6. The day after surgery the animals were immunized with a 20 percent homogenate of human brain emulsified with an equal volume of complete Freund's adjuvant fortified with heat-killed tuberculosis cells (0.1 mg/ml) (Lilly Laboratories, Indianapolis, Indiana). A total of 4 ml of the emulsion was given to each animal; 1-ml portions were injected into the large shoulder and thigh muscles of the fore and hind limbs, respectively. The animals were observed daily for clinical signs of the disease. When the animals died or were killed, autopsies were performed and tissue was obtained for microscopic examination.
7. The assay for myelin basic protein has been described (2). Briefly, 0.05-ml portions of a tenfold concentrated assay buffer (2*M* tris-acetate, *p*H 7.5, containing 10 mg of histone per milliliter) and antiserum at the appropriate concentration were added directly to 0.5 ml of spinal fluid. This mixture was incubated for 1 hour at 37°C; ^{125}I-labeled basic protein (15,000 count/min; specific activity, 3 to 7 μc/μg) were added, and the mixture was incubated for an additional 16 to 24 hours at 4°C. The antibody-basic protein complex was then precipitated with cold ethanol, the pellet and supernatant fraction were separated by centrifugation, and each was assayed for radioactivity. The percentage of ^{125}I-labeled basic protein bound was then determined.
8. Antibody to myelin basic protein was determined by mixing 500 μl of assay buffer (0.2*M* tris-acetate, *p*H 7.5, containing 1 mg per milliliter of histone, and 1 percent fetal calf serum), serum or spinal fluid of the appropriate dilutions, and 10 μl (15,000 count/min) of ^{125}I-labeled basic protein (specific activity, 3 to 7 μc/μg). The mixture was incubated for 24 hours at room temperature; cold ethanol was added, the pellet and supernatant fraction were separated by centrifugation, and each was assayed for radioactivity. The percentage of ^{125}I-labeled basic protein bound was then determined for each dilution.
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Nicotine in Breast Fluid of Nonlactating Women

Abstract. *Using a combination of gas chromatography, mass spectrometry, and selected ion recording techniques, we have identified nicotine and its major metabolite, cotinine, in the breast fluid of nonlactating women smokers. As little as 25 picograms could be measured by using the deuterated variants, [5′,5′-^{2}H]nicotine and [3,3-^{2}H]cotinine, both as internal standards and as carriers in an inverse isotope dilution method.*

It is generally known that human milk may contain a broad spectrum of extraneous chemical substances to which the mother is exposed (*1*). It is less well-known that during the major portion of adult life the nonlactating breast glands of women secrete and reabsorb breast fluid (*2*). Because of this secretory mechanism, the accumulation of potentially harmful exogenous substances in breast tissue and its secretions could pose a serious hazard to the breast epithelium.

Specimens obtained by a standardized nipple aspiration technique enable us to study the physiology, biochemistry, and cytology of secretions from the nipples of nonlactating women (*3*). We have examined nipple aspirates of breast fluid for the presence of foreign compounds to which women may be exposed and have chosen the tobacco alkaloid nicotine for our initial study because both "exposed" and control populations are readily available.

The unambiguous identification of trace amounts of small molecules in the complex milieu of body fluids can be difficult. The concentration of nicotine to be expected is probably similar to that found in plasma (*4*), that is, approximately 10 ng/ml, and the amount of breast fluid obtainable per subject is limited to approximately 50 μl. It follows that the amount of nicotine present in the total sample, if one allows for a 50 percent recovery and a ± 10 percent accuracy, would require the measurement of approximately 250 pg with an accuracy of ± 25 pg.

Table 1. The GC-MS-SIR analysis of standard solutions of [^{2}H]nicotine enriched with unlabeled nicotine. Each injection contained a total of 25 ng of nicotine.

Ratio of weight of nicotine to weight of [^{2}H]nicotine (%)	Observed ion current ratios* (%)	
	Before extraction†	After extraction‡
0.0	2.7	4.3
0.0	2.9	4.5
5.0	7.2	8.4
5.0	7.4	
10.0	11.5	13.3
10.0	11.6	13.9
20.0	20.0	21.9
20.0	20.3	22.1

*Ratio of the ion current at *m/e* 84 to that at *m/e* 86. †Calculation of a regression line: $y = 2.88 + 0.865x$ with $r^2 = 1.000$. ‡Calculation of a regression line: $y = 4.41 + 0.885x$ with $r^2 = 0.998$.

