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Characterisation of textile dust extracts: II Bronchoconstriction in man

J S DOUGLAS, PAMELA G DUNCAN, AND EUGENIJA ŽUŠKIN

From the John B Pierce Foundation Laboratory, New Haven, Connecticut 06519, USA

ABSTRACT Aqueous extracts of cotton bracts induce a concentration dependent decrease in flow rates on partial expiratory flow volume curves. It has been shown that the active substance(s) is stable to moderate heat and to acid or alkaline conditions. Results of dialysis and gel filtration suggest that the active component(s) has a molecular size of about 1000 daltons. The component(s) is not absorbed on to ion exchange resins but is readily removed from aqueous extract by activated charcoal. The bronchoconstrictor agent cannot be steam distilled or extracted by ether. It is readily soluble in water; a single extraction of bracts removes all biologically active material. It is proposed that healthy subjects, responsive to cotton bracts extract, can be used qualitatively to fractionate and quantitatively to assay the active substance(s) in this material.

In many textile mill workers the inhalation of cotton, flax, or hemp dust causes acute bronchoconstriction. Laboratory studies have shown that inhalation of aqueous extracts of cotton bracts[1–5] or hemp dust[3 6] cause similar subjective and physiological symptoms to those experienced by mill workers.[7–9] The purpose of this study was to assess the bronchoconstrictor response to fractions of cotton bracts extract.

Methods

PULMONARY FUNCTION TESTING

Inhalation testing

Two panels of 10 individuals were used. Group A consisted of six men and four women and group B six women and four men. Each subject was required to sign an informed consent form approved by the human investigations committee and to complete a standard MRC respiratory symptom questionnaire.[10] Before the inhalation of each extract to be tested (see below), each subject performed the expiratory manoeuvres described as follows: on each occasion the subject inhaled to about 65% of forced vital capacity (FVC) and performed a forced expiration (partial expiratory flow volume curve, PEFV[11]) then inspired to total lung capacity (TLC) and performed a second forced expiration to generate a maximal expiratory flow volume curve (MEFV) (fig 1). These flow volume curves were recorded using a pneumotachograph integrator device[12] and a Brush 500 high performance XY recorder (Gould Electronics, Oxnard, Calif). The subject was asked to repeat this manoeuvre five times. In two subjects we also determined airway resistance using a constant pressure body plethysmograph.[7 13]

The subject then inhaled an aerosol of 0·9% w/v saline, Tyrode's solution, or one of the extracts for 10 minutes (see results). In addition, three subjects were challenged with a condensed tannin aerosol. This solution was prepared by extracting the tannin with 0·9% w/v sodium chloride. We also challenged three other subjects with methyl piperonylate (0·09% w/v) dissolved in 0·9% w/v saline. Before aerosol inhalation, each extract was filtered through a sterilising filter (Nalgene Gelman Metricel, 0·2 micron porosity, Curtin Matheson Scientific Inc, Boston, Mass). The aerosols were produced with a D30 generator[14] which produced 0·25 ml of aerosol per minute at a disruptive pressure of 15 psi (mean particle size, 0·05 μM). The generated aerosol was temporarily stored in an open ended tube on the inspiratory side of a Collins "J" valve. Subjects in group A repeated the respiratory manoeuvres at 15 minute intervals for up to one hour. Subjects in group B repeated the tests at 20 minute intervals for up to 100 minutes. Our study necessitated the use of subjects who responded to the cotton bracts extract, thus all potential participants were first tested with

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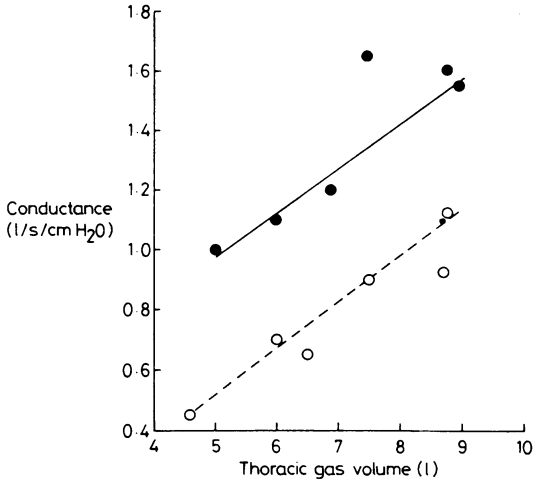

