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BENZO[a]PYRENE METABOLISM IN INDIVIDUAL CELLS

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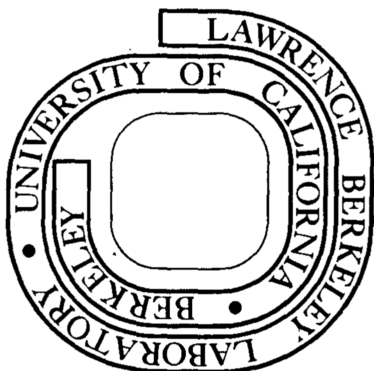

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Benzo[a]pyrene Metabolism in

Individual Cells

ABSTRACT

The conversion of benzo[a]pyrene (BaP) into 7,8,9,10-tetrahydro-7,8,-diol-9,10-epoxy-benzo[a]pyrene(BaP diol-epoxide) in individual cells is measured by flow cytometry. The measurements are based on the alterations that occur in the fluorescence emission spectrum of BaP when it is metabolized at the 7,8,9,10-positions. The appearance of BaP diol-epoxide correlates with the binding of BaP to DNA. The approach has application to the study of genetic susceptibility to chemical carcinogenesis as well as basic studies in the regulation of carcinogen metabolism.

Many chemical carcinogens must be metabolized before they are active(1). In the case of polycyclic aromatic hydrocarbons, this metabolism takes place by an inducible enzyme system called aryl hydrocarbon hydroxylase(AHH)(2). AHH action on the polycyclic aromatic hydrocarbon benzo[a]pyrene(BaP) results in the production of many oxidized products, but the products most active in binding to cellular macromolecules and presumably giving rise to cancer are the epoxides. With BaP, the epoxide that appears most active in mutagenesis(3) and may be the ultimate carcinogenic form is the 7,8,9,10-tetrahydro-7,8-diol-9,10-epoxy-benzo[a]pyrene(BaP diol-epoxide).

Studies have shown that the ability of cells to metabolize polycyclic aromatic hydrocarbons is highly variable(4). In mice much of this variability has been related to genetic factors, and the locus determining the inducibility of AHH has been identified(5). Boobis and Nebert(6) have recently described a positive association of this locus with the binding of BaP to DNA and the susceptibility of mice to chemical carcinogenesis.

In humans, the involvement of genetic factors in AHH induction or chemical carcinogenesis has not been clearly established. Okuda _et al._(7) have studied AHH in monocytes from monozygotic and dizygotic twins and found considerable variation even when cells were harvested from the same individual at different times. They estimated from this study that from 55 to 70% of the variation observed was genetically controlled, and suggested that much of the remaining variation was due to "technical limitations" with the AHH assay which measure the formation of alkali extractible phenols. Other studies with human lymphocytes in culture have also shown great individual variability in the metabolism of BaP, and that persons with lung cancer can more actively metabolize the carcinogen than persons without lung cancer(8). These studies suggest the possibility that individuals may possess different susceptibilities to carcino-

genesis by polycyclic aromatic hydrocarbons depending on their ability to metabolize the compounds to derivatives active in binding to cellular components.

We have developed a rapid and sensitive technique for monitering the cell mediated conversion of BaP to BaP diol-epoxide. The technique should make possible the screening of cells from a large number of individuals to determine their ability to metabolize chemical carcinogens. In addition, individual cells can be compared for their ability to convert BaP to BaP diol-epoxide. The basis for our measurements is the alterations that occur in the fluorescence emission spectrum of BaP when it is oxidized at the 7,8,9, and 10-positions. BaP has a major emission peak at 407 nm, a second peak at 428 nm, and a third peak at 420 nm. The emission spectrum of BaP diol-epoxide is characteristic of a pyrene structure and is unique for BaP metabolic derivatives that are oxidized at the 7,8,9, and 10-positions(9).

When BaP is incubated with mouse liver cells that can actively metabolize the compound, the BaP emission spectrum is converted into the pyrene-like spectrum (Fig. 1). The cells used in this experiment (NMuLi cl 8) have an inducible AHH that peaks in activity 12 hr after adding BaP to the growth medium(10). The spectral changes that occur upon conversion of BaP to BaP diol-epoxide begin appearing between 14 and 17 hr and reach a maximum at 22 hr after addition of BaP to the growth medium. The appearance of measurable fluorescence at 378 nm does parallel the covalent binding of BaP to DNA in these cells(11).

