Electron Microscopic Study of Base Sequence in Nucleic Acids. VIII. Specific Conversion of Thymine into Anionic Osmate Esters*

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ABSTRACT: 3′,5′-Diacetylthymidine (DAT) was treated with OsO_4 in benzene solution to give an addition product containing 1 mole of osmium/mole of DAT. This osmate ester, as well as those formed from cyclohexene and 3-butenoic acid, formed anionic addition compounds with 2 moles of CN^- or SCN^-. An identical addition compound was also formed with thymidine in water if OsO_4 and CN^- ions were added together. Cyanide addition prevented ester hydrolysis. At pH 7 no addition occurred with adenosine or guanosine, and only relatively slowly with cytidine. A similar reaction also occurred with the thymine base of deoxyribonucleic acid, converting it into an altered form carrying one osmium atom and two cyanide ions.

An electron microscopic study of nucleotide sequence requires reagents which can couple heavy atoms selectively to specific bases (Beer and Moudrianakis, 1962). In a quest for such markers we have studied the reaction of OsO_4 with nucleotides, nucleosides, and DNA and have shown that the reaction is specific for thymine (Beer *et al.*, 1966). Similar results have been obtained by Burton and Riley (1966) who have studied the oligonucleotides yielded by diphenylamine degradation. However, the conditions used previously led to a hydrolytic breakdown of the expected thymidine–osmate cyclic ester.

Crigee (1936) has shown that the osmate esters are stable in nonaqueous solvents and that they react with 2 moles of pyridine to form addition products. In this paper we show that the osmate esters form apparently similar addition compounds with 2 moles of aqueous cyanide or thiocyanate and that these products are rather stable toward hydrolysis. Further it is shown that the same stable product can also be obtained in an aqueous solution of OsO_4 containing cyanide ion. Conditions were found under which thymidine was converted into the OsO_4–CN^- addition product. Under these same conditions cytidine reacted only slightly and guanosine and adenosine not detectably. Finally, it is shown that incubation of DNA under similar conditions gives an altered polymer in which the thymine residues are converted into a product carrying one osmium atom and two cyanide ions. The bound anions are important in that through them additional heavy cations might be bound to the altered thymine bases and so permit the recognition of these selectively stained bases in an electron microscopic study of extended DNA polynucleotide chains.

While this work was in progress Seligman and his collaborators (1966) developed a cytological staining reaction based on the binding of a ligand to an osmate ester in aqueous solvent. While the aim of that work was very different from the present one, there are some similarities between the reactions used.

Experimental Details

Materials. Cyclohexene was obtained from Eastman Organic Chemicals. It had a boiling point of 82.5–83.5°. 3-Butenoic acid (practical grade) was obtained from Eastman Organic Chemicals and was purified by vacuum distillation at 17 mm. The fraction with boiling range 68–72° was used. It had a refractive index of 1.4210. Thymidine (dT)[1] was obtained from Calbiochem, dT-2-^{14}C from Schwarz BioResearch Inc., and 3′,5′-diacetylthymidine (DAT) was prepared by the method of Michelson and Todd (1953) and crystallized from ether. Highly polymeric salmon sperm DNA was from Calbiochem. Osmium tetroxide was obtained from Fisher Scientific Co., and ^{14}C-labeled KCN and KSCN were from New England Nuclear Corp. Benzene and toluene were reagent grade from Baker.

Analyses. Osmium content was determined as before (Beer *et al.*, 1966), phosphate by the method of Chen *et al.* (1956). Formation of the osmate ester of DAT was followed by descending chromatography on Whatman No. 3MM paper using Wyatt's (1951) isopropyl alcohol–HCl solvent. Conversion of olefins or their osmate esters into the anionic cyanide addition compounds was followed by electrophoresis on Whatman No. 3MM paper using 2×10^{-2} M sodium phos-

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[1] Abbreviations used: dT, desoxythymidine; T–Os, thymidine–osmate ester; B–Os, 3-butenoic acid–osmate ester; DAT, 3′,5′-diacetylthymidine; DAT–Os, 3′,5′-diacetylthymidine–osmate ester.

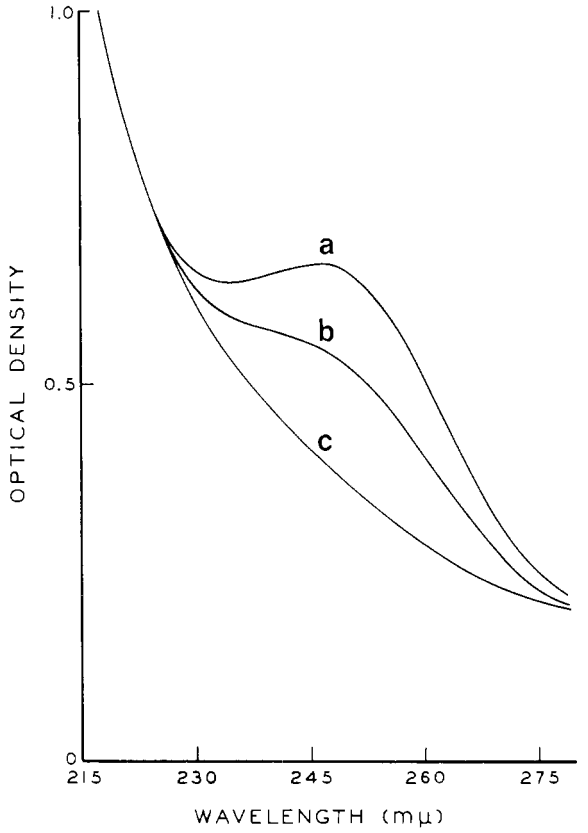

FIGURE 1: Ultraviolet spectra of the butenoic acid–osmate ester in aqueous KCN solutions. Concentration of KCN: (a) 0.06, (b) 0.04, and (c) 0.02 M. Ester concentration is 0.02 M. Solutions diluted 100× in water for spectroscopy.

phate buffer (pH 6.8) with a gradient of approximately 15 v/cm.

