Selective Fluorescence Quenching of Benzo[*a*]pyrene and a Mutagenic Diol Epoxide Derivative in Mouse Cells†

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ABSTRACT: The overlap of the fluorescence spectra of benzo[*a*]pyrene and its metabolites, including an ultimate carcinogen diol epoxide (±)-7β,8α-dihydroxy-9α,10α-epoxy-7,8,9,10-tetrahydrobenzo[*a*]pyrene, impedes the use of fluorescence in studying the impact of these important carcinogens on cell populations. It is shown that in cells certain collisional quenchers can be used to selectively quench the benzo[*a*]pyrene fluorescence, leaving the diol epoxide emission. Because such quenchers work only with close contact over distances much less than the cell dimensions, the preferential solubilities can be used to probe fluorophore locations. Benzo[*a*]pyrene and its diol epoxide derivative were added directly to mouse cells in culture: NMuLi (mouse liver epithelial cells), Balb 3T3 A31 HYF (mouse fibroblast cells), and MSV/MLV Balb 3T3 A31 HYF (Moloney sarcoma virus transformed mouse fibroblast cells). Fluorescence lifetimes of the aromatic hydrocarbons were measured, and fluorescence and flow cytometric studies were made on the effects of various quenchers added to suspensions of these cells. The collisional quencher *n*-octyl iodide, which is lipid soluble, quenches the benzo[*a*]pyrene emission by a factor of six with little change in the diol epoxide emission. At comparable molarity, the ionic quencher, KI, is relatively ineffective and does not offer selective effects, presumably because the I^- does not penetrate the cell. At comparable molarity, methylene iodide is a relatively effective quencher for both aromatic hydrocarbons, but it offers little selectivity. The results were, in general, independent of the particular type of mouse cell strain. The results provide information on the location of the hydrocarbons when added to the cells. Evidence indicates that the selectivity offered by the *n*-octyl iodide arises from the fact that collisions with benzo[*a*]pyrene are much more likely than collisions with the diol epoxide. Consequently, the possible locations of the metabolite under the experimental conditions are: bound in proteins or attached near the polar head groups of lipids in membranes or attached in a nonlipid portion of the cell. The potential for using the relative solubilities of collisional quenchers in separating the fluorescence emissions of polycyclic aromatic hydrocarbons is discussed. Applications of the technique to flow cytometry are demonstrated.

The carcinogenic effects of many chemicals are related to the metabolism of these compounds to reactive intermediates which bind to critical cellular components (Busch, 1974; Miller and Miller, 1974). Such knowledge has led to studies of the nature and chemical structures of these reactive metabolites (ultimate carcinogens) and their metabolic precursors (proximate carcinogens) (Miller and Miller, 1974). The aromatic hydrocarbon, BaP,[1] a ubiquitous air pollutant, possesses potent mutagenic and carcinogenic activity. BaP is metabolized by microsomal enzymes to arene oxides, phenols, and dihydrols; there is now strong evidence (Borgen et al., 1973; Sims et al., 1974; Daudel et al., 1975; Ivanovic et al., 1976; Meehan et al., 1976; Huberman et al., 1976; Yang et al., 1976, 1977) that an ultimate carcinogenic metabolite is the BaP diol epoxide, DE (Figure 1). The enzymatic oxidation of the BaP and the location and the action of DE are of extreme importance in the molecular biology of carcinogenesis.

Fluorescence is a major tool in studies of PAH carcinogenesis, and spectrofluorometric studies have been done in vitro (Geacintov et al., 1976), in vivo (Kodama and Nagata, 1975), and in single-cell systems (Salmon et al., 1974a-c; Tyler et al., 1977).

The fluorescence spectra of BaP and its metabolites overlap, the emission spectrum of DE, for example, being blue shifted about 25 nm from that of BaP. This fact plus the large BaP to DE ratio that exists during initial metabolism limits considerably the general applicability of fluorescence as a tool for studies of the metabolic process and as a possible method of screening for DE exposure. This is a particular problem in flow cytometry, where little information is contained in the weak DE signal which appears as a very small shoulder on the much larger BaP emission.

