Oxidation of Pyrimidine Nucleosides and Nucleotides by Osmium Tetroxide

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1. Pyrimidine nucleosides such as thymidine, uridine or cytidine are oxidized readily at 0° by osmium tetroxide in ammonium chloride buffer. There is virtually no oxidation in bicarbonate buffer of similar pH. Oxidation of 1-methyluracil yields 5,6-dihydro-4,5,6-trihydroxy-1-methyl-2-pyrimidone. 2. Osmium tetroxide and ammonia react reversibly in aqueous solution to form a yellow 1:1 complex, probably OsO_3NH . A second molecule of ammonia must be involved in the oxidation of UMP since the rate of this reaction is approximately proportional to the square of the concentration of unprotonated ammonia. 3. 4-Thiouridine reacts with osmium tetroxide much more rapidly than does uridine. The changes of absorption spectra are different in sodium bicarbonate buffer and in ammonium chloride buffer. They occur faster in the latter buffer and, under suitable conditions, cytidine is a major product. 4. Polyuridylic acid is oxidized readily by ammoniacal osmium tetroxide, but its oxidation is inhibited by polyadenylic acid. Pyrimidines of yeast amino acid-transfer RNA are oxidized more slowly than the corresponding mononucleosides, especially the thymine residues. Appreciable oxidation can occur without change of sedimentation coefficient.

Burton & Riley (1966) have described the oxidation of various nucleosides by osmium tetroxide in dilute aqueous ammonia at 0°. The reaction shows several interesting features. Thymidine is readily oxidized, and uridine or deoxyuridine react about ten times more slowly. The corresponding cytosine nucleosides react more slowly still, and adenosine and guanosine are not attacked. Native DNA reacts much more slowly than heat-denatured DNA. Somewhat similar results have been obtained in an independent study that was directed towards the determination of nucleotide sequences by electron microscopy (Beer, Stern, Carmalt & Mohlhenrich, 1966). Beer *et al.* (1966) performed the oxidation in buffer solutions that did not contain ammonia, and they found it necessary to use higher concentrations of osmium tetroxide, longer reaction times and higher temperatures.

Apart from the potential value of the reaction in sequence analysis, the comparative resistance of native DNA suggested that osmium tetroxide might be used to study the conformation of nucleic acids and the relation between their structure and

biological activity. In exploring this possibility, Burton, Varney & Zamecnik (1966) found that, when amino acid-transfer RNA from *Escherichia coli* is treated with osmium tetroxide, there is a strong correlation between the loss of acceptor activity for various amino acids and the presence of adenine at the middle nucleotide of the corresponding codon in messenger RNA.

The present paper is a further study of the oxidation of nucleosides or nucleotides and includes a study of the reaction with 4-thiouridine, which is a constituent of transfer RNA from *E. coli* (Lipsett, 1965).

EXPERIMENTAL

Chemicals. Aqueous solutions of OsO_4 were prepared and standardized spectrophotometrically (ϵ_{260} 2500) as described by Burton & Riley (1966). 4-Thiouridine disulphide was purchased from Calbiochem Ltd., London, and part was reduced electrolytically at a potential of 1.5 v (Cecil & Weitzman, 1964) to give 4-thiouridine. During reduction, λ_{max} increased to about 328 m μ and then began to fall. The reduction was stopped soon after λ_{max} had reached 328 m μ . The yellow solution was concentrated in a rotary evaporator and chromatographed on Whatman no. 1 paper with 86%

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(v/v) butan-1-ol as solvent. The purified material was stored in frozen aqueous solution at -15° and used within 2 weeks of its preparation. The concentration of 4-thiouridine was determined from the molar extinction coefficient at $331\text{ m}\mu$ and pH 2 (1.7×10^4 ; Lipsett, 1965). During prolonged storage, the extinction at $260\text{ m}\mu$ increased relative to that at $320\text{ m}\mu$. Initially, both at pH 2 and pH 6, the E_{320}/E_{260} ratio was 5.6. Other nucleosides and nucleotides were obtained from Calbiochem Ltd., or from British Drug Houses Ltd., Poole, Dorset. Potassium osmium tetroxide was prepared as described by Clifford & Kobayashi (1960). PolyA* and polyU were obtained from the Miles Chemical Co., Elkhart, Ind., U.S.A. Yeast transfer RNA was extracted from baker's yeast (Distillers Co. Ltd.) with the use of phenol as described by Holley (1963). An aqueous solution was treated with 1 vol. of 2-ethoxyethanol and 0.7 vol. of 20% (w/v) potassium acetate. The precipitate was collected by centrifuging, dissolved in water, reprecipitated by adding ethanol and dried *in vacuo*.

