EFFECTS OF CHEMICALS ASSOCIATED WITH COTTON DUST ON ALVEOLAR MACROPHAGE FUNCTION

P.R. Miles, M.J. Reasor, C.A. Glance, and V. Castranova
National Institute for Occupational Safety and Health and West Virginia University, Morgantown, West Virginia 26505

Abstract

Alveolar macrophages are lung cells which kill and ingest bacteria and foreign particles. Thus, these cells serve as the first line of defense against airborne material. We studied the effects of three natural products associated with cotton, i.e., gossypol, rutin, and catechin, on the function of rat alveolar macrophages. These substances have no effect on cellular volume; i.e., they do not cause membrane leakiness. However, the products do have some inhibitory effects on the following physiological parameters: resting and particle-stimulated oxygen consumption; release of antibacterial substances (reactive forms of oxygen), ingestion of foreign particles; and the activity of acid phosphatase, a lysosomal enzyme which is responsible for digestion of ingested bacteria and/or particles. In general, gossypol is the most toxic of the chemicals tested and catechin is the least toxic. These results indicate that natural products associated with cotton dust can compromise alveolar macrophage function.

Introduction

Byssinosis is an occupational respiratory disease associated with the inhalation of dust by cotton mill workers. This disease is characterized by chest tightness, increased airway resistance, decreased forced expiratory volume (FEV_1), and pulmonary inflammation. However, to date neither the etiologic agent(s) nor the mechanism for the pathogenesis of byssinosis has been determined.

Alveolar macrophages are phagocytic cells found in the alveoli and the small airways of the lungs. Their main function is to engulf inhaled particulates and, thus, cleanse the lungs of debris (1). It is possible that any initial pulmonary response to inhaled cotton dust would involve this cell type. Rylander and Lundholm (2) have reported an increase in the number of alveolar macrophages in the lung after exposure to bacteria commonly associated with cotton. Macrophages exposed to cotton dust have been shown to secrete chemoattractants for leukocytes, resulting in pulmonary inflammation (3). In addition, cotton extracts stimulate the release of prostaglandin F2 (alpha) from alveolar macrophages (4). Since these data demonstrate that alveolar macrophages are affected by exposure to cotton, further investigation concerning these interactions is warranted.

A large number of natural products have been isolated from the cotton plant. These products act in the plant as insecticides, bactericides, fungicides, viricides, and nematicides (5). Included among these products are: terpenoid aldehydes such as gossypol, flavonol glycosides such as rutin, and condensed tannins such as catechin. Bell and Stipanovic (6) have reported that some of these products are found in high concentrations in mill dust; e.g., mill dust contains 600 ppm gossypol and 7% tannins by weight, while containing only trace amounts of rutin. Therefore, it is possible that these compounds may be involved in byssinosis. However, the effects of these natural products associated with cotton on pulmonary macrophages have not yet been extensively studied.

The objective of this investigation was to determine the effects of three natural products from cotton, i.e., gossypol, rutin, and catechin, on functional properties of alveolar macrophages. These properties are cellular volume, oxygen consumption, secretion of reactive oxygen species, lysosomal enzyme activity, and phagocytosis. These data have been reported previously in abstract form (7).

Materials and Methods

Source of Cotton Materials

The cotton dust samples used in this investigation were supplied by Cotton Incorporated. Dust samples were collected through a rotating screen condenser from the drawing and spinning areas of a textile mill which used 100% West Texas cotton. In this study, we used the fraction of the dust containing particles less than 38 μm in diameter.

An aqueous extract of the dust was obtained using the standardized procedure outlined by Cotton Incorporated. Cotton dust (1 gram) was incubated at 4°C for 1 hour in 25 ml of pyrogen-free water. Then this suspension was centrifuged at 10,000g for 10 minutes. The supernatant was collected and put through a 0.45 μm filter. This filtrate was the aqueous cotton extract used in this study.

We also evaluated the effects of three natural products associated with the cotton plant on alveolar macrophages. We tested the effects of gossypol, rutin, and catechin on alveolar macrophage function. Gossypol acetate was obtained from Calbiochem-Behring (La Jolla, CA). Rutin (quercetin-3-β-D-rutinoside) and (+)-catechin were obtained from Sigma Chemical Company (St. Louis, MO).

