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Application of Laser Self-Beat Spectroscopic Technique to the Study of Solutions of Human Plasma Low-Density Lipoproteins†

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ABSTRACT: The technique of self-beat or homodyne spectroscopy has been applied to the determination of the translational diffusion constant of human plasma low-density lipoproteins (LDL). Both power spectrum and autocorrelation methods give equivalent results, but with conventional equipment the power spectrum measurement takes on the order of hours while autocorrelation is accomplished in minutes. The effects of concentration of LDL, scattering angle of the light pH of the solution and buffer concentration were investigated. The diffusion constant obtained, $D_{25,w} = 2.14 \pm 0.09 \times 10^{-7}$ cm²/sec, was in good general accord with conventional measures. The equivalent spherical diameter, obtained from the Stokes–Einstein relationship, 229 ± 10 Å was within a broad band of values given by other techniques.

The translational diffusion constant, D, has been classically used in biochemistry for the determination of the molecular weight of proteins in conjunction with sedimentation data and for the calculation of frictional ratios of proteins. While sedimentation equilibrium methods have become popular for molecular weight determinations of smaller proteins, these methods cannot generally be applied to large particles such as the plasma lipoproteins. The measurement of D has traditionally been done by experiments in which the spreading of the boundary is measured as the protein being studied diffuses into a solvent layer (Longsworth, 1945). The most accurate measurements of D have been by the spreading boundary technique (Gosting, 1956). In recent years, the measurement of the D of macromolecules has been greatly simplified by a technique involving the analysis of laser light scattered from a suspension of the molecules in question (Chu, 1970; Cummins and Swinney, 1970). The Brownian motion of scattering macromolecules causes the scattered beam to be slightly broadened in frequency. This effect has been used by Dubin *et al.* (1967), who obtained physical information about biological macromolecules from the power spectrum of scattered light. While recording the power spectrum requires on the order of hours with conventional equipment, we have been able to obtain comparable results in minutes with the adjunct of autocorrelation. It is the purpose of this communication to describe how laser homodyne spectroscopy may be conveniently applied to the determination of the translational diffusion constant of human plasma low-density liporpoteins (LDL).

Theory

For a selected scattering angle the frequency spectrum of photocurrent is measured from a photodetector exposed to the scattered light. The square of the amplitude of the output of the photodetector at a given frequency, within a constant frequency band, is proportional to the spectral power, $S(f)$, of the photocurrent. For a monodisperse system of scatterers, the component of the power spectrum that arises from the random motions of scatterers can be shown to have a Lorentzian form, *i.e.*

$$S(f) \propto 1/(1 + f^2/f_0^2) \quad (1)$$

This spectral power is in addition to the shot noise of the detector and electrical noise of associated circuits. The Lorentzian band is centered around zero frequency. The half-width frequency at $^1/_2$ the maximum power, f_0, of Lorentzian spectrum is proportional to the translational diffusion constant of the scattering molecules. Specifically, if monochromatic light of wavelength λ is scattered through an angle θ in a medium of index of refraction n by particles of diffusion constant D, then

$$f_0 = 16\pi Dn^2 \sin^2(\theta/2)/\lambda^2 \quad (2)$$

A second method, complementary to that of measuring the power spectrum of the fluctuations in intensity of scattered light, is that of measuring the autocorrelation function of the

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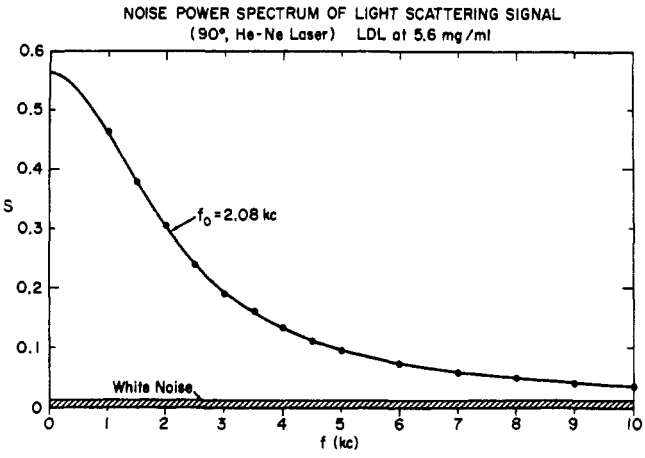