While the spectral measurements described above do allow the comparison of the ability of different populations of cells to metabolize BaP, they do not allow this comparison to be made on a single cell basis. Measurements on single cells would be useful for many purposes; for example, the detection of small populations of cells that actively metabolize BaP in the presence of large numbers of cells inactive in this metabolism. To measure BaP

metabolism in individual cells we have used the combination 351.1 and 363.8 nm lines of an argon-ion laser (Spectra-Physics, Model 171-05) to excite cells as they pass under laminar flow conditions through a flow cytometer(12). The emitted light corresponding to BaP fluorescence was collected at right angles from the laser beam in a photomultiplier tube (PMT) after passing through a 10 nm band pass filter with peak transmission at 410 nm (Baird-Atomic 10-82-9). The BaP diol-epoxide associated signal was collected in a second PMT after filtration through a 10 nm band pass filter centered on 380 nm (Baird-Atomic 10-77-5). A plate glass filter was used as a signal beam splitter directing most of the emitted light to the 380 nm PMT and reflecting approximately 10% of the signal to the 410 nm PMT.

Fig. 2 presents the fluorescence intensity distributions of cells exposed to either (a) BaP or (b) BaP diol-epoxide. The average amount of each compound per cell was measured independently by using either ^{14}C-BaP (Amersham Corp.) or ^{3}H-BaP diol-epoxide (a gift of K. Straub). At the concentrations and instrumental settings used for these measurements there was very little contamination of the signal collected in the 410 nm PMT by signal from BaP diol-epoxide and visa versa. The distributions shown in Figs. 2a and b are non-Gausian presumably reflecting the volume distribution throughout the cell cycle as well as cell clumping. The minimum amount of each compound detectible by fluorescence with the present instrumental configuration is approximately 5×10^6 molecules BaP and 1×10^7 molecules BaP diol-epoxide per cell.

The concentration of the two compounds can be associated on a cell by cell basis by measuring a 3 parameter distribution of 380 nm signal per cell *vs* 410 nm signal per cell *vs* cell number. To make quantitation easier we have reduced the parameters to a ratio of 380 nm/410 nm signal per cell *vs* cell number. The fluorescence ratio was determined by directing the pulses from each PMT into an analogue divider and recording the quotient per cell in the

pulse height analyzer. The fluorescence ratio measurement allows the direct association of the loss of BaP signal to gain of BaP diol-epoxide signal per cell. In the concentrations experiments shown in Figs. 2c and d these fluorescence ratios were measured for two different sets of BaP and BaP diol-epoxide concentrations. The average concentration per cell of each compound was determined by radioactivity measurements as described in the figure legend. A ratio of the mode channel in Fig. 2c with that in Fig. 2d compares favorably with that expected from the average molar concentrations of the two compounds in these cells as measured by radioactivity.

Application of the fluorescence ratio technique to metabolism of BaP in cells growing in culture is shown in Fig. 3. In this experiment two clones of mouse liver cells were exposed to 10 μg/ml BaP for different periods of time. NMuLi cl 7 is a sister clone to NMuLi cl 8, but lacks a highly inducible AHH activity(10). The lack of metabolism of BaP to BaP diol-epoxide by clone 7 is demonstrated by the 380/410 ratio per cell maintaining a low value characteristic of unmetabolized BaP. The distribution of fluorescence intensities per cell at 380 nm with NMuLi cl 8 at 16 hr indicates the appearance of BaP diol-epoxide in the cells (data not shown). As seen in Fig. 3, the 380/410 fluorescence ratio has increased three-fold over the 2 hr point (unmetabolized BaP). With NMuLi Cl 8 the fluorescence ratio reaches a maximum at 20 hr after application of BaP and follows a time course that reflects the covalent binding of BaP to DNA in these cells.

In conclusion, we have taken advantage of the difference in the fluorescence emission spectra of BaP and BaP diol-epoxide to develop a technique that measures the conversion of BaP to BaP diol-epoxide in individual cells. The technique is rapid and very sensitive; and unlike "classical" methods of assaying metabolism of BaP, it follows the production of a product known to be highly mutagenic and carcinogenic. This method should be applicable

to the screening of cells from individuals for susceptibility to polycyclic aromatic hydrocarbon carcinogenesis as well as studies that require the analysis or separation of cells on the basis of their ability to convert BaP to BaP diol-epoxide.