We achieved this degree of sensitivity, specificity, and accuracy by means of an analytical method in which [5′,5′-^{2}H] nicotine (*5*) is used as an internal standard for quantitation and as a carrier in an "inverse isotope dilution" technique.

By using a combination of gas chromatography, mass spectrometry, and selected ion recording (GC-MS-SIR) techniques (*6*), we can, with good precision, measure the ratio of the ions of mass to charge ratios, *m/e*, 84 (the base peak of the mass spectrum of natural nicotine) and of *m/e* 86 (the base peak of the internal standard [^{2}H]nicotine). In this way quantities of nicotine as small as 25 pg can be measured.

Previously, nicotine in body fluids has been assessed by thin-layer chromatography used in conjunction with radioactive isotopes (*7*), gas-liquid chromatography (GLC) (*4*), and atmospheric pressure ionization (API) mass spectrometry (*8*).

Breast fluid samples were obtained from nonlactating volunteers with a history of smoking, by a standardized nipple aspiration technique with a breast pump (*3*) 15 minutes after a single cigarette had been smoked. Nonsmokers were used as controls. Each sample of breast fluid was placed in a 1-ml reaction vial and treated with 5.0 μl of [^{2}H]nicotine standard solution (9.1 ng/μl) in methanol, 100 μl of 1*N* NaOH solution, and 200 μl of dichloromethane (spectroscopic grade, purified by washing 100 ml of solvent two times with 50 ml of 1*N* HCl). All aqueous reagents were made from doubly distilled deionized water purified by washing 100 ml of water twice with 40 ml of dichloromethane.

After being agitated on a vortex mixer for 5 minutes, the vial was centrifuged for 10 minutes and the organic layer was removed to a second tube. This extraction was repeated with another 200-μl portion of dichloromethane; the combined dichloromethane extracts were then extracted once with 200 μl of 1*N* HCl. The aqueous layer was removed, brought to a *p*H of at least 10 with 150 μl of aqueous 2*N* NaOH solution, and the resulting solution extracted with two 300-μl portions of dichloromethane. The combined organic extracts were dried for 1 hour with 5 mg of anhydrous potassium carbonate, and the solution then decanted in portions to a 0.3-ml reaction vial and carefully reduced in volume to about 15 μl under a stream of nitrogen. Absolute ethanol (10 μl) was added, the total volume reduced to about 5 μl, and the entire sample analyzed on the GC-MS-SIR system in a single run.

A gas chromatograph (Infotronics

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Table 2. Analysis for nicotine in the breast fluids from smokers and nonsmokers.

Subject	Smoking history	Breast fluid obtained (μl)*	Ratio of m/e 84 to m/e 86 (%)	Nicotine concentration (ng/ml)*
A	Nonsmoker	42	4.3	0
B	Nonsmoker	102	4.7	0
C	Two packs per day	47	22.2	195
D	One pack per day	90	14.9	60
D	One pack per day	77	11.3	46
E	Approximately one pack per day	45	9.5	59

*Density assumed to be approximately 1.0.

model 2400) fitted with a glass U-tube column (2 m by 2 mm) packed with 2 percent Carbowax (20*M*) and 2 percent KOH was used; the column temperature was kept at 140°C for 1 minute and then increased 5°C per minute. The injection port temperature was 175°C, and the helium flow was 25 ml per minute.

The gas chromatograph was interfaced to a mass spectrometer (AEI MS-12). The mass spectrometric operating conditions were: ion source temperature, 200°C; 8-kv base accelerating voltage; 2.2-kv multiplier voltage; 500-μa trap current; electron energy, 70 ev; resolving power 1200. Selected ion current records at m/e 84 for natural nicotine and at m/e 86 for the [^{2}H]nicotine standard were obtained according to the selected ion recording system (*9*).

The ion current records generally showed a single peak for m/e 86 at 4.5 minutes for the added [^{2}H]nicotine standard, and a single peak for m/e 84 for unlabeled nicotine with a retention time 1 second longer than the [^{2}H]nicotine peak. The ratio of m/e 84 to m/e 86 was determined by measurement of the ion current records, the added [^{2}H]nicotine serving both as an internal quantitation standard and as a carrier. By referring to the standard curve, m/e 84 to m/e 86 ratios as determined by GC-MS-SIR were converted to the amount of nicotine present.

