Determination of Base Sequence in Nucleic Acids with the Electron Microscope. V. The Thymine-Specific Reactions of Osmium Tetroxide with Deoxyribonucleic Acid and Its Components*

Michael Beer, Samuel Stern, Duane Carmalt, and Kenneth H. Mohlhenrich

ABSTRACT: Osmium tetroxide (OsO_4) in neutral aqueous solution at 23 or 55° reacts readily with desoxythymidine 5′-monophosphate (dTMP) very slightly with desoxycytidine 5′-monophosphate (dCMP) and negligibly with desoxyadenosine 5′-monophosphate (dAMP) and desoxyguanosine 5′-monophosphate (dGMP).

Thymine and desoxythymidine (dT) also react, but 5-bromouracil (5-BU) and uracil do so less rapidly. dT (1 mole) consumes 1 mole of OsO_4 giving a mixture of two osmium-containing compounds. The radioactivity of dT-2-^{14}C appears in a substance believed to be 4,5-dihydroxythymidine. OsO_4 reacts predominantly with the thymine base of denatured deoxyribonucleic acid (DNA) but does not react with native DNA.

Recently a method using the electron microscope was proposed for the determination of the sequence of bases in nucleic acids (Beer and Moudrianakis, 1962). The method requires chemical reactions for the base selective attachment of markers to the polynucleotide chains. With this in mind we have undertaken the investigation of the thymine-selective reaction of osmium tetroxide (OsO_4) with deoxyribonucleic acid (DNA) and the bases and nucleotides derived from it. The possible selectivity of this reagent for the components of DNA was suggested by results of Bahr (1953), who observed that color developed when OsO_4 was mixed with thymine in neutral aqueous solution but not with the other bases found in nucleic acids. Bahr, however, reported no reaction with DNA. More recently Burton and Riley (1966) showed that in the presence of ammonia OsO_4 selectively degrades the thymine component of oligonucleotides.

The nature of the reactions of OsO_4 with organic compounds [for a review, see Gunstone (1960)] was first explored by Criegee (1936) who showed that in nonaqueous solvents the reagent can add to C–C double bonds to form a cyclic ester. On hydrolysis these esters give *cis*-glycols. Even in aqueous solution OsO_4 behaves nearly as a nonelectrolyte: its ionization constant is about 10^{-13} (Yost and White, 1928). Thus it might be expected to enter the same reactions. However, the addition product can survive only if its rate of formation is fast relative to its rate of hydrolysis, and on this there appears to be no information.

In this paper we confirm the selectivity of OsO_4 for thymidine at 23° and show that such a selectivity is observed also at 55° under certain conditions of pH. The products obtained by us are however not addition products, indicating that the hydrolysis is too rapid for their survival. At 55° in the presence of low concentrations of buffer DNA remains denatured (Dove and Davidson, 1962). We show that OsO_4 reacts with DNA under such conditions but not with double-stranded DNA.

Experimental Details

Materials. OsO_4 was obtained from Matheson Coleman and Bell and used without purification. Thymine and uracil were obtained from Nutritional Biochemical Corp., Cleveland, Ohio; 5-BU[1] from Calbiochem, Los Angeles, Calif. dT, dT-2-^{14}C, and the desoxynucleoside 5′-monophosphates were obtained from Schwarz Bioresearch, Inc., Mt. Vernon, N. Y. "Highly polymerized" salmon sperm DNA was purchased from Calbiochem.

Reactions with Desoxynucleotides, Desoxynucleosides, and Bases. Reaction mixtures contained 5×10^{-3} M of the base, nucleoside, or nucleotide and 0.01 or 0.02 mg/ml of OsO_4. When ^{14}C-labeled material was used its activity was 1 μc/ml or 2 mc/mmole. In the experiments done at 23° the buffer was 5×10^{-2} M phosphate at pH 7.0; in the experiments done at 55°,

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[1] The following abbreviations will be used: dT, desoxythymidine; dTMP, desoxythymidine 5′-monophosphate; dCMP, desoxycytidine 5′-monophosphate; dGMP, desoxyguanosine 5′-monophosphate; dAMP, desoxyadenosine 5′-monophosphate; 5-BU, 5-bromouracil; dT-2-^{14}C, desoxythymidine-2-^{14}C; EtAB, ethanol–ammonium acetate–borate; AmBer, ammonia–isobutyric acid.