Rate of reaction. Oxidation was followed by the decrease in the ultraviolet absorption of the pyrimidine nucleosides or nucleotides. If an excess of OsO_4 had been used, that remaining after reaction was removed by shaking the reaction mixtures with 20–40 vol. of ice-cold A.R. ether. After centrifugation to separate the two phases, the water layer was frozen by dipping the tubes into liquid N_2 . The ether layer was then discarded and the extraction was repeated with another portion of ether.

Spectrophotometers. A Cary model 14 recording spectrophotometer and a Unicam SP.500 instrument were used.

Isolation of 5,6-dihydro-4,5,6-trihydroxy-1-methyl-2-pyrimidinone (I). 1-Methyluracil (II) (140 mg.; Cyclo Chemical Corp., Los Angeles, Calif., U.S.A.) was dissolved in 4.0 ml. of 0.45N- NH_3 and mixed at 0° with 8.0 ml. of 154 mM- OsO_4 . After 17 hr. at 0° , the excess of OsO_4 was removed by shaking with 30 ml. of ether. Then NH_3 and ether were removed from the aqueous phase by a stream of air bubbles and the solution was then passed through a column (20 cm. $\times$ 2 cm.) of ECTEOLA-cellulose (California Corp. for Biochemical Research, Los Angeles, Calif., U.S.A.). The column was washed with water and 70 ml. fractions were collected. Most of the reduced dark-brown osmium compounds remained on the column. The product was located in the washings by treating 5 μ l. samples with 0.2N- NaOH for 30 min. and using a colour reaction for urea (Coulombe & Favreau, 1963).

Fractions that contained the product were pooled and purified from most of the contaminating unchanged methyluracil (II) by chromatography on a column

(25 cm. $\times$ 2 cm.) of cellulose powder. On washing with water-saturated butan-1-ol, methyluracil was eluted before the product. The fractions containing the product were pooled, chromatographed again in the same way and then passed through a column of Norit A (10 cm. $\times$ 1 cm.) to remove final traces of colour and of methyluracil. This column was washed with water and the combined eluate and washings were concentrated *in vacuo* to 0.5 ml. White crystals were obtained, recrystallized from 0.3 ml. of water and dried to constant weight *in vacuo*. The yield was 25 mg. of 5,6-dihydro-4,5,6-trihydroxy-1-methyl-2-pyrimidinone. Microanalyses were performed by Mid-West MicroLab Inc., Indianapolis, Ind., U.S.A. (Found: C, 36.4; H, 5.5; N, 17.9; O, 38.9. Calc. for $\text{C}_5\text{H}_8\text{O}_4\text{N}_2$: C, 37.5; H, 5.0; N, 17.5; O, 39.9%). The nuclear-magnetic-resonance (n.m.r.) spectrum was measured in D_2O in a Varian 60 spectrometer: CH_3 , δ 3.1 p.p.m.; C-H, δ 4.8 and 5.1 p.p.m. (both doublets, J 4.1 cyc./sec.).

Oxidation of sodium fumarate. Samples were removed from reaction mixtures (for details see the legend to Fig. 6) and immediately shaken with 50 vol. of ice-cold ether to remove any excess of OsO_4 . The mixtures were centrifuged for about 30 sec. to separate the layers quickly, the aqueous phase was frozen by the use of liquid N_2 and the ether layer was discarded. Tartaric acid was determined in the aqueous phase. Other samples (0.15 ml.) were applied to two sheets of Whatman no. 1 paper. One sheet was subjected to electrophoresis in 5% (v/v) formic acid for 6 hr. at 150 v/cm. in the apparatus described by Rushizky & Knight (1960). The other sheet was chromatographically developed in butan-1-ol-formic acid-water (10:2:15, by vol.). The papers were dried and sprayed with 0.05% bromophenol blue in 95% ethanol.