A standard curve was constructed by injecting 3.0 μl of solution in methanol ($\sim$ 9 ng/μl) containing known ratios of nicotine to [^{2}H]nicotine in the range from 0 to 20 percent enrichment directly onto the GC-MS system and measuring the ion current ratios. However, when ion current records from blank experiments on 50 μl of water were analyzed according to the procedure for breast fluid samples, enhanced ion current ratios were obtained, indicating the presence of a contaminant (identified by GC-MS as nicotine) that is picked up during the extraction procedure. Since this contamination was present in a consistent amount in all blank samples, it could be corrected for by constructing a new standard curve based on the ion current ratios obtained from known mixtures of nicotine and [^{2}H]nicotine after they had gone through the described extraction procedure and analysis.

Using this new standard curve [which had the same slope as the original standard curve but a larger intercept (see Table 1)], we calculated the amounts of nicotine in the breast fluid samples (Table 2). These amounts were considerably greater (50 to 200 ng/ml) than those reported in plasma (10 to 20 ng/ml) (*4*). In addition, it was possible to identify the major metabolite of nicotine, cotinine (*10*), in the same breast fluids by using [3,3-^{2}H]cotinine (*5*) as the internal standard and carrier. Cotinine concentrations of 200 to 300 ng/ml were found in breast fluid, and the same concentrations of cotinine were also detected in plasma. Plasma levels of 100 to 600 ng/ml have been reported previously in smokers (*10*).

Nicotine is known to be rapidly metabolized, having a plasma half-life of less than 30 minutes (*4*), whereas cotinine persists in blood for days after an individual stops smoking (*9*). In addition to its presence in the blood of smokers, nicotine has been found in the amniotic fluid of pregnant women smokers (*9*) and in mothers' milk (*1*). The detection of both nicotine and cotinine in breast fluid supports our hypothesis that the secretion of exogenous substances into nonlactating breast glands may be a general phenomenon. In this connection we have found that a variety of classes of substances administered orally or parenterally, including barbiturates (*11*), fatty acids (*12*), and metastable technitium-labeled perthechnetate (*13*), are also rapidly secreted into breast fluids. It is therefore likely that most substances which gain access to the blood will be secreted and concentrated by the "resting" breast (*4*).

In addition to nicotine and cotinine, polycyclic carcinogenic substances in tobacco smoke such as benz[*a*]anthracene, benzo[*a*]pyrene, and methylchrysene (*4*) will probably also reach the breast fluid. The excretion of mutagenic chemicals in the urine of smokers indicates that these compounds are absorbed through the lungs and enter the circulation (*14*). However, their detection in breast fluid would be extremely difficult because of their very low concentrations in blood and breast fluid as compared to nicotine.

Whether nicotine and its metabolites and other substances produce adverse effects on breast epithelium remains to be determined. It is not unlikely that physiological and cytological effects might be produced in breast tissue by constant exposure to hazardous chemicals present in tobacco smoke and from other environmental sources (*15*). This possibility is supported by studies demonstrating a marked increase in the frequency of atypical and proliferative lesions of the breast with advancing age (*16*).

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Particle-Mediated Membrane Uptake of Chemical Carcinogens Studied by Fluorescence Spectroscopy

Abstract. *The fluorescence emissions of chrysene, N-ethylcarbazole, and 1,6-diphenylhexatriene undergo large spectral shifts or changes in quantum yield, or both, upon their uptake from particulates by phospholipid vesicles. This membrane uptake of carcinogen and carcinogen-like molecules by model membranes does not result in any disruption of the lipid bilayers. The fluorescence emission of chrysene, when bound to silica, was found to be sensitive to the surface density of chrysene on the silica. These observations demonstrate the feasibility of using fluorescence spectroscopy to measure the rates of exchange of carcinogens from particulate matter to cell membranes and to characterize the surface distribution of chemical carcinogens on particulate matter. Comparison of the uptake rate of chrysene from the unperturbed crystal state, sonicated crystals, and the silica-adsorbed state demonstrates that the last condition results in the most rapid transport of chrysene into model membranes. This information should prove valuable in understanding the cocarcinogenic effects of particulates and polynuclear aromatic hydrocarbons.*

Humans are often exposed to both asbestos and cigarette smoke, and the health records indicate that these materials are highly cocarcinogenic. For example, asbestos insulation workers have a seven- or eightfold higher probability of dying from bronchogenic carcinoma than persons from the general population. Among these workers, the nonsmokers have no increased disposition to lung cancer whereas the smokers have a 92-fold increased disposition (*1, 2*). Thus it appears that most asbestos-induced lung cancer is the result of the synergistic effects of the polynuclear aromatic hydrocarbons (PAH) in cigarette smoke, and the particulate, asbestos. Some cancers, such as mesothelioma, are probably the result of exposure to asbestos alone (*3*).

