Evidence for Cross-Linking of Polyribonucleotides with 4′-Aminomethyl-4,5′,8-trimethylpsoralen Hydrochloride†

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ABSTRACT: Evidence is presented for the formation of cross-links in base-paired regions of polyribonucleotides by long-wave ultraviolet irradiation in the presence of 4′-aminomethyl-4,5′,8-trimethylpsoralen hydrochloride. The instantaneous reannealing of base-paired structure following heating and fast cooling was taken as evidence for the presence of covalent bridges. Single-strand-specific nuclease S_1 was used as a probe for base-paired structure. This was done with 16S rRNA from *Escherichia coli* and the double-stranded homopolymer poly(I)·poly(C). The photoreaction with poly(I)·poly(C) suggested that the reagent is not pyrimidine specific, contrary to what was deduced more indirectly by others. In the case of 16S rRNA, a procedure was developed for the demonstration of the instantaneously reannealing structure by (1) predigestion of endogenous unpaired loop regions with nuclease S_1; (2) inactivation of the nuclease by diethyl pyrocarbonate; (3) heating and fast cooling; (4) rechallenge of the structure with nuclease S_1. The photoreacted 16S rRNA and poly(I)·poly(C) both had altered structural properties by optical parameters. The spectra showed hyperchromicity with a peak at long (peak at 350 nm) and at short (250 nm and below) wavelengths and a very small hypochromicity at 270 nm. Melting curves at 260 nm showed no significant change in total thermal hyperchromicity, but a large increase in melting temperature. With high levels of the drug, the melting temperature increased by 18 °C for 16S rRNA and by 12 °C for poly(I)·poly(C). When the reaction was limited by lower concentrations of reagent, significant shifts of the entire melting curve became detectable with the initial ratio of drug in the range of 10^{-2} to 10^{-1} mol per base pair for 16S rRNA and in the range of 10^{-3} to 10^{-2} mol per base pair for poly(I)·poly(C). The reaction had no effect on the compact structure which we had shown earlier to be resistant to the spreading conditions of the electron microscopy. However, the compact structure thus observed was no longer susceptible to denaturation by heating in formalaldehyde.

Reagents of the psoralen family have already found wide application in the cross-linking of base-paired structures in DNA for electron microscopy (Cole, 1970, 1971; Dall'Acqua et al., 1971; Cech & Pardue, 1976; Hanson et al., 1976; Shen & Hearst, 1976; Wiesenhahn & Hearst, 1976; Wiesenhahn et al., 1977; Isaacs et al., 1977). The chemical basis for this was firmly established from reactions with bases as model compounds (Musajo et al., 1967a,b; Krauch et al., 1967) and from the physical properties of the cross-linked DNA (Cole, 1970, 1971; Dall'Acqua et al., 1970, 1971). The usefulness of these reagents for fixation in RNA electron microscopy has been limited in the past by their lower reactivity for RNA (see Isaacs et al., 1977) compared with DNA. Isaacs et al. (1977) screened a number of derivatives for their reactivity with RNA. One of them, aminomethyltrioxsalen,[1] was shown to have superior reactivity toward RNA in comparison with the parent compound, trioxsalen. Wollenzien et al. (1978) also used the related compound, hydroxymethyltrioxsalen, as a tool for sealing the hidden breaks in insect 26S rRNA for electron microscopy.

However, in the case of the reaction of aminomethyltrioxsalen with RNA, the evidence for the formation of covalent bridges was the uptake of radioactively labeled reagent into an acid-insoluble product and the preservation of double-stranded structure in electron microscopy (Isaacs et al., 1977). While both of these criteria support the idea of the formation of covalent bridges, alternative explanations are possible. For example, we have found that RNA structures can be surprisingly stable to the denaturing conditions normally used for spreading RNA for electron microscopy (Hochkeppel et al., 1977; Hochkeppel & Gordon, 1978). This, therefore, cannot constitute proof of a covalent linkage. The aim of this work was to show more complete evidence for the formation of covalently cross-linked structure in RNA. From the reaction of the defined double-stranded homopolyribonucleotide poly(I)·poly(C), we showed evidence supporting the idea that the drug is not pyrimidine specific as is the case for the reaction of other drugs of the same family with bases, nucleosides, and nucleotides (Krauch et al., 1967; Musajo et al., 1967b). Finally, we characterized the effect of the photoreaction on several structural parameters.

