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Cleavage of DNA by the Insulin-Mimetic Compound, $NH_4[VO(O_2)_2(phen)]$[†]

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ABSTRACT: The kinetics and mechanism of cleavage of DNA by the insulin-mimetic peroxo-vanadate $NH_4[VO(O_2)_2(phen)]$, pV, are described. In the presence of low energy UV radiation or biologically common reducing agents, pV decomposes into the monomer, dimer, and tetramer of vanadate and an uncharacterized compound of V^{4+} as shown by ^{51}V NMR, ESR, and absorption spectra. The rate of photodecomposition of pV is reduced in the presence of calf thymus DNA, indicating that a decomposition product of the peroxo-vanadate, that is important in the destruction pathway of the complex, is interacting with DNA. This species, probably a short-lived complex of V^{4+}, may also be responsible for the observed catalytic decomposition of pV in the absence of DNA by ascorbate. If closed circular pBR322 DNA is present when the peroxo-vanadate is destroyed by either UV radiation or reducing agents, the polymer may have its sugar-phosphate backbone broken. Closed circular DNA (form I) is converted into nicked circular DNA (form II) and linear DNA (form III). The amounts of the various forms produced as a function of irradiation time and peroxo-vanadate concentration were fit to a kinetic model to derive rate constants for the conversions. The kinetic analysis shows that pV is a single-strand nicking agent which exhibits some base and/or sequence preference. Furthermore, the pH dependences of the rates for conversion of form I to form II and for conversion of form II to form III are different, indicating that the nature of the chemistry at the site of cleavage on DNA influences further cutting by activated pV. Reduced amounts of DNA breakage in the presence of various salts and metal binding ligands indicate that a short-lived reactive complex of V^{4+}, not the V^{4+} species detected by ESR at long irradiation times, is important in the cleavage process. The susceptibility of pV to decomposition by biologically common reducing agents suggests that metabolites of the agent, and not the compound itself, are responsible for its insulin-mimetic effects.

The peptide hormone insulin is widely used to treat diabetes. While the compound is successful in regulating glucose levels in the body, the fact that it must be given by injection stimulated interest in finding alternatives to insulin which can be taken orally. In 1979 it was reported that solutions of vanadate can affect glucose metabolism (Tolman et al., 1979). Since vanadate salts can be administered orally, this report prompted investigation of the insulin-mimetic properties of a large number of vanadium compounds (Shechter 1990; Shechter et al., 1990; McNeill et al., 1992; Shaver et al., 1993, 1995; Posner et al., 1994).

It is known that vanadate, V^{5+}, mixed with hydrogen peroxide can augment the binding of insulin growth factors to rat adipocytes and activate the insulin receptor kinase (Kadota et al., 1987; Fantus et al., 1989). Since addition of hydrogen peroxide to vanadate produces a mixture of compounds (Jaswal & Tracy, 1991), well characterized complexes containing V^{5+} bound to peroxide ligands were synthesized and their insulin-mimetic properties studied (Shaver et al., 1993; 1994, 1995; Posner et al., 1994; Fantus et al., 1994; Bevan et al., 1995). It is known that vanadate is very likely reduced to vanadyl, V^{4+}, in the body (Sakurai et al., 1980). This observation stimulated considerable interest in the insulin-mimetic properties of compounds containing V^{4+} bound to malonate, salicylate, hydroxamate, and other organic ligands (McNeill et al., 1992; Yuen et al., 1993a,b; Dai et al., 1993; Shechter et al., 1992; Orvig et al., 1995).

Both diabetes and cancer are characterized by aberrant metabolic processes, and it is not surprising that vanadium compounds also exhibit antitumor effects. Complexes of V^{5+} which contain peroxo and polycarboxylate ligands show antitumor activity against L1210 leukemia implanted in mice (Djordjevic & Wampler, 1985; Djordjevic, 1995). Toxicities of the compounds vary widely from a few mg/kg up to 160 mg/kg. Although the molecular basis for the antitumor properties is not known, the compounds may undergo decomposition *in vivo*, producing V^{4+} and superoxide. The latter could change the free radical character of the cellular environment, ultimately causing the cell to die (Djordjevic & Wampler, 1985).

Antitumor effects have also been found for compounds containing V^{4+}. Dietary vanadyl sulfate is able to block nitrosourea-induced mammary carcinogenesis in mice (Thompson et al., 1984), and at very low concentrations in cell culture ($\sim 10^{-10}$ M) the compound inhibits the growth of human tumor specimens (Hanauska et al., 1987). Biodistribution studies in rats show that V^{4+} and V^{5+} accumulate in the nuclei of liver cells (Sakurai et al., 1992). It has been proposed that the antitumor effect of V^{4+} is due to its oxidation by cellular hydrogen peroxide, which releases hydroxyl radical. In support of this hypothesis is the observation that vanadyl sulfate and hydrogen peroxide can break Col E1 DNA and that hydroxyl radicals are generated during the cleavage process (Sakurai et al., 1992). The

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vanadyl complex, $[VO(phen)(H_2O_2)_2]^{2+}$, shows high antitumor activity toward human nasopharyngeal carcinoma (Sakurai et al., 1995). The compound may exhibit its cytotoxic effects by binding to DNA and, in the presence of hydrogen peroxide, cleave the polymer.

While vanadium salts have potential for treating serious diseases such as diabetes and cancer, they are also widely taken by those interested in "body building" and muscle development. Vanadyl sulfate in tablet form mixed with vitamins, herbs, and other agents is a dietary supplement available at most health food stores. The product is thought to increase glucose uptake by muscle cells, but the biological fate of the vanadium, and any long term health effects from extended use of the supplements, are unknown (Domingo et al., 1995).

In addition to their importance as pharmaceuticals, vanadium compounds have potential for probing the structure of biological molecules such as proteins and DNA. The simple vanadate oligomers can photocleave myosin, dynein, and tubulin in a site-specific manner (Gibbons & Mocz, 1991; Cremo et al., 1991; Correia et al., 1994). Although the mechanism of cleavage is not completely characterized, photolysis in the presence of vanadate oxidizes specific serine residues of the protein to serine aldehyde. This ultimately causes a break in the peptide backbone of the protein at the site of modification. DNA is also a target for cleavage by vanadium compounds. The antitumor agents, $[VO(phen)(H_2O)_2]^{+2}$ and vanadyl bleomycin, can break the sugar-phosphate backbone of DNA in the presence of hydrogen peroxide (Sakurai et al., 1995; Kuwahara et al., 1985). In the case of vanadyl bleomycin, cleavage most often occurs at GA sequences of DNA.

