Composition Studies on Tobacco

XLVI. Attempts to Modify Selected Biochemical Properties of Cigarette Smoke by the Use of Specially Treated Filters*

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A major problem in the examination of tobacco smoke has been the lack of a rapid, inexpensive test for the biological activity of the smoke samples. Various methods of analysis have been proposed or utilized involving whole animals, tissue cultures or cell homogenates (1, 2). *Dalhamn* and *Rylander* (3) have presented experimental requirements for any toxicological evaluation of tobacco smoke, including *in vivo* systems and long duration exposure to actual smoke rather than topical or other applications of smoke solutions. However, tests based on such requirements would be expensive and would preclude the rapid evaluation that is frequently desirable for determining the filter efficiency.

Since biochemical reactions occur in an aqueous medium, many tests have employed aqueous solutions of tobacco smoke for measurement of the biological activity (4, 5). However, such solutions represent a partial fractionation of the smoke on the basis of solubility. Some components in both the particulate and vapor phases are highly soluble in water, e.g., short-chained aliphatic aldehydes and acids, but other components are essentially insoluble, e.g., terpenes and paraffinic hydrocarbons. Certain components show a variable solubility which is a function of the pH and salt formation, e.g., phenols and long-chained acids or bases. An obvious exception to the apparent relationship between the aqueous solubility and biological effect is the gas carbon monoxide, which is relatively insoluble in water but very soluble in blood, because of reaction with hemoglobin. The effect of smoke solutions on enzyme systems has been examined by several investigators (1, 6–8), since enzymes are basic to cellular function. With the enzyme yeast alcohol dehydrogenase, most of the enzymatic inactivation by whole smoke solutions is attributable to components in the particulate matter (PM) (8). However, the vapor phase (VP) of the smoke appears to interact with the particulate matter in some way so that the inactivating effect of the whole smoke is less than the sum of the separate phases. The amino acid cysteine protects the enzyme against much of the inactivation but shows less effect when added after a period of time (8).

An examination of the effect of the aqueous smoke solutions and known smoke components on lactic and glucose-6-phosphate dehydrogenases has indicated that the enzymes differ in their susceptibility to inactivation by smoke or smoke components (9). This type of inactivation is a complex interaction between the enzyme and smoke components, rather than a reaction with the substrate or cofactors. The partial protective action of cysteine (8) suggests a strong involvement of sulfhydryl deactivation in the mechanism of enzyme inactivation.

Deactivation of sulfhydryls could occur through oxidation to disulfides and sulfenic and sulfonic acids, or through addition, substitution, or alkylation. Studies on the determination of free sulfhydryls in solutions of cysteine after reaction with tobacco smoke indicated that a series of complex reactions occurs and that the smoke also participates in side reactions with the reagent employed, 5,5′-dithiobis(2-nitrobenzoic acid) (Ellman's reagent) (10). The two phases of smoke (VP and PM) differ in their reactions with sulfhydryls and with Ellman's reagent. Incubation studies of smoke solutions with sulfhydryls indicated that the disappearance of sulfhydryl parallels the loss of enzyme activity with yeast alcohol dehydrogenase. Particulate matter contributes to the loss of sulfhydryl observed with whole smoke, and the vapor phase containing thionucleophilic components contributes to the reaction with Ellman's reagent.

Fresh smoke solutions react with both sulfhydryls and disulfides and may shift the equilibrium of the redox pair in either direction. Since the redox pair might also be affected by other oxidation-reduction components present in smoke, the redox potentials of fresh smoke solutions were examined (11). E'_0 values for cigarette smoke solutions, redox dyes, and other redox systems are shown in Table 1. Cigarette smoke with a redox potential in the range of +150 millivolts might be expected to oxidize ascorbic acid and sulfhydryls having a similar range of potentials. Also, smoke would be expected to reduce the redox dye 2,6-dichlorophenol

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Table 1. Oxidation-reduction potentials of selected redox pairs.