A similar synergism exists between PAH and particulates other than asbestos. In order to induce a high incidence of lung cancer in experimental animals, Saffiotti *et al.* (*4*) found it necessary to disperse benzo[*a*]pyrene on the particulate, hematite. In other studies, intratracheal injection of benzo[*a*]pyrene or dimethyl benzanthracene resulted in only low incidences of lung cancer in animals unless asbestos (*5*) or india ink (*6*) were also injected. These particulates did not induce cancers if benzo[*a*]pyrene was not also injected. Similar results were found in cell transformation studies (*7*).

Why do particulates and PAH act as cocarcinogens? Many particulates have the ability to adsorb PAH and thereby become carriers. Asbestos will adsorb the natural oils from the jute bags in which it is often shipped (*8*) and will adsorb benzo[*a*]pyrene out of benzene solution (*5*). Silica, alumina, and other materials with large surface area to volume ratios are used for chromatographic separations of aromatic compounds because of their adsorptive abilities. Soot, a particulate, when isolated from human lungs is found to be depleted of its normally occurring benzo[*a*]pyrene (*9*). One possible explanation of this depletion is that plasma proteins are capable of removing benzo[*a*]pyrene from soot (*10*).

All these data point to the importance of particles in aiding the transport of polynuclear aromatics into cell membranes and thus increasing the availability of these materials for microsomal activation (*11*). Particles may increase the cellular availability of these chemicals by being phagocytosed, by facilitating PAH uptake by cell membranes, or by puncture of the membrane. The relative importance of these pathways is unknown.

The polynuclear aromatics are all highly fluorescent, and their fluorescent properties are sensitive to their surrounding environment. We have utilized these changes in fluorescence spectral properties to measure the uptake rates of chrysene, *N*-ethylcarbazole, and 1,6-diphenylhexatriene off particulates and into phospholipid vesicles.

Figure 1A shows the fluorescence emission spectrum of chrysene when dissolved in ethanol and when coated on porous glass particles which are suspended in buffer. The intensity of the porous glass spectrum fluctuates as a result of the particles settling to the bottom of the cuvette. The spectrum shown represents an average of ten repetitive scans. The first two peaks in the chrysene-ethanol spectrum are not observed for chrysene on porous glass. This phenomenon is probably a result of readsorption of the shorter wavelengths by adjacent ground-state chrysene molecules. Birks and Cameron (*12*) examined 41 carcinogens and related molecules, and many of them showed crystal spectra that were distinct from those observed in solution because of either readsorption or excimer formation. Upon the addition of dimyristoyl-L-α-lecithin vesicles to the suspension of chrysene and porous glass, a slow increase in the ethanol-like spectrum appeared (Fig. 1A; 363 and 383 nm) as a result of transport of chrysene into the lipid bilayer. This transport was only about 5 percent complete after 2 hours. Figure 1B shows the fluorescence emission of chrysene crystals that had been dispersed by sonication and emission spectra of the same sample at various times after the addition of phospholipid vesicles of dipalmitoyl-L-α-lecithin. The similarity of the fluorescence emission spectra of chrysene on porous glass and as dispersed crystals suggests that in both instances the chrysene is present as microcrystals. Chrysene transfer was about 20 percent complete in 2 hours.

Chrysene adsorbed to silica was prepared by a chromatographic procedure (*13*). The shapes of the fluorescence emission spectra were sensitive to the surface density of chrysene on the silica. These spectral changes were not a result of impurities since silica alone showed no significant fluorescence under identical instrumental conditions and the fluorescent material that was extracted from the silica showed an emission spectrum identical to pure chrysene. Figure 1C shows the emission spectrum of chrysene on silica at a concentration of 1.15×10^{-3} g of chrysene per gram of silica. In a series of six such samples the fluorescence emission spectra shifted from the crystal-like spectrum to the ethanol-like spectrum as the chrysene to silica ratio decreased from 3.2×10^{-3} to 0.05×10^{-3}. Also shown in Fig. 1C are the emission spectra recorded after addi-

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