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Methanol Extract of Grain Dust Shows Complement Fixing Activity and Other Characteristics Similar to Tannic Acid

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We have found several similarities between tannic acid and grain dust extract prepared with methanol. Both formed a precipitate with IgG, and these interactions were inhibited by albumin. In addition, both preparations fixed complement; this activity was heat stable and was removed by prior adsorption of the preparations with hide powder. Adsorption with polyvinyl polypyrrolidone reduced the complement-fixing activity of tannic acid but not that of the methanol grain dust extract. The similarities between tannic acid and the methanol grain dust extract are consistent with the presence of a tannin or tanninlike material in grain dust.

Introduction

Grain elevator workers collectively develop increased respiratory symptoms and impaired pulmonary function (*1–5*), both of which are reversible (*6*). The pulmonary function impairment shows exposure-response relationships with the respirable fraction of grain dust (*7*). The available evidence does not support the presence of a type I or type III hypersensitivity mechanism as the basis for these collectively occurring pulmonary function changes (*3,8*). However, elevated levels of α-1 antitrypsin in the sera of grain handlers have been postulated to be due to a nonimmunologic inflammatory response in the lung (*8*). This response may be due to the activation of the complement system in respiratory secretions by inhaled grain dust.

In support of this hypothesis, it has been demonstrated that grain dust, as well as aqueous extracts thereof, activate complement, *in vitro* (*9,10*). Activation occurs both by the alternate and the classical pathways, in a nonimmunologic manner (*11*). Grain dust extract has also been shown to form a nonimmunologic precipitate with the gamma globulin fraction of normal human serum (*12*). Extracts derived from cotton dust have been shown to possess similar complement-fixing (*13,14*) and gamma globulin-precipitating (*15,16*) activities.

The levels of complement-fixing activity in grain dust and cotton dust extracts do not correlate with the levels of gram-negative bacterial endotoxin present in the extracts (*9,14*), suggesting that endotoxin does not make a major contribution to the complement fixing activity of these dusts. However, tannins have been implicated in the gamma globulin-precipitating activity of cotton dust extracts (*15*).

In this paper we describe the possible presence of a tannin or tanninlike material in grain dust, which fixes complement and forms a precipitate with IgG. The approach taken was to compare a methanol extract of grain dust, dried and reconstituted in aqueous solution, with tannic acid. We found that the methanol grain dust extract showed several similarities with tannic acid, and one difference. This difference does not preclude the possibility that grain dust contains a tannin or tanninlike material which fixes complement.

Methods

Settled grain dust was obtained from two grain elevators in Thunder Bay, Canada, which simultaneously handled multiple species of grain including wheat, oats, rye, and barley. The dust was extracted with absolute methanol (Consolidated Alcohols, Toronto, Canada) using a ratio of 1 g of dust to 10 mL of extraction fluid. Methanol was used as the extraction fluid in an attempt to enhance the extraction of tannins and tanninlike materials from the dust (*17*). The mixture was shaken for 24 hr at 4°C, following which it was centrifuged for 30 min at 4°C and 2400*g*. The 35 mL of the supernatant was dried by evaporation under vacuum at 55°C, and the residue was redissolved in two sequential 5-mL portions of deionized water. The two reconstituted fractions were centrifuged for 60 min at 4°C and 25,000*g* to remove the undissolved material. The second fraction was found to contain a higher level of complement-fixing activity than the first and also possessed the ability to

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form a precipitate with IgG. Therefore, the second fraction was employed as the methanol extract of grain dust. Sodium chloride was added to this solution from a 3 M stock, to a final concentration of 0.12 M, and sodium phosphate buffer was added from a 0.4 M stock solution, pH 7.4, to a final concentration of 0.033 M. The extract was passed through a sterile 0.22 μm filter unit (Nalgene) and stored in a sterile glass bottle at 4°C.