FIGURE 1: Power spectrum of light-scattering signal from a suspension of LDL. The LDL concentration was 5.6 mg (as protein) per ml in 0.15 N NaCl containing 0.01% NaEDTA (pH 7.4). The scattered light was observed at 90° to an incident beam from a He–Ne laser. The half-width at half-height of the Lorentzian line is 2.08 ± 0.04 kHz by least-squares analysis of the data from 0.5 to 10 kHz.

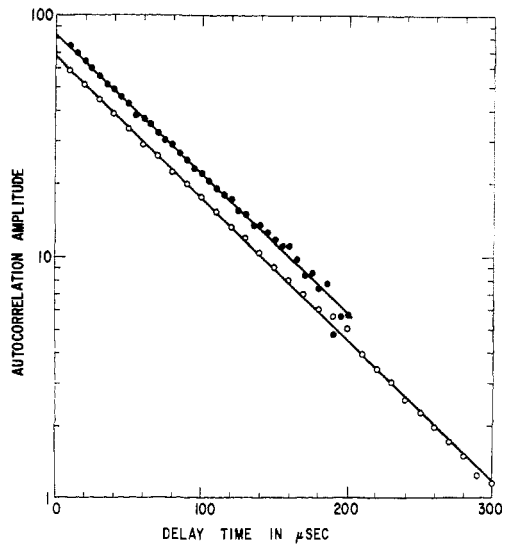

FIGURE 2: Autocorrelation function of light-scattering signal from a suspension of LDL. Curve A: solid data points indicate conditions similar to those for the data of Figure 1. A least-squares fit gives the decay time constant as 75.0 ± 1.5 μsec. Curve B: open data points refer to a result in the second series of measurements for LDL at a concentration 6.0 mg of LDL (as protein) per ml in 0.154 N NaCl–0.01% EDTA (pH 7.5) and 23.8°. The calculated decay time constant in this case is 73.85 ± 0.4 μsec.

intensity fluctuations. The reciprocity and complete equivalence of these two methods of noise analysis are contained in the so-called Wiener–Khintchine (1930) theorem. If $V(t)$ is a voltage proportional to the intensity of scattered light as, for instance, the output of a photomultiplier tube, then for the case outlined above the autocorrelation function is defined as

$$A(\Delta t) = \overline{V(t)V(t + \Delta t)} \tag{3}$$

where the signal at time t is multiplied by a signal at some later time, $t + \Delta t$, and the results averaged over all starting times. For the case treated above of a monodisperse system

$$A(\Delta t) = A_0 \exp(-\Delta t/\tau) \tag{4}$$

where τ is the decay time constant of the fluctuations. This time is simply related to the Lorentzian half-width of the power spectrum by the relationship

$$\tau = \frac{1}{(2\pi f_0)} \tag{5}$$

The diffusion constant obtained either from spectral analysis or from autocorrelation analysis must be reduced to some standard conditions. A particular measurement will give a value strongly dependent on temperature, viscosity, concentration, and charge (Gosting, 1956). It is customary to extrapolate the measured value of D to zero concentration, zero charge, and to pure water.

The Stokes–Einstein equation

$$D = \frac{kT}{3\pi\eta d} \tag{6}$$

directly displays the dependence of D on the variables η, the viscosity, and absolute temperature T. Here k is Boltzmann's constant and d is the diameter of a sphere that would yield the same translational diffusion constant as measured for the molecules under investigation.