Estimation of tartaric acid. The sample was oxidized by periodate to form glyoxylic acid and this was estimated by the method of Zaremski & Hodgkinson (1965), the butanol extraction being omitted. Samples (0.1 ml.) of the reaction mixtures of Fig. 6 were each extracted with 5 ml. of ice-cold water-saturated ether as above and the ether layer was discarded. Water (0.9 ml.) was added to the aqueous layer followed by 0.2 ml. of a freshly prepared solution containing 1% (w/v) NaIO_4 , 0.05N-acetic acid and 0.05M-sodium acetate. After 2 min. at room temperature, 0.5 ml. of conc. HCl was added. Excess of periodate was destroyed by titration with 2% sodium metabisulphite. Iodine was liberated at first, but it reacted further on continuing the addition. About 0.2 ml. of the sulphite solution was required. Resorcinol (0.1 ml. of an aqueous 5% soln.) was now mixed with each sample and the mixtures were heated at 100° for 5 min. After cooling, 3 ml. of 25% (w/v) K_2CO_3 was added with thorough mixing. The extinction was measured 15 min. later at $490\text{ m}\mu$. Standards of 0.05–0.2 μ mole of D-tartaric acid were used.

Estimation of urea and methylurea. The colour reaction with diacetylmonoxime and thiosemicarbazide (Coulombe & Favreau, 1963) was used with appropriate standards. Monomethylurea gives 40% of the colour yield obtained from urea itself.

Base analysis of RNA. Acid hydrolysis (Smith & Markham, 1950) was preferred to alkaline hydrolysis because after the latter method the thymine ribonucleoside and ribonucleotide were contaminated by other unidentified compounds. The RNA (2 mg./ml.) was heated with N-HCl at 100° for 1 hr. and concentrated to dryness in a rotary

* Abbreviations: polyA, polyadenylic acid; polyU, polyuridylic acid.

evaporator. The major components were determined by thin-layer electrophoresis (DeFilippes, 1964). Ribothymidylic acid was present in the uridylic acid band. The content of thymine was determined by isolating larger quantities of the mixed uracil and thymine nucleotides from paper chromatograms in propan-2-ol-conc. HCl-water (13:4:2, by vol.). The mixture was treated with phosphomonoesterase (Burton & Petersen, 1960) and subjected to paper chromatography in aq. 70% (v/v) propan-2-ol. Ribothymidine was obtained as a separate spot and its identity was confirmed by measuring the absorption spectra at several pH values.

RESULTS

Oxidation of the nucleosides and nucleotides of thymine, uracil and cytidine

The rate of oxidation of nucleosides by osmium tetroxide in 0.1–0.4*N*-ammonia was increased by raising the concentration of the nucleoside, the tetroxide or the ammonia (Burton & Riley, 1966). On further examination, no reaction was detected without ammonia, except at higher concentrations of tetroxide and higher temperatures (cf. Beer *et al.* 1966).

In ammonium chloride–ammonia buffers, the rate of reaction was nearly proportional to the square of the concentration of unprotonated ammonia, but it was comparatively unaffected by changes in the concentration of ammonium chloride (Figs. 1 and 2). In other words, for a constant concentration of unprotonated ammonia, changes of the pH or of the concentration of ammonium chloride have relatively little effect on the rate of reaction. These kinetic effects suggest the possibility that the active agent in the oxidation is an uncharged complex formed between one molecule of the tetroxide and two molecules of unprotonated ammonia.

For most subsequent experiments, buffers were used with final concentrations of approx. 0.1*N*-ammonia and 0.5*M*-ammonium chloride. The pH was 8.7 at 25° or 9.6 at 0°.

Reaction between osmium tetroxide and ammonia

Aqueous solutions of osmium tetroxide have a pale-yellow colour that becomes deeper on the addition of ammonium chloride buffer. The colour is changed to brown in more alkaline solutions because of the ionization of the tetroxide (p*K* 12; Sauerbrunn & Sandell, 1953). Although the tetroxide is converted into an osmiate (e.g. KOSO₃N) in strongly alkaline solution, the absorption spectra in ammonium chloride buffer are different from that of potassium osmiate (Fig. 3). The spectrum of osmium tetroxide can be

Fig. 1. Effects of ammonia and ammonium chloride concentrations on the rate of reaction. Initially, the reaction mixtures contained 3.04 mM-UMP and 10.4 mM-OsO₄. The concentration of NH₄Cl was 0.093 *M* for points ○ and □; for all other points it was 0.835 *M*. Concentrations of NH₃ were: ○ and ●, 0.092 *N*; △ and ●, 0.23 *N*; ▲ and □, 0.46 *N*; ■, 3.8 *N*. The temperature was 0°. The ordinate shows *E*₂₇₀ in 1 cm. cells after removal of excess of OsO₄ and 41-fold dilution in 0.1 *N*-HCl.

