Cellular Uptake and Intracellular Localization of Benzo(a)pyrene by Digital Fluorescence Imaging Microscopy

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ABSTRACT Uptake of benzo(a)pyrene by living cultured cells has been visualized in real time using digital fluorescence-imaging microscopy. Benzo(a)pyrene was noncovalently associated with lipoproteins, as a physiologic mode of presentation of the carcinogen to cells. When incubated with either human fibroblasts or murine P388D1 macrophages, benzo(a)pyrene uptake occurred in the absence of endocytosis, with a halftime of ~2 min, irrespective of the identity of the delivery vehicles, which were high density lipoproteins, low density lipoproteins, very low density lipoproteins, and 1-palmitoyl-2-oleoylphosphatidylcholine single-walled vesicles. Thus, cellular uptake of benzo(a)pyrene from these hydrophobic donors occurs by spontaneous transfer through the aqueous phase. Moreover, the rate constant for uptake, the extent of uptake, and the intracellular localization of benzo(a)pyrene were identical for both living and fixed cells. Similar rate constants for benzo(a)pyrene efflux from cells to extracellular lipoproteins suggests the involvement of the plasma membrane in the rate-limiting step. The intracellular location of benzo(a)pyrene at equilibrium was coincident with a fluorescent cholesterol analog, N-(7-nitrobenz-2-oxa-1,3-diazole)-23,24-dinor-5-cholen-22-amine-3β-ol. Benzo(a)pyrene did not accumulate in acidic compartments, based on acridine orange fluorescence, or in mitochondria, based on rhodamine-123 fluorescence. When the intracellular lipid volume of isolated mouse peritoneal macrophages was increased by prior incubation of these cells with either acetylated low density lipoproteins or with very low density lipoproteins from a hypertriglyceridemic individual, cellular accumulation of benzo(a)pyrene increased proportionately with increased [1-14C]oleate incorporation into cellular triglycerides and cholesteryl esters. Thus, benzo(a)pyrene uptake by cells is a simple partitioning phenomenon, controlled by the relative lipid volumes of extracellular donor lipoproteins and of cells, and does not involve lipoprotein endocytosis as an obligatory step.

Benzo(a)pyrene is a common environmental pollutant whose carcinogenic potential depends on oxidative metabolism after cellular uptake (1). Oxygenation and hydration reactions occur in the endoplasmic reticulum to form active carcinogenic compounds, such as diol epoxides, that interact covalently with DNA. Most investigations have been concerned with the enzymatic events of cytochrome P450-dependent metabolic processes, and with the chemical modification of DNA. In vivo, these processes are necessarily preceded by cellular uptake of the hydrophobic precursor hydrocarbon (2). Typically, metabolic studies of benzo(a)pyrene are performed by exposing cells or microsomal fractions to concentrated solutions of benzo(a)pyrene in organic solvents diluted into the culture medium or the reaction mixture. Because of the low aqueous solubility of benzo(a)pyrene, ~10^-8 M (3), little of the hydrocarbon is truly in solution under these conditions and most, if not all, is present in the form of microcrystals (4). The kinetics of desorption of benzo(a)pyrene from microcrystals to lipid bilayers is slow (5). Because solubilization of benzo(a)pyrene in microsomal membranes is obligatory for its further metabolism, this experimental procedure most likely leads to significant underestimation of the rate and extent of metabolism, because there is limited incorporation of benzo(a)pyrene into microsomal preparations or cell membranes.

A physiologic mode of presentation of these hydrophobic compounds is as a solubilized component of a lipid matrix. Studies in vitro (6, 7) and in vivo (8) have shown that
benzo(a)pyrene and dimethylbenzanthracene, respectively, are present in the plasma as noncovalent components of the plasma lipoproteins. The plasma lipoproteins are dynamic macromolecular assemblies of lipids and specific apoproteins and differ in size, density, chemical composition, and metabolic fate (9, 10). In fasting plasma, the most abundant lipoproteins are the protein-rich high density lipoproteins (HDL), which contain about 25 mol% phosphatidylcholine and 50 mol% protein. Low density lipoproteins (LDL) contain 37 mol% cholesteryl ester, whereas very low density lipoproteins (VLDL) are 50 mol% triglyceride. Apolipoprotein B (ApoB) a protein component of LDL, is recognized by specific high-affinity membrane sites on certain cells for receptor-mediated endocytosis (11). Thus, uptake of LDL by these receptors could introduce benzo(a)pyrene and other xenobiotics into the cell interior as components of the lipoprotein.

The lipids and most of the protein components of lipoproteins exchange rapidly among lipoprotein classes (9, 10). Benzo(a)pyrene transfer between LDL is relatively fast, with a halftime of ~200 ms (12). Many factors are known to influence the rates and energetics of spontaneous desorption of lipophiles such as fatty acids, phospholipids, and polycyclic aromatic hydrocarbons from a phospholipid surface. These include the length of acyl chains (13), the nature of the hydrophilic headgroup (13–15), the molecular surface area of aromatic compounds (16), the nature of the hydrophobic environment (17, 18), and the radius of the lipid surface from which desorption occurs (19).

Evidence that cellular uptake of benzo(a)pyrene from lipoproteins occurs by transfer of individual molecules through the aqueous solution has been obtained by Renssen and Shireman (20), who have demonstrated with LDL receptor-negative cells that cellular uptake of benzo(a)pyrene can occur in the absence of LDL endocytosis. These studies, however, did not address the kinetics of passive transfer of benzo(a)pyrene, or the potential contribution in normal cells of either receptor-mediated endocytosis or fluid-phase endocytosis of the lipoprotein carrier. Clarification of these aspects of the dynamics of cellular uptake of benzo(a)pyrene and the mechanism of intracellular and intercellular distribution of benzo(a)pyrene is necessary to understand the overall process of benzo(a)pyrene carcinogenesis. Thus, one objective of this study was to examine the mechanism and kinetics of benzo(a)pyrene entry into cells by direct observation of living cells in real time using digital fluorescence-imaging microscopy. A second objective was to determine the effects of lipoprotein metabolism on the extent of cell uptake and on intracellular distribution of benzo(a)pyrene. An abstract of this work has been published (21).