The pharmacological and biochemical importance of vanadium compounds, and the fact that there are many potential receptor sites for vanadium salts in the body (Chasteen, 1995), prompted us to investigate the reactivity of $NH_4[VO(O_2)_2(phen)]$, where phen is 1,10-phenanthroline, pV, with DNA. The peroxo-vanadate is similar in structure to compounds earlier tested against L1210 leukemia (Djordjevic & Wampler, 1985), and it is also a member of a class of V^{5+} peroxocompounds which exhibit insulin-mimetic effects (Posner et al., 1994; Bevan et al., 1995). In a preliminary report we used ^{51}V NMR and absorption to show that low energy UV light and NADPH decompose pV into vanadate and that DNA present during the decomposition will have its sugar-phosphate backbone broken (Hiort et al., 1995). In this report we study the kinetics and mechanism of the reaction by which pV cleaves pBR322 DNA in the presence of UV light, inhibitors, and chemical reducing agents.

MATERIALS AND METHODS

Materials. Ammonium vanadium oxide (99.995%) was purchased from Johnson Matthey Materials Technology, U.K., and 1,10-phenanthroline was obtained from Aldrich Chemical Co. $NH_4[VO(O_2)_2(phen)]$, pV, was synthesized according to Vuletic and Djordjevic (1973) which is similar to the procedure of Posner et al. (1994). The elemental analysis was performed by Oneida Research Services Inc. Anal. Calcd for $NH_4[VO(O_2)_2(phen)]\cdot 2H_2O$: C, 39.9; H, 4.4; N, 11.5. Found: C, 39.22; H, 4.38; N, 11.22. The purity of the compound in D_2O was checked using ^{51}V NMR. Solutions of pV were freshly prepared for the various experiments and used within 24 h of preparation. The solid was stored for extended periods of time (months) in the dark at room temperature. Concentrations were determined optically at 270 nm using a molar absorptivity of 25 800 M^{-1} cm^{-1}. All absorption spectra were obtained with a Varian Cary 1 spectrophotometer in quartz cells with a 1-cm path length.

pBR322 plasmid DNA was purchased from Boehringer Mannheim Biochemical, USA, and ethanol precipitated prior to use to remove EDTA and Tris present in the storage buffer. Calf thymus DNA, obtained from Sigma Chemical Co. (St. Louis), was used without purification. The concentration of calf thymus DNA, in DNA phosphates, was determined optically at 258 nm using a molar absorptivity of 6 600 M^{-1} cm^{-1}. The stated DNA concentrations are the final concentrations in mixtures. Unless otherwise noted, all studies were carried out in phosphate buffer (0.1 M sodium phosphate, mono- and diionic forms, pH 6.0), hereafter referred to as buffer. Cleavage as a function of pH was studied in 0.1 M phosphate buffer using a number of pH values stated below. The *tert*-butyl alcohol, salts, and chemical activators used in the DNA cleavage studies were reagent grade. Buffers were from Sigma Chemical Co. (St. Louis).

Spectroscopy. One-dimensional ^{51}V NMR spectra were recorded at 65.81 MHz on a Bruker 250 MHz NMR spectrometer using a 10-mm tunable probe either in phosphate buffer containing 50% D_2O or in neat D_2O. Samples were run at ambient temperature with a deuterium lock. Parameters were as follows: pulse angle 90°, sweep width 100 000 Hz, acquisition time 0.005 s, with no relaxation between pulses. The chemical shifts are reported relative to the external standard $VOCl_3$ (0 ppm). No exponential multiplier was used; an 8K data set was collected and zero-filled to 16K. The data were processed on a Sunstation using the NMR 1 software developed by New Methods Research, E. Syracuse, NY.

The ESR spectrum of the V^{4+} species was obtained using a Varian E-9 spectrometer operating at X-band frequency. The instrument settings were as follows: magnetic field, 3220 G; microwave frequency, 9.515 GHz; microwave power, 10 mW; modulation amplitude, 20; scan range, 2000 G; time constant, 0.3 s; scan times, 2 min. Spectra were run at ambient temperature in water. The magnetic field was calibrated using a value of the g tensor for DPPH of 2.0036 (McMillan, 1968).

Photolysis. Samples, contained in Exax brand flint glass test tubes (5-mm path length), NMR tubes (1-cm path length), or quartz cuvettes (1-cm path length), were irradiated at a distance of 30 cm using a Blak Ray 100 W long-wave ($\lambda_{max} = 365$ nm) UV mercury lamp. The relative amounts of UV intensity incident at various points on the area illuminated by the lamp were determined by placing glass test tubes containing identical amounts of pBR322 DNA and pV in buffer at various points on the area of illumination. Analysis of the amounts of cleaved DNA in the tubes using the procedure described below revealed the area of constant illumination intensity. All irradiation experiments were carried out with samples located in this area.

Decomposition of pV. A D_2O solution (4 mL) containing 25 mM pV in the absence or presence of calf thymus DNA (8.3 mM base pairs) in a 10-mm NMR tube was irradiated using low energy UV radiation. The composition of the solutions as a function of irradiation time was determined

using ^{51}V NMR and absorption spectroscopy. The decomposition of pV using chemical reducing agents in the absence of DNA was also studied. A solution containing 0.7 mM sodium ascorbate and 25 mM pV in 4 mL of D_2O was prepared and the composition of the solution as a function of time studied with ^{51}V NMR.

Cleavage of pBR322 DNA by pV Using UV Radiation. The cleavage reactions were carried out in a total volume of 25 μL containing 25 μM pBR322 DNA in buffer. Irradiations were for 30 min using various concentrations of pV: 1, 2, 3, 5, 6, 8, 10, 12, 15, 17, 20, 24, and 32 μM. In a second series, 25 μM DNA in the presence of 10 μM pV was irradiated for various times: 2, 4, 6, 8, 10, 12, 14, 16, 18, 22, 25, and 30 min. The DNA forms present in the reaction mixtures were separated using electrophoresis in a 1% agarose gel. After staining with an ethidium bromide solution (0.5 μg/mL) for 45 min, the gel, without destaining, was placed on a UV transilluminator and photographed with a Polaroid camera using type 55 film. The exposure time was such that the maximum intensity of all bands was in the linear response range of the film (Ribeiro et al., 1989; Prunell et al., 1977). Linearity of measured intensity with DNA concentration was checked by loading different amounts of DNA in the gel and, after electrophoresis, staining, and photography, constructing plots of band intensity versus DNA concentration. The photographic negative was scanned with a Molecular Dynamics (Model 300A) microdensitometer to obtain volume integrations of bands. With these staining conditions, closed circular DNA (form I) stains with the same efficiency as the other two forms of pBR322 DNA (Shubsda et al., unpublished results). Since linear DNA (form III) was not degraded to smaller fragments in any of the experiments, errors due to loading, nonuniform illumination of the gel, and optical effects due to the camera were corrected by normalizing the total intensity of the three DNA forms in a given lane on the gel to a constant value. This correction was less than 15% of the total intensity in a given lane.