Fig. 2. Effects of ammonia concentration on the rate of reaction. Initial reaction velocities (*v* moles min.⁻¹ l.⁻¹) were determined from Fig. 1 and other similar data. Initial concentrations were: ○, 0.835 *M*-NH₄Cl, 3.04 mM-UMP and 10.4 mM-OsO₄. All other values were obtained with 0.74 *M*-NH₄Cl, 0.50 mM-UMP and the following concentrations of OsO₄: ●, 1.75 mM; △, 3.5 mM; ▲, 5.3 mM. The temperature was 0°. The straight line has a slope of 2.

regained by diluting the buffered mixtures with water, whereas the spectrum of potassium osmiate is not influenced by dilution or the addition of ammonia. It is concluded that osmium tetroxide and ammonia react reversibly at about pH 9 to form a yellow complex that is distinct from an osmiate.

The extent of the spectral change on mixing with

Fig. 3. Absorption spectra of osmium tetroxide and potassium osmate in water and the effect of ammonium chloride buffer. OsO_4 (0.30mm): A, in water; B, in 0.49N- NH_3 + 0.32M- NH_4Cl ; C, in 2.5N- NH_3 + 1.6M- NH_4Cl . D is the spectrum of KOsO_3N ; it was the same in all three solutions.

Fig. 4. Reaction between osmium tetroxide and ammonia in aqueous solution: evaluation of dissociation constant, K , at 25°. The mixtures contained 12.6mm- OsO_4 and various concentrations of ammonium acetate buffer in which $[\text{NH}_3] = 5 \times [\text{ammonium acetate}]$. Solvents were: ●, water; ○, aq. 82.4% (v/v) dioxan. The line is drawn as the best fit to the points in water. The ordinate shows $[\text{NH}_3]/(E - E_0)$, where E is the extinction coefficient at 380 mμ in the mixture and E_0 is the extinction coefficient of 12.6mm- OsO_4 in the absence of ammonium acetate buffer.

ammonium chloride buffer depends primarily on the concentration of unprotonated ammonia and only to a small degree on the pH. Since osmium tetroxide is not ionized in the buffers that were used, the yellow complex cannot carry an ionic charge. The composition of this complex was deduced from the absorption spectra of 0.5–20mm-osmium tetroxide in various ammonium chloride buffers, all of which contained a large excess of unprotonated ammonia over the concentration of tetroxide. For given concentrations of unprotonated ammonia, the extinctions were proportional to the total concentration of tetroxide, indicating that the yellow complex contains a single atom of osmium.

Assuming that only a 1 : 1 complex is formed, the extinction coefficient, E , of a particular mixture at any given wavelength would be:

$$E = E_0 + \Delta\epsilon \cdot [\text{complex}] \quad (1)$$

where E_0 is the extinction coefficient of a solution containing the same total concentration of osmium tetroxide but no ammonia and $\Delta\epsilon$ is the difference between the molar extinction coefficients of the complex and of the tetroxide. It can then be shown that:

$$[\text{NH}_3]/(E - E_0) = (K + [\text{NH}_3])/\Delta\epsilon \cdot C \quad (2)$$

where $C = [\text{OsO}_4] + [\text{complex}]$

$$K = [\text{OsO}_4][\text{NH}_3]/[\text{complex}]$$

A plot of $[\text{NH}_3]/(E - E_0)$ against $[\text{NH}_3]$ gives a straight line (Fig. 4), from which K was evaluated to be 0.68mole/kg. of water. The data are closely consistent with eqn. (2) except at the highest concentration of ammonia (10.1molal), where E is about 8% below the expected value. The agreement confirms the original assumption that a 1 : 1 complex is formed, but the slight deviation suggests that a 1 : 2 complex might also be formed at high concentrations of ammonia.

Fig. 4 also shows results from a similar experiment in which the solvent was dioxan–water (4.8 : 1, v/v) mixture. The points lie very close to the line that was drawn from the results in the absence of dioxan. K is found to be 0.50mole/kg. of water in the dioxan mixture. Since a hydrated compound, such as $\text{OsO}_4 \cdot \text{NH}_3$ (Hair & Robinson, 1960), should have given a higher value for K in the dioxan mixture than in water, it is concluded that the 1 : 1 complex has the formula OsO_3NH . This would be an isomer of osmiamic acid, which itself has the structure $\text{OsN}(\text{OH})\text{O}_2$ (Griffith, 1965). OsO_3NH appears to resemble the nitrido-osmates, OsO_3NR , which are formed from primary amines and osmium tetroxide (Milas & Iliopoulos, 1959; Clifford & Kobayashi, 1960).