MATERIALS AND METHODS

Fluorescence Microscopy: The digital fluorescence-imaging microscopy system is described in detail in the preceding article (22). Briefly, the system consisted of a Leitz Diavert inverted fluorescence microscope (E. Leitz, Inc., Rockleigh, NJ), a Hamamatsu Vidicon C1000-12 low light level camera (Hamamatsu Corp., Middlesex, NJ), and a Grinnell 274 image processor

1 Abbreviations used in this paper: acLDL, acetylated low density lipoprotein; Dil, 1,1'-dioctadecyl-3,3',3'-tetramethylindocarbocyanine; HDL, high density lipoproteins; htg, hyperglyceremic; LDL, low density lipoproteins; NBD-cholesterol, N(-7-nitrobenz-2-oxa-1,3-diazole)-23,24-dinor-5-cholen-3-aminoo-36-ol; VLDL, very low density lipoprotein.

(Grinnell Systems Corp., San Jose, CA) with three separate 512 × 480 8-bit memory planes. A Lab Datex LSI 11/23 minicomputer (Data Translation, Inc., Marlboro, MA) was interfaced through a Q-hub to the image processor, a Charles River (Charles River Data Systems, Inc., Framingham, MA) 20 Mbyte hard disk and a Cipher F880 magnetic tape (Cipher Data Products Inc., San Diego, CA) for archival storage. Photographs of the Grinnell images were produced with a Matrix Instruments 35-mm color graphic recorder (Matrix, Inc., Mesa, AZ).

Cells were cultured as described below on 22-cm² × 0.17-mm glass coverslips in tissue culture dishes, and were transferred to Bionique culture chambers (Bionique, Corning, Lake Placid, NY) for experiments on the microscope. Live cells were maintained on the microscope stage at constant temperature (37°C ± 1°C) with constant infusion of 5% CO₂. Cellular fluorescence was measured for 1 s at intervals of 30 s or 1 min. Typical conditions included one to three fluorobins and 12–20 macrophones. Single frames were digitized in 33 ms. To increase the signal-to-noise ratio, each image that was stored was an average of 32 frames acquired at 0.3 Hz. To avoid photofading, excitation light was reduced with neutral-density filters. The absence of photofading was verified by rate constant maps (22). In the microscope field of 111 × 128 µm, pixel size was 0.0625 µm² at the magnification used for this study. Time lapse video recording of the phase-contrast images allowed evaluation of viability of cells by the salutary movement of the phase-dense lysosomes (23).

Lipoprotein Preparation: Lipoproteins were isolated from normal human plasma by ultracentrifugation (24) at the following densities: normal VLDL, ρ < 1.006; LDL, 1.019–1.063; HDL, 1.063–1.21. Lipoprotein-deficient serum was prepared from the fraction with ρ > 1.21. Hypertriglyceremic VLDL (htg VLDL) with an Sf of 100–400 was prepared from plasma of a type IV individual by flotation (25). Acetylated LDL (acLDL) was prepared as described by Basu et al. (26). Lipoproteins were labeled with benzo(a)pyrene, [G-3H]benzo(a)pyrene, or N(-7-nitrobenz-2-oxa-1,3-diazole)-23,24-dinor-5-cholen-3-aminoo-36-ol (NBD-cholesterol) (27) by drying organic solutions of the fluorophore on 200-µm glass beads (Polysciences, Inc., Warrington, PA), and incubating the lipoprotein preparation with the beads overnight at 37°C. Approximately 0.2 µmol of benzo(a)pyrene was incorporated per milligram of LDL protein, which is equivalent to an average of 100 benzo(a)pyrene molecules per LDL. Benzo(a)pyrene content of labeled LDL and VLDL was determined by reversed phase octadecyl-silica high-pressure liquid chromatography in 80% acetonitrile. DiI and rhodamine-123 were obtained from Molecular Probes (Junction City, OR), and acridine orange was purchased from Polysciences, Inc. of NBD-cholesterol has been described (27). Live fibroblasts previously incubated with benzo(a)pyrene were exposed to 5 µg ml⁻¹ rhodamine-123 for 30 min at 37°C. Cells were placed in fresh medium for 10 min, transferred to the stage of the microscope, and viewed immediately. Rhodamine fluorescence was observed using a 560-nm band-pass filter for excitation and a 575-nm cut-off filter for emission. Acridine orange was added to live cells on the stage at a final concentration of 5 µg ml⁻¹. After 2 min, cells were rinsed twice with fresh medium and fixed. Acridine orange fluorescence was viewed immediately with a 480-nm bandpass plus a 495-nm cutoff filter for excitation and a 510-nm cut-off emission filter. Because of organelle movement in live
cells, fixation of cells after labeling with acridine orange was required for covalently bound metabolites. Media samples were separated from labeled lipoproteins by filtration through glass fiber filters. For all double-label experiments, control cells labeled with one of each fluorophore were examined to quantify the appearance of fluorescence through the protocols for the spatial distribution of cellular fluorescence. Therefore, cellular fluorescence measurements were not corrected for any contribution from the medium.

