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The Chemistry of Pseudouridine. VII. Selective Cleavage of Polynucleotides Containing Pseudouridylic Acid Residues by a Unique Photochemical Reaction*

Maria Tomasz and Robert Warner Chambers†

ABSTRACT: Specific cleavage of the oligonucleotide, TpΨpCpGp, at its pseudouridylic acid residue was achieved by irradiation with ultraviolet light

$$\text{TpΨpCpGp} \xrightarrow{h\nu} \text{TpY} + \text{5-formyluracil} + \text{pCpGp}$$

where Y is an unknown fragment derived from the pseudouridylic acid moiety. Examination of the stoichiometry of the reaction indicated that other, nonspecific, photochemical reactions were also occurring. These reactions caused a 15–20% loss in ultraviolet absorbing material, but did not interfere with the detection of the predicted products. Irradiation of purified alanine-soluble ribonucleic acid (s-RNA) followed by gel filtration on a Sephadex G-100 column at 56° gave four partially resolved peaks. According to the nucleotide sequence of yeast alanine-s-RNA, five fragments (55, 39, 37, 21, and 15 nucleotides) could be produced by specific photolysis of the pseudouridylic acid residues. The positions of the peaks emerging from the Sephadex column are consistent with this prediction and the peaks may represent polynucleotides containing 77 (starting material), 55, (39 + 37), and (21 + 15) nucleotides.

During our recent studies on the chemistry of pseudouridine, we discovered a unique photochemical reaction in which pseudouridine 3′-monophosphate (Ψ3′P)[1] (I, R = H) is converted to 5-formyluracil (II), inorganic phosphate, and an unknown fragment, RY (Tomasz and Chambers, 1964). A similar reaction occurs with pseudouridine 3′,5′-diphosphate (Ψ3′,5′DP) (I, R = PO_3^{2-}), while Ψ2′P, pseudouridine 2′,3′-cyclic phosphate, or pseudouridine itself produces only traces of 5-formyluracil under identical conditions. This unusual behavior of Ψ3′P and Ψ3′,5′DP is in sharp contrast to the well-known photochemical reactions of pyrimidine nucleotides which usually involve a reversible

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[1] The following abbreviations will be used: Ψ, pseudouridine (*C* isomer); Ψ3′P, pseudouridine 3′-monophosphate; Ψ3′,5′DP, pseudouridine 3′,5′-diphosphate; C2′(3′)P, cytidine 2′- and 3′-monophosphate mixture; C2′(3′)5′DP, cytidine 2′,5′-diphosphate and cytidine 3′,5′-diphosphate mixture; dT5′P, deoxyribothymidine 5′-monophosphate; T3′P, ribothymidine 3′-monophosphate; ATP, adenosine triphosphate; TpψpCpGp, thymidylylpseudouridylylcytidylylguanylic 3′-acid. Abbreviations according to this system will be used for all other nucleotides.

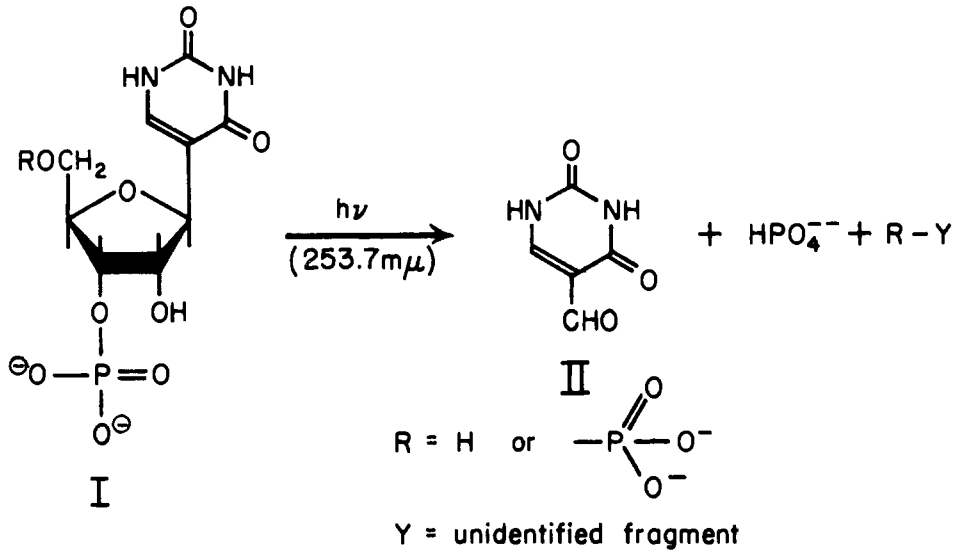

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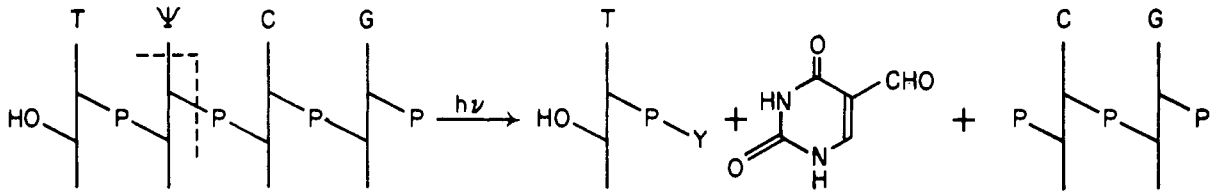
CHART I



