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Wade, Chas. G.

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

Chas. G. Wade, *[‡] David E. Baker[♂] and James C. Bartholomew[♂]

‡ Department of Chemistry, University of Texas at Austin, Austin, Texas 78712

§ Laboratory of Chemical Biodynamics, University of California at Berkeley,
California 94720

- 1 Abbreviations used: BaP, benzo [a] pyrene; DE, (+)7 β , 8 α -dihydroxy-9 α , 10 α -epoxy-7,8,9,10-tetrahydrobenzo [a] pyrene; DMSO, dimethyl sulfoxide; ~~saline~~ GM, 1.5 mM Na₂HPO₄/1.1 mM KH₂PO₄ at pH 7.4 with 1.1 mM glucose, 0.14 M NaCl, and 5 mM KCl; NOI, n-octyl iodide; MBalb, MSV/MLV Balb 3T3 A31 HYF.

ABSTRACT: The overlap of the fluorescence spectra of benzo [a] pyrene and its metabolites, including an ultimate carcinogen diol epoxide (+)7 β , 8 α -dihydroxy-9 α , 10 α -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 of 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 probable reasons for the selectivity offered by n-octyl iodide are developed, including information on the location of the benzo [a] pyrene and the diol epoxide when added to the cells.

The potential for using the relative solubilities of collisional quenchers in separating the fluorescence emissions of polycyclic aromatic hydrocarbons is discussed. The technique should have broad applications in studies of fluorescence of biological systems, including flow cytometry and more conventional fluorescence spectroscopy. In particular, the technique should allow the possible application of flow cytometry to monitor more precisely the role of the metabolism of carcinogens and their precursors in cell populations.

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., 1977; Yang, et al., 1976) that an ultimate carcinogenic metabolite is the BaP diol epoxide DE (Fig. 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.

Fig. 1

Fluorescence is a major tool in studies of PAH carcinogenesis, and spectrofluorimetric studies have been done in both in vitro (Geacintov, et al., 1976) and in vivo (Kodama and Nagata, 1975) systems.

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. Although fluorescence quenching has been an active field of research in chemistry for decades (Stern and Volmer, 1919; Birks, 1970), only recently have applications to biological systems appeared. Most of these have concentrated on intrinsic protein fluorescence; only a few have utilized the possibilities of information from selective quenching. 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 fluorimetric studies of various kinds in biological systems, including flow cytometry and cell sorting (these topics are reviewed by Horan and Wheelless, 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 leads to an increased population of the upper electronic (principally singlet) states (Fig. 2). It is helpful to consider that the excited states manifold consists of singlet and triplet levels which are independent. (This is satisfactory within the resolution of the optical techniques used in this paper, but as demonstrated by Zewail, et al. (1977) this is in general an incorrect view.) Normally, excitation to any excited state above the first (S_1) is quickly followed by relaxation (in 10^{-11} to 10^{-14} sec) to S_1 via internal conversion,

Fig 2

a radiationless conversion of electronic vibrational energy. This leaves a significant excited state population only in the lowest excited singlet state, S_1 . From this state, the energy can be distributed in various well known modes (Birks, 1970) to allow the molecule to return to the ground state:

(a) emission (fluorescence) from the first excited singlet state to the ground state can occur, (b) a radiationless transition from the excited singlet to the ground state can occur, (c) intersystem crossing to the triplet can occur with the possibility of emission (phosphorescence) or of radiationless transitions from the triplet to the ground state. Quenching of fluorescence refers to a decrease in the intensity of the singlet-singlet emission. Mechanistically this can mean that the absorbed energy is transferred to any of the other modes at the expense of singlet emission. Several well known quenching methods exist. Those applicable in the present study are collision quenching (Badley, 1975; Lehrer, 1971; Bieri and Wallach, 1975; Koblin, et al., 1973; Shinitsky and Rivnay, 1977; Borochoy and Shinitsky, 1976) and resonance energy transfer (Wu and Stryer, 1972; Stryer, 1968; Vanderkooi, et al., 1977).

These and other techniques have been applied to biological systems in recent years, but the majority of these studies have concentrated on the intrinsic fluorescence of proteins in an effort to probe the fluorophore locations. Applications to micellar systems (Pownall and Smith, 1974; Papageorgiou and Argoudelis, 1973), to membranes (Badley, 1975; Lehrer, 1971; Bieri and Wallach, 1975; Koblin, et al., 1973; Shinitsky and Rivnay, 1977; Borochoy and Shinitsky, 1976; Burnstein, et al., 1973; Brown, et al., 1977), and to DNA bound systems (Geacintov, et al., 1976) have been reported.

For what follows, it will be helpful to briefly review the properties of the quenchers which will be applied to this study. A more extensive treatment can be found in the book by Birks (1970).