This paper demonstrates that in cells, selective quenching of the BaP signal can be achieved with little attendant change in the DE emission. Recent applications of quenching to biological systems have concentrated on intrinsic protein fluorescence. The present paper is to the authors' knowledge the first to apply selective quenching effects to PAH molecules in cells. The technique should have broad application to fluorometric studies of various kinds in biological systems, including flow cytometry and cell sorting (these topics are reviewed by Horan and Wheeless, 1977). The present results provide information on the locations of the PAH molecules in cells in culture.

Fluorescence Quenching. The absorption of energy in the UV region by these molecules results in an increased population of the lowest lying, excited singlet state. Quenching of fluorescence refers to a decrease in the intensity of the emission from that state. Mechanistically (Birks, 1970), this can mean that the absorbed energy is transferred to any of the other modes (radiationless transitions, intersystem crossing to the triplet, etc.) at the expense of singlet emission. Several well-known quenching methods exist. Those applicable in the present study are collisional quenching and resonance energy-transfer quenching.

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[1] Abbreviations used: BaP, benzo[*a*]pyrene; DE, (±)-7β,8α-dihydroxy-9α,10α-epoxy-7,8,9,10-tetrahydrobenzo[*a*]pyrene; saline GM, 1.5 mM Na_2HPO_4/1.1 mM KH_2PO_4 at pH 7.4 with 1.1 mM glucose, 0.14 M NaCl, and 5 mM KCl; MBalb, MSV/MLV Balb 3T3 A31 HYF; PAH, polycyclic aromatic hydrocarbon.

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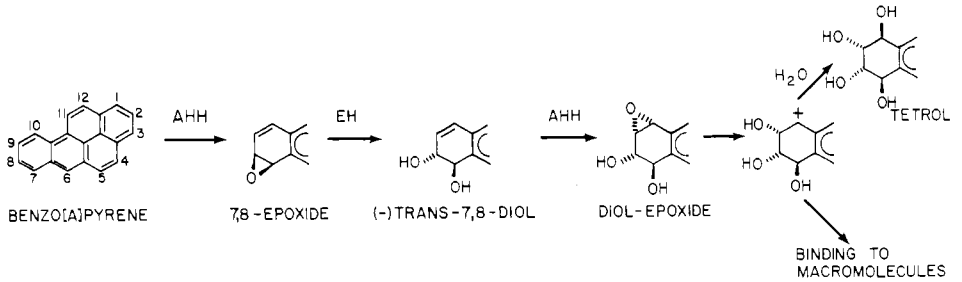

FIGURE 1: Mechanism of one portion of the metabolism of benzo[*a*]pyrene. AHH and EH indicate aryl hydrocarbon hydroxylase and epoxide hydrase, respectively. Triangles and dotted lines indicate that the substituents are toward and away from the viewer, respectively.

Collisional quenching requires a close approach (typically 4–6 Å) or "collision" of the quencher with the fluorophore. Some collisional quenchers (including the heavier halogens) act by promoting intersystem crossing to the triplet state through spin–orbit coupling interactions, while others quench by a charge-transfer interaction (Shinitsky and Rivnay, 1977; Borochov and Shinitsky, 1976). Biological applications of collisional quenching (principally of intrinsic fluorescence of proteins) have been reported for micellar systems (Pownall and Smith, 1974; Papageorgiou and Argoudelis, 1973), membranes (Badley, 1975; Lehrer, 1971; Bieri and Wallach, 1975; Koblin et al., 1973; Shinitsky and Rivnay, 1977; Borochov and Shinitsky, 1976; Burnstein et al., 1973), and DNA-bound systems (Geacintov et al., 1976).

Collisional quenching in isotropic solvents can usually be described by the Stern–Volmer (1919) equation,

$$(F_0/F) = 1 + K_{sv}[Q] \quad (1)$$

where F_0 and F are the fluorescent intensities in the absence and presence, respectively, of the quencher at a molar concentration [Q]. K_{sv} is a constant which is the product of a collisional quenching rate constant, k_q, and the excited-state lifetime, τ, of the fluorophore in the absence of the quencher:

$$K_{sv} = k_q\tau \quad (2)$$

In a heterogeneous population of fluorophores, such as might exist in cells, eq 1 is not usually obeyed.

