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Fluorescence line-narrowing spectrometry: a versatile tool for the study of chemically initiated carcinogenesis*

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Abstract: An important initiating step in the induction of tumors is believed to be the covalent binding of an active carcinogenic species to a cellular macromolecule, e.g. DNA. Therefore, a spectroscopic technique which allows for positive identification of the intact (macromolecular) DNA adduct and/or isolated damaged nucleosides/nucleotides is highly desirable. It is shown that fluorescence line-narrowing spectroscopy (FLNS) is a rapid, versatile, highly sensitive and selective analytical technique, which can be used directly to characterize DNA adducts and isolated nucleosides. FLNS possesses sufficient resolution to distinguish between the major DNA adducts derived from different enantiomers of benzo[a]pyrene diol-epoxide (BPDE). With the present limit of detection (~1 adducted base per 10^8 normal base pairs for 100 μg of DNA), the technique is applicable to *in vivo* samples. Analysis of liver DNA from fish exposed to benzo[a]pyrene (BP) (100 mg BP/kg fish) showed that a major DNA adduct is derived from syn-BPDE.

Keywords: *Fluorescence line-narrowing spectrometry (FLNS); DNA-adduct; benzo[a]pyrene (BP); benzo[a]pyrene diol epoxide (BPDE); carcinogenesis.*

Introduction

For many carcinogenic compounds such as the polycyclic aromatic hydrocarbons (PAH), metabolic activation to electrophilic intermediates is a necessary condition for the production of damage to nucleic acids, and this arises from covalent binding with the metabolite. The current view as regards PAH is that metabolic activation occurs by two pathways: mono-oxygenation to yield bay-region diol-epoxides [1–3]; and one-electron oxidation to produce radical cations [4]. The potent carcinogen benzo[a]pyrene (BP), for example, can be metabolized by both mechanisms in expressing its biological potential.

A firm understanding of the mechanistic aspects of chemical carcinogenesis requires a versatile and rapid bioanalytical technique which is both highly sensitive and selective. Fluorescence line-narrowing spectroscopy (FLNS), a method by which highly characteristic spectra can be obtained, has been shown to possess the required attributes for the analysis of damage to cellular macromolecules (e.g. DNA, globin) resulting from covalent binding between the macromolecule and carcinogenic metabolites of PAH [5–8].

The numerous other applications of FLNS to biological systems include the investigation of haem proteins [9], antenna protein complexes [10], chlorophyll-a [11–13], chlorophyll-b [11, 12, 14], phaeophytin-a [13], protochlorophyll and protophaeophytin [13], bacteriochlorophyll and bacteriophaeophytin [14], etiolated leaves [15], iron-free cytochrome-c [16], and the light-harvesting complex of photosystem I (S. Johnson and G. Small, unpublished data). The present work, however, confines itself to the application of FLNS for the investigation of chemical carcinogenesis.

Formation of an adduct between the genetic material DNA and a chemical agent is thought to be a crucial step in the initiation of carcinogenesis and tumorigenesis [17, 18]. As typical DNA damage levels are low ($\lesssim 1{:}10^8$ base pairs), there has recently been considerable interest in the development of suitably sensitive bioanalytical techniques for damage analysis. As important as high sensitivity is the

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requirement for the requisite high selectivity, which allows for the distinction between structurally very similar DNA adducts.

Advantages also exist for a technique which is applicable to the *intact* (macromolecular) DNA adduct (rather than only to the isolated damaged nucleotide), and which is rapid and non-destructive.

The ideal method for the study of DNA damage should be applicable to small samples of DNA with the low adduction levels found in environmental and occupational samples [3, 19]. Several fluorescence-based techniques have been proposed and utilized [3, 17, 20, 21]. In particular, FLNS has been shown to be an effective technique for the identification and determination of DNA adducts and for the study of various metabolic pathways leading to tumor formation (P. Lu, R. Jankowiak, G.J. Small, M. Nashimoto and U. Varanasi, in preparation; and R. Jankowiak, P. Lu, G.J. Small, S.K. Kim and N.E. Geacintov, *Chem. Res. Toxicol.* (in press) [7, 8, 22]. In the present work, therefore, it is intended to show that FLNS is not only a rapid, broadly applicable, sensitive, and selective technique for the study of chemical carcinogenesis, but also that the present limit of detection permits the technique to be applied to *in vivo* samples.