Fig 1 *Airway conductance data in a man. Conductance volume plots before (●) and after (○) a 10 minute inhalation of single strength cotton bracts extract. Values were determined using a body plethysmograph and were determined 20 minutes after inhalation. For details see methods.*

freeze dried boiled cotton bracts extract. Only those showing a strong positive inhalation test were retained for further participation in the study (see results). Subjects were tested with one extract a week to avoid tachyphylaxis.[1]

Calculation of pulmonary function data

From each curve, at each testing time, we measured the FVC. The values were averaged and 60% of FVC was calculated. Forced expiratory flow rates at 40% FVC were interpolated from both the partial (PEFV) and maximal (MEFV) flow volume curves. These values were determined using TLC as the fixed reference point—that is 40% FVC = TLC (point of maximal inspiration) (fig 1)—60% FVC. FEV_1 was established by a time marker on the axis of the maximal expiratory flow volume curve. At each testing time we determined the mean of the two best—that is, greatest—manoeuvres and expressed the values as a percentage of the control value before aerosol inhalation. Values shown are the means ± SEM. Differences were evaluated using non-normalised data with Student's *t* test for paired variates. A p value of $<0{\cdot}05$ was considered to be significant.

PREPARATION OF TEXTILE EXTRACTS TESTED

Extracts were prepared as described in the accompanying paper[15] and were: boiled, steam distilled, ether extracted, charcoal treated, dialysed, and freeze dried, boiled bracts. In addition the following extracts were prepared.

Steam distillate residue—The steam distillate was prepared as described.[15] The concentrated residue was reconstituted in deionised water in a volume equal to the original bracts extract before steam distillation.

Second extract of bracts—The residue of cotton bracts isolated by filtration after the first buffer extraction was re-extracted with Tyrode's solution as described in the accompanying paper.[15]

Boiled bracts dialysate—Double strength boiled bracts was dialysed in visking tubing for 48 hours against deionised water. The dialysis was carried out in a large beaker and three exchanges of dialysis fluid were made. The dialysate (12 litres) was freeze dried and reconstituted to the same volume as was placed in the visking tubing—that is, equivalent to double strength boiled bracts, using deionised water.

Chromatographic separation of boiled bracts through Sephadex—Boiled bracts (double strength) was applied to columns of Sephadex G10 (75 cm × 2·5 cm). The columns, precalibrated with blue dextran 2000 and sodium chloride, were developed with 0·01M phosphate buffer. Fractions (4 ml) were collected using a fraction collector (Fractomette 200, Buchler Instruments Inc, Fort Lee, NJ). As a control, 0·01M buffer was passed through the column and similarly collected. In preliminary experiments we examined the elution of single, double, and quadruple strength bracts from the column to ensure that the column capacity was not exceeded. Each fraction was examined for (*a*) protein content using Folin phenol reagent,[16] (*b*) hexose content using phenol sulphuric acid,[17] and (*c*) conductivity using a standard conductivity cell and meter (Radiometer, Copenhagen). Based on the outcome of these assays, fractions were pooled, freeze dried, and reconstituted to double strength in deionised water.

Chromatographic separation of boiled bracts through ion exchange resins—Freeze dried double strength boiled bracts extract, adjusted to pH >9·5 with NH_4OH (1N), was passed through a glass column (160 mm × 10 mm, Chromaflex, Kontes Glass, Vineland, NJ) packed with ion exchange resin. The cationic resins used were Amberlite CG50, H^+ form (Mallinckrodt Chemical Works, St Louis, Mo) and Dowex 50W, 200–400 mesh, H^+ form (Sigma Chemical Company, St Louis, Mo). Similarly, the extract, adjusted to pH 3 with acetic acid (2N), was passed through an anionic exchange resin (Dowex 1 × 10, J T Baker Chemical Company, Phillipsburg, NJ). The material which passed straight through the columns was collected, freeze dried, and reconsti-

