Photoelectric properties and detection of the aromatic carcinogens benzo[a]pyrene and dimethylbenzanthracene

(photoelectron microscopy/photoelectron quantum yields/lipid bilayers)

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Communicated by Harden M. McConnell, June 11, 1979

ABSTRACT   The absolute photoelectron quantum yield spectra for benzo[a]pyrene and dimethylbenzanthracene are presented in the wavelength range 180–230 nm. These polycyclic aromatic carcinogens have photoelectron quantum yields of approximately 2×10^{-3} electrons per incident photon at 180 nm. The quantum yields fall off quickly and monotonically at wavelengths longer than 210 nm (5.9 eV). Threshold values for benzo[a]pyrene and dimethylbenzanthracene are 5.25 ± 0.06 eV and 5.27 ± 0.04 eV, respectively. The photoelectron quantum yields of benzo[a]pyrene and dimethylbenzanthracene are several orders of magnitude greater than typical components of biological membranes (amino acids, phospholipids, and polysaccharides). Preliminary micrographs of benzo[a]pyrene and dimethylbenzanthracene sublimed onto poly(L-lysine) and onto dimyristoyl phosphatidylcholine demonstrate the high contrast of small crystallites of carcinogens against a background of membrane components. These results and calculations involving relative contrast factors suggest that the distribution of these carcinogens in biological membranes can be determined by using photoelectron microscopy.

Benzo[a]pyrene and dimethylbenzanthracene are two of the most ubiquitous toxic environmental carcinogens (1). These polycyclic aromatic hydrocarbons are lipid soluble and therefore are concentrated in biological membranes. Benzo[a]pyrene and dimethylbenzanthracene are metabolized to their ultimate carcinogenic forms by a group of membrane enzymes, the mixed-function oxygenase system in the endoplasmic reticulum (2–4). The rate-limiting step for this process is thought to be the oxygenation of carcinogen by cytochrome P-450 (5). Little is known about how aromatic carcinogens distribute in membranes. Many different integral proteins are present, each with lipid-binding sites (6). The carcinogens can in principle bind to a variety of hydrophobic proteins and also distribute between lipid phases present in membranes. The distribution affects the availability of the substrate for binding to the catalytic site of cytochrome P-450 and hence the rate of production of the ultimate carcinogen. At present there is no general microscopic method for determining the distribution of polycyclic aromatic carcinogens in membranes. A possible solution to this problem is to take advantage of an unexploited property of these polycyclic aromatic molecules, the photoelectric effect. Upon absorption of light of sufficient energy, molecules will emit electrons. The number of electrons emitted per photon at a given wavelength of incident light is defined as the photoelectron quantum yield. Large differences in photoelectron quantum yield of biological molecules can be employed as a source of contrast in photoelectron microscopy. High depth resolution and moderately high lateral resolution (7–9) make this technique suitable for the determination of chemical topography of biological membranes. There is to the best of our knowledge no previously published data on the photoelectron quantum yields of benzo[a]pyrene and dimethylbenzanthracene.† In the present study we examine the photoelectric properties of the carcinogens benzo[a]pyrene and dimethylbenzanthracene and investigate the feasibility of detecting small clusters or single molecules of these compounds in biological membranes.

MATERIALS AND METHODS

Materials. Dimyristoyl phosphatidylcholine and poly(L-lysine) were obtained from Sigma Chemical Co. and used without further purification. All solvents were freshly distilled before use. Benzo[a]pyrene was obtained from Aldrich (Gold Label Grade, est. 99%) and 7,12-dimethylbenzanthracene was received from Sigma. Purity of these polycyclic aromatic hydrocarbons was determined by silica gel thin-layer chromatography and elemental analysis. Silica gel thin-layer chromatography of benzo[a]pyrene and dimethylbenzanthracene in several solvents of different polarities indicated no observable impurities as detected by quenching of plate fluorescence or iodine vapor visualization. Elemental analysis. Calculated for $C_{20}H_{12}$: C, 95.21; H, 4.79. Found: C, 94.87; H, 4.61. Calculated for $C_{20}H_{16}$: C, 93.71; H, 6.29. Found: C, 93.47; H, 6.05.

