CONTRAST IN THE PHOTOELECTRIC EFFECT OF ORGANIC AND BIOCHEMICAL SURFACES

A First Step towards Selective Labeling in Photoelectron Microscopy

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ABSTRACT The photoelectric effect can provide the physical basis for a new method of mapping organic and biological surfaces. The technique, photoelectron microscopy, is similar to fluorescence microscopy using incident ultraviolet light except that photoejected electrons form the image of the specimen surface. In this work the minimum wavelengths of incident light required to produce an image were determined for the molecules 3,6-bis(dimethylamino)acridine (acridine orange) (I), benzo[*a*]pyrene (II), *N*,*N*,*N*′,*N*′-tetraphenylbenzidine (III), and copper phthalocyanine (IV). The photoelectron image thresholds for these compounds are 220 (I), 215 (II), 220 (III), and 240 nm (IV), all ±5 nm. Contrast of I–IV with respect to typical protein, lipid, nucleic acid, and polysaccharide surfaces was examined over the wavelength range 240–180 nm. The low magnification micrographs exhibited bright areas corresponding to I–IV but dark regions for the biochemical surfaces. The high contrast suggests the feasibility of performing *extrinsic* photoelectron microscopy experiments through selective labeling of sites on biological surfaces.

INTRODUCTION

The photoelectric effect has the potential of providing new information on the structure and topography of cell surfaces. When a properly prepared specimen is subjected to ultraviolet light it will usually fluoresce. The emitted light is then focused and imaged with conventional glass optics in the powerful technique of fluorescence microscopy (Needham, 1968; Goldman, 1968). If, however, the energy of the incident light is sufficiently high the sample can emit electrons (photoionize) as well as fluoresce. By evacuating the sample chamber, accelerating the electrons, and replacing the glass optics with electron optics, it is possible, in principle, to form an image of any biological surface based on its photoelectric properties. We refer to this technique as *photoelectron microscopy* (Griffith et al., 1972). Photoelectron micros-

copy bears little resemblance to conventional or scanning electron microscopy but it may be viewed as a natural extension of fluorescence microscopy. The two essential differences are that photoelectron microscopy is a surface technique and the ultimate resolution is related to the wavelength of electrons rather than the wavelength of visible light.

In a recent paper, we reported the first low magnification photoelectron images of organic and biological surfaces (Griffith et al., 1972). The organic surfaces examined were grid patterns of acridine orange, fluorescein, and benzo[*a*]pyrene on a Butvar-coated support. The initial biological sample imaged was sectioned rat epididymis, principally because the well-characterized morphology can be viewed at low magnifications. These initial experiments demonstrate the feasibility of mapping biological surfaces according to differences in ionization potentials of molecules on or near the surface. They do not, of course, provide assurance that this approach will be successful at high magnifications. A number of biophysical studies must be performed, and certain technical advances (e.g., tunable ultraviolet lasers) are necessary to achieve this goal. Of particular importance is the search for specific photoionization labels that will lead to extrinsic photoionization of biological surfaces. In this paper we report preliminary observations on the image contrast of possible probes as a function of wavelength of the incident light.

EXPERIMENTAL

Materials and Sample Preparation

Acridine orange (I), benzo[*a*]pyrene (II), *N,N,N′,N′*-tetraphenylbenzidine (III), and copper phthalocyanine (IV) were purchased from Matheson Coleman & Bell (Cincinnati, Ohio), Sigma Chemical Co. (St. Louis, Mo.), Eastman Organic Chemicals Div. (Eastman Kodak Co., Rochester, N. Y.), and Research Organic/Inorganic Chemical Corporation, (Sun Valley, Calif.), respectively. Saltfree soybean trypsin inhibitor was obtained from Worthington Biochemical Corp. (Freehold, N. J.). Calf thymus DNA, containing 80.8% DNA, 2% RNA, and 0.22% protein by analysis, was purchased from Mann Research Labs. Inc. (New York). Ficoll (synthetic polysaccharide of approximate mol wt 400,000) was obtained from Sigma Chemical Co. Egg lecithin (95%) was obtained from BDH Chemicals Ltd. (Poole, England). Butvar (polyvinyl butyral) was purchased from Polysciences, Inc. (Warrington, Pa.). All chemicals were used without further purification. The standard photoelectron microscope sample mount is a stainless steel or gold-plated copper rod, 6.35 mm in diameter, 2.54 cm long and has a protruding ring near the upper end to position the rod in the sample stage assembly. To suppress photoemission from the sample rod, the lower end (sample end) was coated with Butvar. This was done in the following way: a microscope cover slip was dipped into a 1.0% solution of Butvar in chloroform, coating the glass with a thin polymeric film; after trimming, the film was floated off the glass into water and then onto the end of the sample rod.

