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Osmium-Labeled Polynucleotides. The Reaction of Osmium Tetroxide with Deoxyribonucleic Acid and Synthetic Polynucleotides in the Presence of Tertiary Nitrogen Donor Ligands†

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ABSTRACT: Osmium tetroxide in the presence of pyridine or 2,2′-bipyridine has been found to react completely with the pyrimidine moieties (thymine, uracil, and cytosine) in polynucleotides. Pyrimidine osmate ester moieties, L_2OsO_4-pyrimidine, were formed. The OsO_4 has added across the 5,6 double bond and L = pyridine or ½-bipyridine. The pyridine derivatives were not stable and decomposed slowly after the OsO_4-pyridine reagent was removed by gel chromatography. Labeled poly(uridylic acid) lost osmium completely during gel chromatography unless the eluent contained a high concentration of pyridine. The products formed between OsO_4-bipyridine and polynucleotides were much more stable and the Os label was retained during and after gel chromatography. Both the OsO_4-pyridine and OsO_4-bipyridine reagents reacted more rapidly than the OsO_4-CN^- reagent.

The biological importance and utility of polynucleotides derivatized by inertly attached heavy metals have recently been summarized (Daniel and Behrman, 1976; Marzilli, 1977; Dale and Ward, 1975). Osmium labeling of polynucleotides has proved particularly useful and has found application in both electron microscopic (Highton et al., 1968; Whiting and Ottensmeyer, 1972) and x-ray crystallographic (Rosa and Sigler, 1974; Schevitz et al., 1972; Kim et al., 1972) investigations of nucleic acids.

Osmium tetroxide selectively degrades pyrimidine bases of nucleic acids (Beer et al., 1966; Burton and Riley, 1967). In the presence of added ligands, L = CN^-, pyridine, ½-bipyridine, etc., stable osmate ester derivatives are formed containing the moiety L_2OsO_4-pyrimidine (Highton et al., 1968; Subbaraman et al., 1971; Daniel and Behrman, 1975, 1976). For L = pyridine, recent crystallographic investigations have

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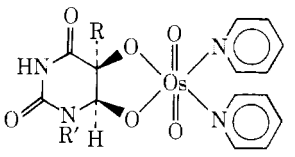
demonstrated the overall geometry I for thymine (R = CH_3;



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R′ = H) and 1-methylthymine (R = R′ = CH_3) derivatives (Neidle and Stuart, 1976; Kistenmacher et al., 1976). Behrman and his co-workers have extensively explored the kinetics of formation, the chemical properties, and the stability of osmate esters of monomeric nucleic acid species as well as esters of dinucleoside monophosphates (Clark and Behrman, 1975; Subbaraman et al., 1971–1973; Ragazzo and Behrman, 1976; Daniel and Behrman, 1975, 1976). The results so obtained suggested that the OsO_4–pyridine reagent would retain the selectivity toward pyrimidines of the OsO_4–CN^- reagent used previously in studies of nucleic acids (Highton et al., 1968; di Giamberardino et al., 1969; Whiting and Ottensmeyer, 1972) and offer the advantage of lower temperatures and shorter reaction times. However, no study was available on the reaction of the promising OsO_4–pyridine reagent with nucleic acids other than tRNAs and therefore we undertook such an investigation. Our finding of inadequate stability of the OsO_4–pyridine product led us to explore also the reaction of OsO_4–bipyridine with polynucleotides. In all cases investigated here, the reaction of the OsO_4 reagent forms an adduct on the 5,6 double bond of the pyrimidine. Such "heterocyclic osmate esters" are to be contrasted with the "sugar osmate esters" which are formed when Os(VI) reagents add to the ribose 2′,3′-hydroxyl groups of nucleosides (Marzilli, 1977; Daniel and Behrman, 1976).

Materials and Methods

Calf thymus DNA (type I) (A, 28%, G, 22%) and ribo- or deoxyribopolynucleotides (potassium or sodium salt) were obtained from Sigma. 2,2′-Bipyridine, 2-(dimethylaminomethyl)-3-hydroxypyridine, 8-hydroxyquinoline-5-sulfonate, bathophenanthrolinedisulfonic acid, disodium salt, hydrate, and 3-(2-pyridyl)-5,6-diphenyl-1,2,4-triazine-*p,p′*-disulfonic acid, disodium salt, trihydrate were from Aldrich. Osmium tetroxide was from Fisher Scientific Co. Sephadex beads (G-50–150) were from Sigma. Other chemicals were reagent grade.

