Stimulation of Iron(II) Bleomycin Activity by Phosphate-Containing Compounds†

Richard M. Burger,* Susan Band Horwitz, and Jack Peisach

Departments of Molecular Pharmacology, Cell Biology, and Molecular Biology, Albert Einstein College of Medicine of Yeshiva University, Bronx, New York 10461

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ABSTRACT: Orthophosphate and phosphate derivatives including pyrophosphate, hexametaphosphate, ATP, ADP, and inositol hexaphosphate enhance the extent of DNA degradation by iron(II) bleomycin. These phosphate-containing compounds increase both the release of free nucleic base and that of base propenals which are DNA cleavage products, probably by enhancing the efficiency with which Fe(II) is recruited into the drug. Phosphate action occurs during drug activation prior to the attack on DNA. In addition, phosphates affect the stability of the activated drug complex, overcome the inhibition observed with high concentrations of DNA, and reduce the size of the DNA fragment necessary for reacting with the drug. Phosphate derivatives bind to iron(II) bleomycin and alter its optical spectrum. An analysis of titration data for pyrophosphate and inositol hexaphosphate indicates that each phosphate compound binds to more than one iron(II) bleomycin molecule. With ATP, ADP, and 2,3-diphosphoglycerate, only a single phosphate-containing compound binds to the ferrous drug complex. The affinity for ATP is sufficiently high as to suggest that the ternary complex formed in vitro may exist physiologically.

The bleomycins are a family of structurally similar glycopeptide antitumor antibiotics that degrade DNA in cells and in vitro (Umezawa et al., 1966a,b; Suzuki et al., 1969). DNA damage includes single- and double-strand scission (Suzuki et al., 1969) which is accompanied by the formation of base propenals[1] (Kuo & Haidle, 1974; Povirk et al., 1978; Burger et al., 1980, 1982; Giloni et al., 1981). Other DNA damage, without scission, results in the release of free nucleic bases (Haidle et al., 1972; Povirk et al., 1977; Burger et al., 1982) accompanied by deoxyribose modification (Burger et al., 1980; Giloni et al., 1981; Wu et al. (1983). DNA damage is thought to be responsible for the drugs' cytotoxicity (Suzuki et al., 1969), mutagenicity (Moore, 1978), and induction of DNA repair and related processes (Gudas & Pardee, 1975; Moore 1978; Smith & Oishi, 1978; Baluch et al., 1980). In vitro, BLM activity requiring Fe(II) and O_2 proceeds through the formation of activated BLM (Burger et al., 1981), a species formed rapidly by single-electron reduction of an initially formed O_2–Fe(II)–drug complex (Burger et al., 1979b) (eq 1). This reduction is believed to be carried out by Fe(II)·BLM

$$\text{Fe(II)·BLM} + O_2 \rightarrow O_2\text{·Fe(II)·BLM} \quad (1)$$

$$O_2\text{·Fe(II)·BLM} + \text{Fe(II)·BLM} \rightarrow \text{activated BLM} + \text{Fe(III)·BLM} \quad (2)$$

(Kuramochi et al., 1981) which itself is oxidized to Fe(III)·BLM (eq 2). Activated BLM subsequently attacks DNA, yielding base propenal and free nucleic base.

$$\text{activated BLM} + \text{DNA} \rightarrow \text{Fe(III)·BLM} + \text{base propenal} + \text{free nucleic base} \quad (3)$$

As an overall summary of the single turnover reaction in air (Burger et al., 1982) (eq 4), four Fe(II)·BLM molecules are

$$4\text{Fe(II)·BLM} + 2O_2 + \text{DNA} \rightarrow 4\text{Fe(III)·BLM} + \text{base propenal} + \text{free nucleic base} \quad (4)$$

required for each DNA scission, since half the Fe(II)·BLM is oxidized during activation (eq 2) and about half the activated bleomycin initiates base release rather than base propenal formation (eq 3). It is usually found, though, that more than four Fe(II)·BLM molecules are required for each base propenal produced and, further, that the ratio of base propenal to base released is dependent on O_2 concentration (Burger et al., 1982).

DNA cleavage by iron bleomycin has been observed in a variety of pH buffers, but orthophosphate buffer, while not required for drug action, has been reported to enhance activity by 2-fold or more (Sausville et al., 1978). Phosphate compounds such as ATP were known to enhance DNA degradation by bleomycin (Takeshita et al., 1976). When Fe(II) was shown to be a necessary cofactor for the DNA cleavage reaction in assays requiring external reductants, it was suggested that the stimulation by ATP could arise from trace iron contamination (Sausville et al. 1976). We now report that ATP and several other phosphate derivatives have potent effects on bleomycin activity, even when iron is plentiful.

Materials and Methods

Materials. Bleomycin sulfate (Blenoxane), a gift of Bristol Laboratories, contained approximately 60% bleomycin A_2, 30% bleomycin B_2, and 10% various other bleomycin congeners. It was dissolved in water and standardized by optical titration (Sausville et al., 1978): small volumes of Mallinckrodt analytical reagent grade ferrous ammonium sulfate solution were added to bleomycin in aerobic 10 mM Hepes buffer, pH 7.0. This standardized solution displayed an $\epsilon_{291} = 1.7 \times 10^4$ M^{-1} cm^{-1}. It is important that solutions of ferrous ammonium sulfate in water be prepared at concentrations of about 20 mM, on the day of use, as more dilute solutions tend to hydrolyze and oxidize. Calf thymus DNA (Worthington) was dissolved

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* Address correspondence to this author at the Department of Molecular Pharmacology.