Tannic acid, a hydrolyzable gallotannin, obtained from Near East gall nuts, was purchased from Sigma Chemicals (St. Louis, MO). A solution of 10 mg/mL tannic acid in deionized water was used in the double-diffusion experiments. For the complement-fixation experiments, an isotonic preparation of 5 mg/mL tannic acid in phosphate-buffered saline, pH 7.4, was prepared fresh daily, as required.

Complement-fixation assays were performed by using a method adapted from that of Kabat and Mayer (*18*). Serial two-fold dilutions of the methanol grain dust extract or tannic acid, in a volume of 0.5 mL, were incubated at 37°C for 2 hr with 0.5 mL of 6 C'H50 units/mL of guinea pig complement (Gibco Laboratories, Grand Island, NY). Following the incubation, 0.5 mL of 2% sensitized sheep red blood cells was added, and the incubation was continued at 37°C for 1 hr further. The tubes were centrifuged for 10 min at 4°C and 400*g*, and the absorbance of the supernatants at a wavelength of 540 nm was determined. The fractional hemolysis was calculated by dividing this absorbance by the absorbance of the same concentration of sensitized sheep red blood cells hemolyzed osmotically. Results are given by the endpoint dilution for 50% hemolysis, determined from a plot of percent hemolysis versus the reciprocal dilution of grain dust extract or tannic acid. The phosphate-buffered saline neither fixed complement nor caused hemolysis.

Double diffusion was performed in 0.8% agarose (Indubiose, Fisher Scientific, Toronto, Canada) prepared in phosphate-buffered saline, pH 7.4, containing 0.1% sodium azide. The templates had six peripheral wells which were 2 mm apart and 2.5 mm from the center well; all wells were 2.5 mm in diameter. The plates were read after standing at 4°C for 48 hr.

Chromatographically purified pooled normal human IgG was purchased from Cappel Laboratories (Malvern, PA), and was employed at a concentration of 10 mg/mL. This solution was found to be free from contamination by IgM, IgA, and albumin by double diffusion against antisera to these proteins.

Crystallized human serum albumin was purchased from Sigma Chemicals (St. Louis, MO) and was used at a concentration of 100 mg/mL in phosphate-buffered saline, pH 7.4. This solution was found to be free from contamination by IgG, IgM, and IgA by double diffusion against goat antisera to the immunoglobulin heavy chain isotypes.

Adsorption with polyvinylpolypyrrolidone (PVP, from BDH Chemicals, Toronto, Canada) was performed at room temperature using a ratio of 1 g of the insoluble adsorbent to 10 mL of sample. The mixture was shaken for 2 hr, following which it was centrifuged for 15 min at 4°C and 2400*g*. The supernatant was filtered through Whatman No. 54 hardened filter paper and was tested immediately for complement-fixing activity. Controls consisted of the same samples similarly treated in the absence of PVP.

A similar protocol was followed for adsorption with hide powder (Sigma Chemicals, St. Louis, MO), except that the adsorption ratio was 1 g of adsorbent to 20 mL of sample.

Results

Both the methanol grain dust extract and tannic acid formed a precipitate with IgG on double diffusion (Fig. 1). In both cases, precipitate formation with IgG was inhibited by albumin, as demonstrated by the decrease in the length of the precipitate when albumin was present in the adjacent wells.

Both the methanol grain dust extract and tannic acid fixed complement (Table 1). Equal amounts of complement were fixed by 0.026 mg/mL tannic acid and a 1:56 dilution of the grain dust extract.

The complement-fixing activity of both the methanol grain dust extract and tannic acid was stable when the preparations were heated on a boiling water bath for 10 min (Table 1).

Adsorption of the methanol grain dust extract with polyvinylpolypyrrolidone (PVP) did not decrease its level of complement fixing activity (Table 1). However, adsorption of tannic acid with PVP removed more than 90% of the complement-fixing activity of this preparation.

Adsorption of the methanol grain dust extract with hide powder eliminated the ability of the extract to fix complement (Table 1). Adsorption of the tannic acid solution with this compound also removed more than 90% of its complement-fixing activity.