DNA solutions were prepared and denatured as described by Highton et al. (1968). Ultraviolet and visible absorption spectra were recorded using a Cary 17 spectrophotometer. Sephadex columns were calibrated with blue dextran and $CoCl_2$.

Pyridine (7.8 ml), concentrated phosphoric acid (25 μl), and deionized distilled water (2.03 ml) were added to 0.145 ml of OsO_4 solution (0.21 M). This OsO_4–pyridine solution (pH 7.1, 5 ml) was then mixed with 10 ml of denatured DNA solution (approximately 0.6 mg/ml) in 0.01 M phosphate buffer at pH 7.0. The resulting reaction mixture contained 1 mM OsO_4, 3 M pyridine, and about 1 mM of DNA. Aliquots (1 ml) allowed to react at room temperature for known times were eluted through a Sephadex G-50–150 column with deionized distilled water. The polymer fraction was collected and analyzed for osmium and phosphate contents.

Reaction mixtures containing 3 mM OsO_4, 3 M pyridine, and about 1 mM of polynucleotide were made by mixing an equal volume of an OsO_4–pyridine solution and of a solution of polynucleotide. The solution pH was adjusted to neutral with concentrated hydrochloric acid. Aliquots (1 ml) of the reaction mixture were purified as above and the polymer fraction was analyzed.

A 1:1 OsO_4–bipyridine solution containing 18 mM in each species was prepared before each reaction. Reaction mixtures were obtained by mixing an equal volume of this OsO_4–bipyridine solution and of a solution of polymer. The pH of the resulting solution was measured and, if necessary, adjusted to neutral with concentrated hydrochloric acid. Aliquots of the reaction mixture were purified and the polymer fraction was analyzed as above. This procedure was also used for the reaction of nucleic acid polymers with OsO_4 and other bidentate ligands.

Stock reagent solutions for total phosphate analysis were prepared as described by Murphy and Riley (1962). Sodium antimonyl tartrate instead of potassium antimonyl tartrate was used. The color-developing reagent solution was freshly prepared for each series of determinations by dissolving ascorbic acid (52.8 mg) in 3 ml of deionized distilled water and then adding 5 N H_2SO_4 (5 ml), ammonium molybdate solution (1.5 ml), and sodium antimonyl tartrate solution (0.5 ml). This solution was stored in the cold and was stable for only 24 h. The procedure used by Ames and Dubin (1960) for determination of total phosphate content was modified. An aliquot (usually 100 μl) of the solution to be assayed was transferred to an acid-washed test tube and a 10% solution of $Mg(NO_3)_2$ in ethanol (60 μl) was added. The tube was placed in an oven to evaporate the solvent, and then heated over a strong flame until NO_2 fuming ceased. The tube was cooled to room temperature, and 0.3 ml of 1 N HCl was added; after about 1 h, 1.38 ml of deionized distilled water and 0.32 ml of color-developing reagent solution were then added. The blue color usually developed in less than 5 min at room temperature. The optical density of the assay solution at 882 nm was determined after half an hour. The procedure was calibrated by determining phosphate content of a freshly prepared standard solution of KH_2PO_4 of known concentration.

Osmium content was determined by the Na_2O_2 method of Beer et al. (1966). Two approximate but more rapid methods were also used to determine osmium content in the preliminary stages of this investigation. The KOH method was based on the procedure used by Ayres and Wells (1950). Aliquots (0.7 ml) were transferred to analytical tubes and 15 μl of 1 N KOH was added to each tube. These tubes were heated in boiling water for 15 min, and 0.2 ml of 12 N H_2SO_4 and 20 μl of 10% thiourea solution were then added. The tubes were heated in boiling water again for 15 min to develop the color. The volume of the assay solution was brought to 2 ml before the visible spectrum between 700 and 400 nm was recorded. This method, which was not applicable for those Os-containing products with bidentate ligands, was calibrated by determining the osmium content of a solution of OsO_4 of known concentration in 0.01 M KOH. The KCN method used essentially the same procedure as that of the KOH method, except that 10 μl of 0.2 M KCN solution was added to each tube and, after 1 h, 1 N KOH was added. The KCN method was calibrated by determining the osmium content of a stock solution of $Na_4[OsO_4(CN)_2(5'\text{-TMP})]$ which had been synthesized in this lab. The concentration of osmium in this stock solution was determined in turn by the Na_2O_2 method.