Experimental Procedure

Materials. Ribosomal subunits from *E. coli* were prepared according to Gordon & Ramjoué (1977). The RNA from the 30S subunits was extracted with phenol–sodium dodecyl sulfate as described by Hochkeppel et al. (1976). Aminomethyltrioxsalen was a generous gift of Peter Wellauer, ICRS, Lausanne. It had been synthesized according to Isaacs et al. (1977). Poly(I)·poly(C) was from Boehringer, Mannheim. The molecular weight of the 16S rRNA was assumed to be 0.56×10^6 (Fellner, 1974). The mean molecular weight of the poly(I)·poly(C) sample used here was determined by polyacrylamide gel electrophoresis using complete denaturing conditions in 99% formamide (Merck, cleaned over mixed bed resin prior to electrophoresis) according to Jost & Pehling (1976). The mean molecular weight was 3.5×10^5, and this was heterodisperse, with a distribution having half-maximum at $\pm 1 \times 10^5$.

Photoreaction Conditions. For the purpose of presenting the reaction conditions as a ratio of reagent to base pairs, the following parameters were assumed: ϵ_{260} per mol of base pair $= 2.6 \times 10^4$ for 16S rRNA and 1.3×10^4 for poly(I)·poly(C).

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[1] Abbreviations used: aminomethyltrioxsalen, 4′-aminomethyl-4,5′,8-trimethylpsoralen hydrochloride; poly(I)·poly(C), poly(riboinosinic acid), poly(ribocytidylic acid), hydrogen bonded; Tricine, *N*-tris(hydroxymethyl)methylglycine; SSC buffer, 0.15 M NaCl, 15 mM sodium citrate, 1 mM EDTA, pH 7.4.

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The 16S rRNA was assumed to be 60% base paired and the poly(I)·poly(C) 100%. The ϵ_{250} per mol of aminomethyltrioxsalen (2.5×10^4) was taken from Isaacs et al. (1977) and confirmed by us. The RNA (25 μg/mL in 30 mM Tricine buffer, pH 7.8) was irradiated at 365 nm for 30 min in the following setup. A Hanau high intensity UV lamp (Model TQ, 150-W) was used. The radiation was filtered with a 40% (w/w in H_2O) $Co(NO_3)_2$ solution; temperature was regulated at 10 °C. The lamp assembly and the sample were further cooled by immersion in ethanol temperature regulated at 0 °C. The sample in a quartz cuvette was stirred with a magnetic stirring bar. The irradiated samples were then precipitated with 2 volumes of ethanol and 50 mM NH_4Ac and centrifuged for 30 min at 10000 rpm (Sorvall SS34 rotor), the precipitate was dissolved in SSC buffer, and the dissolved samples were dialyzed for 8 h against SSC buffer to remove any unbound reagent. No significant change in 260-nm absorbancy was observed, and yields were reproducible through this procedure at ±5%.

Nuclease Digestion. Nuclease S_1 (Seikagaku Kogyo Co. Ltd., Tokyo, Japan) digestion of RNA was carried out by dilution of the RNA into a buffer containing 0.3 M NaCl, 0.03 M NaAc, and 0.003 M $ZnCl_2$, pH 4.5, and with 10 μg of RNA/100-μL sample, except where otherwise indicated. Ribosomal RNA samples were all predigested with 2000 units of enzyme/sample for 5 min at 40 °C to eliminate endogenous hairpin loops, and the reaction was stopped with 1 μL of diethyl pyrocarbonate. The samples were then heated for 3 min at 100 °C and rapidly cooled on ice. To each sample 3000 units of nuclease S_1 was added immediately and the digestion continued for 25 min at 40 °C. Poly(I)·poly(C) was treated similarly, but with the omission of the predigestion step, and with 500 units of nuclease S_1 per μg of poly(I)·poly(C) for 40 min. The digested RNA was then precipitated with 10% Cl_3CCOOH and centrifuged in a Sorvall SS34 rotor for 30 min at 10000 rpm. The sediment containing the nuclease-resistant RNA fragments was resuspended in 5% Cl_3CCOOH and heated for 15 min at 100 °C to solubilize all RNA. Any material remaining precipitated was removed by centrifugation (10000 rpm, 30 min). The absorbance at 260 nm of the supernatant was determined and compared with controls from which nuclease S_1 had been omitted. In experiments with poly(I)·[^{125}I]poly(C), the poly(C) had been labeled according to Cory & Adams (1977) and a procedure essentially identical with that used for the optical measurement was followed, except that radioactivity in the 10% Cl_3CCOOH pellet was determined.

