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June 1983

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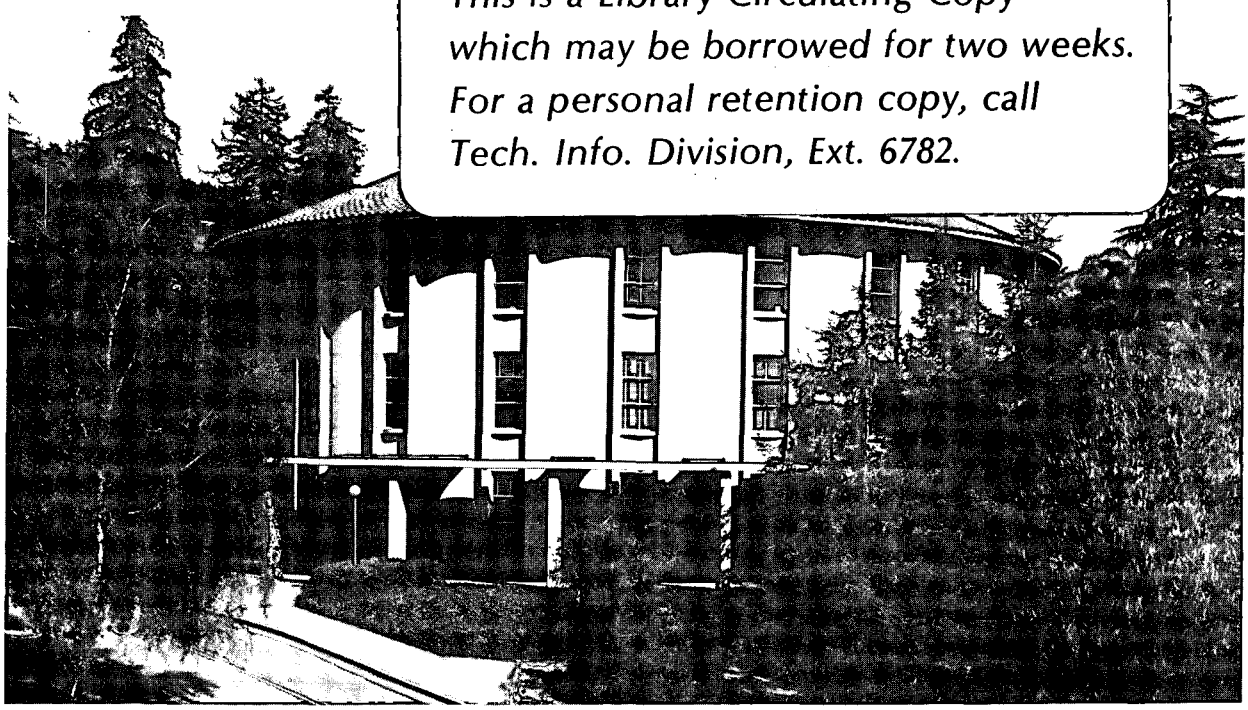

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FLOW CYTOMETRIC MEASUREMENT OF THE METABOLISM OF BENZO[A]PYRENE BY MOUSE LIVER CELLS IN CULTURE[1]

James C. Bartholomew, Charles G. Wade[2], Kathleen K. Dougherty[3]

Laboratory of Chemical Biodynamics
Lawrence Berkeley Laboratory
University of California
Berkeley, California 94720
USA

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[2]Present Address: IBM Instruments Inc
San Jose, California

[3]Present Address: Chevron Research
Richmond, California

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SUMMARY

The metabolism of benzo[a]pyrene in individual cells was monitored by flow cytometry. The measurements are based on the alterations that occur in the fluorescence emission spectrum of benzo[a]pyrene when it is converted to various metabolites. Using present instrumentation the technique could easily detect 1×10^6 molecules per cells of benzo[a]pyrene and 1×10^7 molecules per cell of the diol epoxide. The analysis of C3H 10T 1/2 mouse fibroblasts growing in culture indicated that there was heterogeneity in the conversion of the parent compound into diol epoxide derivatives suggesting that some of variation in sensitivity to transformation by benzo[a]pyrene may be due to differences in cellular metabolism. The technique allows sensitive detection of metabolites in viable cells, and provides a new approach to the study of factors that influence both metabolism and transformation.

INTRODUCTION

Benzo[a]pyrene(BaP) is a polycyclic aromatic hydrocarbon that must be metabolized by the cell before it becomes mutagenic or carcinogenic[1-3]. This metabolism takes place by an inducible enzyme system called aryl hydocarbon hydroxylase(AHH)[4-6]. Studies have shown that the ability of cells to metabolize BaP is highly variable[7-14]. In mice, much of this variability has been related to genetic factors, and the locus determining the inducibility of AHH has been identified[15]. Boobis and Nebert[16] have 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.[17] 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. 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[18, 19]. These studies suggest the possibility that individuals may possess different susceptibilities to carcinogenesis by polycyclic aromatic hydrocarbons depending on their ability to metabolize the compounds to derviatives active in binding to cellular components. Other factors besides genetic such as position in the cell cycle and differentiation state of the tissue[20] have been shown to influence carcinogen metabolism, and so metabolism may even vary from cell to cell within a tissue.

Because of the importance of cellular metabolism in defining the activity of chemicals like BaP in carcinogenesis, and because of the variability of this metabolic potential, careful analysis of the metabolism of such chemicals becomes a necessary part of any risk assessment. With such a complex metabolic profile as depicted in Figure 1 for BaP, the detailed study of the quantities of individual metabolites in different types of tissues or even in different individuals has been difficult. Normally, such an analysis involves the extraction of metabolites from the cells followed by quantification of the compounds by techniques such as HPLC. However, many of the metabolites bind covalently to cellular components making quantitative extraction not possible with present procedures. Extraction techniques are also time consuming and necessarily lead to an averaging of the metabolites over all the cells in a tissue preparation.