Fluorescence line-narrowing and related processes

Fluorescence line narrowing (FLN) is a low-temperature technique which can eliminate or significantly reduce the contribution of site inhomogeneous line broadening, Γ_{inh}, to spectral profiles [23–26]. A site inhomogeneously broadened absorption profile is the convolution of a very large number of individual site absorptions possessing a homogeneous linewidth Γ. At liquid helium temperatures $\Gamma < 0.1\ cm^{-1}$, while for amorphous molecular hosts such as glasses or polymers $\Gamma_{inh} \simeq 300$–$500\ cm^{-1}$. Classical broad-band excitation of all the energetically inequivalent sites which contribute to the inhomogeneously broadened profile results in an equally broad fluorescence spectrum. Such spectral resolution does not allow for satisfactory distinction between substitutional isomers of a given PAH, or of closely related DNA adducts for example, since their S_1 states typically differ only by 10–30 cm^{-1} from each other. However, selection of a narrow isochromat of the absorption profile using a laser of width $\Delta\nu \ll \Gamma_{inh}$ can result in dramatic line narrowing provided that certain conditions are met. First, the impurity concentration must be sufficiently low to preclude site randomization due to intermolecular energy transfer on a time scale comparable to the excited state lifetime. In addition, the fluorescence observed must originate from the electronic state being excited — a consequence of the fact that site energy distribution functions for different electronic states are generally not correlated. The narrow isochromat of the state selected by the laser thus maps onto a much broader "polychromat" in another excited electronic state.

For practical applications of FLN to molecular systems, the origin or low vibronic transitions of the lowest excited singlet state (S_1) are generally excited. For origin excitation, the resonance fluorescence (origin) transition occurs at the laser frequency and is, therefore, not a reliable analytical line. Utilization of vibronic excitation is significantly better with respect to characterization and selectivity. Figure 1 depicts an absorption spectrum in which there are two overlapping vibronic transitions to fundamental excited state vibrations 1_α and 1_β. To the left is an energy level diagram in which the slanted

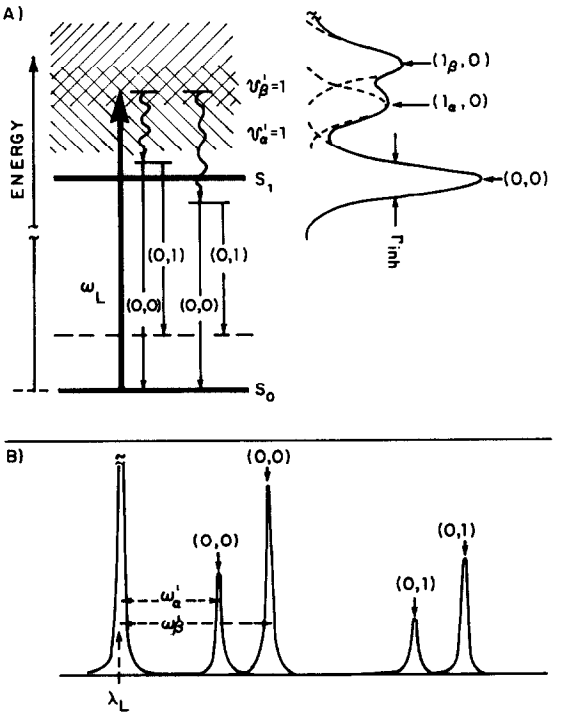


Figure 1
Schematic representation of laser site selection in FLNS (see explanation in text).