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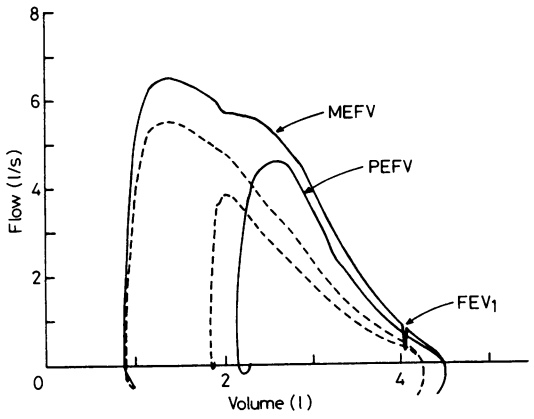

Fig 2 *Flow volume curves in a woman. Partial expiratory flow volume (PEFV) and maximal expiratory flow volume (MEFV) curves are shown before (–) and 80 minutes after (– – – –) a 10 minute inhalation of double strength boiled bracts extract. Time marker showing FEV_1 is also shown. See methods for details.*

tuted to double strength using deionised water. The cationic resins were then washed with 3N HCl and the anionic resin with 3N NH_3. These eluates were adjusted to pH 7 and clarified by centrifugation at 5000 rpm for 15 minutes.

Results

Inhalation of aerosolised saline, Tyrode's solution, and some of the aqueous extracts (see below) induced no measurable lung function changes in either group of subjects. By contrast, the inhalation of an aerosol of bracts extract induced bronchoconstriction in normal healthy subjects (figs 1 & 2). Pulmonary function changes occurred in both central and peripheral airways—that is, flow rates at both high and low lung volumes and airway conductance were significantly affected (figs 1 & 2). Flow rates on the PEFV curve were the most sensitive indicator of a positive inhalation test. Reductions in flow on MEFV curves and of FEV_1 and FVC were also observed but were smaller. Conductance was

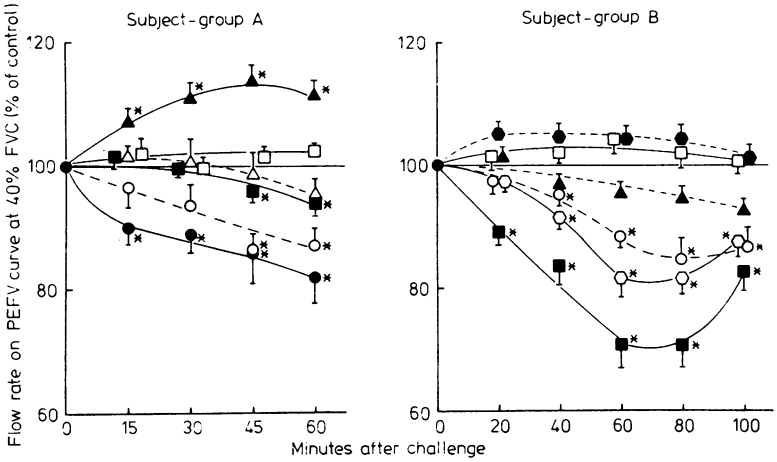

Fig 3 *Pattern of response after inhalation of cotton bracts extracts. Subjects, selected as described, performed prescribed pulmonary function tests (see methods). They then inhaled one of various preparations of cotton bracts for 10 minutes from a Dautrebande D30 generator. After inhalation pulmonary function tests were again measured at 15 min (left panel) or 20 min (right panel) intervals. Extracts tested and number of subjects used (N) were as follows: group A: ● single strength boiled bracts, (8); ○ freeze dried single strength boiled bracts, (5); △ ether extract of boiled bracts, (8); ▲ steam distillate of cotton bracts, (7); ■ residue of steam distillate, (5); □ charcoal treated boiled bracts, (7). Similarly, in group B: ○ freeze dried single strength boiled bracts, (8); ■ freeze dried double strength boiled bracts, (9); □ charcoal treated boiled bracts, (7); ▲ 2nd leaf extraction, (8); ʘ double strength dialysate of boiled bracts, (8); ● boiled bracts after 48 hour dialysis (5). Data shown are mean values ± SEM of normalised values. *Values significantly different from control flow rates using Student's t test for paired variates.*

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altered as much as flow but was not used as the sole measure of response to aerosols (see discussion).