Resonance energy-transfer quenching can occur when the absorption spectrum of the quencher overlaps the emission spectrum of the fluorophore (Birks, 1970; Wu and Stryer, 1972; Forster, 1948, 1959; Vanderkooi et al., 1977). This interaction depends on the sixth power of the separation and typically provides interactions over 30–80 Å, much larger distances than those involved in collision quenching.

The results in this paper focus upon collisional quenchers. A later publication will treat applications of resonance energy transfer.

Rationale. Because collisional quenchers work only with close contact over distances much less than the cell dimensions, one can utilize the preferential solubility of quenchers to probe fluorophore locations. An ionic quencher, such as I^-, which is soluble in the aqueous phase, will be a relatively ineffective quencher for fluorophores located in the lipid portion of the cell membrane or in the interior of the cell. A lipid-soluble quencher, such as a long-chain alkyl halide, will effectively quench materials in lipids but not those in other regions, such as, for example, tryptophans buried in proteins. By using a variety of quenchers with differing, but known, solubilities, the location of fluorophores can be determined. Such a rationale has been used to probe the locations of photoaffinity labels of proteins in cells (Klip and Gitler, 1974) and to probe the locations of tryptophan fluorophores in proteins and in membranes (Bieri and Wallach, 1975; Koblin et al., 1973; Shinitsky and Rivnay, 1977; Borochov and Shinitsky, 1976).

Materials and Methods

Cells. Balb 3T3 A31 HYF cells were derived by clonal selection of Balb 3T3 A31 mouse fibroblasts. The continuous line of MBalb-transformed cells was produced by infection of the nontransformed cell line with the Moloney strain of murine sarcoma virus (MSV/MLV) as reported earlier (Millard and Bartholomew, 1977).

PAH additions were made when the cells neared saturation densities (approximately 2×10^6 cells, nontransformed strains, and 1×10^7 cells, transformed strains, per 100-mm tissue culture dish). The hydrocarbons (BaP and/or DE) in dimethyl sulfoxide (1 mg/mL) were added to achieve a concentration of 2 μg/mL of growth medium and 0.5% dimethyl sulfoxide. After incubation for approximately 1 h, the medium was removed, and the cells were washed twice with 5 mL of 1× saline GM buffer, carefully removed with a rubber policeman, and washed into a centrifuge tube with the buffer. After centrifugation at 2 krpm for 2 min, the supernatant was decanted, and the cells were resuspended in fresh buffer. Coulter counter measurements (two per sample) were used to adjust the cell concentrations (usually 200 000 cells/mL) for the fluorometric and fluorescent lifetimes measurements.

Chemicals and Apparatus. BaP was obtained from Aldrich Chemical Co. (San Leandro, Calif.) and purified as previously described (Landolph, et al., 1976). The DE, synthesized by K. Straub (Meehan et al., 1977), was a mixture of the cis and trans forms. Aqueous potassium iodide solutions were made up as needed from reagent-grade stocks. For the organohalogen quenchers, great care was taken in an effort to prevent the formation of I_3^- which occurs upon extended exposure to light (Lehrer, 1971). This ion absorbs relatively strongly near 300 nm and with considerably less intensity at 340 nm. If present, this ion may give spurious results when excitation in that region is used. The organohalogen compounds were fractionally distilled under vacuum and refrigerated in dark containers. Just before use, the quenchers were dissolved in dimethyl sulfoxide at the appropriate concentrations (up to 100 mg/mL) and stored at −70 °C. During experiments, the quencher solutions were stored in dark containers in an ice bath. Even this care often failed for CH_2I_2, the decomposition of which appears to be accelerated in dimethyl sulfoxide even in the absence of light.