Spectra and Melting Curves. These were in a Beckman Acta III recording spectrophotometer with a heated cuvette holder and temperature sensor in a cuvette. The RNA renaturation was also routinely recorded following return to the ambient temperature. Melting curves were routinely performed at 260 nm.

Electron Microscopy. This was carried out following exactly the procedure of Wellauer & David (1973), as previously used by us (Hochkeppel & Gordon, 1978). For complete denaturation, the samples were heated for 20 min at 63 °C in 3% formaldehyde (buffered with 30 mM triethanolamine, pH 7.4) prior to electron microscopy.

Results

Spectral Analysis. Since the presence of excess aminomethyltrioxsalen, or photoproducts thereof, may significantly perturb structure, it was first necessary to set up conditions for rigorously removing the excess reagent. This is especially important because the drug probably acts by intercalating

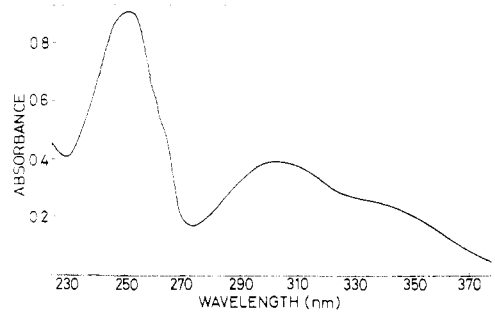

FIGURE 1: Spectrum of aminomethyltrioxsalen in H_2O. Concentration was 12 μg/mL.

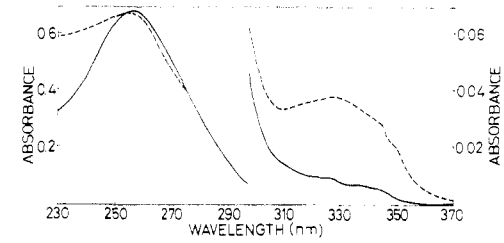

FIGURE 2: Spectra of 16S rRNA; irradiated (dashed) or nonirradiated control (solid). The right-hand half of the figure is on an expanded scale. Photoreaction was with 8 mol of reagent/base pair. Excess reagent was removed as described under Experimental Procedure.

between the stacked bases (Cole, 1970) and the noncovalently bound reagent would severely perturb the structure. The spectrum of aminomethyltrioxsalen (Figure 1) showed a peak at 300 nm, a region of very low nucleic acid absorbancy. This is then useful for monitoring the physical removal of noncovalently bound drug. In the absence of irradiation, the noncovalently bound drug did indeed bind rather strongly. The procedure described under Experimental Procedure, of combined ethanol precipitation and dialysis, was necessary to remove the last trace. The solid curve in Figure 2 then shows the spectrum of such a nonirradiated sample of 16S rRNA. This is not significantly different from samples where the RNA had been irradiated in the absence of drug (too close to be plotted separately). Also, when the drug was irradiated in the absence of RNA, the 300-nm peak was reduced, and no new features appeared. From the spectral data we conclude that less than 0.1% of the noncovalently bound drug remains in the RNA. The broken curve in Figure 2 shows the spectrum following irradiation. It was dramatically altered. There was a large hyperchromic effect with peak at 330 nm and another at 250 nm and below. Further there was a very small but reproducible hypochromic effect at 270 nm. It is clear, by inspection of Figure 1 and 2, that the spectrum of the irradiated sample cannot be the result of the simple addition of the spectra of aminomethyltrioxsalen and RNA: the psoralen spectrum had a peak at 300 nm (Figure 1), whereas the photoreacted RNA had a peak at 350 nm (Figure 2). We can conclude that the structure of the RNA is grossly altered by the cross-linking reaction, presumably the formation of psoralen adducts.

We performed identical experiments with poly(I)·poly(C). The spectral changes found (Figure 3) were qualitatively similar to those found with 16S rRNA. This leads us to suspect that both 16S rRNA and poly(I)·poly(C) have the same gross structural alteration. But spectral data does not permit us to make any hard conclusions concerning the nature of the structural alteration.