In order to show that the reactive species responsible for cutting was short-lived, irradiations of pV in the absence of DNA at various times were carried out. Addition of DNA to the irradiated sample immediately after its removal from the irradiation device did not result in DNA strand scission (data not shown).

The efficiency of photocleavage at various values of pH (4.5, 5.0, 5.5, 6.0, 6.5, and 7.0) in 0.1 M phosphate buffer was studied by irradiating solutions containing 25 μM pBR322 DNA and 0, 5, 10, and 15 μM pV, at each value of pH, for 30 min. Cleavage experiments in buffer (pH 6.0) were also performed using 20 μM pV and 25 μM pBR322 DNA in the presence of trapping agents and salts. The salts, NaCl (0−1500 mM) or NaI (0−1500 mM), and the trapping agents, *tert*-butyl alcohol (0−4 mM) and thiourea (0−4 mM), were added prior to pV and the resulting solutions irradiated for 30 min.

Cleavage of pBR322 DNA with pV Using Chemical Activators. Cleavage of pBR322 DNA (25 μM) with pV (10 μM) in buffer was carried out in the dark using the chemical activators NADPH (0−10 mM), glutathione (0−10 mM), and L-cysteine (0−10 mM). After 30 min, the reactions were quenched by ethanol precipation, the sample was redissolved in distilled water, and the product distribution was determined using quantitative agarose gel electrophoresis. While sodium ascorbate could also activate pV to cleave DNA, the ability of ascorbate to cleave DNA in the absence of pV complicated quantitative studies with this reducing agent.

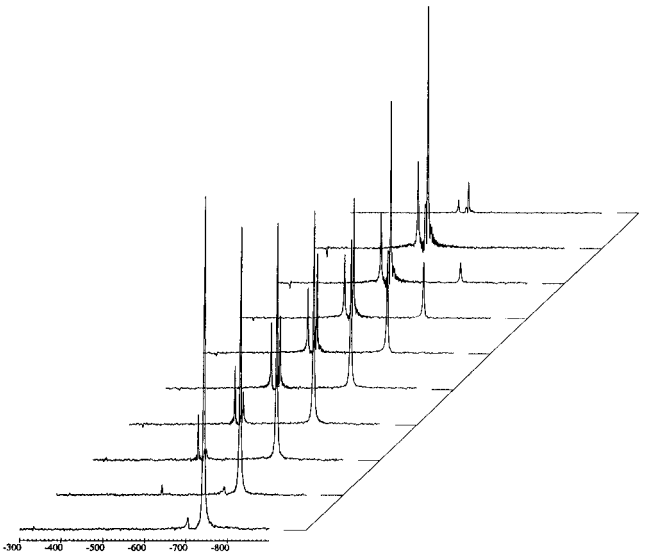

FIGURE 1: Analysis by ^{51}V NMR of the photodecomposition of 25 mM pV in D_2O. The peak at −744 ppm corresponds to pV while the signals at −558, −571, and −576 ppm are due to the monomer, dimer, and tetramer of vanadate, respectively. The samples were irradiated for 0, 45, 85, 120, 180, 225, 270, 335, 480, and 600 min.

RESULTS AND ANALYSIS

The first experiments described had the goal of characterizing the decomposition of pV induced by UV radiation or various biochemical agents. In particular, information was sought about intermediate and final products of decomposition.

Photolysis. Figure 1 shows the ^{51}V NMR spectrum of pV in D_2O before (bottom panel) and after different times of irradiation (t_{irr}). The complex exhibits a single narrow ($\Delta\delta = 4$ ppm) peak located at −744 ppm arising from the diamagnetic V^{5+} ion. When the pV solution is irradiated with UV light, the metal complex is slowly converted into three new diamagnetic V^{5+} species at −576, −571, and −558 ppm, which have earlier been assigned to the tetramer (cyclic $V_4O_{12}^{2-}$, V_4), dimer ($H_2V_2O_7^{2-}$, V_2) and monomer ($H_2VO_4^-$, V_1) of vanadate, respectively (Crans, 1994). The ratio between the products changes, as expected, in favor of the tetramer as the irradiation time is increased. As decomposition occurs, the yellow color of the solution fades, gas evolution is observed, and the solution becomes slightly alkaline, pH = 8.4. The fact that these are the only vanadium species observed, even at $t_{irr} = 25$ h (results not shown) when the signal for pV has completely disappeared, shows that the only diamagnetic end products in the photoreaction are simple vanadates. At very long irradiation times ($t_{irr} > 25$ h) the ^{51}V NMR base line becomes very distorted, indicating the presence of a paramagnetic V^{4+} species in solution. The solution also changes color from yellow to light brown, and a fine dark precipitate forms (possibly $VO(OH)_x$).

When calf thymus DNA is present in a D_2O solution containing pV ([pV]/[DNA] = 3), the photoconversion of pV into the simple vanadates is slowed significantly (see Figure 2). This indicates that at least one of the species

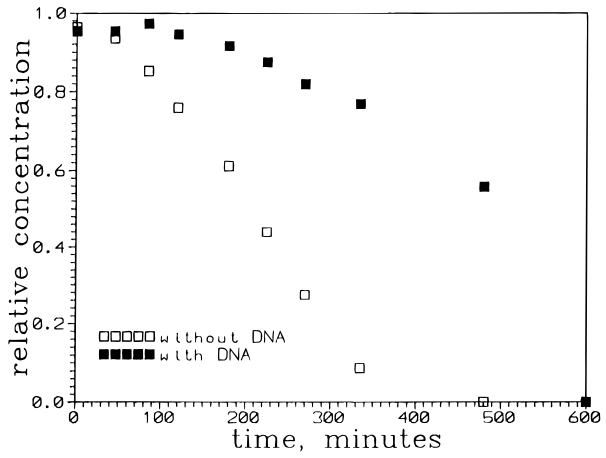

FIGURE 2: Relative concentration of pV (25 mM) as a function of irradiation time in the absence (spectrum shown in Figure 1) and presence of calf thymus DNA, [pV]/[DNA] = 3. Relative concentration was determined using ^{51}V NMR from the area of the signal at −744 ppm for pV.