Preparation of Cells

Male Sprague-Dawley rats (200-250 gm) were anesthetized with sodium pentobarbital (0.2 gm/kg body weight) and exsanguinated by cutting the renal artery. Alveolar macrophages were obtained by pulmonary lavage using an ice-cold, Ca^{++}-free, phosphate-buffered solution (145 mM NaCl, 5 mM KCl, 1.9 mM NaH_2PO_4, 9.35 mM Na_2HPO_4, 5.5 mM glucose (pH = 7.4)) (8). The cells were separated from the lavage fluid by centrifugation at 500g for 5 minutes and washed once in phosphate- buffered medium containing 1mM $CaCl_2$. The number of alveolar macrophages in each sample was determined with an electronic cell counter (Coulter Instrument Co., Hialeah, FL). Cells were preincubated for 15 minutes at 37°C in the absence or presence of cotton dust, aqueous extracts of cotton dust, or chemicals prior to measurements.

Measurements of Functional Properties

Oxygen consumption was measured with an oxygraph equipped with a Clark electrode. Alveolar macrophages (4×10^6 cells) were preincubated at 37°C for 15 minutes in phosphate-buffered medium (1.75 ml) in either the absence or presence of cotton material. The cell suspension was then transferred to a temperature controlled chamber (37°C) and oxygen consumption measured for ten minutes. In some cases, cells were exposed to particles by adding zymosan (6 mg/ml) to the suspension in the oxygraph chamber at zero time.

The mean cell volumes of alveolar macrophages were determined from aliquots of the cell suspensions. Cellular volume was measured using a Channelizer cell sizing attachment to the Coulter electronic cell counter.

Chemiluminescence (CL) was measured as counts per minute in the tritium channel of a liquid scintillation counter operated in the out-of-coincidence mode (9). Alveolar macrophages (4×10^6 cells) were preincubated in 5 ml of phosphate-buffered medium at 37°C for 15 minutes in either the presence or absence of cotton materials before CL was measured and the maximum counts per minute determined. In these experiments, zymosan (6 mg/ml) was added immediately before the CL measurement (zero time).

Phagocytotic rate was quantified by using a suspension of diisodecyl phthalate containing oil-red O and coated with opsonized E coli lipopolysaccharide (10). Alveolar macrophages (2×10^7 cells) were added to 2 ml of phosphate-buffered medium containing 1.26 mM $CaCl_2$ and 1 mM $MgCl_2$. This suspension was preincubated at 37°C for 15 minutes in either the presence or absence of cotton chemicals. Uptake was then initiated by the addition of 0.5 ml of oil suspension. At 0 and 15 minutes, 0.5 ml samples were taken and uptake terminated by dilution with 4.5 ml of a saline solution (154 mM NaCl and 1 mM N-ethyl maleimide). These samples were then centrifuged and the cells washed three times with saline solution to remove extracellular oil-red O. The ingested oil-red

0 was then extracted from the cells with 1.2 ml of dioxane and the optical density determined at 525 nm.

Alveolar macrophages (4 x 10^5 cells) were suspended in 200 µl of Ca-free phosphate-buffered medium and were ruptured by the addition of 50 µl of 1% Triton X-100 to release the lysosomal enzymes. These samples were then preincubated at 37°C in either the presence or absence of cotton chemicals for 15 minutes prior to the initiation of the enzyme reaction by the substrate. Acid phosphatase was measured at pH = 5.0 using p-nitrophenyl phosphate as substrate (11), ß-glucuronidase was measured at pH = 5.0, using p-nitrophenyl-ß-D-glucuronidate as substrate (12), and ß-N-acetyl-glucosaminidase was measured at pH = 4.5 using p-nitrophenyl-N-acetyl-B-D-glucosaminide as substrate (13).

Superoxide anion release was measured directly by determining the reduction of cytochrome c (14). Alveolar macrophages (4 x 10^6 cells) were added to 5 ml of phosphate-buffered medium containing 0.12 mM cytochrome c. This suspension was preincubated at 37°C for 15 minutes in either the presence or absence of cotton material. Then a zero-time sample was taken, centrifuged at 5000g for 5 minutes at 2°C, and the optical density of the supernatant measured at 550 nm with a spectrophotometer. Later, a 30 minute time sample was taken and the optical density measured. The amount of superoxide-dependent cytochrome c reduction is proportional to the difference in optical densities measured over this 30 minute period. Zymosan (6 mg/ml) was added to the suspension at zero time to study the effects of particle exposure. To be certain that reduction of the cytochrome c was due to superoxide, experiments were done in the presence and in the absence of superoxide dismutase.