Collisional quenching requires a close approach (typically 4-6 Å) or "collision" of the quencher with the fluorophore. Both dynamic (often diffusion controlled) and static (formation of a nonfluorescent complex) models have been developed. Some collisional quenchers act by promoting intersystem crossing to the triplet state through spin orbit coupling interactions; examples include the heavier halogens (Badley, 1975; Lehrer, 1971; Burnstein, et al., 1973; Brown, et al., 1977; Pownall and Smith, 1974; Papageorgiou and Argoudelis, 1973), heavy metals such as Cs^+ (Brown, et al., 1977; Altekar, 1974), and nitroxide groups (Bieri and Wallach, 1975; Koblin, et al., 1973). Since spin orbit coupling increases with atomic number, this mechanism for halogens and metals is often referred to as the "heavy atom" effect. Another general mechanism in the collisional quenching category is quenching by a charge transfer interaction, the quaternary pyridinium and picolinum ion salts (Shinitsky and Rivnay, 1977; Borochoy and Shinitsky, 1976) being examples. Regardless of the particular mechanism, the degree of quenching in isotropic solvents can almost invariably 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, equation (1) is not usually obeyed; Lehrer (1971) has presented more generalized versions applicable to heterogeneous systems where several sites are available to the fluorophores.

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 collision 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} wall 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 photo-affinity 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; Shinitzky and Rivnay, 1977; Borochoy and Shinitzky, 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 DMSO (1 mg/ml) were added to achieve a concentration of two micrograms per ml of growth medium and 0.5% DMSO. After incubation for approximately one hour, the medium was removed, the cells were washed twice with 5 ml of 1X 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 minutes, the supernatant was decanted, and the cells were resuspended in fresh buffer. Coulter counter measurements (2 per sample) were used to adjust the cell concentrations (typically 200,000 cells/ml) for the fluorometric and fluorescent lifetimes measurements.

Chemicals and Apparatus. BaP was obtained from Aldrich Chemical Co. (San Leandro, California) 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 organo-halogen 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 organo-halogen compounds were fractionally distilled under vacuum and refrigerated in dark containers. Just before use, the quenchers were dissolved in DMSO at the appropriate concentrations (up to 100 mg/ml) and stored at -70°C . During experiments, the DMSO 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 DMSO 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 DMSO was added to control cell solutions; this usually had a negligible effect on the intensities, however, since typical experiments involved the addition of $100\mu\text{l}$ of quencher solution to approximately 3 ml of solution in a cuvette.

Emission spectra were taken on Perkin-Elmer Hitachi spectrofluorimeters Model MPF-2A and MPF-3A. The latter instrument was used to record the corrected excitation and corrected emission spectra. 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/sec 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 lifetimes 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

The corrected excitation and corrected fluorescence emission spectra of BaP, DE, and the protein fluorophores, all taken in cell suspensions, are given in Fig. 3. Spectra of the transformed and nontransformed cells were identical within experimental error. It is important to note that resonance energy transfer occurs between the proteins and the PAH molecules if excitation below ca. 300 nm is used. This causes the BaP emission in cells to be stronger for excitation at 290 nm than at 340 nm. Just the reverse behavior occurs in isotropic solution. Excitation at 340 nm avoids the possibility of energy transfer.

The Stern-Volmer constant, K_{SV} (eqn. 1), for each quencher with each PAH in isotropic solution (DMSO) is shown in Table I. The KI data obeyed eqn. 1 up to concentrations of $[Q] = 0.2$ M. For the organo-halogen quenchers, positive deviations from linearity occurred at concentrations ≥ 2 mM, indicating possible complex formation. The lower concentration limiting slope was used to calculate the K_{SV} values in Table I. A somewhat unusual finding was that the quenching for BaP was dependent upon wavelength, becoming

Fig 3

more effective at 290 nm than at 340 nm. This may be due to a variety of factors: I_3^- absorption, quencher decomposition, or complex formation. Because all cell studies on BaP were done with excitation at 340 nm, this effect was not investigated further.

The K_{sv} values (in DMSO) are slightly larger at 320 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 $[Q]$. In cells, 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 discussion in Fahey, et al., 1976). 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 than BaP for a given $[Q]$ 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.

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 sufficient 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 $[Q]$. 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 microns, and an average density per cell of 1.07 gm/cm^3 ; hence each cuvette contains 10^{-7} moles of phospholipids. Thus, in these experiments (200,000 cells/ml) the molar ratio of NOI:phospholipids:BaP is approximately 1750:40:1 for a 1 mM NOI solution in the cuvette. For lipid soluble quenchers, the effective "molarity" in the lipid portions of the cells is obviously much higher. Consequently the number of cells per ml is an important parameter when comparing results, and it is listed in what follows.