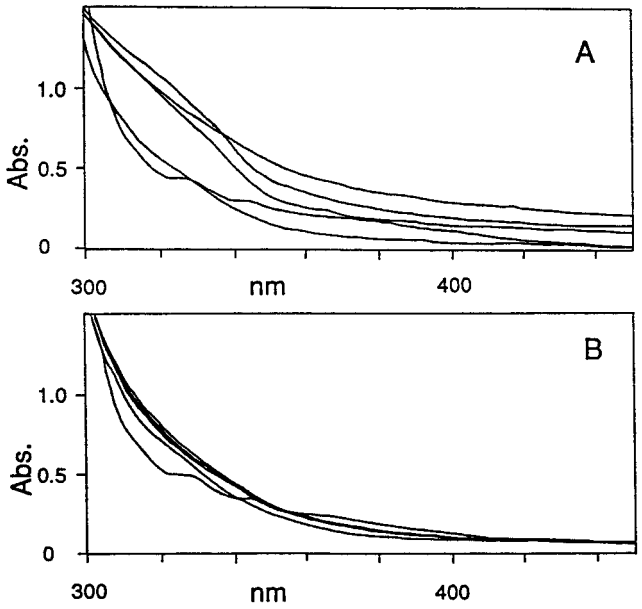

FIGURE 3: Absorption spectra of photolyzed pV in H_2O. (A) 0.35 mM pV alone. Individual spectra correspond to irradiation times (t_{irr}) of 0, 105, 170, 215, and 600 min in order of increasing absorbance at 450 nm, respectively. (B) 0.35 mM pV plus 0.7 mM calf thymus DNA. t_{irr} = 0, 105, 170, 215, and 600 min in order of increasing absorbance at 320 nm, respectively.

involved in the decomposition of pV is interacting with DNA. At a time for which ~25% of pV had decomposed in D_2O without DNA, no vanadate had formed in the presence of DNA. After 480 min, when no pV could be detected in the absence of DNA, ~60% of the V^{5+} in the solutions containing DNA remained as pV (Figure 2). No new ^{51}V NMR active species are formed in the pV/DNA solutions, either before irradiation or as a result of irradiation, as judged by the number of peaks and their chemical shifts. This shows that neither pV nor the simple vanadates interact with DNA, which is expected since these are all negatively charged species.

Irradiation of an aqueous solution of pV causes the shape and intensity of the peroxo LMCT bands in the region 310−345 nm (ϵ_λ ~900 cm^{-1} M^{-1}) to change considerably, as shown in Figure 3A, indicating that the vanadium complex undergoes a major chemical change as the result of photoirradiation. During the first hour, an isosbestic point is observed at ~330 nm. With increasing irradiation time, isosbestic behavior is lost, the absorption increasing monotonically over the region 340−600 nm. After 10 h of irradiation, the absorption is nearly twice as intense as the original spectrum. We attribute the new absorption to a V^{4+} complex formed by the photodecomposition of pV.

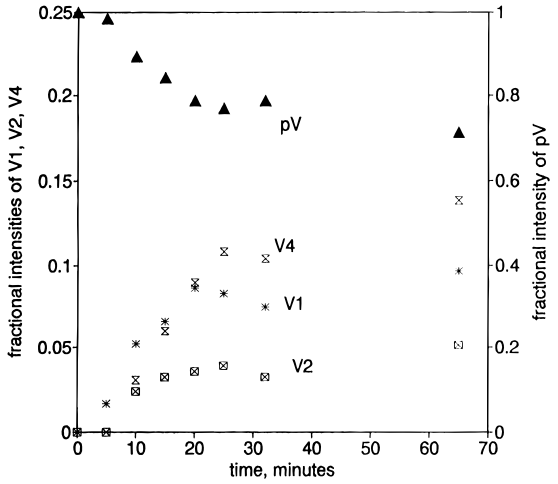

FIGURE 4: Fractional intensities of pV, V_1, V_2, and V_4 as a function of time after the addition of 0.7 mM sodium ascorbate to 25 mM pV in water. Fractional intensities were determined using ^{51}V NMR from the area of a signal divided by the sum of the areas of all signals.

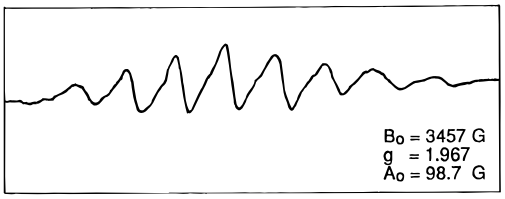

FIGURE 5: ESR spectrum of a V^{4+} complex produced by photodecomposition of 25 mM pV in water.

Decomposition by Ascorbate. Addition of 0.7 mM sodium ascorbate to 25 mM pV in D_2O also causes decomposition of the peroxo-vanadate into the simple vanadates. The decomposition was found to be catalytic with regard to ascorbate; one ascorbate is able to convert about 10 pV complexes into the vanadates. A plot of the area of the ^{51}V NMR signal from the V^{5+} complexes versus time shows that the reaction is complete after 30 min at room temperature, (Figure 4). Moreover, the rate of disappearance of pV is the same as the rates of appearance of V_1, V_2, and V_4, and at the end of the observation time the NMR base line is nonlinear, indicating the presence of V^{4+} (spectra not shown).

The fact that a V^{4+} complex is produced in the photodecomposition of pV was established using ESR. When pV (25 mM in H_2O) is irradiated extensively (>12 h), an ESR spectrum having the characteristic eight line pattern for the electron on V^{4+} interacting with the nuclear spin of ^{51}V ($I = {}^7/_2$) appears (Figure 5). The values obtained for the *g*-tensor and hyperfine coupling constant for the V^{4+} complex were 1.967 and 98.7 G, respectively. A V^{4+} species with similar ESR parameters has been observed in the V^{5+} catalyzed decomposition of H_2O_2 in water (Bonchio et al., 1994).

