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MASTER

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IV. PROGRESS REPORT

A. Overview

The goals of this project were to obtain information about the conformation of the adducts formed when polycyclic aromatic carcinogen metabolites react covalently with DNA. In order to achieve this goal, several spectroscopic techniques were developed, or adapted, specifically for the purpose of determining the orientation and the nature of the microenvironment of the aromatic carcinogen-chromophore complexed to DNA. As a result of these investigations, it became apparent that it would be interesting as well to study the kinetics of the interactions of different isomeric reactive metabolites of polycyclic aromatic hydrocarbons with DNA under

controlled *in vitro* conditions. This work has been started recently and, since it has not yet been published, will be described in greater detail in this progress report. The work already published will be mentioned here only briefly in summary-form, while the details may be found either in the reprints attached, or in the other publications cited.

B. Summary of Physico-chemical Methods Utilized

(1) Fluorescence spectroscopy - standard excitation and emission profiles, decay profiles and lifetimes, quenching techniques. These methods provide information on the type of binding site (intercalation or outside binding mode) of the complexed aromatic polycyclic molecules. (Described in Photochem. Photobiol. 29, 223 (1979); Biochem. Biophys. Res. Comm. 88, 782 (1979)).

(2) Electric Linear dichroism. This technique provides an average orientation angle of the bound chromophore with respect to the axis of the DNA helix. (Described in Biochemistry 17, 5256 (1978) and J. Am. Chem. Soc. 102, 5661 (1980)).

(3) Polarized Phosphorescence spectroscopy of mechanically oriented carcinogen-DNA complexes. Electric linear dichroism techniques provide information about transition moment vectors oriented within the plane of the complexed aromatic molecules. In some cases knowledge of this orientation does not unequivocally provide information about the orientation of the planar aromatic chromophores with respect to the DNA axis. The phorphorescence emission of planar polycyclic aromatic hydrocarbons, however, is polarized perpendicular to the plane; thus by exciting the phosphorescence with polarized light, and measuring the anisotropy of the phosphorescence emission, information about the orientation of the normal to the plane of the complexed polycyclic molecule is obtained. Thus, a second direction of the bound chromophores can be obtained and its orientation with respect to the axis of the DNA helix can be assessed more exactly.

This method is still under development at this writing. The phosphorescence emission emanating from benzo(a)pyrene diol epoxide-DNA adducts oriented in stretched polyvinyl alcohol films is indeed polarized. Careful mathematical analysis of the linear dichroism and phosphorescence polarization data does provide the information necessary to deduce the absolute orientation of the carcinogen complexed to oriented DNA.

(4) Triplet Flash Photolysis spectroscopy. The triplet excited states of complexed polycyclic molecules have been shown to be sensitive probes of their microenvironment. Preliminary results were reported in 1976 (J. Am. Chem. Soc. 98, 6144 (1976)). We have recently improved and refined a flash photolysis apparatus which allows for the measurement of triplet excited state dynamics at extremely low concentration of chromophores ($\sim 10^{-8}$M). Using these techniques we have shown that non-covalently bound aromatic chromophores dissociate from DNA and bind again to DNA on a timescale of less than one millisecond (described below).

(5) Stopped-flow kinetic measurements in the absorption and fluorescence modes. These measurements were started this year in order to investigate the kinetics of binding and reaction of benzo(a)pyrene diol epoxide with DNA. A preliminary report describing

the kinetics has been published (Biochem. Biophys. Res. commun. 92, 1335 (1980). Preliminary results obtained so far are extremely encouraging, but considerable more work is needed to utilize this method effectively.

C. Structure of Benzo(a)pyrene Diol Epoxide-DNA Adducts

Benzo(a)pyrene (BP) is the most widely studied polycyclic aromatic hydrocarbon (PAH) environmental carcinogen. In living cells BP is metabolized to at least 35 different phenolic, quinone and epoxide products. One of these, as mentioned previously, trans-7,8-dihyroxyanti-9,10-epoxy-7,8,9,10-tetrahydrobenzo(a)pyrene (anti-BPDE) binds covalently to DNA in living cells and is believed to be the ultimate carcinogenic form of BP in vivo. In this adduct, which accounts for ~90% of the total binding, a covalent bond is formed between the C-10 position of BPDE and the exocyclic amino group of guanine.

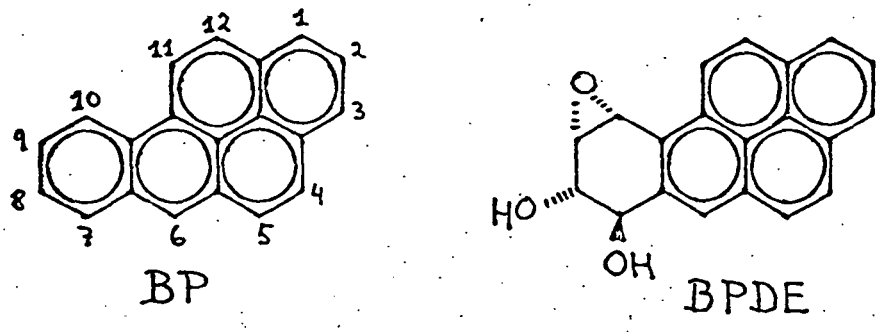

Fig. 4 Chemical structure of BPDE and of benzo(a)pyrene (BP) showing the numbering system.

During the years 1975-1976 we studied the reaction pathways of BP in cells in tissue culture. This involved the isolation, purification and analysis of the carcinogen-modified nucleic acid fractions of the cells. We developed a special low temperature fluorescence technique which permitted us to detect extremely low quantities of reacted PAH molecules. Our work helped to confirm that BPDE is in fact the major metabolite of BPDE which binds covalently to DNA in vivo (12). We then proceeded to study the conformation and physico-chemical properties of the BPDE-DNA adducts obtained in in vitro reactions. The long range goal is to establish what properties of the covalent adducts determine their biological properties, and what the differences are between those adducts whose presence correlates with tumorigenic action, and those which do not correlate with such activity.

Using some of the spectroscopic techniques described above, we found that planar PAH molecules such as BP can bind to DNA by two different mechanisms. One of these is the well-known intercalation mechanism in which the planar aromatic ring-system slides in between adjacent base pairs of DNA. The second mechanism involves an "outside" binding mechanism in which the PAH molecule lies in one of the

external grooves of the DNA molecule. In contrast to the intercalation structure, in the outside binding mode, the plane of the PAH molecule tends to be parallel to the axis of the DNA helix. It was established that in the covalent BPDE-DNA complex, the pyrene-like moeity is located on the outside of the DNA helix. (25,36,37). This is somewhat surprising since the intercalation sites are hydrophobic in nature, while the external sites are hydrophilic. The binding of the hydrophobic BPDE at interior intercalation sites would appear, a priori, to be preferable to an outside binding mechanism. The biological significance of the outside-binding conformation of the BPDE-DNA adduct is not yet known.