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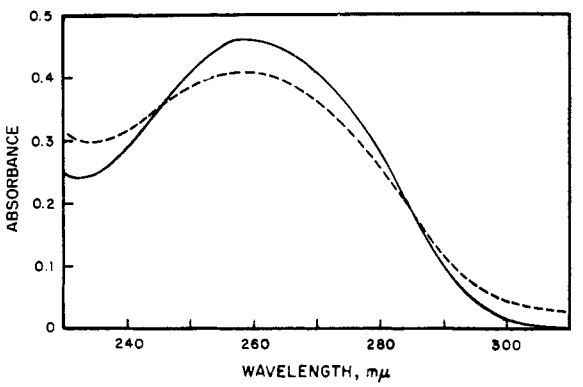

FIGURE 1: Ultraviolet absorption spectra of TpψpCpGp before (———) and after (----) irradiation.

nucleophilic addition to the 5,6 double bond of the pyrimidine ring (for an excellent discussion of these reactions, see McLaren and Shugar, 1964a). This addition may lead to hydration (Moore and Thomson, 1955), dimerization (Beukers and Berends, 1960), or reduction (Cerutti., *et al.*, 1965) depending upon the compound and the conditions employed. The mechanism underlying the unique behavior of Ψ3'P represents an interesting problem in organic photochemistry.

This reaction is also interesting from a biochemical point of view. It has been shown that the ability of soluble ribonucleic acid (s-RNA) to accept amino acids is lost on irradiation with ultraviolet light (Scott and Turter, 1962; Svenson and Nishimura, 1964; Fawaz-Estrup and Setlow, 1964; Zachau, 1964; Gottschling and Zachau, 1965). The evidence also suggests that either uridylic acid or pseudouridylic acid residues are involved in the inactivation process (Fawaz-Estrup and Setlow, 1964). It has also been reported that the pseudouridylic acid residues in s-RNA are destroyed when the RNA is irradiated with ultraviolet light (Lis and Allen, 1961). It is of interest, then, to see if the photolytic reaction described above is responsible for these changes.

Finally, if the photolysis reaction described above occurs with pseudouridine moieties in a polynucleotide chain, cleavage of the chain at that point should occur. If this reaction were really specific for pseudouridine, it should have great potential as a tool for studies on the structure and the function of s-RNA. However, since the other nucleotides occurring in s-RNA are known to undergo photochemical reactions (McLaren and Shugar, 1964a) it was important to examine the photolysis of pseudouridine residues in a simple oligonucleotide of known structure before attempting to apply the reaction to the more complicated s-RNA. It became possible to do this when the tetranucleotide, thymidylyl-pseudouridylylcytidylylguanylic 3'-acid (TpΨp-CpGp), was made available to us through the generosity of Dr. Robert W. Holley. This paper describes the first specific, nonenzymatic cleavage of an oligonucleotide and some preliminary experiments on the photolysis of purified alanine-s-RNA.

Results and Discussion

Photolytic Cleavage of TpΨpCpGp. On the basis of the known behavior of Ψ3′P (Chart I), it was predicted that TpΨpCpGp should undergo photolytic cleavage as shown in Chart II.

In order to test this prediction, a dilute, aqueous solution of the tetranucleotide was irradiated with ultraviolet light under the conditions shown in the first line of Table IV. The solution was then heated at 80° for 5 min in order to reverse any photochemical hydration of cytosine residues that had occurred (Sinsheimer, 1957). As shown in Figure 1, there was only a small difference between the ultraviolet absorption spectrum of the starting material and that of the irradiated, unheated material. An aliquot of the reaction mixture was fractionated by paper electrophoresis. The product distribution as shown in Figure 2 was obtained. These products were characterized as follows:

Spot B was shown to be 5-formyluracil by comparison with an authentic standard by electrophoresis and by paper chromatography in two solvent systems (Table II). In addition, it gave the characteristic yellow color with the *o*-dianisidine spray and its ultraviolet spectra at pH 2 and 12 were identical with those of authentic material.