During measurements, the quenching solutions were added with stirring to the cell solutions in the cuvette. Equilibrium distribution of the lipid-soluble quenchers was established within a few minutes. An equivalent amount of dimethyl sulfoxide was added to control cell solutions; this usually had a negligible effect on the intensities, however, since typical

TABLE I: Stern-Volmer Constants, K_{sv}, of Polycyclic Aromatic Molecules 10^{-7} M in Dimethyl Sulfoxide (λ_{exc} 340 nm).

quencher	BaP (λ_{em} 407)	DE (λ_{em} 380)
CH_2I_2	300 ± 50	250 ± 50
KI	91 ± 5	69 ± 5
n-$C_8H_{17}I$	7.6 ± 1.0	4.6 ± 7

TABLE II: Fluorescence Lifetimes of PAH Molecules in Cells.[a]

strain (or solvent)	BaP (ns)	DE (ns)
NMuLi	28 ± 5	76 ± 10
Balb 3T3 A31 HYF	23 ± 5	74 ± 13
MSV/MLV Balb 3T3 A31 HYF	24 ± 5	112 ± 20
p-dioxane (in air)	34	50
p-dioxane (in argon)	50	98

[a] Measured at 200 000 cells/mL. No effort was made to remove oxygen from cell samples.

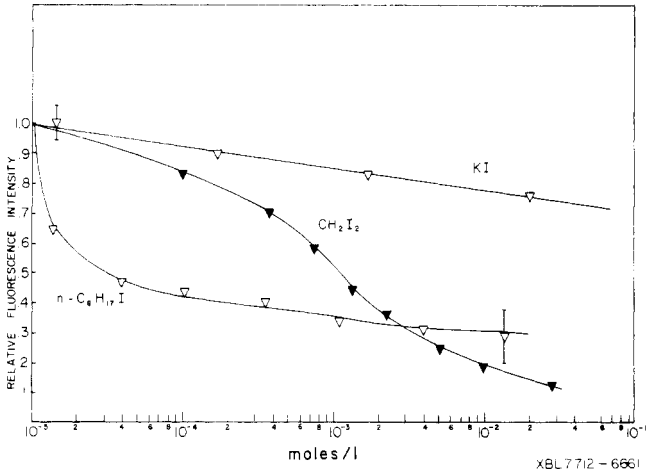

FIGURE 2: Quenching of BaP in MBalb. The cell density was 2×10^5 cells/mL, and excitation was at 340 nm.

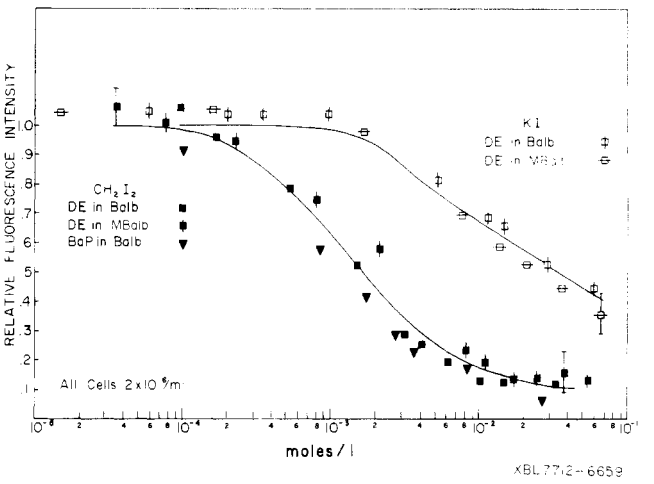

FIGURE 3: Quenching of DE and BaP present individually in cells.

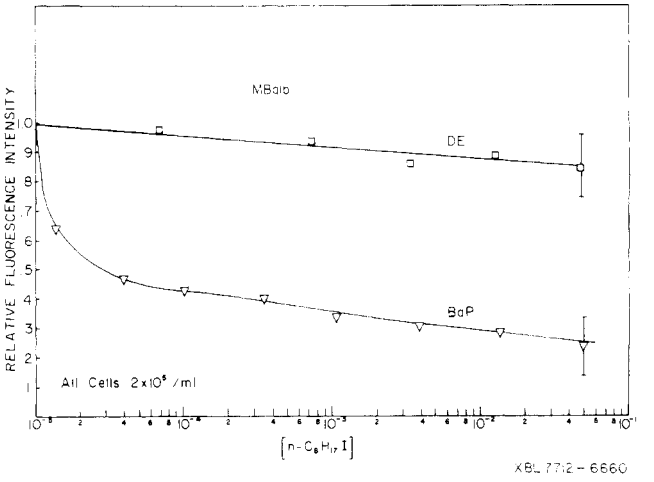

FIGURE 4: Comparison of quenching efficiencies with DE and BaP present individually in MBalb cells.

experiments involved the addition of 100 μL of quencher solution to approximately 3 mL of solution in a cuvette.