[1] Abbreviations: base propenal, the DNA degradation products of the form 3-(pyrimidin-1-yl)-2-propenal and 3-(purin-9-yl)-2-propenal; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonate; Mops, 3-(*N*-morpholino)propanesulfonate; BLM, bleomycin; EPR, electron paramagnetic resonance.

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either in 20 mM Hepes buffer, pH 7.0 or 8.2, or in 20 mM NaCl, pH 5, and standardized optically by using an $\epsilon_{260} = 6.6 \times 10^3$ M^{-1} cm^{-1}. *Staphylococcus aureus* plasmid pRN8008 was prepared from strain RN3268 (Carleton et al., 1984) by the procedure of Guerry et al. (1973), modified by the substitution of 0.1 mg/mL lysostaphin (Sigma) for lysozyme (S. J. Projan and R. P. Novick, personal communication). The DNA was labeled with tracer amounts of [1′,2′,*methyl*-^{3}H]-thymidine (Amersham) furnished in the culture medium. The ethanol-precipitated product was dissolved in 20 mM Hepes buffer, pH 7.0, and standardized optically; its specific activity was 68 Ci/mol total nucleotides.

Drug Reactions and Activity Assays. Incubations of DNA with bleomycin were conducted in 10 mM Hepes buffer, pH 7.0 or 8.2, at room temperature or at 4 °C, with concentrations of 40 μM Fe(II), 34 μM bleomycin, and 200 μM DNA, unless otherwise stated. The reactions were initiated either by addition of Fe(II) to buffered, aerobic bleomycin or by addition of aerobic buffer to anaerobic solutions of buffered Fe(II)·BLM (Burger et al., 1979a). Orthophosphate, DNA, or both were added when desired, either before or after the reaction's initiation, as previously described (Burger et al., 1981).

DNA scission was assayed indirectly as described (Burger et al., 1979a) with 2-thiobarbituric acid, which reacts with base propenals (Giloni et al., 1981), stoichiometric products of the DNA scission reaction (Burger et al., 1982), to produce an intensely colored derivative [$\epsilon_{532} = 1.6 \times 10^5$ M^{-1} cm^{-1} (Waravdekar & Saslaw, 1959)]. Drug reactions were terminated by the addition of the thiobarbituric acid reagent (Burger et al., 1979a) which was further acidified with HCl when high concentrations of phosphate or phosphate derivatives were used. Addition of phosphate to assay mixtures after completion of the drug reaction did not affect the assay.

The amounts of thymine propenal and free thymine released were determined by reversed-phase thin-layer chromatography of the degradation products of DNA labeled with [^{3}H]thymidine (Burger et al., 1980). At the end of the reaction DNA was removed from completed reaction mixtures by precipitation with 70% ethanol in a dry ice/ethanol bath for 5 min, followed by 1-min centrifugation in a Fisher Micro centrifuge at 4 °C. Seven microliters of the supernatants was applied to Analtech RPS 0.25-mm plates for ascending chromatography with 2 M ethanol and 18 mM sodium phosphate buffer, pH 7.0, for 1 h at 4 °C. Chromatograms were divided into 4-mm segments which were scraped from the support plate for scintillation counting as previously described (Burger et al., 1980).

EPR Spectrometry. EPR spectra were obtained on a Varian Model E-12 spectrometer operated at 77 K with 10-mW microwave power and equipped with an NMR gauss meter and a Systron Donner frequency counter.

Samples of iron bleomycin for EPR spectroscopy were prepared as previously described (Burger et al., 1981), in 50% (v/v) ethylene glycol buffered at pH 7.0 with 10 mM Hepes. EPR samples of activated bleomycin were usually prepared at 4 °C by adding Fe(II) to aerobic solutions of bleomycin, buffer, ethylene glycol, and, sometimes, DNA, phosphate, or both. These samples were then transferred into 3-mm quartz EPR tubes and frozen in liquid nitrogen, all within 30 s unless otherwise stated. EPR species were quantitated as described (Burger et al., 1981) by using the signal amplitudes at $g = 1.89$ and $g = 4.3$ for Fe(III)·BLM and at $g = 1.94$ for activated bleomycin. The line shapes of these features did not vary under the conditions used except for the supression of a $g = 1.95$ shoulder by DNA (Burger et al., 1981). The method was calibrated with bleomycin which had been standardized with ferrous ammonium sulfate and then air oxidized.