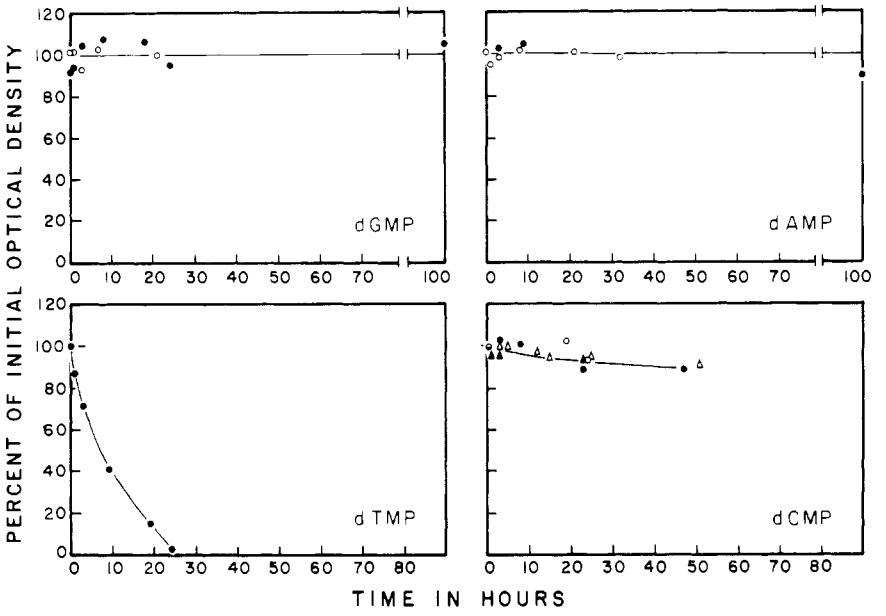

FIGURE 1: Reaction of desoxynucleotides with OsO_4 at 23°. Reaction mixtures contained 0.08 M OsO_4 and 0.01 M nucleotide in 5×10^{-2} M phosphate at pH 7.0. Reaction mixtures were analyzed by paper electrophoresis and the unreacted nucleotides eluted and determined spectrophotometrically. The curves give the consumption of nucleotides as a function of time.

10^{-2} M acetate buffer was used between pH 3.5 and 5.0 $\times$ 10^{-2} M cacodylate buffer at pH 7.0 and 10^{-2} M phosphate buffer at pH 8.0.

Equal portions of the reaction mixture were sealed in scrupulously clean identical Pyrex tubes and placed in a water bath. At intervals tubes were removed, broken open, and 20-μg portions were spotted on 3MM paper for electrophoresis or chromatography or on Eastman Chromagrams for thin layer chromatography. For electrophoresis 0.03 M sodium acetate–acetic acid buffer was used at pH 3.5 with 10^{-4} M EDTA. In the chromatography the ammonia–isobutyric acid solvent of Krebs and Herns (1952) and the ethanol–ammonium acetate–borate solvent of Klenow and Lichtler (1957) were used.

The distribution of radioactivity on the paper was determined by scanning with Nuclear-Chicago actigraph. Spots were eluted in 10^{-2} N HCl and the amount of unreacted material was determined spectrophotometrically.

The occurrence of a reaction and its rate could also be determined by drying, under vacuum, 20 μg of the reaction mixture to eliminate the volatile OsO_4 and redissolving the residue in 2.0 ml of 10^{-3} M phosphate buffer at pH 7 for assay by spectroscopy. In the resulting solution the major ultraviolet light absorbing component at wavelengths >240 mμ was the unreacted nucleotide or base. This was suggested by the fact that the shape of the absorption curves was changed only at very short wavelengths during the course of the reaction. It was shown that this more rapid assay gave essentially the same reaction rates as the one mentioned earlier.

