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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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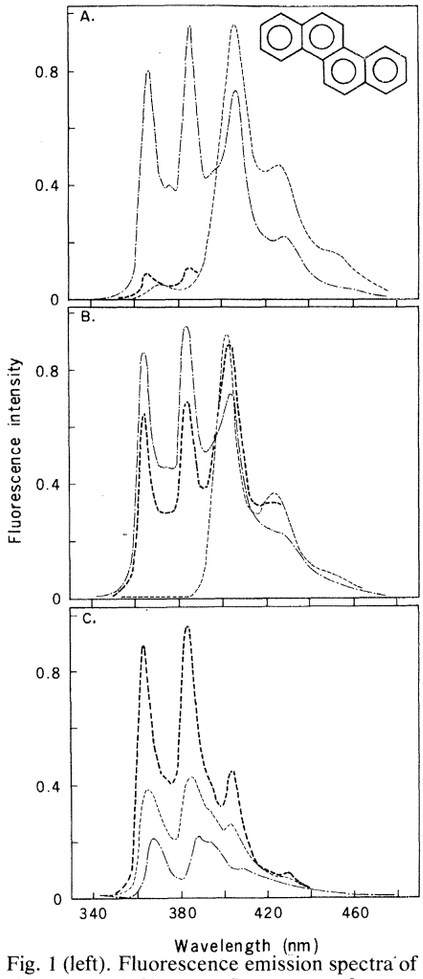

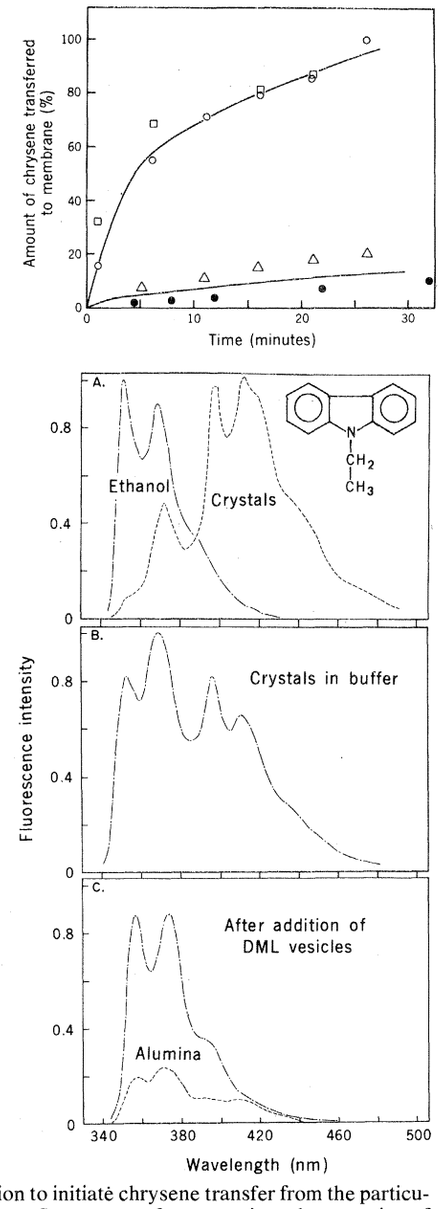

Fig. 1 (left). Fluorescence emission spectra of chrysene. (A) Chrysene fluorescence from an ethanol solution (– · –), from an aqueous suspension of chrysene-coated porous glass beads (- - -), and from this same aqueous suspension 30 minutes after the addition of lipid vesicles (- - -). The 10 ml of aqueous solution contained 4 mg of coated beads. The coating density was 0.046 g of chrysene per gram of porous glass. Dimyristoyl-L-α-lecithin vesicles (1 ml, 10 mg/ml) were added to the suspension to initiate chrysene transfer from the particulate. The solutions were held at 37°C. (B) Chrysene fluorescence from a sonicated suspension of 95 μg of chrysene in 10 ml of buffer (- - -). Dipalmitoyl-L-α-lecithin vesicles (2 ml, 10 mg/ml) were added to this suspension. The emission spectra were obtained at 6 hours (- - -) and 24 hours (– · –) after the lipid addition. The solution temperature was 40°C. (C) Fluorescence emission of chrysene adsorbed to silica. The chromatographic procedure is described in (*13*). The chrysene content is 1.15×10^{-3} g of chrysene per gram of silica. The particulate (10 mg) was suspended in 10 ml of buffer at 31°C. The recording of each spectrum took 5 minutes. To initiate the membrane uptake of chrysene, dipalmitoyl-L-α-lecithin vesicles (1 ml, 10 mg/ml) were added to the suspension. The spectra shown were recorded before lipid addition (– · –), 10 minutes after lipid addition (- - -), and after heating the sample to 50°C for 10 minutes and then cooling to 31°C prior to recording the spectrum (- - -). Fig. 2 (top right). Membrane uptake rates of chrysene. Chrysene (11 μg) was used in all runs. The uptake of chrysene was rapid with both dimyristoyl-L-α-lecithin (□) and dipalmitoyl-L-α-lecithin (○) when the chrysene was added in the silica-adsorbed form. The fluorophore-particulate weight ratio was 1.15×10^{-3} g of chrysene per gram of silica. Membrane uptake was slower from sonicated chrysene crystals by dimyristoyl-L-α-lecithin (•) and for microcrystals formed by the evaporation of benzene from benzene solution of chrysene (△). In all runs 1 ml of a vesicle solution (10 mg/ml) was added to initiate chrysene uptake. All the solutions were held at 40°C. Fig. 3 (bottom right). Fluorescence emission spectra of *N*-ethylcarbazole. (A) *N*-Ethylcarbazole in ethanol (– · –) and in the dry crystalline state (- - -). (B) A suspension of *N*-ethylcarbazole crystals in buffer. The relative intensities of the ethanol-like and water-like portions of this spectrum fluctuate as a result of the crystals floating to the top of the sample, thus removing them from the observation area. (C) A suspension of alumina coated with *N*-ethylcarbazole. The spectrum in the absence of dimyristoyl-L-α-lecithin vesicles (- - -) was taken after 1 hour of equilibration with buffer. Coated alumina (5.4 mg, 0.05 g of *N*-ethylcarbazole per gram of alumina) was suspended in 10 ml of buffer. Dimyristoyl-L-α-lecithin vesicles (1 ml, 10 mg/ml) were added to initiate transfer. The spectrum, with lipid (– · –), was recorded 1 hour after the lipid addition. The excitation wavelength was 330 nm, temperature 5°C; other details as in (*13*).