Instantaneous Reannealing of Photoreacted RNA. The most convincing direct evidence elsewhere for the presence of covalently cross-linked hydrogen bonded structures either in

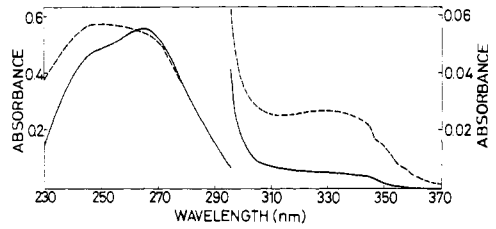

FIGURE 3: Spectra of poly(I)·poly(C); irradiated (dashed) or nonirradiated control (solid). Photoreaction was with 4 mol of reagent/base pair. Other details as in Figure 2.

Table I: Reannealing of Nuclease S_1 Resistant Fragments in Photoreacted 16S rRNA and Challenge with Nuclease S_1[a]

	irradiation	heat, fast cool	S_1 resistant fraction: % of total	S_1 resistant fraction: % of unheated control
(1)	−	−	25	100
(2)	−	+	9	36
(3)	+	−	37	100
(4)	+	+	27	73

[a] Photoreaction and digestion conditions were as described under Experimental Procedure. The cross-linking was with 8 mol of reagent/mol of base pair. In controls where the predigestion step (see Experimental Procedure) was omitted, approximately 60% of the RNA was resistant to S_1 digestion with or without heating and fast cooling. These data are from a representative experiment, and variations between individual experiments were less than ±5%.

trioxsalen cross-linked DNA (Cole, 1970, 1971) or in giant hairpin RNA (Lazzarini et al., 1975) has been the demonstration of the presence of a structure which instantaneously reannealed following heating and fast cooling. This was at concentrations kinetically unfavorable for the re-formation of hydrogen bonds in intermolecular interactions. There is an intrinsic difficulty in applying this approach to 16S rRNA, because it presumably contains endogenously extensive hairpin loops (Fellner, 1974) which would anyhow rapidly reanneal. We have circumvented this difficulty by predigesting the RNA with single-strand-specific nuclease S_1, to remove the non-base-paired structure, including the turns at the ends of the hairpins. As described in detail under Experimental Procedure, the nuclease S_1 was inactivated by addition of diethyl pyrocarbonate; the material was heated and fast cooled (which also destroys the diethyl pyrocarbonate) followed by a second challenge with ribonuclease S_1. The material was then analyzed for S_1-resistant fragments.

The results of this analysis for 16S rRNA are shown in Table I. First, from comparison of the controls not subject to heating and fast cooling, it can be seen that there is more nuclease S_1 resistant material after the photoreaction than before (lines 1 and 3). This was presumably due to the presence of more hydrogen-bonded structure under the mild conditions of photoreaction than under the conditions optimal for nuclease S_1 challenge (40 °C, low pH). More importantly, the cross-linking resulted in a doubling of the fraction of double-stranded RNA which remained nuclease S_1 resistant following heating and fast cooling (right-hand column, Table I). This is evidence for the presence of cross-links in double-stranded regions.

We also sought evidence for the presence of interstrand cross-links in poly(I)·poly(C) by the same procedure, except that the predigestion step was not necessary. Table II summarizes the results of two typical experiments. Experiment 1 in Table II was done under the same concentration conditions as for Table I. The photoreacted material became almost as

Table II: Reannealing of Photoreacted Poly(I)·Poly(C) and Challenge with Nuclease S_1[a]

poly(I)·poly(C) (μg/mL)	drug (mol/mol of base pair)	irradiation	heat, fast cool	S_1 resistant fraction (% of total)
expt 1				
(1) 100	4	−	−	80
(2) 100	4	−	+	46
(3) 100	4	+	−	84
(4) 100	4	+	+	78
expt 2				
(1) 0.35	0.1	−	−	81
(2) 0.35	0.1	−	+	8
(3) 0.35	1	+	−	78
(4) 0.35	1	+	+	68
(5) 0.35	0.1	+	−	75
(6) 0.35	0.1	+	+	52

[a] Photoreaction and digestion conditions were as described under Experimental Procedure. The reaction was with the concentrations of drug and poly(I)·poly(C) indicated. Experiment 1 was based on absorbancy measurements and experiment 2 on radioactivity with ^{125}I-labeled poly(C). The level of iodination specific activity corresponded to ~20 iodine residues/molecule of poly(I)·poly(C).

