Oxidation of Ribonucleic Acids with *m*-Chloroperbenzoic Acid

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Reaction conditions are described under which treatment with *m*-chloroperbenzoic acid yields *N*-oxides of poly(adenylic acid) and poly(cytidylic acid), but not of poly(uridylic acid), poly(guanylic acid), or poly(inosinic acid). The interaction of poly(A) *N*-oxides with poly(U) and poly(C) *N*-oxides with poly(I) decreases with increasing degree of oxidation. The rates of oxidation of adenine and cytosine bases in poly(A)·poly(U) and poly(I)·poly(C) are less than the rates of oxidation of the same bases in poly(A) and poly(C), but oxidation does not occur selectively in single-stranded regions, as reported previously for oxidation with monoperoxyphthalic acid. Oxidation of a natural double-stranded RNA occurs at adenine, cytosine, and guanine units.

N-OXIDATION of a number of pyrimidine and purine bases, nucleosides, and nucleotides with peroxy-acids has been reported.[1-8] The primary site of oxidation is the most basic tertiary nitrogen atom,[9] and the presence of an electron donor, such as an amino-group, on the adjacent carbon atom stabilizes the *N*-oxide formed.[5] Thus in the case of adenine, cytosine, and uracil derivatives *N*(1)-oxides are obtained. With guanine derivatives *N*(3)-oxides are obtained.[8] The preferred reagent for oxidation of polynucleotides has in the past been monoperoxyphthalic acid,[10-15] which had to be specially prepared, assayed, and stored at −20° in ether.[16]

As part of a continuing programme for the preparation of chemically modified double-stranded ribonucleic acids for evaluation as potential antiviral agents, we have investigated the oxidation of synthetic homopolyribonucleotides and polyribonucleotide duplexes, and the double-stranded RNA obtained from a mycophage,[17] with *m*-chloroperbenzoic acid, a commercially available stable peroxy-acid.

RESULTS AND DISCUSSION

N-Oxidation of Homopolyribonucleotides.—Of the homopolyribonucleotides examined, only poly(A) and poly(C) give *N*-oxidation products under the described conditions, and poly(A) reacts more quickly (Table 1). The absence of evidence for the *N*-oxidation of poly(G) is surprising in view of the oxidation that occurs at guanine residues in double-stranded RNA under identical conditions and previous evidence that guanine derivatives yield *N*(3)-oxides.[8] However, it is possible for poly(G) to form hydrogen-bonded aggregates[18] and this could result in protection of N(3) in the polynucleotide against *N*-oxidation.

N-Oxidation of poly(A) and poly(C) decreases the ability of the polynucleotides to complex with poly(U) and poly(I), respectively. In experiments in which the *N*-oxidized polynucleotides are annealed with the complementary polynucleotide (Table 2), both the hyperchromicity observed upon thermal denaturation and

TABLE 1

Oxidation of polyribonucleotides

Polyribonucleotide [a]	*m*-Chloroperbenzoic acid (mg)	Reaction time (min)	Absorption ratio [b]	% Oxidation
Poly(A)	0		0·23	0
	50	5	0·43	5
	250	5	1·05	18
	500	5	2·03	40
	500	20	3·63	76
	500	60	4·32	91
Poly(C)	0		1·03	0
	500	5	1·23	5
	500	20	1·52	13
	500	60	1·99	24

[a] The u.v. spectra of poly(U), poly(G), and poly(I) were unchanged after exposure of the polyribonucleotide to *m*-chloroperbenzoic acid (500 mg) for 60 min. [b] For poly(A) derivatives the absorption ratios quoted are A_{232}/A_{257} values and for poly(C) derivatives A_{224}/A_{271} values. [c] Calculation by extrapolation from corresponding absorption ratios reported previously (F. Cramer and H. Seidel, *Biochem. Biophys. Acta*, 1964, **91**, 14): poly(A), 0·23; poly(A) 39·5% *N*-oxide, 2·0; poly(C), 1·03; poly(C) 100% *N*-oxide, 4·96.

T_m of the resultant double-stranded complexes decrease with increasing levels of oxidation until at the highest levels no complexing occurs.