D. Triplet Probe Techniques.

Triplet excited states of polycyclic aromatic molecules, because of their relatively long lifetime (millisecond range), can provide important information about (1) the local microenvironment of the PAH molecule, and (2) the binding dynamics to DNA. In the former case (1), the results should be complimentary to those obtained from fluorescence spectroscopy, in which the singlet excited state lifetime (100 nanosecond range) is the probe of the PAH molecule's local environment. In case (2) information is obtained on the rates and dynamics of the association and dissociation of PAH-DNA complexes in aqueous solutions. The concentration of triplet excited PAH molecular states following flash excitation can be monitored in two different ways. In the case of certain polynuclear dyes, the triplet (T_1) decays by populating the first excited singlet (S_1), which subsequently decays by fluorescence emission. This emission has a lifetime characteristic of the triplet decay time and decays in the millisecond time range. It is therefore called delayed fluorescence. In order for this $T_1 \rightarrow S_1$ transition to occur, the energy gap $\Delta E = E_S - E_T$ must be of the order of kT. This is the case for many aromatic dyes. In the case of polycyclic aromatic hydrocarbons such as benzo(a)pyrene, pyrene, or their metabolic derivatives, $\Delta E \gg kT$, and delayed fluorescence is not observed. In these cases the triplets are monitored by flash photolysis techniques in which a flash of light excites the molecules to their T_1 states, while a second source of light is used to monitor transient changes in the absorption due to T_1 (absorption to higher triplet states). The two methods of monitoring triplets can thus be summarized as follows:

Delayed fluorescence: $T_1 \xrightarrow[\text{(slow)}]{kT} S_1 \longrightarrow$ Fluorescence (fast)

Flash photolysis: $T_1 \xrightarrow{h\nu} T_n$ (absorption)

We have shown that either method gives the same triplet lifetime.

(1) Dynamics of the Binding of Polycyclic Aromatic Molecules to DNA.

we have sought to develop a method for measuring the rates of binding to, and dissociation from DNA, of PAH molecules. An understanding of the time scales involved in these dynamic processes are important for an understanding of the mechanisms of interaction of BPDE with DNA, specifically the possible formation of physical (non-covalent) complexes which may preceed covalent binding.

Since BPDE is chemically unstable, we have sought to utilize inert unreactive polycyclic molecules to probe the dynamics of binding processes to DNA in general. The acridine dyes, proflavine (PF) and acridine orange (AO) are ideal for this purpose. Their mode of binding to DNA (intercalation) is well characterized, and we have utilized the delayed fluorescence method to monitor the decaytime of the triplets of these dye molecules.

We have discovered that the intercalated PF and AO molecules move in and out of their binding sites on the DNA molecule (in aqueous buffer solution at room temperature) with rates 10^4 s^{-1}. This conclusion is based on the following observations:

(1) The triplet lifetimes of PF and AO depend strongly on the concentration of the DNA in solution. This is demonstrated in fig. 5.

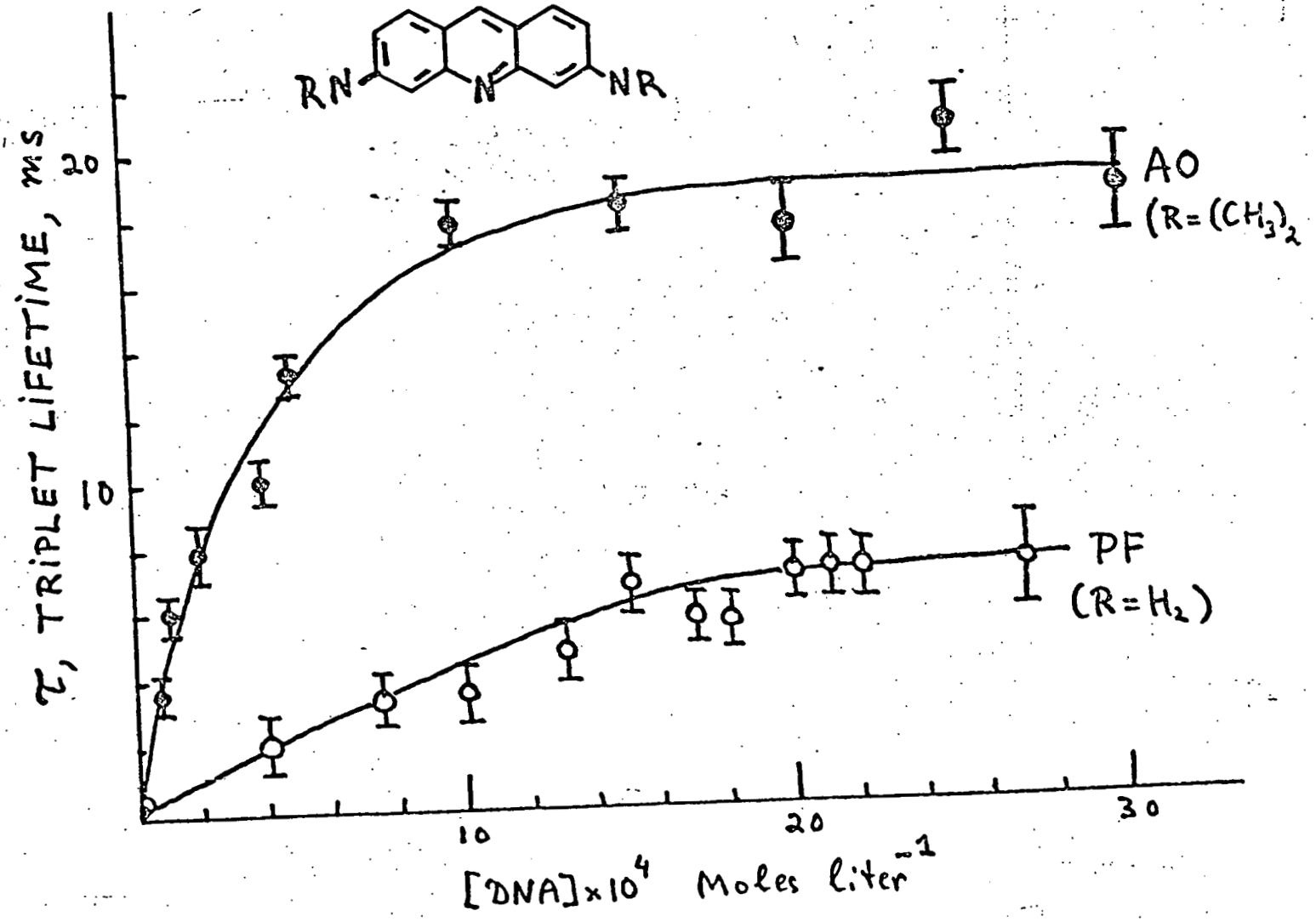

Fig. 5 Triplet lifetimes as a function of DNA concentration. AO...acridine orange; PF...proflavine.