Reactions of OsO_4–CN^- with DNA. Highly polymerized salmon sperm DNA (50 mg) was dissolved in 50 ml of water, 1 ml of 0.25 M EDTA (pH 8) was added, the solution was stored in the cold overnight, dialyzed twice against 10^{-2} M NaCl, and then diluted with two volumes of water. The DNA solutions were denatured by heating for 10 min in boiling water followed by rapid cooling in ice water. This denatured DNA solution was added to an equal volume of a solution containing 0.20 M OsO_3 and 0.20 M $K^{14}CN$ was adjusted to pH 7.0 with HCl. Reaction mixtures were incubated at 55° in sealed, Pyrex tubes.

The reacted polymer was purified on Sephadex G-75, precipitated in the cold by ethanol, redissolved at a concentration of 1 mg/ml, and centrifuged at 2000*g* for 10 min. Aliquots of the resulting DNA solution were used to determine the phosphate content, the osmium content, the cyanide uptake, and the abundance of the bases in the reacted DNA.

The uptake of cyanide was determined by spotting solutions of purified DNA on filter paper and counting in a liquid scintillation counter or an Actigraph recorder. To estimate the number of moles of cyanide from the number of counts, aliquots of the tetraoxide–cyanide reagent were also spotted and counted. Hydrolysis was carried out by the two-step method of Daly *et al.* (1950). This involves a mild hydrolysis with methanol–HCl for the determination of the purines and a separate more drastic hydrolysis in 5 N HCl for the pyrimidines. Perchloric acid and formic acid hydrolyses could not be used successfully in the presence of osmium-containing products. Also hydrolysis by DNase and snake venom phosphodiesterase proved unsuccessful. Bases were determined by descending chromatography on Whatman 3MM paper using Wyatt's (1951) isopropyl alcohol–HCl solvent.

Results

1. The Osmate Ester of 3-Butenoic Acid. PREPARATION OF B–Os. To a 1% solution of OsO_4 in toluene was added 0.02 ml of 3-butenoic acid. Within minutes the solution turned greenish-brown and a precipitate was formed. The mixture was left open in a fume hood to volatilize solvent and unreacted OsO_4, leaving a dark precipitate. Analysis for osmium content indicated the addition of 1 g-atom of osmium/mole of butenoic acid (Table I).

TABLE I: Stoichiometry of Osmium Addition Determined by Analysis of Reaction Product.

Reaction Product	% Os (by wt) Determined	Theor % Os (by wt) for 1:1 Addition
B–Os	52.0 ± 2.1	56.0
C–Os[a]	60.9 ± 2.6	56.5
DAT–Os	28.9 ± 7.7	32.8
DAT–Os–CN	19	30

[a] Cyclohexene–osmate ester.

REACTION OF B–Os WITH CYANIDE. The osmate ester was found to be very soluble in aqueous solutions of potassium cyanide; the rate at which it went into solution increased with cyanide concentration.

In Figure 1 are shown ultraviolet spectra of solutions containing the same concentration of B–Os, but different KCN concentrations. The spectra were taken 1 hr after the addition of KCN, when the material was completely in solution at all KCN concentrations. The peak at 245 mμ increased as the cyanide concentration was increased. Thus the cyanide appears to be reacting with the product of the reaction of 3-butneoic acid and OsO_4.

On electrophoresis B–Os did not move from the origin. However, when cyanide was added, a new

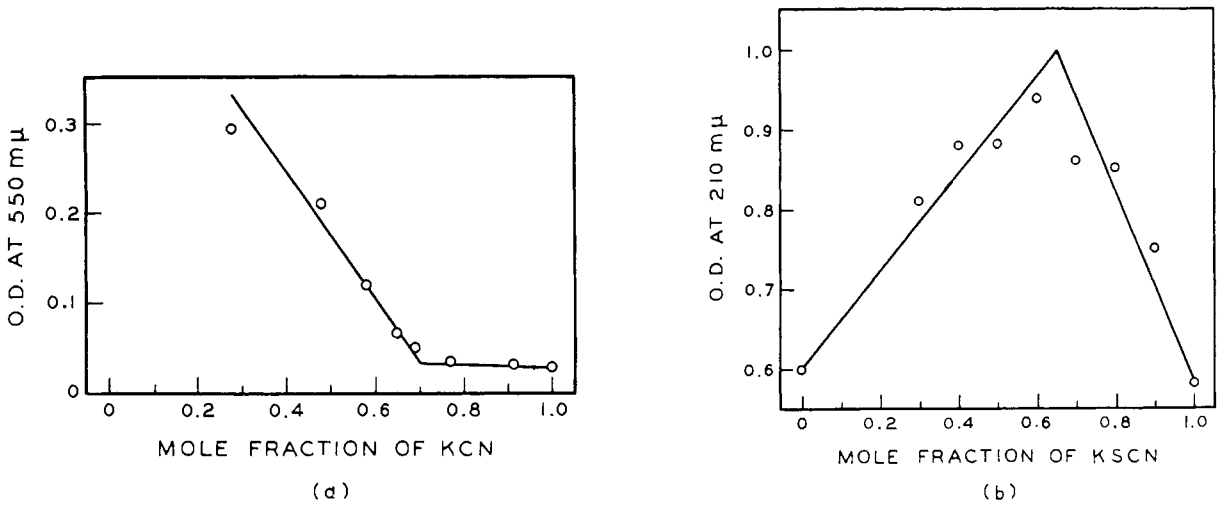

FIGURE 2: Stoichiometry studies. (a) Stoichiometry of addition of CN^- ions to the butenoic acid–osmate ester. Position of break in curve indicates about 2 moles of KCN/mole of osmate ester. (b) Stoichiometry of addition of SCN^- ions to the butenoic acid–osmate ester. Position of break in curve indicates between 1.5 and $2^1/_3$ moles of KSCN per mole of osmate ester.

material appeared that moved toward the anode. The different bands were not studied further; however, they were examined in detail in the analogous reaction of DAT^+ to be described below.