Reactions with DNA. Studies of the reaction of OsO_4 with DNA were carried out at 23 and 55°. In the former case stock solutions containing 0.1 mg/ml of DNA were made up in 0.03 M phosphate buffer at pH 7.0. The reaction mixtures were obtained by mixing equal volumes of the DNA stock solution and 0.16 M solution of OsO_4 in the same buffer.

For the studies at 55° 1.33×10^{-4} M cacodylate buffer was used at pH 7.0. This dilute buffer was chosen on the basis of the studies of Dove and Davidson (1962) in the hope of obtaining complete denaturation at the lowest temperatures. Briefly 1 mg/ml of DNA dissolved in 5×10^{-3} M EDTA at pH 7.0 was diluted with 10 volumes of buffer and denatured by heating for 10 min in water at 90° and quickly cooling in ice water. Then an equal volume of 0.16 M OsO_4 in cacodylate buffer was added to make up the reaction mixture. In all cases water distilled in quartz was used and glassware and dialysis tubing were handled as described by Dove and Davidson. Our reaction mixture differs from the medium of Dove and Davidson by the presence of 0.08 M OsO_4. This solute, however, is only very slightly ionic as was suggested by the electrical conductivity experiments of Hofman *et al.* (1913) and the equipartition experiments of Yost and White (1928). We have also measured the electrical conductivity of OsO_4 and found that when it was added to 1.33×10^{-4} M cacodylate buffer the conductivity was only doubled and was four times less than at buffer concentrations of 1.33

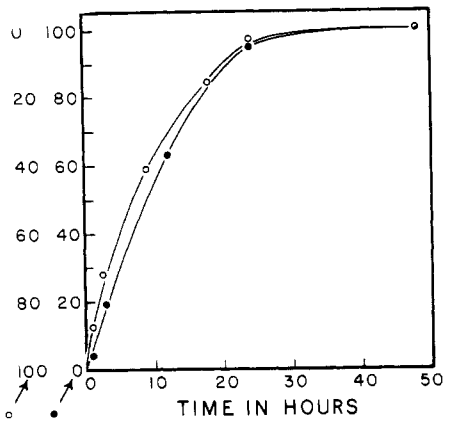

FIGURE 2: Comparison of the rate of development of absorption at 500 mμ with the rate of disappearance of thymidine 5'-monophosphate. Reaction mixture as in Figure 1. -●-, optical density of reaction mixture as a per cent of the final optical density. -○-, per cent of unreacted dTMP.

TABLE I: Electrical Conductivity of OsO_4 Solutions and Buffer Solutions.

Soln (N)	μmhos
Water distilled in a quartz still	1
OsO_4 (0.08) in water	7
Cacodylate (1.33×10^{-4}) buffer	6
Cacodylate (1.33×10^{-3}) buffer	60
OsO_4 (0.08) in cacodylate (1.33×10^{-4}) buffer	15

$\times 10^{-3}$ M. The data given in Table I suggest that the concentration of ionic species contributed by the OsO_4 is not great relative to 10^{-4} M and that the presence of the reagent does not greatly change the melting temperature of the DNA.

After reaction, excess OsO_4 was removed by dialysis at 0°. The reacted DNA was then precipitated by ethanol, and hydrolyzed by formic acid as described by Wyatt and Cohen (1953) and the base composition determined by chromatography (Wyatt, 1951).

Determination of Osmium. Our method for determining osmium was based on that of Dwyer and Gibson (1951) but was scaled down to allow the determination of 0.1 mg of the metal in its organic compounds. Enough of the compound to be assayed was weighed out to give 0.1–0.2 mg of Os and placed in a 14 × 100 mm Pyrex test tube. Approximately 0.1 g of finely powered Na_2O_2 was added with care that all of the compound was covered with the Na_2O_2 to ensure complete reaction. The tubes were immediately placed in an oven at 450° for 15 min. Then they were allowed to cool, 2 ml of distilled water was added, and the tubes were warmed