Emission spectra were taken on Perkin-Elmer Hitachi spectrofluorometers Model MPF-2A and MPF-3A. The latter instrument was used to record the corrected spectra. Excitation at 340 nm was used, path lengths were 10 mm and typical slit widths were 6 nm. Absorption spectra were taken on a Cary Model 118 instrument.

The flow cytometer is the one used previously (Hawkes and Bartholomew, 1977). Cells traversed the flow chamber at approximately 500 cells/s in a stream that intersected the beam of an argon ion laser (Spectra Physics Model 171) tuned to 351.1 and 363.8 nm.

Fluorescence lifetime measurements were done on an instrument described elsewhere (Hartig et al., 1976). All spectroscopic experiments were done at ambient temperature (about 22 °C).

Results and Discussion

Fluorescence emission spectra of BaP and of DE in transformed and in nontransformed cells were identical within experimental error. It is important to note that resonance energy transfer occurs between the protein fluorophores (which emit ~330 nm) and the PAH molecules if excitation below ca. 300 nm is used. Excitation at 340 nm (used for all data herein) avoids the possibility of energy transfer.

The Stern-Volmer constant, K_{sv} (eq 1), for each quencher with each PAH in isotropic solution (dimethyl sulfoxide) is shown in Table I. The KI data obeyed eq 1 up to concentrations of about 0.2 M. For the organohalogen quenchers, positive deviations from linearity occurred at concentrations $\gtrsim$2 mM, indicating possible complex formation. The lower concentration limiting slope was used to calculate the K_{sv} values in Table I.

The K_{sv} values (in dimethyl sulfoxide) are slightly larger at 340 nm for BaP than for DE for each quencher. Thus, in isotropic solution one expects BaP to be quenched slightly more effectively for a given quencher concentration. In cells, however, the absence of a quantitative theory prevents the extraction of a term analogous to K_{sv}. The development of a detailed model for the quenching in cells is hampered by several factors. In two dimensions (as in a lipid bilayer), diffusional relationships are, in fact, quite different from those in three dimensions (see the discussion in Fahey et al., 1977). Furthermore, the locations of the PAH molecules in the cells are obviously very important. The danger of extrapolating from isotropic Stern-Volmer cases to cells can be seen if one considers the fluorescence lifetimes in cells (Table II) where the fluorescence lifetimes of DE are more than twice as long as those of BaP. Assuming comparable k_q values and identical locations, one would predict that DE would be quenched more