We have been developing methods that do not involve extraction for obtaining metabolic information from individual cells exposed to BaP. The approach is to utilize the changes that occur in the fluorescence emission spectra of cells as they convert BaP into metabolites. We have

made these measurements in a flow cytometer and have obtained information about population heterogeneity of BaP uptake and metabolism on a single cell level.

MATERIALS AND METHODS

Cell Culture

The C3H 10T 1/2 cells are mouse fibroblasts capabile of metabolizing BaP[21-22]. The medium used to grow the cells was Eagle's minimal medium[23] (GIBCO, Grand Island, N.Y.) containing 10% donor calf serum (Flow Laboratories, Rockville, MD) and 10 ug/ml insulin (Schwartz/Mann, Orangeburg, N.Y.). All cells were judged free of mycoplasma by incorporation of H^3-thymidine (20.1 Ci/mM; New England Nuclear, Boston, MA) into the nucleus of the cells and not the cytoplasm[24]. Stock cultures were maintained by subculturing the cells twice weekly at a cell density of $1 \times 10^4/cm^2$. All cell cultures were actively growing at the time of addition of test compounds.

Chemical Additions

The BaP and metabolites of BaP were added to the culture medium from a stock solution (1.0 mg/ml) in DMSO (Matheson, Coleman, and Bell, Los Angeles, CA). The final concentration of DMSO was 0.5% in the controls and test cultures. BaP was obtained from the Aldrich Chemical Company, Inc., Milwaukee, Wis., and the metabolites were obtained through the NCI Standard Chemical Carcinogen Reference Repository.

The spectra of all compounds were determined in a Perkin-Elmer MPF-3A (Norwalk, CT) spectrofluorometer. The compounds were added to cells and allowed to equilibrate for 1 hr prior to harvesting the cells. The cells were resuspended in isotonic buffer at 1×10^6 per ml and the spectra were measured. All spectra were recorded with slit widths of 4 nm.

Flow Cytometry

The flow cytometer used in these experiments has been described by Hawkes and Bartholomew[25]. Cells traversed the flow chamber at approximately 500 cells/sec in a stream that intersected the beam of an argon ion laser (Model 171 Spectra Physics, Inc. San Jose, Ca) tuned to 351.1 and 363.8 nm. The typical power output at these wavelengths for these experiments was approximately 100 mW. The fluorescent light prior to reaching the photomultipliers(PMTs) was split using a Corning 0-51 glass filter (Corning, Corning, N.Y.) to reflect approximately 10% of the beam to one PMT and 90% to a second PMT tube. Band pass filters(Baird-Atomic, Bedford, Mass.) having approximately 10 nm bandwidths were selected to analyze for signals characteristic of BaP or its metabolites. In the two parameter measurements, BaP fluorescence was recorded with the PMT receiving only 10% of the emitted light since its concentration in these experiments was in excess over the metabolites. In all experiments, the cells to be analyzed were harvested

by trypsinization and passed directly through the flow cytometer without further processing. The data from each analysis was stored, plotted and analyzed by use of VAX 780 computer (Digital Equipment Corp., Maynard, Mass.).

Radioactivity Measurements

C^{14}-BaP(59 Ci/mole) was obtained from Amersham Corporation (Arlington Heights, Il.) and unlabelled BaP from Aldrich Chemical Company (Milwaukee, Wis.). H^3-BaP diol epoxide (1.3 Ci/mmole) was kindly provided by Dr Ken Straub. Samples were counted in Aquassure liquid scintillation cocktail(New England Nuclear, Boston, Mass.).

RESULTS

Fluorescence Monitoring of BaP Metabolism

AHH metabolism of BaP results in the production of many oxidized products only some of which are shown in Figure 1. The metabolites most active in binding to cellular macromolecules are the epoxides. With BaP, the epoxide that binds most efficiently to DNA 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) [26-30].

The metabolism of BaP by cells generally produces compounds that are still fluorescent, but have alterations in their spectra when compared to that of the parent compound. Figure 2 presents some of the spectra of different metabolites present as individual compounds in C3H 10T 1/2 mouse fibroblasts. The spectra were obtained by exposing 2×10^6 cells growing in culture to 1 ug/ml of the authentic metabolite. The derivatives of BaP which have added a single hydroxyl group without disturbing the aromaticity are characterized by an emission spectrum shifted to longer wavelengths(427 nm) relative to BaP. Whereas; the diol or diol epoxide derivatives gave emission spectra shifted to shorter wavelengths(390 and 380 nm respectively).

In Figure 3, the emission spectra of C3H 10T 1/2 cells and medium which have been treated with BaP for 1 to 48 hr are shown. The 1 hr incubation is long enough for the cells to accumulate BaP to equilibrium, but not long enough to induce the enzymes required for metabolism[20]. At 1 hr, the spectra of both the cells and the medium looks very similar to that of unmetabolized BaP. After 24 hr incubation, the BaP has been metabolized and the resulting spectra indicate the presence of phenols and BaP diol epoxides in both the cells and the medium. It should be pointed out that based on spectral characteristics, it is not possible to distinguish BaP diol epoxide from the tetrol which is derived from the BaP diol epoxide. The medium contains more of all the metabolites than the cells, and the ratio of signal characteristic of phenols to that of BaP diol epoxides is higher in the medium than in the cells. This suggests, not suprisingly, that cells bind the BaP diol epoxide more efficiently than the phenol. By 48 hr there is very little evidence of signal from unmetabolized BaP either in the medium or in the cells. In the medium, the major signal comes from the phenols, but a significant BaP diol epoxide signal was present. The cells clearly contain compounds fluorescing with the characteristics of BaP diol epoxide, and also contain a significant phenol signal. Although the fluorescence spectra give only a qualitative indication of the types of metabolites present, the general features of the metabolism have been confirmed by HPLC analysis of metabolites extracted from both the medium and the cells (data not shown).