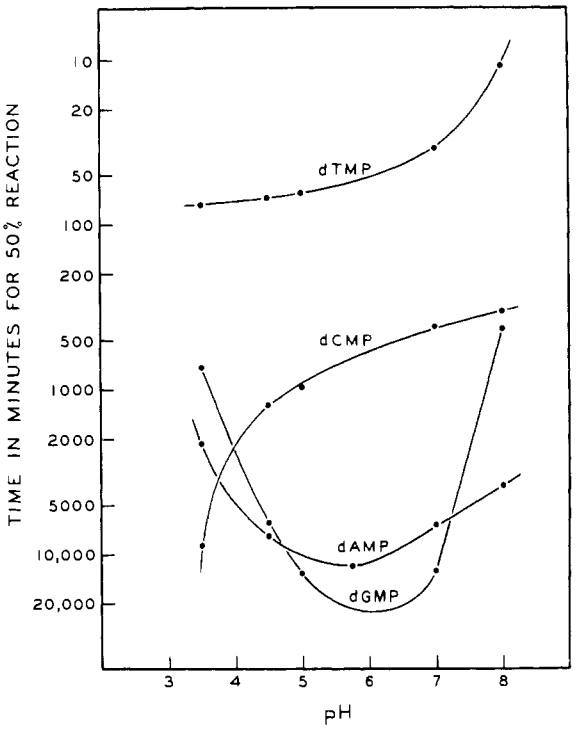

FIGURE 3: Comparison of rates of reactions of desoxynucleoside monophosphates with OsO_4 at 55° at different pH values. At neutral pH TMP reacts about 10 times faster than CMP which again is about 10 times faster than AMP and GMP.

to dissolve all solids. HBr (50%) (2 ml) and 1 ml of 1 N HCl were added and the contents were mixed well; then 0.1 ml of 15% thiourea solution and 0.1 ml of 0.1 M stannous chloride in 2.5 N HCl solution were added; the tubes were placed in boiling water for 15 min and then cooled. The spectrum was determined between 400 and 700 mμ. The procedure was calibrated by determining the Os content of known volumes of a 20% solution of OsO_4 in carbon tetrachloride.

Results

Selectivity of the Reaction with Desoxynucleotides. The reactions of OsO_4 with the desoxynucleotides were studied by following the disappearance of the characteristic ultraviolet absorption. The rate was determined at 23°, pH 7.0, and at 55°, pH 3.5–8. The concentration of OsO_4 was 0.08 M and that of the nucleotides 5×10^{-3} M.

The results of the studies at 23° are given in Figure 1. They indicate that after 25 hr dTMP is about 95% reacted when dCMP is only 10% consumed. Within experimental error there is no loss of the purine nucleotides.

During the reaction a purple color develops rapidly with dTMP, more slowly with dCMP, and not at all with the purine nucleotides. The rate of development of this

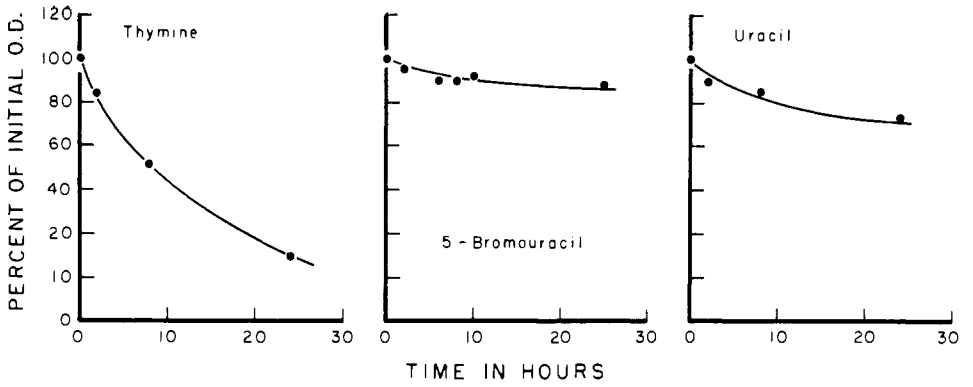

FIGURE 4: Comparison of rates of reactions of thymine, 5-bromouracil, and uracil with OsO_4 at 23°, pH 7.0, in 5×10^{-2} M phosphate buffer.