Experimental Section

Low-density lipoproteins (LDL) were isolated from the plasma of normal fasting subjects by ultracentrifugal flotation in salt solutions between densities 1.019 and 1.063 g per cm^3 (Havel *et al.*, 1955). Several preparations of LDL were used. Each preparation reacted with antisera to human plasma LDL but not with antisera to HDL, albumin or other plasma proteins. The preparations gave a single band of β mobility on immunoelectrophoresis when tested against rabbit antibodies to human plasma and had the chemical composition expected for normal LDL (Havel *et al.*, 1955). One set of measurements compared autocorrelation and spectrum analysis methods. Another set of measurements was done at a later time on a different fresh preparation of LDL using purely autocorrelation analysis in order to quantify the measured D value of LDL to standard conditions.

In initial experiments, a 7.5-mW He–Ne laser source (Spectra Physics Model 123) was used (Figures 1 and 2A) while in later experiments a 15-mW He–Ne laser (Spectra Physics Model 124A) was employed (Figures 2B and 3–6). The laser beam was focused onto a quartz scattering cell containing a suspension of LDL. The light was collected from a narrow range of angles about 90° and was imaged on the surface of a photomultiplier tube (RCA 7265). The photocurrent was analyzed both by autocorrelation (Princeton Applied Research 101A analog correlation computer), and by analysis of the power spectrum. Autocorrelation functions were displayed on an X–Y recorder. For the power spectrum analysis, a wave analyzer was used in place of the autocorrelator. The wave analyzer gave an output proportional to the amplitude of the fluctuations in scattered light within a pass band of 200 Hz. The output was effectively given a ten second time constant by reading it on a digital voltmeter with that integration time. To obtain a quantity proportional to the noise power, this output was squared.

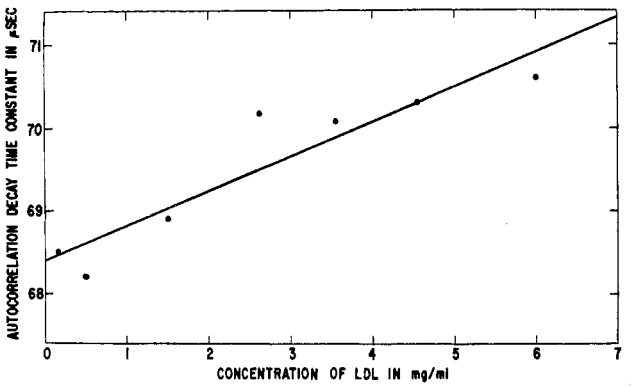

FIGURE 3: Autocorrelation decay time constant corrected to 25° for varying LDL concentrations in 0.154 N NaCl solutions containing 0.01% EDTA (pH 7.5). The solid line is a least-squares fit to the data and gives an intercept of 68.4 ± 0.3 μsec.

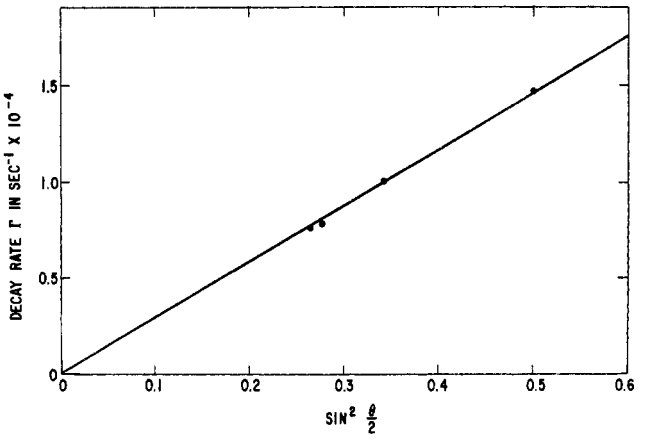

FIGURE 4: Decay time constant as a function of $\sin^2(\theta/2)$ for 0.4 mg/ml of LDL in 0.154 N NaCl (pH 7.5)–0.01% EDTA, corrected to 25°.