Flow Cytometry of Cells Treated with BaP and BaP Diol Epoxide

Adaptation of the fluorescence measurement techniques seen with a large population of cells to the single cell level can be done by using a flow cytometer. Figure 4a shows the fluorescence emission histogram of BaP in cells analyzed by flow cytometry after 1 hr exposure to BaP and measuring the fluorescence intensity with a band pass filter centered at 410 nm. The fluorescent signal was correlated in this experiment with the average amount of BaP per cell as determined by scintillation counting of an aliquot of cells labeled with ^{3}H-BaP of a known specific activity. The average amount of BaP per cell present in this experiment was approximately 1 x 10^6 molecules/cell. In the same manner, cells were treated with BaP diol epoxide and the histogram of fluorescence intensity/cell at 380 nm was recorded(Figure 4b). The level of BaP diol epoxide in this sample was approximately 1 x 10^7 molecules/cell. With the present experimental system, the sensitivity was limited by background signal from untreated cells. Much of this signal may be due to endogenous fluorescence from NAD(P)H[31]. The shape of the fluorescence distributions for either BaP or BaP diol epoxide did not change appreciably as the concentration of either compound was raised through 2 orders of magnitude from that used to obtain the data in Figure 4. Both the mean and the mode of the distributions was a linear function of the average amount of compound per cell as measured by radioactivity until the cells became saturated with hydrocarbons. Using this procedure we determined that saturation with these compounds depended on the amount and type of serum in the medium as well as the cells being studied(data not presented).

In general, during metabolism of BaP both BaP and its metabolites are present. To compare the relationship between the measurement of different relative concentrations of BaP and BaP diol epoxide in cells we compared the ratio of fluorescence at 380 and 410 nm with the known average concentration of compound per cell calculated from determining the amount of radioactivity present in an aliquot of cells. Figure 4 c and d presents the fluorescence ratios obtained from two different imput ratios of BaP and BaP diol epoxide. Figure 5 shows that the relationship between fluorescence ratio and molar ratio is not linear over the range of concentrations tested. The fluorescence ratio is lower than expected from the radioacivity measurements when the level of BaP diol epoxide is high relative to BaP. This may be due to the secondary emission bands of BaP diol epoxide contributing to the signal intensity at 410 nm with these high molar ratios. Of course, the secondary emission signals could be subtracted from the 410 nm signal based on the contribution expected from the measured 380 nm intensity.

As seen in the spectra presented in Figure 3, most of the metabolism that occurs by 24 hr can be identified as shifting the fluorescence emission peak at 410 nm to either 380 nm or 427 nm characteristic of metabolism towards either the BaP diol epoxide or phenols respectively. Measuring the ratio of these two signals on a per cell basis gives an indication whether the population is homogeneous in the type of metabolic pattern it follows. Figure 6 shows the results from analyzing a population of C3H 10T 1/2 cells determining this ratio after they had been treated with BaP for 1 and 24 hr. At 1 hr the 380 nm/427 nm

signal was very low reflecting the low amount of BaP diol epoxide present in the cells at this time. The ratio increases dramatically after 24 hr of incubation reflecting the metabolism that has occurred to both BaP diol epoxide and the phenol derivatives. There is heterogeneity in the measurements with some cells in the population having a very high ratio indicating extensive metabolism to the BaP diol epoxide with little production of phenols.

When two parameter measurement are made on cells exposed to BaP using flow cytometry the relative proportion of metabolites going down the "Diol Epoxide" pathway verses the "Phenol" pathway can be determined in single cells. In these experiments we compared the fluorescence intensity at 380 nm with that at 427 nm. As seen in Figure 3, emission at these wavelengths contribute most significantly to the spectra of cells which have metabolized BaP for 24 hr. The bivariate distributions obtained from these cells is shown in Figure 7. This figure is a composite of samples analyzed 1 hr and 24 hr after addition of BaP to C3H 10T 1/2 cells. At 1 hr most of the signal along the log 427 nm axis comes from the secondary emission signal of the BaP. The 380 nm signal is low and very dispersed and comes from endogenous fluorescence in the population. The lower intensity distribution below that from the 1 hr sample is from cells not treated with BaP which were mixed with the BaP treated cells after harvesting. After 24 hr the log 427 nm signal is primarily from the major emission from phenols(see Figure 3). After the 24 hr incubation period, the log 380 nm signal increased considerably indicating metabolism through the BaP diol epoxides. The distribution along the log 380 nm axis is more dispersed than along the log 427 nm axis suggesting more heterogeneity of metabolism to diol epoxide compounds per cell than to phenols.

DISCUSSION

The techniques described demonstrate that fluorescence spectral analysis of a polycyclic aromatic hydrocarbon like benzo[a]pyrene as it is being metabolized by cells in culture can give information regarding the qualitative nature of the metabolism. Precise quantitative determination of the metabolites is not possible without considerably more knowledge about the multitudinous interactions of the metabolites with all the cellular components. However, both the spectra and the fluorescence histograms do contain indications of the quantities of the metabolites present. The procedure was used here to study BaP metabolism, but can be used with any other compound that changes its spectral properties upon metabolism, or which can be marked with a fluorescent tag.

The application of flow cytometry to the detection of classes of metabolites in individual cells is possible with the use of narrow band pass filters centered in the spectral range that different types of metabolites fluoresce. The sensitivity of the system, while not maximized in our hands, is comparable to that obtained when analyzing metabolites by standard extraction and HPLC analysis.

A major advantage of the flow technique over extraction methods is that individual cells are analyzed and so population heterogeneity can be determined under different experimental conditions. For example, it is conceivable to couple the metabolite measurement with DNA content determination and relate metabolism of BaP to the cell cycle position. Also, such experimental variables as the relationship of medium components to the heterogeneity of BaP uptake and metabolism can be determined. Since the cells are not fixed for the analysis, the technique may allow metabolic parameters to be related directly to transformation frequencies. Cells with high ratios of BaP diol epoxide to other metabolites could be sorted to determine whether they also have a high transformation frequence when related to the rest of the population. Such direct correlations are not possible with classical methods of analysis for metabolites nor with immunological reagents being used to detect carcinogens[32-34]. It should be pointed out that the concentration of BaP used in these experiments is similar to that used in standard transformation studies.