Sodium carbonate (70 mg) was added to 1 ml of concentrated DNA,Os,Py[1] solution. The resulting solution was left at room temperature for 2 days and then passed through a Sephadex G-50–150 column. The polymer fraction was pooled and evaporated to dryness under vacuum. The residue was

TABLE I: Os/P Ratios of the Reaction Products of Nucleic Acid Polymers with OsO_4–Pyridine.

Nucleic Acid Polymer	Reaction Time	Os/P Ratio[a]
Poly(C)	24 h	(1.10); (1.02, 1.12, 1.08)[b]
	48 h	(1.07, 1.03, 1.04, 1.01);[b] (1.00, 1.03, 1.05, 0.95)[b]
	9 days	(0.97, 0.89, 1.06, 1.07)
Denatured DNA	1 h	(0.30, 0.29)
	2 h	(0.38, 0.38)
	3 h	(0.46, 0.47)
	24 h	(0.49, 0.53, 0.49)
	45 h	(0.50, 0.47)
	5 days	(0.36, 0.31)
	7 days	(0.34, 0.30, 0.36)[c]
	9 days	(0.36, 0.29)
Poly[d(AT)]	12 h	(0.35, 0.42)[c]
	24 h	(0.35, 0.40);[c] (0.43, 0.40)[b]

[a] Os content determined by the Na_2O_2 method unless otherwise noted. [b] Os content determined by the KOH method. [c] Os content determined by the KCN method.

dissolved in 1 ml of water. An aliquot (200 μl) of this solution was transferred to a sealable tube which was then placed in an oven to evaporate the solvent. This procedure was repeated until all the solution had been dried in one tube. Concentrated perchloric acid (25 μl) was added to the residue; the tube was then sealed and heated in boiling water for 1 h (Marshak and Vogel, 1951). After cooling, approximately 60 μl of 4 N KOH was added to neutralize the perchloric acid digest and this neutralized solution (10 μl) was spotted on a cellulose thin-layer plate (Eastman Chromatogram Sheet 13254, cellulose adsorbent with fluorescent indicator). As a control, an aliquot (5 μl) of a stock solution containing 5 mM of each of four nucleic acid bases (A,G,C,T) was also spotted. The plate was placed in a jar saturated with the eluting solvent (MeOH–HCl–H_2O, 7:2:1) and developed for 35 min. The separated bases were readily detected by UV irradiation.

Results

1. Reaction of OsO_4–Pyridine with Nucleic Acid Polymers. Poly(C). Poly(C) was reacted with OsO_4–pyridine at room temperature and neutral pH for at least 24 h. The reaction product, poly(C),Os,Py, purified on Sephadex, is an Os-containing polynucleotide with Os/P ratio equal to 1 within experimental error. The Os/P ratios obtained at different times for this reaction are shown in Table I. There was no change in osmium uptake after 24 h. However, after Sephadex purification, these Os-containing products (Os/P = 1) are not stable in aqueous solutions. The color of such solutions was found to change from an initial light yellow to a dark-violet color after several days. Elution of this dark-violet solution through Sephadex decreased the Os/P ratio of the polymer fraction (Table II).

Poly(U). The reaction product of poly(U) with OsO_4–pyridine, purified as above, contained no osmium. The UV spectra for the column purified 1 and 24 h reaction products are shown in Figure 1. The purified 24-h reaction product has an UV spectrum similar to that described by Burton and Riley (1967) for an Os-oxidized poly(U) product obtained from the reaction of poly(U) with OsO_4 in ammonium chloride buffer and purified by dialysis. When an aliquot from a similar reaction mixture (1.5 M pyridine, 24-h reaction) was eluted with the same concentration of pyridine on Sephadex, the polymer fraction showed considerable osmium content (Os/P = 0.6). This result suggests that the osmium was bound to poly(U) before chromatography on Sephadex and that the bound osmium was removed by the column support.

TABLE II: Decomposition of Poly(C),Os,Py in Aqueous Solution.