Results and Discussion

We attempted to monitor the effects of cotton dust or aqueous extracts of cotton dust on alveolar macrophages. However, measurement of oxygen consumption or superoxide anion release proved impossible. We found that both cotton dust or the extract consume large amounts of oxygen even in the absence of alveolar macrophages. This apparent oxygen consumption may be due to bacterial contamination. However, autoclaving the samples does not eliminate this artifact. An artifact was also found in the superoxide anion assay. In this case, both cotton dust and cotton extracts cause reduction of cytochrome c in the absence of alveolar macrophages. Such an artifact invalidates this assay.

Since studies with cotton dust and extracts proved impossible, we investigated the effects of natural products associated with cotton dust on alveolar macrophages. Exposure of cells to 10^{-4}M catechin, rutin, or gossypol for 15 minutes at 37°C has no effect on cellular volume (Table 1). These data suggest that short-term exposure to cotton chemicals does not affect membrane integrity; i.e., the cells do not become leaky and swell.

The effects of cotton chemicals on oxygen consumption at rest and after exposure of cells to zymosan particles are shown in Table 2. Short-term exposure to catechin or rutin at concentrations as high as 10^{-4}M does not affect oxygen consumption of resting or particle-stimulated alveolar macrophages. In contrast, gossypol does adversely affect cellular viability. Alveolar macrophages exposed to particulates seem more susceptable to gossypol exposure than cells at rest. It is not known why particle-stimulated cells are more sensitive to gossypol.

A major function of alveolar macrophages is the secretion of reactive compounds which act to kill inhaled bacteria. Release of these reactive compounds can be determined by monitoring chemiluminescence generated by alveolar macrophages. Catechin, rutin, and gossypol are potent inhibitors of chemiluminescence (Table 3). This inhibition increases with increasing doses of cotton chemicals. The dose-response curve for catechin is shown in Figure 1. These data were analyzed to determine ED_{50} values, i.e., the dose which causes 50% inhibition of chemiluminescence. The potency of the products in inhibiting the release of reactive forms of oxygen from alveolar macrophages is gossypol > rutin > catechin.

Bacterial killing is also affected by the action of lysosomal enzymes. We monitored the effects of the natural products from cotton on the enzyme activity of acid phosphatase, ß-glucuronidase, and ß-N-acetyl-glucosaminidase (Table 4). Of the enzymes tested, only the activity of acid phosphatase was inhibited. Gossypol was the most potent inhibitor of the three products tested while catechin was ineffective.

Since cotton mill workers may be exposed to relatively high levels of dust, it is vital that their alveolar macrophage are capable of engulfing these particulates and, thus, clear it from the lungs. The effects of exposure to cotton chemicals on the phagocytotic activity of alveolar macrophages are shown in Table 5. The data indicate that all three products can inhibit phagocytosis. Their potency is gossypol > rutin > catechin.

In summary, our data indicate that natural products associated with cotton dust can adversely affect the function of rat alveolar macrophages. However, these compounds result in specific rather than generalized toxicity to alveolar macrophages. For example, they inhibit particle-stimulated respiratory burst activity, i.e., chemuluminescence and particle-stimulated oxygen consumption. Membrane integrity is not affected. The sequence of toxicity for these products is gossypol > rutin > catechin.

Other investigators have found that cotton bract extracts affect alveolar macrophages in a specific manner rather than simply killing the cells. Greenblatt and Ziprin (15) have shown that aqueous extracts of bract do not alter membrane integrity monitored by trypan blue exclusion, yet they inhibit chemiluminescence. These data agree with our results for cotton chemicals shown in Table 1 and Table 3. In addition, cotton extracts have also been shown to stimulate the production of prostaglandins by alveolar macrophages (16). Therefore, exposure to cotton does not result in inhibition of all cellular functions, but is rather specific in inhibiting the respiratory burst of macrophages in response to foreign substances and the phagocytosis of particulates. These data from our investigation suggest that exposure of cotton mill workers to cotton dust may compromise the ability of alveolar macrophages to kill bacteria. Bacterial endotoxin has been implicated in the development of byssinosis (17). Therefore, any decrease in the ability of alveolar macrophages to engulf cotton dust and kill its associated bacteria may increase the residence time of these substances in the lung and exasperate the problem. In addition, these workers may be prone to pulmonary infections of all types.