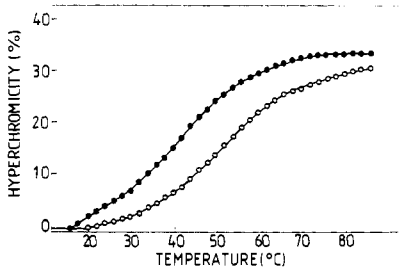

FIGURE 4: Shift in melting profiles between photoreacted and nonirradiated control 16S rRNA. Melting curves of nonirradiated (●—●) and irradiated (○—○). Photoreaction was with 0.8 mol of drug/mol of base pair. Excess of drug was removed as described under Experimental Procedure.

resistant to nuclease S_1 digestion after heating and fast cooling (line 4) as the unheated material (line 3). There was, however, a background in the nonirradiated control which was almost 50% of the total poly(I)·poly(C) (line 2). In order to reduce this background, experiments were performed with much lower concentrations of poly(I)·poly(C) with the use of ^{125}I-labeled material. The nuclease S_1 resistant material was then determined as radioactivity rather than absorbancy, but expressed in the same units. Under these conditions (Table II, experiment 2), the spontaneous reassociation of the nonirradiated control then was reduced to 8% of the total poly(I)·poly(C) (line 2), while the irradiated sample remained resistant to heating and fast cooling (line 4) at this lower concentration. Also, only a slight reduction in the resistant fraction resulted from a tenfold reduction in the ratio of drug to poly(I)·poly(C) (line 4 and line 6). In another experiment, the resistant fraction remained the same with poly(I)·poly(C) photoreacted with initial concentration between 1 and 8 mol per base pair.

Thermal Hyperchromicity. For the above instantaneous reannealing experiments to be significant, it is implicit that the base-paired structures must still be capable of melting. This was in fact the case. The thermal hyperchromicity curves are given in Figure 4 for 16S rRNA and Figure 5 for poly(I)·poly(C). In both cases, the net hyperchromicity was not significantly altered by the photoreaction, but the melting temperatures were shifted dramatically upward. In all experiments, the hyperchromic change was quantitatively re-

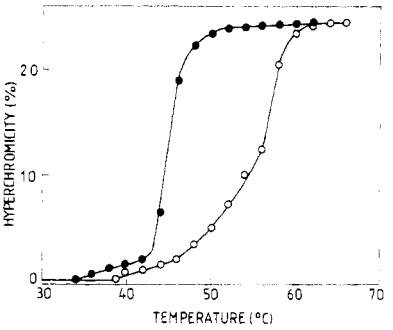

FIGURE 5: Shift in melting profiles between photoreacted and nonirradiated control poly(I)·poly(C). Melting curves of nonirradiated (●—●) and irradiated (○—○). Photoreaction was with 4 mol of drug/mol of base pair. Excess of drug was removed as described under Experimental Procedure.

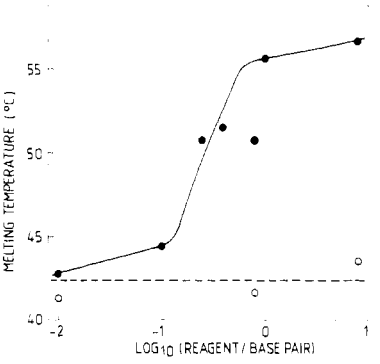

FIGURE 6: Effect of amount of aminomethyltrioxsalen in photoreaction on the melting temperature of 16S rRNA. Melting curves as in Figure 4 were carried out, and the temperature of half-maximal hyperchromic effect was determined. Experimental points: filled circles. Nonirradiated controls: open circles. Data were accumulated from many separate experiments. Scatter was much less in individual experiments. Log plot was used in order to include a wide range of concentrations.

versed on recooling. Thus, the effect was due to reversible melting of hydrogen bonds.

The experiments of Figures 4 and 5 were done at high initial concentration of drug (≥1 mol/mol of base pairs) in order to maximize the resultant effects. However, if the resulting photoproduct were an interstrand cross-link, we might expect stabilization of structure remote from the actual site of the photoreaction. We, therefore, carried out a series of melting curves over a wide range of concentrations of drug. The effect becomes significant at initial concentration of drug between 10^{-2} and 10^{-1} mol per base pair for 16S rRNA (Figure 6) and between 10^{-3} and 10^{-2} for poly(I)·poly(C) (Figure 7). This was obviously a consequence of the longer stretches of continuously base-paired regions in poly(I)·poly(C) compared with 16S rRNA. This implies that, if the photoproduct is a single cross-link, it can affect the stability of stretches of at least 100 base pairs. At all ratios of drug/base pair, the curves shifted as a parallel family (not shown).