Discussion

Tannins are a heterogenous group of naturally occurring, high molecular weight plant compounds which are structurally based on a phenolic subunit (*19*). An important property of tannins is their ability to interact nonspecifically with proteins through the formation of stable hydrogen bonds involving the phenolic hydroxyl groups as hydrogen donators (*19*). The presence of tannins has been demonstrated in sorghum grain and barley (*20*), and thus, it is possible that tannins may be present in grain dust. In this paper we have shown that an extract of grain dust prepared with methanol, the conventional solvent used for the preparation of tannin from sorghum grain (*17*), has several properties which are similar to those of tannic acid.

Both the methanol extract of grain dust and tannic acid formed a precipitate with pooled normal human IgG and in both cases precipitate formation was partially inhibited by human serum albumin (Fig. 1). The inhibition suggests that the complexes formed with albumin

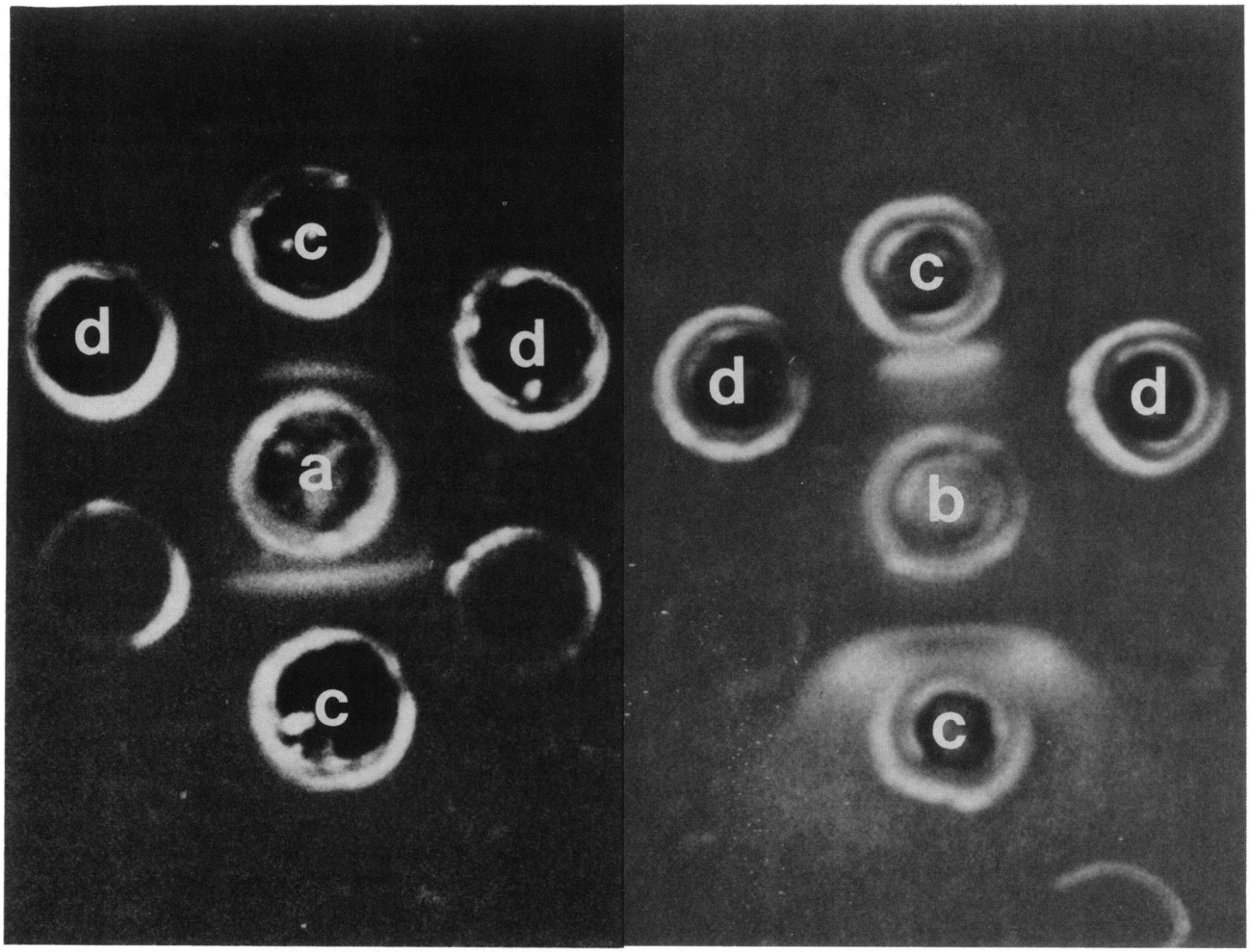

FIGURE 1. Interactions of methanol grain dust extract and tannic acid with IgG and albumin. Double diffusion results obtained with: (*a*) methanol grain dust extract; (*b*) tannic acid (10 mg/mL); (*c*) IgG (10 mg/mL); (*d*) albumin (100 mg/mL).

Table 1. Complement fixation by methanol grain dust extract and tannic acid.

	End point dilution for 50% hemolysis	
	Methanol grain dust extract	Tannic acid (5 mg/mL)[a]
Untreated	1:56	1:194
Unheated	1:30	1:194
Heated (100°C; 10 minutes)	1:33	1:194
Unadsorbed	1:48	1:178
PVP adsorbed	1:56	1:7
Hide powder absorbed	[b]	1:11

[a] Starting concentration of tannic acid.
[b] Complement fixing activity not detected.

are soluble. The ability of the methanol grain dust extract to interact with two different proteins is consistent with the presence of a nonspecific protein-binding component, such as a tannin or tanninlike material.

Another kind of protein with which both the methanol grain dust extract and tannic acid were shown to interact are the complement proteins (Table 1). Although the complement fixation assay employed in our study does not necessarily indicate complement activation, it has previously been shown that grain dust activates complement and causes the release of chemotactically active fragments (*11,21*).