To quantify cellular uptake of [3H]benz[a]pyrene from lipoproteins, cells were separated from labeled lipoproteins by filtration through glass fiber filters. Filter assays were performed with fibroblasts suspended by trypsinization or with P388D1 cells suspended by rinsing the plate with medium. Cells were suspended in 1.5–2 ml of either 0.15 M saline or culture medium without serum. Cell suspensions were stirred continuously with small magnetic bars. Temperature was measured with a Bailey digital thermometer (Bailey Instruments Co., Inc., Saddle Brook, MA). Small volumes of [3H]benz[a]pyrene-labeled lipoproteins were added to cell suspensions. At appropriate intervals, 50-μl aliquots were removed and added to 5 ml of buffered solution containing 0.15 M NaCl, 0.04 M Tris, 0.3 mM EDTA, and 5 mg ml⁻¹ bovine serum albumin. Bovine serum albumin reduced nonspecific binding of benz[a]pyrene-labeled LDL to the filters. For each experiment, a solution containing only [3H]benz[a]pyrene LDL was filtered to determine the background binding. In the absence of cells, filters bound between 2 and 5% of total counts. Filters were counted in scintillation fluid without further processing. Determination of cellular protein bound to filters indicated that the numbers of cells trapped were highly reproducible. As the uptake approached equilibrium, the relative differences in amounts of benz[a]pyrene entering cells were small. To establish that differences in cellular [3H]benz[a]pyrene at the later time points were significantly above background levels, aliquots of the reaction mixture were counted without filtering and compared with the cellular radioactivity at equilibrium.

Fluorescence kinetic assays were performed in an SLM 8000 spectrophotometer (SLM Instruments, Inc., Urbana, IL) with stirred cell suspensions in a temperature-regulated cuvette holder. Solutions of 1-palmitoyl-2-oleoyl-phosphatidylcholine vesicles containing benz[a]pyrene plus a fluorescence quencher, N-(2,4-dinitrophenyl)-N,N-diocetylacylamine (16) were added to suspensions of fibroblasts or P388D1 cells and benz[a]pyrene fluorescence intensity was recorded as a function of time using excitation and emission wavelengths of 365 nm and 407 nm, respectively. Analysis of kinetic data was performed by nonlinear least squares regression with respect to a monoeponential function. Data points were manually entered into an Apple II Plus microcomputer (Apple Computer, Inc., Cupertino, CA). The analytical program involved a reiterative procedure and stringent criteria for convergence. Results of the analysis were evaluated by comparative plots of fitted curves and measured data points, standard deviation of data points from the fitted line, standard deviations for rate constant, and initial and final values, and analysis of correlation of error in these parameters.

RESULTS

Normal human fibroblasts metabolized benz[a]pyrene very slowly, if at all, as illustrated in Fig. 1 and Table I. Thus, the interpretation of increase in cellular fluorescence, as an accurate index of cellular uptake of benz[a]pyrene, is not complicated by the formation of metabolic products. Benz[a]pyrene metabolism was studied in parallel with C3H/10 T½ mouse fibroblasts, which have been shown to have significant benz[a]pyrene metabolic capacity (35). C3H/10 T½ cells produced fourfold more metabolites per milligram of cell protein in 24 h than did normal human fibroblasts. Less than 5% of the radioactivity was present as metabolites in
Benzo(a)pyrene metabolism in cultured cells. Normal human fibroblasts (□) and C3H/10T½ murine fibroblasts (○) were incubated at 37° C in the presence of 50 μg/ml LDL containing [G-3H]benzo(a)pyrene. A total of 10 nmol benzo(a)pyrene was added to each plate. For each cell type, duplicate dishes were incubated for 2 h at 4° C. Approximately 2% of total counts were identified as metabolites in these control plates. Data are expressed as total nanomoles of metabolites recovered from cells and media, corrected for the values at 4° C, and normalized per milligram of cell protein.

**TABLE I**

| Cell type                | Incubation time* | BP in cells | BP in media | % of original radioactivity |
|--------------------------|------------------|-------------|-------------|----------------------------|
| Normal human fibroblast  | 4°C              | 0.11        | 0.58        | 2.5                        |
|                          | 37°C             | 0.14        | 1.14        | 4.3                        |
|                          | 10               | 0.14        | 0.91        | 3.8                        |
|                          | 24               | 0.25        | 1.06        | 4.8                        |
| C3H/10T½                | 2                | 0.14        | 0.68        | 2.3                        |
|                          | 10               | 0.19        | 1.40        | 4.3                        |
|                          | 24               | 0.33        | 1.99        | 5.6                        |

BP, benzo(a)pyrene.

* Time in hours.

fibroblast dishes, in contrast to 12% of total benzo(a)pyrene in C3H/10T½ dishes in 24 h. P388D, cells were also examined for metabolic activity in a similar fashion, with negative results (data not shown).

Benzo(a)pyrene was presented to cells as a noncovalent component of LDL. Benzo(a)pyrene was solubilized in the hydrophobic core of lipoproteins, as demonstrated by its fluorescence spectrum in LDL (Fig. 2). When the same amount of benzo(a)pyrene in ethanolic solution was injected into aqueous buffer, the spectrum revealed that most of the benzo(a)pyrene was present as microcrystalline aggregates.

The cellular benzo(a)pyrene mass was obtained by quantification of [G-3H]benzo(a)pyrene uptake. Fig. 3 shows the result of cell filtration assays in which 1 × 10⁶ cells in stirred suspensions were exposed to increasing concentrations of LDL labeled with [G-3H]benzo(a)pyrene. At the highest level of benzo(a)pyrene-LDL used, 5 mg of LDL protein·ml⁻¹ and 1 mM benzo(a)pyrene, cellular accumulation of benzo(a)pyrene from LDL was ∼1.5 × 10⁻⁵ mol benzo(a)pyrene per cell. With an estimated cell volume based on dimensions of 40 × 30 × 10 μm, this corresponds to an average cellular concentration of ∼150 μM. Benzo(a)pyrene was not uniformly distributed throughout any cell, however, and as will be described, often distinctly accumulated in regions comprising only a fraction of the total cell volume.

The time-dependent increase in the cellular fluorescence of benzo(a)pyrene is plotted in Fig. 4 for a typical experiment in which cells were exposed to 50 mg·mil⁻¹ benzo(a)pyrene-LDL. Mean intensity values and intensities corresponding to the brightest intracellular areas were within the limits of detection and the linear response range of the video camera as determined by the fluorescence intensities of ethanolic solutions containing 0.1–200 μM benzo(a)pyrene.