Twenty of the 26 volunteers tested gave a positive inhalation test to single strength bracts on the first challenge and were asked to participate in the study. In all subjects the pulmonary response was slow in onset, taking at least 60 minutes to reach maximal effect (fig 3 and 4). Preliminary testing showed that the response was reproducible provided that challenges were spaced at least three days apart. The magnitude of the response to bracts extract was not the same in all subjects, the decrease in flow rate ranged from 11·4% to 45% with single strength bracts (fig 5). When bracts extract was freeze dried and reconstituted in deionised water the biological activity was unchanged. Boiling of the extract caused precipitation of large quantities of material. This resulted in a much "cleaner" extract which retained all of its bronchoconstrictor activity. We therefore boiled all bracts extracts used in subsequent studies. Double strength bracts extract induced a significantly greater bronchospasm than single strength bracts in every subject—that is the response was dose related and could be used as the basis for a quantitative bioassay of all the fractions tested (fig 5; see below). A second extraction of bracts did not yield more biological activity. Charcoal treated bracts caused no change in PEFV curves (fig 3). The ether extract of bracts was also pharmacologically inert whereas the steam distillate induced a significant bronchodilation in all the sub-

Recovery of bronchoconstrictor activity

Fractionation method	*Fraction tested*	*% recovery of activity**
Dialysis	Dialysed bracts	0
	Dialysate	64·7 ± 5
Ether extraction	Ether extract	0
Steam distillation	Distillate	Bronchodilator
	Residue	47 ± 4
Sephadex	Void volume	0
Chromatography	Conductivity peak	81·3 ± 13
Weak cation	Unabsorbed material	80·0 ± 8
Exchange resin	Acid eluate	0
Strong cation	Unabsorbed material	85·0 ± 9·5
Exchange resin	Acid eluate	0
Strong anion	Unabsorbed material	85·0 ± 11·9
Exchange resin	Ammonia eluate	0
Charcoal treated	Supernatant	0

*Recovery was determined from dose response curves and is the ratio of the units of activity recovered after fractionation to the units of activity in the bracts extract before fractionation. Values are mean ± SEM.

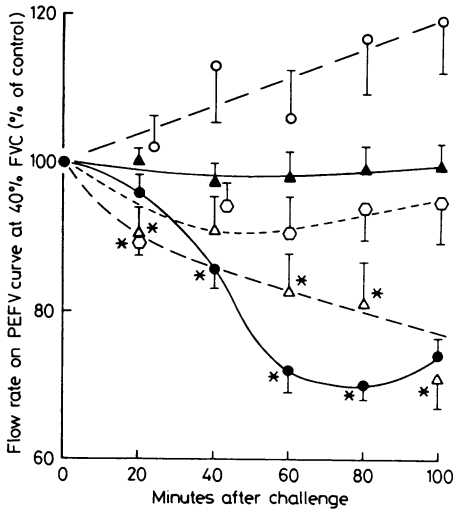

Fig 4 *Pattern of response after inhalation of cotton bracts extracts (fig 3 for details). Double strength boiled bracts fractions tested and number of subjects (N) were: ▲ Sephadex G10 void volume double strength, (6), ● weak cationic, ● strong cationic (4), and ● strong anionic (4) (data combined); ○ weak cationic acid eluate, (3); ○ strong cationic acid eluate (4) (data combined) and ○ strong anionic 3N NH_3 eluate (4). Also shown is the activity of the conductivity peak (△) eluted from the sephadex G10 column (4) (fig 6 for details).*