Direct analysis of the metabolites of BaP per cell by flow cytometry has some unique applications to studies of population heterogeneity and its relation to experimental parameters. The analysis is probably not as sensitive as immunological detection of metabolites, nor as specific as HPLC analysis of extracted samples. However, the sensitivity and selectivity coupled with the viability of the cells after analysis is high enough to contemplate many unique experiments.

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FIGURE LEGENDS

Figure 1. Metabolites of Benzo[a]pyrene.

Figure 2. Fluorescence Spectra of Benzo[a]pyrene Metabolites.

Growing C3H 10T 1/2 mouse fibroblast cells(2×10^6 were exposed to 1.0 ug/ml authentic metabolite for 1 hr prior to harvesting the cells by trypsinization. The metabolites were each dissolved in DMSO prior to addition. The spectra were taken as described in the text.

Figure 3. C3H 10T 1/2 Metabolism of Benzo[a]pyrene.

Growing C3H 10T 1/2 mouse fibroblast cells(2×10^6 were exposed to 1.0 ug/ml benzo[a]pyrene for the times indicated. At the end of the incubation times the medium was removed and saved. The cells were washed and trypsinized from the dishes, and the spectra were measured by exciting at 360 nm and recording the emission as described in the text.

Figure 4. Detection of Benzo[a]pyrene and Benzo[a]pyrene Diol Epoxide in Individual Cells.

Cells were exposed for 1 hr to known amounts of radioactive benzo[a]pyrene(a), benzo[a]pyrene diol epoxide(b), or mixtures of the two compounds (c and d). The cells were removed from the dishes by trypsinization and the amount of each compound was determined in a known number of cells by liquid scintillation counting.

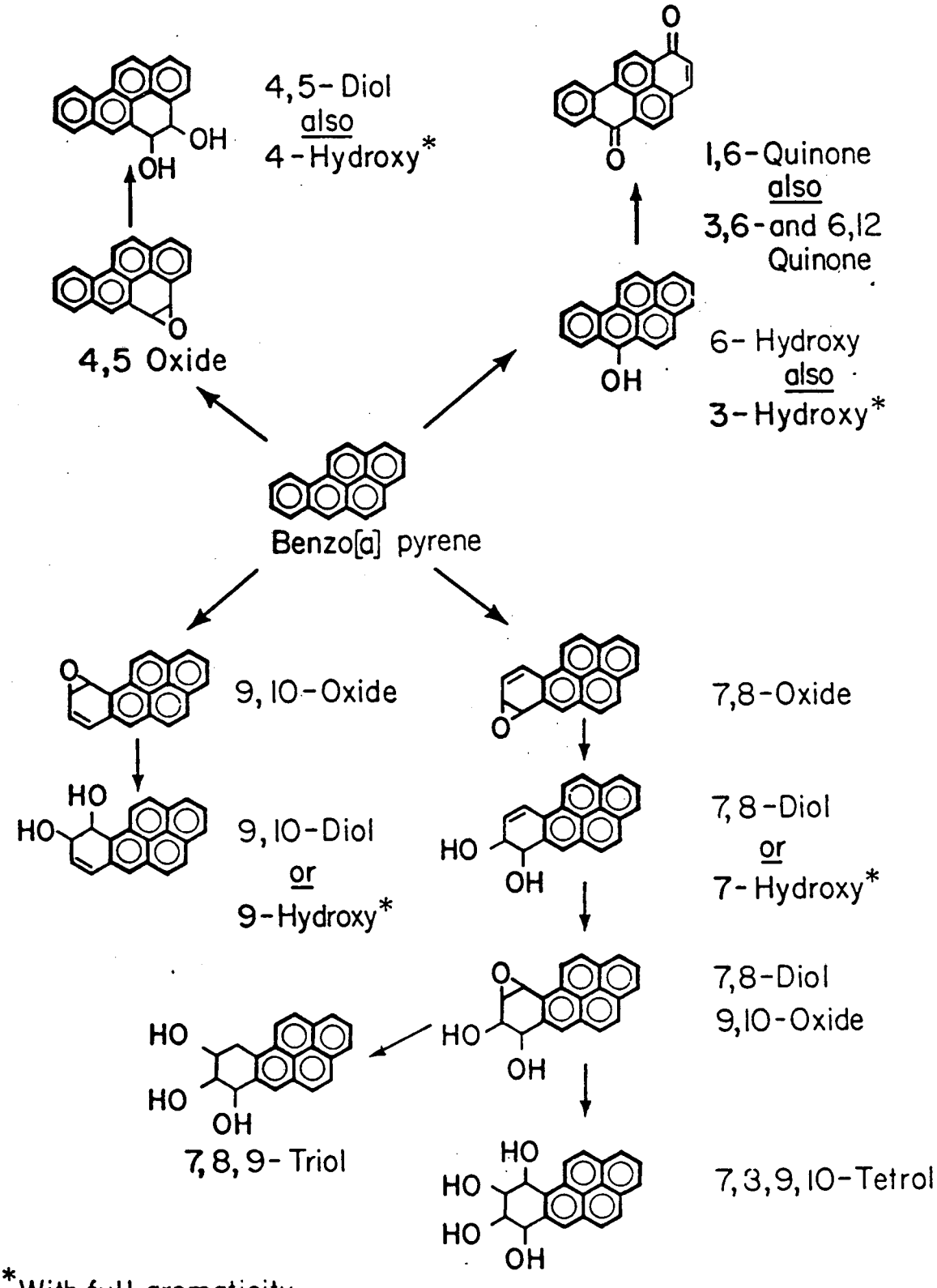
Figure 5. Standardization of the Fluorescence Ratio Measurements.

Cells were exposed to different amounts of benzo[a]pyrene and benzo[a]pyrene diol epoxide as described for Figure 4, and the average amount of each compound in the cells was determined by radioactive measurements.

Figure 6. Ratio of 380 nm/427 nm Fluorescence from C3H 10T 1/2 Cells Metabolizing Benzo[a]pyrene.

Figure 7. Bivariate Plots of Log 380 nm Signal vs 427 nm Signal in Cells Exposed to Benzo[a]pyrene.