Spot C had the same mobility as the starting material on electrophoresis and paper chromatography (solvent A). In addition, its nucleotide composition, determined by alkaline hydrolysis and fractionation of the resulting nucleotides by paper chromatography, gave the following values (expressed as equivalents/mole of tetranucleotide): G2′(3′)P, 1.09; Ψ2′(3′)P, 0.72; C2′(3′)P, 1.00; T2′(3′)P, 1.02. The reason for this low Ψ2′(3′)P value is not known. However, Zamir *et al.* (1965) have reported a similar low value for Ψ2′(3′)P in their analysis of TpΨpCpGp.

Spot D had a greater mobility than TpΨpCpGp on electrophoresis (see Figure 2). This is consistent with the expected compound, pCpGp, which has the same net charge as the tetranucleotide at pH 7 (−5), but is considerably smaller. In order to confirm its structure the material was isolated from spot D and hydrolyzed with alkali. The hydrolysis products were identified as a mixture of cytidine 2′,5′- and 3′,5′-diphosphates (C2′(3′),5′DP) (1.0 equiv) and G2′(3′)P (0.86 equiv) by comparison with authentic standards. Hydrolysis with RNAase produced similar results.

Spot A had an electrophoretic mobility about one-third that of standard U2′(3′)P (Figure 2). This is consistent with the structure TpY which would have half the charge of U2′(3′)P at pH 7, but would run less than half as fast because of the Y group. The ultraviolet absorption spectrum of the material in spot A was similar to that of T3′P (Griffin *et al.*, 1958). Alkaline hydrolysis of the material in spot A gave T2′(3′)P identified by comparison of its electrophoretic, chromatographic, and spectral properties with T2′(3′)P isolated from an alkaline hydrolysate of TpΨpCpGp. On the basis of this evidence, the material in spot A was assigned the structure TpY, where Y is an unknown

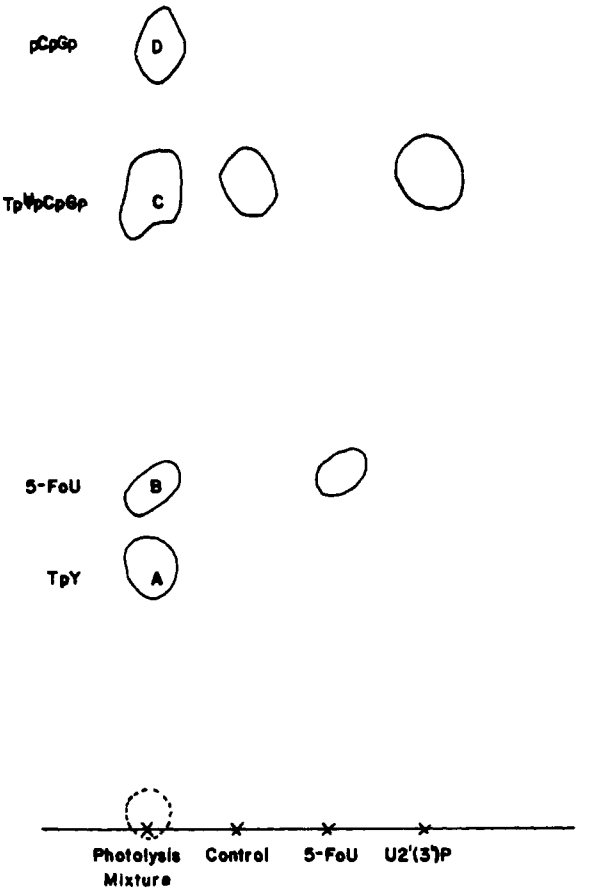

FIGURE 2: Electrophoresis of irradiated TpψpCpGp (1000 v, 2 hr, 0.05 M Na_2HPO_4–NaH_2PO_4, pH 7.2). Control = TpψpCpGp.

fragment derived from the ribose moiety of the pseudouridine residue in the tetranucleotide. Isolation of this fragment indicates that ultraviolet irradiation labilizes the 3′-ester link in the pseudouridylic acid moiety without directly affecting the 5′-ester bond.

These results establish that TpΨpCpGp can be cleaved specifically at its pseudouridine residues by ultraviolet light at 253.7 mμ. This procedure provides a simple method for determining the structure of pseudouridine-containing oligonucleotides such as TpΨpCpGp.

Side Reactions. Although no ultraviolet absorbing products other than those predicted could be found, a 15–20% decrease in absorbance was detected after irradiation of the tetranucleotide. This indicated that side reactions involving the destruction of the heterocyclic ring were occurring. This was confirmed by quantitative analysis of the reaction mixture by paper electrophoresis. When corrected for the amount of starting material recovered (40%), only 50% of the expected pCpGp and 30% of the expected TpY were found.

This loss of absorbance was examined further by irradiation of the individual nucleotides contained in

TABLE I: Irradiation Behavior of Control Substances.