The uptake of benzo(a)pyrene from lipoproteins into cells was independent of endocytosis of the lipoprotein carrier, even in cells that expressed receptors for LDL. Evidence for this was shown by the different rates of uptake of benzo(a)pyrene and dil when both probes were incorporated into the same lipoprotein. Because of the differences in spectral properties of the two probes, their cellular fluorescence was quantified independently. Cellular uptake of benzo(a)pyrene oc-
receptors for HDL (11), were exposed to HDL labeled with benzo(a)pyrene transferred from quenched vesicles to cells. The rate constants determined with this method was about 0.29 min⁻¹ at 25°C. Experiments with cells in suspension performed with either fibroblasts or P388D1, macrophages gave identical results. Thus, regardless of method or cell type used, uptake of benzo(a)pyrene into cells at room temperature occurs with a halftime of ~2 min. Only digital fluorescence-imaging microscopy, however, demonstrated the topographic heterogeneity of the intracellular location of benzo(a)pyrene.

The sampling area of this imaging microscopy system at the object plane was ~0.0625 μm² per pixel. Analysis of the increase in fluorescence intensity with time was performed on single pixels or small numbers of pixels corresponding to discrete intracellular locations and organelles. This analysis of the increase in benzo(a)pyrene fluorescence at discrete subcellular locations in individual cells did not identify any significant differences in rate constants for benzo(a)pyrene accumulation. However, due to a combination of the limited modulation transfer function of the camera (36) and optical diffusion of fluorescence in a light-scattering medium (i.e., cytoplasm), this question cannot be resolved without the application of computer-based mathematical deblurring procedures (36).

The amount of benzo(a)pyrene in different cellular locations varied greatly as determined by fluorescence intensity (Figs. 5, 7, and 8). For reference, the relative fluorescence intensities of 10 μM benzo(a)pyrene at the emission maximum of 405 nm were 1.0, 1.1, 1.3, and 2.0 in acetonitrile, ethanol, cyclohexane, and 1-palmitoyl-2-oleoyl-phosphatidylcholine vesicles, respectively. Because benzo(a)pyrene fluorescence was a linear function of concentration, and the relative fluorescence intensity of benzo(a)pyrene in different hydrophobic environments was similar, these results indicated that benzo(a)pyrene partitioned selectively into discrete subcellular locations.

FIGURE 4

Cellular accumulation of benzo(a)pyrene. Medium containing 50 μg·ml⁻¹ benzo(a)pyrene-labeled LDL was added to viable human fibroblasts on the microscope stage. Fluorescence intensity in a single field was sampled at the indicated times. Temperature was maintained at approximately 37°C. Neutral-density filters attenuated excitation light to 1% of maximum. Mean intensities over the field (I) and upper range values for the field (11) are plotted as a function of time. Arrows at the abscissa designate limits of detection and range of linearity as determined by calibration with ethanol-solutions of benzo(a)pyrene, which were 0.1 and 200 μM, respectively.

The rate constant for cellular uptake of benzo(a)pyrene was 0.21 min⁻¹ at 21°C. Stirred cell suspensions were also mixed with LDL containing [G-³H]benzo(a)pyrene. Aliquots taken at selected time intervals were rapidly filtered through glass fiber filters to trap cells and associated benzo(a)pyrene. The rate of cellular uptake of benzo(a)pyrene faster or to a greater extent than noninduced cells. Cells fixed with 2% formaldehyde also accumulated benzo(a)pyrene from lipoproteins with the same rate and extent to produce the same pattern of intracellular localization as did viable cells examined in parallel. Cellular uptake and distribution of benzo(a)pyrene is therefore a spontaneous physical event that does not require metabolic processes, and the rate is not influenced by the presence or absence of receptors for the lipoprotein carrier.

The rate of cellular uptake of benzo(a)pyrene has been determined by three different techniques. Table II summarizes the rate constants for incorporation of benzo(a)pyrene by living and fixed cells as a function of temperature, lipophilic donor, and method of analysis. With digital fluorescence-imaging microscopy, the fluorescence intensity of each pixel in the image was measured. Fig. 9 shows the time-dependent increase in mean cellular fluorescence. The data were analyzed by nonlinear least squares regression with respect to a monoexponential function. The rate constants for cellular accumulation was 0.39 min⁻¹ at 37°C and 0.25 min⁻¹ at 23°C.

To confirm the rate constants measured by fluorescence microscopy, cell suspensions were mixed with 1-palmitoyl-2-oleoyl-phosphatidylcholine vesicles containing benzo(a)pyrene and a nonexchangeable quencher, N-2,4-dinitrophenyl-N,N-diocadecylamine. Increase in fluorescence intensity as a function of time was followed in a fluorimeter as benzo(a)pyrene transferred from quenched vesicles to cells.

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LDL labeled with both benzo(a)pyrene and diI delivered both probes to cells simultaneously, but spatial analysis showed that the intracellular locations of the two fluorophores were distinctly different. The left panel of Fig. 10 (top left image) shows a fibroblast labeled with both diI and benzo(a)pyrene. The corresponding phase images are shown in the right panel of Fig. 10. The fluorescence images were viewed simultaneously by storing each image in a separate memory plane. Superimposition of the benzo(a)pyrene fluorescence image on the diI fluorescence image demonstrated unequivocally the distinct locations of the two fluorophores.