To learn more about the interaction of the pV photolysis products with DNA, pV was irradiated in the presence of

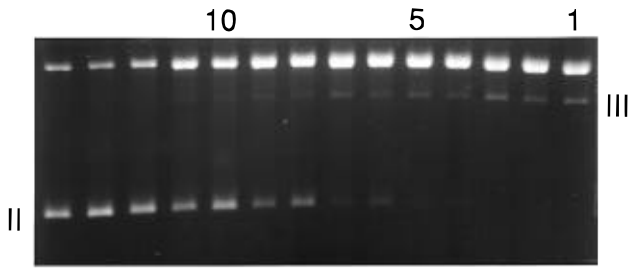

FIGURE 6: Photograph of an agarose gel stained with ethidium bromide showing cleavage of pBR322 DNA by pV as a function of irradiation time in 0.1 M phosphate buffer, pH 6. The forms of pBR322 DNA are shown at the sides of the photograph. Lane 1, 25 μM DNA alone; lane 2, 25 μM DNA alone, UV radiation (30 min); lanes 3−14, 25 μM DNA, 10 μM pV, UV radiation, irradiation times, increasing right to left, are given in the Materials and Methods section.

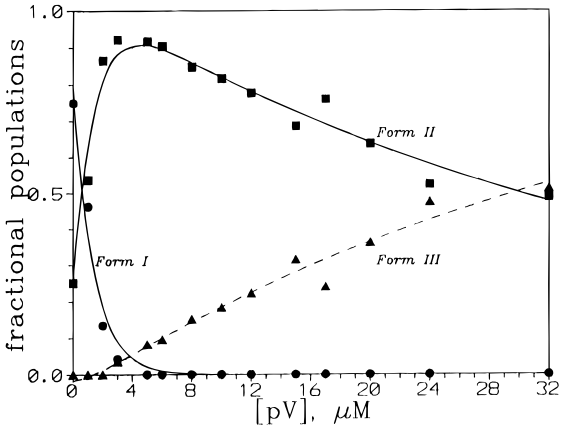

FIGURE 7: Kinetic analysis of photocleavage of 25 μM pBR322 DNA as a function of the concentration of pV in 0.1 M phosphate buffer, pH 6. The experimental points are fit to curves calculated using the kinetic model.

pBR322 DNA which exists in three forms. As shown in Figure 6, photoirradiation of samples containing pBR322 plasmid DNA and pV at various times results in the conversion of the closed circular form (form I) to the nicked circular (form II) and the linear (form III) forms. From the relative amounts of the forms, information about cleavage rate constants may be derived.

Analysis of DNA Cleavage Kinetics. The amounts of DNA formed as a function of pV concentration and time were found by measuring the fluorescence intensity of DNA in agarose gels stained with ethidium bromide. The observed intensities and fits to the data using the kinetic model are shown in Figures 7 and 8.

In deriving the kinetic model, we assume that chemical or photochemical activation of pV produces a species V* which is responsible for the cutting. We consider that V* may interact with the DNA by three second-order reactions: (a) it may nick form I (supercoiled) DNA, converting it into form II (nicked circular) DNA, with rate constant k_1 (single-strand cutting); (b) it may convert form II DNA, which has at least one break in one strand, into form III (linear) DNA by cutting the other strand sufficiently close to the preexisting nick, with rate constant k_2; (c) it may convert form I DNA directly to form III DNA with rate constant k_4 (double-strand cutting). The conversion of form II DNA to form III actually involves many second-order reactions because the conversion rate depends on how many nicks are present in form II DNA; k_2 should be taken as an average or effective rate constant.

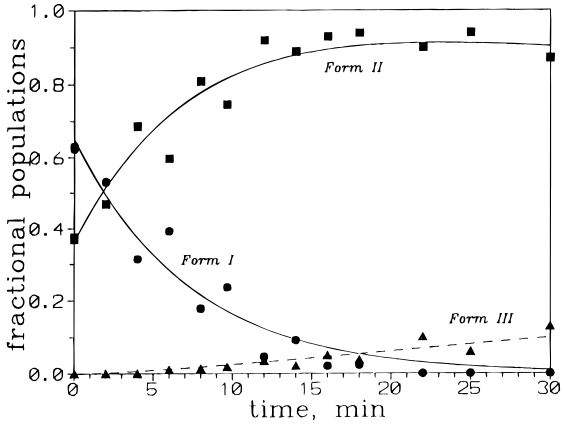

FIGURE 8: Kinetic analysis of photocleavage of 25 μM pBR322 DNA by 10 μM pV as a function of irradiation time (gel shown in Figure 6). The experimental points are fit to curves calculated using the kinetic model.

Eventually, linear DNA may be cut by V* producing smaller DNA fragments, with rate constant k_3, but there is no evidence for this occurring in the present case. If V* is destroyed only by its interaction with DNA, its concentration varies with time according to the following equation:

$$\frac{d[V^*]}{dt} = -(k_1[I][V^*] + k'_2[II][V^*] + k_4[I][V^*]) \equiv -\alpha[V^*] \quad (1)$$

The quantity k'_2, which is the rate constant for single-strand nicking of form II DNA by V*, should be much larger than k_2 because few of these nicking events occur close enough to a preexisting nick to cause conversion of form II to form III; one may expect k'_2 to be closer in size to k_1 than to k_2. Other processes which destroy V* would add a term, $-k_0[V^*]$, to the above expression for $d[V^*]/dt$. Equation 1 shows that [V*] decreases monotonically.

If the rate constants are about the same size, $\alpha = k_1[I] + k'_2[II] + k_4[I]$ will be approximately constant. Also, α is approximately constant for early times, when the concentrations of the three forms of DNA have not changed much; then, $[V^*] = [V^*]_0(1 - ct)$, with $[V^*]_0$ the initial concentration of V* and c a constant. In addition, [V*] will remain essentially constant (and α will be $\sim$0) if $[V^*]_0$ is much larger than the concentration of DNA cutting sites, and there are no mechanisms for destruction of V* other than DNA cleavage. In all of these cases, $[V^*]/[V^*]_0$ varies with time in a manner independent of $[V^*]_0$, i.e.

$$\frac{d \ln [V^*]}{dt} = -f(t)$$

Integrating,

$$[V^*] = [V^*]_0 e^{-F(t)} \quad (2)$$

where

$$F(t) = \int_0^t f(t')\, dt'$$

Now the concentration of form I DNA obeys

$$\frac{d[I]}{dt} = -(k_1 + k_4)[V^*]_0 e^{-F(t)}[I]$$

which, on dividing both sides by [I], can be integrated to

give

$$\ln([I]/[I]_0) = -[V^*]_0(k_1 + k_4)\, G(t) \qquad (3)$$

where

$$G(t) = \int_0^t e^{-F(t')}\, dt'$$

If the expression for [I] is substituted into the equation for d[II]/dt, one finds

$$[II] = [II]_0 e^{-[V^*]_0 k_2 G(t)} - \frac{k_1[I]_0}{k_1 + k_4 - k_2}[e^{-[V^*]_0(k_1+k_4)G(t)} - e^{-[V^*]_0 k_2 G(t)}] \qquad (4)$$

The differential equation for d[III]/dt may be treated similarly. One sees that the operative parameters are rate constants times $G(t)$, rather than the rate constants themselves.