(2) At all DNA concentrations, the decay of the triplets is strictly exponential. If there were two different microenvironments for the polycyclic molecules, i.e. dye molecules bound to DNA as well as free in solution, the decay would be a superposition of two exponential decays Since this is not observed, there must be a rapid exchange between bound and unbound sites.

The triplet lifetimes in aqueous solutions of PF and AO (unbound) is $\lesssim 0.15$ms. Therefore, the rates of exchange between bound and unbound sites must exceed $(0.15\times10^{-3})^{-1}s^{-1}$ or $10^4 s^{-1}$ since only one exponential decay is observed at all concentrations.

The observed decay time, τ , shown in fig. 5, may be represented by the equation

$$\tau = \frac{\tau_f + \tau_b K[DNA]}{1 + K[DNA]} \qquad (1)$$

The DNA concentration is denoted by (DNA), and K^{-1} is defined as the DNA concentration for which the observed decay time is the average of τ_f and τ_b, where τ_f and τ_b are the lifetimes of free and bound triplets respectively.

The fraction X of free, unbound dye molecules can be estimated from the relation

$$\frac{1}{\tau} = \frac{X}{\tau_f} + \frac{1-X}{\tau_b} \qquad (2)$$

and utilizing the data shown fig. 5. The lifetimes of the bound molecules can be estimated from this figure as well by considering the decay times in the region of high DNA concentration, in which additional DNA has no longer any influence on the triplet lifetime ($X \longrightarrow o$).

Based on these results, it is likely that molecules such as BPDE also bind physically to DNA and dissociate from their binding sites at rates of the order of $10^4 s^{-1}$. This would explain how BPDE can first bind physically to DNA by intercalation, and how it can subsequently react covalently at an external binding site (see below). We propose to carry out experiments analogous to those shown in fig. 5 for BPDE and its tetraol hydrolysis product as well, but using triplet flash photolysis, since BPDE does not exhibit delayed fluorescence.

2. Triplet flash photolysis.

We have improved a commercially available flash photolysis apparatus (Xenon Corp.), to the extent that we can detect triplet excited states at close to $\sim 10^{-8}$M concentration of PAH molecules. Using a 20cm pathlength cell, we can detect changes in transmittance (due to transient triplet excited states) as small as 0.04%, with a time resolution of $\sim 20\,\mu$s. The improvements consisted of the following:

(1) Extremely high stability monitoring light source.
(2) Installation of high quality monochromator (McPhearson), baffles and lens systems to reduce stray light levels.
(3) Signal averaging using a Biomation transient recorder coupled to a memory-scope (Didac).

The purpose of monitoring the triplet excited states of PAH molecules bound to DNA is to obtain information on the mode of binding, intercalative, or non-intercalative, to DNA. We have thus studied the triplet excited states of the following molecules bound to DNA, or, in appropriate cases, as free molecules in solution:

(1) Pyrene bound physically (non-covalently) to DNA.
(2) The tetraol derived from BPDE bound physically to DNA.
(3) Covalent adducts derived from the reaction of BPDE with DNA (BPDE-DNA complex).

The principle of the technique involves two factors:

(I) When pyrene, or the tetraol (BPT) derived from the hydrolysis of the diolepoxide BPDE, bind to DNA by an intercalation mechanism, the triplets are completely quenched. Thus, intercalated pyrene-like chromophores do not give any flash photolysis signal. If Ag^+ ions are added (about 0.1 ion per DNA base pair), the triplet signal due to these intercalated chromophores reappears (54). This is due to the heavy-atom effect exerted by silver ions, and the fact that silver ions, by binding to the DNA bases (guanine mostly), disrupt the quenching action of these bases. Thus, based on observations of this type, intercalation binding modes can be distinguished from outside binding modes.

(II) Polycyclic aromatic molecules which are intercalated, are less accessible to molecular oxygen, than those molecules which are bound to the outside of the DNA, or are free in solution. Since oxygen is an effective quencher of triplet excited states, the lifetime of the triplets (τ) is reduced in the presence of oxygen (concentration $[O_2]$) according to the Stern-Volmer equation (3) where τ_o is the triplet lifetime when the oxygen concentration is zero, and K′ is a

$$\frac{1}{\tau} = \frac{1}{\tau_o} + K'[O_2] \qquad (3)$$

constant whose value depends on the accessibility of the polycyclic molecule to oxygen. For intercalated polyclic aromatic molecules, K′ has a value of $\sim 10^8 M^{-1}s^{-1}$, while for free molecules $K' \simeq (1\text{-}2) \times 10^9 M^{-1}s^{-1}$, which is about ten times larger. Using the samples listed above, the following triplet lifetimes (τ_o) and Stern-Volmer quenching constants K′ were obtained:

	τ_o, ms	$K'(O_2)$, $M^{-1}s^{-1}$
BPT in cacodylate buffer, 25°C)	3.1	1.2×10^9
BPT-DNA, Physical complex	0.60 and 3.1	1.1×10^9
BPT-DNA + A_g^+ physical complex	0.20	1×10^8
BPDE-DNA, covalent complex	1.3	1.2×10^9
BPDE-DNA + A_g^+, covalent complex	1.3(no change)	1.2×10^9

These results show that when either BPT or BPDE are bound to DNA, the triplet lifetimes are decreased from about 3 to 1 ms. Furthermore, in the case of the physical BPT-DNA complexes, there are two components, the longer one is due to uncomplexed BPT, while the shorter one is due to BPT bound to the outside of DNA (55); in the absence of silver, the intercalated BPT gives no triplet signal (to be described in detail in a forthcoming publication), while this signal reappears in the presence of A_g^+. This intercalated species, in presence of A_g^+ gives a quenching constant $K \approx 10^8$, as expected, while without silver ions only the free and outside-bound BPT contribute to the triplet signal and exhibit a value of $K \approx 10^9 M^{-1} s^{-1}$.

In the case of the covalent BPDE-DNA complex, silver ions have no effect, and the oxygen quenching constant $K \approx 10^9 M^{-1} s^{-1}$. This result is consistent with the non-intercalation model of the covalent adduct. This result obtained from triplet flash photolysis is thus fully consistent with the data obtained from electric linear dichroism and fluorescence spectroscopy data which showed that the pyrene chromophore is located on the outside of the DNA helix, probably in the minor groove.

3. Utility of Triplet-Probe Techniques.

Finally, the triplet photolysis method gives complementary information to the data obtained from fluorescence spectroscopy. However, in the case of benzo(a)pyrene metabolic derivatives bound to DNA, the aromatic pyrene residue exhibits an unusually long fluorescence lifetime (100-200ns), and thus lends itself particularly well to fluorescence studies. In the case of other polycyclic hydrocarbons (e.g. benz(a)anthracene), the fluorescence liftime is much shorter, and thus fluorescence spectroscopy may not be as useful a tool as it turned out to be for BPDE and BPT. In such cases triplet-probe techniques will be able to provide the same kind of information about the binding mode of these polycyclic aromatic molecules to DNA.