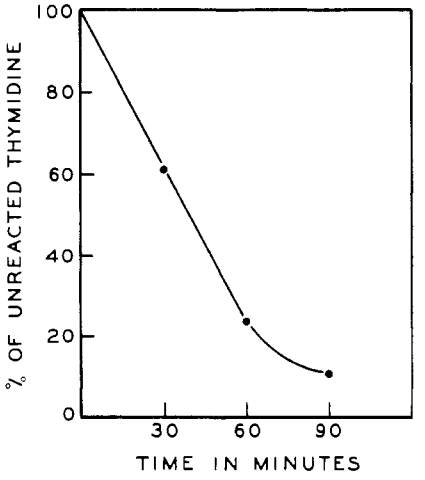

FIGURE 5: Rate of reaction of desoxythymidine with OsO_4 at 55° in 10^{-2} M cacodylate buffer at pH 7.0.

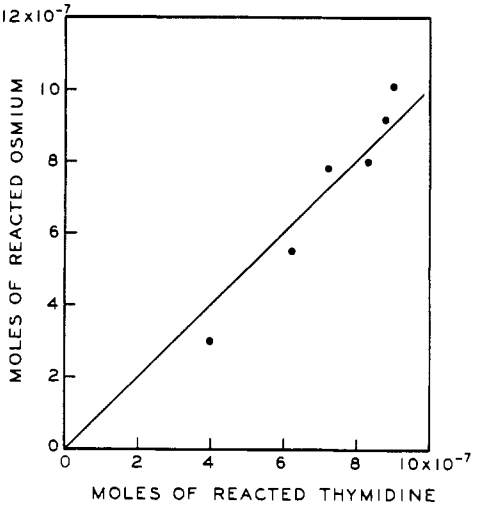

FIGURE 6: Stoichiometry of the reaction of OsO_4 with desoxythymidine. During the reaction the number of moles of desoxythymidine which have reacted equals the number of moles of osmium rendered nonvolatile.

color correlates well with the rate of consumption of the nucleotide, as is clear for dTMP from Figure 2. Here the development of absorption at 500 mμ is compared with the consumption of dTMP.

The data for 55° are summarized in Figure 3. It is clear that the selectivity observed at room temperature is largely maintained at 55°. dTMP again reacts much more rapidly than the other desoxynucleotides. Also there is some variation in the rate of reaction with pH. For dTMP there is a gradual increase by a factor of nearly 10 from pH 3.5 to 8. With dCMP there appears to be a sharp loss of reactivity below pH 4.5 suggesting that the protonated species does not react. On the other hand both dAMP and dGMP appear to react more rapidly in the protonated form as suggested by the increase in reactivity at low pH. Finally, the rapid rise in reactivity of dGMP at pH 8 suggests that the negatively charged species is also more reactive. Under conditions where the bases are uncharged dTMP appears to react about 10 times faster than dCMP and this in turn more than ten times faster than the purines. The specificity of the reaction for dTMP appears greatest in the pH range 4.0–7.0.

Reactions of OsO_4 with Thymine, 5-Bromouracil, and Uracil. The reactivities of the three bases thymine, 5-BU, and uracil were compared at pH 7.0 at 23° in 0.03 M phosphate buffer. It is clear from the data in Figure 4 that thymine reacts several times faster than the other two bases and that 5-BU reacts most slowly. Thus the substitution of a methyl group for the 5-H atom of uracil increases the reactivity, while the bromine atom has the opposite effect. This suggests that the specificity of OsO_4 would be less in ribonucleic acid (RNA) than in DNA. Also the substitution of 5-BU in DNA for the thymine would lead to a slower reaction and lower specificity.

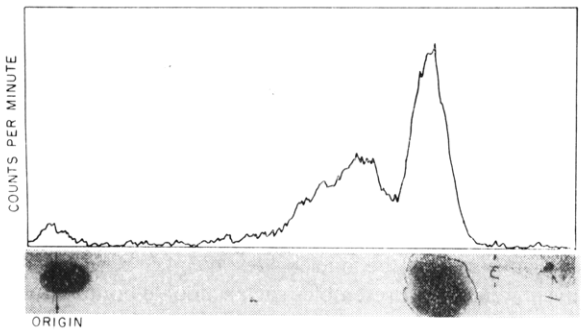

FIGURE 7: Chromatograph of reaction mixture containing desoxythymidine and OsO_4 at 55°, pH 7, spotted on Eastman Chromagram after 40 min of reaction. Ascending chromatography with EtAB as solvent. Chromatograph photographed under ultraviolet light. Only two dense spots near origin are colored and contain Os. Leading spot is ultraviolet absorbing but colorless.

