

COMPOSITION STUDIES ON TOBACCO. XXXVII. INHIBITION OF LACTIC, ALCOHOL AND GLUCOSE-6-PHOSPHATE DEHYDROGENASES BY CIGARETTE SMOKE AND COMPONENTS THEREOF¹

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The action of whole cigarette smoke and its vapor and particulate phase on the three enzymes, lactic, alcohol, and glucose-6-phosphate dehydrogenase, varies from marked inhibition to no effect. No high degree of correlation between the degree of inhibition and the sensitivity of the enzymes to selected classical —SH deactivating reagents can be demonstrated for yeast alcohol dehydrogenase, lactic dehydrogenase (from rabbit muscle) or glucose-6-phosphate dehydrogenase (from yeast). However, the role of —SH deactivation as a major mechanism of smoke inhibition cannot be discounted. Comparisons of the inhibitory patterns of smoke phases and with certain known constituents of smoke suggest that interaction of smoke constituents occurs, resulting in altered rates of inhibition.

Several mechanisms have been proposed to explain the observed *in vitro* inhibition of enzymes by cigarette smoke (3-7, 8, 9, 13.) Most of these mechanisms envision a single component or limited group of smoke components as the responsible agents. Considering the complex composition of smoke and the presence therein of common enzymatic inhibitors, e.g. phenol, hydrogen cyanide, etc., it would appear that such explanations are simplifications and data supporting the contention that enzymatic inhibition is complex have been published recently (1).

The complexity of enzymatic inhibition by smoke is illustrated by the findings shown in Table 1. The cited values were obtained by collecting smoke or individual smoke phases in phosphate buffer. The samples were then analyzed in a system containing the enzyme, yeast alcohol dehydrogenase (YADH), and its cofactor, nicotinamide adenine dinucleotide (NAD). The rate of reduction of NAD at 340 μ was used to obtain the data. The initial rate of inhibition is a measure of the degree of classical competitive, noncompetitive and mixed inhibition. The subsequent rate of inhibition is termed "inactivation" and is a reflection of a number of enzyme-inhibitor or enzyme-inhibitor-substrate reactions (14). The data in Table 1 show that the initial and subsequent inhibitory rates are entirely different for whole smoke and its individual phases. Also, it is apparent that interactions of vapor and particulate phases occur, resulting in an inhibitory activity for the whole smoke which is different than that expected from a summation of the inhibitions of

the individual phases. Reconstitution of smoke by combining of both phases has confirmed this interaction (1). Thus, it is evident that a series of reactions between inhibitors and enzyme is occurring, resulting in a complex pattern. In addition, the effect of cysteine in altering the rate of inactivation shows that reactions with —SH groups may be a part of the overall inhibitory process with this enzyme.

The data in Table 1 are pertinent in establishing the complexity of enzymatic inhibition but are valueless in determining the contribution of specific smoke constituents. To examine this point, a number of common smoke components were tested at the levels in which they occur in smoke (4,12). The findings are summarized in Table 2. In addition to the smoke components, known reagents that react with —SH groups were included to observe the degree of sensitivity of the enzyme to such reagents: N-ethylmaleimide (NEM), iodoacetic acid (IAA), p-chloromercuribenzoate (PCMB) and hydrogen peroxide. The data show that the saturated and unsaturated aldehydes are particularly inhibitory for YADH. This may be due to reactions with —SH groups in which hemithioacetals, sulfides and related products are formed (*vide infra*). Other workers have isolated a cyclic addition product of acetaldehyde and cysteine (2-methyl-L-thiazolidine-4-carboxylic acid) when cigarette smoke was bubbled through a cysteine solution, (3). YADH is known to be particularly sensitive to —SH deactivating reagents (14) and a correlation is seen between this sensitivity

Table 1. Inhibition of yeast alcohol dehydrogenase by cigarette whole smoke and phases thereof.

Sample	Cigarette filter	Cysteine added ^b	Initial inhibition (% of V_0)	Subsequent inactivation rate ^c	
				0-75 min	76-90 min
Whole smoke	None	—	87	—82	+2
Whole smoke	"	+	89	—6	
Vapor phase	"	—	88	—17	+7
Vapor phase	"	+	90	+8	
Particulate phase	"	—	93	—77	+6
Particulate phase	"	+	94	—12	
Whole smoke	Multiple ^a	—	95	—55	0
Whole smoke	Multiple ^a	+	90	+3	

^a Activated carbon-cellulose acetate filter.

^b At 0 min.

^c Rate of enzymatic inactivation expressed as change in % activity for the specified interval. Cysteine (100 μ moles) added at 75 min in tubes containing no cysteine at 0 min. V_0 is the initial velocity at zero time of the control (no smoke solutions added). Data shown indicate representative findings of 5 independent experiments.

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Table 2. Degree of inhibition of yeast alcohol dehydrogenase (YADH) lactic dehydrogenase (LDH) and glucose-6-phosphate dehydrogenase (GPDH) by sulfhydryl reagents, cigarette smoke, smoke phases and smoke components.