E. Reaction Mechanisms of BPDE with DNA .. fluorescence techniques.

Since last year we have sought to elucidate the reasons for which BPDE binds covalently to the outside of DNA, rather than by an intercalation mechanism. For this reason we have studied the reaction kinetics of BPDE with DNA under various reaction conditions *in vitro*. We have utilized stopped-flow fluorescence and absorption techniques in order to study the modes of reactions of BPDE in aqueous solutions in the absence and in the presence of DNA under physiological conditions of pH ($\approx$7.0). This work will now be summarized.

1. Hydrolysis and covalent binding to DNA. BPDE is a diol epoxide and is thus very unstable in aqueous solutions, particularly at low pH. When BPDE is added to an aqueous buffer solution of pH > 7.0 it hydrolyzes to its tetraol (BPT); this reaction is complete within 90 minutes in 5 mM sodium cacodylate buffer solution (pH = 7.1) at 25°C. The hydrolysis reaction can be conveniently monitored by fluorescence methods since BPT is fluorescent, while BPDE is not. In the presence of DNA, BPDE can react covalently with DNA as well. Thus, hydrolysis and covalent binding constitute a pair of competing reaction pathways. This is summarized in Fig. 6 below.

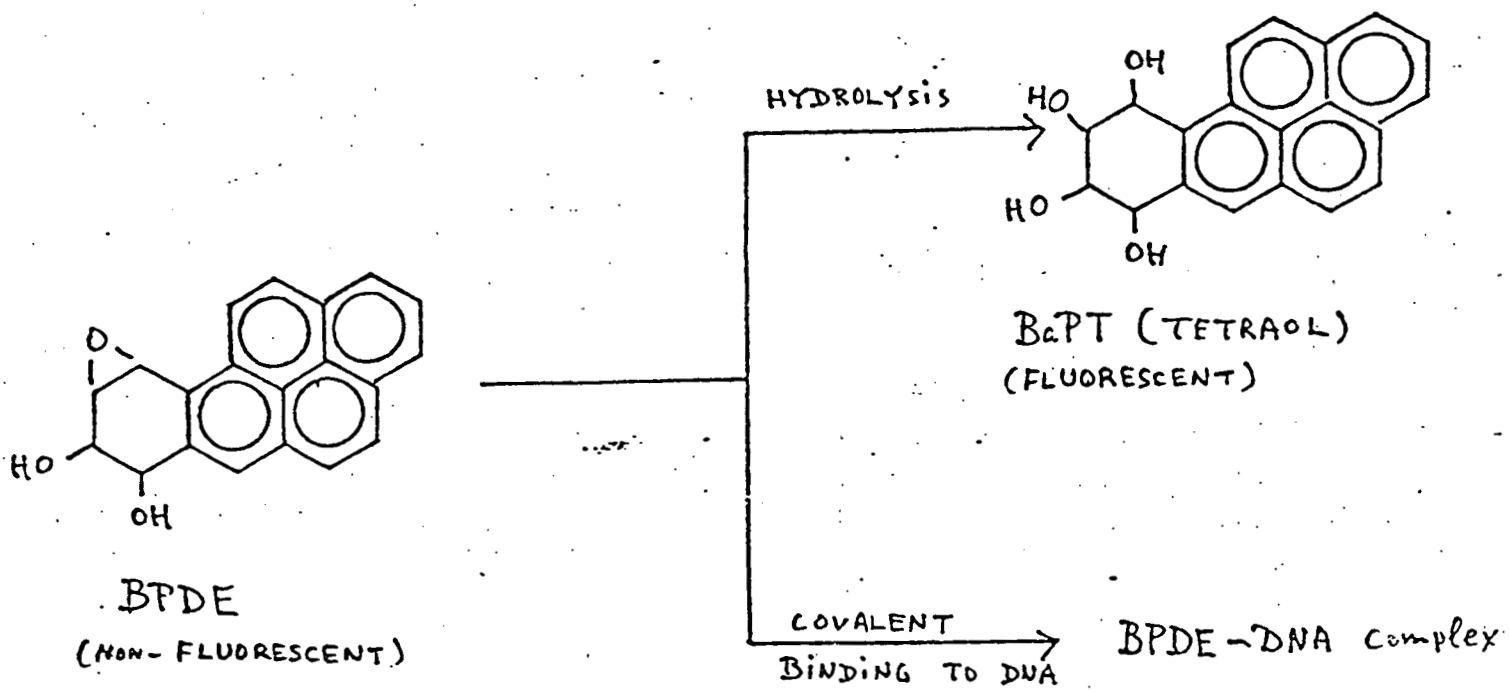

Fig. 6. Hydrolysis and covalent binding reaction pathways of BPDE

The BPT tetraol is chemically inert and does not form a chemical bond with the bases of DNA. However, it does bind physically (non-covalently) to DNA by both an intercalation (site I) and an outside binding (site II) mechanism. The absorption spectra of BPT bound to DNA at these sites (Fig. 7) were deduced from a combination of fluorescence, absorption and equilibrium dialysis methods, which are described in detail elsewhere. (55). A reprint of this paper is attached.