The adenine and cytosine bases in the double-stranded complexes poly(A)·poly(U) and poly(I)·poly(C) are partially protected against *N*-oxidation by hydrogen bonding to the complementary base. The rates of oxidation are lower with the complexes than with poly(A) and poly(C) under identical conditions, and in the case of poly(A)·poly(U) little difference is observed between the products obtained after 20 and 60 min

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TABLE 2

Annealing of oxidized polyribonucleotides with complementary polyribonucleotides

Polyribonucleotide pair [a]	Hyperchromicity (%) [b]	T_m/°C	Absorption ratio [c]
Poly(A)·poly(U)	59·8	59	0·26
Poly(AO^{5})·poly(U)	58·6	56	0·35
Poly(AO^{18})·poly(U)	58·4	44	0·67
Poly(AO^{40})·poly(U)	18·0	Gradual melting 30—40°	1·16
Poly(AO^{76})·poly(U)	0		1·95
Poly(AO^{91})·poly(U)	0		2·30
Poly(I)·poly(C)	75·0	63	0·66
Poly(I)·poly(CO^{5})	66·1	57	0·72
Poly(I)·poly(CO^{13})	25·6	Gradual melting 30—50°	0·80
Poly(I)·poly(CO^{24})	0		0·93

[a] Poly(AO^{x}) denotes poly(A) with x% of the adenylic acid residues oxidized and poly(CO^{x}) poly(C) with x% of the cytidylic acid residues oxidized (see Table 1). [b] The hyperchromicities of poly(A)·poly(U) derivatives were determined at 260 nm and those of poly(I)·poly(C) derivatives at 250 nm. On slow cooling all materials for which hyperchromicities are quoted completely reannealed. [c] For poly(A)·poly(U) derivatives the absorption ratios quoted are A_{232}/A_{260} values, for poly(I)·poly(C) derivatives A_{224}/A_{250} values. Ratios were determined at 100°, conditions of zero hypochromicity.

TABLE 3

Oxidation of double-stranded polyribonucleotides [a]

Polyribonucleotide	Reaction time (min)	Absorption [b] ratio	Oxidized [c] nucleotide % reaction	Hyper [d] chromicity (%)	T_m/°C
Poly(A)·poly(U)	20	0·93	A, 31	59·6	53·5
	60	0·94	A, 32	60·7	52·5
Poly(I)·poly(C)	20	0·70	C, 5	71·1	60
	60	0·78	C, 11	65·9	60

[a] *m*-Chloroperbenzoic acid (500 mg) was used for 20 mg of the double-stranded polyribonucleotide. [b] Absorption ratios and conditions were as described in Table 2, footnote *c*. [c] Calculated by extrapolation from absorption ratios given in Table 2. [d] Determined as described in Table 2, footnote *b*.

(Table 3). Poly(A) and poly(U) can form a triple-stranded complex, poly(A)·2poly(U),[19] and it is possible that as poly(A) is oxidized the proportion of unchanged adenine bases associated with two complementary uracil bases increases and the rate of oxidation is further reduced.

N-Oxidation of Double-stranded RNA.—The results obtained on *N*-oxidation of double-stranded RNA are summarised in Table 4. Initial reaction occurs rapidly and is accompanied by a substantial increase in the absorption at 230 nm and a decrease in T_m. The reaction then slows and the differences observed between the products obtained after 1, 2·5, and 5 h are relatively small. Gel chromatography on Sepharose 2B indicates that for oxidations of up to 5 h duration little degradation of the nucleic acid occurs. After 16 h a highly oxidized single-stranded product of low molecular weight is obtained.

On polyacrylamide gel electrophoresis the 1, 2·5, and 5 h oxidation products all show a single sharp band only just entering the gel, whereas the double-stranded RNA starting material has a higher mobility and is separated into three bands.[17] This is interpreted as evidence of aggregation. Partial denaturation of DNA has been demonstrated to result in aggregation.[20]

Although the T_m of double-stranded RNA in the solvent system used for the oxidations is 84°, 7° lower than in 0·15M-sodium chloride, no denaturation occurs at 20°. *m*-Chloroperbenzoic acid does not therefore selectively oxidize bases located in single-stranded regions. The greatly decreased reaction rate after 1 h may be due to aggregation of the partially oxidized nucleic acid. Further oxidation eventually leads to disintegration of the aggregates and a highly oxidized single-stranded product.