The stoichiometry of the reaction of B–Os with cyanide was determined spectrophotometrically as follows. B–Os (3 mg) was weighed out in each of eight test tubes. Various volumes of 0.2 M KCN in water were added so that the mole fraction of the KCN in the mixture ranged from 0.25 to 0.9. A control tube contained only KCN. After thorough mixing to dissolve all the B–Os in the concentrated KCN solution, water was added to give a total concentration of B–Os plus KCN of 10^{-4} M for each solution. Spectra were determined in the range from 350 to 650 mμ and the optical density at 530 mμ was plotted as a function of the mole fraction of KCN. The results are given in Figure 2a. The sharp break in the curve occurs approximately at 0.7 mole of KCN or a mole ratio of 0.7:0.3 or $2^1/_3$ moles of KCN/mole of osmate ester. Within experimental error the data suggest that 2 moles of KCN react with 1 mole of ester.

REACTIONS OF B–Os WITH THIOCYANATE AND CYANATE. With solutions of KCSN, B–Os gave products which in electrophoresis moved toward the anode. The stoichiometry of the reaction was determined by a procedure similar to the one described above with KCN. Spectra were obtained in the range from 200 to 350 mμ and the optical density at 210 mμ was plotted against the mole fraction of KSCN. The results are given in Figure 2b. The sharp break in the curve occurs between 0.6 and 0.7 mole of KSCN, suggesting that 2 moles of KSCN react with 1 mole of ester. When B–Os was dissolved in solutions of sodium cyanate (NaNCO), no change in the electrophoretic mobility was found, suggesting no reaction.

2. The Osmate Ester of Cyclohexene. To 1 ml of a 3% solution of OsO_4 in toluene was added 0.03 ml of cyclohexene. The solution rapidly turned black. It was left open overnight in a fume hood to permit evaporation of solvent and unreacted OsO_4 leaving a black noncrystalline solid. Analysis for osmium (Table I) indicated the addition of one osmium atom per cyclohexene molecule. In electrophoresis at pH 6.8 the product did not move. However, in the presence of cyanide it was converted into a new substance that mi-

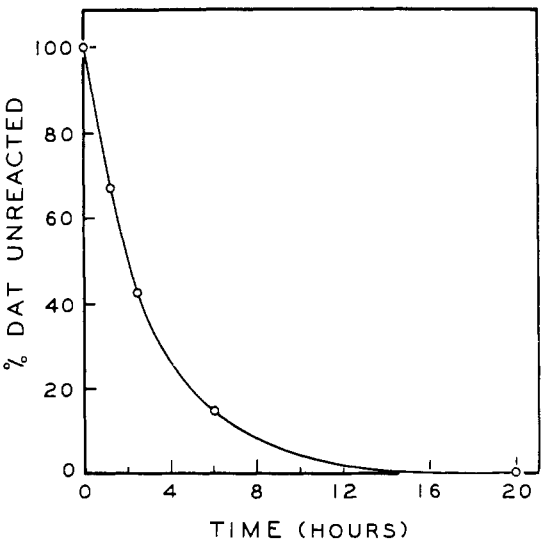

FIGURE 3: The rate of reaction of 3′,5′-diacetylthymidine (DAT) with OsO_4 in benzene at 55°. Concentration of DAT was 5 mg/ml and of OsO_4 1 g/ml.

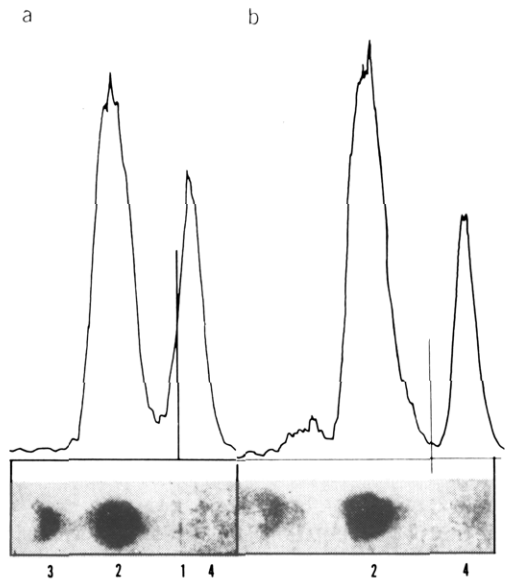

FIGURE 4: Electrophoretic pattern (bottom) and corresponding scan of radioactivity of ^{14}C-labeled DAT–Os dissolved in 0.4 M KCN. Vertical line indicates origin. Anode is to the left, cathode to the right. Numbers below electrophoretic pattern are used to identify spots (see text). (a) Immediately after dissolving in KCN; (b) 170 hr after dissolving.