Fig. 4 shows the quenching data in cells for BaP and for protein fluorophores. Quenching of the protein fluorophores by KI and by NOI (Fig. 4b)

is not very effective presumably because the quenchers cannot reach the fluorophore locations. This is consistent with the usual findings (Badley, 1975; Lehrer, 1971) for quenching in proteins where I^- concentrations of typically a few tenths M are required. CH_2I_2 however provides good quenching. It has seldom been used in earlier studies of protein quenching.

(Fig 4A),

For BaP_A excitation at 340 nm does not result in energy transfer from the protein fluorophores, so quenching reflects directly the "collisional" contact with the BaP. The most effective quenching arises from NOI 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 NOI) 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 Fig. 5. 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 NOI, however. Fig. 6 shows the relative quenching of the BaP in cells (407 nm emission) and of DE in cells (378 nm emission) both excited at 340 nm. The selectivity offered by NOI is evident. An even more striking demonstration of the selectivity of NOI quenching is in Fig. 7 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 (Fig 7B),

(Fig. 7C)

As the NOI concentration is increased, the BaP emission is successively quenched and a well resolved DE spectrum is left.

Fig 4

Fig 5

Fig 6

Fig 7

The effects of collision quenchers on flow cytometric studies of BaP in NMuLi cells are shown in a representative fashion in Fig. 8. Equivalent amounts of quenchers (1 mg/ml in DMSO) were added to cell suspensions, which were then run through the flow cytometer. With no BaP present, the cells show only weak fluorescence (Fig. 8b). BaP fluorescence gives a strong fluorescence intensity (Fig. 8a) which is quenched to varying degrees by various quenchers (Figs. 8c-8j). These data are not meant to be quantitative but rather to show the potential of the use of quenchers in such studies.

Fig 8

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 NOI. The implication of Figs. 5-7 is thus that the PAH/ molecules are in different locations in the cell insofar as the collision possibility with the NOI 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 NOI and the BaP are in the lipid regions of the cell 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 NOI 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 NOI 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 wall that the NOI 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 NOI contact. Because CH_2I_2 is less hydrophobic than NOI, 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.

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Table I. Stern-Volmer constants K_{sv} of polycyclic aromatic molecules
 10^{-7} M in DMSO.

Quencher	BaP ($\lambda_{em} = 407$)		DE ($\lambda_{em} = 380$)
	$\lambda_{ex} = 290$	$\lambda_{ex} = 340$	$\lambda_{ex} = 340$
CH_2I_2	1600 ± 200	300 ± 50	250 ± 50
KI	77 ± 5	91 ± 5	69 ± 5
n-C ₈ H ₁₇ I	90 ± 20	7.6 ± 1.0	4.6 ± 7

Table II. Fluorescence lifetimes of PAH molecules in cells. Measured at 200,000 cells/ml.

Strain	BaP (nsec)	DE (nsec)
NMuLi	28 ± 5	76 ± 10
Balb 3T3 A31 HYF	23 ± 5	74 ± 13
MSV/MLV Balb 3T3 A31 HYF	24 ± 5	112 ± 20

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Figure Captions

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

Fig. 2. Simplified diagram of emission and radiationless transitions in aromatic molecules.

Fig. 3. Corrected absorption (---) and corrected emission (—) in cells of BaP, DE, and fluorophores in proteins. Instrument gain is different in the three cases.

Fig. 4. Quenching of BaP (A) and protein fluorophores (B) in MBalb. The cell density was 2×10^5 cells/ml and excitation was at 290 nm.

Fig. 5. Quenching of DE and BaP present individually in cells. Excitation was at 340 nm.

Fig. 6. Comparison of quenching efficiencies with DE and BaP present individually in cells. Excitation was at 340 nm.

Fig. 7 (a). Fluorescence emission spectra of DE and of BaP present individually in cells.

Fig. 7 (b). Fluorescence emission spectrum from DE and BaP when both are present in cells.

Fig. 7 (c). Selective quenching of BaP when both BaP and DE are present in cells. The vertical bars indicate the approximate BaP intensity as determined in a separate experiment in cells containing BaP only. All spectra obtained with excitation at 340 nm.

Fig. 8. Effects of various quenchers on BaP in NMuLi cells as given by flow cytometry. A. BaP alone; B. No BaP; C. NOI; D. n-Hexyl-Br; E. Bromobenzene; F. Ethyl-I; G. 1-Br-Phenanthrene; H. 1,5-Dibromo Pentane; I. α -I-Naphthalene; and J. α -Br-Naphthalene.

Fig. 1

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Fig. 2

Fig. 3

Fig. 4

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Fig. 5

Fig. 6

Fig. 7 a

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Fig. 7b

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Fig. 7c

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Fig. 8

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