parallel lines indicate the site inhomogeneous broadening for the $(1_\alpha,0)$ and $(1_\beta,0)$ transitions. The laser excitation frequency (ω_L) is chosen to excite different isochromats belonging to the $\nu'_\alpha = 1$ and $\nu'_\beta = 1$ levels. For example, ω_L selects an isochromat associated with $\nu'_\beta = 1$ that lies near the bottom of its distribution, while that associated with $\nu'_\alpha = 1$ lies near the top of its distribution. Because vibrational relaxation occurs on the picosecond timescale, it precedes the fluorescence which originates from the zero-point vibrational level. Vibrational relaxation is indicated by the downward wiggly arrows which illustrate that fluorescence originates from two different isochromats that are linked to the two vibrations initially excited. The resulting fluorescence spectrum has the appearance of Fig. 1(B) in which the laser line (ω_L) lies to high energy of all fluorescence bands. Fluorescence from the two isochromats results in a doubling of the origin or (0,0) transitions as well as the (0,1) vibronic transitions. An important point is that the displacements between ω_L and the (0,0) bands provide the excited state vibrational frequencies (ω'_α and ω'_β) while the displacements between the (0,0) and (0,1) bands yield ground state vibrational frequencies.

With the notion of isochromat selection established by FLN photochemical hole burning (PHB) followed logically. It is apparent that if the species giving rise to the absorption is photoreactive, isochromat selective photobleaching can be engineered [27].

Non-photochemical hole burning (NPHB) does not require photoreactivity of the absorbing species. What is required is a host with a "faulty memory" for its pre-excitation configuration around the absorber. That is, upon completion of the ground state to excited state to ground state cycle, the host configuration must have changed and this should be more or less permanent if persistent holes are to be produced [27, 28]. Because hole burning processes result in a loss of absorbers at the excitation energy it is important, for high sensitivity measurements, not only to be aware of this possibility but also to be able to minimize such processes where they do occur. For example, the authors have shown recently [8] that one can minimize the deleterious effects of NPHB by: synchronous scanning of excitation and detection wavelength (thus continuously changing the isochromat excited); by use of a slightly higher temperature (up to ~20 K); and by restoring the original inhomogeneous distribution through broad-band (white-light) excitation [8].

Experimental

The equipment needed to perform FLN can be divided into four major components: a spectrally narrow excitation source, an optically accessible low-temperature sample chamber, a dispersion device and a detection system. Such instrumentation for FLN experiments has been described in detail previously [8] but, for completeness, it will be reviewed here. A block diagram of the FLNS instrumentation is shown in Fig. 2. The excitation system employed consists of two basic parts, an excimer laser and a dye laser. The excimer laser is a Lambda Physik model EMG 102 MSC. A Zero Drift Control (ZDC) manufactured by Lambda Physik (model EMG 97) is used to provide low temporal jitter between the laser and sync-out pulse. The FL-2002 dye laser is typically used with a linewidth of 2 cm^{-1}. The dye laser is scanned with a Lambda Physik FL 512A scan controller.

Fluorescence from DNA adducts in glasses was collected at an angle of 90° to the excitation beam and focused into a 1-m McPherson 2061 monochromator (F = 7.0) having a reciprocal linear dispersion of 0.416 nm mm^{-1}.

In the case of thin-layer chromatography (TLC) plates the laser excitation beam was directed at an angle of ~30° to the normal of the TLC plate in order to reduce specular reflection of the beam onto the entrance slit of the monochromator. All experimental data reported in this paper were obtained at 4.2 K in a 3 l double-nested glass total-immersion Dewar (manufactured by Pope Scientific).

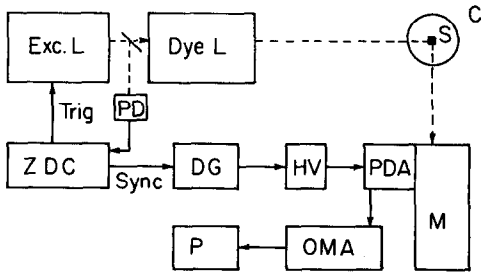


Figure 2
Block diagram of present FLNS instrumentation showing excimer laser (Exc. L), dye laser (Dye L), cryostat (C), sample (S), photodiode (PD), photodiode array (PDA), monochromator (M), optical multichannel analyzer (OMA), and printer (P). See text for discussion.

The output beam of the dye laser was formed to the shape of the sample (cross section 2 × 10 mm for glass matrices, 5 × 5 mm for TLC plates).