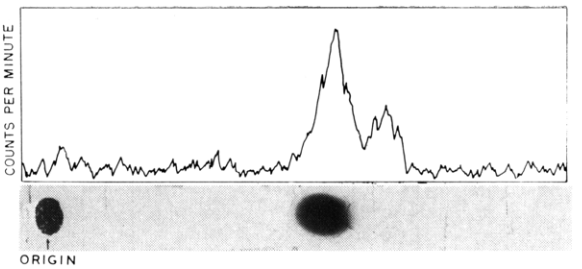

FIGURE 8: Chromatograph of reaction mixture containing desoxythymidine and OsO_4 at 55°, pH 7, spotted on Eastman Chromagram after 2 hr of reaction. Ascending chromatography with AmBu as solvent. Chromatograph photographed under visible light. Leading spot is colorless but absorbs ultraviolet light. Second spot colored and contains osmium.

Stoichiometry of the Reaction of Desoxythymidine with OsO_4. Thymidine undergoes a reaction similar to the one found for dTMP when heated at 55° in an aqueous solution of 0.08 M OsO_4 buffered with cacodylate at pH 7.0. Again purple develops as the characteristic ultraviolet absorption of thymidine diminishes. The rate of the disappearance of nucleoside is given in Figure 5. It is similar to that observed for dTMP under the same conditions.

The stoichiometry of the reaction was determined by taking advantage of the fact that OsO_4 is volatile whereas the other compounds of osmium are not. After reaction the unreacted OsO_4 was sublimed away under vacuum and the amount of nonvolatile osmium determined in the residue. Pumping overnight through a cold trap with a mechanical pump seemed sufficient; further pumping did not change the results of the osmium analyses. The accumulation of nonvolatile osmium is plotted as a function of the consumed dT in Figure 6. The number of moles of osmium rendered nonvolatile equals the number of moles of dT reacted. Thus 1 mole of OsO_4 reacts with 1 mole of dT.

Products of the Reaction of dT and dTMP. In an attempt to gain insight into the nature of the products formed, the reaction mixture was studied by chromatography and paper electrophoresis. In these experiments the nucleoside and nucleotide were labeled with ^{14}C in the C-2 position. A typical chromatogram obtained with EtAB, the ethanol–ammonia–borate solvent of Klenow and Lichtler (1957), together with its radioactivity, is shown in Figure 7. The rapidly moving ultraviolet-absorbing spot must be unreacted dT since its mobility is the same as that of dT and its ultraviolet absorption and radioactivity decrease together during the course of the reaction. The slower spot has very much less ultraviolet absorption and contains the radioactivity lost from the dT spot during the course of the reaction. In this region of the chromatograms no osmium was detected. This spot is clearly some reaction product containing the C-2 of the pyrimidine ring.

Near the origin two dark spots were observed. One did not move at all during chromatography and contained almost no radioactivity but approximately one-half of the osmium rendered nonvolatile by the reaction. The other spot moved very slightly during chromatography and contained the remainder of the osmium but none of the radioactivity.

When AmBu, the ammonia–isobutyric acid solvent of Krebs and Herns (1952), was used the results were only slightly different. A typical chromatogram together with its radioactivity scan is shown in Figure 8. Again the radioactivity decreases in the region where unreacted thymidine is found and reappears in a new slower moving spot. In this same region is also found the moving osmium-containing spot. Indeed the overlap is so close that it raises the possibility that the radioactivity and the osmium occur as an addition compound. However, when identical experiments were run with the nucleotide, the two radioactive spots moved slower while the osmium-containing spot had the same mobility as with the nucleoside. This suggests that the osmium and the pyrimidine are not found as an addition product. On paper electrophoresis of the reaction mixture the colored spot containing the osmium moved as an anion to a spot where no radioactivity was found; all the radioactivity moved slightly as a cation.