Time[a]	Os/P Ratio
0	(1.0)[b,c]
4 days	(0.60)[b]
5 days	(0.75)[c]
12 days	(0.72, 0.64)[c]

[a] Time at room temperature before Sephadex purification. [b] Os content determined by the KOH method. [c] Os content determined by the Na_2O_2 method.

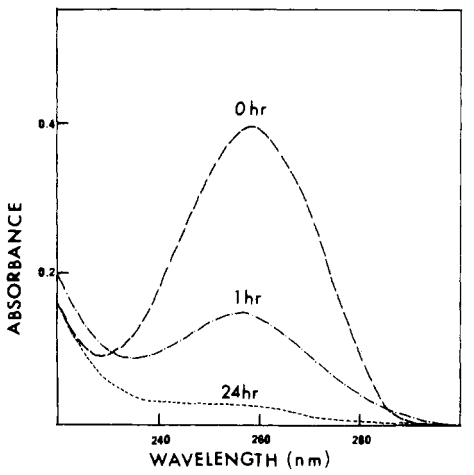


FIGURE 1: UV spectra of partially (— · —·) and completely (- - -) reacted poly(U) products with OsO_4–pyridine. The reaction mixture contained 3 mM OsO_4, 2 M pyridine, and 1.8 mM poly(U) at pH 7.1. The polymer concentration is 0.42 mM in all three spectra. The spectrum of unreacted poly(U) (— — —) is illustrated for comparison.

Denatured DNA. Denatured DNA reacts with OsO_4–pyridine at room temperature and neutral pH to form an Os-containing product, DNA,Os,Py, with Os/P ratio reaching 0.5 within 24 h (Table I). Reaction times longer than 3 days yielded products with lower Os/P ratios. The reacted DNA can be purified on Sephadex without apparent loss of bound osmium. However, the color of solutions containing such column purified products also gradually changed to dark violet as was observed for purified poly(C),Os,Py. Perchloric acid hydrolysis was not successful with OsO_4–CN^- reacted DNA product (Highton et al., 1968). In order to perform TLC analysis of the bases in DNA,Os,Py, osmium was removed from DNA,Os,Py. The bis(pyridine) osmate ester of thymine has been found to convert into thymine glycol at room temperature in saturated sodium carbonate solution (Subbaraman et al., 1971). A solution containing DNA,Os,Py was concentrated by rotary evaporation, then saturated with Na_2CO_3, left

[1] Abbreviations used: DNA,Os,Py and poly(C),Os,Py, the column purified reaction products of denatured DNA or poly(C) with OsO_4–pyridine, Os/P ratios equal to 0.5 and 1.0, respectively; DamHP, 2-(dimethylaminomethyl)-3-hydroxypyridine; NaHQS, sodium 8-hydroxyquinoline-5-sulfonate; Na_2BPDS, bathophenanthrolinedisulfonic acid, disodium salt; Na_2DPTADS, 3-(2-pyridyl)-5,6-diphenyl-1,2,4-triazine-*p,p'*-disulfonic acid, disodium salt; UV, ultraviolet; TLC, thin-layer chromatography; poly(U), poly(uridylic acid); poly(C), poly(cytidylic acid); poly(A), poly(adenylic acid); poly(G), poly(guanylic acid).

TABLE III: Os/P Ratios of the Reaction Products Formed between OsO_4-Bipyridine and Nucleic Acid Polymers.

Nucleic Acid Polymer	Reaction Time	Os/P Ratio[a]
Poly(U)	24 h	(0.97)
	4 days	(0.83, 0.91, 1.00)
Poly(C)	24 h	(0.86); (1.00)
Poly(A)	24 h	(0.00)
Poly(G)	24 h	(0.00)
Denatured DNA	24 h	(0.48, 0.48, 0.55, 0.49)
	3 days	(0.50)
	13 days	(0.49, 0.47, 0.49)
Native DNA	21 h	(0.45, 0.44)

[a] Os content determined by the Na_2O_2 method.