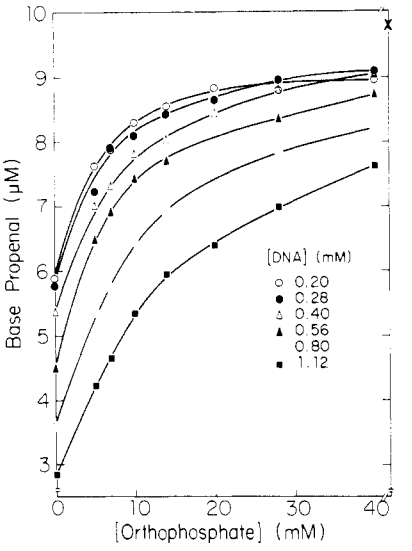

FIGURE 1: Effects of orthophosphate and DNA on bleomycin activity. Reaction mixtures (200 μL) contained 34 μM bleomycin, 20 mM Hepes buffer, pH 7.0, plus sodium phosphate, pH 7.0, and DNA as indicated. Reactions were initiated by addition of 40 μM Fe(II), were run at room temperature for $\geq 1/2$ h, and then were assayed for base propenal with 2-thiobarbituric acid as described under Materials and Methods. The yield of base propenal extrapolated to infinite phosphate concentration is indicated (×).

Optical Spectrometry. Optical spectra were obtained on a Cary Model 14 spectrophotometer. Samples of Fe(II)·BLM were prepared anaerobically in Thunberg cuvettes as previously described (Burger et al., 1979a). Anaerobic additions of phosphates and other materials were made in small volumes of argon-purged, concentrated solutions by Hamilton syringe through septum stoppers. When repetitive additions were to be made, the Fe(II)·BLM sample received anaerobically prepared 10 mM dithionite immediately after the initial spectrum was recorded. Spectra obtained with dithionite present were checked by adding the titrant in a single aliquot to duplicate samples of Fe(II)·BLM lacking dithionite.

The complexation of Fe(II)·BLM by phosphate derivatives (P) was analyzed for K, the equilibrium constant, and n, the apparent molecularity of the reaction with respect to Fe(II)·BLM, as given by

$$K = \frac{[P\cdot(Fe(II)\cdot BLM)_n]}{[P][Fe(II)\cdot BLM)]^n}$$

The optical titration data were fit by least-squares criteria to an equation expressing the absorbance at any concentration of the variable titrant (i.e., [P]) in terms of K, n, the absorbances of the complexed and uncomplexed drug species, and the concentration of Fe(II)·BLM employed (Peisach & Mims, 1977).

Results and Discussion

Phosphate Specificity and Potency. The stimulation of Fe(II)·BLM activity by phosphate compounds was assayed by the production of base propenal from DNA. A 2–3-fold stimulation of drug activity by orthophosphate is shown in Figure 1. Free nucleic base yield was similarly enhanced. Thus, there is a net increase in drug activity induced by orthophosphate and not merely an enhancement of one reaction product at the expense of the other.

The specificity of orthophosphate in enhancing base propenal yields was demonstrated by comparison with the effects of

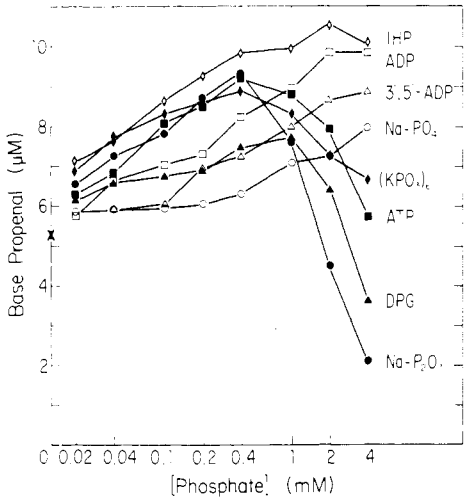

FIGURE 2: Effects of phosphate-containing compounds on bleomycin activity. Reactions were conducted as described for Figure 1 except that all mixtures contained 0.2 mM DNA and the indicated phosphate compounds. IHP and DPG denote inositol hexaphosphate and 2,3-diphosphoglycerate, respectively, and (×) denotes no addition. The abscissa scale, concentration of phosphate-containing compounds, is logarithmic.

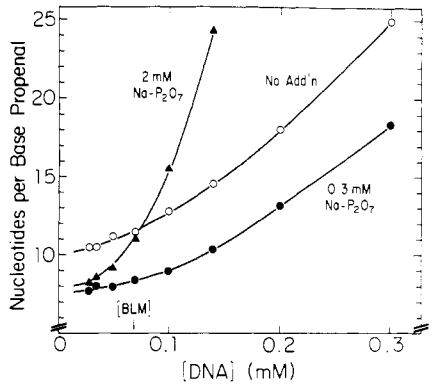

FIGURE 3: Effects of pyrophosphate on limit digests of DNA by bleomycin. Reactions mixtures (200 μL) contained 68 μM bleomycin, 10 mM Hepes buffer, pH 7.0, 80 μM Fe(II), and DNA plus no addition (○), 0.3 mM pyrophosphate (●), or 2 mM pyrophosphate (▲). Reactions were initiated by the addition of Fe(II) to reaction mixtures at twice their final concentrations but lacking DNA. After the 2-s period required for drug activation, an equal volume of DNA was added, and the mixtures were incubated for more than 20 min at room temperature and then assayed for base propenal. Since base propenal production is stoichiometric with DNA strand scission (Burger et al., 1982), the average oligonucleotide product size (ordinate) is calculated as the quotient of DNA nucleotides base per propenal. An equivalent series with 0.3 mM inositol hexaphosphate (data not shown) gave the same results as with 0.3 mM pyrophosphate. Using 20 mM orthophosphate resulted in products approaching nine nucleotides per scission (data not shown).

other anions such as chloride and sulfate. Although all three anions enhance bleomycin activity, the drug is far more sensitive to orthophosphate. With 0.2 mM DNA, the concentrations of anions giving half-maximal stimulations, 4 mM orthophosphate, 8 mM sulfate, and 32 mM chloride, are too different to attribute to nonspecific ionic strength effects. However, at concentrations exceeding 100 mM, all three salts are inhibitory, probably due to the decrease of drug binding to DNA observed at high salt concentrations (Chien et al., 1977; Dabrowiak, 1982).

