

THE ALKYLATING ACTIVITY OF CIGARETTE SMOKE

Activité alcoylante de la fumée de cigarette.

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SUMMARY

Cigarette smoke condensate has alkylating activity when tested by a highly sensitive procedure using the nucleophile, 4 (4'-nitrobenzyl) pyridine, as the reagent. The condensate has a relatively low level of activity which is equivalent to about 20 γ of 2-iodobutane per cigarette. When the condensate is fractionated by conventional procedures, the activity is distributed throughout all fractions; however, the bulk of the activity is found in the neutral substances and in the water-soluble fraction remaining after removal of non-polar acids, bases and neutrals.

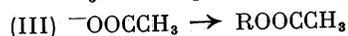
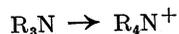
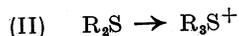
RÉSUMÉ

Quand elle est testée par un procédé très sensible utilisant le nucléophile, 4(4'-nitrobenzyl) pyridine, comme réactif, la fumée de tabac présente une activité alcoylante. Le condensat a un taux d'activité relativement bas qui est équivalent à environ 20 γ de 2-iodobutane par cigarette. Quand le condensat est fractionné suivant des procédés classiques, l'activité est répartie dans toutes les fractions; toutefois, le gros de l'activité se trouve dans les substances neutres et dans la fraction hydrosoluble restant après élimination des acides, bases et neutres non polaires.

The term «alkylation» usually refers to displacement of a hydrogen atom in a molecule by an alkyl group (I).



However, the term may also include addition of an alkyl group to a molecule containing an atom in a lower valence state (II) or to an anion (III).



Alkylation is a key step in many normal and pathological biochemical processes, e.g. the biosynthetic methylation of nornicotine to nicotine and the induction of cellular mutation by nitrogen and sulfur mustards. Since **Preussmann** (1965) and **Sawicki et al.** (1963) have shown that cigarette smoke gives positive tests for alkylating activity, it is of interest to learn more about this property.

To provide a background, some further discussion of alkylation is desirable. Table 1 lists some common types of alkylating agents. All of these types act by yielding an alkyl or substituted alkyl group under the influence of a solvent or another molecule which has relatively strong affinity for the positively charged migrating group, i.e. in a nucleophilic reaction. Reactions IV and V show two general types of mechanisms involved in alkylation. In both mechanisms we obtain the same product. In a simplified version of the first case (IV), the oxirane ring opens under the influence of solvent and a carbonium ion is obtained.

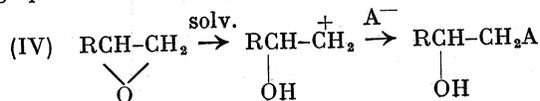
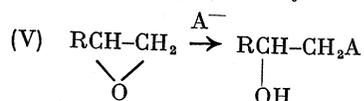


TABLE 1

Types of Alkylating Agents.

Alkyl Halides
Sulfuric and Sulfonic Acid Esters
Phosphoric Acid Esters
Ammonium and Sulfonium Compounds
2-Chloroethyl Sulfides and Amines
Ethylene Imines
Epoxides
β -Lactones
Activated Olefinic Compounds

This then reacts with the nucleophile (A^-) which becomes alkylated. This process is the familiar S_N1 . In the second case several forces are operative in splitting the ring but the specific structure of the nucleophile plays a major role and the latter reacts with the agent