A 200-mesh copper electron microscope grid, 3.05 mm in diameter (Ernest F. Fullam, Inc., Schenectady, N. Y.), was dipped in a 1.0% solution of Butvar in chloroform filling all of the open area with Butvar. Two parallel rows of holes in the grid were then opened (with three rows of Butvar-filled holes between the two rows of open holes, leaving the two rows

of open holes approximately 400 μm apart) using a fine needle under a dissecting microscope. The grid was held tightly against the Butvar-coated sample rod, and the organic compounds were then vacuum evaporated (using a 2-inch diffusion pumped system operated at 5×10^{-5} Torr) through the grid from an aluminum oxide crucible.

Aqueous solutions (5 mg/ml) of soybean trypsin inhibitor, calf thymus DNA, and Ficoll were prepared (the egg lecithin was prepared as a briefly sonicated lecithin-water [5 mg/ml] dispersion), and the biochemical samples were deposited on the sample rod between the rows of organic dye using a very fine camel hair brush.

The Photoelectron Microscope

A schematic diagram of the photoelectron microscope (PEM) is shown in Fig. 1. This is the ultrahigh vacuum microscope used previously (Griffith et al., 1972) except for two modifica-

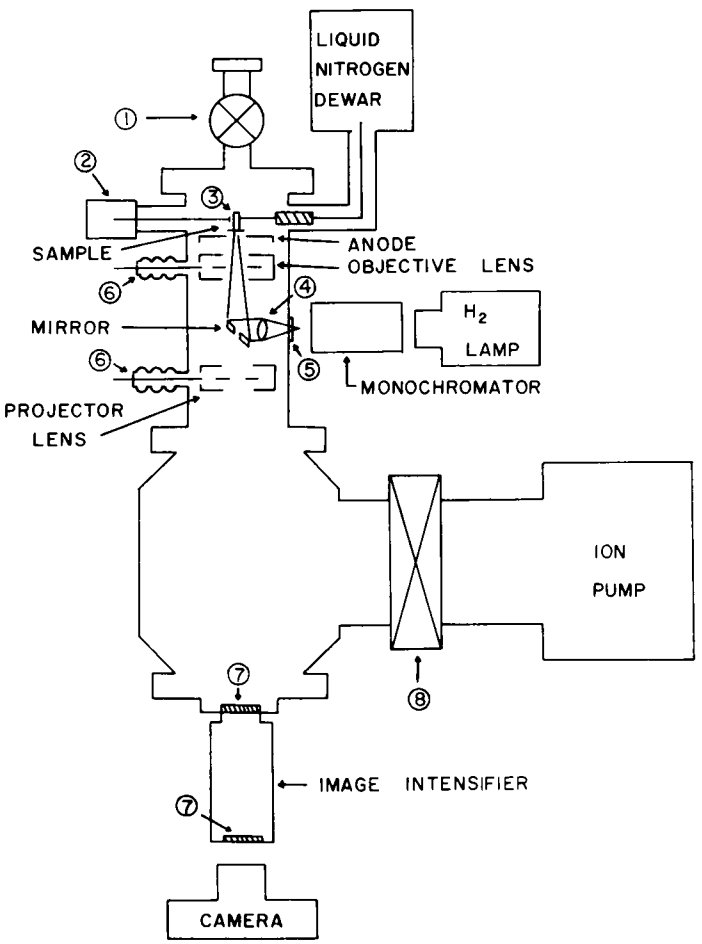

FIGURE 1 Simplified diagram of the photoelectron microscope. (1) Viton-sealed straight-through valve; (2) stage translator; (3) sample rod; (4) magnesium fluoride lens; (5) magnesium fluoride window; (6) high voltage ceramic feed-throughs; (7) aluminized phosphor screen on a fiber optic window; (8) 15.24 cm (6 inch) Viton-sealed gate valve.

tions. The first modification is the substitution of a Varo Corporation 40 mm three-stage image intensifier of the phosphor-photocathode type for the microchannel plate image intensifier (Varo Corp., Electron Devices Div., Garland, Tex.). The second modification is the placement of a Bausch & Lomb 200–400 nm high intensity grating monochromator (Bausch & Lomb Inc., Scientific Instrument Div., Rochester, N. Y.) between the hydrogen lamp and the PEM. The PEM was designed for use with a laser and the monochromator and lamp are a temporary measure to be used until tunable far ultraviolet lasers become available. The ultimate resolution of the PEM is estimated to be on the order of 30–40 Å, which is sufficient to map the positions of specific proteins in biological membranes. The present magnification is limited by the excitation source and not by the electron optics. With the available monochromator and lamp combination, the magnification is limited to $\times$ 100 because of the low light intensity and very weak images. The magnification range of $\times$ 10–$\times$ 100, however, is ideal for contrast studies because inexpensive 200-mesh copper electron microscope grids may be used to form the test patterns of organic compounds.