Identification of which cellular structures accumulate benzo(a)pyrene was achieved with a series of experiments using different organelle-specific fluorescent probes. Endocytosis of LDL delivers diI to lysosomes (10). Because diI is positively charged at low pH, it accumulates in these acidic compartments. To confirm the localization of diI, fibroblasts were grown in lipoprotein-deficient medium and then incubated with diI-labeled LDL for 2–4 h. Live cells were then exposed to 5 μg·ml⁻¹ acridine orange to identify acidic compartments (37). The left center image of Fig. 8 (left) shows the coincidence of DiI and acridine orange fluorescence, indicating co-localization in acidic intracellular compartments. Benzo(a)pyrene fluorescence was not associated with

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FIGURE 5 Cellular accumulation of benzo(a)pyrene by viable cells. 50 μg·mL⁻¹ LDL containing either dil or benzo(a)pyrene was added in medium to cells on the microscope stage at 37°C. At the times indicated, cells were exposed for 1 s to excitation light attenuated to 1% for benzo(a)pyrene and 60% for dil. Cells had been preincubated for 2 d in lipoprotein-deficient medium to induce the expression of LDL receptors. (Left images) Benzo(a)pyrene fluorescence at 0.017, 1, 2, and 4 min, respectively. (Right) The isometric projections (22) illustrate the pixel-by-pixel time-dependent increases in intensity values for benzo(a)pyrene fluorescence. The corresponding digital images are immediately to the left of each projection.
FIGURE 6  Cellular accumulation of LDL by viable cells. The experimental conditions are described in the legend to Fig. 5, except the excitation light was attenuated 60% to observe Dil without photobleaching. (Left images) Dil-LDL fluorescence at 5, 15, 30, and 60 min, respectively. (Right) The isometric projections (22) illustrate the pixel-by-pixel time-dependent increases in intensity values for dil fluorescence. The corresponding digital images are immediately to the left of each projection.
FIGURES 7 and 8  Comparison of benzo(a)pyrene uptake from HDL and LDL. Normal human fibroblasts on glass coverslips were preincubated in lipid-deficient medium for 2 d, and then incubated for 4 h at 37°C with medium containing either LDL or HDL. The lipoproteins were labeled with both dil and benzo(a)pyrene. Final benzo(a)pyrene concentration was 2.1 μM. Dil labeling was ~6 molecules per HDL and ~18 per LDL. LDL concentration was 30 μg·ml⁻¹ and HDL concentration was 182 μg·ml⁻¹. Cells were washed three times with 0.04 M Tris (pH 7.4) containing 5 mg·ml⁻¹ bovine serum albumin, fixed, and placed in a Bionique chamber for viewing. Excitation light was attenuated to 5% for benzo(a)pyrene uptake and to 40% for dil-LDL uptake. The derivative images were obtained by subtraction of the appropriate shifted images from the original images and displaying the differences. The shifted images were created by incrementing the x,y coordinates of each pixel by 3 to the right and 3 to the top. Fig. 7 (left images) (top to bottom) Benzo(a)pyrene fluorescence, the derivative image of benzo(a)pyrene fluorescence, and the phase image of the cells, respectively. (Right images) (top and bottom) dil fluorescence of LDL, and the derivative image of dil fluorescence, respectively. Fig. 8 (left images) (top to bottom) Benzo(a)pyrene fluorescence, the derivative image of benzo(a)pyrene fluorescence, and the phase image of the cells, respectively. (Right images) (top and bottom) dil fluorescence of HDL, and the derivative image of dil fluorescence, respectively.
acridine orange fluorescence (Fig. 10, left bottom image).

The location of benzo(a)pyrene fluorescence was also compared with that of two other vital fluorescence probes, rhodamine-123 which is specific for mitochondria (38), and a cholesterol analog, NBD-cholesterol (27). From inspection of the images in the left panel of Fig. 10, it is apparent that

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**Figure 9** Kinetics of benzo(a)pyrene uptake by viable fibroblasts. At time zero, medium containing 50 µg·ml⁻¹ benzo(a)pyrene-labeled LDL was added to fibroblasts at 37°C on the microscope stage. Open circles represent mean fluorescence intensities for the field containing 1–3 cells at the indicated times. The solid line represents analysis of the data with respect to a monoexponential function. The rate constant for uptake was 0.39 min⁻¹.
FIGURE 10 Comparison of spatial distribution of benzo[a]pyrene fluorescence in fibroblasts with fluorescence of Dil, acridine orange, rhodamine-123, and NBD-cholesterol. Fibroblasts induced for LDL receptors were incubated for 6 h at 37°C in the presence of 50 μg·ml⁻¹ LDL containing both benzo[a]pyrene and dil. Cells were washed 2 times with 0.15 M NaCl and fixed with 2% formaldehyde before viewing. Labeling procedures used with the other fluorescent probes are described in Materials and Methods. To compare the distribution of two fluorescent probes, the phase images for the respective fluorescent images were superimposed by computer alignment of two memory planes. This procedure was necessary to correct misalignment originating from the nonparfocal nature of the filter cubes. Either a red or blue-green color was assigned to the two image channels for display of the fluorescence images identified below. With this combination of pseudocolors, regions of overlapping intensity in each image appear in white. The horizontal cursor identifies the pixel rows, the fluorescence intensity values of which are displayed as either a red or white intensity profile at the bottom of each panel. (Left page) (left top images) Dil (red color and red intensity profile), benzo[a]pyrene (blue-green color and white intensity profile). (Left center images) Dil (red color and red intensity profile), acridine orange (blue-green color and white intensity profile). (Left bottom images) Acridine orange (red color and red intensity profile), benzo[a]pyrene (blue-green color and white intensity profile). (Right top images) Rhodamine-123 (red color and red intensity profile), benzo[a]pyrene (blue-green color and white intensity profile). (Right bottom images) NBD-cholesterol (red color and red intensity profile), benzo[a]pyrene (blue-green color and white intensity profile). (Right page). The phase images correspond to their respective superimposed fluorescence images on the facing page. Bar, 10 μm.
with bovine serum albumin. Cells were then incubated for 1 h with "acceptors"

Materials and Methods. The fluorescence intensity of cells on

- a, fluorescence microscopy; b, spectrofluorimetry; c, [G-3H]benzo(a)pyrene

cell extract.