EXPERIMENTAL

Materials.—Homopolyribonucleotides and double-stranded homopolyribonucleotide complexes were purchased from P-L Biochemicals, Milwaukee. The isolation and properties of the mycophage double-stranded RNA have been described previously.[17] *m*-Chloroperbenzoic acid was purchased from Ralph N. Emanuel Ltd.

Oxidations.—The polyribonucleotide (20 mg) was dissolved in 0·4M-potassium acetate, pH 8·2 (20 ml), and a solution of *m*-chloroperbenzoic acid in ethanol (10 ml) was added. The solution was maintained at 20° for the desired reaction time and then ethanol (60 ml) was added. The precipitated product was separated by centrifugation and washed with ethanol (2 × 100 ml).

TABLE 4

Oxidation of double-stranded ribonucleic acid [a]

Reaction time (h)	A_{230}/A_{258}	Hyperchromicity [b] (%)	T_m/°C [b]	Base ratios A	U	C	G	% Base oxidised [c] A	U	C	G
0	0·45	33·3	91	0·97	1·04	0·98	1·00	0	0	0	0
1	0·57	34·6	79	0·80	1·00	0·74	0·72	20	0	26	28
2·5	0·61	34·6	77·5	0·75	1·00	0·70	0·72	25	0	30	28
5	0·67	34·1	75								
16	2·22	<10									

[a] Oxidations were carried out using *m*-chloroperbenzoic acid (500 mg) for 20 mg of RNA. [b] Determined at 260 nm in 0·03M-sodium chloride containing 1% ethylene glycol. [c] Calculated from the base ratios. The total absorption values obtained for uridylic acid from the *N*-oxidized products were the same as those from the same amount of the original RNA, indicating that no reaction occurs at uracil bases.

Annealing of Oxidized Homopolyribonucleotides.—Equimolar quantities of solutions of the polynucleotides in 0·15M-sodium chloride were annealed at 20° for 2 h. Prior experimentation had established that when annealing was

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carried out for longer periods, the thermal denaturation characteristics of the resultant complexes did not change.

U.v. Data.—U.v. measurements were determined for solutions in 1 cm cells with a Pye-Unicam SP 800B spectrometer equipped with an SP 877 electrically heated cell holder and an SP 876 Accuran temperature programme controller. Unless otherwise stated, the hyperchromicities and T_m values were measured for solutions in 0·15M-sodium chloride, pH 7. The solution temperature was elevated by 0·5° per min. The recorded T_m is the temperature at which the materials exhibited 50% of the total hyperchromicity.

Gel Filtration Chromatography.—For gel filtration a Sepharose 2B column 90 cm long, internal diameter 2·5 cm, volume *ca.* 450 ml, was used. The column was eluted in an upward direction with a solution containing sodium chloride (0·15M), Tris buffer (0·05M), and magnesium chloride (0·005M), pH 7·5, at 0·4 ml min^{-1}. Fractions (7·2 ml) were collected.

Electrophoresis.—4% Polyacrylamide gels containing 0·04% bisacrylamide were prepared in glass tubes, 4 mm internal diameter. Running buffer was Tris (0·04M), sodium acetate (0·02M), ethylenediaminetetra-acetic acid (0·002M), pH 7·8. Electrophoresis was carried out at 5 mA per tube for 1—3 h. The gels were stained with Methylene Blue.

Base Ratios.—Base ratios for double-stranded RNA oxidation products were determined by hydrolysis in 0·3N-sodium hydroxide at 37° for 18 h. The hydrolysate was neutralized with ammonium chloride and the mixture of nucleoside 2′,3′-phosphates was separated on a Dowex 1 × 8 column (200—400 mesh) eluted with increasing concentrations of hydrochloric acid.[21] Cytidylic acids were eluted with 0·003N-, adenylic acids with 0·005N-, uridylic acids with 0·007N-, and guanylic acids with 0·1N-acid. The fractions containing each nucleotide were combined and the nucleotide concentration was calculated from its u.v. absorption. *N*-Oxides of both purines and pyrimidines are degraded by both aqueous alkali and acid to products which do not absorb in the u.v.,[15] and so cannot be determined directly. The values recorded are the averages of three independent determinations.

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