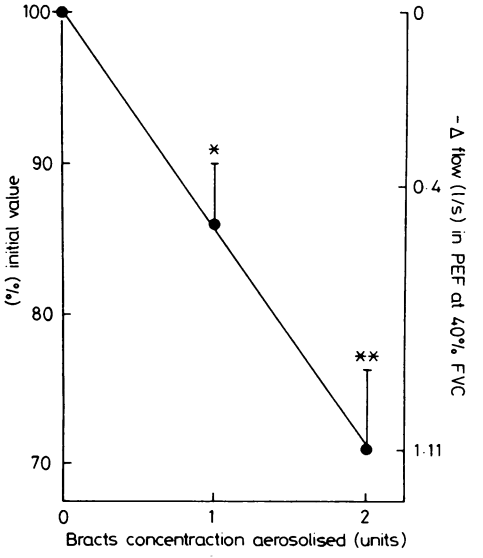

Fig 5 *Dose response curves to boiled cotton bracts extract. Boiled cotton bracts extract was prepared as described in methods, then freeze dried. Residue was reconstituted to single (1 unit) or double (2 units) strength. Subjects inhaled aerosols (for 10 minutes) of each concentration. Tests were spaced one week apart. Response to extracts was determined from PEFV curves 80 min after inhalation. Values are mean ± SEM from nine subjects. *Values significantly different from control (Student's t test for paired variates, $p<0·01$). **Values significantly different from response to one unit bracts extract ($p<0·01$).*

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jects tested (fig 3). The residue from steam distillation had about 50% of the original bronchoconstrictor activity. Dialysis of the extract showed that the active constituent(s) was of small molecular size since we measured about 65% of the original biological activity in the dialysate while the dialysed material was biologically inert (table). Neither the tannin extract nor methyl piperonylate (0·09% w/v) induced bronchoconstriction in vivo. The bronchoconstrictor activity was not removed by column chromatography using cationic or anionic exchange resins (fig 4; table). No additional biological activity was recovered if these columns were developed with acid or alkali (fig 4; table).

Gel filtration chromatography using Sephadex G10 separated the constituents of bracts on the basis of molecular size. The elution profiles of blue dextran and sodium chloride showed that the void volume of the column was between 0 ml and 170 ml and the total exclusion volume between 240 ml and 280 ml. The position of the peaks seen when single, double, or quadruple strength bracts were chromatographed was similar indicating that the column capacity had not been exceeded. The pigmented material in the bracts extract was eluted in the void volume of the column between 140 ml and 160 ml. This peak absorbed visible light at 625 nM and gave positive reactions in the Folin and Hexose assays. It was, however, biologically inert (fig 4). Several materials were eluted between the end of the void volume and the total exclusion volume (170–240 ml). The test results for protein residues and hexose were positive. Although there was no absorption at 625 nM, the substance(s) present absorbed light at 280 nM. About 80% of the biological activity was eluted between 170 ml and 240 ml. Measurements of conductivity between 170 ml and 240 ml showed the presence of two peaks, one eluting between 170 ml and 200 ml, the other eluting at the total exclusion volume of the column (fig 6). Most of the biological activity was associated with the fraction showing a rise in conductivity, although it should be noted that this fraction (170–200 ml) also contained positive tests for protein residues and hexose.

Discussion

Evaluation of environmental conditions in the workplace can be rapidly and quantitatively assessed by measuring the bronchoconstrictor reponse to textile dusts. Exposure to textile dusts of >10 years' duration may induce a permanent and irreversible impairment of lung function and there is a correlation between dust concentration and the severity of the disease.[18] Based on these observations, it has been proposed that (*a*) workers showing acute reactions should be moved to less dusty areas since they would be considered "at risk" and (*b*) fine dust levels (<0·7 μM) should be reduced to <0·1 mg/mm.[18] These are arbitrary criteria for establishing controls. It has never been established that there is an absolute and progressive relationship between the acute symptoms (grades 0–1) and the chronic forms (grades 2–3) of byssinosis.[19,20] Thus while functional measurements may help to reduce the incidence of acute symptoms, they may have no impact on the development of chronic, disabling forms of byssinosis. Epidemiological studies show that there is a higher incidence of byssinosis when low quality cotton is processed. Therefore a standard based on dust levels or particle size alone does not take into account dust quality. More realistic and enforceable standards can only be set when the causative agent(s) in raw cotton is identified and can be quantitatively assayed.