Results[1]

The noise power spectrum is a Lorentzian line superimposed on a small white-noise component (Figure 1). The latter is frequency independent and is attributed to a shot noise from the photomultiplier. The apparent value of f_0 for LDL at a protein concentration of 5.6 mg/ml is 2.08 ± 0.04 kHz (Figure 1) from a least-squares analysis of the data from 0.5 to 10 kHz. This value should be corrected for the effect of the finite bandwidth of the receiver. We have performed a numerical calculation of this point and find the true half-width to be lower by only 0.2% under the experimental conditions employed. For 25° and 0.154 N NaCl the diffusion constant from this result is 1.89×10^{-7} cm²/sec and the equivalent sphere diameter is deduced as 255.6 Å after a correction for viscosity of the saline solution compared to pure water (International Critical Tables, 1928).

Whereas the point by point recording of the power spectrum requires about 3 hr, the same signal could be analyzed within 2 min using the technique of autocorrelation. The autocorrelation function, for conditions identical with those of Figure 1, is given in Figure 2 (curve A). Here the least-squares fit to the data gives τ = 75.0 ± 1.5 μsec. The apparent diffusion constant (D) for 25° is 1.93×10^{-7} cm²/sec in the saline solution and the equivalent sphere diameter is 250.6 Å for pure water. These measurements indicate the equivalence of the two modes of analysis as expected from the Wiener–Khintchine theorem.

In order to evaluate the effects of concentration, charge and possible contamination by larger molecular weight aggregates or dust particles, a systematic study was performed in which the concentration of LDL, the pH, the scattering angles, or the salt concentration was varied. For these measurements autocorrelation alone was used for analysis of scattered light and the autocorrelation function recorded for 90° scattering. A typical result, at 23.8° is shown in Figure 2, curve B. For this measurement a least-squares fit to a single exponential gives τ 73.85 ± 0.4 μsec. We consider that the results of the experiments shown in Figure 2 (curves A and B) are in good agreement, since curve B was obtained with a better signal to noise ratio (a 15 mW *vs.* 7.5 mW power source) and with a different preparation of LDL which was ultrafiltered prior to spectroscopic measurements. A series of measurements (Figure 2, curve B) gives an average value for the decay time constant of 70.61 ± 0.75 μsec extrapolated to 25°. The effect of LDL concentration on the observed decay time constant is shown in Figure 3. (All results shown henceforth are corrected to 25° for comparison purposes.) The LDL suspension was diluted in 0.154 N NaCl containing 0.01% EDTA (pH 7.5). From the data we may infer that at zero concentration of LDL and for these conditions, the decay time constant would be 68.4 ± 0.3 μsec.

The geometry of our system precluded measurement of the decay constant over a wide range of angles. We have, nevertheless, attempted to verify the dependence of the decay rate Γ (*i.e.*, $\Gamma = 1/\tau$) on the scattering angle. In the absence of high molecular weight constituents or dust particles, Γ should be strictly proportional to $\sin^2 \theta/2$ as seen from eq 2 and 5. If relatively large contaminants are present, however, their effect on the scattered light characteristics becomes more severe at smaller scattering angles because the cross-section for scattering light for large particles increases as the scattering angle decreases. In the range of angles studied, we see no departure from a strict proportionality of Γ on $\sin^2 \theta/2$ as shown in Figure 4.

Large particle effects can be noticed experimentally by other means. First, the autocorrelation function may not be a single exponential function of time. In particular, large clumps will cause slow fluctuations and an elevated base line will appear for the measured autocorrelation function. Secondly, large "speckle" pattern (forward scattered laser light intensity) changes can be observed as large particles intersect the light beam. When either of these effects were noticed, we filtered the solution through 0.22 μ Millipore filters. This treatment removed excess slow fluctuations and allowed fairly reproducible results as indicated by the data for a variety of different suspensions and conditions over a 4-day period. We noted that a measurable effect of large particles occurred only when the slow fluctuations occurred more frequently than about a few seconds out of an integration time of 100 sec.