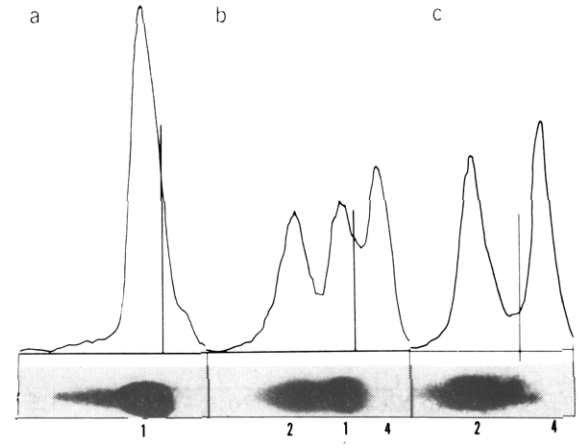

FIGURE 5: Electrophoretic pattern (bottom) and corresponding scan of radioactivity of ^{14}C-labeled DAT–Os dissolved in 0.08 M KSCN. Vertical line indicates origin. Anode is to the left, cathode to the right. Numbers below electrophoretic pattern are used to identify the spots (see text). (a) Immediately after dissolving in KSCN; (b) 21 hr later; (c) 170 hr later.

grated toward the anode. In this way the behavior of this ester was like that of butenoic acid.

3. The Osmate Ester of 3,'5'-Diacetylthymidine. To study the reactions of thymidine, analogous to those described above, we had to render it soluble in a nonaqueous solvent. This was done by converting it into 3',5'-diacetylthymidine (DAT), using the method of Michelson and Todd (1955).

PREPARATION OF DAT–Os ESTER. DAT was reacted with OsO_4 in benzene. Solutions containing 0.5 g/ml of OsO_4 and 2.5 mg/ml of DAT were incubated at 55° in sealed, glass tubes. The reacted material formed a black precipitate. The extent of the reaction at different times was inferred from the loss of DAT. Aliquots of the solution were chromatographed to separate unreacted DAT, which was eluted in water and its amount was determined from the optical density at 267 mμ. The reaction was found to be 95% complete in 8 hr (Figure 3).

Two methods were used to determine the stoichiometry of the reaction of OsO_4 with DAT. First, 12.5 mg of DAT and 0.25 g of OsO_4 were incubated in 5 ml of benzene at 55° for 40 hr. Aliquots of the solution were chromatographed, and the spots were eluted in water. From their spectra the reaction was seen to be 90% complete. The solution was dried to a constant weight. The increase in weight over a control containing no DAT was 10.9 mg. This control was necessary since there was a slight reaction of OsO_4 with benzene, resulting in a nonvolatile precipitate. The increase in mass corresponded to the addition of 1.1 moles of OsO_4/mole of DAT.

The second method for determining the stoichiometry of reaction involved direct analysis for osmium on DAT–Os. The results again indicated the addition of one osmium atom per DAT (Table I).

ADDITION OF CYANIDE TO DAT–Os. The addition of cyanide to the osmate ester in aqueous solution was followed by electrophoresis. In the absence of cyanide a single purplish spot (1 in Figure 4a) close to the origin was observed. When enough KCN was added to this solution to give a final concentration of 0.4 M KCN with a CN^-:DAT mole ratio of 20:1, a second and third spot (2 and 3 in Figure 4) appeared in electrophoresis. Both moved faster toward the

TABLE II: Addition of CN^- Ions to DAT–Osmate Ester.

Expt	% DAT with Bound CN^- Ions	Mole Ratio CN^-:DAT
1[b]	73	
2	72	2.0:1
3	76	
4	68	1.8:1
5		2.0:1[a]

[a] Per cent DAT with bound CN^- ions used for this calculation was taken as 72%, the average of the four other experiments. [b] All five experiments are identical and electrophoretic analysis was performed immediately after CN^- addition.

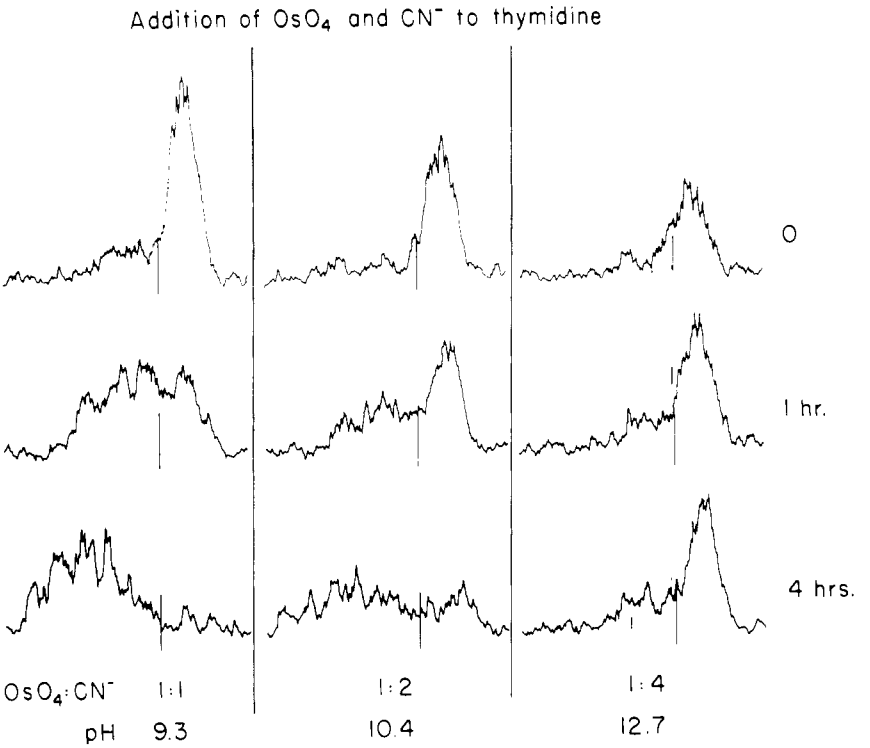

FIGURE 6: Reaction of thymidine-^{14}C with 0.1 M OsO_4 in the presence of (a) 0.1 M KCN, (b) 0.2 M KCN, and (c) 0.4 M KCN at 55°. Aliquots of the reaction mixture were subjected to electrophoresis at pH 7 and the strips were scanned with a counter. The traces show activity along the strip. Vertical line indicates the origin; anode was to the left, cathode to the right. Most rapid conversion of thymidine into an anonic product occurred in case a.