Substance Irradiated	Loss of A_{260}^{7} (%)[a]	Assay for P_i after Irrad	Other Observations
C2′(3′)P	11[b]	Negative	No UMP was formed[c]
G2′(3′)P	11	Negative	
dT5′P	9	Negative	
5-Formyluracil	47	...	No increase of ultraviolet absorption after 16 hr at pH 1[d]
Mixture of C2′(3′)P, G2′(3′)P, ψ3′P, and dT5′P	25	...	ψ3′P completely disappeared from the reaction mixture
TpψpCpGp	15–20	...	40% TpψpCpGp remained

[a] $\{[(A_{260}^{7}$ before $h\nu) - (A_{260}^{7}$ after $h\nu$ + heat)]/$(A_{260}^{7}$ before $h\nu)\} \times 100$, measured directly in the reaction mixture. [b] Measured after heating for 5 min at 80° to reverse any hydration that might have occurred (Sinsheimer, 1954). [c] Paper chromatography of the irradiated sample in solvent A. [d] This treatment causes dehydration of photochemically hydrated U2′(3′)P (Sinsheimer, 1954).

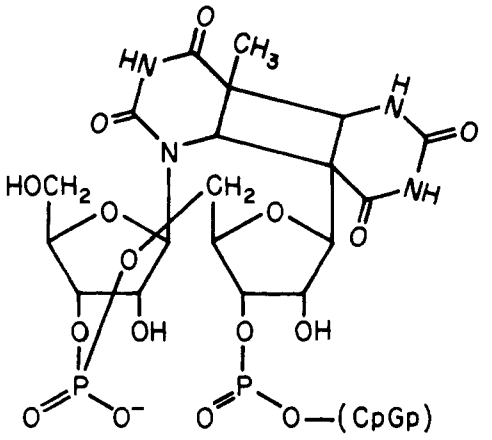

FIGURE 3: Hypothetical dimer formed between the T and ψ residues in TpψpCpGp.

TpΨpCpGp. As shown in Table I, C2′(3′)P, G2′(3′)P, and deoxyribothymidine 5′-monophosphate (dT5′P)[2] each showed approximately a 10% loss in absorbance. Irradiation of 5-formyluracil produced a 47% loss in absorbance. This loss was irreversible by heating at 80° for 5 min or by allowing the reaction mixtures to stand for 16 hr at pH 1 (Sinsheimer, 1954).

When the individual reaction mixtures, after separate irradiation of dT5′P or C2′(3′)P or G2′(3′)P, were examined by paper chromatography, only starting material could be detected by ultraviolet light. Irradiation of 5-formyluracil, on the other hand, produced two additional ultraviolet-absorbing compounds which have not been identified, but which have R_F values identical with those of 5-carboxyuracil and 5-hydroxymethyluracil, respectively, in solvent B.

[2] T2′(3′)P was not available in sufficient quantity for this experiment.

Finally, a mixture of dT5′P, C2′(3′)P, G2′(3′)P, and Ψ3′P was irradiated under the same conditions that had been used for TpΨpCpGp. A 25% loss in absorbance (Table I) occurred. This is similar to the loss with the tetranucleotide (15–20%). It is interesting that dT5′P, C2′(3′)P, and G2′(3′)P, but no Ψ3′P could be detected in the reaction mixture by paper chromatography. Thus, Ψ3′P reacts completely when it is part of an equimolar mixture of dT5′P, C2′(3′)P, and G2′-(3′)P, but it is only partially photolyzed when it is present as an integral part of TpΨpCpGp (Table I).

The nature of the irreversible loss of absorption that accompanies the photolytic cleavage of TpΨpCpGp is not clear. We have considered the obvious possibility that dimerization occurred between the adjacent thymine and pseudouridine residues to give the compound such as the one shown in Figure 3. If such a dimer formed, cleavage of the tetranucleotide at the pseudouridine residue would be prevented since dimerization leads to the destruction of the chromophore absorbing at 253.7 mμ. Therefore, dimerization should lead to a new tetranucleotide. No evidence of heterogeneity in the tetranucleotide fraction was found. Furthermore, dimerization would lead to an equivalent loss in both the pseudouridylic and thymidylic acid residues. Thus, alkaline hydrolysis of the tetranucleotide fraction should lead to low values for both T2′-(3′)P and Ψ2′(3′)P, relative to C2′(3′)P and G2′(3′)P. A low value for Ψ2′(3′)P (28% below theory) was indeed found after alkaline hydrolysis of the tetranucleotide recovered from the photolysis mixture, but the T2′-(3′)P content was normal. Taking into account the low values for Ψ2′(3′)P which have been obtained both by us and by others (Zamir, 1964) as well as the demonstrated destruction of ultraviolet absorption of the individual nucleotides, we conclude that dimerization did not occur to any significant extent.