cholesteryl ester, respectively, in the neutral lipid fraction of the

open and closed bars represent the amount of triglyceride and

and arrows designate mean intensity values for the entire field. The

striped bar represents upper range intensity values for each image,

coverslips was digitized with a gray scale of 0-255. Height of the

[GSH]benzo(a)pyrene-LDL. Quantification of [14C]-oleate and

peritoneal macrophages were incubated for 38 h with 50 pg.m1-1

benzo(a)pyrene uptake, and benzo(a)pyrene fluorescence. Mouse

TABLE II

Rate Constants for Uptake and Efflux of Benzo(a)pyrene by
Human Fibroblasts

| Temperature | k | Cell condition Method* |
|------------|---|------------------------|
| °C | min⁻¹ |
| Donors |
| LDL | 37 | 0.39 (±0.04) | live a |
| 22 | 0.29 (±0.03) | fixed a |
| 23 | 0.25 (±0.08) | live b |
| HDL | 37 | 0.34 (±0.08) | live a |
| 21 | 0.32 (±0.04) | fixed a |
| 26 | 0.29 (±0.03) | live b |
| VLDL | 26 | 0.33 (±0.04) | live b |
| POPC vesicles | 21 | 0.21 (±0.03) | live c |
| Acceptors |
| HDL | 22 | 0.26 (±0.04) | fixed a |
| POPC | 37 | 0.34 (±0.03) | live c |

The values in parentheses are standard deviations of data points from their fitted curves. The vesicles were 1-palmitoyl-2-oleylphosphatidylcholine (POPC).

* a, fluorescence microscopy; b, spectrofluorimetry; c, [G-3H]benzo(a)pyrene filter assay.

FIGURE 11 Relationship of cellular neutral lipid content, [G-3H]-benzo(a)pyrene uptake, and benzo(a)pyrene fluorescence. Mouse peritoneal macrophages were incubated with 38 h with 50 µg.ml⁻¹ LDL, acLDL, or hgt VLDL, in addition to [1-14C]olate complexed with bovine serum albumin. Cells were then incubated for 1 h with [G-3H]benzo(a)pyrene-LDL. Quantification of [14C]-ulate and [G-3H]-benzo(a)pyrene in cells grown in parallel is described in Materials and Methods. The fluorescence intensity of cells on coverslips was digitized with a gray scale of 0-255. Height of the striped bar represents upper range intensity values for each image, and arrows designate mean intensity values for the entire field. The open and closed bars represent the amount of triglyceride and cholesteryl ester, respectively, in the neutral lipid fraction of the cell extract.

TABLE III

Effect of Intracellular Lipid on Benzo(a)pyrene Accumulation

| Lipo-protein in | Triglyceride | Cholesteryl ester | Benzo(a)pyrene Fluorescence |
|-----------------|--------------|------------------|-----------------------------|
| preincubation medium | [1-14C]olate mg⁻¹ protein | [G-3H]-BP mg⁻¹ protein | intensity |
| LDL | 67 | 0.8 | 10 | 13 | 66 |
| acLDL | 85 | 66 | 12 | 18 | 150 |
| hgt VLDL | 210 | 3 | 15 | 46 | 240 |

Rhodamine-123 and benzo(a)pyrene did not distribute in the same intracellular location (top right), but that NBD-cholesterol and benzo(a)pyrene were distinctly co-localized (bottom right).

The co-localization of benzo(a)pyrene and the fluorescent cholesterol analog suggests that benzo(a)pyrene partitions selectively into cytoplasmic lipid droplets. Partitioning of benzo(a)pyrene into intracellular lipid droplets is not unexpected, in view of its lipophilic nature. Moreover, benzo(a)-pyrene has been used previously as a cytochemical stain for lipid (39). Because of the partitioning of benzo(a)pyrene, intracellular accumulation of lipid should produce increased benzo(a)pyrene accumulation. To demonstrate this relationship, mouse peritoneal macrophages were incubated with acLDL (34) or hgt VLDL (25), lipoproteins that produce large intracellular accumulations of cholesteryl esters or triglycerides in these cells, respectively. Cells incubated with acLDL incorporated ~6.6 pmol and 8.5 pmol.mg⁻¹ protein of [1-14C]olate into cholesteryl esters and triglycerides, respectively. Cells incubated with hgt VLDL incorporated 3.0 pmol and 210 pmol.mg⁻¹ protein [1-14C]olate into cholesteryl esters and triglycerides, respectively. The amount of benzo(a)pyrene incorporated by these cells increased with increased lipid accumulated, measured by [G-3H]benzo(a)pyrene and by cellular fluorescence intensity (Fig. 11; Table III). After preincubation with acLDL, 24 nmol benzo(a)pyrene was accumulated per nanomole [1-14C]olate esterified. After preincubation with hgt VLDL, cells accumulated 34 nmol benzo(a)pyrene per nanomole [1-14C]olate esterified.

When LDL labeled with [G-3H]benzo(a)pyrene was added to cell suspensions containing increasing concentrations of unlabeled LDL, the rate constants for benzo(a)pyrene uptake were unchanged. However, the net amount of cellular benzo(a)pyrene decreased as a function of increasing amount of unlabeled LDL added to the extracellular solution (Fig. 12). Thus, the equilibrium distribution of benzo(a)pyrene into cells was controlled by the relative lipid volume of cells and extracellular particles.

DISCUSSION

Mechanism of Cellular Uptake

The importance of partitioning and solubility characteristics of carcinogenic compounds on their distribution and metabolism has been largely ignored. Consideration of the
physical properties of polycyclic aromatic hydrocarbons suggests that it is reasonable to expect benzo(a)pyrene metabolism would be influenced by its lipid solubility and partitioning into membranes and lipid droplets. It has been noted (40-42) that the apparent $K_m$ measured for cytochrome P450 substrates is dependent on concentrations of membranes or cells. Recently, Backes et al. (43) addressed the mechanism of this phenomenon by calculating microsomal partition coefficients for a series of aromatic substrates of increasing molecular size. Nemoto and Takayama (44) found that the addition of serum to microsomes resulted in large increases in benzo(a)pyrene metabolism, at least in part due to increased solubilization of benzo(a)pyrene.