Fluorescence was detected using a Tracor-Northern TN-6134 intensified blue-enhanced gateable photodiode array (PDA). Gated detection of the fluorescence was accomplished using a Lambda Physik EMG-97 zero drift control (ZDC) to synchronize the sync-out pulse to within a few nanoseconds of the excimer light pulse, a Berkeley Nucleonics Corporation model 8010 pulse generator was used as a delay generator (DG) to set the delay (0–1 s) between the laser and the detector's temporal observation window, and an Avtech model AVL-TN-1 high voltage pulse generator (HV) was employed to set the width of the observation window (5–120 ns). All data handling was performed using a Tracor-Northern TN-6500 optical multichannel analyzer (OMA).

DNA adducts were dissolved in water to give a DNA concentration of ~1 mg ml^{-1}. The low modification level DNA adducts were observed under these conditions. The higher modification level samples (>1:10^3) were diluted with the glycerol–water (gly–H_2O) glass formulation to make these samples <10^{-5} M in adduct concentration. Typical sample volumes were 30 μl.

For studies utilizing TLC plates (Whatman KC 29 reversed-phase, 200 μm) as the sample matrix, the samples of the C8dG-BP were diluted in water to a concentration <10^{-4} M. Five μl of the sample solution was spotted onto a TLC plate using a 50-μl glass syringe. Prior to spotting, the TLC plates were washed by developing them overnight in methanol (Altech, Deerfield, IL) followed by drying. Following the application of the sample, the plates were allowed to dry. The plates were attached to a sample holder and cooled to low temperature in an optical Dewar.

The preparation of the covalent adducts derived from the chemical reaction of (+)-anti-BPDE, (−)-anti-BPDE and syn-BPDE with DNA is described elsewhere [29–31, 33]. Since BPDE–DNA adducts are known to be unstable and to dissociate to the tetraols (BPT), particularly in the presence of light [32, 33], special precautions were taken to minimize the contributions from BPT molecules to the overall fluorescence of solution of BPDE–nucleic acid adducts. The synthesis of C8dG-BP by electrochemical oxidation has been described [34].

English sole were collected by otter trawl from a non-industrialized area of Puget Sound, WA, and were held in flowing seawater (28%) for up to 2 weeks for acclimatization. Fish were fed with a 1:1 mixture of ground clams and krill, containing gelatin as a binder. Neither PAHs nor PCBs were present at detectable concentrations in the food. English sole (47 ± 11 g) were injected parenterally in a sinus near the fin ray with 100 mg BP/kg body wt; the BP was dissolved in Emulphor 620–acetone (1:1, v/v) and administered at 1 ml/kg body wt. Control fish were given an injection of solvent vehicle only. Liver was sampled from English sole at 4-day post-exposure. The liver samples were immediately frozen in liquid nitrogen and then stored at −80°C.

Hepatic DNA was isolated using the method of Bender *et al.* [35] with some modifications described in detail elsewhere [36].

Results and Discussion

The structures of the isomeric anti- and syn-diol epoxide derivatives of benzo[a]pyrene are shown in Fig. 3. In the syn-isomer (syn-BPDE) the benzylic hydroxyl group and the epoxide oxygen atom are on the same face, whereas in the anti-diastereomer (anti-BPDE) these groups are on opposite faces of the molecule. Since each diestereomer may exist as a pair of enantiomers, four stereoisomers of BPDE are possible. Methods for the stereospecific syntheses of anti- and syn-BPDE were developed by the groups of Harvey [29–31] and Jerina [37].

All four stereoisomers of BPDE have been tested for carcinogenicity [33]. The (+) and (−) enantiomers of BPDE, in particular, provide a striking example of structure–biological activity relationships. In this paper it will be demonstrated that the spectra of the DNA adducts derived from the binding of the (+) and (−)-anti-BPDE and syn-BPDE to native DNA are sufficiently different to permit distinction between the adducts.