When orthophosphate is used alone or in combination with other buffers such as Hepes and Mops (data not shown), its effect is not compromised. Therefore, orthophosphate must be considered active in enhancing drug activity rather than merely reversing a putative inhibition by other buffers.

Orthophosphate analogues such as arsenate and vanadate stimulate at comparable concentrations. Much more potent are the phosphate derivatives pyrophosphate, hexametaphosphate, ATP, ADP, 3′,5′-ADP, 2,3-diphosphoglycerate, and inositol hexaphosphate (Figure 2). Under the conditions of Figure 2, the half-maximal stimulation observed with orthophosphate is 4 mM. Under comparable reaction conditions the various phosphate derivatives have a half-maximal stimulation of only 0.05 mM. No stimulatory effect of AMP or creatine phosphate was seen at concentrations up to 10 mM. Although orthophosphate shows activity, phosphate derivatives containing at least two phosphate moieties are more active.

Like orthophosphate, phosphate derivatives stimulate both free nucleic base and base propenal production. The phosphate compounds that stimulate (Figure 2) fall into two groups; those indicated by open symbols remain stimulatory at higher concentrations while those indicated with closed symbols become inhibitory at higher concentrations. For example, pyrophosphate, a member of the second group, becomes inhibitory at 2 mM so that both base propenal and free nucleic base production is decreased, while ADP, a member of the first group, does not inhibit, even at the highest concentrations studied.

pH Dependence of Phosphate Effect. The enhancement of drug activity by orthophosphate, and the other anions too, is influenced by pH. In the absence of orthophosphate, the drug activity at pH 7.0 is only about 65% of that at pH 8.2. With ~10 mM phosphate present, there is approximately equal activity at either pH. Thus, phosphate counteracts the inhibition at suboptimal pH and explains the broad activity optimum seen near pH 7 (Sausville et al., 1978).

Antagonism by DNA. When DNA is provided in excess of 5 times the bleomycin concentration, reaction yields are lowered and the reaction rates slowed (Burger et al., 1981). The effect of orthophosphate concentration on base propenal production at several DNA concentrations is shown in Figure 1. Extrapolation of the data to yields at infinite phosphate concentration indicates that phosphate completely reverses the inhibition due to DNA. The double-reciprocal plots of activity enhancement as a function of phosphate concentration were linear at every concentration of DNA employed. Although orthophosphate increases the yield of base propenal, it does not reduce the inhibition in the rate of its formation observed in the presence of DNA.

It is difficult to assess the activity of bleomycin in the absence of DNA, but the effects of phosphate derivatives are apparent even at the lowest DNA concentrations tested. For example, at the lowest DNA:drug ratios shown in Figure 3, both 0.3 mM pyrophosphate and 2.0 mM pyrophosphate reduce the minimal size of oligonucleotide capable of interacting with the drug from about 10 in Hepes buffer ("no addition" curve) to about 8. A similar reduction with 0.3 mM ATP, orthophosphate, and inositol hexaphosphate is observed. ADP is less effective than ATP. Although the effects of 0.3 and 2 mM pyrophosphate are the same at low DNA concentrations, inhibition by 2 mM pyrophosphate becomes apparent as the DNA is increased.

At optimal DNA and phosphate concentrations the effect of orthophosphate and phosphate derivatives is to enhance reactivity, although the drug differs considerably in its sensitivity. Table I shows the yield of base propenal from mixtures of drug with two different concentrations of DNA nucleotide, in the presence or absence of stimulatory levels of orthophosphate, and various phosphate derivatives. At both DNA concentrations all phosphate derivatives are effective in in-

Table I: Effects of DNA Concentration on Bleomycin Activity with Phosphates Present[a]

additions	base propenal (μM) produced with 200 μM DNA	600 μM DNA
none	4.3	3.2
10 mM orthophosphate	13.2	8.5
0.2 mM pyrophosphate	12.8	6.8
0.2 mM inositol hexaphosphate	15.1	9.3
1.0 mM ADP	11.2	6.2
0.4 mM ATP	11.2	6.0

[a] Reaction mixtures (200 μL) contained 40 μM bleomycin, 40 μM Fe(II), 10 mM Hepes buffer, pH 7.0, and either 200 or 600 μM DNA plus the additions of the phosphate derivatives indicated. Reactions were initiated by addition of Fe(II), incubated at room temperature for $\geq^1/_2$ h, and then assayed for base propenal as described under Materials and Methods.