RESULTS AND DISCUSSION

The organic molecules chosen for study are acridine orange (I), benzo[*a*]pyrene (II), *N*,*N*,*N*′,*N*′-tetraphenylbenzidine (III), and copper phthalocyanine (IV) (see Fig. 2). Acridine orange was selected because of its importance in fluorescence microscopy and fluorescence spectroscopy (Thompson, 1966). The mechanism of binding of acridine orange, particularly to DNA, has been well-studied (Dusenbery and Uretz, 1972). The second molecule, benzo[*a*]pyrene is a chemical carcinogen of current interest (Daudel and Daudel, 1966; Cavalieri and Calvin, 1971). Preliminary patterns of both I and II were observed previously without the monochromator (Griffith et al., 1972). The remaining two molecules were chosen because they evidently have low ionization potentials. Liquids or compounds that could not be sublimed were avoided in order to minimize difficulties in sample preparation.

Patterns of organic and biochemical samples were made as indicated in the lower part of Fig. 3. The purpose of the experiment is to determine the relative contrast of

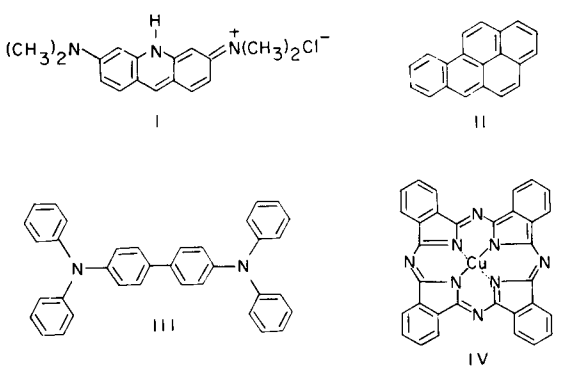

FIGURE 2 Structures of the organic molecules examined in the photoelectron microscope in this study: (I) acridine orange; (II) benzo[*a*]pyrene; (III) *N*,*N*,*N*′,*N*′-tetraphenylbenzidine; (IV) copper phthalocyanine.

I–IV with respect to typical biochemical surface components: protein, lipid, nucleic acid, and starch. Soybean trypsin inhibitor, egg lecithin, calf thymus DNA, and Ficoll were arbitrarily chosen as specific examples of these classes of biomolecules. Samples were prepared of I–IV with each biomolecule, for a total of 16 samples. Images of each sample were photographed as a function of the wavelength of incident light by advancing the monochromator in 5- or 10-nm steps over the range 240–180 nm. Typical photoelectron micrographs are shown in Fig. 3. In each case the image disappeared immediately as the lamp was switched off. The images were deflected when electrostatic or magnetic fields were brought near the apparatus. There is no doubt that these images are formed by electrons photoejected from the samples by ultraviolet light.

In Fig. 3, the most interesting feature of the micrographs is the absence of any image of the biochemical samples. Above the thresholds the images of the organic patterns were always bright and easily seen without time exposures. The biochemical patterns, however, were not visible on the phosphor screen. With long time exposures (30 s) it was possible to detect faint images of some biochemical samples at 180 nm. Thus, the contrast between the organic samples and biochemicals is striking. There may be a dilution effect, particularly in the protein sample. For example, if one or two of the many amino acids present has the same quantum yield as, say, acridine orange, the protein sample would still appear dark compared with the pure acridine orange grid pattern. This question can be resolved later by measuring the quantum yields of all of the common amino acids. In this study no attempt has been made to examine the dependence of contrast on sample thickness or temperature so that some effects of sample conductivity cannot be ruled out. The principal physical basis for contrast is almost certainly the relative ionization potentials and photoelectric quantum yields of the organic and biochemical surfaces.

The photoelectron image thresholds of I–IV are listed in Table I. The image thresholds were determined by advancing the monochromator dial in 5 nm steps to shorter wavelengths after recording the micrographs (3 s exposure, $\times$ 35). The image threshold was operationally defined as the longest wavelength setting at which the image could be seen on the micrograph. This definition is, of course, arbitrary but it is useful in relative measurements. It corresponds to an absolute quantum yield of about 10^{-5}–10^{-6} electrons/quantum of incident light. The increase in brightness is quite noticeable as the wavelength is scanned through the threshold, and the measurements are reproducible. The lamp intensity measured at the exit slit of the monochromator was found to be very nearly constant over the wavelength range 240–180 nm and no lamp excitation curve corrections were applied to the data of Table I.