These data are used to identify the major adduct observed in fish liver DNA from English sole exposed to 100 mg BP/kg of fish. However, because of space limitations, it is not

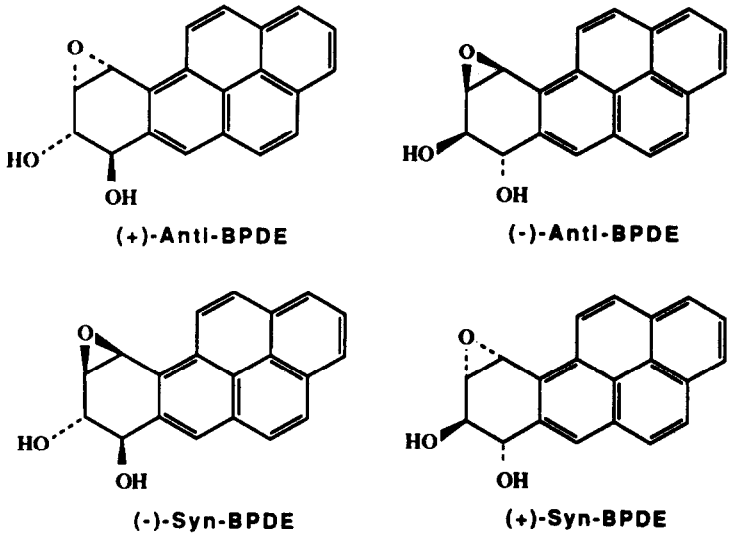


Figure 3
Structures and absolute stereochemistry of the anti- and syn-diastereomers of the benzo[a]pyrene trans-7,8-dihydrodiol 9,10-epoxide (BPDE).

intended to discuss the implications of these findings on the mechanistic aspects of chemical carcinogenesis from PAH. Rather, the focus will be on illustrating the sensitivity and selectivity of FLNS using a wide variety of samples.

FLN spectra of the (+)-anti-BPDE–DNA, (−)-anti-BPDE–DNA and syn-BPDE–DNA adducts are shown in Fig. 4 (B, C and D, respectively). For all these spectra, excitation into a vibronic band was employed in order to detect the intense fluorescence origin. These spectra were obtained with the standard gly–H_2O glass at T = 4.2 K, λ_{exc} = 371.6 nm. It is noteworthy that FLN can readily distinguish between the adducts derived from BPDE.

It is well known that the composition of the (−) adducts is chemically more heterogeneous than that of the (+) adducts [38, 39]; the (+) enantiomer binds mainly to N2 of deoxyguanine (N2-dG) (~94% of adducts), while the (−) enantiomer gives rise to 58% of N2-dG, 21% of O6-dG, and 18% of N6-deoxyadenine adducts [38]. Therefore, the FLN spectra in Fig. 4(B, C and D) must correspond to the major DNA adducts derived from (+)-anti-BPDE, (−)-anti-BPDE and syn-BPDE, respectively. Moreover, by combining FLNS and fluorescence excitation spectroscopy with the fluorescence spectra obtained under non-line-narrowing conditions (with and without quencher, e.g. acrylamide), it is also possible to distinguish between different adducts from the anti- and syn-diasteromers of BPDE (cf. R. Jankowiak *et al.*, paper in preparation). Thus

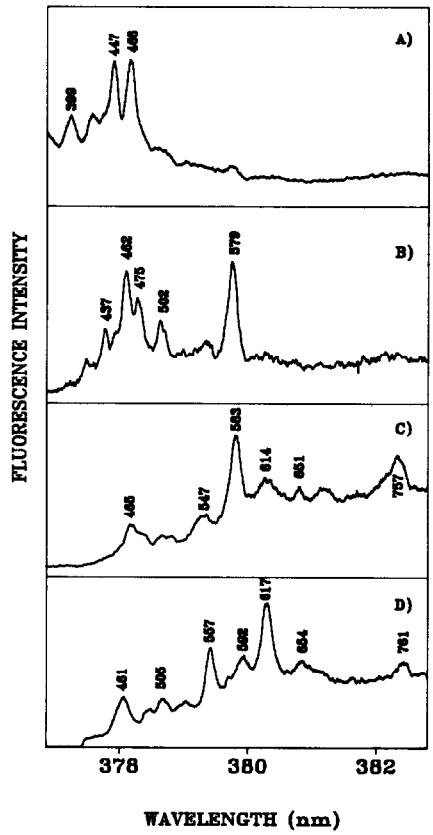
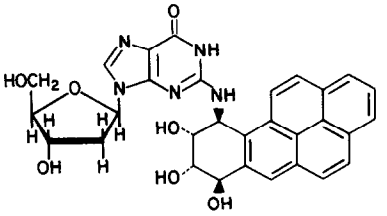