anode than spot 1. Spot 2 was brown and spot 3 was yellow. Using ^{14}C-labeled DAT, about 70% of the activity (Table II) was shown to be in spot 2. Under these conditions, the remainder was found in a colorless spot (4 in Figure 4) which did not absorb ultraviolet light and was on the cathode side of the origin (Figure 4).

In controls containing no DAT a spot in the same position as spot 3 appeared, apparently resulting from a product formed by the reaction of OsO_4 with benzene. With DAT-^{14}C no radioactivity was ever found in this region.

Using ^{14}C-labeled KCN, spot 4 was shown to contain no CN^- ions, and since it was colorless, very likely it contained no osmium. Further, as it had no ultraviolet absorption, it was not aromatic and was assumed to be the glycol resulting from the hydrolysis of the DAT–osmate ester.

The osmium released by the 30% hydrolysis of the ester was assumed to be in spot 3, together with the products from the reaction of OsO_4 with benzene. The major component of spot 2 was considered to be the DAT–osmate ester, complexed with CN^-. Radioactivity was found in this spot when either DAT-^{14}C or KCN two-thirds was used. A fourfold increase in time of electrophoresis did not resolve it into two or more spots.

Parallel quantitative runs indicated the presence of 2 moles of cyanide/mole of DAT in spot 2 (Table II). A rather poor analysis for osmium on material eluted from this spot was obtained. It showed two-thirds osmium atoms for every DAT molecule (Table I). The low value is believed to result from experimental error.

To demonstrate that the coincidences in the position of the ^{14}C activities in DAT and in CN^- together with the color indicated a single compound, we also analyzed the system by chromatography using three different solvent systems. These were propanol–H_2O (85:15) (pH 7.1), butanol–H_2O (85:15) (pH 7.7), and ethanol–ammonium borate (70:30) (pH 9). In each of the three solvents the labeled CN^- ions, DAT, and the color moved at the same rate, showing they were components of a single compound.

The reaction of aqueous KCN with DAT–Os is rapid. Electrophoresis (Figure 4) carried out immediately after mixing the reagents indicated that the DAT–Os had already been converted into the rapidly moving product. In the subsequent 170 hr the electrophoretic pattern was essentially unchanged. The product was, therefore, stable in this medium. The fraction of material in spot 4 still constituted about 30% of the counts, when the pH of the reaction mixture was reduced from 11.3 to 9.0.

ADDITION OF THIOCYANATE TO DAT–Os. Potassium thiocyanate also reacted with the osmate ester of DAT

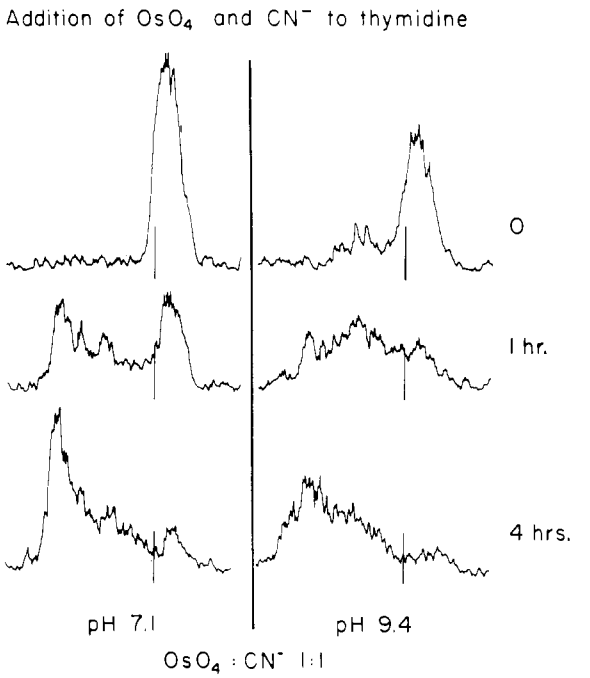

FIGURE 7: Reaction of thymidine-^{14}C with 0.1 M OsO_4 in the presence of 0.1 M KCN with pH adjusted to (a) 7.1 and (b) 9.4. Explanation of curves as above. No sharp dependence of pH is found in the rate of conversion of thymidine into the anionic product.

giving the anionic spot 2 of Figure 5. Clear reactions were obtained with 0.02 M DAT when 0.08 M KSCN was used. However, with 0.4 M KSCN much of the DAT remained at the origin. Parallel runs using ^{14}C-labeled DAT and ^{14}C-labeled KSCN indicated that the material in spot 2 contained about 2 moles of SCN^-/mole of DAT. In this respect the reaction with SCN^- resembled that with CN^-.