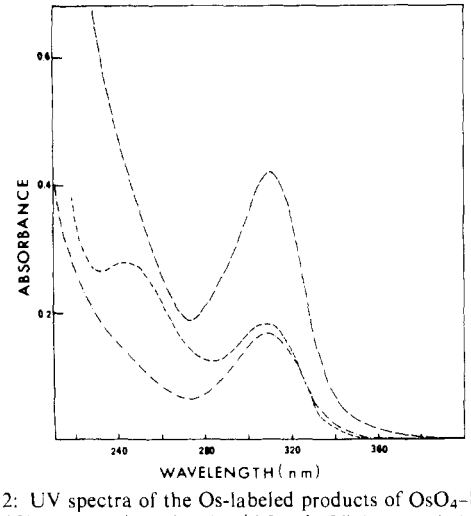


FIGURE 2: UV spectra of the Os-labeled products of OsO_4-bipyridine with poly(C) (— — —), 3.78×10^{-4} M, poly(U) (— · — ·), 1.53×10^{-4} M, and denatured DNA (- - -), 2.84×10^{-4} M. The reaction time was 24 h.

at room temperature for 2 days, and finally purified on Sephadex. The polymer fraction so obtained did not contain osmium. Thin-layer chromatography of the perchloric acid hydroxylate of this Os-free DNA exhibited only two spots corresponding to G and A.

Poly [d(AT)]. The reaction of poly[d(AT)] with OsO_4-pyridine was carried out at 65 ± 1 °C in a sealed tube for 12 or 24 h. The Os/P ratios, shown in Table I, are only approximate as osmium content in these products was not determined by the Na_2O_2 method.[2]

2. Reaction of OsO_4-Bipyridine with Nucleic Acid Polymers. Reactions of OsO_4-bipyridine with nucleic acid polymers were carried out at room temperature and neutral pH. The reaction time was usually 24 h and the reaction products were purified on Sephadex. Table III gives the Os/P ratios obtained for the reaction of OsO_4-bipyridine with four polyribonucleotides (poly(C), poly(U), poly(A), poly(G)) and with both denatured and native DNA. The UV spectra of the purified Os-containing reaction products are shown in Figure 2. A characteristic maximum at 310 nm occurs in all the spectra.

[2] Os content determined by the two approximate methods was usually 10 to 15% low compared with that obtained by the Na_2O_2 method. These two approximate methods gave similar osmium analyses for Os-containing polydeoxyribonucleotide products. For Os-containing polyribonucleotide products, the results obtained by the KCN method were considerably lower than those obtained by the KOH method.

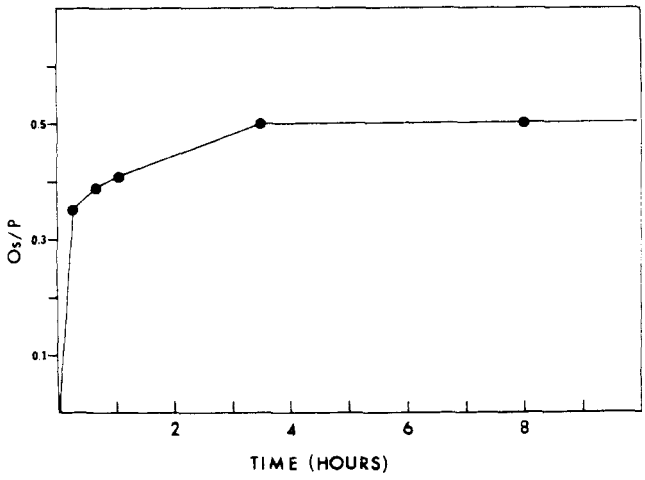


FIGURE 3: The time dependence of the Os/P ratio during reaction of denatured DNA with OsO_4-bipyridine. The reaction mixture contained 9 mM OsO_4, 9 mM bipyridine, 1.7 mM DNA at pH 6.8.

TABLE IV: Os/P Ratios of the Reaction Products of Nucleic Acid Polymers with OsO_4-BPDS.

Polymer	Reaction Time (h)	Os/P Ratio[a]
Poly(U)	24	0.97
	69	0.94
	168	0.90
Poly(C)	24	0.30
	46[b]	0.31
	70[b]	0.36
	70[c]	0.74
	137[c]	0.90
Denatured DNA	24	(0.50, 0.46)
	46	(0.48, 0.47)

[a] Os content determined by the Na_2O_2 method. [b] Reaction mixture contained 8 mM OsO_4, 8 mM BPDS, 2 mM poly(C), pH 7.0. [c] Reaction mixture contained 27 mM OsO_4, 24 mM BPDS, 2 mM poly(C), pH 6.8.