This figure is a composite of data from cells analyzed after 24 hr or 1 hr exposure to BaP. The cells were harvested as described in the text. The initial concentration of benzo[a]pyrene was 1 ug/ml. Prior to analyzing the 1 hr sample cells which were not exposed to benzo[a]pyrene were added to the sample and their location in indicated on the plot.



*With full aromaticity

XBL 799-5020

Fig. 1

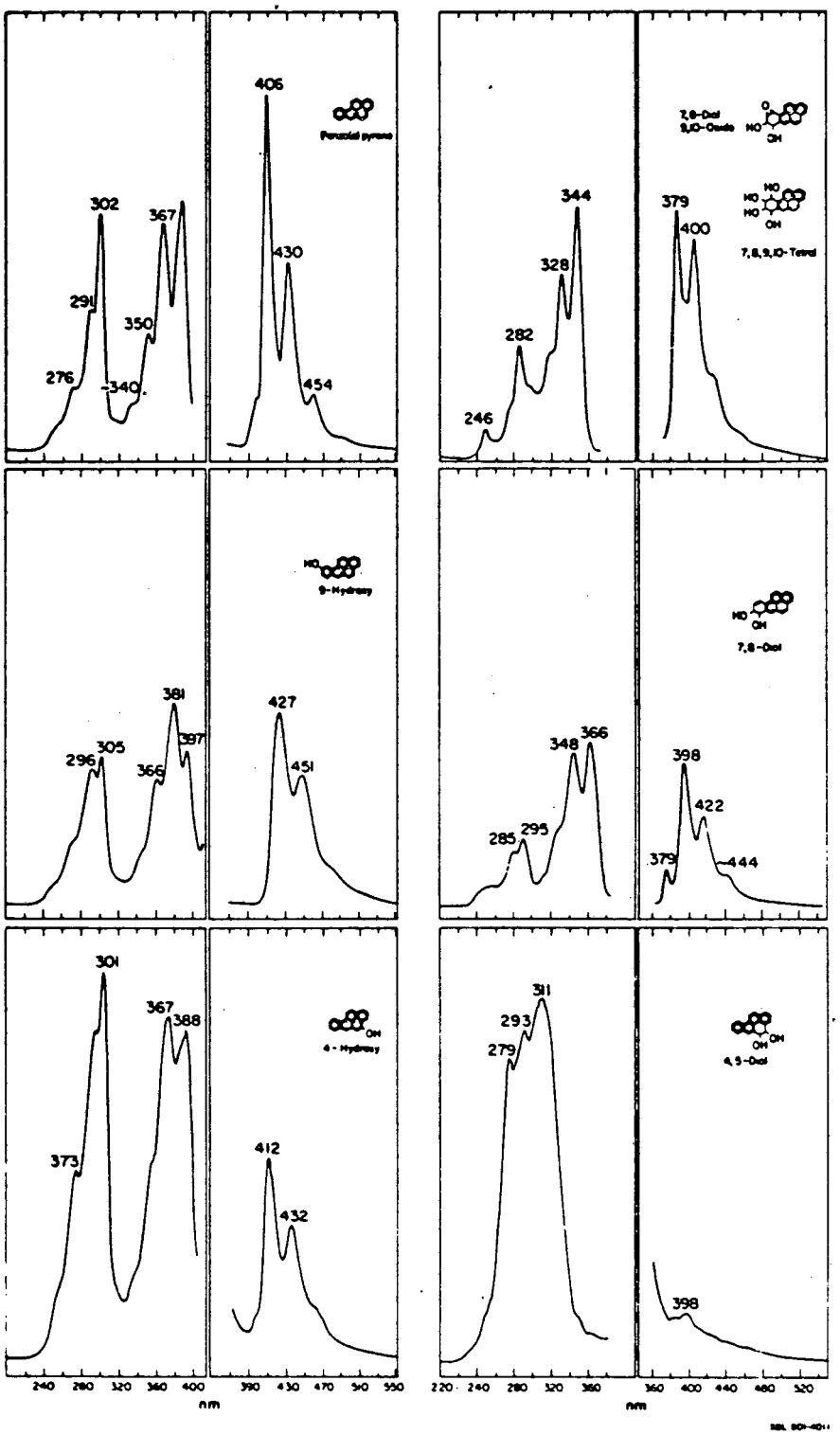

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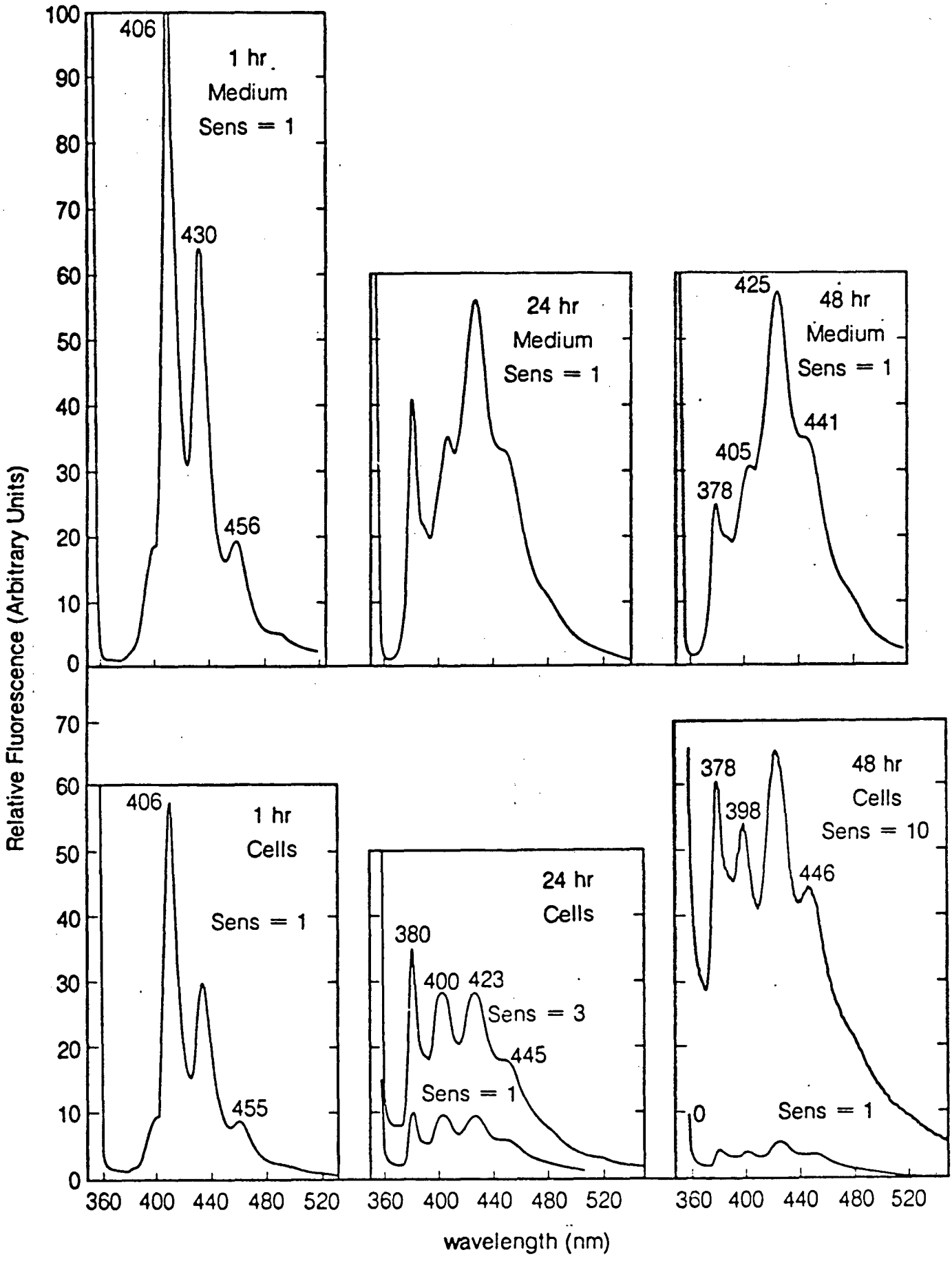
Fig. 2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Sens = 1
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Fig. 3

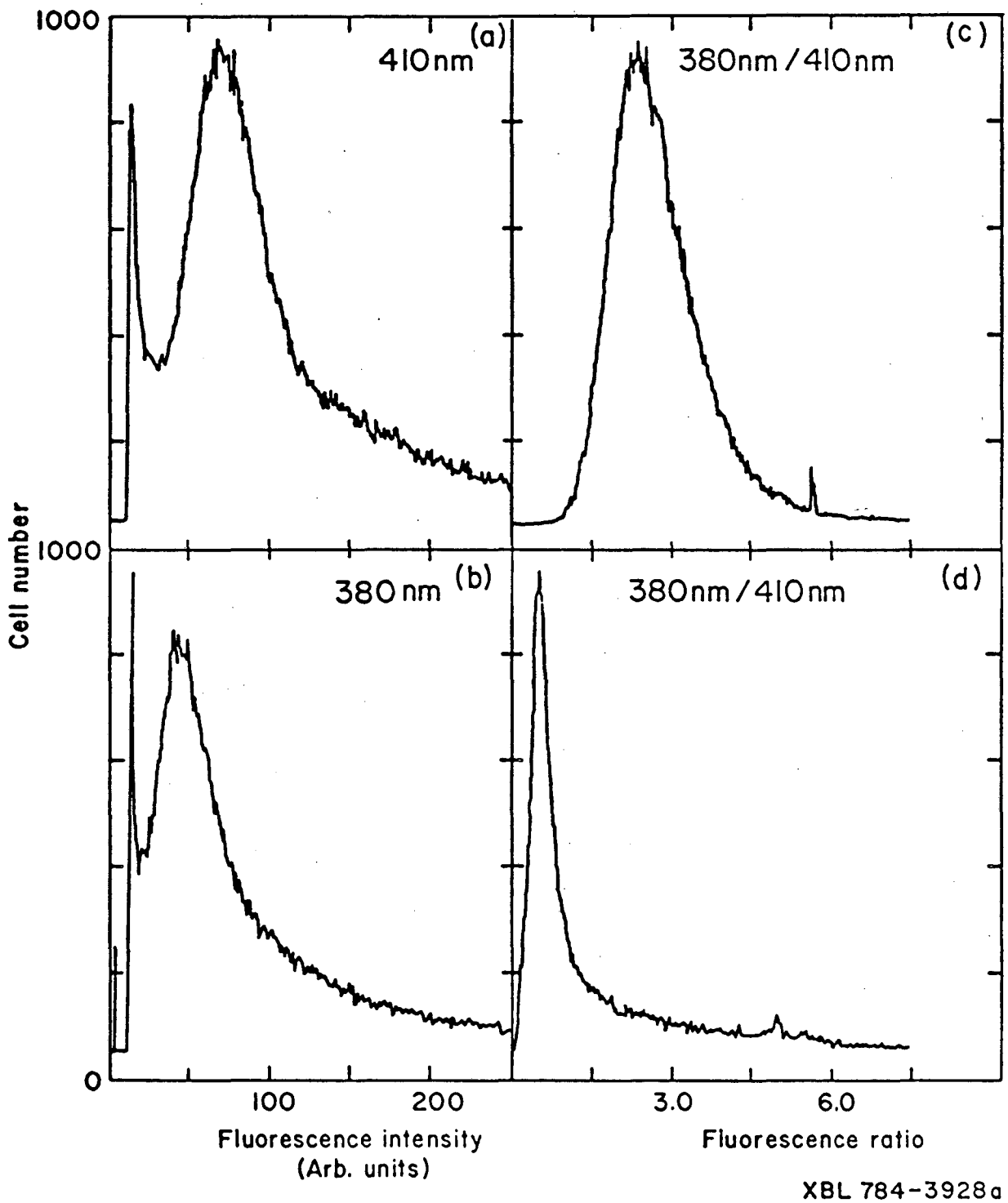
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Fig. 4