In this study we have developed an assay system that mimics the acute byssinotic response. Inhalation of an aqueous extract of cotton bracts causes a gradually increasing bronchoconstriction that takes up to one hour to reach its peak (figs 2 & 3). This pattern distinguishes the response from other nonspecific reactions which are generally both rapid in onset and of short duration. Conductance changes, which also occur, reflect constriction in large and central airways. We used PEFV curves to assess responses to inhaled extracts to be sure that our subjects had peripheral airway responses since, according to Bouhuys and van de Woestijne,[7] these individuals would be more likely to develop chronic byssinosis. The response rate of our subjects (77%) is similar to that seen in industrial environments.[5] Testing large numbers of fractions was not practical since at least one week between challenges was required to avoid tachyphylaxis and so simple fractionations yielding two components were therefore decided on. This enabled the testing of both components of each separation to (*a*) eliminate the possibility of false positives, (*b*) ensure that the biological activity was not destroyed by the fractionation procedure, and (*c*) ensure that the biological activity was fully accounted for. Based on our data, a bracts extract can be boiled, chromatographed via ion exchange resins and Sephadex G10, and a fraction of molecular weight between 100 and 1000 daltons isolated. This refined material when freeze dried and reconstituted has almost as much biological activity as the original boiled bracts extract (table). Sephadex chromatography shows that the biologically active component, the conductivity peak (fig 6), is clearly distinct from byssinosan, tannins, methyl piperonylate, and some other components

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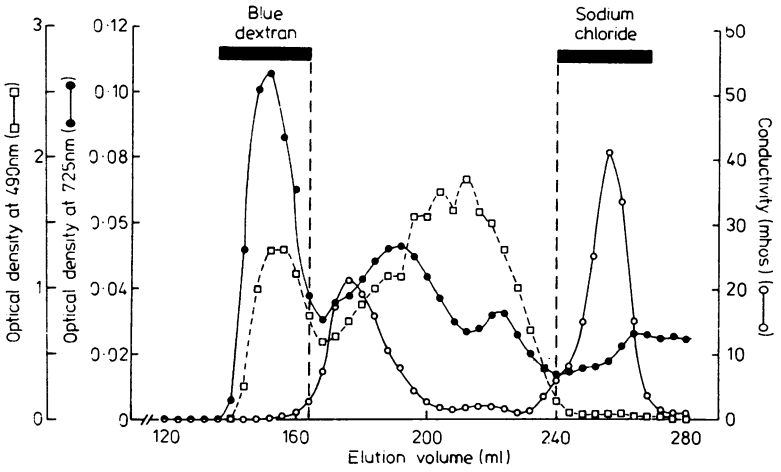

Fig 6 *Elution profile of boiled bracts from Sephadex G10. Four millilitres of quadruple strength boiled bracts were applied to a Sephadex G10 column. Solid horizontal bars show column calibrations—namely, a blue dextran marker designating end of void volume (0–170 ml) and sodium chloride peak labelling total exclusion volume of column (240–280 ml). Conductivity (○), carbohydrate content expressed as absorbance at 490 nM, and protein residues expressed as absorbance at 725 nM for samples from each 4 ml aliquot are shown.*

which have been associated with byssinosis.[21–29]

Our data show that the agent(s) is highly water soluble. Steaming of cotton bales has resulted in reduced biological activity,[30] and our results suggest that a vigorous washing of cotton bales before processing might be a simple and effective way to control the acute symptoms of byssinosis. On a more quantitative level, the assay of a particular class of substance in the conductivity peak, amino-acids or sugars, for example, determined using simple colorimetric procedures might correlate with the bronchoconstrictor activity in vivo in man. Previously published data suggest that hexose determinations in the conductivity peak fraction would be an appropriate starting point.[31]

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Requests for reprints to: Dr James S Douglas, John B Pierce Foundation Laboratory, 290 Congress Ave, New Haven, Connecticut.

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