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FIGURE LEGENDS

1. Fluorescence Emission Spectra of Mouse liver Cells Treated with 10 ug/ml BaP.

   BaP was added from a 1 mg/ml stock solution in DMSO (final DMSO concentration of 1%). At the indicated times cells on the dish were washed 2 times with Saline GM (1.5 mM Na_2HPO_4/1.1 mM KH_2PO_4 at pH 7.4 containing 1.1 mM glucose, 0.14 M NaCl and 5 mM KCl), scraped from the dish and suspended at a density of approximately 2 x 10^5 cells/ml in Saline GM. Emission spectra were taken on a Perkin-Elmer Hitachi spectrofluorometer Model MPF-2A. Excitation was at 340 nm and the path length was 10 mm with a typical slit width of 6 mm. The vertical scale is different for each spectrum.

2. Flow Cytometry Analysis of Cells Labeled with BaP and BaP Diol-Epoxide.

   ^{14}C-BaP and/or ^{3}H-BaP Diol-Epoxide was added to cultures of mouse liver cells. The average concentration of the compounds per cell was determined after exposure for 1 hr by washing and scraping the cells as described in Fig. 1, filtering a portion of the cells onto Millipore membrane filters (HAWP 025 00), oxidizing the filters (Packard Tri-carb), and counting the ^{3}H and/or ^{14}C by liquid scintillation spectroscopy (Beckman LS9000). Flow cytometry was carried out as described in the text.

   a. Fluorescence distribution at 410 nm of cells labeled with BaP to an average concentration of 8.2 x 10^6 molecules per cell.
   b. Fluorescence distribution at 380 nm of cells labeled with BaP diol-epoxide to an average concentration of 1.7 x 10^7 molecules per cell.

c) Ratio of BaP diol-epoxide to BaP fluorescence per cell at an input ratio of the compounds calculated from radioactivity measurements of 4.52.

d) Ratio of the BaP diol-epoxide to BaP fluorescence per cell at an input ratio of the compounds calculated from radioactivity measurements of 0.82.

To compare the radioactivity measurements with the fluorescence ratio determinations, the mode fluorescence ratio from sample c was divided by that for sample d to give a value of 4.26. The radioactivity measurement for the two samples was 4.52/0.82 = 5.51.

3. Kinetics of BaP Metabolism in Individual Cells.

NMuLi cl 7 and cl 8 cells growing in culture were treated as described in Fig. 1. The mode fluorescence ratio of the 380/410 distribution was determined at each time point and the values expressed relative to the unmetabolized BaP ratio at zero time.