The rate of reaction with SCN^- was, however, rather slow. After 21 hr 30–50% of the radioactivity of DAT had been converted into the material of spot 2, 20% was still in spot 1 with the remainder in spot 4. This latter material carried the radioactivity of DAT but not of SCN^- and was colorless. As with the CN^- reaction, it is believed to be the 4,5-glycol of DAT. Interestingly, incubation for 170 hr. did not decrease the proportion of spot 2 indicating that the SCN^- addition product is stable and the presumed glycol is probably not formed from it but rather directly from the cyclic ester.

4. Reactions of Thymidine in Aqueous Solutions Containing both OsO_4 and KCN. Having established that the thymidine osmium cyanide addition product is stable, we sought procedures for its production under conditions that are compatible with DNA.

^{14}C-labeled thymidine was added to 0.1 M solutions of OsO_4 containing various amounts of KCN in water. At varying times aliquots of the reaction mixture were analyzed by paper electrophoresis and the radioactivity was determined by scanning.

Concentrations of 0.1, 0.2, and 0.4 M KCN gave reaction mixtures having pH 9.3, 10.4, and 12.7, respectively. The electrophoretic behavior of thymidine in these is shown in Figure 6. It is seen that in all cases the nucleoside is converted into a product that moves as an anion. This conversion was most rapid with 0.1 M KCN at pH 9.3 and slowest with 0.4 M KCN at pH 12.7. When the reaction mixture contained 0.1 M of both the OsO_4 and KCN, the pH appeared to have little effect on the rate of conversion into the anionic product. Figure 7 shows that the rate is not much changed between pH 7.1 and 9.4.

5. Identification of the Osmate Ester–Cyanide Complex Formed in Aqueous Solution with That Formed from the Ester Made in Anhydrous Solution. The osmate ester of DAT made in benzene as described in section 3 was treated with KCN in an aqueous solution. It was run in electrophoresis in parallel with DAT treated in an aqueous solution with OsO_4 and cyanide at pH 7 as described in section 4. ^{14}C-labeled thymidine was used in both. The mobility of the two products was the same. The two compounds were also run together in chromatography in isopropyl alcohol–H_2O (85:15), butanol–H_2O (85:15), and EtAB, the ethanol–ammonium borate buffer of Klenow and Lichtler (1957).

In all three chromatographic runs the products of the aqueous and nonaqueous system had the same mobilities when followed either by using ^{14}C-labeled thymidine or ^{14}C-labeled cyanide. In addition the products having these mobilities were colored indicating the presence of osmium. The identity of mobility in each experiment and the presence of thymidine, osmium, and cyanide in the products was taken as an indication that the products are in fact identical.

6. Hydrolysis of Thymidine Osmate Ester in the Absence of Cyanide. Some understanding was gained of the rate of breakdown of the thymidine osmate ester in the absence of cyanide. Thymidine was incubated at pH 6.1, 7.7, and 0.8 in 0.2 M OsO_4 at 55°. After incubation an aliquot was analyzed by paper electrophoresis. To the remainder was added $^1/_{25}$ volume of 5 M KCN and the mixture was left at room temperature for 24 hr and analyzed by electrophoresis. The results are shown in Figure 8. Two observations are important. First, it is clear that some electrophoretically mobile compound is produced even in the absence of CN^- if the times of incubation are very long (43 hr, line a at pH 7.7) or the pH is high (1.5 hr, line a at pH 9.8). These compounds are not the same as the ones obtained previously in the presence of cyanide: their rate of formation is nearly 100 times slower at pH 7 and they clearly do not contain cyanide, since no cyanide is present in these systems. These compounds probably result from the interaction of osmate esters with glycols formed by ester hydrolysis. At high pH the glycols could chelate with the osmium as was suggested by Crigee (1936).

The second observation from this experiment indicates that, in room temperature incubation of thymidine for 24 hr even at pH 6.1, the OsO_4–CN^- reagent

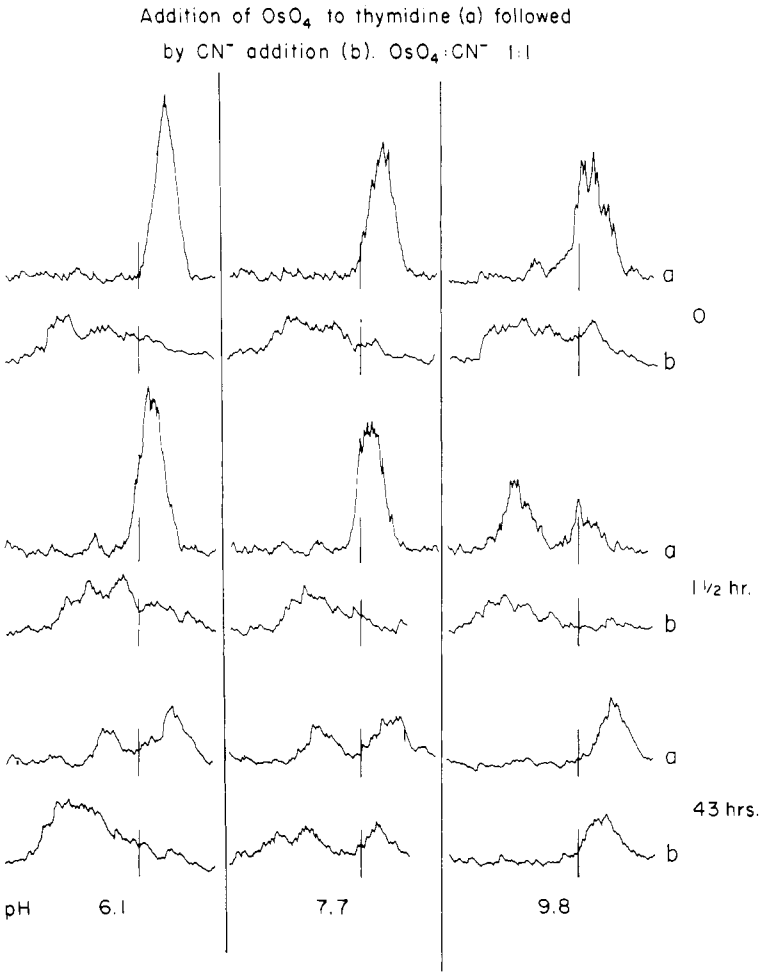

FIGURE 8: Reaction of thymidine-^{14}C with 0.1 M OsO_4 at 55°. (a) After reaction KCN was added to give 0.1 M and the reaction mixture was incubated for 24 hr at room temperature for 24 hr. (b) Reaction mixture contained 0.1 M KCN.