tion of vesicles of dipalmitoyl-L-α-lecithin. Chrysene transfer from silica was complete in 30 minutes. These data demonstrate the possibility of using the changes in the spectral properties of chrysene to measure the rates at which chrysene can leave a particulate and enter a cell membrane. By using the increase in fluorescence intensities at 363 nm we quantitated the rates of chrysene uptake (Fig. 2). The result of these experiments demonstrate that chrysene enters phospholipid bilayers much more rapidly from the silica-adsorbed state than from a microcrystalline state. Additional work is required to determine how the many types of particulates (for example, asbestos, hematite, and cotton fibers) can alter the cellular availability of PAH. We expect this technique to be applicable to most classes of particulates.

Chrysene is not unique in showing spectral changes upon exchange between the particulate bound or membrane bound state. Figure 3A shows the emission spectra of *N*-ethylcarbazole when dissolved in ethanol and when in the dry crystalline state. Again, the first two emission bands were readsorbed in the crystals. The shifts in the crystal spectrum were not a result of contamination since this spectrum remained unchanged after five additional recrystallizations, and this material was found to be chromatographically pure in three different solvent systems. Figure 3B shows the emission spectrum of *N*-ethylcarbazole crystals in buffer. In this case both an ethanol-like and crystal-like spectrum is observed. The ethanol-like spectrum increased in intensity over a period of about 20 minutes, most probably because of the limited but significant solubility of the carbazole in water. Also shown are the spectra of this carbazole coated on alumina, before and after addition of lipid (Fig. 3C). The increase in intensity at 372 nm, upon *N*-ethylcarbazole incorporation into the bilayer, permits easy measurement of membrane uptake. Membrane uptake of *N*-ethylcarbazole off alumina was complete in 1 hour. Additional lipid had no effect on the fluorescence intensity after the 1-hour equilibration. The more rapid uptake of *N*-ethylcarbazole was probably a result of its higher water solubility. 1,6-Diphenylhexatriene was nonfluorescent when coated on glass beads (3 to 8 μm in diameter) but highly fluorescent after transport into lipid vesicles. This phenomenon will permit easy determination of the membrane uptake rates of this last molecule.

In the interpretation of the observed spectral changes it is essential to know

whether these changes occur as a result of the transport of aromatic hydrocarbons into membranes or as a result of the lipids adsorbing onto the particulates. This adsorption process is likely to cause disruption of the spherical bilayer. We investigated the integrity of the vesicles in the presence of particulates by two means. The first involved release of the fluorogenic probe, umbelliferyl phosphate, from the internal volume of the vesicles (*14*). Umbelliferyl phosphate is only weakly fluorescent, whereas the hydrolysis product, umbelliferone, is highly fluorescent. The negative charges on umbelliferyl phosphate prevent diffusion across lipid bilayers, and this material cannot escape unless the bilayers are disrupted. The fluorogenic probe was trapped in the internal volume of dimyristoyl-L-α-lecithin vesicles by sonication, and the external probe was removed by dialysis. Alkaline phosphatase was added to the external media, rapidly cleaving any umbelliferyl phosphate that was released. The vesicles were disrupted by boiling, then more alkaline phosphatase was added which produced a large increase in fluorescence intensity. None of the particulates used in these studies caused any disruption of the phospholipid vesicles.