These results suggest that the loss of absorption is due to nonspecific photolytic destruction of all base components of the tetranucleotide. It is important to note

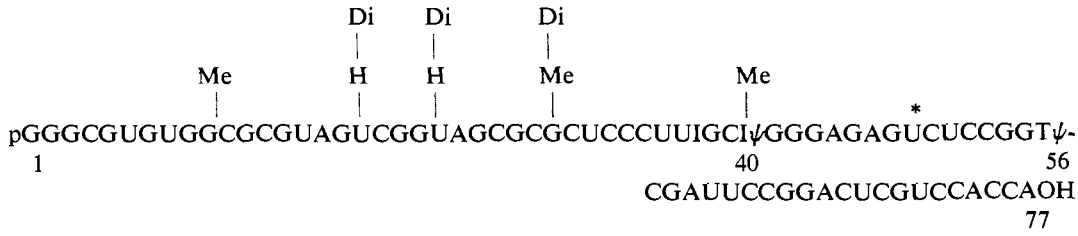

FIGURE 4: Nucleotide sequence of an alanine-s-RNA isolated from yeast (Holley *et al.*, 1965a). The symbol Di-H-U is abbreviation for 5,6-dihydrouridine residues; * marks the position where 25% of the uridine residues are replaced by 5,6-dihydrouridine residues.

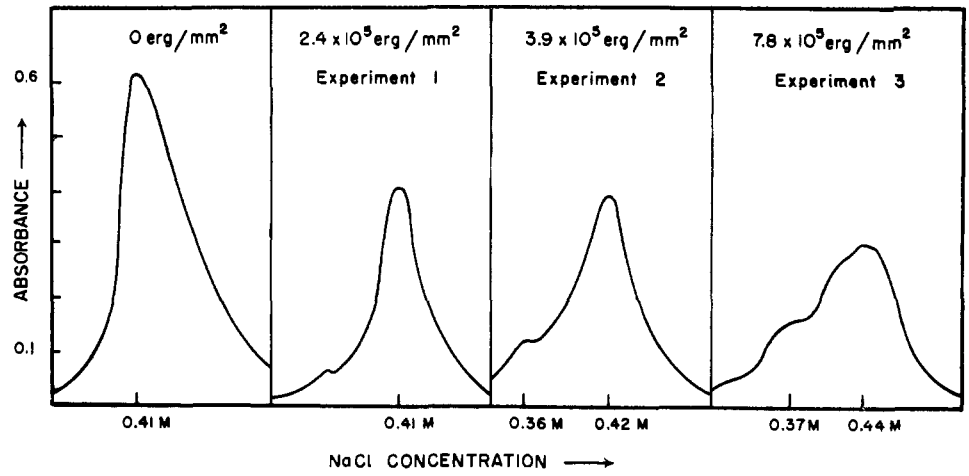

FIGURE 5: Chromatography of irradiated alanine-s-RNA on DEAE-cellulose. See Methods and Table IV (expt 1–3) for irradiation conditions. After irradiation (5 A^{7}_{260} units) the reaction mixture was concentrated to dryness and taken up in 2.5 ml of 0.1 M Tris buffer, pH 8.3, and heated at 85° for 10 min. Urea (2.1 g) was added and the volume was adjusted to 5 ml with water. The mixture was chromatographed on a 0.3 × 58 cm column of DEAE-cellulose (previously equilibrated with 0.05 M Tris buffer, pH 8.1, + 7 M urea) using a linear gradient (60 ml of 0.05 M Tris buffer, pH 8.1, which was 7 M in urea, in the mixer; 60 ml of buffer which was 7 M in urea and 0.6 M in NaCl in the reservoir). The flow rate was 0.27–0.35 ml/min and 1.1-ml fractions were collected.

that the extent of these side reactions is fairly small and the only ultraviolet absorbing products found were those predicted on the basis of a specific cleavage at the pseudouridine moiety.

Photolytic Cleavage of s-RNA. The success of the photolysis reaction with TpΨpCpGp encouraged us to extend the reaction to s-RNA. The brilliant work of Holley and his collaborators (Holley *et al.*, 1965a,b) in establishing the structure of an alanine-s-RNA from yeast allows us to predict the fragments which should be produced if photolysis of this s-RNA were specific for the pseudouridine residues. The pseudouridylic acid moieties occur as the 40th and 56th nucleotides counting from the pG end (Figure 4). Cleavage at these two residues would produce three fragments containing 39, 15, and 21 residues, respectively. In addition, single cleavages of the s-RNA would produce two fragments containing 55 and 37 residues, respectively. Thus, a maximum of five fragments would be expected from specific photolysis of alanine-s-RNA. Again, through the generosity of Dr. Holley, it was possible to carry out some preliminary experiments to test these predictions.