Indicator dyes	E_0' (volts)	Biologicals
Quinhydrone (pH 1) ⟶	+ 0.699	
	+ 0.682 ⟵	Hydrogen peroxide
	+ 0.260 ⟵	Cytochrome C
2,6-Dichlorophenol indophenol ⟶	+ 0.219	
		Cigarette smoke
Toluylene blue ⟶	+ 0.115	
Phenazine methosulfate	+ 0.080	
		Ascorbic acid
Methylene blue ⟶	+ 0.011	
		Sulfhydryls
Tetrazolium blue ⟶	— 0.080 ⟵	Flavins
		NAD/NADH
		Ethanol/ acetaldehyde
Neutral red ⟶	— 0.325	
		Ferredoxin
	— 0.420 ⟵	Hydrogen electrode

indophenol, or cytochrome C, and such reductions are experimentally observed. Electrometric determinations done concurrently with colorimetric determinations have indicated a disparity in the apparent contribution of vapor phase to the redox potential by the two methods: a greater reducing capacity is observed for vapor phase by electrometric determination. These differences reflect the greater quantity of reductants in particulate matter resulting in a buffering type action with the dye, which is itself a redox pair (12).

From the above considerations, it is apparent that sulfhydryl groups play a key role in some of the biological effects of cigarette smoke. In the present study, the use of cigarette filter additives containing added sulfhydryl groups has been examined from the standpoint of changes in smoke composition and properties.

MATERIALS AND METHODS

Commercial 85 mm cigarettes with a combination charcoal-cellulose acetate filter were altered by removal of the charcoal and replacement with the test materials. The latter consisted of cotton fabrics prepared from cellulose treated to introduce sulfhydryl and/or ion exchange groups therein. The samples were furnished by Dr. Truman L. *Ward,* Southern Marketing and Nutrition Research Division, ARS, U.S.D.A., New Orleans, Louisiana. The sulfhydryl group is incorporated into the cellulose by reaction of 1-chloro-2,3-epithiopropane, with the hydroxyl group on C_2, C_3, or C_6 of the glucose subunit (13). Filter samples were prepared by mincing of the cotton cloth samples and insertion of weighed amounts into the compartment from which the charcoal had been removed. All cigarettes were conditioned before use and smoked under standard conditions (35 ml volume, 2-second puff, 1 puff per minute). The smoke from one cigarette was bubbled through 10 mls of aqueous buffer (0.05 M phosphate, pH 7.4). During smoking the changes in pH and redox potential were monitored electrometrically (12). After completion of the smoking of the test cigarette, the buffer solution was removed and 0.5 ml aliquots used for the assays. The aqueous smoke solutions were tested for their effect on enzyme inactivation, reaction with Ellman's reagent (disulfide), with cysteine solution (sulfhydryl) and for reduction of the redox dye 2,6-dichlorophenol indophenol (DCIP). In addition, the contents of aldehydes and sulfides were determined, and ultraviolet absorption spectra (200 to 400 nm) were measured. The assays, modified for these experiments, are given below:

Yeast Alcohol Dehydrogenase Inhibition

When the concentration of phosphate buffer is not otherwise specified, it was 0.05 M, pH 7.4. Smoke solution (2.5 mls) was mixed with an equal volume of enzyme solution (10 μg/ml phosphate buffer) and incubated at 25° C. Aliquots (1.0 ml) were removed and added to the reaction mixture [400 μmoles ethanol, 200 μmoles nicotinamide adenine dinucleotide (NAD), 50 μmoles pyrophosphate buffer; pH 8.8, total volume 2.0 ml] in a cuvette, and the rate of NADH formation was read at 340 nm. The smoke-enzyme mixture was examined for activity at 0, 15, 30, and 45 minutes after mixing. Controls (2.5 mls phosphate buffer in place of smoke solution) were run at the same time. Enzyme activities were converted to percentage of initial control values.