Figure 4
Comparison of the FLN spectra of three different DNA adducts (B, C, D) with a mixture of (BPT + DNA) (A). All spectra were obtained in the standard gly–H_2O glass at T = 4.2 K, λ_{ex} = 371.6 nm. A, (BPT + DNA) mixture; BPT concentration $<10^{-5}$ M; DNA $\sim10^{-4}$ M. B, (+)-anti-BPDE–DNA; 0.5% bases modified; C, (−)-anti-BPDE–DNA; 1.5% bases modified; D, syn-BPDE–DNA; concentration ~1 adduct in 10^7 bases. The FLN peaks are labelled with their corresponding excited-state vibrational frequencies (in cm^{-1}).

the spectra in Fig. 4(B and C) establish that FLNS displays sufficient resolution to distinguish between the major DNA adducts derived from BPDE, e.g. N2-dG from the (+) and (−) enantiomeres. The structure of this major nucleoside adduct (labelled also as 2-NH_2-dG) is shown in Fig. 5 for the anti-diastereomer. Figure 6 shows an excitation spectrum presumably of the above mentioned major N2-dG adduct in this DNA sample. This spectrum was obtained for (+)-anti-BPDE–DNA sample at T = 4.2 K for the observation wavelength, λ_{obs} = 378.5 nm, which is selective for this type of adduct. The detection bandwidth was 4 cm^{-1}. The spectrum yields the excited state vibronic frequencies in the region of ~800–1600 cm^{-1} and these are in good agreement with FLN data obtained with vibronic excitation. Because of the high selectivity of FLN, small contributions from tetraol (BPT) modes (T, 1332; T, 1442; and T, 1517 cm^{-1}) are also observed. These modes were identified on the basis of the excitation spectrum of BPT in DNA taken separately (spectrum not shown). The question of whether this weak contribution of BPT in Fig. 6 is also observed in Fig. 4 should be considered. To this end the FLN spectrum for (BPT + DNA) is shown in Fig. 4(A), obtained under the same conditions



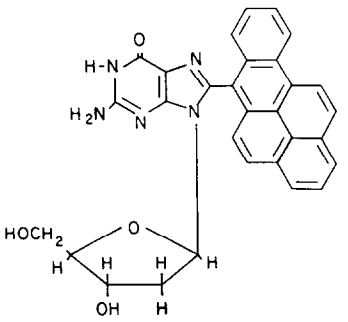


Figure 5
Structures of 2-NH_2-dG and C8dG-BP nucleoside adducts formed by covalent binding to DNA in two different metabolic activation pathways.

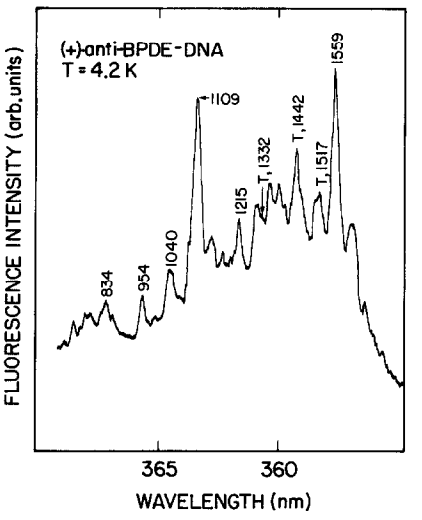


Figure 6
Excitation spectrum of (+)-anti-BPDE–DNA adducts in the gly–H_2O glass at 4.2 K, obtained for the observation wavelength (λ_{obs}) equal to 378.5 nm. λ_{obs} was selective for the major nucleoside adduct (2-NH_2-dG); T corresponds to BPT mode. Peaks are labelled (in cm^{-1}) with their excited-state vibrational frequencies.

the spectrum may be due to free BPT and to non-covalently complexed BPT. Its comparison with the spectra in Figure 4(C, B and D) establishes that the contribution of BPT fluorescence in these samples is negligible. Moreover, these data demonstrate that the distinction between the unbound tetraols and their respective DNA adducts is possible since the energy of S_1 state of tetraols is typically higher by ~100–150 cm^{-1} [7, 8].