Sample Preparation. Benzo[a]pyrene and dimethylbenzanthracene were each prepared for photoelectron quantum yield measurements by depositing a 50 mg/ml solution in benzene onto a carbon-coated stainless steel sample rod and allowing it to air dry. Some samples were prepared by sublimation of the carcinogen onto the surface of a water-cooled sample rod. No difference in quantum yield between these two preparative procedures was detectable. Photoelectron quantum yield spectra presented in this report represent the mean of 15 independent measurements for each compound.

Samples for photoelectron microscopy were routinely prepared as follows. A thin (ca 100 nm) layer of carbon was evaporated onto the surfaces of stainless steel sample rods, providing a nonreflective surface (11). On this surface was placed either a drop of dimyristoyl phosphatidylcholine (0.1 mg/ml, chloroform) or a drop of poly(L-lysine) (10 mg/ml, H_2O). The poly(L-lysine) rods were washed with distilled water after a few mintues and the excess water was drawn off with lens tissue. After air drying, the sample rod was observed in the photoelectron microscope to ensure that the biochemical substrate evenly coated the sample rod. Benzo[a]pyrene or dimethylbenzanthracene was sublimed onto the biochemical substrate through a 300 mesh copper grid. After sublimation, the sample rod was immediately placed onto the cooled stage of the photoelectron microscope and the instrument was evacuated to high vacuum before observation.

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† Some preliminary photoelectron data on benzo[a]pyrene were reported by this laboratory (8, 10).

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Proc. Natl. Acad. Sci. USA 76 (1979)   4181

Instrumentation. Photoelectron quantum yield spectra were obtained with an ultrahigh vacuum photoelectron instrument as described (10). The reference compound was purified metal-free phthalocyanine, whose absolute photoelectron quantum yield spectrum is known (12). Photoelectron microscopy was performed with an ultrahigh vacuum photoelectron microscope built at the University of Oregon (cf. ref. 13). The present study was performed by using 100-W Cd/Hg-doped xenon lamps (Advanced Radiation Corp., Santa Clara, CA) as an UV light source. The sample stage was cooled by using liquid nitrogen with an external Dewar flask.

Beam current measurements were made as follows. The photoelectron image of a thin film of benzo[a]pyrene was focused on a phosphor screen in the photoelectron microscope. The phosphor material had been spread on a tin oxide-coated piece of glass. A shielded electrical lead connected the phosphor screen to a Keithly 620 electrometer. The phosphor was held at +20 V with respect to the grounded housing by a battery. Beam current measurements were made at several magnification settings and corrected for dark current by shuttering the UV lamps.

RESULTS AND DISCUSSION

Quantum Yields. The photoelectron quantum yields for benzo[a]pyrene and dimethylbenzanthracene are shown in Figs. 1 and 2. This parameter is not related to the familiar fluorescence quantum yield. We have also measured the photoelectron quantum yield spectra for a variety of biological molecules including amino acids (14), phospholipids (15), sac-
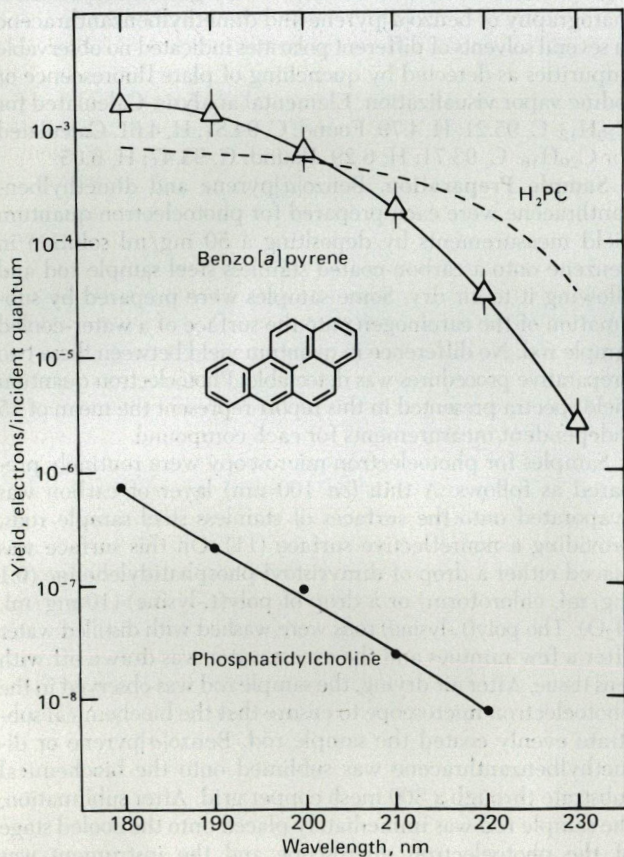