Reaction with Disulfides and Sulfhydryls

Two mls of a 0.01 M stock solution of 5,5'-dithiobis(2-nitrobenzoic acid) (Ellman's reagent) were diluted to 100 mls with phosphate buffer. One ml was employed for assays.

For reaction of smoke with disulfides, an 0.5 ml aliquot of the smoke solution was mixed with 1.0 ml of Ellman's reagent solution and 1.5 mls of distilled water. The absorption at 412 nm was determined after 15 and 30 minutes incubation at 25° C with appropriate controls.

For reaction of the smoke with sulfhydryls, 0.5 ml aliquot of smoke solution was mixed with 1.0 ml of dilute cysteine solution (10^{-4}M) and 0.5 ml phosphate buffer. After 30 minutes incubation at 25° C, 1.0 ml of Ellman's reagent solution was added and the absorption at 412 nm was determined 30 seconds after mixing. The control blank (0.5 ml buffer in place of smoke) should have an optical density at 412 nm of 0.4 to 0.6. The observed change in optical density at this wave length multiplied by 6.56×10^{-4} converts the values to μmoles reactive material per cigarette.

DCIP Dye Reduction

Smoke solution (0.5 ml) was added to 2.0 mls of 2,6-dichlorophenol indophenol (2.7×10^{-4}M) and 0.5 mls phosphate buffer. Absorbance at 615 nm was determined at 15 and 30 minutes after the addition of the

smoke solution. The change in optical density at 615 nm may be converted to micromoles of reducing materials per cigarette by the factor 1.24×10^{-4}.

Aldehyde Content

Aldehydes were determined enzymatically with yeast alcohol dehydrogenase. Smoke solution (0.5 ml of a 0.005 % solution) was added and the difference in absorbance at 340 nm between the smoke and control samples determined at 15 and 30 minutes. Acetaldehyde solutions (1.68×10^{-4}M) were used for standards. The decrease in absorption at 340 nm may be converted to micromoles of aldehyde/cigarette by the factor 9.678.

Sulfide Assay

A modification of Miller's methylene blue procedure was employed to determine the sulfide concentration. Three sets of combined reagents (14) were used. *Reagent A* contained zinc acetate (0.022 M) and sodium hydroxide (0.073 M). *Reagent B* contained N,N-diethyl-p-phenylenediamine hydrochloride (0.0036 M) and ferric chloride (0.066 M) in HCl (4.3 M). *Reagent C* consisted of water-saturated n-butanol. The stock sulfide was 2.3 millimolar. For the sulfide assay, 0.5 ml of smoke solution was added to a tube containing 2.0 mls of *Reagent A*. The tube was shaken and 0.7 mls of *Reagent B* was added, followed by 1.5 mls of *Reagent C*. The tubes were shaken to mix the layers, and were incubated with occasional shaking for 45 minutes. The tubes were centrifuged and 1 ml of the butanol layer was read at 665 nm against a butanol blank. Concentration of sulfide was determined by comparing results to a standard curve.

RESULTS AND DISCUSSION

Five types of cotton fabrics were tested. These included: plain cotton, cotton with added sulfhydryl groups, cotton with diethylaminoethyl (DEAE) groups, cotton with both DEAE and sulfhydryl groups, and a cotton with added amino groups (aminized) (13). These samples were compared to a commercial cigarette control containing a combination charcoal-cellulose acetate filter. The complete removal of the material from the inner charcoal compartment was observed to alter the pressure drop, introducing another possible variable. To control this, four different weights of additives were examined for each type: 40, 50, 60, and 70 mgs; the filters containing 50 mgs of cotton additives produced about the same pressure drop as the 120 mgs of charcoal in the commercial cigarettes.