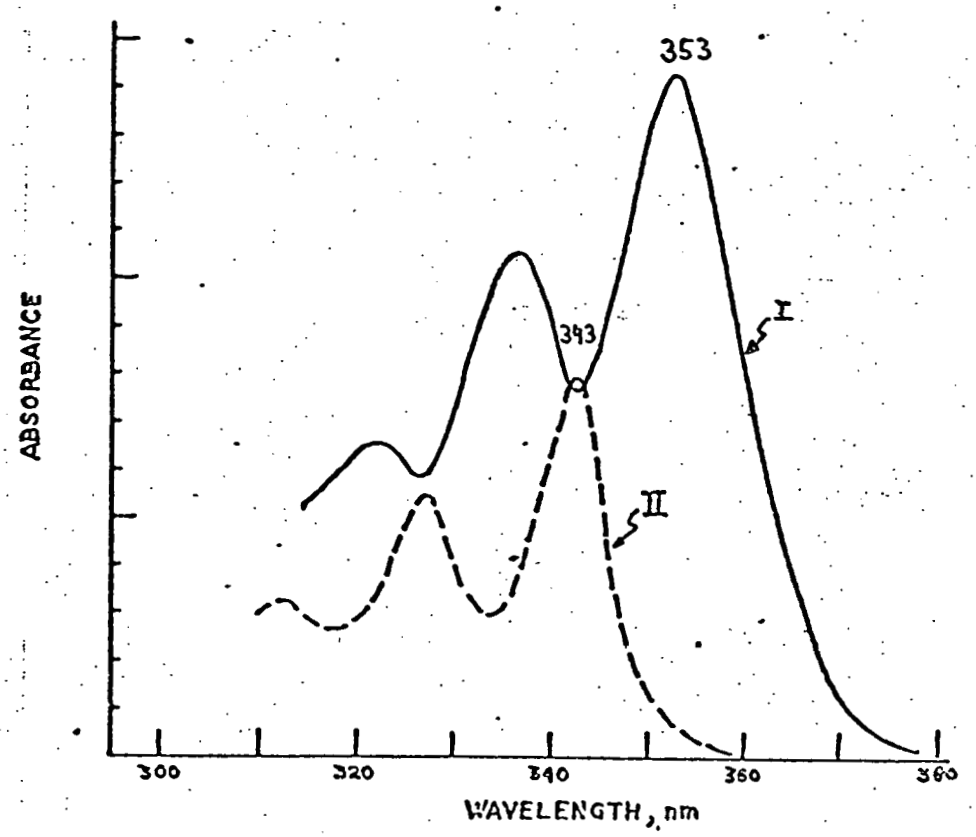

Fig. 7. Absorption spectra of BPT bound to DNA at site I (intercalated tetraol) and site II (externally bound tetraol).

The site II spectrum displays a peak at 343 nm, and is identical to the absorption spectrum of BPT dissolved in buffer solution in the absence of DNA. The site I spectrum is red-shifted by 10 nm and displays a peak at 353 nm. Such red-shifts are characteristic of intercalated PAH molecules. In addition, we have shown (55) that the fluorescence yield of BPT at site I is totally quenched, which is also characteristic of intercalation complexes. BPT at site II displays normal fluorescence characteristics (similar to the fluorescence in buffer solution without any DNA present).

In Fig. 8 the time course of the two different reactions, covalent binding of BPDE to DNA, and hydrolysis of BPDE to BPT monitored by the fluorescence yield increase, are shown for different concentrations of DNA.

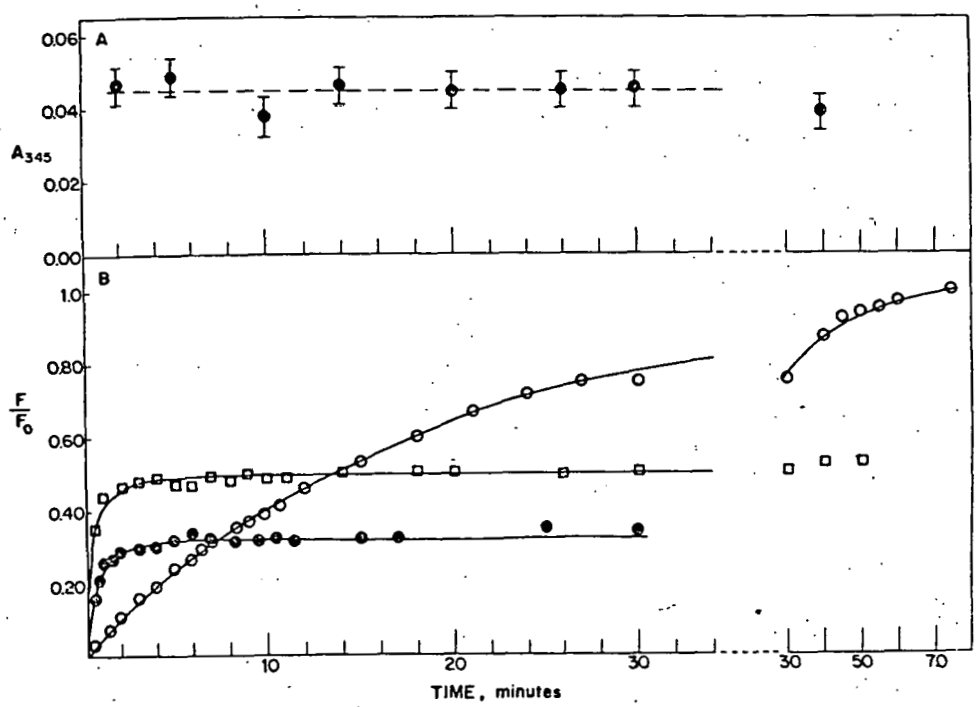

Fig. 8 (A) Time course of covalent binding of BPDE to DNA, as measured by absorbance at 345 nm (after thoroughly extracting the DNA mixture with organic solvents, only covalently bound PAH products remain). $[DNA] = 2.1 \times 10^{-3}M$.

(B) Hydrolysis of BPDE (non-fluorescent) to BPT (fluorescent)-relative fluorescence intensity as a function of time; (a) $[DNA] = 0$; (b) $[DNA] = 7.6 \times 10^{-4}M$; (c) $[DNA] = 1.52 \times 10^{-3}M$. At t=0, BPDE is mixed into the DNA solutions.

As is evident, in the absence of DNA, BPDE persists for more than 70 minutes. In the presence of DNA, however, the decomposition of BPDE is markedly accelerated. Hydrolysis is complete within less

than two minutes; the covalent binding is constant after two minutes, or more, showing that after this time interval there is no longer any diol epoxide present. At 0°C, both the hydrolysis and the covalent binding reactions are sufficiently slowed down (the reaction reaches its limiting value after 7 minutes) so that the rate of fluorescence increase and the time dependence of the covalent binding can be compared. At this lower temperature there is indeed a direct relationship between these two quantities, confirming the above conclusion that the fluorescence yield is an indirect indication of the amount of unreacted BPDE remaining; when this yield reaches its maximum value after some time, the concentration of BPDE is zero.

The electric linear dichroism spectra (ΔA) of the reaction mixture before extraction with an organic solvent (negative ΔA, solid line), and after extraction (positive ΔA, dashed line), are shown in Fig. 9.

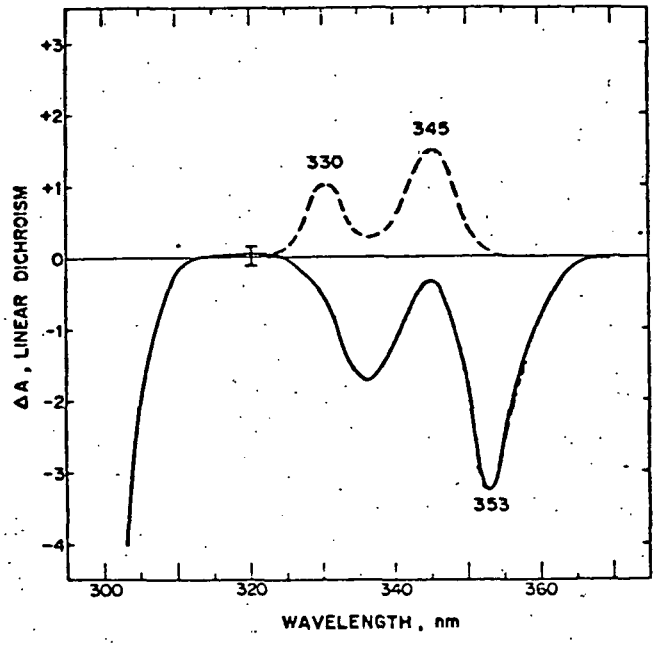

Fig. 9. Electric linear dichroism spectra of aqueous BPDE-DNA aqueous solutions at equilibrium. ___, before extraction, with organic solvents, $\Delta A < 0$, due to physically bound tetraols mostly; --- after extraction, $\Delta A > 0$ due to covalently bound BPDE only.