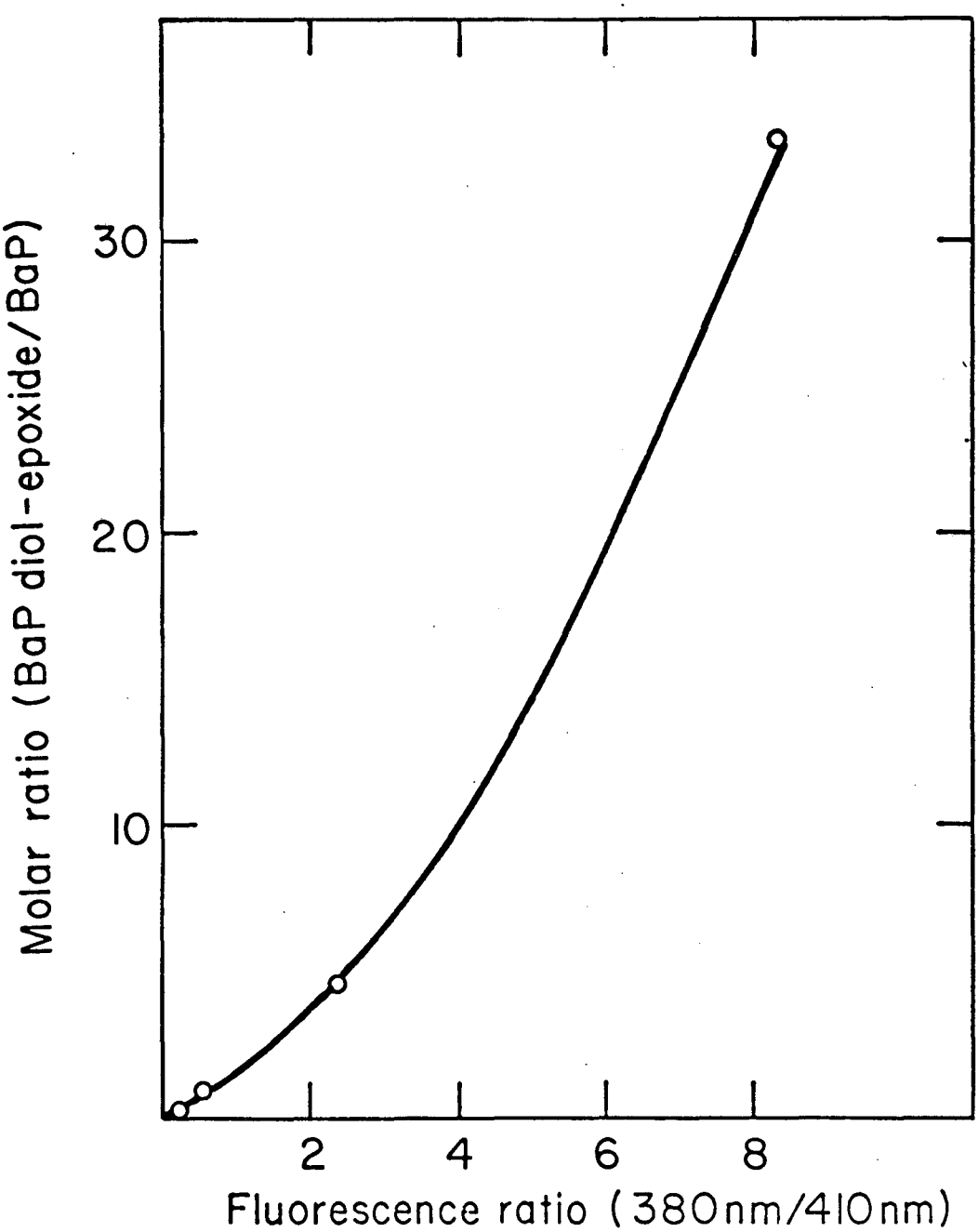

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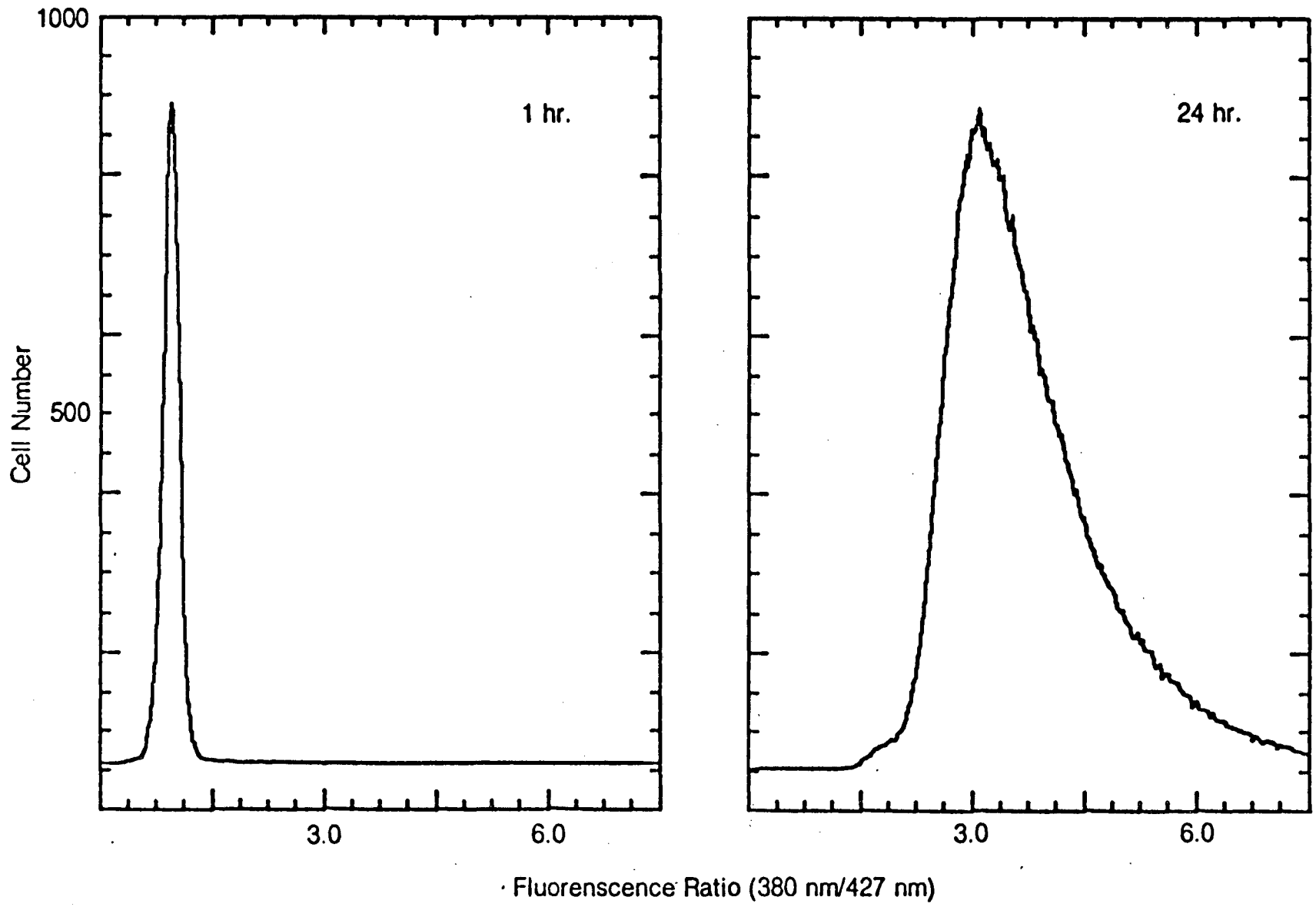
Fig. 5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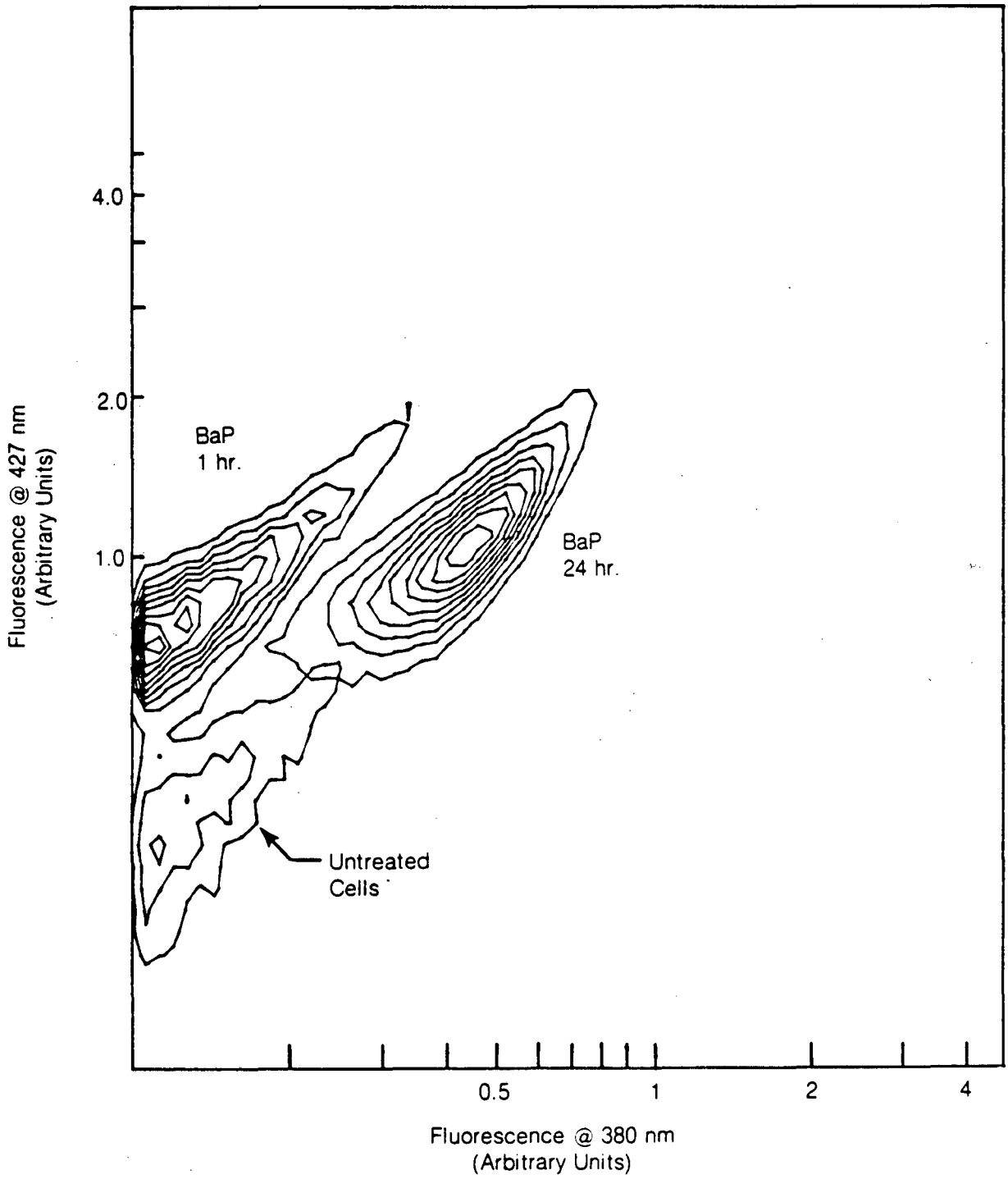
6.0
3.0
6.0
Fluorenscence Ratio (380 nm/427 nm)

Fig. 6



XBL 836-332

Fig. 7

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TECHNICAL INFORMATION DEPARTMENT
LAWRENCE BERKELEY LABORATORY
UNIVERSITY OF CALIFORNIA
BERKELEY, CALIFORNIA 94720