Measurement of Rate Constants. In the present experiments, $[V^*]_0$ is not known, only the initial concentration of pV. The active species V* is generated from pV, and probably destroyed by interaction with oxygen and other species in solution as well as with DNA. It seems reasonable to consider V* as an unstable intermediate, so that d[V*]/dt is small compared to the rate of creation or the rate of destruction of V*. Since the rate of destruction is proportional to [V*] and the rate of creation is proportional to [pV], [V*] is proportional to [pV], say $[V^*] = k[pV]$. Of course, [pV] itself decreases with time during digest. In the expressions for [I], [II], and [III] above, $[pV]_0$ may be substituted for $[V^*]_0$, with the understanding that $G(t)$ is actually k times the true $G(t)$, where k is less than unity. If α is assumed to be small, $kG(t)$ is replaced by kt in the expressions.

This means that one cannot obtain the rate constants without additional assumptions. The quantities determined, which we refer to as k_1 and k_2, are actually k times the actual rate constants. However, ratios of rate constants, which are often of interest, can be obtained from experiments in which initial concentration of cleavage agent is varied with digest time t kept fixed, or from experiments in which t is varied with the concentration of cleavage agent held fixed.

A set of experiments of each type has been performed, with results given in Figures 7 and 8. The smooth curves in the figures correspond to intensities calculated according to the theoretical model given above. Values for the initial amounts of the various DNA forms ($[I]_0$, $[II]_0$, $[III]_0$) and three rate constant parameters were found to minimize the sum of the squares of the deviations between calculated and experimental intensities. In the variable-concentration experiments, the rate constant parameters are $k_1G(t)$, $k_2G(t)$, and $k_4G(t)$. In the variable-time experiments, we assumed $G(t)$ equal to t, so the rate constant parameters were $k_1[V^*]_0$, $k_2[V^*]_0$, and $k_4[V^*]_0$, with $[V^*]_0 = 10\ \mu M$. It was found that the fit to the experimental intensities was not much worsened by assuming $k_4 = 0$ in both experiments, which shows that there is no appreciable double-strand cutting. With $k_4 = 0$ (so there are five parameters: $[I]_0$, $[II]_0$, $[III]_0$, and two rate constants), the sum of the squares of the deviations was 0.072 for the variable-time experiments. For the variable-concentration experiments, the sum was much less, only 0.047, which suggests that the assumption $G(t) \sim t$ is not completely valid.

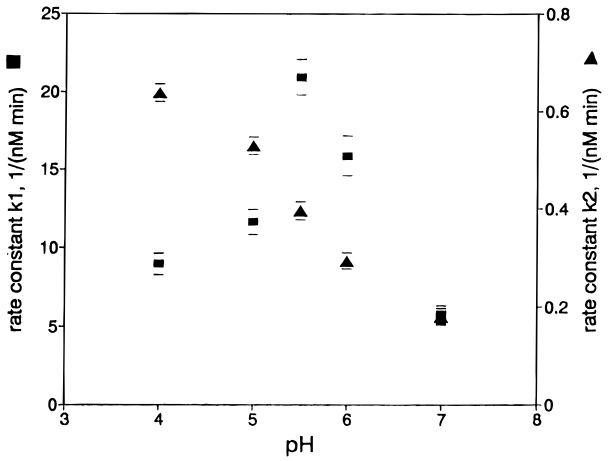

FIGURE 9: pH dependence, in 0.1 M phosphate buffer, of the rate constants for photocleavage of closed circular (form I) into nicked circular (form II), k_1, and form II into linear (form III), k_2, pBR322 DNA by pV.

The results for the rate constants from the variable-time experiments were $k_1 = 13.8 \pm 3.2\ (nM)^{-1}\ min^{-1}$ and $k_2 = 0.42 \pm 0.10\ (nM)^{-1}\ min^{-1}$, assuming $G(t) \sim t$. From the variable-concentration experiments, we obtain $k_1G(t) = 720 \pm 123\ (nM)^{-1}$ and $k_2G(t) = 24.7 \pm 8.9\ (nM)^{-1}$; assuming $G(t) \sim t = 30$ min makes k_1 and k_2 24.0 ± 4.1 and $0.82 \pm 0.29\ (nM)^{-1}\ min^{-1}$, respectively, in fair agreement with the variable-time results. The ratio $k_1/k_2 = 29.1 \pm 10.5$, from the variable-concentration results, agrees well with the ratio from the variable-time results; 33.3 ± 7.6. All errors are standard deviations from the nonlinear least-squares fitting procedure.

pH Dependence of Cleavage Rates. Fractions of forms I, II, and III were measured for four different pV concentrations [pV], at each of five values of pH. The value of k_1G (proportional to the rate constant k_1) was extracted by least-squares fitting the fraction of form I, f_I, to an exponential, $Ae^{-b[pV]}$, for each pH; since there is little double-strand cutting (direct conversion of form I to form III DNA), $b = k_1G$. The uncertainty was estimated by changing one of the measured values of f_I by the estimated error and observing the change in the calculated value of b. To obtain values of k_2G, we note that, for small values of f_{III},

$$f_{III} = f_{II}^{\ 0}(k_2G)[D]$$

where f_{II}° is the initial value of f_{II}, the fraction of form II, [D] is the total DNA concentration, and G is as earlier defined. Thus, we fitted the values of f_{III} to a straight line and divided the slope by the initial fraction of form II. The uncertainty was calculated as for k_1.

The dependences of k_1G and k_2G (referred to for brevity as k_1 and k_2) on pH are shown in Figure 9. The behaviors are obviously quite different: k_2 decreases linearly with pH whereas k_1 passes through a maximum. These parameters are referred to as "rate constants", but the true second-order rate constants are obtained from the values given for these parameters by dividing by the DNA concentration, by a function of the irradiation time (30 min), and by the ratio of [V*] to [pV].