The data from a representative run with a control cigarette are shown in Table 2. With a dilute buffer solution as employed here, the alteration in the pH will affect the change in the redox potential. Consequently, all redox potentials have been corrected for the effect of pH change (Nernst equation) with the assumption that a 59 millivolt change corresponds to a unit pH change when the oxidation and reduction involves proton shifts (12). The decrease noted is the change in millivolts from the initial potential of +238 millivolts. With the protocol employed, the data for each cigarette were obtained under identical conditions, and may be compared on the basis of the weight of tobacco smoked or on the optical density of the aqueous solution at 260 nm. Unfortunately, there is no satisfactory method for quantitating the smoke present in the various aqueous samples. The optical absorbance is an attempt to provide a measure of the content of nicotine and phenols present and generally reflects the contribution of the particulate phase.

Table 2. Representative data — control cigarette (combined charcoal-cellulose acetate filter).

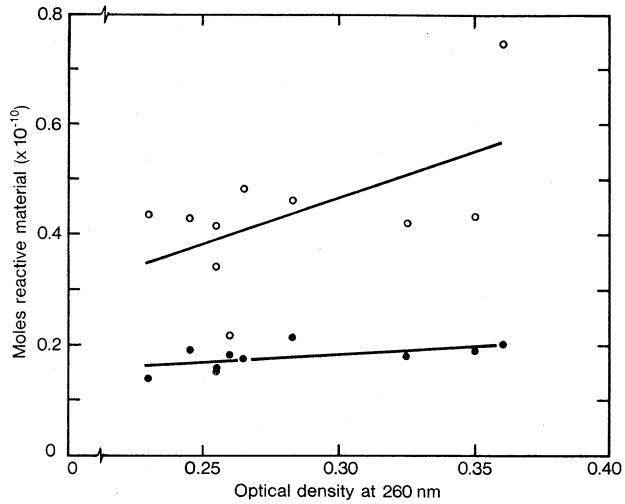
Weight of tobacco smoked (gms)	0.716
Decrease in Eh during smoking (mv)	170
Decrease in pH during smoking	0.49
Eh decrease corrected for pH change (mv)	199
O.D. at 260 nm (1-20 dilution)	0.260
DCIP reductants (μμmole/cigarette)	10.0
DTNB reactive material (μμmoles/cigarette)	16.4
Sulfhydryl reactive material (μμmoles/cigarette)	19.7
Total aldehydes (μmoles)	2.71
Enzyme* inactivation after 45 minutes incubation (% of zero time control)	87.9

* Yeast alcohol dehydrogenase

A comparison of the effect of the various filter additives on the redox potential change, redox dye reduction, disulfide, and sulfhydryl tests is shown in Table 2. These data reflect unweighted averages from at least three separate additive weights and demonstrate general trends. Replicate determinations on the same samples agree within ± 3 %. Determinations between samples of the same class may show greater variations from differences in the amount of the particulate matter produced. As experimental conditions did not allow a determination of total particulate matter (TPM) on the aqueous samples, the value could not be corrected for this factor. The redox potentials were measured electrometrically using one cigarette and corrected for the pH effect by the Nernst equation (12). A comparison of these relative standings (arranged in order of increasing reducing ability measured electrometrically) with those for the redox dye reduction indicates a slight difference in the order of the first three additives. The reaction of the smoke with the disulfide DTNB shows the same relative order as the first column, but the relative reactivities with sulfhydryls follow a quite different order.

Some general comments should be made concerning the implications of some of these results. The vapor phase of the smoke contains the gases that can react with disulfides by thionucleophilic displacement: hydrogen cyanide and hydrogen sulfide (10). The vapor phase is also much more effective than the particulate matter in reducing the pH of an unbuffered solution, presumably because of its content of formic acid and apparent lack of weak base buffers. In addition, the vapor phase contains the low molecular weight aldehydes. Therefore, in whole smoke determinations, the vapor phase would be expected to contribute to changes in pH and alde-

Figure 1. Representative scatter plots showing relationship between the optical density at 260 nm of cigarette smoke solutions and the reactivity of such solutions with 2,6-dichlorophenol indophenol (DCIP) (●) and 5,5'-dithiobis-(2-nitrobenzoic acid) (○). Solid lines indicate least squares plot for two relationships.