The effects of pH are indicated in Figure 5. Here the scatter in the data is evidently larger than for any of the other measurements. The decay time constant for each pH value was determined rather quickly after adjusting for the pH and it may be that thermal equilibrium was not achieved and improper temperature corrections were assumed. Nevertheless, there is a general small increase of D with decreasing pH. There may even be a dip in the value of τ at the isoelectric

[1] Please note that all statistical values are given ±2 standard deviations.

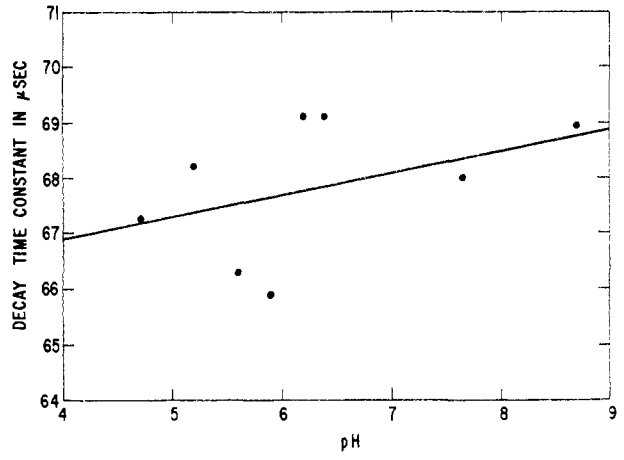

FIGURE 5: Decay time constant as a function of pH of 0.4 mg/ml of LDL in 0.154 N NaCl-0.01% EDTA (pH 7.5), corrected to 25°. The standard deviation of the data about the solid line is 1.3 μsec.

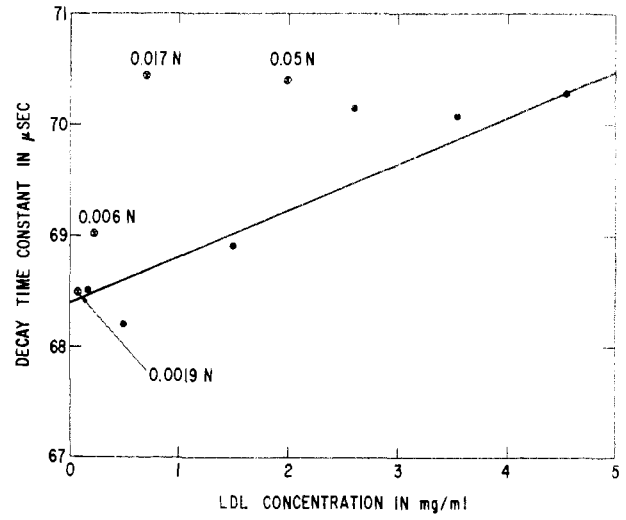

FIGURE 6: Comparison of the decay time constant dependence on LDL concentration for varying salt concentration. Solid data points and solid line were previously displayed in Figure 3. Open data points of Figure 6 have been corrected to correspond to the viscosity of the 0.154 N NaCl solution. The salt concentration for each of the open data points is indicated.

point, pH 5.5 (Oncley *et al.*, 1950); however, the scatter in the data is too large to ascertain whether the dip is real or not. We will use the least-squares fitted line of Figure 5 as indicative of the pH correction to be made (in order to interpret the diffusion constant for zero change) and twice the standard deviation of the data points about that line as an estimate of the error in that correction. Thus, from pH 7.6 to 5.5, the isoelectric point, there is a decrease in the decay constant of 0.8 ± 2.6 μsec.