In our initial experiments,[3] alanine-s-RNA was irradiated at three different dose levels as shown in expt 1–3 in Table IV. The reaction mixtures were fractionated on DEAE-cellulose columns in the presence of 7 M urea (Tomlinson and Tener, 1963). The results are shown in Figure 5. Even at the lowest dose level a shoulder on the leading edge of the s-RNA peak can be seen clearly. As the dose was increased, the area under the shoulder increased and eventually (expt 3; *cf.* Table IV) the sharp peak, representing starting material, broadened considerably. These results suggested that fragments were being formed, but they did not rule

[3] These experiments were carried out in Dr. R. W. Holley's laboratory at Cornell University. We are grateful to Dr. Holley and Dr. Jean Apgar for their help and hospitality.

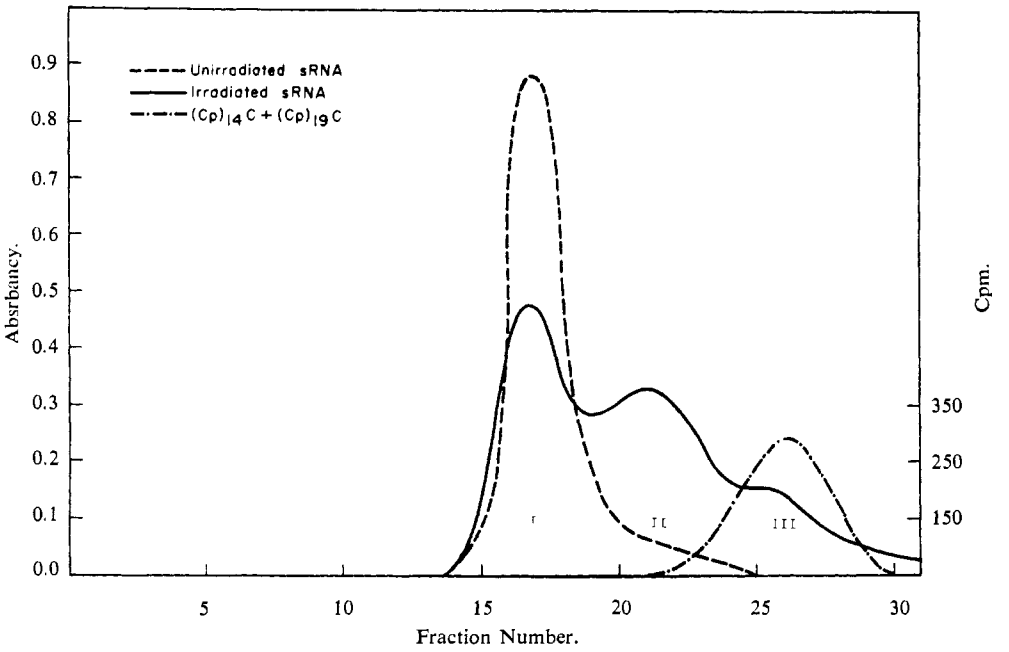

FIGURE 6: Chromatography of irradiated alanine-s-RNA on Sephadex G-100 at 56°. The reaction was carried out using the conditions shown in Table IV. The mixture was evaporated to dryness and the residue was dissolved in 1 ml of 0.2 M Tris buffer, pH 8.3, before heating at 85° for 10 min. The solution was applied to a Sephadex G-100 column which had been equilibrated at 56° with 0.01 M phosphate buffer, pH 7.6, containing 0.1 M NaCl. Elution was carried out with the same solution. In this experiment the column was 0.9 × 64 cm, the flow rate was 9.1 ml/hr, and the fractions were 1.1 ml. The elution required 4 hr. The column was calibrated in separate experiments with unirradiated alanine-s-RNA (3.3 $A^{?}_{260}$ units in 1 ml of eluent) and with a mixture of [^{14}C]-labeled $(Cp)_{14}C$ (175 cpm) and $(Cp)_{19}C$ (144 cpm) in 1 ml of eluent. The radioactivity was measured in a scintillation counter using the scintillation mixture described by Bray (1960).

out the possibility that the change in elution pattern was due simply to a change in the structure of the RNA molecule rather than to an actual fragmentation. Because of the small amount of material available, rechromatography was not practical and complete resolution of the shoulder material from the main peak could not be achieved under several different chromatographic conditions.

If photolysis of alanine-s-RNA occurs as expected, then it should be possible to separate the fragments into three or possibly four fractions by gel filtration on Sephadex G-100. Figure 6 shows the results of an experiment designed to test this hypothesis. The column was calibrated with unirradiated alanine-s-RNA and with a mixture of polycytidylic acids containing 15 and 20 residues (broken curves). After photolysis of alanine-s-RNA the pattern shown by the solid curve was obtained. Three partially resolved peaks are clearly visible. Comparison of these peaks with the markers indicates that they correspond to the positions expected for the predicted fragments; *i.e.*, peak I, starting material and the 55 nucleotide fragment; peak II, the 36 and the 39 nucleotide fragments; peak III, the 15 and the 21 nucleotide fragments. It should also be noted that the two polycytidylic acids do not separate under these conditions so that no fractionation of the fragments containing the 15 and 21 nucleotides (peak III) would be expected.