The molar extinction coefficients at this wavelength were determined to be 11 000, 10 800, and 6200 cm^{-1} M^{-1} for the OsO_4-bipyridine-reacted poly(C), poly(U), and denatured DNA products, respectively. A time study was carried out for the reaction between denatured DNA and the OsO_4-bipyridine reagent. The Os/P ratio vs. reaction time is plotted in Figure 3. Clearly, the reaction product is an Os-containing DNA with Os/P ratio equal to 0.5.

3. Reaction of Nucleic Acid Polymers with OsO_4 and Other Bidentate Nitrogen Donor Ligands. The reactions of nucleic acid polymers with OsO_4 and two negatively charged bidentate nitrogen donor ligands, BPDS and DPTADS, were carried out as above. The results are summarized in Table IV. The OsO_4-BPDS reagent yielded Os-containing products for poly(U) and denatured DNA, with Os/P ratios equal to 1.0 and 0.5, respectively, by 24 h. These ratios remained the same for reaction times longer than 24 h. Poly(C) was less reactive and required longer time periods and higher concentrations of OsO_4-BPDS. There was, however, no osmium found in the polymer fraction after reacting poly(U) with OsO_4-DPTADS for up to 11 days. Similar reactions of poly(U) or denatured DNA with OsO_4-DamHP and OsO_4-HQS resulted in the formation of polymeric osmium species in addition to the Os-bound nucleic acid polymers. The reacted poly(U) or DNA products could not be

TABLE V: Ultraviolet Spectral Data on Osmium-Labeled Polynucleotides.

Polymer	Ligand	Os/P	λ_{max} (nm)	λ_{min} (nm)	ϵ_{max} (M^{-1} cm^{-1})	$\epsilon_{max}/\epsilon_{min}$
Poly(U)	bipy	1.0	310	272	10 800	2.68
Poly(C)	bipy	1.0	310	272	11 000	2.29
DNA	bipy	0.5	310, 250	282	6 200	1.47,[a] 1.50[b]
Poly(U)	BPDS	1.0	290	255	37 170	1.66
DNA	BPDS	0.5	2.88	255	21 330	1.28
Poly(C)	BPDS	0.9	285	255	32 200	1.25
DNA	py	0.5	255	235	7 220	1.32
Poly(C)	py	1.0	250	240	12 750	1.01

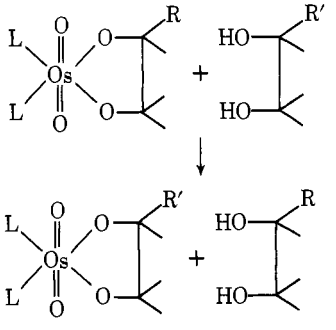
[a] $\epsilon_{310}/\epsilon_{min}$. [b] $\epsilon_{250}/\epsilon_{310}$.

separated from these unidentified polymeric osmium species by Sephadex chromatography. Elution of aliquots of a control solution in which no nucleic acid polymer was present also revealed the formation of polymeric Os-containing products, as evidenced by Sephadex chromatography.

Discussion

The extensive studies of Behrman cited in the introduction on the addition of OsO_4 to nucleic acid bases of nucleosides, nucleotides and dinucleoside monophosphates in the presence of pyridine prompted our investigation of the applicability of this reagent to the labeling of polymers. Although our results suggest that the selectivity of this reagent toward pyrimidine bases is maintained during reaction with DNA, we also observed considerable instability of the osmium label and a decrease in the Os/P ratio with time. Unless pyridine was present at high concentration in the eluent, it was not possible to purify osmium-labeled poly(U) and retain any of the label. Even with pyridine present, the osmium label was not completely retained.

The bis(pyridine) osmate esters have been shown to undergo transesterification reactions (eq 1) much more readily than



(1)

comparable bipyridine osmate esters (Daniel and Behrman, 1975, 1976). The facility of the transesterification reaction depends on the nature of the organic part of the ester as well. The exchange reaction is inversely proportional to L ligand concentration. Since these characteristics of the transesterification reaction on small molecules paralleled the loss of osmium label from polynucleotides, we extended our investigation to include bidentate chelate ligands.