In most metabolic studies, either in purified reconstituted P450 systems or in cell culture, benzo(a)pyrene is usually added as a concentrated solution in organic solvent (45, 46). The observation of crystalline benzo(a)pyrene in lysosomes of fibroblasts, which involved the addition of benzo(a)pyrene at high concentrations in dimethylsulfoxide to the culture medium, most likely involved phagocytosis of particulate hydrocarbon. Spectral analysis of benzo(a)pyrene (3, 4) demonstrates that benzo(a)pyrene is dispersed as microcrystals by this method. Lakowicz et al. (5) have shown significantly slower rates of solubilization of benzo(a)pyrene from microcrystals to membranes, compared with the rates for benzo(a)pyrene solubilized by adsorption to particulate material. Thus, any kinetic data for benzo(a)pyrene metabolism involving this mechanism of presentation is therefore difficult, if not impossible, to interpret. In this study we have avoided this experimental ambiguity by presenting cells with benzo(a)pyrene solubilized in the hydrophobic core of plasma lipoproteins.

It has been shown (6) that benzo(a)pyrene in plasma is associated with lipoproteins, and to the greatest extent with LDL in normolipemic plasma. By exposing both living and fixed cells to LDL containing benzo(a)pyrene, we have shown by digital fluorescence microscopy that the cellular uptake of benzo(a)pyrene occurs at the same rate and is therefore independent of LDL endocytosis. This conclusion supports the work of Remsen and Shireman (20), who compared benzo(a)pyrene uptake from LDL by both normal and receptor-negative fibroblasts, at single time points. Although these authors did not exclude low affinity receptor-independent pathways, they correctly concluded that a passive diffusion process was responsible for benzo(a)pyrene transfer to the plasma membrane. In the present study, digital fluorescence microscopy provides topographic information about the intracellular location of benzo(a)pyrene. Thus, mechanistically, passive transfer appears to be solely responsible for redistribution of benzo(a)pyrene from extracellular lipoproteins to the plasma membrane, and from the plasma membrane to intracellular compartments. The time course of uptake and the intracellular localization of benzo(a)pyrene are clearly independent of that of the lipoprotein particle as indicated by the nontransferable fluorescent lipoprotein probe, dil.

The results from this investigation are not consistent with the conclusions reached by Shu and Byrum (47). These authors reported that the amount of benzo(a)pyrene excreted in the bile of rats injected with benzo(a)pyrene noncovalently associated with lipoproteins was dependent on the lipoprotein donor with which benzo(a)pyrene was originally associated. However, equilibration of benzo(a)pyrene among plasma lipoprotein classes occurs on a millisecond time scale (13). In that this equilibration is two orders of magnitude faster than benzo(a)pyrene entry into cells, rate constants for cell uptake and release of benzo(a)pyrene should be independent of the identity of the original lipoprotein donor, which we observe.

**Intracellular Location of Benzo(a)pyrene**

Intracellular location of benzo(a)pyrene has been compared with that of various fluorescent probes with known intracellular locations. These experiments indicate that benzo(a)pyrene is not predominantly located in acidic compartments or mitochondria, but concentrates in cytoplasmic lipid droplets (48, 49), as expected from its solubility properties and intracellular coincidence with a cholesterol analog. It should be noted that benzo(a)pyrene fluorescence is conspicuously absent from the nucleus. It is not possible, at present, to distinguish between fluorescence quenching and the presence of benzo(a)pyrene concentrations that are below the limits of detection. It seems unlikely that quenching accounts for the absence of benzo(a)pyrene fluorescence. Studies involving removal and replacement of nucleic acids and treatment of tissue with ethanol or buffers did not produce measurable effect on fluorescence intensity (50, 51).

Other attempts to identify the intracellular location of benzo(a)pyrene have employed subcellular fractionation (52-57). This method gives questionable results because of the rapid rate of benzo(a)pyrene partitioning. Autoradiography of subcellular locations of polycyclic aromatic hydrocarbons carcinogens suggest that these compounds associate with cell nuclei (57-59). However, the comprehensive study by Shires (50) of the effects of various preparative procedures showed that benzo(a)pyrene fluorescence in the nucleus cannot be detected except when ethanol, citrate, or acetate is used. Moreover, nuclear fluorescence was detected by Stora (60) in tissue sections from rats treated with benzo(a)pyrene only after 43 d. No nuclear fluorescence was detected before that time. These latter experiments involved embedding fixed tissue sections in paraffin, and deparaffinizing before viewing. Inasmuch as the solvents used for these experiments would remove all noncovalently bound benzo(a)pyrene, the nuclear fluorescence observed by Stora was not that of benzo(a)pyrene, but more likely a covalently immobilized metabolite of benzo(a)pyrene. The predominant intracellular location for benzo(a)pyrene reported in our study is consistent with its partitioning characteristics.

Transfer of benzo(a)pyrene between lipoprotein particles is two to three orders of magnitude faster than uptake by cells. The fact that the rate constants for cell uptake are the same, regardless of carrier size, suggests that the slow step in the entry of benzo(a)pyrene into cells occurs after the initial desorption process. Identification of the slow step in the process of cell uptake of benzo(a)pyrene is still under investigation, but it appears that the plasma membrane is the site of the rate-limiting step in the accumulation of polycyclic aromatic hydrocarbons by cells.

Increasing the concentration of extracellular unlabeled LDL does not alter the rate constant for benzo(a)pyrene uptake, but does decrease the net amount of benzo(a)pyrene entering cells. By contrast, increasing the amount of lipid present in cells produces increased cellular benzo(a)pyrene accumulation. Moreover, it is apparent that benzo(a)pyrene uptake by cells is a spontaneous transfer process from a donor hydrophobic particle to cellular lipid compartments. Spontaneous aqueous phase transfer is controlled by partitioning of the transferring species between one hydrophobic compartment and another. The extent of transfer of benzo(a)pyrene

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into cells is determined by the equilibrium distribution between the relative hydrophobic volumes inside and outside the cell. Increasing cellular lipid content in vivo could result in increased benzo(a)pyrene accumulation in tissues and indicates a possible relationship between tissue lipid deposits and carcinogenesis.