Our results indicate that significant impairment of macrophage function can occur after exposure to as little as 10^{-6}M gossypol or rutin. Maximal effects were observed at 10^{-4}M. Bell and Stipanovic (6) have determined the levels of various chemicals associated with cotton. Relatively high levels of gossypol have been found in mill dust. Large quantities of tannins such as catechin have also been found in mill dust. In contrast, rutin was found at only trace levels. Our data indicate that catechin is relatively inactive and rutin, while active, is not found at high levels. However, gossypol is found at 600 ppm in mill dust (6) and dust levels in mills are now regulated at 200 mg/m^3 (18). Therefore, workers may be exposed to µg quantities of gossypol during the workday. These levels exceed the doses used in our study. Therefore, exposure to gossypol may be a major source of concern for cotton mill workers.

Acknowledgements

The authors would like to thank Dr. Robert R. Jacobs for supplying samples of characterized cotton dust. This investigation was supported in part by the Interagency Agreement, NIOSH-USDA 58-7B30-M28.

References

1. Green, G. M. 1970. Amberson Lecture: In defense of the lung. Am. Rev. Respir. Dis. 102:691-700.

2. Rylander, R. and M. Lundholm. 1978. Bacterial contamination of cotton and cotton dust and effects on the lung. Brit. J. Ind. Med. 35:204-207

3. Rylander, R. and P. G. Holt. 1983. Macrophages-neutrophil-platelet interaction as a mechanism for byssinosis. Proc. Seventh Cotton Dust Res. Conf. P.J. Wakelyn and R.R. Jacobs, eds. pp 32-33.

4. Fowler, S. R., R. L. Ziprin, M.H. Elissable, Jr., and G.A. Greenblatt. 1981. The etiology of byssinosis possible role of prostaglandin $F_{2\alpha}$ synthesis by alveolar macrophages. Am. Ind. Hyg. Assoc. J. 42:445-448.

5. Bell, A. A. and R. D. Stipanovic. 1978. Biochemistry of disease and pest resistance in cotton. Mycopathologia. 65:91-106.

6. Bell, A. A. and R. D. Stipanovic. 1983. Biologically active compounds in cotton: an overview. Proc. Seventh Cotton Dust Res. Conf. P.J. Wakelyn and R.R. Jacobs, eds. pp. 77-80.

7. Castranova, V., C. A. Glance, and M.J. Reasor. 1983. The effects of chemicals associated with cotton dust on rat alveolar macrophage function. The Physiologist 26:A-125.

8. Myrvik, Q. N., E. S. Leake, and B. Fariss. 1961. Lysozyme content of alveolar and peritoneal macrophages from the rabbit. J. Immunol. 86:133-136.

9. Miles, P. R., V. Castranova, and P. Lee. 1978. Reactive forms of oxygen and chemiluminescence in phagocytizing rabbit alveolar macrophages. Am. J. Physiol. 235:C103-C108.

10. Cox, J. M. and T. P. Stossel. 1976. Measurement of phagocytosis by macrophages. In vitro methods in cell mediated and tumor immunity. B. R. Bloom and J. R. David, eds. Academic Press, NY pp. 363-368.

11. Turnbull, J. M. and M. W. Neil. 1969. The osmotic stability of lysosomes from adult and foetal guinea-pig liver tissue. Biochem. J. 111:503-507.

12. Lockard, V. G. and R. E. Kennedy. 1976. Alterations in rabbit alveolar macrophages as a result of traumatic shock. Lab. Invest. 35:501-506.

13. Sellinger, O. Z., H. Beaufay, P. Jacques, A. Doyan, and C. DeDuve. 1960. Tissue fractionation studies. Intracellular distribution and properties of β-galactosidase in rat liver. Biochem. J. 74:450-456.

14. Babior, B. M., R. S. Kipnes, and J. T. Curnette. 1973. Biological defense mechanisms: the production by leukocytes of superoxide, a potential bacterial agent. J. Clin. Invest. 52:741-744.

15. Greenblatt, G. A. and R. L. Ziprin. 1979. Inhibition of luminol-dependent chemiluminescence of alveolar macrophages by possible etiological agents of byssinosis. Am. Ind. Hyg. Assoc. J. 40:860-865.