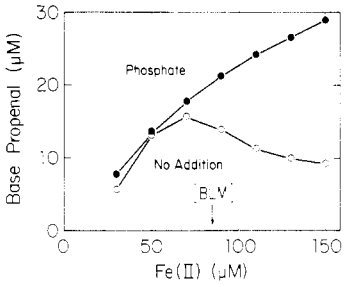

FIGURE 4: Effect of orthophosphate on iron utilization for bleomycin action. The relationship between Fe(II) in the reaction and base propenal yield is shown in the absence (○) or presence (●) of 20 mM sodium phosphate, pH 7.0. Reaction mixtures (100 μL) also contained 85 μM bleomycin, 10 mM Hepes buffer, pH 7.0, 0.5 mM DNA, and Fe(II) as indicated. Reactions were initiated by addition of Fe(II) and assayed for base propenal after $^1/_2$ h incubation at room temperature. Reactions initiated by O_2 addition gave the same results.

creasing the base propenal yield, but less so at the higher DNA concentration.

Effect of Iron Concentration. The effect of phosphates on bleomycin activity is also strongly influenced by the amount of Fe(II) provided (Figure 4). In a reaction mixture containing 85 μM bleomycin and with a low level of Fe(II), neither orthophosphate, pyrophosphate, nor inositol hexaphosphate has much effect on drug activity. As the Fe(II):drug ratio approaches and exceeds 1:1, the effects of phosphates become conspicuous; bleomycin activity remains roughly dependent on Fe(II) concentration. Even at DNA:drug ratios as high as 18:1, a similar effect of phosphate is observed. In contrast, in the absence of phosphates, excess Fe(II) appears to inhibit drug activity, especially at moderate ratios of DNA:drug, i.e., 6:1.

Since all of the forgoing experiments were initiated by the addition of Fe(II) to aerobic reaction mixtures of drug, DNA, buffer, and phosphate, it was unclear which components interact in what order. Therefore, these experiments were repeated, adding O_2 last to otherwise complete reaction mixtures. In either case the same results were obtained: permitting drug, iron, phosphate, and DNA to equilibrate before admitting O_2 does not alter effects of Fe(II) and phosphate-containing compounds on the ensuing reaction.

It is suggested that phosphate affects the utilization of Fe(II) by the drug. It may do this by preventing the oxidation of excess Fe(II) at neutral pH or by facilitating the substitution of Fe(II) for the Fe(III) already sequestered by the drug. This latter idea is borne out in the following study. When Fe(II) is added to an already cycled reaction mixture containing Fe(III)·BLM, only 50% of the activity obtained by adding Fe(II) to metal-free bleomycin is observed. Carrying out the

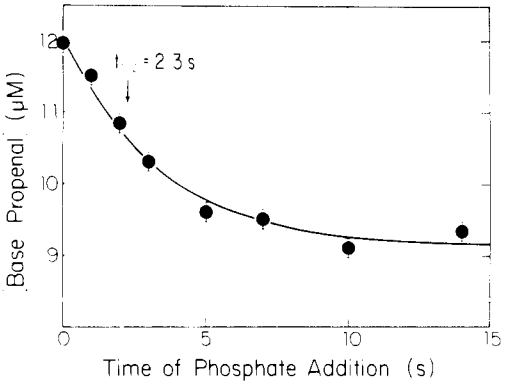

FIGURE 5: Duration of bleomycin sensitivity to stimulation by orthophosphate. Reaction mixtures contained 40 μM Fe(II), 34 μM bleomycin, 150 μM DNA, and 7 mM Hepes buffer, pH 7.0. The reaction was initiated by the addition of 40 μM Fe(II) followed by 17 mM phosphate, added at the times indicated. After $\geq^1/_2$ h incubation at 4 °C, base propenal was assayed as described under Materials and Methods. The curve represents first-order kinetics with the indicated $t_{1/2}$, and is fit to the data by least-squares criteria. The error bars indicate the standard mean deviation.

Table II: Effects of Orthophosphate on the Yield of Activated Bleomycin[a]

reaction mixtures prepared with BLM (μM)	Fe(II) (μM)	P_i (mM)	yield of activated BLM (μM)
170	140	0	36
170	140	20	42
170	220	0	42
170	220	20	87

[a] Reaction mixtures (360 μL) contained 170 μM bleomycin (BLM), 1 mM DNA, 10 mM Hepes, pH 7.0, and 50% (v/v) ethylene glycol plus the indicated concentrations of orthophosphate (P_i) and Fe(II). After Fe(II) addition samples were incubated for 30 s at 4 °C and frozen for EPR spectrometry as described under Materials and Methods.

reaction in phosphate buffer increases this to 80%.

Kinetic Locus of Phosphate Action. A series of experiments were performed to demonstrate the time at which phosphates must be added to stimulate drug activity. Hence, reactions were initiated by Fe(II) addition followed at intervals of 1–30 s by phosphates. Figure 5 shows that the increase of product formation produced by phosphate addition is not observed if phosphate is added a few seconds after the addition of iron. The $t_{1/2}$ for loss of sensitivity to phosphate is about 2 s at 4 °C. This is much earlier than the attack of DNA and the breakdown of activated bleomycin, both of which occur with a $t_{1/2}$ of 90 s, but is comparable to the $t_{1/2}$ of the formation of activated bleomycin at 2 °C, 6 s (Burger et al., 1979b).