Electron Microscopy. The original aim for the drug was for use in stabilization of base-paired regions of RNA for electron microscopy. We have already shown conditions for significantly stabilizing structure in the electron microscope without cross-linking (Hochkeppel et al., 1977; Hochkeppel & Gordon, 1978). We, therefore, investigated whether any additional features were revealed by the cross-linking. In the earlier studies (Hochkeppel et al., 1977; Hochkeppel & Gordon, 1978), we had shown that the compactness of the molecule seen in the electron microscope was a measure of the amount of significant structure, as evidenced by the independent biochemical parameter of preservation of protein

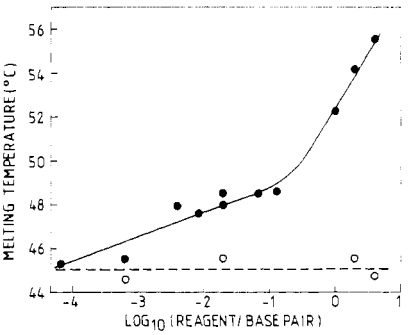

FIGURE 7: Effect of amount of aminomethyltrioxsalen in photoreaction on the melting temperature of poly(I)·poly(C). Details as for Figure 6.

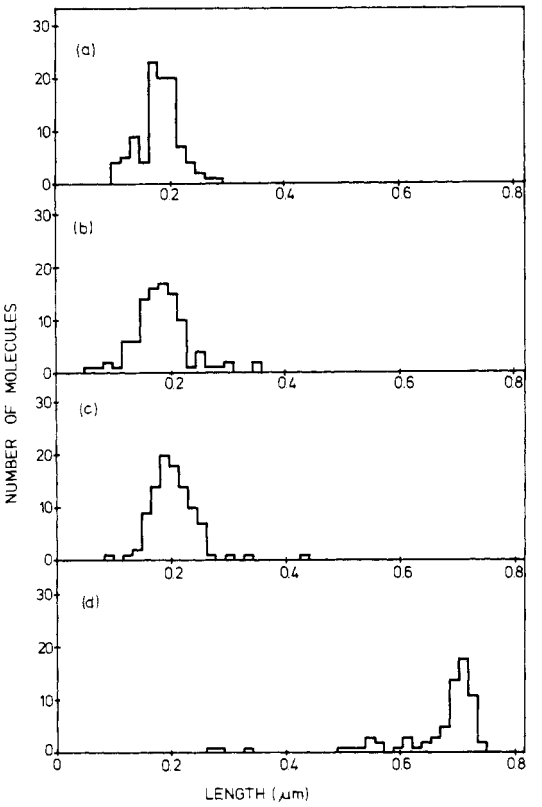

FIGURE 8: Electron microscopy of photoreacted 16S rRNA and controls. Histograms of the length distributions of (a) photoreacted, (b) photoreacted and formaldehyde denatured, (c) nonirradiated control, and (d) formaldehyde-treated nonirradiated control.

binding sites. Figure 8 shows the histograms of such structure; in fact, the size distribution was identical for the irradiated (Figure 8a) and nonirradiated control (Figure 8c). This confirms the significance of our earlier structure determinations by electron microscopy; the nonirradiated control (Figure 8c) gives the same mean length for phenol-extracted 16S rRNA as we reported before, in the absence of drug (Hochkeppel et al., 1977; Hochkeppel & Gordon, 1978). In the previous study, heating in formaldehyde yielded full length molecules as is confirmed here for the nonirradiated control (Figure 8d). The photoreaction rendered the structure completely resistant to formaldehyde denaturation (Figure 8b).