hyde, sulfide, and disulfide levels. The particulate matter contributes more to the reduction values determined colorimetrically, to the reaction with sulfhydryls, to enzyme inactivation, and to the optical density of an aqueous solution at 260 nm. Both phases contribute to the change in redox potential, as measured electrometrically, with a slightly greater contribution from the vapor phase. Scatter plots (Figure 1) also appear to indicate some correlation between the optical density at 260 nm, and the degree of enzyme inactivation and the sulfhydryl reactivity. There appeared to be no correlation on the scatter plots of the optical density at 260 nm with the weight of tobacco smoked, or with the content of aldehydes. A slight correlation was also apparent between the optical density at 260 nm and the total change in redox potential and the sulfide content. These observations may be a reflection of the content of particulate matter present. In this aspect, the optical density of a diluted solution of tobacco smoke may provide a rapid method for quantitation of particulate matter present.

The chemically treated cotton did not appear to offer any significant benefits over the control filter materials. Filtration has been shown to be a combination of both mechanical separation of particles and a removal of volatile materials by chemical reactions. The treated cottons were not optimized for mechanical filtration, as they had been processed into fibers and fabrics. Consequently, the degree of filtration contributed by gross removal of particles might be estimated from the plain cotton samples. In Table 3, the plain cotton filter is more effective in reducing the total change in redox potential and in reactions with the redox dye 2,6-dichloro-phenol indophenol and sulfhydryls than the treated cottons. This was contrary to the expected results and may have been due to changes in the mechanical filtration characteristics of the cotton resulting from the chemical treatment. The treated cloths did show some alterations in mechanical tests for abrasion when compared to plain cotton controls (13). Organoleptically, the cotton filters containing sulfhydryl groups had a strong garlic odor which came through when the cigarettes were smoked. Another factor that may have contributed to the results was a slow reaction rate for the sulfhydryl groupings on the treated cottons. The time of filter dwell is in the range of seconds, whereas our previous results have indicated that the sulfhydryl reactivity with smoke may take 15 minutes or longer (10). Consequently, the compounds reacting with sulfhydryl groups are not selectively removed by the filter. In general, the filters which decrease the particulate phase will decrease the content of the materials which react with the enzymes, the redox dyes, and sulfhydryl compounds. The ability of the charcoal filter to reduce the vapor phase content may be noted in the lower values for DTNB reactivity in Table 3.

Table 3. Effect of filter additives upon redox potential change, and reactivity of smoke with redox dyes, disulfides, and sulfhydryl compounds.

Filter additive	Total decrease in E (mv)	DCIP reactive	DTNB reactive	Sulf-hydryl reactive
Charcoal	206	14.1*	19.0*	19.7*
Plain cotton	215	08.1	26.4	13.3
DEAE cotton	223	12.2	35.2	28.5
Plain + sulfhydryl	244	15.9	38.4	31.3
DEAE + sulfhydryl	250	16.7	40.9	33.2
Aminized	253	16.9	42.9	19.0

* ($\times 10^{-12}$ moles per cigarette)

SUMMARY

Aqueous solutions of cigarette smoke produced by collecting smoke in dilute buffer were monitored during puffing for alterations in redox potential and pH. These solutions were examined subsequently for their inhibitory effect on yeast alcohol dehydrogenase, their reaction with model disulfide and sulfhydryl compounds, and their reaction with the colorimetric oxidation-reduction indicator 2,6-dichlorophenol indophenol. These properties were selected to indicate possible biochemical reactions of smoke, and to distinguish between alternative theories for observed enzyme inactivation. Previous experiments had indicated that smoke constituents that react with sulfhydryls might be responsible for such behavior and that such constituents might be removed selectively by filter agents. Cellulose cottons treated to introduce sulfhydryl and/or ion-exchange groups were employed as filter additives, and the smoke solutions were examined for alterations in their reactivity with the above tests. Sulfhydryl reactivity appears to be too slow for effective selective filtration of such reactants from tobacco smoke. The various tests are evaluated on the basis of the contribution of the two phases; the vapor phase contains the components affecting pH, aldehyde content, and reactions with disulfides, and the particulate matter phase contains the components mainly affecting the remaining tests.

