.C.B. diet and injected iron-dextran (125 mg. I.P. after induction of porphyria).

There were two rats in group I, three in group II, and six each in groups III, IV, and V. 24-hour urinary porphyrins were estimated by the method of Schwartz et al. (1960). At necropsy, done the day after the final 24-hour urine collection, fragments of liver were examined under ultraviolet light for the characteristic red fluorescence of porphyrins. Sections of liver were also stained for iron.

Patients

We have observed excellent clinical and biochemical responses in six patients with P.C.T. (four with and two without demonstrable hæmosiderosis) whom we have treated by multiple venesections, but we are only presenting in detail the data on the three patients we have studied most thoroughly.

Patient 1.—A 65-year-old woman, admitted to hospital in November, 1966, first showed signs and symptoms of P.C.T. in 1961. She maintained that she was a sworn teetotaler. She had had diabetes mellitus for at least 20 years, and was on insulin. The patient could not relate the skin lesions to sunlight exposure, but believed that when she had tried an oral hypoglycaemic agent (tolbutamide) several years before they had become worse. She had taken iron orally for vague reasons for a brief period in 1954; she had never had a blood-transfusion. She had had a hysterectomy and oophorectomy in 1947; no hormone replacement therapy had been given. She claimed to have had hepatitis in 1925. The family history was non-contributory, except for a diabetic mother. On physical examination there was considerable periocular suffusion, and there were multiple erosions over the head and extremities including the shoulders, scattered tense vesicles on the forearms, hypertrichosis of the face and arms (with patchy non-scarring alopecia of the frontal scalp), and sclerodermoid changes in the neck and upper arms. The liver was slightly enlarged. Pertinent laboratory data included a hæmatocrit of 42%, a serum iron and iron-binding-capacity (Fe/FeB.C.) of 200/248 µg./100 ml., and a negative Watson-Schwartz test for urinary porphobilinogen (P.B.G.). 24-hour urinary porphyrin excretion (Schwartz et al. 1960) was 2271 µg. uroporphyrin, and 106 µg. coproporphyrin. Skin biopsies revealed subepidermal vesiculation and considerable dermal sclerosis; iron-staining for excess tissue iron was negative. Light microscopy of a marrow

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