FIG. 1. Photoelectron quantum yield spectrum of benzo[a]pyrene (△). Vertical bars represent the maximum estimated error of the measurements. Also shown are the photoelectron quantum yield spectra for the experimental reference standard, metal-free phthalocyanine (---, H₂PC) (12), and phosphatidylcholine (●) (15).
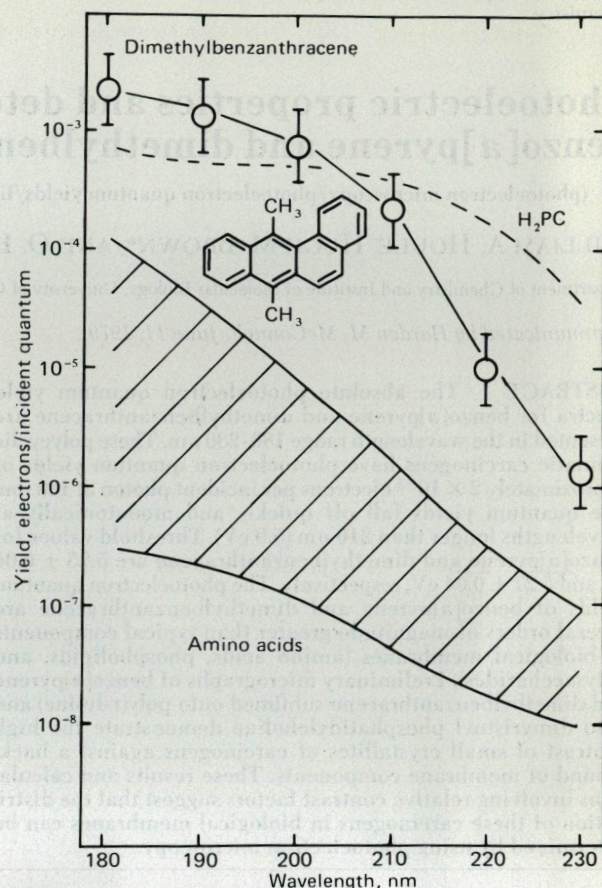

FIG. 2. Photoelectron quantum yield spectrum of dimethylbenzanthracene. Included in this figure are the photoelectron quantum yield spectrum of metal-free phthalocyanine (H₂PC) (12) and the band of spectra for the amino acids and their homopolymers (14).

charides (16), hemes (17), chlorophylls (18), and tetracene (14). For reference, Fig. 1 shows the photoelectron quantum yield for phosphatidylcholine (15), while Fig. 2 shows the photoelectron quantum yield for the amino acids and their homopolymers (14).

Threshold Values. Threshold values for benzo[a]pyrene, dimethylbenzanthracene, and tetracene were calculated by using the following equation:

$$Y^{1/3} = C(h\nu - E_{th}), \qquad [1]$$

in which Y is the photoelectron quantum yield, $h\nu$ is the energy of the incident light, E_{th} is the threshold energy, and C is a constant of proportionality (19, 20). Threshold values were obtained from extrapolating to zero quantum yield plots of $Y^{1/3}$ versus $h\nu$ as illustrated for the carcinogens in Fig. 3. The threshold value of tetracene was determined in a similar manner from its photoelectron quantum yield obtained previously in this laboratory (14).