Fig. 5. Selectivity of the reaction in ammonium chloride buffer, pH 8.7. Initial concentrations were: 17.8 mM-OsO₄, 1 mM-nucleoside, 0.11 N-NH₃ and 0.59 N-NH₄Cl. The nucleosides were: Δ , thymidine; $\bullet$, uridine; $\square$, deoxyuridine; $\circ$, cytidine; $\blacktriangle$, pseudouridine. Extinctions were measured in 0.1 N-HCl at 280 m μ for cytidine and 267 m μ for other nucleosides. The ordinate shows $(E - E_{\infty}) / (E_0 - E_{\infty})$, where E is the extinction after treatment, E_0 is the initial value and E_{∞} is the final value (30 min. for thymidine; 18 hr. for other nucleosides).

Selectivity of the reaction

The reaction of several nucleosides in ammonium chloride buffer solution is shown in Fig. 5. The relative rates of reaction are similar to those found previously in 0.4 N-ammonia (Burton & Riley, 1966). 5-Ribosyluracil (pseudouridine) is slowly attacked at about the same rate as cytidine. Uridine reacts 4.5 times as fast as cytidine. Ribonucleosides react 1.4–1.8 times as fast as the corresponding deoxynucleosides. Thymidine reacts 13 times as fast as deoxyuridine.

Thiopyrimidine compounds react much more quickly than any of these nucleosides. With 4-thiouridine, at least, the reaction is more complex and there is a different type of dependence on the concentration of ammonia. The oxidation of 4-thiouridine and of its disulphide is described below.

Product of the oxidation of 1-methyluracil

The crystalline product was isolated after oxidation in dilute ammonia as described in the Experimental section and it was found to contain C, N and O in the atomic proportions 5:2:4. The ultraviolet absorption in the range 250–290 m μ was virtually abolished, but there was strong absorption at 230 m μ . The n.m.r. spectrum in deuterium oxide showed one methyl group and two C-H protons (δ 4.8 and 5.1) that cannot be assigned to an aldehydic proton (δ approx. 9.5 in deuterium oxide) but can be assigned to H₍₅₎ and H₍₆₎ respectively of the lactam tautomer of 5,6-dihydro-4,5,6-tri-

Fig. 6. Oxidation of sodium fumarate by osmium tetroxide. Disodium fumarate (8 mM) was treated with 1.4 mM-OsO₄ at 0° and pH 9.5: $\circ$, in 8 mM-KHCO₃ + 2 mM-K₂CO₃; $\bullet$, in 12.5 mM-NH₄Cl + 12.5 mM-NH₃.

hydroxy-1-methyl-2-pyrimidone (I). (The lactim formulation is given purely because of convenience in indexing.)

This compound would give methylurea on alkaline hydrolysis and, in confirmation, it gave an appropriate colour reaction (Coulombe & Favreau, 1963) after it had been treated with 0.2 N-sodium hydroxide at 37°. The intensity of colour was the same whether hydrolysis was performed for 30 min. or for 1 hr., and it was equivalent to that given by 91% of the theoretical amount of monomethylurea.

Although osmium tetroxide normally effects *cis*-hydroxylation of ethylenic double bonds, it is conceivable that structure (I) may racemize at C₍₆₎ via an open-chain form.

Action of ammonia in the oxidation of fumarate

Zelikoff & Taylor (1950) studied the oxidation of fumaric acid and its salts by aqueous osmium tetroxide in the absence of ammonia. However, this reaction is not essentially different from the oxidation of pyrimidines because it also occurs much faster in ammonium chloride buffer than in bicarbonate buffer (Fig. 6). The product had the same R_f and electrophoretic mobility as tartaric acid. Hydroxyaspartic acid, which is a conceivable product in the ammonium chloride buffer, was not detected.

Oxidation of polyuridylic acid and RNA

PolyU is readily oxidized by osmium tetroxide in ammonium chloride buffer. After extended reaction, a sticky brown precipitate was formed, but

Table 1. *Action of alkali on oxidized polyuridylic acid*

PolyU (0.8mg./ml.) was oxidized at 0° for 60 min. by 10mM-OsO₄ in 0.6M-NH₄Cl+0.12N-NH₃. Excess of OsO₄ was removed by ether extraction without freezing or centrifuging. After dialysis against water, total P was determined on one portion (for details see Burton & Petersen, 1960). Other samples were treated with 0.2N-NaOH at 37° for different times and urea was determined immediately afterwards.