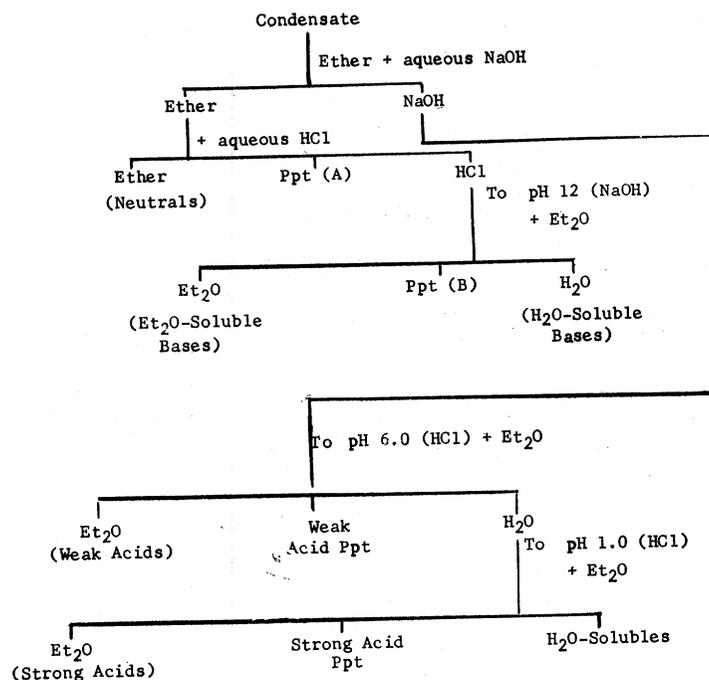


directly without formation of a carbonium ion. This is the familiar S_N2 mechanism. The nucleophile can be any proton acceptor such as a reagent in an analytical method for determining alkylating activity or a key metabolite in a living cell exposed to the agent. The particular mechanism of alkylation will differ with the nature of the alkylating agent and with environmental conditions. The key points in the mechanisms are that, in the S_N1 , the nature of the solvent is a key factor in determining the reactivity of the agent but, in the S_N2 , solvent characteristics are not as important as the nature of the alkyl acceptor, i.e. the nucleophile.

Analytical methods for determining alkylating activity are based on the degree of alkylation of a nucleophile, i.e. the reagent. In the work reported here, the method of **Sawicki et al.** (1963) was employed using 4-(4'-nitrobenzyl) pyridine as the reagent. The smoke sample is dissolved in 1:1 acetophenone-diethylene glycol monoethyl ether, the reagent is added and the mixture is heated at 180°C for exactly 3-1/2 minutes. After cooling, cyclohexylamine is added, and the resulting chromogen is read at 570 m μ . Because of the sensitive nature of the test it is necessary to eliminate all sources of interfering substances in reagents, solvents, etc. which are used in the fractionation of the condensate and the analytical determination. The diethylene glycol monoethyl ether is redistilled and the lower boiling distillate is discarded. Azeotropic hydrochloric acid prepared by distillation of diluted 37% acid is used in the fractionation. Other solvents must be checked for extraneous activity and appropriately purified. Daily blanks must be run to insure validity of sample readings. The test is highly sensitive, giving molar absorptivities of greater than 100,000 for such well-known alkylating agents as l-iodobutane. When used to analyse cigarette smoke condensates the method has a variability of about $\pm 10-15\%$, which is satisfactory for the screening purposes intended in the present work.

Smoke condensate was prepared by smoking domestic commercial cigarettes under conditions generally employed in the United States: puff volume, 35 ml; puff duration, 2 seconds; puff interval, 1 per minute; and butt length, 20 mm. The smoke was collected in traps cooled in solid carbon dioxide, the collected condensate was fractionated as shown in Figure 1 (p. 1021). In this separation, all precipitates, including tarry substances, were recovered; this is in contrast to many published methods of separation in which mention is only infrequently made of the appearance of such precipitates on fractionation and of the ultimate disposition of same.

The alkylating activity of unfractionated smoke appears to be relatively weak. A level of activity equivalent to about 20 γ of 2-iodobutane per cigarette was obtained with the condensate studied here. The variation among samples of smoke condensate prepared under similar conditions from the same cigarettes was within the error of the method. Fractionation of the smoke gave the pattern of distribution of activity and weights shown in Table 2. The weight distribution is somewhat different from some distributions reported in the literature. In our experience, the percentages of «neutrals», «water-soluble acids» and other empirical smoke fractions obtained from a condensate will vary markedly with how the fractionation is conducted and, of course, with the type of cigarette. Alkylating activity is described in Table 2 in two ways. The «specific» activity is a measure of the alkylating capability of a fraction per unit weight of sample; more exactly, it is the absorbance per



20 mg. of the fraction at the absorption maximum. «Total» activity refers to percentage of total activity of the condensate found in the fraction under discussion.

TABLE 2
Distribution of Alkylating Activity in Major Smoke Fractions.