Extraction of the aqueous reaction mixture with an organic solvent (ether) removes all of the physically bound PAH molecules, so that only covalently bound products remain after the extraction. It is shown in detail elsewhere (37) that ΔA is negative for intercalation complexes (binding site I), and is positive for external binding site (II). Figure 9 thus demonstrates that the BPT tetraols bind preferably by an intercalation mechanism, a fact which has been verified (55), and that the covalent adduct has a site II (external) conformation.

We now return to the question of the accelerated decomposition of BPDE in the presence of DNA, shown in Fig. 8(IB). One might suppose that this accelerated reaction rate of BPDE in the presence of

DNA is due to the covalent reaction of BPDE with DNA, a reaction pathway which is not available in a DNA-free buffer solution. However, we have shown that only 7% or less of the BPDE initially added to the DNA solutions reacts covalently with the bases. Thus, the primary effect of the DNA is to catalyze the BPDE to its tetraol BPT; if it were not for this remarkable catalytic effect, the reactivity of BPDE with DNA by covalent bond formation might be considerably more extensive than the 0.07-0.25% modification levels obtained in the experiments of Fig. 8A (% modification refers to the percentage of the DNA bases which have reacted with BPDE by covalent bond formation).

In order to gain insight into the mechanisms of covalent binding of BPDE to DNA, and of the catalytic effect of DNA on the hydrolysis of BPDE, we studied the dependence of these processes on DNA concentration. However, since the lifetime of BPDE in the presence of DNA is less than a few minutes (Fig. 8B) we utilized a stopped-flow rapid mixing apparatus to study the reaction kinetics.

2. Rates of hydrolysis of BPDE as a function of DNA concentration. Since the appearance of BPT due to hydrolysis of BPDE gives rise to an increase in the fluorescence yield, we measured the hydrolysis rate of BPDE by means of the fluorescence stopped flow technique. In this method, two solutions, one containing BPDE only (in buffer), and the other one containing DNA only, are mixed rapidly by a special hydraulically operated plunger apparatus (Aminco-Bowman). Since the mixing time is about 5 ms, the reaction can be followed at longer times only. A typical set of results which are obtained at different DNA concentrations are shown in Fig. 10.

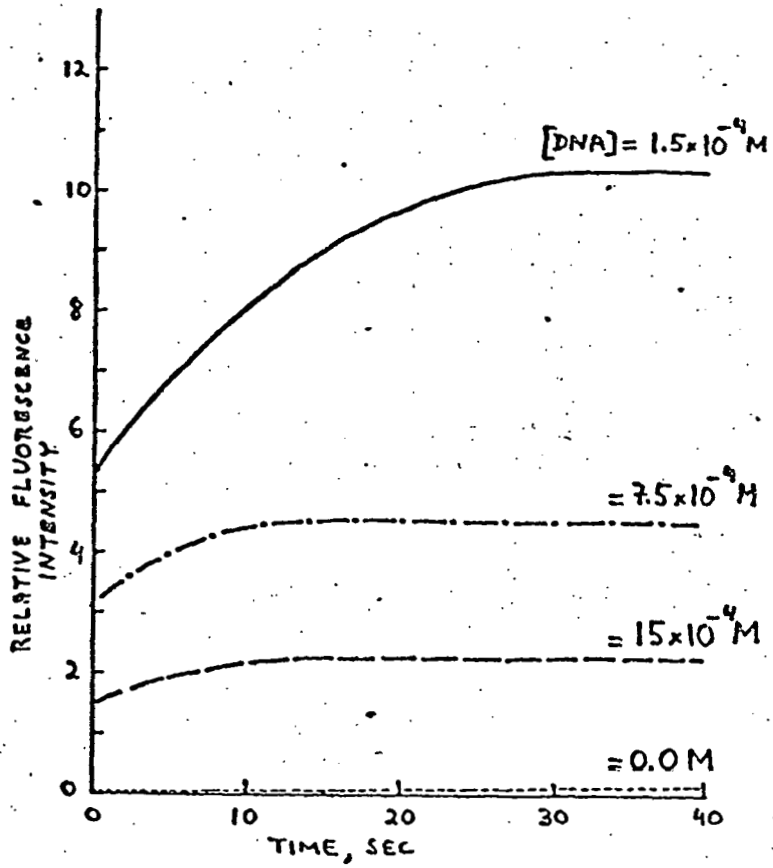

Fig.10. Rapid mixing stopped-flow fluorescence experiments. Two solutions, one containing BPDE in buffer only, the other containing DNA in buffer, are rapidly mixed at t = 0. As BPDE is hydrolyzed to BPT the fluorescence intensity increases. Note the comparatively low rate of hydrolysis at zero DNA concentration (fresh BPDE sample, see text)

The initial fast rise at t = 0 is due to the presence of BPT (due to partially hydrolyzed) BPDE in our samples even before mixing. BPDE is difficult to store in stable form and hydrolyzes to tetraols gradually upon standing. The increase in the fluorescence signal for $t > o$ is due to further hydrolysis of BPDE to BPT catalyzed by DNA. These rates are not affected by the presence of hydrolysis products. When freshly prepared samples of BPDE are used, the initial fluorescence yield at t = 0 is negligible (as indicated by the [DAN] = 0 curve in Fig. 10).

As suggested already by the similar (but slower mixing time!) experiments in Fig. 8B, the time required for complete hydrolysis of BPDE decreases with increasing DNA concentration. The steady-state fluorescence level decreases with increasing DNA concentration as well; this particular result is easily understood in view of our previous findings that when BPT binds to DNA by intercalation (site I), its fluorescence is completely quenched. (55). Thus, DNA catalyzes the hydrolysis of BPDE to BPT, which subsequently binds to DNA and which then gives rise to the decreased fluorescence yield. As the DNA concentration is increased, less free, fluorescent BPT is present.

The slow phase of the fluorescence rate increase follows first-order kinetics, and the rate constant is dependent on the DNA concentration (Fig. 11); it is therefore a pseudo-first-order rate constant. The shape of this curve is such that the rate constant reaches a constant value at DNA concentrations of $\approx 7.5 \times 10^{-4}$M.

The shape of this curve is reminiscent of a binding curve in which the concentration of bound adducts, C_b, varies with DNA concentration as follows:

$$C_b = C_o \frac{K[DNA]}{1 + K[DNA]} \qquad (4)$$

where C_o is the total amount of ligand present (bound plus unbound), and K is the equilibrium constant.