Time in alkali (min.)	Urea (mole/g.atom of P)
0	0.08
2	0.60
5	0.78
20	0.93
45	0.94
100	0.95

Table 2. *Oxidation of polyuridylic acid and of polyadenylic acid+polyuridylic acid by osmium tetroxide*

The polynucleotides were treated with OsO₄ at 0° at the following final concentrations: OsO₄, 13mM; polyU, 0.44mg.atom of P/l.; polyA, 2.47mg.atom of P/l.; NH₄Cl, 0.6M; NH₃, 0.12N; pH 9.6 at 0°. After reaction for the indicated times, excess of OsO₄ was removed by ether extraction and the polynucleotides were dialysed against water. Total P and the urea released on treatment with 0.2N-NaOH at 37° for 30 min. were then measured.

Time of reaction with OsO ₄ (min.)	Urea released by alkali/total P (molar ratio)		
	PolyU	PolyA	PolyU+polyA
5	0.27	—	0.029
10	0.66	—	0.026
60	0.95	0.045	0.045

this redissolved and most of the brown colour was lost during dialysis against water. The dialysed material had lost the original absorption peak at 260mμ. Dilute alkali released 95% of the theoretical amount of urea for complete oxidation (Table 1).

PolyA inhibited the oxidation of polyU (Table 2). The work of Stevens & Felsenfeld (1964) indicates that the double-stranded polymer, poly(A+U), should have been formed in preference to the triple-stranded poly(A+2U) because of the low temperature and the excess of polyA. In a separate experiment, the extinction was measured in the range 230–280mμ after successive additions of 5N-ammonia to poly(A+U) in 0.6M-ammonium chloride at 22°. Denaturation did not commence until the pH had reached 9.9 (cf. Cox, 1966) and hence the double helix must have been the stable form at pH 9.6 and 0°.

Table 3. *Base composition of RNA treated with osmium tetroxide*

Yeast transfer RNA (3mg./ml.) was oxidized by 16mM-OsO₄ at 0° for 4 hr. in 0.55M-NH₄Cl+0.11N-NH₃. Excess of OsO₄ was removed by ether extraction and the RNA was dialysed as described by Burton *et al.* (1966). Results are given as moles/mole of adenylic acid. No corrections are made for the presence of minor nucleosides other than thymine riboside.

	Control (no OsO ₄)	Treated with OsO ₄	Extent of reaction (%)
Guanine	1.49	1.55	—
Cytosine	1.55	1.36	12
Uracil	0.87	0.70	20
Thymine	0.12	0.08	33

It thus appears that uracil residues in a double-stranded nucleic acid are resistant to oxidation by osmium tetroxide. In support of this view, polyA did not prevent the oxidation of polyU in 0.1N-ammonia solution. Also, native DNA is known to be resistant whereas denatured DNA is attacked (Burton & Riley, 1966; Beer *et al.* 1966). Although the results in Table 2 indicate that polyA is slowly attacked, no effect was observed on the ultraviolet absorption of the polymer. The small amount of apparent urea may be an artifact due to interference by residual osmium oxides in the colour reaction.

On prolonged oxidation of amino acid-transfer RNA, there is a change of base composition that parallels the selectivity of the reaction towards free nucleotides (Table 3). The rates of reaction in the RNA are much slower and this difference is greatest for the thymine residues. Most of these are present in the sequence G-T-Ψ-C-G, which is common to many species of RNA (Zamir, Holley & Marquisee, 1965). Perhaps the conformation at this site of the RNA hinders the action of osmium tetroxide on the thymine. Yoshida & Ukita (1966) have reported that the pseudouridine residue in this sequence is resistant to cyanoethylation.

Effect on molecular weight. The sedimentation coefficient of oxidized and unoxidized transfer RNA was examined at a concentration of 0.005% in the Beckman model E analytical ultracentrifuge at 39780 rev./min. and with ultraviolet optics. No effect was found of oxidation for up to 3 hr. under the conditions of Table 3.

Oxidized polyU was examined by schlieren optics at 66000 rev./min. and at a concentration of 1% in a solution containing M-sodium chloride, 2mM-tris-hydrochloric acid buffer, pH 7.3, and mM-magnesium acetate. A sample that had been 14% oxidized showed exactly the same sedimentation pattern as the original polyU. Both were heterogeneous with an *S* value of 5.1s calculated from the

peak. The pattern from a completely oxidized sample indicated that some degradation had occurred, but the probability of chain scission must have been much less frequent than the oxidation of uracil residues.