16. Greenblatt, G. A. and R. L. Ziprin. 1983. The effect of dust and bract extracts on alveolar macrophage prostaglandin production and cockroach hindgut muscle contraction. Proc. Seventh Cotton Dust Res. Conf. P. J. Wakelyn and R. R. Jacobs, eds. pp 86-89.

17. Olenchock, S.A., R.M. Castellan, J.B. Cocke, D.J. Rodak, J.L. Hankinson, and J.C. Mull. 1983. Endotoxins and acute pulmonary function changes during cotton dust exposures. Proc. Seventh Cotton Dust Res. Conf. P.J. Wakelyn and R.R. Jacobs, eds. pp 70-71.

18. Strobel, G. A. 1983. The OSHA cotton dust standard. Proc. Seventh Cotton Dust Res. Conf. P.J. Wakelyn and R.R. Jacobs, eds. pp 4-6.

Table 1. Effects of Catechin, Rutin and Gossypol on the Mean Cell Volumes (MCV) of Alveolar Macrophages*

Treatment (conc)	MCV (% Control)
Control	100
Catechin (10^{-4}M)	101(±5)
Rutin (10^{-4}M)	102(±4)
Gossypol (10^{-4}M)	102(±5)

*Rat alveolar macrophages were preincubated in the presence of absence of each compound for 15 minutes at 37°C before measurement of mean cell volumes using an electronic cell sizer. The mean cell volume of control cells was 1105(±46)μ^3. Values are means of five experiments ± SEM.

Table 3. Effects of Catechin, Rutin, and Gossypol on Zymosan-induced Chemiluminescence (CL) in Alveolar Macrophages*

Treatment	Maximal Inhibition(%)	ED_{50}(M)
Control	0	-
Catechin	75(±3)	$5.5(\pm3.6)\times10^{-5}$
Rutin	84(±1)	$7.5(\pm4.2)\times10^{-5}$
Gossypol	98(±)	$2.7(\pm0.2)\times10^{-5}$

*The cells (4×10^6 cells in 5 ml of phosphate-buffered medium) were incubated in the presence or absence of each compound for 15 minutes at 37°C before the measurements were made. Zymosan (6 mg/ml) was added to the cell suspension just prior to measurement of chemiluminescence with a liquid scintillation counter set in the out-of-coincidence mode. The ED_{50} is the concentration of compound which produced 50% inhibition. The maximal inhibition was produced at a final concentration of 10^{-4}M for each compound. The numbers shown are mean values for five experiments ± SEM.

Table 4. Effects of Catechin, Rutin, and Gossypol on Lysosomal Enzyme Activities in Alveolar Macrophages*

Treatment (Conc.)	Lysosomal Enzyme Activity (% Control) Acid Phosphatase	β-Glucuronidase	β-N-Acetyl-Glucosaminidase
Control	100	100	100
Catechin (10^{-4}M)	100(±6)	92(±2)	93(±3)
Rutin (10^{-4}M)	69(±5)	97(±2)	96(±3)
Gossypol (2×10^{-5}M)	72(±5)	94(±5)	98(±4)

*The cells (4×10^5 cells in 200μl of phosphate-buffered medium) were ruptured with 50μl of 1% triton X-100. Lysates were incubated in the presence or absence of each compound for 15 minutes at 37°C prior to measurement of lysosomal enzyme activity. The control rates of enzyme release were: 258(±13)nmoles/min.·10^7 cells for acid phosphatase, 63(±3)nmoles/min.·10^7 cells for β-glucuronidase, and 152(±18)nmoles/min.·10^7 cells for β-N-acetylglucosaminidase. The numbers shown are mean values for three to five experiments ± SEM.

Table 5. Effects of Catechin, Rutin, and Gossypol on Phagocytotic Rate in Alveolar Macrophages*

Treatment	Maximal Inhibition(%)	ED_{50}(M)
Control	0	-
Catechin	2(±1)	-
Rutin	38(±8)	$>10^{-4}$
Gossypol	63(±1)	$2.3(\pm0.4)\times10^{-5}$

*The cells (2×10^7 cells in 2 ml of phosphate-buffered medium) were incubated in the presence or absence of each compound for 15 minutes at 37°C before the measurement of uptake of diisodecyl phthalate particles containing oil red 0. Determination of oil red 0 ingestion was monitored spectrophotometrically at 525 nm after extraction from the cells with 1.2 ml of dioxane. The ED_{50} is the concentration of compound which produced 50% inhibition. The maximal inhibition was produced at a final concentration of 10^{-4}M for each compound. The control value for uptake of oil red 0 is 0.35(±.03) mg oil/10^7 cells·15 min. The numbers shown are mean values for five experiments ± SEM.