Since these separations require approximately 4 hr at 55° it was possible that fragmentation was being produced not by photolysis, but by heat. In order to rule out this possibility, a photolysis mixture was fractionated on Sephadex G-100 in the presence of 7 M urea at room temperature. The elution pattern (not shown) was similar to that shown in Figure 6.

A more recent experiment at the same dose level, but on a larger scale and using a longer column, is shown in Figure 7. Here another peak (Ib) in the correct position for the 55 nucleotide fragment can be seen clearly.

It was of considerable interest to see if any of the peaks contained biologically active material. In a separate run at lower dose level the pattern shown in Figure 8 was obtained. The material in peak I was still able to accept alanine, but the specific activity of the peak tube was only 40% that of the starting material.

These experiments, although encouraging, do not prove that a specific cleavage of alanine-s-RNA at its pseudouridylic acid residues occurred under the conditions described here. In view of the known resistance of synthetic polyribonucleotides to chain cleavage by ultraviolet irradiation (Marmur *et al.*, 1961) and the results described above, it seems unlikely that non-

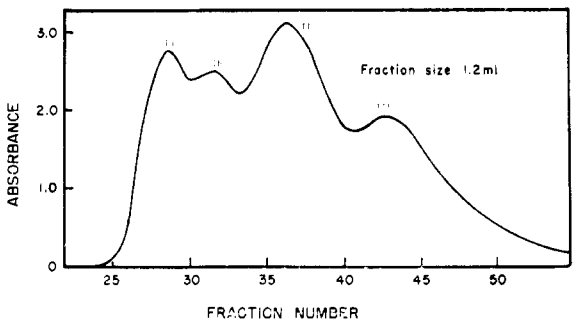

FIGURE 7: Chromatography of irradiated alanine-s-RNA on Sephadex G-100 at 56°. The reaction was similar to that described in Figure 6 except on a large scale and $D_{av} = 1.6 \times 10^6$ ergs/mm^2. The column was 0.9 × 115 cm and was monitored continuously with a Gilford multiple sample absorbance recorder using a 0.5-cm flow-through cell obtained from A. H. Thomas and Co.

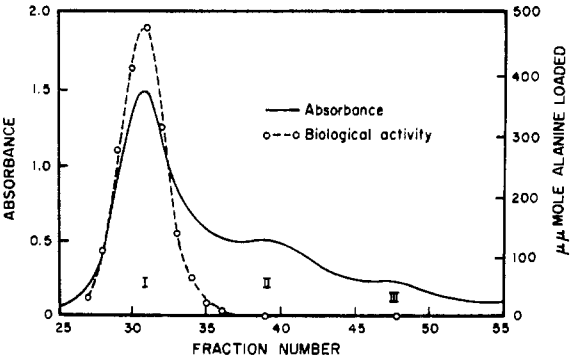

FIGURE 8: Effect of irradiation on alanine acceptor activity. For dose see Table IV; column as in Figure 7.

specific cleavage occurred. However, it still remains to obtain pure fragments and show that their structures are consistent with the structure of alanine-s-RNA. These experiments, which are in progress, should also establish whether any photochemically induced changes in other nucleotide residues occurred. These results should be particularly interesting in terms of the loss in activity of s-RNA on irradiation (Scott and Turter, 1962; Svenson and Nishimura, 1964; Fawaz-Estrup and Setlow, 1964; Zachau, 1964; Gottschling and Zachau, 1965).

Experimental Section

Materials. Samples of the tetranucleotide, TpΨpCpGp (Zamir *et al.*, 1965), and purified yeast alanine-s-RNA (Apgar *et al.*, 1962) were generously provided by Dr. Robert W. Holley. The samples gave single spots on paper chromatography in solvent A and on electrophoresis (see Table II). It is of interest that the tetranucleotide was partially decomposed on chromatography in solvent B. Three ultraviolet absorbing spots were observed on the paper chromatograms and the strongest spot was resolved into two components on electrophoresis. This result was unexpected in view of the frequent use of solvent B for the purification of oligonucleotides (*e.g.*, Nishimura *et al.*, 1963).

5-Formyluracil was synthesized by the method of Cline *et al.* (1959), with the modifications described elsewhere (Tomasz *et al.*, 1965). Yeast G2′(3′)P was purchased from Pabst Laboratories, Milwaukee, Wis. dT5′P and C2′(3′)P were obtained from Schwarz Bioresearch, Orangeburg, N. Y. Ψ3′P was prepared according to Cohn (1961) and C2′(3′)5′DP was prepared by the method of Hall and Khorana (1955). Ribonuclease was purchased from Worthington Biochemical Corp., Freehold, N. J. Samples of $(Cp)_{14}C$ and $(Cp)_{19}C$ were provided by Dr. Wendell Stanley, Jr.