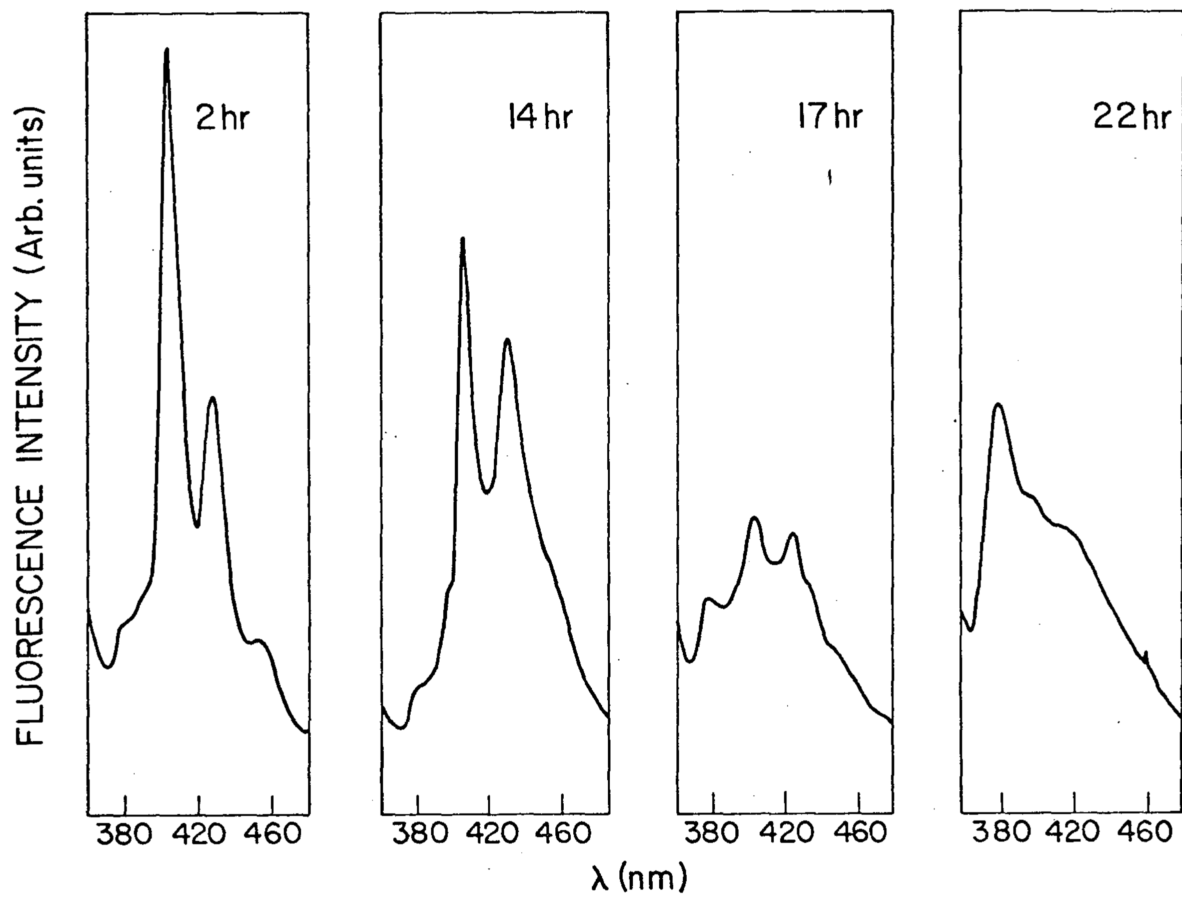

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Figure 1

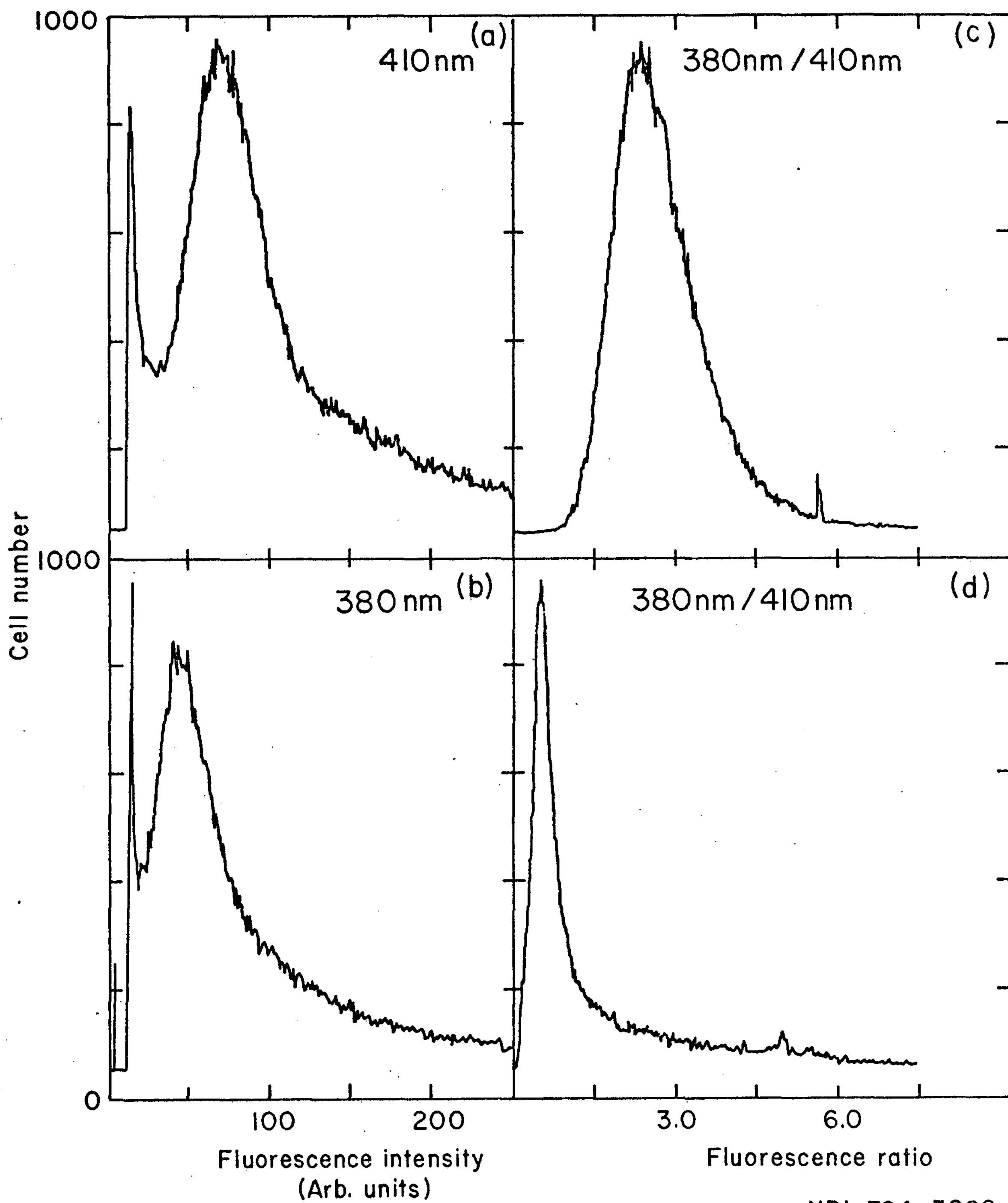

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Figure 2