Threshold values for benzo[a]pyrene and dimethylbenzanthracene can be determined from their photoelectron quantum yield spectra by using Eq. 1. Threshold values, or the minimum amount of energy required to stimulate emission from the solid state, were determined to be 5.25 ± 0.06 eV for benzo[a]pyrene and 5.27 ± 0.04 eV for dimethylbenzanthracene. As a control we have also measured the photoelectron quantum yield for another polycyclic aromatic hydrocarbon, tetracene. The threshold value obtained from our data for this molecule, 5.22 ± 0.08 eV, agrees well with values reported by other workers using independent techniques (5.24 eV, ref. 24; 5.25 eV, ref. 25; 5.31 eV, ref. 26).
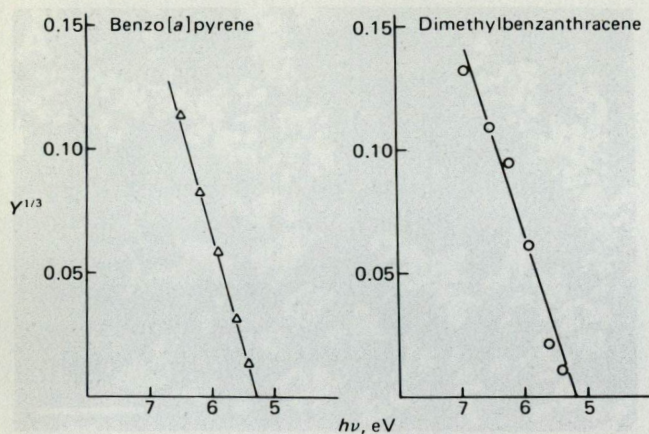
FIG. 3. Plots of photoelectron quantum yield to the $1/3$ power versus energy of the incident light for benzo[a]pyrene (*Left*) and dimethylbenzanthracene (*Right*).

Contrast Ratios. The quantum yield curves suggest that benzo[a]pyrene and dimethylbenzanthracene should exhibit high photoelectric contrast in biological membranes. The photoelectron quantum yields of molecules do not correlate with carcinogenicity, but they do provide a method of detecting these aromatic carcinogens. The brightness or intensity ratio, I, between a resolution element containing benzo[a]pyrene and a lipid background is a function of the photoelectron quantum yields and the fractional area occupied by benzo[a]pyrene. Thus

$$I = \frac{f_2 Y_2 + (1 - f_2) Y_1}{Y_1}, \quad [2]$$

in which f_2 is the fractional area occupied by benzo[a]pyrene in the resolution element, $1 - f_2$ is the fractional area occupied by the lipid in the resolution element, Y_2 is the quantum yield of benzo[a]pyrene, and Y_1 is the quantum yield of the lipid. The fractional area occupied by the benzo[a]pyrene molecules is easily calculated from the expression $f_2 = nA_B/\pi r^2$, in which n is the number of benzo[a]pyrene molecules in the resolution element, A_B is the cross-sectional area of a benzo[a]pyrene molecule, and r is the radius of the resolution element.

For a pure patch of benzo[a]pyrene in a lipid background, $f_2 = 1$, and thus I becomes the ratio of the quantum yields of benzo[a]pyrene and lipid. In this case the patch of benzo[a]-pyrene is 1000 times as bright as the lipid background. The other limiting case is a single molecule of benzo[a]pyrene in a resolution element. The theoretical resolution limit of the current photoelectron microscope is on the order of 5 nm. The dimensions of a benzo[a]pyrene molecule as determined from Corey–Pauling-Koltun (CPK) space-filling models are 13 Å $\times$ 9 Å $\times$ 3.5 Å, which yields an average cross-sectional area of 65 Å^2. With these values, Eq. **2** shows that a single benzo[a]-pyrene molecule in a resolution element would be approximately 30 times as bright as a lipid background. Similar results are obtained for dimethylbenzanthracene.

Whether the carcinogen molecule is surrounded by other carcinogens or by lipids and proteins should have very little effect on its photoemission properties. In previous work on hemes, for example, it was shown that the photoelectron quantum yield curve of hemoglobin is consistent with the quantum yield curves of pure heme and protein, taking into account the fractional area of heme present in hemoglobin (17). Photoemission is a rapid process and we observe no quenching effects such as those commonly encountered in fluorescence.