converts it into an anionic product. This is clear from a comparison of lines a and b at 0 hr in Figure 8. This conversion does not occur in the absence of cyanide and is probably the room temperature analog of the Os–CN^- reaction discussed in section 4 above. The inability to convert all the slow ^{14}C-containing material into the anionic product when incubating with Os–CN^- shows that the ^{14}C is no longer in the form of unreacted thymidine. This is the case after 43 hr at pH 7.7 and already after 1.5 hr at pH 9.8. An appreciable fraction of the thymidine has been converted into an unreactive product, presumably 4,5-dihydroxythymidine.

7. Selectivity of the Reaction of Os–CN^- with the Deoxynucleotides. Reaction mixtures were made up of each of the nucleotides and 0.1 M OsO_4 and 0.1 M KCN with the pH adjusted to 7. The reaction mixtures were incubated at 55°. At various times aliquots were removed for analysis by electrophoresis. The data for 5-hr incubation are shown in Figure 9. It is clear that thymidine is largely converted into the more negative product, guanosine, and adenosine seem unchanged, while cytidine has been converted into the extent of 10–20%. Thus this reaction of OsO_4–CN^- has very much the same rate and selectivity as does the aqueous reaction of OsO_4 in the absence of cyanide as found previously (Beer *et al.*, 1966).

8. Reactions of DNA with OsO_4–CN^-. Salmon sperm DNA was heat denatured and then incubated in sealed, Pyrex tubes at 55° in the presence of 0.1 M OsO_4–0.1 M $K^{14}CN$ at pH 7.0. At various reaction times from 0 to 12 hr, aliquots were purified by chromatography on Sephadex G-75. For DNA so treated we determined the optical density, the osmium content, the radioactivity (and hence the CN^- uptake), and the abundance of unreacted bases per mole of phosphate.

The results on the osmium uptake and cyanide uptake of the DNA which had been treated with the OsO_4–CN^- reagent are given in Table III. The former determination led to highly reproducible results. After 7 hr when about 0.29 mole of osmium/mole of phosphate had been bound to the DNA, the reaction continued only very slowly.

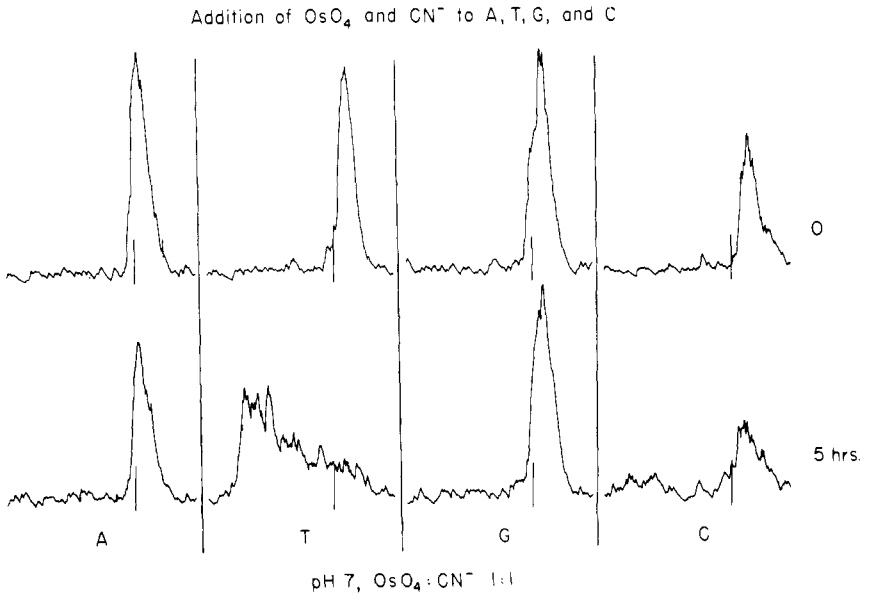

FIGURE 9. Reaction of ^{14}C-labeled deoxynucleosides with 0.1 M OsO_4 and 0.1 M KCN at pH 7, 55°. After reaction aliquots of mixture were analyzed by electrophoresis and the paper strips were scanned for radioactivity. Vertical lines indicate the origin. The mobility of adenosine and guanosine was unaltered, while essentially all of the thymidine was converted into parts involving components.

TABLE III: Osmium Content and Cyanide Content and Reacted DNA.

Duration of Reaction (hr)	Moles of Os/Mole of DNA Phosphate	Moles of CN^-/ Mole of DNA P	Moles of CN^-/ Mole of Os
0	0.03 ± 0.01		
3	0.15 ± 0.01	0.33 ± 0.03	2.2 ± 0.2
7	0.29 ± 0.01	0.52 ± 0.12	1.8 ± 0.4
12	0.30 ± 0.01	0.36 ± 0.12	1.2 ± 0.4

Our measurements of the uptake of cyanide were less reproducible. This may result from some loss during the purification of the DNA. Within the variation observed, it was found that in the early stages of the reaction 2 moles of cyanide were bound/mole of osmium. At longer times, however, there appeared a significant decrease in the cyanide to osmium ratio.