It is of interest to compare these data with literature values of ionization potentials and photoelectron quantum yields. Unfortunately, very little information is available. We were unable to locate any ionization potential data for acridine orange. The first ionization potential of benzo[*a*]pyrene is estimated to be 7.2 eV (172 nm)

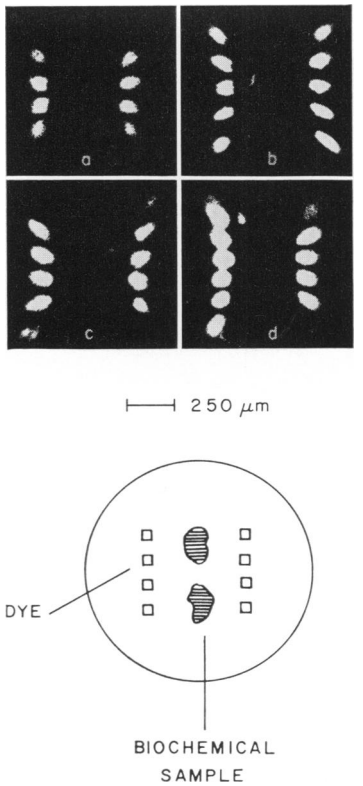

FIGURE 3 Photoelectron micrographs of samples consisting of both an organic dye and a biochemical sample deposited on thin films of Butvar. The samples were placed on the sample rods as indicated in the schematic diagram at the bottom of the figure. (*a*) Acridine orange (I) and soybean trypsin inhibitor; (*b*) benzo[*a*]pyrene (II) and calf thymus DNA; (*c*) *N*,*N*,*N*′,*N*′-tetraphenylbenzidine (III) and Ficoll; (*d*) copper phthalocyanine (IV) and soybean trypsin inhibitor. All micrographs were approximately × 35. The wavelength of the exciting radiation was 210 nm for samples (*a*), (*c*), and (*d*); 200 nm for sample (*b*). The sample stage temperature was about 80–100°K and the stage voltage was −10 kV in all experiments. Exposure times were 3.0 s for micrographs (*a*), (*c*), and (*d*) and 2.0 s for (*b*). The pincushion distortion in the images is caused by the three-stage image intensifier.

TABLE I

PHOTOELECTRON IMAGE THRESHOLDS*

Compound	Threshold
	nm
Acridine orange (I)	220
Benzo[*a*]pyrene (II)	215
N,*N*,*N*′,*N*′-Tetraphenylbenzidine (III)	220
Copper phthalocyanine (IV)	240

* The experimental accuracy in the threshold measurements is estimated to be ±5 nm.

from an indirect method based on optical data of charge-transfer complexes in solution (Briegleb and Czekalla, 1959). The ionization potentials of N,N,N',N'-tetraphenylbenzidine apparently have not been reported. A solid sample of N,N,N',N'-tetramethylbenzidine, however, has a photoionization threshold of 4.71 eV (263 nm), defined as the point where the photoelectron quantum yield is 10^{-8} electrons/quantum of incident light (Batley and Lyons, 1968). The photoionization threshold of solid copper phthalocyanine is on the order of 5.0 $\pm$ 0.1 eV (248 nm), (Pope, 1962; Batley and Lyons, 1968). Photoelectric data on biomolecular surfaces are rare. Setlow (1960) has observed a photoelectric effect in the far ultraviolet for a dry ribonuclease film. More recently Berger (1969) measured the relative photoelectron quantum yield for the bacteriophage ϕx-174 DNA over the wavelength range 253.7–58.4 nm. The sharp increase in relative quantum yield occurs at about 9 eV (138 nm). The purpose of this study was to relate the photoionization data to inactivation of infectious DNA by ultraviolet light. The study is of interest here because it suggests relatively high ionization potentials for DNA. Our results are, therefore, consistent with the literature that is available. It should also be noted that the voltage across the sample in the conventional experiment is 0–200 V/cm whereas in the PEM experiments it is 10^4–10^5 V/cm, so that the latter may yield slightly lower thresholds. Thus, in order to understand the PEM image contrast it will be necessary to collect ionization potential data as a function of field strength. The high field strengths are required in PEM in order to achieve sufficient resolution for high magnification experiments (Grivet, 1965; Griffith et al., 1972).

In summary, the essential point of this work is that organic molecules do exist that have appropriate photoelectric properties to serve as labels in photoelectron microscopy. It is probable that *intrinsic* photoionization of biological surfaces under vacuum ultraviolet radiation will yield useful information, but the potential of *extrinsic* photoionization using specific labels cannot be ignored. This study is to be regarded as a first step towards the development of labeling techniques in PEM. We have not yet shown, for example, that isolated labels coupled to biomolecules will yield favorable contrast, nor has the depth resolution been examined. Nevertheless, it is encouraging that images observed under present low-illumination conditions are stable. The contrast did not fade. In these experiments there were no difficulties with sample charging, although of course this may become troublesome under high illumination and high magnification conditions.

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