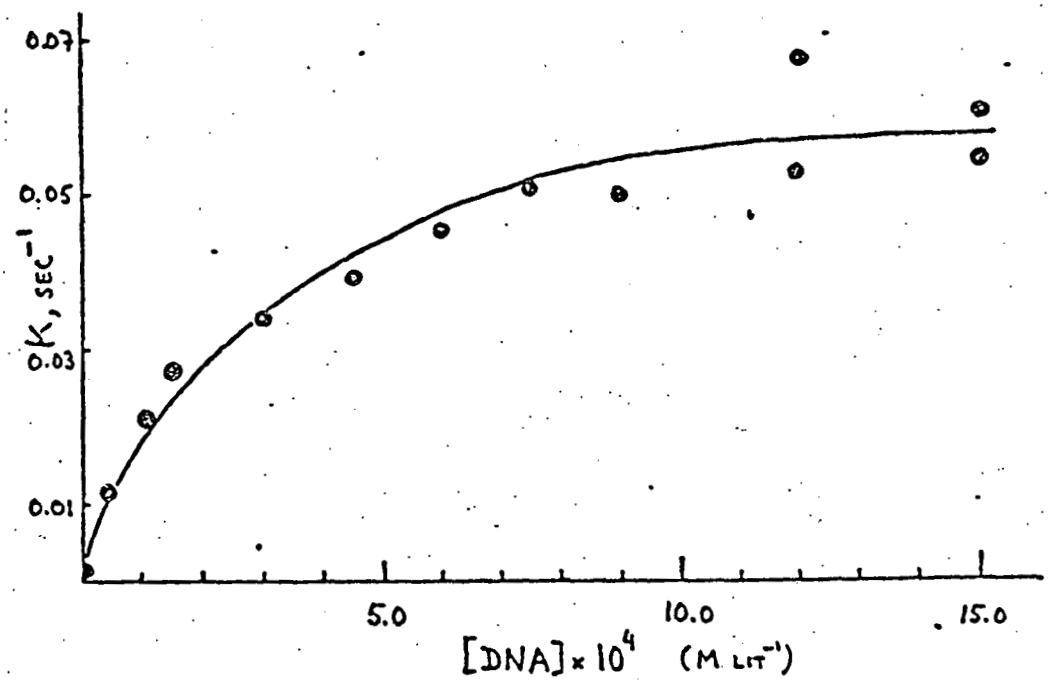

Fig. 11. Dependence of pseudo-first-order rate constant (K) on DNA concentration. DNA-catalyzed hydrolysis of BPDE to its tetraol BPT. (Data from experiments as shown in Fig. 10).

We thus conclude that the hydrolysis reaction (Fig. 8B) is mediated by a complex formation between DNA and BPDE , which then results in the hydrolysis of the latter to BPT.

This conclusion suggests that additional information might be obtained by utilizing the stopped-flow apparatus in the absorption mode.

3. Physical binding of BPDE to DNA by intercalation. We have shown that the tetraol BPT, when it physically binds to DNA by intercalation, displays a red-shifted absorption maximum at 353 nm. It is reasonable to suppose that the diol epoxide might also bind physically to DNA by such an intercalation mechanism, even though covalent binding does not occur at such sites (I).

We have measured the rate of appearance of the absorbance peak at 353 nm as a function of time in our stopped-flow apparatus. The results are shown in Fig. 12.

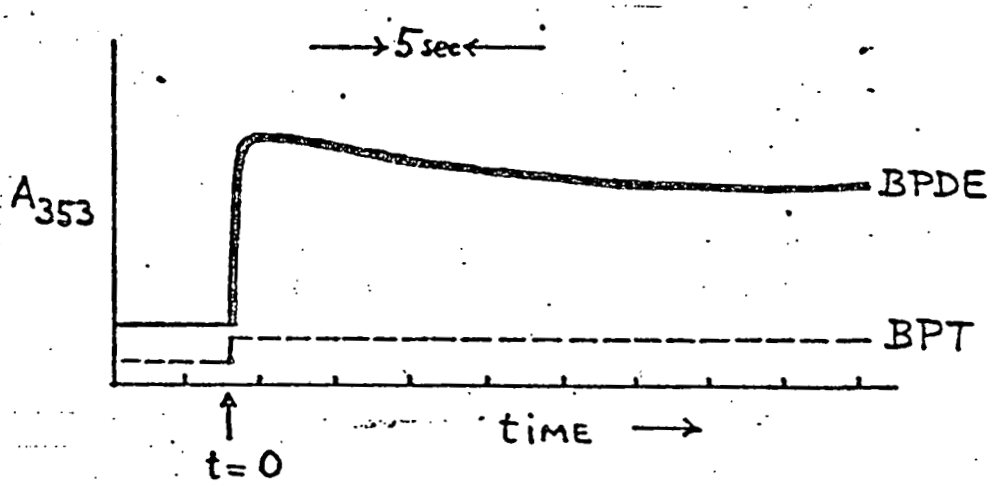

Fig. 12. Appearance of absorbance at 353 nm (A_{353}) due to intercalation of either the diol epoxide (BPDE) or its tetraol (BPT) in separate mixing experiments (at t = 0). concentration of BPDE and BPT=3.5 x 10^{-6}M; [DNA] = 1.5 x 10^{-4}M.

Within 5 ms of the mixing time, there is a rapid increase in absorbance at 353 nm, followed by a small but definite decrease which levels off at about 20-30 sec. This slow phase is reminiscent of the one observed in the time dependent fluorescence curve (top curve in Fig. 10). The rapid phase is due to the rapid intercalation of BPDE into DNA. In the case of BPT only the fast intercalation phase is observed, while the slow phase in the BPDE curve is due to the conversion of BPDE to BPT. It appears from Fig. 12 that BPDE binds more strongly to DNA than BPT, since the same concentrations were used for both molecules (the extinction coefficients of BPT and BPDE are the same within 5%).

The results shown in Fig. 12 indicate that binding of both BPT and BPDE occur on timescales faster than 5 ms.

The dependence on DNA concentration of A_{353} in the BPDE-DNA mixing experiment of Fig. 12 has also been studied, and the results are shown in Fig. 13.