Photoelectron Statistics. It is useful to calculate how many electrons must be emitted from a resolution element in order to visualize it against background noise. In an electron optical system the point at which the signal-to-noise ratio, SNR, is most critical occurs at the sample surface (21). The SNR is given by the formula

$$SNR = \frac{n_2 - n_1}{(n_2 + n_1)^{1/2}} \quad [3]$$

(22). Let n_1 be the number of electrons emitted from a resolution element consisting of the lipid background and n_2 be the number of electrons emitted from a resolution element containing benzo[a]pyrene. A satisfactory image can be produced by a SNR of 6 (22). The relative values of n_1 and n_2 can be determined from the intensity ratio, I, in Eq. **2**. For a pure patch of benzo[a]pyrene $n_2 = 1000n_1$. Solving for n_2 using this relationship in Eq. **3** and setting SNR = 6 shows that emission of 36 electrons would be required from a resolution element containing pure benzo[a]pyrene to visualize it against a lipid background. For a single molecule of benzo[a]pyrene in a 5-nm resolution element $n_2 = 30n_1$. In this case, emission of about 40 electrons would be required to visualize a resolution element containing a single benzo[a]pyrene molecule. The same results are obtained for dimethylbenzanthracene.

Beam Current Measurements to Determine if Multiple Emission Is Possible. Measurements of the beam current from a thin film of benzo[a]pyrene were taken over a 5-hr period. Under continuous illumination no significant change in beam current was detected. The sample was held at liquid nitrogen temperature to prevent sublimation of the benzo[a]pyrene.

Molecular emission rates can be determined from beam current measurements. When the field of view was a 34-μm-diameter circle, the beam current was 14 pA. The current density at the sample surface was calculated to be 1.5 μA/cm^2. This is equivalent to 10^{13} electrons emitted per cm^2 of sample surface per second. Because the energy of the photoemitted electrons is low, only those electrons emitted at or near the surface have sufficient energy to escape into the vacuum. Typical escape depths range from 1 to 3 nm (cf. ref. 8). Assuming the dimensions of the benzo[a]pyrene molecule given in the previous section, and that all emitted electrons originate in the top 2 nm, it follows that each molecule emits an electron on the average every 50 sec.‡ At this emission rate, the fact that the beam current did not change over 5 hr requires that each molecule emit 360 times.

Implications for Biological Studies. Photoelectron quantum yield is defined as the number of electrons emitted per incident photon of light at a given wavelength. Photoelectron quantum yield spectra shown in Figs. 1 and 2 demonstrate that benzo[a]pyrene and dimethylbenzanthracene are among the most photoemissive organic compounds measured. Benzo[a]pyrene and dimethylbenzanthracene are 100 to 5000 times as photoemissive as typical membrane components such as amino acids, phospholipids, and polysaccharides in the wavelength range 180–240 nm (14–16). Benzo[a]pyrene and dimethylbenzanthracene also have much higher photoelectron quantum yields than purines and pyrimidines in this wavelength range (ref. 23 and unpublished data). At wavelengths longer than 240 nm the photoelectron quantum yields of these molecules quickly fall below the level of detection. There is no significant yield of electrons in the visible region of the spectrum. At wavelengths shorter than 180 nm the photoelectron quantum yield curves begin to converge. Thus the region of the spectrum from 180 to 240 nm is optimal for detecting these aromatic chemical carcinogens.

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‡ New UV optics under development will increase the light intensity by a factor of 100. Thus, the molecules should emit electrons on the order of 1 per sec, assuming no conductivity limitations.

The high photoelectron quantum yields and relatively low threshold energies suggest the possibility of detecting small clusters of carcinogen or carcinogen-rich phases in membranes by using a photoelectron microscope. *In vivo* concentrations of carcinogens are low, so we consider first the limiting case, the feasibility of detecting single molecules of benzo[a]pyrene. The dimensions of a benzo[a]pyrene molecule (13 Å × 9 Å × 3.5 Å) are less than the resolution limit of a photoelectron microscope (5 nm). However, on the basis of the quantum yield spectrum, a single molecule of benzo[a]pyrene in a resolution element would be approximately 30 times as bright as a lipid background in a photoelectron microscope.§ At the other limit, a pure patch of benzo[a]pyrene the size of a resolution element would be 1000 times as bright as a lipid background. These contrast ratios are sufficiently high to visualize a resolution element containing benzo[a]pyrene in a lipid bilayer.

Given that a resolution element containing benzo[a]pyrene exhibits sufficient contrast to be seen in a lipid matrix, the emission statistics must be considered. Calculations presented above indicate that emission of about 40 electrons from a resolution element is needed for detection at 5-nm resolution over the concentration range of interest (pure patch to a single molecule of carcinogen). For a pure patch, emission of one electron per carcinogen molecule would be sufficient, whereas in the limiting case of a single carcinogen molecule per resolution element, this molecule would have to emit 40 times. Measurements of the beam current from a thin film of benzo[a]pyrene molecules over time show that single molecules of benzo[a]pyrene can photoemit an electron at least several hundred times. There was no significant decrease of the beam current over a time period of several hours. Evidently the cathode supplies an electron to the benzo[a]pyrene molecule after each photoionization event.