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REFERENCES

1. Gelboin, H. V. (1980). Benzo(a)pyrene metabolism, activation, and carcinogenic role and regulation of mixed-function oxidases and related enzymes. Proc. Natl. Acad. Sci. USA 77(4):121-123.

2. Bergsagel, J. (1981). Reversible induction of cytochrome P-450 in rat liver by benzo(a)pyrene and benzo(a)pyrene-7,8-diol. J. Biol. Chem. 256:14757-14762.

3. Weinberger, R., and L. J. C. Love. 1984. Luminescence properties of polycyclic aromatic hydrocarbons on colloid or microcrystalline suspensions. Spectrochim. Acta 40A:49-55.

4. Lakowicz, J. R., D. R. Bevan, and S. C. Rieker. 1980. Transport of a carcinogen, benzo(a)pyrene, from particulates to lipid bilayers. Biochim. Biophys. Acta 639:243-258.

5. Shu, H., P. G. Vischer, and F. A. Nichols. 1983. Benzo(a)pyrene uptake by human plasma lipoproteins in vitro. Cancer Res. 43:1234-1237.

6. Chen, T. C., W. A. Bradley, A. M. Gott, and J. W. S. Anderson. 1979. Binding of the carcinogenic hydrocarbon 3,4-benzpyrene to lipoprotein lipids and cholesterol esters. Biochim. Biophys. Acta 594:265-275.

7. Selkirk, J. K., R. G. Croy, J. P. Whitlock, and H. V. Gelboin. 1975. In vitro metabolism of benzo(a)pyrene by human liver microsomes and lymphocytes. Cancer Res. 35:3651-3656.

8. Guth, E. B., and C. Heidelberger. 1982. Metabolic activation of benzo(a)pyrene by transformable and nontransformable NIH 3T3 fibroblasts in culture. Cancer Res. 42:2970-2974.

9. Castellan, K. R. 1979. Digital Image Processing. John Wiley & Sons, Inc., New York. 257-260.

10. Rehbein, E. P., I. Marcus, and N. K. Gaitanas. 1965. Dynamics of acridine orange-cell interaction. II. Die-induced ultrastructural changes in multicellular bodies (acridine orange particles). J. Cell Biol. 21:49-62.

11. Simmers, A. B., W. L. Hui, and M. W. Berns. 1982. Laser-stimulated fluorescence of submicrometer regions within single mitochondria of trypanosome-infected mammalian cells in culture. Proc. Natl. Acad. Sci. USA 79:466-470.

12. Shu, H. P., and E. N. Bynum. 1983. Systemic excretion of benzo(a)pyrene in the control and 3-methylcholanthrene treated rat. J. Lipid Res. 24:1785-1789.

13. Backes, W. L., R. E. C. Thierry, B. Serrou, and P. Viallet. 1981. Slower step of the polycyclic aromatic hydrocarbons metabolism: kinetic data from microspectrofluorometric techniques. Biomed. Chem. 21:4023-4030.

14. Doody, M. C., J. P. Whitlock, J. T. Kao, and L. C. Smith. 1980. Mechanism and kinetics of transfer of a fluorescing fatty acid between single-walled phospholipid vesicles. Biochemistry. 19:108-116.

15. Massey, J. B., A. M. Gott, and J. H. Pownall. 1982. Kinetics and mechanism of the spontaneous transfer of fluorescent phospholipids between apolipoprotein-phospholipid reconstituted after the polar headgroup. J. Biol. Chem. 257:544-548.

16. Smith, L. C., B. J. Massey, J. T. Sparrow, A. M. Gott, Jr., and J. H. Pownall. 1983. Structure and dynamics of human plasma lipoproteins. In Supramolecular Structure and Function of Biological Membranes. G. Pfaltz and J. N. Herak, editors. Plenum Press, New York. 205-243.

17. Brown, M. S., J. R. Faust, and L. C. Goldstein. 1975. Role of the low-density lipoprotein receptor in regulating the content of free and esterified cholesterol in human fibroblasts. J. Cell. Biol. 75:783-793.

18. Smith, L. C., M. C. Doody. 1981. Kinetics of benzo(a)pyrene transfer between human plasma lipoproteins. In Chemical Analysis and Biological Fate of Polynuclear Aromatic Hydrocarbons. M. Cooke and A. J. Dennis, editors. Battelle Press, Columbus, OH. 615-624.

19. Pownall, H. J., D. L. Hickson, and L. C. Smith. 1983. Distribution of light scattering and radioactivity in reconstituted lipoprotein lipids. Biochim. Biophys. Acta 665:538-545.

20. Shu, H. P., and J. C. Smith. 1982. Kinetics of transfer of pyrene and n-acetyl-2-[1-(pyrene)-butanol]-[2-[1-pyrene]-butanol] between human plasma lipoproteins. Biochemistry. 21:4023-4030.

21. Rennert, J. F., and R. B. Shireman. 1981. Effect of low-density lipoprotein on the incorporation of benzo(a)pyrene by cultured cells. Cancer Res. 41:3179-3185.

22. Benson, M. A., A. M. Gott, and L. C. Smith. 1982. Lipoprotein lipids: kinetics of uptake of benzo(a)pyrene and of low-density lipoprotein by living cells. J. Cell Biol. 97(2), Pt. 2, 1147-1148.

23. Benson, M. A., A. M. Gott, and J. C. Smith. 1982. Distribution of hydrophobic microsomal matrix: selective heterogeneity of phosphorylating rate constants in individual cells. J. Cell Biol. 100:1309-1323.

24. Williams, M. C., and L. Pasan. 1978. The visualization of fluorescent proteins in living cells by video-intensification microscopy. (VICM). Cell 13:501-507.