A brief examination of salt concentration effects is shown in Figure 6. A suspension of LDL at a protein concentration of 6.0 mg/ml in 0.154 N NaCl containing 0.01% EDTA (pH 7.5) was successively diluted with distilled water. Figure 6 shows the observed decay constant after each dilution. In order to compare to the earlier results of τ *vs.* LDL concentration in an isotonic salt solution (Figure 3), we scale the observed results in Figure 6 for viscosity changes due to the presence of salt (calculated from International Critical Tables). For very low LDL concentrations, the observed τ is seemingly unaffected by a decrease in salt concentration. There appears to be an increase in τ when salt concentration is reduced at an LDL concentration of approximately 1 mg of protein/ml. These results are offered as further evidence that the effects of LDL charge on the diffusion constant at low LDL concentrations are small and will be adequately encompassed by a 2.6 μsec (*i.e.*, 2 standard deviations) uncertainty in the value of τ.

From Figure 3 we deduce τ at zero LDL concentration to be 68.4 μsec. The correction to pure water from 0.154 N NaCl reduces this value by 1.0135 (due to viscosity of 0.154 N NaCl compared to water at 25°, International Critical Tables) to 67.5 ± 0.5 μsec. The reduction of this value to the isoelectric point gives finally $\tau_{25,w}$ + 66.7 ± 2.8 μsec, where the subscripts refer to water and 25°. Here we also assume ±0.5° error in temperature. The diffusion constant is then, $D_{25,w}$ = 2.14 ± 0.01 × 10^{-7} cm^2/sec and the diameter of the equivalent sphere is 229 ± 10 Å.

Discussion

We have applied the technique of self-beat or homodyne spectroscopy to the determination of the translational diffusion constant of LDL. Both the power spectrum and the autocorrelation methods give equivalent results but, with conventional equipment, the power spectrum analysis takes on the order of hours while autocorrelation is accomplished in minutes. The D so obtained ($D_{25,w}$ = 2.14 ± 0.09 × 10^{-7} cm^2/sec) is in good general accord with more conventional measures while the equivalent spherical diameter, 229 ± 10 Å is within a broad band of values given by other techniques.

The results may be compared with earlier measurements of diffusion constant and the particle diameter. Pedersen (1947) measured a value of $D_{20,w}$ of 1.7 × 10^{-7} cm^2/sec (equivalent to a $D_{25,w}$ of 1.95 × 10^{-7} cm^2/sec) while Toro-Goyco (1958) reported $D_{20,w}$ of 1.85 × 10^{-7} cm^2/sec ($D_{25,w}$ = 2.12 × 10^{-7} cm^2/sec). A recent study by Fisher *et al.* (1971) gives values of $D_{25,w}$ that range from 1.9 × 10^{-7} to 2.2 × 10^{-7} cm^2 per sec at the concentration we employed. In addition, they found a weak concentration dependence of the diffusion constant such that their value extrapolated to zero concentration was $D_{25,w}$ = 2.17 × 10^{-7} cm^2/sec.

We used the Stokes–Einstein relationship to infer an equivalent hydrated spherical diameter for the LDL. Our value of 229 ± 10 Å is in agreement with that of 226 Å as inferred from the measurements of Fisher *et al.* (1971). Other measurements give quite a wide range of values. Sedimentation velocity experiments have led to values of 185 Å (Oncley *et al.*, 1947) and 196 Å (Toro-Goyco, 1958) while a gel filtration method has given an equivalent diameter of 258 Å (Margolis, 1967). Recent electron microscopic studies with negatively stained preparations have shown spherical particles with a narrow range of diameters of 216–220 Å (Forte *et al.*, 1968; Gotto *et al.*, 1968), and to a range 219–290 Å.

Our results for D are seen to be in close accord with those of earlier investigators. The ease and relative precision of the laser technique we employ makes it possible to easily investigate any differences in LDL geometry that might exist among individuals or in the course of disease.