Table 1: IC_{50} Values[a] of Substances Affecting the Cleavage of pBR322 by pV

	IC_{50} ($\times mM^{-1}$)		IC_{50} ($\times mM^{-1}$)
Activators		Inhibitors[b]	
NADPH	0.92	NaCl	550
glutathione	0.11	NaI	8
cysteine	0.038	*tert*-butyl alcohol[c]	3.2, 2.9
		thiourea[c]	0.65, 0.61

[a] For activators, this is the concentration of an agent necessary to decrease the concentration of form I pBR322 DNA to half of its initial value. For inhibitors, this is the concentration of an agent necessary to produce a concentration of form I pBR322 DNA half that in the absence of cleavage. [b] The method of activation was UV light. [c] Two series of experiments.

Activators and Inhibitors of DNA Cleavage. The vanadium compound can be activated by NADPH, glutathione, and cysteine to produce DNA breakage. To determine the efficiency of a chemical activator of pV, the fraction of form I DNA was measured after a 30 min digest in the presence of various concentrations of the activator. This was least-squares fit to an exponential function of c, the activator concentration, i.e., $f_I = Ae^{-bc}$. The value of c required to reduce f_I to half its value before digest, IC_{50}, was calculated as $\ln(2)/b$. The values of IC_{50} are given in Table 1.

As the concentration of the reducing agent increases, f_I, the fraction of form I DNA, decreases, except for cysteine. Although f_I can be reduced to zero by 1 mM cysteine, it increases again for higher cysteine concentrations. The fraction of form II after digest correspondingly increases for cysteine concentrations below 1 mM and decreases subsequently as does the fraction of form III DNA. Thus, cysteine acts as an *activator* at low concentrations, but as an *inhibitor* at higher concentrations.

Certain agents, NaI, NaCl, *tert*-butyl alcohol, and thiourea, inhibit the ability of pV to photochemically degrade DNA. We measured the fraction of form I after a 30 min digest as a function of c, the concentration of the agent. This fraction increases from zero (complete cleavage) for low values of c, eventually leveling off at a maximum for a high concentration of agent (minimal cleavage or complete inhibition). To characterize the inhibitors quantitatively, we fit f_I as a function of c to the equation

$$f(c) = \frac{Ae^{bc}}{C + e^{bc}}$$

by choosing the parameters A, C, and b to minimize the sum of the squares of the deviations between measured and calculated f_I. We then calculate the concentration giving 50% inhibition, IC_{50}, as $b^{-1}\ln(C)$. The resulting IC_{50} values for the inhibitors are given in Table 1.

DISCUSSION

The Cleavage Mechanism. UV light and chemical reducing agents decompose pV into the monomer, dimer, and tetramer of vanadate (Figures 1, 3, and 4). After extensive degradation, an uncharacterized complex of V^{4+} can be detected using ESR (Figure 5). The initial complex produced in the photoreduction of pV is a V^{4+} compound with one of the peroxide ligands of pV oxidized to superoxide, $O_2^{\bullet-}$ (Djordjevic et al., 1988; Shinohara & Nakamura, 1989). Although the fate of this species is unknown, it could lose superoxide and/or peroxide, ultimately forming an aquated V^{4+} compound. This complex would be more substitution labile than pV and it probably catalyzes the decomposition of pV into vanadate using an electron transfer/ligand exchange process similar to that which occurs for complexes of Pt^{4+} in the presence of catalytic amounts of Pt^{2+} (Bailey & Johnson, 1969; Ellison et al., 1961). That this catalytic mechanism may be operating is suggested by the decomposition of pV in the presence of ascorbate (Figure 4). The amount of pV which decomposes to the vanadates is ~10 times the amount of ascorbate. Since reduction of pV to V^{4+} by ascorbate is expected to be stoichiometric, the initial V^{4+} compound produced in the reduction or a related species must catalyze the decomposition of pV into vanadate. The fact that not all of the pV is converted into vanadate indicates that pathways also exist for inactivation of the catalytic species, one of which is interacting with DNA. This is evident from the lower rate of photodecomposition of pV in the presence of DNA (Figure 2). The simplest explanation for the reduced rate is that DNA serves as a quenching agent for the species, a complex of V^{4+}, which is involved in the decomposition pathway of pV. It is likely that the same species causes breakage of DNA. Although ESR shows the presence of a V^{4+} complex at extended irradiation times in the absence of DNA (Figure 5), this complex is probably *not* responsible for breakage. Irradiation of pV for various times followed by addition of DNA does not result in DNA breakage, indicating that the species responsible for cutting is short-lived (Material and Methods).

Since both UV light and chemical reducing agents are able to activate pV, and $O_2^{\bullet-}$ is generated only in the photoreaction, superoxide ion is probably not the species responsible for DNA breakage. This is consistent with earlier studies by Graham et al. (1980), who showed that free superoxide ion is relatively unreactive toward DNA.

Hydroxyl radical has been implicated in the cleavage of DNA by vanadyl compounds in the presence of hydrogen peroxide. The antitumor agent, $[VO(phen)(H_2O)_2]^{2+}$, and the complex between the anticancer drug bleomycin and vanadyl sulfate both catalyze the decomposition of hydrogen peroxide to hydroxyl radical (Sakurai et al., 1992, 1995; Kuwahara et al., 1985). If DNA is present, hydrogen peroxide mixtures of the compounds break the sugar-phosphate backbone of the polymer. Although the cleavage mechanism has not been characterized, hydroxyl radical can be trapped in these reactions, suggesting that it is the species responsible for breakage. However, vanadyl-bleomycin and probably also $[VO(phen)(H_2O_2)_2]$ bind to DNA and it may be that a peroxo-V^{4+} species bound to the polymer is responsible for breakage. It is known that the vanadyl/peroxide system can produce trapped hydroxyl radical not only by the usual route of interaction of the free radical with the trap but also through direct interaction of another metal complex bearing a radical with the trap (Carmichael, 1990; Butler et al., 1994). The events taking place in the vanadyl/hydrogen peroxide system probably resemble those occurring with pV in the presence of UV light or reducing agents. However, in the case of pV, peroxide is already bound to the metal ion and it is only necessary to reduce V^{5+} to V^{4+} by chemical or photochemical means in order to produce species which can damage DNA.