It is concluded that fairly extensive oxidation can occur without change of molecular weight.

Oxidation of 4-thiouridine

In the absence of ammonia. 4-Thiouridine is readily oxidized by osmium tetroxide in sodium bicarbonate buffer at pH 9 as shown by the absorption measurements in Figs. 7 and 8. From the spectra there is no suggestion that uridine ($\lambda_{\max}$ 262m μ) is a major product.

Formation of cytidine by oxidation in the presence of ammonia. When osmium tetroxide was added to 4-thiouridine in ammonium chloride buffer, the fall of ultraviolet absorption was more rapid and more extensive than in sodium bicarbonate buffer (Fig. 7). It was accompanied by an increase in the extinction at 270m μ and there was an isosbestic point at 284m μ (Fig. 9). At the equivalence point, 1 mole of 4-thiouridine reacted with an average of 2.8 moles of osmium tetroxide (Fig. 10), and the resulting solution showed an absorption maximum at 268m μ with a small peak at 310m μ (Fig. 9). When more tetroxide was added there was no

Fig. 7. Reaction of osmium tetroxide and 4-thiouridine. Mixtures were prepared at 24° to contain 50 μ M 4-thiouridine, buffer and 137 μ M-OsO₄, which was added last. The curves show E_{320} as a percentage of the initial extinction, which was calculated from the value obtained before the addition of OsO₄. Buffers were: A, 5 mM-Na₂CO₃+5 mM-NaHCO₃, pH 9.0; B, 0.093 M-NH₄Cl plus 0.021 N-NH₃; C, 1.86 M-NH₄Cl+0.42 N-NH₃.

Fig. 8. Action of osmium tetroxide on 4-thiouridine in the absence of ammonia. A, Spectrum of 57 μ M-4-thiouridine in 5 mM-Na₂CO₃+5 mM-NaHCO₃; B, similar mixture also containing 50 μ M-OsO₄ at 8 min. after preparation; C, as B, but at 50 min. (after a further 100 min. there was a further decrease of 0.02 in the extinction at the peak and an increase below 305 m μ amounting to 0.05 at 260 m μ); D, mixture containing 100 μ M-OsO₄ read after 150 min.; E, mixture containing 134 μ M-OsO₄ read at 120 min. or 150 min.

Fig. 9. Absorption spectra of 4-thiouridine treated with different amounts of osmium tetroxide in ammonium chloride buffer. Spectra were measured 2 min. after the addition of OsO₄. The final volume was 2.0 ml., containing 110 μ moles of 4-thiouridine, 3.72 m-moles of NH₄Cl and 0.84 mole of NH₃. Amounts of OsO₄ were: A, none; B, 104 μ moles; C, 208 μ moles; D, 318 μ moles.

Fig. 10. Stoichiometry of reaction between osmium tetroxide and 4-thiouridine in ammonium chloride buffer. Data were obtained from those of Fig. 9 and other similar experiments.

indication of any further reaction. After acidification there was a maximum at $280\text{m}\mu$ with a small shoulder at $310\text{m}\mu$ and a minimum at about $255\text{m}\mu$. These spectra suggested that cytidine was present, and this possibility was investigated by oxidizing 4-thiouridine by osmium tetroxide (2.8 mol.prop.) in ammonium acetate buffer, pH 8.7, freeze-drying and examination by paper chromatography. The oxidized samples showed a spot with the same R_f value as authentic cytidine in each of the two solvents that were tried, namely aq. 70% (v/v) propan-2-ol and propan-2-ol-conc. hydrochloric acid-water (13:4:2, by vol.). Eluates from the unknown spot had ultraviolet spectra similar to those of cytidine at pH values 2, 4.5, 7 and 12. The yields of cytidine were between 60 and 70% of the original 4-thiouridine. A similar yield was obtained in another experiment in which a large excess of osmium tetroxide was used (26 moles/mole of 4-thiouridine).

The subsidiary absorption maximum at $310\text{m}\mu$ (curve D in Fig. 9) is not due to cytidine. It might be due to a reduction product of osmium tetroxide since a similar peak was obtained after the reaction of sodium sulphite with 1 mol.prop. of osmium tetroxide.