Acknowledgments

We thank Dr. C. P. Bean of General Electric for his good offices in linking our laboratories and for the aid with the manuscript, and Drs. Donald S. Fredrickson and Henry D. McIntosh for advice and encouragement. In addition, we are

indebted to C. M. Penney for allowing us the use of his laser facilities in the preliminary experiments. Mrs. M. V. Doyle of the same laboratory has been most helpful in treating the experimental data.

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Electron Paramagnetic Resonance Study of Iodine-Induced Radicals of Benzo[*a*]pyrene and Other Polycyclic Hydrocarbons†

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ABSTRACT: Free radicals have been postulated previously as intermediates in the chemical linkage of the environmental carcinogen benzo[*a*]pyrene to nucleic acids when activated by iodine. Electron paramagnetic resonance studies indicate the presence of benzo[*a*]pyrene radicals in benzene, methanol and cyclohexane solution induced by iodine. These radicals are quenched by pyrimidine, purine, nucleosides, imidazole, and other nitrogenous compounds but not by alcohol, aldehyde, or water. These results strongly support the proposal that radicals of benzo[*a*]pyrene are involved in the chemical reaction between the hydrocarbon and nucleic acids in the presence of iodine. The electron paramagnetic resonance studies on the steady-state radical concentration of 14 polycyclic hydrocarbons formed in the presence of iodine indicate that, in general, the carcinogenic compounds such as benzo[*a*]pyrene, 7,12-dimethylbenzanthracene, 3-methylcholanthrene, etc., have a much higher concentration of radicals than the noncarcinogenic compounds such as benzo[*e*]pyrene, benzanthrene, pyrene, naphthacene, etc. There are one or two exceptions. The steady-state radical concentrations of these compounds do not correlate well with their ionization potentials, though the compounds having low ionization potentials do tend to yield higher concentration of radicals.

The scientific and public health significance of polycyclic hydrocarbon carcinogenesis has been generally well recognized (Bergman and Pullman, 1969). In the preceding papers of this series (Lesko *et al.*, 1969; Hoffmann *et al.*, 1970), our laboratory reported that B[*a*]P[1] and other carcinogenic polycyclic hydrocarbons react specifically with DNA and polynucleotides in the presence of I_2 in aqueous or aqueous ethanol systems. Under similar conditions noncarcinogenic analogs react with nucleic acid to a much lesser extent. It was postulated that radicals of B[*a*]P and other hydrocarbons may serve as reaction intermediates (Lesko *et al.*, 1969; Hoffmann *et al.*, 1970). Earlier, several investigators had proposed the radical cation of B[*a*]P as the intermediate in the reaction of B[*a*]P with pyridine or nucleic acid bases in a solid-phase system activated by I_2 vapor (Rochlitz, 1967; Wilk and Girke, 1969). In 1960, Szent-Györgyi *et al.* reported the existence of radicals of a number of compounds including B[*a*]P when activated by I_2.

We report in this paper electron paramagnetic resonance (epr) studies on formation and reactivities of radicals of B[*a*]P and related polycyclic hydrocarbons induced by I_2 in various organic solvent systems. The results strongly support the previous proposal that radicals of B[*a*]P and other hydrocarbons are formed in the presence of I_2, a mild oxidative system, and that these radicals participate in the chemical reaction with nucleic acid. The relationship of these findings

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[1] Abbreviations used are: B[*a*]P, benzo[*a*]pyrene; B[*e*]P, benzo[*e*]pyrene; MCA, 3-methylcholanthrene; DMBA, 7,12-dimethylbenz[*a*]anthracene; 5′-mmtr-(2′,3′)-ipr-guanosine, 5′-monomethoxytrityl-(2′,3′)-isopropylideneguanosine; 5′-mmtr-(2′,3′)-ipr-adenosine, 5′-monomethoxytrityl-(2′,3′)-isopropylideneadenosine.