

morpholine. Our identification is based not only on retention time and "spike" experiments, but also on comparison of GLC-MS spectra with a spectrum of authentic material (Singer et al., 1975) (Figure 2). We could find no evidence of morpholine in any water source available to us (detection level was about 0.07 ppb); therefore morpholine was not being introduced in water used during the analysis nor as a contaminant of our reagents. Moreover, it was present in samples of fish from local fresh water (including one of the local water sources checked), as well as in all the commercially prepared samples.

The morpholine is apparently not formed artifactually during our isolation procedure since none was detectable when the steam-distillation pot contained, for example, diethanolamine, choline, lecithin, etc. The natural source of the morpholine is still not clear. It should be noted that its ubiquitous occurrence is relevant to our concern about in vivo formation of nitrosamines. *N*-Nitrosomorpholine is produced in good yield at stomach pH, both in vitro (Fan and Tannenbaum, 1973) and in vivo (Sander and Bürkle, 1969; Sander et al., 1968), and is a potent liver carcinogen in the rat and mouse (Bannasch and Müller, 1964).

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Naturally Occurring Nitrosatable Amines. II. Secondary Amines in Tobacco and Cigarette Smoke Condensate

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The previously described method for isolation and identification of naturally occurring secondary amines has been applied to tobacco and cigarette smoke condensate ("tar"). Pyrrolidine and dimethylamine are the predominant amines found in both substances.

The high incidence of human lung cancer which has been correlated with cigarette smoking cannot be accounted for by the amounts of known carcinogens which have been identified in cigarette smoke condensate ("tar"). A possible source of other carcinogens is the in vivo ni-

triosation of the amines from the mainstream smoke to produce carcinogenic *N*-nitrosamines. These compounds have been detected in cigarette smoke condensate (Roades and Johnson, 1972; Neurath, 1969; McCormick et al., 1973; Wynder and Hoffmann, 1968), but only at the parts per billion level. [*N*-Nitrosornicotine has been found in chewing tobacco at a much higher level (Hoffmann et al., 1974).] It has been established that an appreciable portion of the inhaled smoke is swallowed and retained in the

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Table I. Amines in Unburned Tobacco

Amine	Concentration		
	NCI exptl cigarette code 9, this report, ^a ppm	Burley tobacco, (Bush, 1970), ^b ppm	Latakia tobacco, (Irvine and Saxby, 1969) ^c
Dimethylamine	4	9-75 ^d	+ ^e
Methylethylamine	ND ^f	<0.1-3 ^d	+
Diethylamine	<0.1	<0.1-35 ^d	+
Methyl- <i>n</i> -propylamine	0.1		
Methylisopropylamine	ND		+
Methyl- <i>n</i> -butylamine	0.3		+
Methylisobutylamine	ND		+
Methylisoamylamine	ND		+
Di- <i>n</i> -propylamine	<0.1		+
<i>n</i> -Propylisopropylamine	ND		+
Di- <i>n</i> -butylamine	ND		+
Di- <i>sec</i> -butylamine	ND		+
Δ ³ -Pyrrolidine	ND		+
Pyrrolidine	2		
Δ ³ -Piperidine	3		+
Piperidine	ND		
Morpholine	0.3		

^a *p*-Toluenesulfonamides. ^b Dinitrobenzene derivatives from dinitrofluorobenzene. ^c Trifluoroacetamides. ^d Range depends on species. ^e + = present. ^f Not detected.

stomach (Kendrick, J., unpublished), where nitrosation of ingested amines is known to occur (Sander and Burkle, 1969; Mysliwy et al., 1974; Sander et al., 1968).

We have, therefore, applied our analytical procedure for the estimation of naturally occurring nitrosatable secondary amines (see Singer and Lijinsky, 1976) to samples of unburned tobacco and cigarette smoke condensate.

EXPERIMENTAL SECTION

All solvents and reagents were of reagent grade and were used as received. Melting points are uncorrected.

Quantitative and qualitative GLC and GLC-MS were carried out as previously described (Singer and Lijinsky, 1976).

The *p*-toluenesulfonamides for use as authentic standards were prepared by standard procedures (Shriner et al., 1956) and had melting points or boiling points in accord with literature values.

Cigarette smoke condensate was prepared by Meloy Laboratories, Inc., Springfield, Va. Approximately 14000 NCI code 75 cigarettes were smoked on a P&I smoking machine. The smoke condensate was removed from the glass trapping system with 10 l. of acetone, and concentrated HCl (100 ml) was added. The suspension, concentrated in vacuo to constant weight, yielded 227 g of cigarette smoke condensate.

Approximately 10 g of this condensate was tagged with [¹⁴C]dimethylamine and distilled with steam until about 1 l. had distilled. The residue was made basic with 20% NaOH and 50 ml of saturated Ba(OH)₂ (to control foaming) and then distilled with steam into 2 N HCl (5 ml). This distillate was processed as previously described (see Singer and Lijinsky, 1976).

Cigarettes for analysis of unburned tobacco were NCI experimental code 9, obtained through the courtesy of Dr.