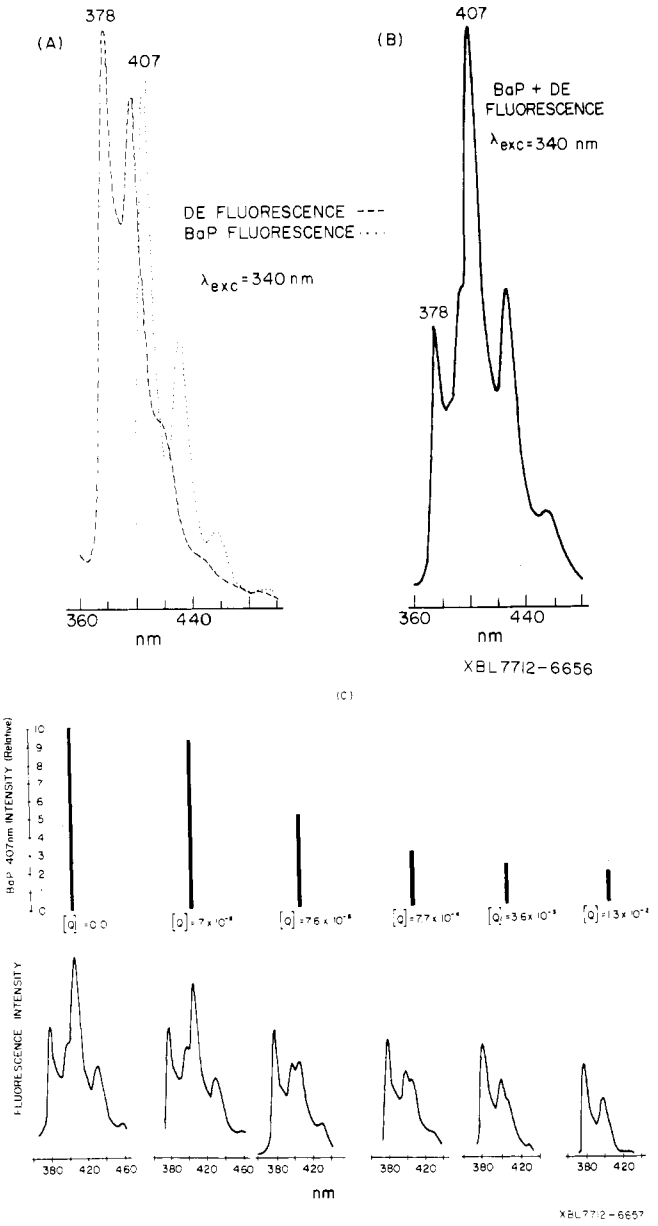

FIGURE 5: (A) Fluoresence emission spectra of DE and BaP present individually in cells. (B) Fluorescence emission spectrum of DE and BaP when both are present in cells. (C) Selective quenching of BaP when both BaP and DE are present in cells and n-$C_8H_{17}I$ is added at a molar concentration [Q]. The vertical bars indicate the approximate BaP intensity as determined in a separate experiment in cells containing BaP only.

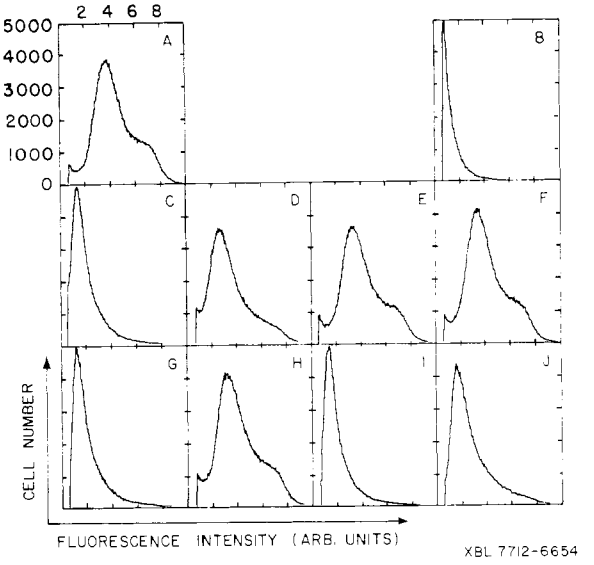

FIGURE 6: Effects of various quenchers on BaP in NMuLi cells as given by flow cytometry: (A) BaP alone; (B) no BaP; (C) n-$C_8H_{17}I$; (D) n-hexyl bromide; (E) bromobenzene; (F) ethyl iodide; (G) 1-bromophenanthrene; (H) 1,5-dibromopentane; (I) α-iodonaphthalene; (J) α-bromonaphthalene. The vertical and horizontal scales are, in general, arbitrary, but those for BaP alone (A) have been labeled.

than BaP for a given quencher concentration and, furthermore, that DE would be quenched more effectively in MBalb than in Balb. As will be demonstrated and discussed in more detail below, such behavior is not observed.

It is interesting and perhaps significant that the DE lifetime is longer in the transformed than in the nontransformed strain. If generally true, this would be a useful parameter in cell-transformation studies. However, the measurements were done in the presence of ambient oxygen, and this may have some effect on the values, as shown for p-dioxane (Table II).

Although three cell strains were used, no significant differences between strains were found for PAH quenching. Consequently, in what follows, although the particular strain is identified in each case, the results are meant to be representative.