Effect of Orthophosphate on Yield of Activated Bleomycin. Since the DNA-cleaving activity of bleomycin is embodied in activated bleomycin, the effect of phosphate on the yield of this species was quantitated. Activated bleomycin reaction mixtures prepared with different amounts of Fe(II), with or without phosphate, were examined by stop-freeze EPR spectroscopy. When more drug than Fe(II) was used, phosphate had little effect on the yield of activated bleomycin (Table II) or on base propenal formation (Figure 4). When more Fe(II) than bleomycin was introduced into reaction mixtures, additional activated bleomycin was formed and more base propenal produced, but only when phosphate was present.

Interactions of Phosphate Derivatives with Fe(II)·BLM. Fe(II) reacts with bleomycin to produce a 1:1 pink complex (λ_{max} 476 nm) (Sausville et al., 1976). Anaerobic reaction mixtures of Fe(II)·BLM containing phosphate derivatives

Table III: Spectrophotometric Titrations of Fe(II)·BLM[a]

sample	λ_{max} (nm)	ϵ_{mM} (mM^{-1} cm^{-1})	isosbestics with spectrum of Fe(II)·BLM (nm)	binding constant (mM)	molecularity (n)
Fe(II)·BLM	476	0.38			
plus ATP	406	0.28	390, 437	0.04	1.0
plus GTP	*b*		468, 560	0.17	1.0
plus 2,3-DPG	*b*		470, 580	0.5	1.0
plus ADP	385[c]		470	1.6	1.1
plus IHP	385	0.42	392, 450	1.7	1.3
plus pyrophosphate	415	0.44	390, 457	30	1.6
plus orthophosphate	380[c]		443	100	1.0

[a] Titrations were performed and analyzed for the apparent affinity constant and the molecularity of bleomycin per ligand, as described under Materials and Methods and in the legend to Figure 6. Data were analyzed from absorptions at 510 nm, the spectral region showing greatest change after ligand addition. New absorption maxima are seen with ATP, inositol hexaphosphate (IHP), and pyrophosphate, but GTP and 2,3-diphosphoglycerate (2,3-DPG) resulted in featureless end absorptions. [b] Maxima are absent in the range 350–650 nm. [c] A shoulder, rather than an absorption maximum, is observed.

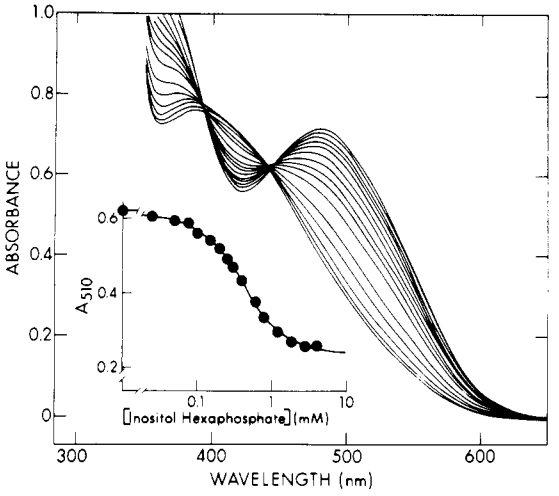

FIGURE 6: Spectrophotometric titration of Fe(II)·BLM with inositol hexaphosphate. Small volumes of anaerobic 25 or 200 mM inositol hexaphosphate, pH 7.0, were added successively to a solution of 2 mM Fe(II), 2.1 mM bleomycin, 20 mM Hepes buffer, pH 7.0, and 10 mM sodium dithionite, in a septum-stoppered Thunberg cuvette of 1-cm path length. The spectra depicted were obtained (in order of decreasing A_{510}) with inositol hexaphosphate concentrations of 0, 25, 50, 75, 100, 150, 200, 250, 300, 400, 600, 800, 1200, 1800, 2800, and 4000 μM. The inset is a computer-generated curve fit, as described under Materials and Methods, by an iterated least-squares regression, to the A_{510}.

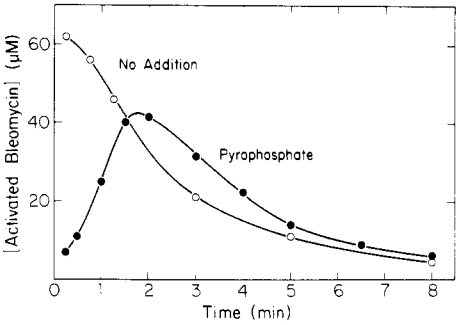

FIGURE 7: Effect of pyrophosphate on kinetics of bleomycin activation. Activated bleomycin was determined by stop-freeze EPR spectrometry as described under Materials and Methods. Reaction mixtures (400 μL) contained 0.2 mM Fe(II), 0.21 mM bleomycin, 1 mM DNA, 10 mM Hepes buffer, pH 7.0, and 50% (v/v) ethylene glycol plus 0.25 mM sodium pyrophosphate (●) or no addition (○). Reactions were initiated by Fe(II) addition and run at 4 °C for the times indicated prior to immersion in liquid nitrogen. The disappearance of activated bleomycin corresponds to the formation of Fe(III)·BLM as shown in Burger et al. (1981) (data not shown).