The stability of the osmium label in osmium tetroxide treated polynucleotides containing bipyridine was dramatically greater than that for polymers containing pyridine. Whereas the osmium label was lost completely on Sephadex purification of poly(U) treated with OsO_4–pyridine, full retention of the label was found for OsO_4–bipyridine-treated poly(U). Furthermore, in the absence of the OsO_4–bipyridine reagent, there was no appreciable loss of osmium label from reacted poly(U), even after 4 months.

The great stability of the bipyridine osmate esters of nucleic acids is obviously advantageous. However, our results indicate that the OsO_4–bipyridine reagent is probably too reactive to serve as a selective reagent for thymine (see Figure 3). This lower selectivity of the OsO_4–bipyridine reagent compared with the OsO_4–pyridine was also observed (Ragazzo and Behrman, 1976) in studies comparing the reactivity of N^6-(Δ^2-isopentenyl)adenine with thymidine.

There are a number of practical approaches for overcoming the problem of selectivity. One feasible approach which is currently being pursued in our laboratories (S. Rose, C. H. Chang, M. Beer, and L. G. Marzilli, unpublished results) is to modify the C residues in DNA so as either to make these residues unreactive toward OsO_4–bipyridine or make the residues add more than one Os per C residue. A second approach, which will be discussed in detail next, is to alter the chelate ligand so as to introduce a negative charge. Such negatively charged reagents would generally be expected to attack the negatively charged polynucleotides less readily.

Although several negatively charged chelate ligands have been studied (see Results), we will limit this discussion to the most promising reagent BPDS. After one day, the OsO_4–BPDS reagent labeled poly(U) completely. After 1 day, the pyrimidine bases in DNA were completely labeled. However, poly(C) was labeled to only 30%. After increasing the concentration of the OsO_4–BPDS reagent, it was possible to label poly(C) to ca. 90%. The BPDS-containing labeled polymers appear to be more stable than the pyridine-containing polymers and, thus, the OsO_4–BPDS reagent shares with OsO_4–bipyridine the desirable characteristic of stability of labeling. These negative chelates have several possible advantages; additional heavy metals might be introduced to neutralize the charge and the negatively charged osmium labels will repel each other and, therefore, might be separated better on grids than the neutral osmium labels.

We have assumed throughout this discussion that the nature of the osmium label on the polynucleotides corresponds exactly to the structure of the monomers. The electronic absorption spectrum of the bipyridine-labeled polymers (Table V) in the region of about 310 nm provides support for this assumption and in addition appears to be a useful spectral region for monitoring the labeling reaction and the stability of the labeled polymer. The extinction coefficient of the bipyridine osmate esters in the monopolymer appears to be about 11 000 M^{-1} cm^{-1}. DNA contains 50% pyrimidine bases and the apparent extinction coefficient is 6200 M^{-1} cm^{-1} or 12 400 M^{-1} cm^{-1} per pyrimidine moiety. The absorbance of the unreacted purine

bases is negligible at these wavelengths. Behrman has also noted this spectral feature in nonpolymeric bipyridine osmate esters (Daniel and Behrman, 1975). The extinction coefficients found for a number of derivatives were all in the range of 12 200 to 13 700 M^{-1} cm^{-1} and this band could be attributed to a transition in the coordinated bipyridine ligand after literature assignments (McWhinnie and Miller, 1969). This similarity in spectral properties and the obvious parallels between the chemistry of the monomeric and polymeric osmate esters strongly suggest that the nature of the osmium moieties in the polynucleotides studied here is similar to that observed and characterized previously for monomeric compounds. The exact nature of the osmate ester of cytosine derivatives is not yet fully established, however.

In summary, bis(pyridine) osmate esters of nucleic acids are probably marginally stable for use in electron microscopic studies of nucleic acid sequences by the visualization of single heavy atoms. Bipyridine osmate esters are considerably more stable and, since feasible alternatives are possible for overcoming the low selectivity of the OsO_4–bipyridine label, this latter reagent (or that of a similar but charged ligand) appears to be a most promising method for multiple site derivatization of nucleic acids. Behrman has already suggested this reagent for single site labeling. However, the lability of the pyridine in bis(pyridine) osmate esters (Marzilli, et al., 1976) does offer the attractive advantage of permitting ligand exchange reactions at osmium.

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