Photoelectron micrographs of polycyclic aromatic carcinogens are shown against uniform backgrounds of polypeptide and phospholipid in Fig. 4. These micrographs are not at the highest resolution because the photoelectron microscope is still under development. The current resolution is about 25 nm. The micrographs, however, demonstrate the essential features of photoelectric properties presented in this paper. Fig. 4A is a photoelectron micrograph of dimethylbenzanthracene on an even film of poly(L-lysine). The photoelectron quantum yield of poly(L-lysine) is typical of amino acids and their polymers. Fig. 4B shows benzo[a]pyrene on an even film of the lipid dimyristoyl phosphatidylcholine. The carcinogens are present as small crystals. The largest crystals are approximately 1000 nm. The smallest are no more than 25 nm. Any clusters smaller than this would appear to be about 25 nm because of the resolution limit of the microscope. Both carcinogens exhibit marked photoelectron contrast with these biochemical backgrounds, in accord with their photoelectron quantum yield spectra.

In conclusion, the absolute photoelectron quantum yields of benzo[a]pyrene and dimethylbenzanthracene have been measured. The high photoelectron contrast of these carcinogens with biochemical molecules, the ability of these carcinogens to photoemit repeatedly, and these preliminary micrographs suggest the feasibility of employing the photoelectron microscope to study the distribution and phase behavior of benzo[a]-pyrene and dimethylbenzanthracene in biological membranes.

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§ If the carcinogen were distributed in an asymmetric manner and buried in the lower half of the bilayer, the intensity ratio would be attenuated by a factor of about 3, judging from monolayer studies on chlorophyll (27). In this case, the intensity ratio for a single molecule in a resolution element would still be high enough to visualize the carcinogen, making it possible to investigate the asymmetry of the carcinogen distribution.
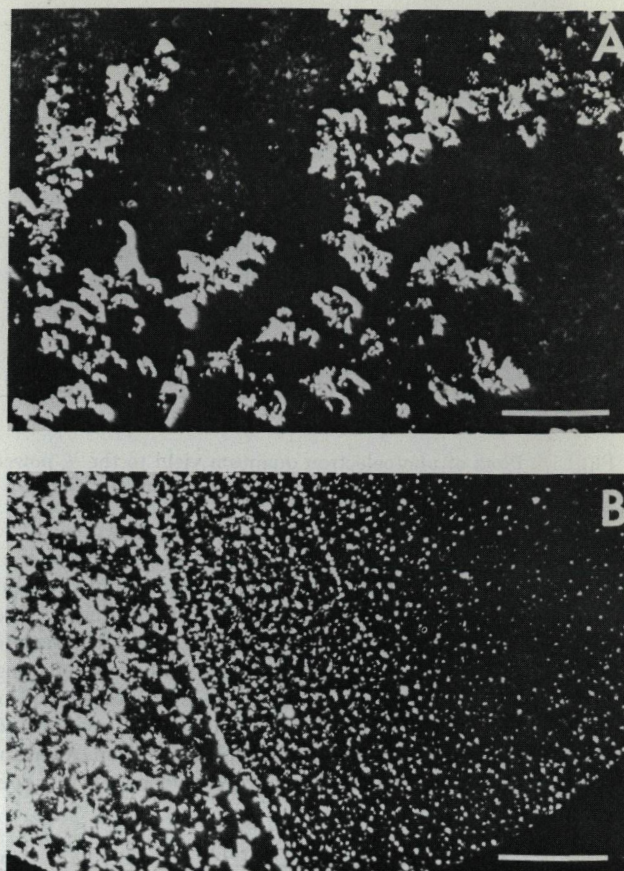

FIG. 4. (*A*) Photoelectron micrograph of dimethylbenzanthracene sublimed in a vacuum onto a thin layer of poly(L-lysine). (*B*) Benzo[a]pyrene sublimed onto a thin layer of dimyristoyl phosphatidylcholine. The bar represents 5 μm in both micrographs.

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