It was important to characterize all these model adducts by FLN because the spectra obtained could be expected to be important for positive identification of DNA adducts formed *in vivo*. Therefore, the analysis of liver DNA from fish exposed to 100 mg of BP/kg body wt was undertaken. Of particular interest was whether or not the expected N2-dG adduct from (+)-anti-BPDE is formed. The pertinent result is presented in Fig. 7 showing the FLN spectra of fish liver DNA obtained at T = 4.2 K for λ_{exc} = 369.6 nm. Despite the very low damage level of this sample (1 adduct in 10^7 to 10^8 bases), the FLN spectrum exhibits a good signal-to-noise ratio. As expected, a major adduct is derived from BPDE, indicating the importance of the mono-oxygenation mechanism. However, the adduct is not derived from (+)-anti-BPDE (as might be expected) but rather from syn-BPDE. This is established by comparing the fish liver DNA

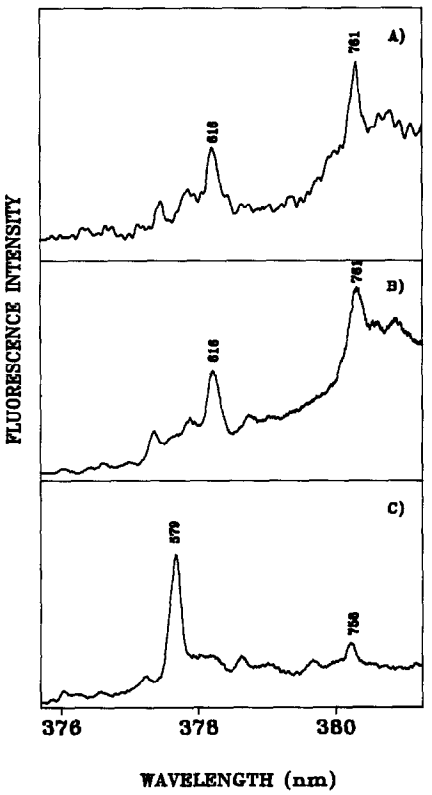


Figure 7
FLN spectrum of fish-liver DNA extracted from fish (English sole) exposed to 100 mg BP/kg of fish. The modification in the sample is approximately 1 adduct in 10^7 to 10^8 nucleotides (A). B and C show FLN spectra of standard syn-BPDE–DNA adduct with a modification level of approximately 1 adduct in 10^7 bases (determined radiometrically), and at (+)-anti-BPDE–DNA with a modification level of 1 adduct in ~200 bases, respectively. Conditions: λ_{exc}, 369.6 nm; T = 4.2 K; gly–H_2O glass.

spectrum of Fig. 7(A) with the FLN spectra of syn-BPDE and (+)-anti-BPDE–DNA in Fig. 7. Full vibrational analysis of these spectra will be presented elsewhere (cf. R. Jankowiak *et al.*, paper in preparation). Even though the major adduct is derived from syn-BPDE, weaker contributions from (+)-anti- and (−)-anti-BPDE–DNA adducts cannot be excluded. Although anti- and syn-BPDE are both strongly mutagenic in bacterial and mammalian cells, anti-BPDE shows generally greater activity than syn-BPDE in most tests [40]. Therefore, the high proportion of syn-BPDE–DNA adducts in English sole exposed to the high dosage of BP utilized in these experiments presents an interesting problem. It is noteworthy, however, that similar results were obtained recently by Varanasi *et al.* [36] by using ^{32}P-postlabelling, who showed that while fish exposed to 2–15 mg BP/kg body wt contained mainly the anti-BPDE–DNA adduct, those fish exposed to 100 mg BP/kg body wt had high proportion of syn-BPDE–DNA adducts.