The destruction of bases during the reaction was followed by chromatography of the hydrolysate of the reacted DNA. The results are given graphically in Figure 10. It is clear that the major base affected is thymine and it is 90% destroyed in about 4 hr. During the same time the loss of cytosine is about 10% and the purines are unaffected within experimental error.

The loss of the pyrimidines occurred at the same rate as the uptake of osmium suggesting that these two effects are manifestations of the same reaction (Figure 11). Indeed, the data presented here are compatible with the hypothesis that in denatured DNA the reactions are those which would be expected on the basis of the results described earlier in this paper for the nucleosides. These suggest that thymine is most readily attacked and in the reaction it is converted into a new product which carries 1 g-atom of osmium and 2 moles of cyanide/mole of base.

Discussion

In the first part of this paper we report the preparation of the OsO_4 addition products of thymidine using benzene as a solvent. This reaction could be carried out only after the nucleoside was acetylated to enhance its solubility in the organic solvent. The addition product contained 1 g-atom of osmium/mole of thymidine. It could react with cyanide or thiocyanate to form a product which behaved as an anion in water and contained 2 moles of cyanide (or thiocyanate)/ mole of thymidine. In this reaction thymidine resembled two simple olefins, 3-butenoic acid and cyclohexene.

The cyanide addition product was formed rapidly in

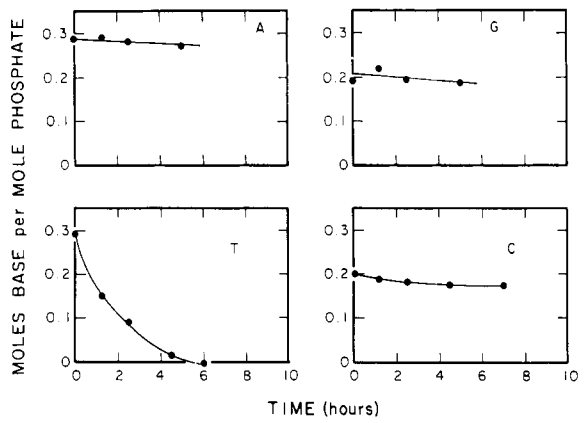

FIGURE 10: Disappearance of the bases of salmon sperm DNA in the presence of 0.1 M OsO_4 and 0.1 M KCN at pH 7, 55°. The thymine (90%) disappears in 6 hr while only about 10% of the cytosine is lost and negligible amounts of the purines.

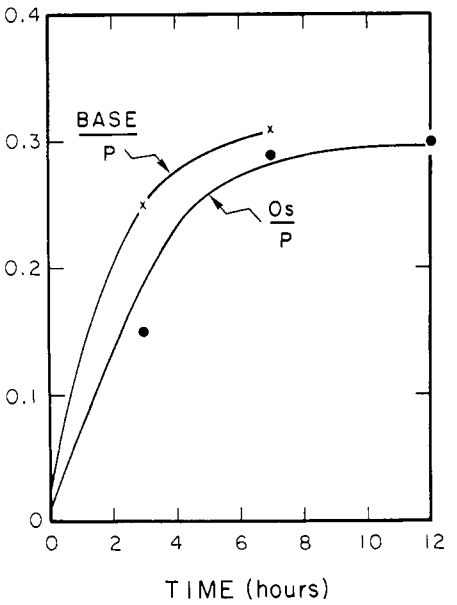

FIGURE 11: Binding of osmium to DNA and destruction of bases in DNA during reaction wth 0.1 M OsO_4 and 0.1 M KCN at pH 7, 55°. Similarity in progress of two reactions suggested processes are related.

70% yield whereas addition of thiocyanate was much slower and a gave poorer yield. Rather surprisingly the yield was not sensitive to the pH of the cyanide addition reaction. Both compounds were remarkably stable in the presence of excess cyanide or thiocyanate. Their stabilities in the absence of these ions can only be guessed from the sharpness of the bands in electrophoresis and chromatography. Clearly in a few hours no extensive breakdown occurred.

The addition compound behaved as an anion. We have no direct evidence on the magnitude of its charge, but we believe that it was a dianion on the following grounds. If instead of CN^- the ligands were pyridine, the complex had no mobility in electrophoresis suggesting zero charge (unpublished data). Replacing the two neutral pyridine ligands by two charged ones would then give a doubly negative anion.

Subsequently conditions were found for producing the same addition product of osmium and cyanide in an aqueous system. The mechanism of this reaction is unclear. When cyanide and OsO_4 are mixed, a pronounced and immediate color change occurs indicating an interaction. Indeed Krauss and Schrader (1928) have presented evidence for a tetracyanide complex with OsO_4. If this is the predominant form of the cyanide ion in our system, then only one-fourth of the OsO_4 in the reaction mixture becomes so altered. The rate of the reactions reported here for the aqueous system are very similar to those found in the absence of CN^-. This suggests that the first step in the reaction is the attack by OsO_4. To explain the formation of the complex, one would have to postulate that cyanide is much more strongly bound to the osmate esters of olefins than to OsO_4 itself.

The purpose of the present study was to develop reactions which would make possible the attachment of heavy atoms to thymine specifically. This aim seems to have been realized. One osmium atom is bound together with two negatively charged groups. These, it is believed, can be used to bind additional heavy cations. Electron microscopic studies of DNA molecules so stained are now in progress.

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