Fraction	Weight (%)	Alkylating Activity	
		Specific*	Total (%)
Smoke Condensate	100	.32	100
Neutrals	35	.31	34
: Et ₂ O - Soluble	6.8	.18	3.7
: H ₂ O - Soluble	1.6	.44	2.2
: Ppt. A	.4	1.1	1.5
: Ppt. B	.1	.70	.2
Acids	11	.19	6.5
: Weak, Et ₂ O - Soluble	6.8	.40	8.5
: » , Ppt.	4.2	.21	2.8
: Strong, Et ₂ O - Soluble	2.3	.88	6.5
: » , Ppt.	32	.84	84
: » , H ₂ O - Soluble (Found)**	—	.34	34
: » , H ₂ O - Soluble (Calc.)	—		

* Absorbance/20 mg of fraction

** See text.

The total alkylating activities of the various fractions were greater than that of the original smoke condensate. Much, if not all, of the excess activity was probably due to the contribution of artifacts formed in the water-soluble acidic fraction during separation. In the scheme, this fraction represents the material remaining in the aqueous layer after

removal of the ether-soluble acids. In working up this fraction, the aqueous layer, which contains approximately 0.1 N HCl and some NaCl, is evaporated to dryness *in vacuo* at a temperature not greater than 25°C. Even under these relatively mild conditions, some hydrohalogenation of double bonds of acids may occur yielding trace amounts of halogen acids which have alkylating activity. Such trace amounts would undoubtedly respond to the highly sensitive test. Experiments with pure crotonic and maleic acids have shown that alkylating activity can be generated from these compounds under these conditions. Thus, the high values reported in Table 2 for the water-soluble strong acid fraction («Found») may be a reflection of artifact formation. If we calculate a value for this fraction by difference [Table 2, «(Calc.)»], a 60% reduction in the observed activity of the fraction is obtained. It should be noted that water-soluble substances other than strong acids may be found in this fraction.

The bulk of the alkylating activity was found in the neutral and water-soluble strongly acidic fractions. The precipitates which formed on acidification of the alkaline extract and the ether-soluble weak acids showed about the same general level of activity. The remainder of the activity was distributed throughout the fractions. Specific alkylating activity varied over a six-fold range with Precipitate A giving the highest value followed by the precipitate which forms on adjusting the pH of the alkaline extract from 6.0 to 1.0 (Table 2, «Acids: Strong, Ppt.»).

Further fractionation of the neutrals yielded the data shown in Table 3. In this scheme, the ether-soluble neutrals were initially partitioned between cyclohexane and 90% methanol in water. The cyclohexane solubles were then extracted with nitromethane. This is essentially the procedure used to obtain a subfraction of condensate relatively rich in polynuclear aromatic hydrocarbons. In the data shown in Table 3, some weight and activity could not be accounted for possibly due to losses in volatile material. The bulk of the alkylating activity appeared in the cyclohexane fraction but the highest specific activity was found in the aqueous methanol solubles. The entire pattern of distribution of activity in all fractions suggests that a number of different alkylating agents occurs in smoke rather than a single active compound which is distributed throughout the fractions as a result of differential solubility alone.

TABLE 3
Distribution of Alkylating Activity in Neutral Subfractions.

Subfraction	Weight (%)*	Alkylating Activity	
		Specific	Total(%)*
MeOH - Water Solubles	10	.57	18
Cyclohexane »	77	.29	70
Nitromethane »	6.8	.39	8

* Percentage of total neutrals

Some comment should be made regarding the smoke components possibly responsible for the alkylating activity. Methyl chloride is a well-known smoke constituent (**Johnstone and Plimmer**, 1959) possessing alkylating activity, but this compound was probably not present in the above fractions since it would have been volatile during solvent removal. Epoxides have not been reported in smoke, but the occurrence of violazanthin, an epoxydiol xanthophyll, in green (but not aged) leaf is known (**Wright et al.**, 1959) and the presence of epoxides (**Wynder and Hoffmann**, 1964) in leaf oxidation products (**Swain et al.**, 1961; **Stedman et al.**, 1962) has been postulated. Several ether (not epoxy) derivatives of macrocyclic terpenes have been found in smoke (**Rowland et al.**, 1964) but these may have little or no alkylating activity. No β -lactones have been reported in smoke although two epimeric γ -lactones (α - and β -levantenolide) have been found (**Cook and Rodgman**, 1962). Compounds having activated ethylenic groups with significant alkylating activity may exist in smoke, although no evidence of this has appeared to date. Of even greater importance, it is possible that some of the alkylating activity may be derived from an extraneous source, such as halogenated hydrocarbons or phosphate esters employed as pest control agents during growth of the plant.