Component ^a	Level ^b		Degree of inhibition ^c		
	μg	μmoles	YADH	LDH	GPDH
Whole smoke	—	—	+++	+	0
Vapor phase	—	—	+++	+	0
Particulate phase	—	—	+++	+	0
Nicotine	1700	10.5	+++	+	0
Acrolein	92	1.64	+++	+	0
Acetaldehyde	740	16.8	+++	+	0
Crotonaldehyde	15	0.21	+++	+	0
Formaldehyde	53	1.76	+++	+	0
Phenol	100	1.06	0	+	0
Furfural	100	1.04	+	+	0
Sulfur dioxide	30	0.47	+++	+	+
IAA	—	0.01	+++	N.E.	+
PCMB	—	0.001	+++	+	+
NEM	—	0.005	+++	N.E.	+
H ₂ O ₂	—	0.085	+++	+++	0

^a IAA = iodoacetic acid, PCMB = *p*-chloromercuribenzoate, NEM = *N*-ethylmaleimide.

^b Whole smoke, vapor phase and particulate phase added in amounts equivalent to 1 cigarette.

^c Strong inhibition = +++, weak inhibition = +, no inhibition = 0. N.E. = not examined. Acetone, methanol, pyridine, acetonitrile and nicotinonitrile do not inhibit. Data based on reaction rate for 0-60 min interval after addition of test solution to the enzyme solution.

Table 3. Influence of acetaldehyde on the reaction rate of disulfide scission by hydrogen cyanide.

Component	Absorbance ^a
HCN	0.640
Acetaldehyde(Ac)	0
HCN + Ac	0.270

^a Absorbance at 412 mμ after 30 sec reaction with 5,5'-dithiobis(2-nitrobenzoic acid).

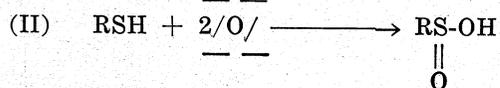
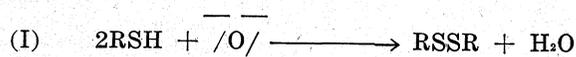
and the degree of inhibition of YADH by cigarette smoke and individual phases.

Lactic dehydrogenase (LDH) is an important enzyme that is involved in anerobic metabolism and found in most tissues including lung tissues. Lactic dehydrogenases from different cellular or tissue locations are known to possess different properties. Generally, lactic dehydrogenases from rabbit muscle, beef heart and beef brain are less sensitive to PCMB than YADH (14). The LDH employed in the present study was obtained from rabbit muscle and was significantly inhibited by PCMB and hydrogen peroxide (Table 2). However, the degree of inhibition of LDH by whole smoke and particulate phase was much less than that of YADH. The vapor phase was noninhibitory. Thus, the correlation between susceptibility to -SH deactivating reagents and the inhibitory activity of cigarette smoke is not as evident with LDH as with YADH. When selected smoke components were tested for inactivation of LDH (Table 2), strong inhibition was observed for the four aldehydes, nicotine and sulfur dioxide just as in the case of YADH. These findings confirm earlier indications that smoke components interact to influence the observed patterns of inhibition. Enzymes may differ markedly in their susceptibility to the products of such interactions.

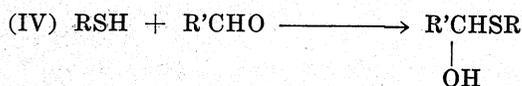
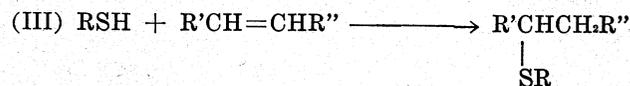
The third dehydrogenase examined was glucose-6-phosphate dehydrogenase (GPDH), which is a key enzyme in the production of high energy intermediates for the *in vivo* synthesis of fats and proteins. Generally, GPDH is relatively insensitive to -SH deactivating reagents (14). The patterns of inhibition of GPDH (from yeast) by whole smoke, individual phases, and smoke components are shown in Table 2. Only weak

inhibition by the vapor phase of smoke, the saturated aldehydes, nicotine, sulfur dioxide and most of the -SH deactivating reagents is evident. Particulate phase and whole smoke show no inhibition with this enzyme. These findings were also confirmed by experiments in which the inhibition of bovine erythrocyte GPDH activity was measured in red blood cells exposed to whole smoke and individual phases.

Although reactions between enzymatic -SH groups and smoke constituents are not exclusively responsible for the overall inhibition by whole smoke, such reactions may be of major importance in inhibitions involving enzymes that are especially sensitive to -SH reagents. The partial protective action of cysteine suggests the strong involvement of -SH deactivation in the case of YADH. The specific reaction involving -SH groups may be oxidations, additions, substitutions and alkylations. Weak oxidants can form disulfides (I) and strong oxidants can produce sulfinic (II) and sulfonic acids. Quinones, polyphenols and possibly the brown pigments in smoke condensate are illustrations of such oxidants (10).