Recently FLNS has been used to characterize five BP-nucleoside adducts synthesized by one-electron oxidation of BP (in the presence of guanosine, deoxyguanosine and deoxyadenosine, respectively) [34]. The results were used to prove that a major depurination adduct from the binding of BP to DNA in rat liver nuclei is 7-(benzo[a]pyren-6-yl) guanine [22]. This, together with the results presented above for the fish liver DNA, demonstrates that FLNS has significant potential for the characterization and determination of DNA adducts formed *in vivo* by metabolites that yield bound chromophores which are fluorescent.

Finally, a brief discussion is presented on the application of FLNS to nuceloside adducts sorbed onto TLC plates. In the ^{32}P-postlabelling procedure [41] DNA adducts are hydrolysed to nucleotides which are then subjected to two-dimensional TLC. Differences between *in vivo* and *in vitro* adducts have been noted [41]. For *in vivo* samples, the nucleotide adduct radiograms often do not display the patterns expected on the basis of the mobilities of standard adducts [41, 42]. With a view towards structural characterization of TLC spots the authors recently demonstrated that FLN is applicable to nucleoside adducts sorbed onto TLC plates [43].

As an example, Fig. 8 shows that FLN spectrum of the C8dG-BP nucleoside adduct (see Fig. 5) on a TLC plate. The spectrum was obtained with vibronic (1,0) excitation at λ_{ex} = 395.7 nm, and shows FLN origin multiplet structure. The origin components are labelled with values (cm^{-1}) corresponding to their displacements from the laser frequency. As discussed, these displacements yield the excited-state frequencies of the fundamental vibrations pumped by the laser. The FLN spectrum of Fig. 8 is very similar to that observed for C8dG-BP in a glass [22]. A calibration curve for this adduct yielded a detection limit of ~10 without optimization of conditions. A detection limit of ≤1 fmol was projected. In the Randerath ^{32}P-postlabelling procedure an additional complication arises when the developed TLC plate exhibits diffuse multicomponent zones. This was exactly the case observed recently in the investigation of liver DNA from fish living in polluted areas

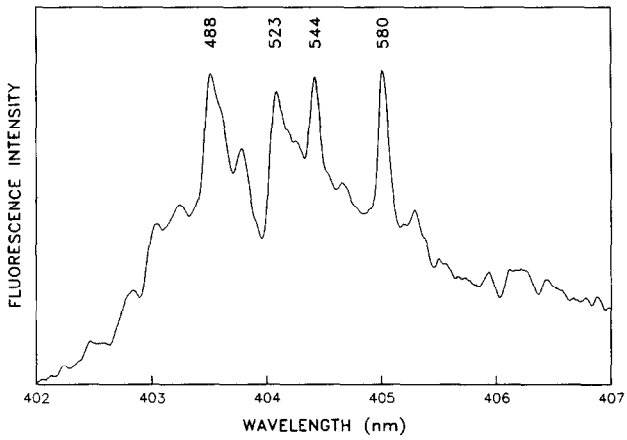


Figure 8
FLN spectrum of the C8dG-BP nucleoside adduct sorbed onto a KC18 reversed-phase TLC plate. Conditions: λ_{exc}, 395.7 nm; *T*, 4.2 K. The numbers above the peaks correspond to excited-state vibrational frequencies (in cm^{-1}).

[42, 44]. Therefore, a spectroscopic technique which allows for resolution of such a zone into constituent components would be particularly valuable.

The authors believe that the interfacing of FLNS and ^{32}P-postlabelling will yield a methodology that will be very useful for the understanding of the initial mechanistic aspects of chemical carcinogenesis.

In conclusion, results presented in this paper and in other publications (cf. R. Jankowiak *et al.*, papers in preparation) demonstrate that FLNS is a rapid, versatile, highly sensitive, and highly selective analytical technique which can directly analyse carcinogenic metabolites, and depurinated and macromolecular DNA adducts from *in vitro* and *in vivo* exposures to PAH. Additionally, FLNS can be coupled to the ^{32}P-postlabelling procedure to yield a high-resolution methodology for the identification of nucleotide adducts sorbed onto TLC plates.

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