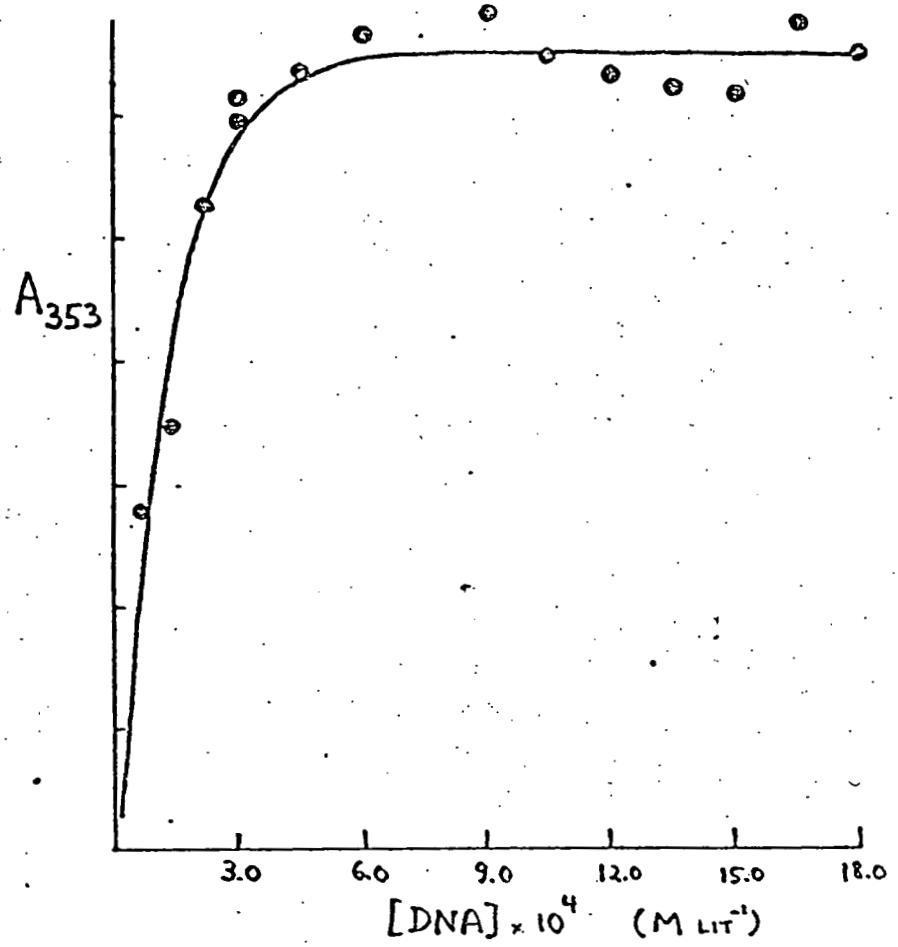

Fig. 13. Intercalation of BPDE into DNA as monitored by the appearance of the absorption at 353 nm (A_{353}) as a function of DNA concentrations. (Stopped-flow absorption experiment).

Again, the shape of a typical complex formation curve (equation (4)) is apparent, exhibiting the typical saturation behavior at high DNA concentrations.

4. Covalent binding of BPDE: concentration dependence. Both, the slow and the fast hydrolysis mechanisms of BPDE catalyzed by DNA appear to be mediated by physical complex formation between BPDE and DNA. It is therefore of interest to establish the DNA concentration dependence of the formation of the covalent adduct. A typical result is shown in Fig. 14.

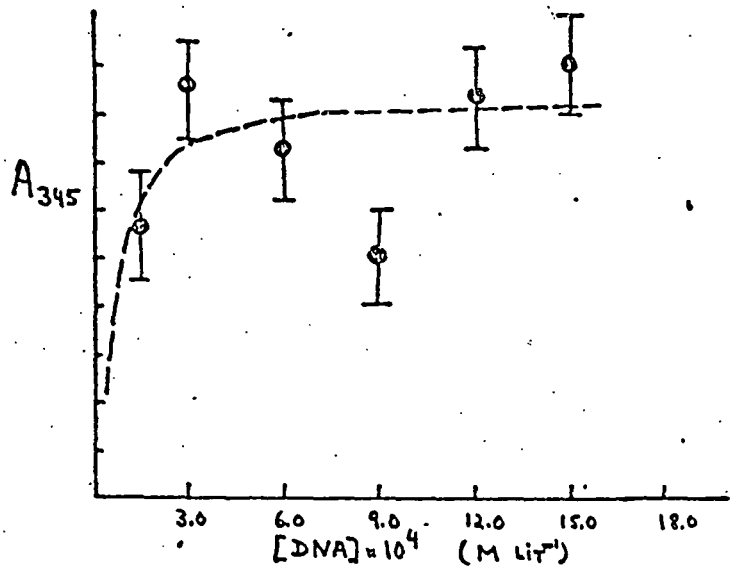

Fig. 14. Extent of covalent binding of BPDE (as measured by the absorbance maximum at 345nm of the covalently bound BPDE moeity, A_{345}) as a function of DNA concentration

While the error bars in this experiment are quite large, it appears that saturation of binding does occur at high concentration. This is not too different from the physical intercalation binding curve of Fig. 11. Thus physical binding of BPDE precedes covalent bond formation.

F. Conclusions

One hypothesis for the mechanism of covalent reaction between BPDE and DNA is that this process is preceded by intercalation, i.e. a non-covalent complex formation. If this is the case, it is not clear how the covalent adduct which is subsequently formed, winds up at the external binding site as is observed experimentally. A more attractive hypothesis is that when BPDE is rapidly mixed with DNA, non-covalent binding occurs at both sites I (intercalation) and site II (external); rapid hydrolysis to BPT occurs at site I without covalent bond formation, while covalent adduct formation occurs at site II. The reasons for these differences may well be due to stereoselective factors for these two types of binding sites. It is evident that additional information will be required to differentiate between these two possibilities.

G. PUBLICATIONS RESULTING FROM THIS CONTRACT

(a) "Fluorescence Properties of a Benzo(a)pyrene 7,8 Dihydrodiol 9,10-oxide-DNA Adduct. Conformation and Effects of Intermolecular DNA Interactions". T. Prusik and N.E. Geacintov. Biochem. Biophys. Res. Commun. 88, 782 (1979).

(b) "Kinetics of Hydrolysis to Tetraols and Binding of Benzo(a)pyrene - 7,8-Dihydrodiol - 9,10-Oxide and its Tetraol Derivatives to DNA. Conformation of Adducts". N.E. Geacintov, V. Ibanez, A.G. Gagliano, H. Yoshida and R.G. Harvey, Biochem. Biophys. Res. Commun. 92, 1335 (1980).

(c) "Physical Binding of Tetraols Derived from 7,8-Dihydroxy-9,10-epoxybenzo(a)pyrene to DNA". V. Ibanez, N.E. Geacintov, A.G. Gagliano, S. Brandimarte and R.G. Harvey. J. Am. Chem. Soc. 102, 5661 (1980).

In preparation

(d) "Physico-chemical Properties of Covalent Benzo(e)pyrene Diol Epoxide-DNA Adducts. Spectroscopic Studies of Conformation". A.G. Gagliano, N.E. Geacintov, v. Ibanez and R.G. Harvey to be submitted to Photochem. Photobiol. (11/80).

(e) "The Triplet Excited State as a Probe of the Binding Dynamics of Polynuclear Aromatic Dyes to DNA in Solution". N.E. Geacintov, T. Kolubaev and J. Waldmeyer, to be submitted to Biophys. J. (12/80).