Such compounds as sulfides and thiohemiacetals can be formed by addition reactions with unsaturated compounds (III) and aldehydes (IV) known to be present in smoke.



Alkylating agents in smoke (11) may react with -SH groups to form a variety of products, e.g. (V).



In addition, a number of inhibitory mechanisms not involving -SH groups can be visualized: protein denaturation; chelation of essential metallic ions; deactivation of cofactors such as NAD; other reactions with substrates or products therefrom; and reactions with amino, imidazole and disulfide bonds or groups in proteins.

The problem is complicated by the interaction of smoke constituents forming products which may differ markedly in their rate of inhibition or be entirely noninhibitory. Hydrogen cyanide rapidly cleaves disulfide bonds and the reaction (VI) can easily be measured using 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB) as the sulfur compound (2) (Table 3).



Acetaldehyde alone has no effect on DTNB, while the combination of this aldehyde and HCN shows reduced reactivity toward DTNB presumably due to cyanohydrin formation. Considering the complex nature of smoke it is not unexpected that the overall inhibitory process will be complex and vary quantitatively with different enzymes.

EXPERIMENTAL

Cigarette Smoke. Commercial 85 mm nonfilter ciga-

rettes were smoked mechanically under standard conditions: puff rate, one per min.; puff volume, 35 ml; and puff duration, 2 sec. The smoke was collected in 0.067 M phosphate buffer, pH 7.4. To collect the vapor phase of smoke, a Cambridge filter was inserted between the butt end of the cigarette and the wash bottle containing buffer. To test the particulate phase, the Cambridge filters used to collect VP were suspended in phosphate buffer and the filtrate from this suspension was employed. In all cases, the collection of whole smoke or phases thereof was arranged so that each ml of buffer contained the material from 1 cigarette. The pH of the smoke solutions differed by no more than 0.1 unit from that of the control buffer in all cases.

Enzymatic inhibition. For yeast alcohol dehydrogenase (Worthington Biochemicals Corp.)² the smoke solution was mixed with an equal volume of enzyme solution (10 $\mu\text{g}/\text{ml}$ buffer). Aliquots were removed at intervals and added to the reaction mixture. The latter consisted of 200 μmoles ethanol, 300 μmoles NAD and 50 μmoles pyrophosphate buffer (pH 8.8) in a total volume of 2.0 ml. The rate of NADH formation was followed at 340 $\text{m}\mu$.

The lactic acid dehydrogenase (LDH) assay measured the rate of disappearance of NADH on reduction of pyruvate to lactate. The reaction mixture consisted of 1 μmoles pyruvate, 0.2 μmoles NADH, and 81 μmoles phosphate buffer (pH 7.4) in 2.0 ml total volume. The absorbance at 340 $\text{m}\mu$ was adjusted to 0.900 before the addition of a 1.0 ml aliquot of the smoke—LDH mixture containing 2.0 μg enzyme. LDH from rabbit muscle (Worthington Biochemicals, Inc.) was employed.

For the assay of glucose-6-phosphate dehydrogenase (yeast) (Calbiochem), the reaction mixture consisted of 2.1 μmoles glucose-6-phosphate, 1.64 μmoles NADP, 75 μmoles tris (hydroxymethyl) aminomethane hydrochloride buffer (pH 7.5) and 16.5 μmoles magnesium sulfate in 2.0 ml. Aliquots (1.0 ml) of the smoke-enzyme mixture (10 μg enzyme) were added to the reaction mixture and the reaction rate at 340 $\text{m}\mu$ was determined.

Reaction with 5,5'-dithio-bis(2-nitrobenzoic acid (DTNB) (2). A mixture of 0.2 μmoles of DTNB and 30 μmoles of phosphate buffer (pH 8.0) in a 2.0 ml volume was added to a cuvette. The test solutions (1.0 ml) were added to the cuvette and mixed well. After 30 sec of reaction the absorbance at 412 $\text{m}\mu$ was read.

²Mention of a specific commercial item does not imply endorsement by the department over other similar items not mentioned.

SUMMARY

The action of whole cigarette smoke and its vapor and particulate phase on the three enzymes, lactic alcohol, and glucose-6-phosphate dehydrogenase, varies from marked inhibition to no effect. No high degree of correlation between the degree of inhibition and the sensitivity of the enzymes to selected classical -SH deactivating reagents can be demonstrated for yeast alcohol dehydrogenase, lactic dehydrogenase (from rabbit muscle) or glucose-6-phosphate dehydrogenase (from yeast). However, the role of -SH deactivation as a major mechanism of smoke inhibition cannot be discounted. Comparisons of the inhibitory patterns of smoke phases and with certain known constituents of smoke suggest that interaction of smoke constituents occurs, resulting in altered rates of inhibition.

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