appeared yellow. This yellow color was dependent on drug and Fe(II) and was characteristic of the phosphate compound used. For example, the spectrum of the inositol hexaphosphate complex (Figure 6) has a λ_{max} of 385 nm and isosbestic points with the spectrum of Fe(II)·BLM near 392 and 450 nm (Table III). Analysis of the optical titrations of Fe(II)·BLM with inositol hexaphosphate, for example, (Figure 6, insert) gives an association constant of 1.7 mM with an apparent n for Fe(II)·BLM of 1.3. For pyrophosphate the association constant is 30 mM and an n of 1.6. By this analysis, the ratio of drug complexed to either of these phosphate compounds is greater than unity. Therefore, more than one Fe(II)·BLM reacts with a single phosphate derivative. For ATP, GTP, and 2,3-diphosphoglycerate, the n value for the titration is unity (Table III), suggesting that each phosphate-containing ligand reacts with only a single Fe(II)·BLM molecule. It should be noted that the affinity for ATP exceeds that for pyrophosphate by nearly 3 orders of magnitude.

Different phosphate substituents have strong effects on bleomycin binding and activity. Thus, ADP and inorganic pyrophosphate differ considerably in their binding to Fe(II)·BLM and in the spectra of their drug complexes (Table III) as well as in their potency in enhancing drug activity (Figure 2). ATP and GTP also have large differences in these effects (Table III) although they differ only in nucleic base moieties.

Orthophosphate (Table III) and arsenate (data not shown) also interact with Fe(II)·BLM to produce similar spectral changes, but these anions appear to bind weakly. It should be noted that 10 mM AMP does not affect the optical spectrum of Fe(II)·BLM, nor does it affect the activity of the drug as do other phosphate derivatives (Figure 2). Addition of DNA produced a spectral change similar to that resulting from phosphate or arsenate additions, but a titration approaching completion was limited by the ability to manipulate viscous DNA solutions.

Kinetics of Drug Activation. When pyrophosphate (Figure 7) or inositol hexaphosphate (data not shown) is present in Fe(II)·BLM reaction mixtures, the formation of activated bleomycin at 4 °C is sufficiently slowed to be readily monitored by stop-freeze EPR spectroscopy. In the absence of pyrophosphate, the EPR spectrum of activated bleomycin is maximal within the first 15 s after Fe(II) addition. With 0.25 mM pyrophosphate or inositol hexaphosphate present (data not shown), the EPR spectrum of activated bleomycin appears much more slowly and achieves a steady-state level in about 1.5 min. The lag in the appearance of activated bleomycin once again supports the view that phosphate derivatives affect some reaction step prior to that yielding activated bleomycin.

Duration of Sensitivity to DNA Inhibition. As noted in eq 2, the activation of bleomycin is believed to be a bimolecular process (Kuramochi et al., 1981). This reaction is strongly inhibited by high DNA concentrations (Albertini et al., 1982)

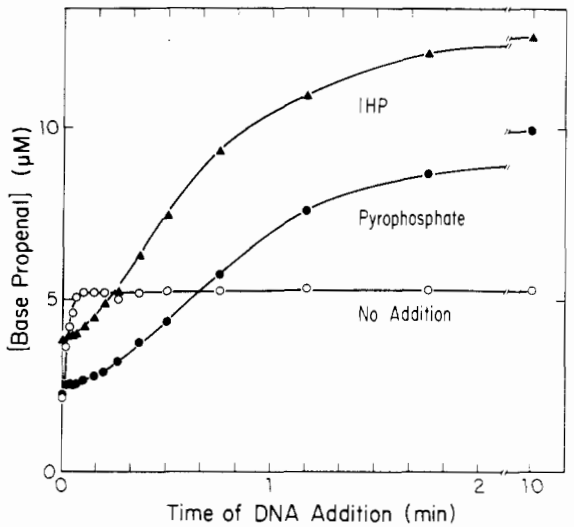

FIGURE 8: Bleomycin sensitivity to inhibition by DNA is prolonged by inositol hexaphosphate (IHP) and pyrophosphate. Reaction mixtures (125 μL) initially contained 80 μM Fe(II), 68 μM bleomycin, 0.4 mM DNA, and 10 mM Hepes buffer, pH 7.0 plus 0.3 mM inositol hexaphosphate (▲), 0.3 mM sodium pyrophosphate (●), or no addition (○), with the Fe(II) added last to initiate the reaction. The initial concentration of DNA is not inhibitory. At the indicated times an equal volume of 1.2 mM DNA and 10 mM Hepes, pH 7.0, was added, and incubation was continued for $^1/_2$ h at 4 °C followed by assay of base propenal, as described under Materials and Methods.

but weakly by low concentrations, and this inhibition is no longer operative once activation has taken place (Burger et al., 1981). When DNA is present at high concentrations in the initial reaction mixture (Figure 8, "no addition" curve), the yield of base propenal is not as great as when high concentrations of DNA are added after 10 s, when drug activation is complete. After 10 s excess DNA has no effect on the yield of base propenal. In comparison, with inositol hexaphosphate or pyrophosphate present, the time that the reaction remains sensitive to high concentrations of DNA is markedly increased. Even when this DNA is added 2 min after the Fe(II), inhibition is still observed. Pyrophosphate and inositol hexaphosphate (but not orthophosphate) therefore prolong the period when the reaction is sensitive to DNA inhibition.