Composition of ammonium chloride buffer. The rate of change of extinction at $320\text{m}\mu$ was approximately proportional to the concentration of unprotonated ammonia (Table 4) and was essentially independent of the concentration of ammonium chloride. At low concentrations of ammonium chloride the fall of extinction at $320\text{m}\mu$ (Fig. 7),

Table 4. Oxidation of 4-thiouridine at different concentrations of ammonia and ammonium chloride

The reaction mixtures were similar to those described in the legend to Fig. 7. The third column shows the time required for E_{320} to fall from 0.25 to 0.20. The inverse of this time is taken as a measure of the rate. In all cases, E_{320} was 0.90 initially and eventually became 0.10.

Concn. of NH_3 (N)	Concn. of NH_4Cl (M)	t (time required) (sec.)	Relative rate ($1/t$)
0.48	2.10	4.8	0.208
0.34	0.42	9.6	0.104
0.24	1.05	9.0	0.111
0.096	0.42	27	0.037
0.096	1.70	23	0.043
0.024	0.10	128	0.008

Fig. 11. Time-course of formation of cytidine as indicated by the extinction at $270\text{m}\mu$. The reaction mixture was the same as that for curve B of Fig. 7.

occurred more quickly than the increase at $270\text{m}\mu$ (Fig. 11). Nevertheless the final absorption spectra were identical at all concentrations of ammonia used. Two reaction stages could be separated by allowing the tetroxide and thiouridine to react in bicarbonate buffer before adding ammonium chloride buffer. The final spectrum was closely similar to that obtained by oxidation in the presence of ammonia.

Oxidation of 4-thiouridine disulphide by osmium tetroxide

When the disulphide was oxidized by osmium tetroxide in ammonium chloride buffer, the

absorption spectrum of the solution was different from that after the oxidation of 4-thiouridine. The maxima occurred at the same wavelengths, but the peaks were less defined and the E_{310}/E_{270} ratio was 0.9 compared with 0.26 in the reaction mixture with the thiouridine.

DISCUSSION

Action of ammonia in the oxidation at C=C bonds. In aqueous solution the rates of hydroxylation of olefinic bonds in either pyrimidines or fumarate is greatly stimulated by ammonia. Osmium tetroxide and ammonia react reversibly to form a 1:1 complex, probably OsO_3NH , but a second molecule of ammonia must be involved in the oxidation reaction because UMP is oxidized at a rate that is approximately proportional to the square of the concentration of unprotonated ammonia.

Oxidation of 4-thiouridine. The oxidation of this compound is very rapid, especially when ammonia is present together with the osmium tetroxide. The ammonia can react with an oxidized intermediate so that the principal final product is cytidine. Presumably the sulphur is displaced by ammonia after it has been oxidized to a better leaving group, e.g. $-\text{SO}_2\text{H}$. The spectral changes indicate that the rate of this complex reaction is approximately proportional to the concentration of ammonia. Hence a low, but definite, concentration of ammonia will favour the conversion of 4-thiouridine into cytidine relative to the hydroxylation at the 5,6-double bond of uridine. Under the conditions used to study the oxidation of thiouridine, hydroxylation of the 5,6-double bonds in uridine and cytidine occurs very slowly.

The stoichiometry of the oxidation (2.8 moles of osmium tetroxide/mole of 4-thiouridine) cannot be explained without further investigation of the end products. The reaction is obviously complex and it has not been possible to obtain a quantitative conversion of 4-thiouridine into cytidine.

Action on RNA. Thymine, uracil and cytosine residues in transfer RNA are oxidized in the same relative order as the free nucleosides, but their oxidation is limited by the secondary structure of the nucleic acid. Presumably most of the nucleotides that are attacked are in single-stranded sections of the molecules. Much of any 4-thiouridine in the RNA may conceivably be converted into cytidine when the oxidation is performed in ammonium chloride buffer. Burton *et al.* (1966) found that oxidation of *E. coli* transfer RNA resulted in a rapid loss of several amino acid acceptor activities and especially of those for phenylalanine,

glutamic acid and lysine. Further work is needed to establish whether these inactivations are due to the conversion of thiouridine into cytidine.

Ultracentrifugal measurements did not indicate any breakage of the polynucleotide chains during the oxidation of transfer RNA or of 14% of the uracil residues in polyU. Complete oxidation of polyU did, however, cause some degradation. Cerutti, Miles & Frazier (1966) have shown that the 5,6-dihydrouracil residues in partially reduced polyU do not form base pairs with adenine and are not induced to enter into helix formation by neighbouring unmodified uracil residues. Hence it is not to be expected that the oxidized residues will be able to enter into the Watson-Crick type of double helix.

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