Another general finding is that in cells, the data are not linear in a Stern-Volmer plot, an indication that several PAH locations may be involved. An analysis similar to that of Lehrer (1971) was not attempted, since the data point density was not suffioient to provide the extraction of meaningful results from a fitting procedure. The particular plotting method used to illustrate the effect in what follows was chosen merely because it is the easiest to visualize relative intensities as a function of quencher concentration. This corresponds to no theory. The quencher concentrations are given in moles per liter in the *cuvette* solution. Estimates of concentrations of lipid-soluble quenchers in the cells can be made if one assumes a total phospholipid fraction in the cells of 0.03 (Barnum et al., 1950), a cell diameter of 20 μm, and an average density per cell of 1.07 g/cm^3; hence, each cuvette contains 10^{-7} mol of phospholipids. Thus, in these experiments (200 000 cells/mL) the molar ratio of n-octyl iodide/phospholipids/BaP is approximately 1750:40:1 for a 1 mM n-octyl iodide solution in the cuvette. For lipid-soluble quenchers, the effective "molarity" in the lipid portions of the cells is obviously much higher than in the solution. Consequently, the number of cells per milliliter is an important parameter when comparing results, and it is listed in what follows.

Figure 2 shows the quenching data in cells for BaP. The most effective quenching arises from n-$C_8H_{17}I$, which is lipid soluble and can encounter the BaP. KI does not penetrate the lipid region to any great extent and thus quenches poorly. Methylene iodide is also lipid soluble (though more water soluble than n-$C_8H_{17}I$) and, in part because it has a relatively large Stern-Volmer constant for BaP, it is a relatively good quencher. As will be seen, however, it does not offer selectivity.

Typical quenching data for DE with KI and CH_2I_2 are in Figure 3. Some BaP data with CH_2I_2 are included to demonstrate that, although it is an effective quencher, CH_2I_2 is not selective. Compared to BaP, DE shows markedly different quenching with n-$C_8H_{17}I$, however. Figure 4 shows the relative quenching of the BaP in cells (407-nm emission) and of DE in cells (378-nm emission). The selectivity offered by n-$C_8H_{17}I$ is evident. An even more striking demonstration of the selectivity of n-$C_8H_{17}I$ quenching is in Figure 5, which shows the fluorescence spectra of cells containing both DE and BaP.

Excitation at 340 nm excites both molecules, and the emission spectrum reflecting the presence of both is shown (Figure 5B). As the quencher concentration is increased (Figure 5C), the BaP emission is successively quenched and a well-resolved DE spectrum is left.

The effects of collision quenchers on flow cytometric studies of BaP in NMuLi cells are shown in a representative fashion in Figure 6. Equivalent amounts of quenchers (1 mg/mL in dimethyl sulfoxide) were added to cell suspensions, which were then run through the flow cytometer. With no BaP present, the cells show only weak fluorescence (Figure 6B). BaP fluorescence gives a strong fluorescence intensity (Figure 6A) which is quenched to varying degrees by various quenchers (Figure 6C–J). These data are not meant to be quantitative but rather are meant to show the potential of the use of quenchers in such studies.

Conclusions

The differences in the Stern–Volmer constants (Table I) and the excited-state lifetimes (Table II) for BaP compared to DE are not sufficient to explain the selective quenching of *n*-$C_8H_{17}I$. The implication of Figures 2–5 is thus that the PAH molecules are in different locations in the cell insofar as the collision possibility with the *n*-$C_8H_{17}I$ is concerned. It is not possible to determine the precise locations with the present data, but some educated guesses may be made. It is almost certain that the *n*-$C_8H_{17}I$ and the BaP are in the lipid regions of the cells, with the BaP probably located near the ends of the lipid chains as found in phospholipid vesicles (Galla and Sackman, 1974; Cheng and Thomas, 1974; Sontar et al., 1974; Vanderkooi and Callis, 1974). It is probable that the iodine of the *n*-$C_8H_{17}I$ is located near the ends of the lipid chains as well. The DE location is more difficult to estimate. Because it is a very reactive species, it is quite probable that some of the DE is bound in the outer cell membrane. If this is so, then it must be located at some site which does not allow a "collision" with the *n*-$C_8H_{17}I$ or with KI but does allow one with CH_2I_2. Such a site might be inside the proteins or perhaps on some moiety at a position sufficiently near the exterior or interior surface of the membrane that the *n*-$C_8H_{17}I$ does not easily contact it. Also, some of the DE might be bound in a nonlipid region of the cell which would also serve to prevent *n*-$C_8H_{17}I$ contact. Because CH_2I_2 is less hydrophobic than *n*-$C_8H_{17}I$, it is more likely to be found near the head groups of the lipids where it could contact DE.