Table II. Secondary Amines in Cigarette Smoke Condensate

Amine	Concentration			
	This report ^a ppm	μg/cigarette	Neurath et al., 1966, ^b ppm	Pailer et al., 1967 ^c
Dimethylamine	110	1.8	15	+ ^d
Methylethylamine	26	0.42	3	+
Diethylamine	ND ^e		0.4	+
Methyl- <i>n</i> -butylamine	ND		} 2.5	+
Methylisobutylamine	ND			
Di- <i>n</i> -propylamine	ND			+
<i>n</i> -Propylisopropylamine	ND			+
<i>n</i> -Butylisobutylamine	ND			+
<i>N</i> -Methylaniline	ND			+
Δ ³ -Pyrrolidine	ND			+
Pyrrolidine	240	3.9	36	+
Δ ³ -Piperidine	} 16	0.03	0.8	+
Piperidine			1.4	+
2-Methylpyrrolidine	ND		0.8	+
Dimethyl- or ethylpyrrolidine	ND			+
Morpholine	<5	0.08		

^a Derivative, *p*-toluenesulfonamides. ^b Derivative, 4'-nitroazobenzene 4-carboxylic acid amides. ^c Derivative, trifluoroacetamides. ^d + = present. ^e Not detected.

M. R. Guerin, Analytical Chemistry Division, ORNL. Portions of the tobacco from these cigarettes (ca. 100 g) were processed as previously described (Singer and Lijinsky, 1976).

RESULTS AND DISCUSSION

The results for unburned tobacco are summarized in Table I along with the previous literature values; results for cigarette smoke condensate are shown in Table II. The values for dimethylamine are corrected for the recovered [¹⁴C]dimethylamine, but these recoveries were good (70-100%). The values for the other amines are uncorrected and should be assumed to be minimum values. Previous studies (Singer and Lijinsky, 1976) found that about 50% of added morpholine was recovered.

This adaptation of the Hinsburg procedure gave remarkably clean amine fractions. GLC-MS indicated that all of the peaks eluting between the internal standards (Figure 1) were tosylamides. The separation is not absolute, however, as can be seen by the presence of nicotine and several primary amines in the secondary amine fraction but these can be readily distinguished by their MS fragmentations (Singer et al., 1975).

The results are in qualitative agreement with previous work, but the values for the amines in the cigarette smoke condensate are appreciably higher than Neurath's (Neurath et al., 1966). The differences may be due in part to the choice of different tobaccos for analysis, or to higher recoveries in the current procedure, or to a combination of these factors.

The *N*-nitroso derivatives of all of the amines found are known carcinogens. The biological implications are of potential importance, since in vivo nitrosation in the stomach has been established for a number of amines,

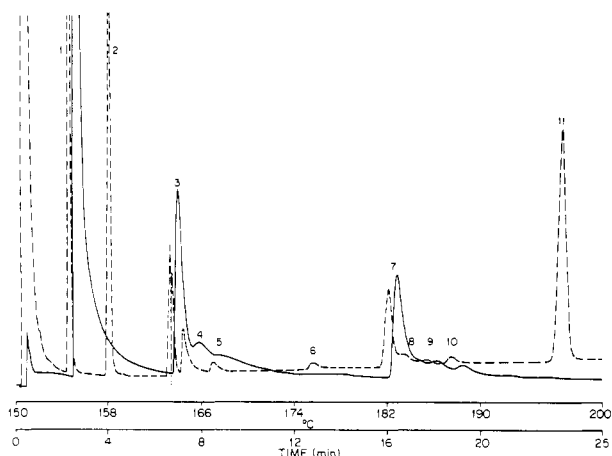


Figure 1. GLC of cigarette smoke condensate amines as tosylamides, 10 ft \times 2 mm i.d. 5% SE 30/Gas-Chrom Q (80–100), 150 to 200 $^{\circ}$ C at 2 $^{\circ}$ C/min: (peak 1) nicotine; (peak 2) acenaphthene (internal standard); (peak 3) dimethylamine; (peak 4) methylamine; (peak 5) methyl-ethylamine; (peak 6) *n*-butylamine; (peak 7) pyrrolidine; (peak 8) unknown; (peak 9) morpholine; (peak 10) piperidine + Δ^3 -piperidine; (peak 11) benzyloxynaphthalene (internal standard); F.I.D. (---); C.E.C.D. (—).

including the cyclic amines pyrrolidine (Mysliwy et al., 1974) and morpholine (Sander et al., 1968).

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Isolation and Biological Activity of the Pigments of the Mold *Epicoccum nigrum*

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Besides flavipin and humic acid, new types of pigment (pigments A and B) have been isolated from cultures of the mold *Epicoccum nigrum*. Pigments A and B are alcohol-soluble dark orange-red solids which are yellow in dilute solution. Ethanol solutions show a very characteristic absorption maximum at 429 nm and a shoulder at 450 nm. Acidification results in a decrease in absorbance and a shift in the maximum to 390 nm. In aqueous solution the pigments interconvert, A appearing more stable, and give rise to fluorescent products. Exposure of solutions of the pigments to direct sunlight causes a rapid bleaching and the formation of fluorescent products. Flavipin strongly inhibited the growth of *Chlorella pyrenoidosa* while pigment A and humic acid showed no effect. Pigment A strongly inhibited the growth of *Bacillus megaterium* while flavipin and humic acid had little effect.

Epicoccum nigrum is a red pigment-producing mold which grows on and spoils a variety of agricultural products. The carotenoids β -carotene, γ -carotene, rhodoxanthin, and torularhodin (Gribanovski-Sassu and Foppen, 1967) and the yellow pigment flavipin (3,4,5-

trihydroxy-6-methyl-*o*-phthalaldehyde) (Bamford et al., 1961) have been identified among the pigments produced by the mold. In addition, brown polymeric "humic acids" consisting of phenolic substances and amino acids have also been isolated from *E. nigrum* cultures, especially from old cultures (Foppen, 1969; Martin et al., 1967). Naumann (1911) described a purple-red pigment produced by this mold, when grown on rice, which was soluble in ethanol and methanol, difficultly soluble in water, and insoluble in the less polar solvents, and which showed a weak absorption band at 448–468 nm and end absorption in the

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