The second method we used to examine vesicle integrity in the presence of particulates depended on both the ability of the lanthanides to shift the frequencies of nuclear magnetic resonances and the impermeability of intact lipid bilayers to these triply charged ions. Europium chloride, when added to preformed lecithin vesicles, binds only to the external phosphates. The proximity of Eu^{3+} to the outer surface choline residues results in a shift in their resonance frequency (*15*). Disruption of the lipid bilayer results in the inner and outer shifts becoming equivalent. None of the particulates used in this study affected the europium-induced separation of the inner and outer choline resonances. We conclude that particulate contact with dimyristoyl and dipalmitoyl lecithin vesicles was not disruptive in our studies.

This study demonstrates the feasibility of using fluorescence spectroscopy for measuring the exchange of PAH between particulates and cell membranes. The sensitivity of the fluorescent properties of these aromatic hydrocarbons to their local environment should allow the preparation of particulates with known surface distributions of carcinogens. The study of coated particulates should prove useful in understanding how the mode of carcinogen administration to experimental animals affects the cellular availability and carcinogenicity. In addition, such knowledge should provide a rational basis for understanding the synergistic effects of polynuclear aromatic hydrocarbons and particulates.

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13. Fluorescence spectra were obtained with a spectrofluorometer (SLM Instruments). Samples were placed in a cuvette (2 by 2 cm) which in turn was placed in a cuvette holder equipped with a magnetic stirrer and a thermostat. The samples could thus be observed while the particles were kept in suspension by continuous stirring. Scattered light at the excitation wavelength did not affect our measurements because we used the appropriate optical filtration. The excitation wavelength was 305 nm, and the excitation and emission band-passes were 8 and 2 nm, respectively. Phospholipid vesicles were prepared by sonication of lipid in buffer, followed by centrifugation at 48,000*g* for 1 hour. Unless otherwise stated, all experiments were conducted at 40°C. The buffer was 0.01*M* tris, 0.05*M* KCl, *p*H 7.5. Umbelliferyl phosphate (Polysciences), as the sodium salt, was incorporated into vesicles by cosonication of phospholipid and umbelliferyl phosphate, followed by dialysis to remove the external fluorophore. Dimyristoyl-L-α-lecithin and dipalmitoyl-L-α-lecithin (both from Sigma) were used without further purification. The *N*-ethylcarbazole, chrysene, and 1,6-diphenylhexatriene were from Aldrich. The *N*-ethylcarbazole was recrystallized six times from absolute ethanol, and the chrysene was recrystallized from benzene. Porous glass was obtained from Corning (200 to 350 m^2/g), glass beads (3 to 8 μm) from Polysciences, acid-washed alumina from Merck, and silica gel G preparative thin-layer plates from Analabs. *N*-Ethylcarbazole was coated onto the particulates from ethanol, 1,6-diphenylhexatriene from acetone, and chrysene from benzene; the solvent was then removed under vacuum with continual mixing. Silica with adsorbed chrysene was prepared by chromatographing chrysene on preparative thin-layer plates, with cyclohexane and benzene (80:20 by volume) as the solvent system; this was followed by mechanical removal of the chrysene band. The fluorophore content of the particulates was determined by solvent extraction and subsequent optical density measurements.
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β-Glucuronidase Catalyzed Hydrolysis of Benzo[*a*]pyrene-3-Glucuronide and Binding to DNA

Abstract. *β-Glucuronidase catalyzes the hydrolysis of benzo*[a]*pyrene-3-glucuronide to 3-hydroxybenzo*[a]*pyrene. During the enzymatic hydrolysis, a benzo*[a]*pyrene derivative is formed which binds to DNA to a far greater extent than either the 3-hydroxybenzo*[a]*pyrene or its glucuronide. These results suggest that conjugates of benzo*[a]*pyrene may be converted by β-glucuronidase at intracellular and organ sites distal to the initial sites of oxygenation and conjugation of benzo*[a]*pyrene to activated intermediates that are possibly carcinogenic.*

Benzo[*a*]pyrene is a carcinogenic hydrocarbon commonly found in the environment (*1*). The toxic, mutagenic, and carcinogenic effects of this hydrocarbon are dependent on metabolism by the mixed function oxidases and metabolically related enzymes (*2*). Benzo[*a*]-pyrene is enzymatically converted to epoxides and at least four phenols and three quinones. The epoxides are also converted to dihydrodiols (*3*). The oxygenated metabolites are also converted to glutathione (*4*), glucuronide (*5*), and sulfate conjugates (*6*). Recent studies have demonstrated the metabolic formation of a diol epoxide of benzo[*a*]pyrene, *r*-7,*trans*-8-dihydroxy-*trans*-9,10-oxy-7,8,9,10-tetrahydrobenzo[*a*]pyrene which is highly mutagenic (*7*) and binds very actively to nucleic acids (*8, 8a*).

The water-soluble conjugates of benzo[*a*]pyrene, such as the glucuronides, have been generally viewed as detoxification products. These products, largely formed in the liver, are transported systemically to remote organs and are excreted in the urine or feces (*9*). When benzo[*a*]pyrene is administered to animals it is excreted in the bile, feces, and urine in the form of water-soluble conjugates, many of which have been identified as glucuronides and sulfates (*9*). Wa-

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