Selective quenching is a potentially valuable technique in biomedical applications of fluorescence and flow cytometry. Use of a selective quencher would permit cell population studies on the basis of metabolite production rather than on the distribution of the parent compound. It should be possible using this technique to rapidly measure the efficiency of conversion of proximate carcinogens into mutagenic derivatives.

Further applications could include screening for exposure on the basis of metabolic products of workers in those occupations in which a large BaP exposure is known.

It should be noted, however, that the studies in this paper were done by adding the PAH molecules to the cells. More work would be required to elucidate in detail applications of quenching to metabolically produced DE, for example, although the BaP quenching should be the same. Utilizing the solubility properties of quenchers should help in identifying the location of DE under such conditions.

Acknowledgments

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Phosphorus-31 Nuclear Magnetic Resonance Studies of Active Proton Translocation in Chromaffin Granules†

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ABSTRACT: ATP hydrolysis and proton translocation in chromaffin granules were followed using ^{31}P nuclear magnetic resonance. The intragranular pH affects the resonance frequency of the γ-phosphate of granular ATP. By measuring frequency vs. pH in solutions which simulate the intragranular matrix, this may be calibrated to give quantitative pH measurements. The pH in the resting granule is 5.65 ± 0.15. This drops by 0.4 to 0.5 pH unit when ATP is added externally and protons are actively pumped into the granules. Because of differences in the composition and pH of the internal and external solutions, the resonances of internal and external nucleotides and P_i can be distinguished. Consequently, ATP hydrolysis and changes in internal pH may be observed simultaneously and continuously in a single sample of chromaffin granules. From the measured buffering capacity of a reconstituted intragranular solution, pH changes were converted into an absolute number of protons translocated. The net proton flux (protons translocated/ATP hydrolyzed) was about 1.0 immediately after external ATP addition but fell toward zero as the pH gradient increased to a new steady state. These ^{31}P NMR results agree with intragranular pH measurements determined from methylamine distribution and with H$^+$/ATP stoichiometries calculated from pH changes observed in the external medium.

Phosphorus nuclear magnetic resonance has proved to be a powerful method for studying metabolism in working systems, because pH and concentrations of such important compounds as ATP, ADP, AMP, and P_i can be monitored continuously in a single sample. ^{31}P NMR[1] has been exploited to follow pH and metabolite levels in perfused and ischaemic tissues such as muscle (Dawson et al., 1977), heart (Garlick et al., 1977), and kidney (Sehr et al., 1977). At the cellular level, intracellular pH has been measured in respiring and anaerobic cells of *Escherichia coli* (Navon et al., 1977). At the level of the organelle, ^{31}P NMR is a potentially valuable technique for bioenergeticists investigating the role of pH gradients in energy coupling. ^{31}P NMR can simultaneously monitor concentrations of phosphate compounds and the internal pH in organelles which contain or can be loaded with a high concentration of an appropriate phosphate compound.

Chromaffin granules, the catecholamine storage vesicles of the adrenal medulla (for a review, see Winkler, 1976), contain high concentrations of ATP (0.1 M), adrenaline (0.55 M), and chromogranin protein (120 mg/mL). The ATP, while its function in vivo is unknown, provides a convenient indicator of internal pH. The pK_a of the γ-phosphate is close to the intragranular pH, and the resonance frequency shifts markedly with the degree of protonation. Moreover, the granules possess an electrogenic, inwardly directed, proton-translocating ATPase, which hydrolyzes extragranular but not intragranular ATP. We have shown that this proton pump can change the intragranular pH and shift the resonance frequency of the γ-phosphate of intragranular ATP (Casey et al., 1977; Njus and Radda, 1978). We report here the use of ^{31}P NMR to follow simultaneously proton translocation and ATP hydrolysis in chromaffin granules.

Materials and Methods

Chromaffin granules were isolated from bovine adrenal

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[1] Abbreviations used are: ATPase, adenosine triphosphatase; Hepes, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; Mes, 2-(*N*-morpholino)ethanesulfonic acid; NMR, nuclear magnetic resonance; ppm, parts per million.

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