The complement-fixing activity of both the methanol grain dust extract and tannic acid were shown to be heat stable (Table 1). This property is consistent with a tannin and is not compatible with most other possible sources of complement fixing activity in grain dust. For example, proteins such as fungal protein antigens, staphylococcal protein A, lectins, or proteolytic enzymes would be expected to be denatured under these conditions. Bacterial endotoxins cannot be excluded on the basis of this finding, since these are known to be relatively stable to heat (*22*). Nevertheless, it has previously been shown that endotoxin is not likely of be the major complement fixing source in grain dust since endotoxin levels do not correlate with this activity(*9*).

It is known that phenolic compounds, including tannins, can be removed from solution by adsorption with polyvinylpolypyrrolidone (PVP), although not all phen-

ols may be adsorbed (*23,24*). Tannic acid was adsorbed by PVP while the complement-fixing component of the methanol grain dust extract was not (Table 1). This finding suggests that either the complement-fixing component of grain dust in not a tannin, or, if it is, then it is one that does not bind to PVP, possibly because of steric restrictions within the molecule or because the interaction is blocked by the presence of grain proteins present in the extract.

Another substance that is known to bind at least some tannins is hide powder (*25*). This material is composed of dried, pulverized, and slightly chromated bovine skin and is employed by leather chemists for the determination of polyphenols in tanning liquors (*25*). Adsorption of tannic acid with hide powder had a similar effect as adsorption with PVP (Table 1). However, unlike PVP, the hide powder succeeded in removing all of the complement-fixing component of the grain dust extract. This finding, which is consistent with the presence of a tannin or a tanninlike material in grain dust, is not necessarily inconsistent with the result obtained using PVP, since there is no comparative information of this type based on different known tannins.

Thus, our results show several similarities between tannic acid and methanol grain dust extract, which are consistent with the hypothesis that grain dust contains a tannin or tanninlike material. The difference between tannic acid and the methanol grain dust extract does not preclude this possibility.

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