Thus it appears that there are three major products of the reaction of thymidine and OsO_4. One is a compound containing the C-2 of thymidine, possessing little ultraviolet absorption, having an R_F in AmBu and EtAB somewhat lower than that of thymidine. This product is believed to be 4,5-dihydroxythymidine obtained by hydrolysis of the 4,5-addition product of thymidine with OsO_4. The other compound contains osmium, moves as a negative ion, and is probably the osmate ion. The third is an insoluble osmium-containing compound probably obtained by the breakdown of the osmate ion.

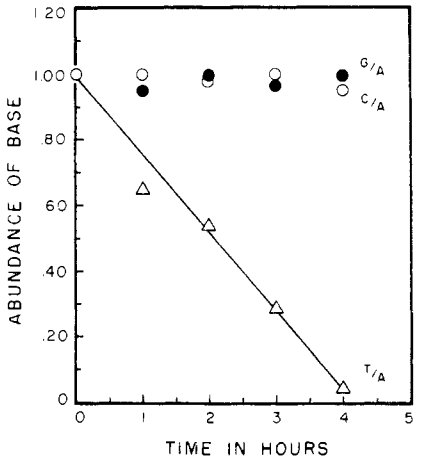

FIGURE 9: Selective destruction of thymine in denatured DNA in the presence of OsO_4. Ordinate gives variation in ratio of unreacted base to unreacted adenine, with the ratio adjusted to unity at the beginning of the reaction. Buffered with 1.33×10^{-4} M cacodylate at pH 7.0, 55°.

Reactions of OsO_4 with DNA. The reactions of OsO_4 with DNA were studied at pH 7.0 in two types of experiments: (1) at 23° using native DNA. (2) at 55° under conditions of low ionic strength with DNA which had been heated to 80° and then rapidly cooled.

The progress of the reaction was followed by paper chromatography of the hydrolysate. The decrease in the abundance of a base relative to adenine is considered a measure of the reaction of that base. Such an approach implies the assumption that adenine is unaffected in the reaction. This appears justified by the results on the nucleotides where dAMP reacted to a negligible extent. Also the ratio of guanine to adenine is constant, a fact which would be consistent with their reaction only if they had the same rate. At 23° in undenatured DNA the base ratios remained constant during 74 hr, suggesting no reaction was occurring.

When the reaction mixture was kept at 55° in cacodylate buffer at a concentration low enough to ensure that the DNA was denatured the disappearance of bases resembled that found for the nucleotides (Figure 9). Thymidine decayed with a half-life of about 2 hr. When its abundance had decreased to about 10% of its original value about 90% of the cytosine remained. The ratio of guanine to adenine was unchanged within experimental error, suggesting a negligible loss of these bases. Apparently under these conditions the thymine bases of DNA are degraded selectively as they are in mononucleotides, though the rate of breakdown is slower in the polymer by a factor of about 2.

Discussion

OsO_4 reacts much more rapidly with dTMP than with dCMP while dAMP and dGMP hardly react. A similar reaction is found with dT and it consumes 1 mole of OsO_4 for 1 mole of dT reacted. This fact is consistent with the notion that in dT one bond is susceptible to reaction. OsO_4 reacts most readily with aliphatic C–C double bonds. Bromination and oxidation experiments indicate that in the nucleosides the 4,5 bond of the pyrimidines most resembles such a double bond. Therefore, the selective reaction of OsO_4 with dT is consistent with its known specificity. The product however is not an addition product suggesting that in an aqueous solvent hydrolysis occurs too rapidly for its survival. In analogy with the hydrolysis of other osmic esters the product is assumed to be 4,5-dihydroxythymidine.

The experiments carried out with DNA indicate that with denatured DNA the thymine base is selectively destroyed. We believe that it is also converted to 4,5-dihydroxythymine, though no proof is presented here. Finally, it should be noted that OsO_4 appears to be a reagent which discriminates between denatured and double-stranded DNA in that it reacts with the former but not the latter.

Acknowledgment

Dr. Kenneth Burton has kindly shown us, prior to publication, the manuscript of his work on the reactions of OsO_4 with nucleic acids.

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