Discussion

The work described here is all consistent with the idea that the photoproduct was an interstrand cross-link in either 16S rRNA or poly(I)·poly(C). The re-formation of double-stranded structure, insensitive to the exonucleolytic activity

of S_1, following heating and fast cooling, was consistent with the concentration-independent, zero-order reannealing reaction described elsewhere for self-complementary regions within one molecule (see, for example, Cole, 1970, 1971; Lazzarini et al., 1975). In nonirradiated controls where there was clearly no cross-linking, the background was concentration dependent (Table II). While it could be argued that increased resistance to nuclease S_1 was due to monoadduct base modification, this seems unlikely since similar resistance was found over an 80-fold range of concentrations of the drug (Table II and results described in the text). This corresponds to a range where there is significant variation in the degree of substitution as shown from the effect on melting temperature (Figure 7). It seems most unlikely that the poly(I)·poly(C) would be inactivated as a substrate for an endonuclease at such low levels of substitution.

It could also be argued that the presence of a monoadduct base modification would increase the helix stability and, hence, give the effects on the melting curves seen here (Figures 6 and 7). Such is the case for poly(I)·poly(BrC). However, in that case, the effect of the bromination on the helix stability is linear with respect to the degree of substitution and is insignificant when the degree of substitution falls below 10% (Howard et al., 1969). Here we find no linear relationship, and the stabilization is significant at lower than 10^{-2} mol of drug added per mol of base pair. It should be pointed out that the value of 10^{-2} is an upper limit to the possible number of cross-links. Isaacs et al. (1977) showed that, under conditions similar to ours, there was approximately 50% uptake of the drug into covalent structures. The proportion of diadducts may be even lower.

The electron microscopy data also support the idea of covalent cross-links in the 16S rRNA. While we have shown elsewhere (Hochkeppel et al., 1977; Hochkeppel & Gordon, 1978) that this spreading procedure preserves some significant structure which can be quantitated as the visible contour length of the molecules, this structure was *always* denaturable by formaldehyde. Any monoadduct base modification could also stabilize double helical structure under the electron microscopy spreading conditions, but only a covalent modification could make the structure resist denaturation in formaldehyde at high temperature. This is the case here (Figure 8).

If we have indeed achieved interstrand cross-linking in poly(I)·poly(C), we need to account for the failure of all previous workers to recognize the photoreactivity of psoralen reagents with purines as well as with pyrimidines. One reason is that all conclusions concerning the specificity were based on reactions with bases, nucleosides, or nucleotides (Musajo et al., 1967a,b; Krauch et al., 1967) and not with polynucleotides. In fact, in one report a lower but significant level of reaction with poly(A) was obtained (Krauch et al., 1967). Another could be the uniqueness of the aminomethyl derivative used here. The base specificity of this particular derivative had not been investigated previously. The uniqueness of the aminomethyl derivative is the presence of a charged group absent from other compounds investigated (cf. Musajo & Rodighiero, 1972; Isaacs et al., 1977). This feature may account for both the superior reactivity toward RNA (Isaacs et al., 1977) and the possible lack of base specificity described here. The photoreacted RNA melts, even at the highest ratio of drug to RNA; we saw no systematic decrease in net thermal hyperchromicity. This suggests that, if cross-linked, the hydrogen bonds still are capable of melting. The melting curves shifted to higher temperatures as a parallel family of curves (not shown). This also implies that there was no selectivity of the reaction for more stable structures in the case of 16S rRNA. Even at the lowest levels of cross-linking giving a detectable effect, the entire curve was shifted.

The high reactivity of aminomethyltrioxsalen for base-paired structures in RNA (Isaacs et al., 1977) is a double-edged sword in electron microscopy. If all the base-paired structure is preserved (e.g., 60% for 16S rRNA; Fellner, 1974), the details will be below the resolving power. Wollenzien et al. (1978) circumvented this problem by limiting the cross-linking reaction in ionic conditions where only the most stable structure was preserved. We have dealt with the analysis of such unresolvable structures in detail elsewhere (Hochkeppel et al., 1977; Hochkeppel & Gordon, 1978) by using the overall molecular size observed as a net measure of the amount of structure. Our approach is confirmed by data presented here which show that the compactness of the structure of the rRNA was not affected by the photoreaction, at least for the case of phenol-extracted RNA. For these reasons, and because the data presented here suggest that regions remote from the site of cross-link may be stabilized, care is clearly important in the interpretation of the electron microscopy data.

Finally, the possible ramifications of double-stranded RNA as in interferon inducer (recent reviews: Pitha & Hutchinson, 1977; DeClerq, 1978) has not escaped our attention. In preliminary experiments we found that cross-linked poly(I)·poly(C) has relatively unaltered interferon-inducing ability (J. Gordon, H. K. Hochkeppel, and A. Schuerch, manuscript in preparation).