Interactions of Phosphates with Activated Bleomycin. Phosphates and DNA have no effect on yield of DNA breakdown products when they are added subsequent to the formation of activated bleomycin. This would be evident in the rate of base propenal formation, which is concurrent with activated bleomycin decay, except that DNA, like the other phosphate compounds employed in this study, also accelerates these processes. Alternatively, the decay of activated bleomycin may be monitored in the absence of DNA by periodically adding aliquots of a reaction mixture to DNA and observing the decreasing yields of base propenal. Thus, the potential for producing base propenal is seen to decay with first-order kinetics (Figure 9), and this rate is doubled or tripled by the presence of 20 mM orthophosphate. With 0.2 mM pyrophosphate, a level that stimulates bleomycin activity (Figure 2 and the ordinate intercept of Figure 9), no effect on the breakdown of activated bleomycin is observed. However, when the level of pyrophosphate is raised to 0.5 mM, there is a marked increase in the rate of decay of activated bleomycin which is comparable to that observed with 20 mM orthophosphate (data not shown).

CONCLUSION

Phosphates have multiple effects on bleomycin and its re-

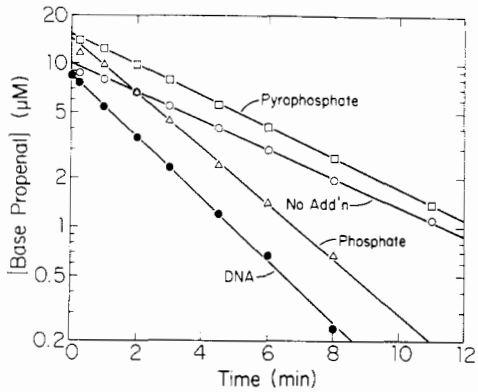

FIGURE 9: Kinetics of activated bleomycin decay: effects of phosphate compounds on the loss of drug activity in forming base propenal. Semilogarithmic plots depict the residual ability to form base propenal in reaction mixtures supplemented with 20 mM sodium phosphate (△), 0.2 mM sodium pyrophosphate (□), 0.22 mM DNA (●), or no addition (○). Residual activity was assayed at the times indicated by adding reaction mixture aliquots to DNA and assaying for base propenal 20 min later (△, □, ○). Those reaction mixtures containing DNA throughout (●) were assayed for residual activity at the times indicated by assaying base propenal and subtracting this value from that obtained in reactions permitted to reach completion 20 min later. Prior to DNA addition, 100-μL reaction mixtures contained 69 μM bleomycin, 80 μM Fe(II), and 20 mM Hepes buffer, pH 7.0, plus 20 mM orthophosphate (△), 0.2 mM pyrophosphate (□), or no addition (○). After DNA addition, 200-μL reaction mixtures contained 0.22 mM DNA, 34 μM bleomycin, 40 μM Fe, and 20 mM Hepes buffer plus 10 mM orthophosphate (△), 0.1 mM pyrophosphate (□), or no addition (○, ●). All incubations were at 4 °C.

actions. We have sought to define the mechanistic locus whereby phosphates enhance the yield of drug-induced DNA degradation and to distinguish this from other interactions of drug and phosphates. Kinetic analysis indicates that phosphates must be present prior to drug activation in order to increase DNA breakdown (Figure 5). Phosphates, like DNA, slow down drug activation (Figure 7) and, at the same time, alter the period in which DNA can inhibit this reaction (Figure 8). It is suggested that complex formation of Fe(II)·BLM with phosphate compounds may in part be responsible for these effects. The acceleration of activated bleomycin decay requires higher concentrations of phosphates (Figure 9), which may be added subsequent to drug activation.

The yield of activated bleomycin and of DNA degradation products is enhanced by phosphates whenever the Fe(II) employed is equal to or exceeds the amount of bleomycin in the reaction mixture. Phosphate-containing compounds bind to Fe(II)·BLM with an avidity that approximates their effect on DNA degradation. They probably enhance the availability of Fe(II) to bleomycin, possibly by protecting the Fe(II) from hydrolysis to hydroxides and consequent autoxidation during the period preceding drug activation.

Do phosphates play a role in bleomycin activity in vivo? The concentrations of phosphates reported in nonmuscle tissues of animals are about 2 mM ATP, 1 mM ADP, 3 mM orthophosphate, and 0.01 mM pyrophosphate (Williamson & Brosnan, 1974). These concentrations of ATP and ADP strongly stimulate drug activity in vitro, although orthophosphate is only moderately stimulatory while pyrophosphate is almost without effect (Figures 1 and 2). ATP and ADP therefore seem likely to have a role in bleomycin activity in cells, possibly by mobilizing or protecting Fe(II) as they appear to do in vitro. In addition, physiological levels of ATP far exceed the association constant with Fe(II)·BLM, suggesting that in vivo there may be an ATP·Fe(II)·BLM complex. Such a complex, when formed, would slow down drug activation

(Figure 7) and prolong the time in which the drug is inhibited by DNA (Figure 8).

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Registry No. ATP, 56-65-5; ADP, 58-64-0; GTP, 86-01-1; inositol hexaphosphate, 83-86-3; diphosphoglyceric acid, 14265-44-2; orthophosphoric acid, 14265-44-2; pyrophosphoric acid, 2466-09-3; blenoxane, 9041-93-4.

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