Another question of interest concerns the persistence of alkylating activity in smoke after condensation and storage. Since nucleophiles are present as natural components of smoke, e.g. pyridine and derivatives thereof, one might ask why these do not react with the alkylating agents also present. Of course, it is entirely possible that such reactions have proceeded before the condensate is analyzed and that we are measuring only a small, re

sidual part of the original activity. If this has not occurred, the answer may lie, at least in part, in the nature of the S_N1 and S_N2 mechanisms discussed above. For S_N1 reactions, the reaction rates are more rapid in solutions with solvents of high dielectric constants, and cooled condensate does not appear to fulfill these conditions. For S_N2 reactions, alkylation is a function of the degree of nucleophilicity of the nucleophile, and the analytical reagent may be more active in this respect than the nucleophiles naturally present in smoke. All that is certain at present is that smoke condensate collected at low temperature in the conventional manner contains weak but detectable alkylating activity.

The assistance of R.L. Chrzanowski in this study is acknowledged.

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DEBATE

- Elmenhorst.** 1) Did you investigate the question if different types of tobacco or different treated tobacco mixtures have different alkylating activity in smoke?
2) And if you repeat the experiment of estimating the alkylating activity on one kind of condensate for example, how large do the results differ?
- Stedman.** 1) We were able only to do the work described and this took a bit of a time as it was. The work required considerable method development that I did not go into. We had to spend a lot of time in developing a solvent since all fractions of the smoke condensate must be soluble in the same solvent. We had to spend a lot of time in purifying the reagents since some of them gave alkylating activity due to trace impurities.
2) We did not analyze enough samples to get a standard deviation, but I would judge that the results would vary by about 10 or 15%. This is good enough for one to obtain general picture as we tried to do here.
- Elmenhorst.** Is it necessary to do this reaction at 80°C or could it be done at a lower temperature?
- Stedman.** We have not investigated this with smoke condensate but Sawicki has studied this exhaustively with known compounds. Alkylation does take place at lower temperatures but at a reduced rate. You may look at this paper in Analytical Chemistry to obtain the details. Also, Sawicki lists several nucleophiles which can be used for determining alkylating activity. However, NBP is the only one we tried.
- Keith.** The formation of these precipitates is of course well known to all of us, this does seem to be somewhat time dependent process and I wonder whether the alkylating activity of the smoke is changing with time? Have you measured smoke samples that you have obtained for period of time and see whether you find any differences there?
- Stedman.** I believe that we did spot check a few samples after they had been fractionated and stored, and we found the values were within the range of what we got initially. Regarding the precipitates, we did considerable work with the weak acid precipitate and we tried various ways of getting it out with respect to varying the time and pH. Whether these variables have anything to do with the alkylation pattern I do not know. We did not find too much difference when varying these experimental conditions as far as the physical nature of the precipitate is concerned, but this may have nothing to do with alkylation per se.
- Keith.** Have you attempted to correlate this alkylating activity with biological responses of any type?
- Stedman.** Not yet. We are undertaking some work at the present time to bioassay all those fractions but the final data will not be available for a year and a half and preliminary findings in 6 months or so.
- Hausermann.** It is a question concerning the artifacts adopted in the procedure. Did you

also recombine all fractions to reconstitute their original condensate and make again the overall alkylating activity of the recombined fractions?

Stedman. Yes, we did it, but unfortunately I cannot recall our results. We did this work some 9 months ago. Generally, I do not think that we got 100% back, but if we had lost most of the activity it would have been impressed on my mind. I do recall that there is some mechanical difficulty in reconstituting these fractions.

Moseley. Did I understand that the alkylating substances were more concentrated in the fractions rich in polycyclic hydrocarbons or was it the reverse?

Stedman. The nitromethane soluble fraction is not the major subfraction of the total neutrals, with respect to percentage weight, total alkylating activity or specific alkylating activity.

Neurath. To my knowledge, Sawicki, Preussmann and you did use the same conditions for the reaction with NBP. Couldn't one fear that alkylation even by hydrocarbons with labile alkyl groups could occur? Did you or the other authors make trials with such type of substances to clarify this point?

Stedman. Such reactions are still alkylations, which the method measures. Alkylation of the reagent by activated olefinic compounds may possibly occur. Sawicki investigated a wide range of compounds for responses and perhaps he tested such olefins. One should realize that we are using a chemical method in this work and, tacitly, trying to compare this with what might happen *in vivo*, since we are interested in genetic effects. The entire system of kinetics might be different in the *in vitro* and *in vivo* systems.