While this manuscript was in the process of revision, a publication appeared describing the cross-linking of heterogeneous nuclear RNA by aminomethyltrioxsalen in intact HeLa cells (Calvet & Pederson, 1979). They also used the "snapback" to RNase resistance as the criterion for cross-linking.

Acknowledgments

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Fructose 1,6-Bisphosphatase from Rabbit Liver. [^{18}O]Phosphate–H_2O Exchange as a Probe of the Catalytic Mechanism†

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ABSTRACT: Fructose 1,6-bisphosphatase (FBPase, EC 3.1.3.11, D-fructose-1,6-bisphosphate 1-phosphohydrolase) has been found to catalyze the solvent exchange of ^{18}O from initially highly enriched inorganic phosphate (P_i). The exchange occurs with either Mg^{2+} or Mn^{2+} as the cofactor. The exchange proceeds negligibly in the presence of P_i alone but is greatly stimulated by the presence of fructose 6-phosphate, the second product of the FBPase reaction. A theoretical treatment of ^{18}O exchange from P_i is presented, based upon the ability to determine the ^{18}O isotopic distribution in a P_i sample. The analytical technique permits the determination of the percentage of P_i molecules in a sample which contain none, one, two, three, or four ^{18}O atoms per P_i molecule, in addition to the total atom percent ^{18}O of the sample. Comparative data are presented, illustrating the sensitivities and precision of an ^{18}O-shifted ^{31}P NMR technique and the mass spectrometric method, in which the latter is shown to be approximately 10^2–10^3 times more sensitive and considerably more precise in the determination of low abundance species. The theoretical treatment defines the conditions under which more than one ^{18}O atom can be exchanged per interaction of a P_i molecule with an enzyme. The quantitative features of exchange time courses, expressed in terms of a rate of exchange/rate of dissociation partition coefficient (k_x/k_{off}), are described. Dissociation of P_i from either Mn^{2+}– or Mg^{2+}–FBPase under equilibrium isotope exchange conditions is sufficiently slow so that exchange of more than one ^{18}O can occur per protein–ligand interaction: k_x/k_{off} = 1.4–2.0. However, during steady-state hydrolysis of fructose 1,6-bisphosphate in [^{18}O]H_2O, only one oxygen of the P_i produced becomes equilibrated with the solvent, predicting a $k_x/k_{off} \leq 0.01$. Such results suggest that either (1) the intermediates involved in equilibrium exchange are different than those involved in hydrolysis or (2) the state of occupancy of the active sites in the enzyme tetramer may control the apparent dissociation rates. However, the observed equilibrium exchange suggests a possible net reversal by FBPase of the hydrolysis reaction.

Fructose 1,6-bisphosphatase (FBPase,[1] EC 3.1.3.11, D-fructose-1,6-bisphosphate 1-phosphohydrolase) catalyzes the hydrolysis of fructose 1,6-bisphosphate (fru-1,6-P_2) to fructose 6-phosphate (fru-6-P) and inorganic phosphate (P_i). Phosphorus–oxygen bond cleavage occurs during hydrolysis, as inferred from information available from other phosphohydrolases and from ^{18}O incorporation experiments in which hydrolysis of fru-1,6-P_2 is carried out in [^{18}O]H_2O (Pontremoli et al., 1965).

Two points concerning this catalysis have not been established to date: (1) whether fru-1,6-P_2 hydrolysis by FBPase proceeds through a phosphoryl enzyme intermediate and (2) whether the reaction is reversible—i.e., if fru-1,6-P_2 can be formed from fru-6-P and P_i. We have addressed experiments toward testing these two points by utilizing oxygen isotope exchange reactions catalyzed by FBPase. Analysis for ^{18}O

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[1] Abbreviations used: FBPase, fructose 1,6-bisphosphatase; fru-1,6-P_2, fructose 1,6-bisphosphate; fru-6-P, fructose 6-phosphate; P_i, inorganic phosphate; P_n, inorganic labeled with ^{18}O, n denoting the number of ^{18}O atoms per phosphate molecule, e.g., P_3 contains three ^{18}O atoms per phosphate molecule; NMR, nuclear magnetic resonance; GC–MS, gas chromatograph–mass spectrometer.

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