Table 2. Effects of Catechin, Rutin, and Gossypol on Oxygen Consumption in Alveolar Macrophages*

	Cells at rest		Cells exposed to zymosan	
Treatment	Max. Inhibition(%)	ED_{50}(M)	Max. Inhibition	ED_{50}(M)
Control	0	-	0	-
Catechin	0(±6)	-	6(±2)	-
Rutin	8(±5)	-	1(±9)	-
Gossypol	38(±10)	$>10^{-4}$	56(±8)	$6.6(\pm1.8)\times10^{-5}$

*The cells (3×10^6 cells in 1.65 ml of phosphate-buffered medium) were preincubated in the presence or absence of each compound for 15 minutes at 37°C before the measurements were made. Oxygen consumption was measured over a 10-minute period. Zymosan (6 mg/ml) was added to the cell suspension just prior to measurement of oxygen consumption with a Clark electrode. The ED_{50} is the concentration of compound which produced 50% inhibition. Maximal inhibition for each compound was measured when the final concentration of cotton chemical was 10^{-4}M. The control rates of oxygen consumption were 282(±58) and 476(±35) nmoles/10^6 cells·hr for resting and zymosan-stimulated cells, respectively. The numbers shown are mean values for five experiments ± SEM.

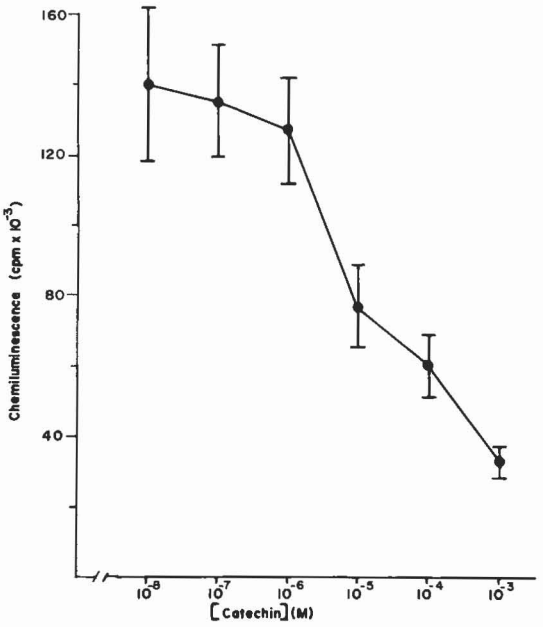

Figure 1. The effect of catechin on the maximal generation of zymosan-induced chemiluminescence by rat alveolar macrophages. Cells (4×10^6 cells in 5 ml of phosphate-buffered medium) were incubated in the presence of various concentrations of catechin for 15 minutes at 37°C prior to the addition of zymosan (6mg/ml) and the measurement of chemiluminescence with a liquid scintillation counter set in the out-of-coincidence mode. Points are mean values of five experiments while bars represent standard errors of the mean.

Price: $25.00

COTTON DUST

Proceedings of the Eighth Cotton Dust Research Conference
Beltwide Cotton Production Research Conferences
Atlanta, Georgia, January 9-10, 1984

Sponsored by
National Cotton Council
and
The Cotton Foundation

P. J. Wakelyn, National Cotton Council
and R. R. Jacobs, Cotton Incorporated, Editors

Proceedings published by:
National Cotton Council, Memphis,TN and
Cotton Incorporated, Raleigh, NC 1984

Library of Congress Cataloging in Publication Data

Cotton Dust Research Conference (8th : 1984 : Atlanta, Ga.)
Cotton dust.

Bibliographies: p.
1. Cotton dust--Toxicology--Congresses. 2. Byssinosis --Congresses. 3. Cotton manufacturer--Hygienic aspects-- Congresses. 4. Cotton manufacture--Dust control-- Congresses. 5. Cotton dust--Composition--Congresses. I. Wakelyn, P.J. (Phillip J.), 1940- . II. Jacobs, R. R. (Robert R.), 1948- . III. National Cotton Council of America. IV. Cotton Foundation (Memphis, Tenn.). V. Title.
RA1242.C82C68 1984 616.2'44 84-8268
ISBN 0-9613408-0-0

PRINTED IN THE UNITED STATES OF AMERICA