ZUSAMMENFASSUNG

Wässerige Lösungen von Cigarettenrauch, hergestellt durch Einleiten in verdünnte Pufferlösungen, wurden darauf untersucht, ob sich das Redoxpotential und die Wasserstoff-Ionen-Konzentration im Laufe des Abrauchvorganges ändern. Die Lösungen wurden anschließend auf ihre Hemmwirkung auf die Hefe-Alkoholdehydrogenase, auf ihre Reaktion mit Modell-Disulfid- und -Sulfhydrylverbindungen sowie auf ihre Reaktion mit dem kolorimetrischen Oxydations- und Redoxindikator 2,6-Dichlorphenol-Indophenol untersucht. Diese Eigenschaften wurden mit dem Ziel untersucht, mögliche biochemische Reaktionen des Rauches aufzudecken und zwischen alternativen Theorien zur Erklärung der beobachteten Desaktivierung von Enzymen zu unterscheiden. Frühere Arbeiten hatten gezeigt, daß bestimmte Inhaltsstoffe des Rauches in einer Reaktion mit Sulfhydrylverbindungen für eine solche Wirkungsweise verantwortlich sein könnten und daß solche Stoffe durch spezielle Filter selektiv entfernt werden könnten. Vorbehandelte Baumwollzellulose mit Sulfhydryl- und/oder Ionenaustauschgruppen wurde als Filteradditiv verwendet, und die Rauchlösungen wurden auf Änderung ihrer Reaktivität in den genannten Tests untersucht. Bei Verwendung von Sulfhydrylverbindungen laufen die Reaktionen für eine selektive Filtration aus dem Tabakrauch zu langsam ab. Die verschiedenen Tests wurden mit beiden Phasen des Gesamtrauches durchgeführt. Die Gasphase enthält Komponenten, die den pH-Wert, den Gehalt an Aldehyden und die Reaktionen mit Disulfidverbindungen beeinflussen. Die Partikelphase weist Komponenten auf, die sich vor allem in den übrigen Tests auswirken.

RESUME

On analyse des solutions aqueuses de fumée de cigarettes, obtenues en récupérant la fumée dans un tampon dilué, afin d'observer les modifications en potentiel redox et pH. On a examiné ces solutions successivement quant à leur effet inhibitoire sur le déhydrogénase alcoolisé de levure, leur réaction avec les composés homologues de disulfide et sulfhydryle, et leur réaction avec l'indicateur colorimétrique d'oxydation-réduction, le 2,6-dichlorophénol indophénol. On a sélectionné ces propriétés pour faire ressortir les réactions biochimiques possibles de la fumée, et afin d'y voir clair entre les différentes théories d'inactivation enzymatiques. Des expériences antérieures avaient démontré que les constituants de la fumée qui réagissent avec les sulfhydryles pourraient être responsables de cette réaction, et qu'on pourrait éliminer sélectivement ces constituants par des agents filtrants. On a employé de l'ouate de cellulose traitée de façon à introduire le sulfhydryle et/ou les échangeurs d'ions dans le filtre, et on observe les changements de réactivité des solutions de fumée dans les tests précités. La réactivité au sulfhydryle semble trop lente pour obtenir une filtration sélective de ces réactifs de la fumée de tabac. On établit les différents tests en se basant sur la contribution de chaque phase: la phase vapeur contient les particules affectant le pH, la concentration en aldéhyde et les réactions avec les disulfides, et la phase particulaire contient les composés affectant les autres tests.

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