The involvement of a metal complex in the breakage process is supported by the effects of various inhibitors on

the cleavage reaction. As we showed earlier, dissolved oxygen gas inhibits the photocleavage reaction (Hiort et al., 1995). Since oxygen could oxidize a V^{4+} species to V^{5+}, its presence in solution would reduce the amount of reactive intermediate available for breakage. Table 1 shows that thiourea stongly inhibits cleavage in the reactions activated with UV light. As a good metal binding ligand (Huheey et al., 1993), thiourea probably prevents the reactive intermediate from binding directly to DNA by blocking metal coordination sites necessary for binding. Table 1 shows that I^- is strongly inhibitory while Cl^- is not. Iodide is a better metal binding ligand than chloride, especially toward ions in lower oxidation states, so it may inhibit the reaction like thiourea, by binding to sites on a V^{4+} complex, blocking the interaction of the compound with DNA.

The inhibitory effect of tertiary butyl alcohol, a poor metal binding ligand, is not consistent with a metal based intermediate as the species responsible for breakage. However, the alcohol is an effective trap for radicals. A number of different radicals have been implicated in the decomposition of peroxovanadates in water (Bronchio et al., 1994), and it may be that a radical pathway also exists for DNA breakage.

As shown in Table 1, the reducing agents cysteine, glutathione, and NADPH can be used to activate pV. The effectiveness of cysteine is probably related to the fact that it is the smallest of the three reducing agents and can thus attack the vanadium ion in the sterically crowded peroxovanadate (Shaver et al., 1994). This would be the initial step in the reduction to produce the reactive V^{4+} complex. Although cysteine effectively induces strand scission at low concentrations, it *inhibits* cleavage at concentrations above ~1 mM. Since cysteine is a good metal binding ligand, it probably inhibits cleavage at high concentration in the same way that thiourea inhibits breakage in the photoactivation reaction, i.e., by direct interaction with a reactive V^{4+} complex. Clearly, additional work on both the nature of the metal species present in solution and the chemistry taking place on DNA will be necessary in order to fully characterize the mechanism by which pV is able to cleave DNA.

Kinetics of DNA Cleavage by pV. The results of the kinetic studies provided information on the mechanism of the cleavage reaction. As we have earlier shown (Kishikawa et al., 1991; Sehlstedt et al., 1994), quantitatively analyzing the cleavage kinetics of a agent using closed circular DNAs can give valuable information on the ratios of rate constants for cutting the various DNA forms and in some instances the values of the rate constants themselves. Since pV itself does not bind to and cleave DNA, it is not the species directly responsible for cleavage. Rather it must be activated to a species, V* (a complex of V^{4+}), which cleaves DNA using the mechanism outlined above. Obtaining rate constants for the cleavage of the various forms of pBR322 DNA would require knowledge of how the concentration of V^* is related to [pV], which can be measured.

By analyzing the variable-time experiments, we obtained $k_1 = 13.8\ (\text{nM})^{-1}\ \text{min}^{-1}$ and $k_2 = 0.42\ (\text{nM})^{-1}\ \text{min}^{-1}$. From the variable-concentration experiments, we derive $k_1 = 24.0\ (\text{nM})^{-1}\ \text{min}^{-1}$ and $k_2 = 0.82\ (\text{nM})^{-1}\ \text{min}^{-1}$. The fair agreement between the two sets of values implies that $F(t)$ (see eqs 1−4) is not large for $t < 30$ min, the duration of the digests in the variable-concentration experiments. This means that the concentration of V* does not change much during the digest time of 30 min.

The ratio of rate constants, k_1/k_2, is ~31. Note that k_1 is the rate constant for single-strand cleavage of form I DNA and k_2 the rate constant for single-strand cleavage of form II DNA at positions sufficiently near (~10 base pairs) a preexisting cut on the opposing strand to transform form II to linear form III DNA. If cleavage were random, and if the rate of single-strand cleavage of form II were comparable to that of form I, the value of k_1/k_2 for pBR322 DNA, which has a 4.3 kb genome, should be ~1000. Since the ratio is much less than this value, pV probably exhibits some base and/or sequence specificity, i.e., cleavage is not random on the genome.

Figure 9 shows that the values of k_1 and k_2 depend differently on pH. The rate constant for cleavage of form I to form II, k_1, exhibits a maximum at pH ~5.5. This suggests that the amount of the activated species producing breakage is greatest at this pH and/or the chemistry on DNA leading to the break is most effective at this pH. Figure 9 also shows that it becomes more difficult to make form III DNA from form II as the pH is raised. Since form III can only be produced by cuts which are sufficiently close to one another on opposing strands (within ~10 base pairs), the chemical change accompanying cleavage on one strand may be inhibiting the cleavage agent from binding to and cleaving the opposing strand. A change in pH may change the state of ionization at the breakage site which would affect the binding of a charged species nearby, so the pH dependence of k_2 suggests that the agent responsible for breakage may be a charged species such as a metal complex.

Biological Significance. In this work we show that the insulin-mimetic compound pV, in the presence of UV light or biologically common reducing agents, breaks DNA. If the compound is able to cross the cellular membrane and enter the nuclear envelope, it could be activated by reducing agents present in the envelope to cleave DNA in the cell. Whether or not DNA breakage actually occurs in cells which have been exposed to pV has not yet been determined. However, as we show in this report, the complex is readily degraded by biological reducing agents and light, and perhaps metabolites of pV, and not the compound itself, are present in the biological system. Thus, with pV as well as with other vanadium compounds (Crans et al., 1995; Macara et al., 1980; Sakurai et al., 1981), modification by agents present in the biological system may be important in the molecular mechanism of their insulin-mimetic effects. To unravel the mechanisms by which pV and related vanadium compounds are able to exhibit insulin-mimetic or antitumor effects will require additional knowledge of their stability and reactivity with important cellular targets such as proteins and DNA.

The potential of pV as a footprinting agent or structural probe for DNA has not gone unnoticed. Similar to $[Cu(phen)_2]^{2+}$ and related tris-bidentate chelates (Sigman, 1986; Barton, 1986), the species responsible for cutting has a phenanthroline ring which may intercalate into DNA (Satyanaryana et al., 1993). In addition, the compound is relatively stable as a solid and its concentration in solution can be accurately determined using absorbance. Exposure of solutions of pV to UV radiation produces a short-lived intermediate which cleaves DNA. As this study shows, the amount of cleavage by pV is readily controlled, suggesting that the compound may be useful in quantitative footprinting studies. Future work will focus on the cleavage specificity of the agent and on its ability to accurately measure ligand−

DNA binding constants in the quantitative footprinting experiment.

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