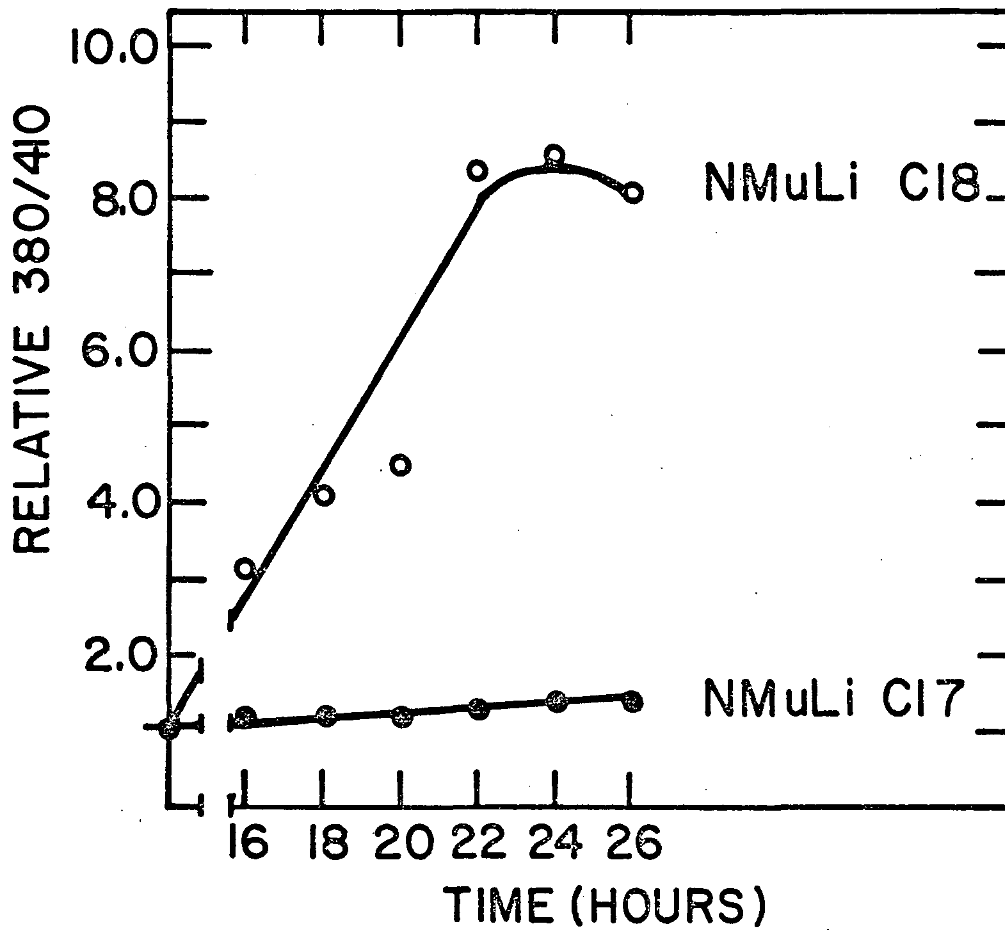

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Figure 3

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