TABLE II: Paper Chromatography and Electrophoresis.

Compound	R_F Values in Solvent[a] A[b]	B	C	Electrophoretic Mobilities Relative to U2′(3′)P[c]
TpψpCpGp	0.10			1.0
pCpGp	0.18			1.22
TpY	...			0.41
5′-Formyluracil	0.68	0.62		0.53
G2′(3′)P	0.39		0.26	
C2′(3′)P	0.63		0.40	
T2′(3′)P[d]	...		0.45	
ψ3′P	0.27		0.32	
C2′(3′), 5′DP	0.27		0.19	
dT5′P	0.55		0.40	
U2′(3′)P	0.39			
5-Carboxyuracil		0.36		
5-Hydroxymethyluracil		0.76		

[a] The proportion of each component is given in terms of volume. Solvent A, isobutyric acid–0.5 M NH_4OH (10:6); solvent B, 95% ethanol–1 M NH_4OAc (7:3); solvent C, 1-propanol–concentrated NH_4OH–H_2O (11:7:2). [b] Two "solvent fronts" were visible under a ultraviolet lamp. R_F values are calculated on the basis of the one closer to the origin. [c] The mobility of U2′(3′)P was usually about 18 cm. The buffer was 0.05 M Na_2HPO_4–NaH_2PO_4, pH 7.2. [d] Isolated from an alkaline hydrolysate of TpψpCpGp by paper chromatography.

Methods. Paper chromatography was carried out on Whatman 40 paper using the descending technique. The ultraviolet absorbing areas were detected with a Mineralite lamp (Model SL). The R_F values are recorded in Table II. Paper electrophoresis was carried out on Whatman 3MM paper at pH 7.2 and 1000 v for 2 hr using an EC Apparatus Co. unit (Swarthmore, Pa.).

The mobilities of various substances relative to U2′-(3′)P are recorded in Table II. For quantitative measurements the paper strips were washed with distilled water and dried prior to use in order to reduce the blank values. For preparative purposes a volatile buffer, 0.1 M $Et_3NH^+HCO_3^-$ adjusted to pH 7.2 with CO_2, was employed. 5-Formyluracil travels faster in this buffer (mobility 0.95) than in the phosphate buffer described in Table II. The reason for this is not clear, but it may be due, at least in part, to pH changes in the bicarbonate buffer. It should be noted that our spectral data indicate that the pK of 5-formyluracil is close to 7.

Ultraviolet absorbing compounds were isolated on a preparative scale from paper chromatograms and electrophorograms by cutting out the ultraviolet absorbing areas, cutting them into small pieces, and eluting them with several portions of water. The pH of the combined eluates was adjusted, whenever necessary, to 6 with washed Dowex 50W-H^+ (50–100 mesh). Quantitative analysis of ultraviolet absorbing components was achieved by cutting the appropriate area from the paper, eluting it with 2 ml of 1 M NH_4OH, and measuring the absorbance of the eluate against an appropriate paper blank. The extinction coefficients used for quantitative calculations are listed in Table III.

TABLE III: Extinction Coefficients.[a]

Compound	ϵ^7_{260}	ϵ^{11b}_{λ}
dT5′P	8,600[c]	$7{,}540_{267}$
C2′(3′)P	7,600[d]	$9{,}000_{270}$
ψ2′(3′)P	7,760[e]	$7{,}355_{286}$
G2′(3′)P	11,800[d]	$11{,}800_{260}$
TpψpCpGp	35,760[f]	$34{,}015_{260}$
pCpGp	19,400[f]	$19{,}400_{260}$
5-Formyluracil	7,040[g]	

[a] Superscripts refer to pH, subscripts to wavelength in mμ. All measurements were made in 1-cm cuvets. [b] Determined by measuring the $A^7_{260}/A^{11}_{\lambda}$ ratio and using ϵ^7_{260}, given in the preceding column, for the appropriate calculation. In case of the first three substances λ represents λ^{11}_{max}. [c] Fredericq *et al.* (1961). [d] Volkin and Cohn (1954). [e] Determined by measuring the $A^7_{262}A^2_{260}$ ratio and using ϵ^7_{262} 7900 (Shapiro and Chambers, 1961) for the appropriate calculation. [f] Obtained by summing the ϵ^7_{260} values of the individual mononucleotide components. No correction for hyperchromicity was applied. [g] Determined by measuring the A^2_{275}/A^2_{260} ratio and using ϵ^7_{275} 11,850 (Cline *et al.*, 1959) for the appropriate calculation.

TABLE IV: Irradiation Conditions of Various Experiments.[a]

Substance Irradiated	Method of Irradn	I_0 (erg/mm² per sec)	Time of Irradn (hr)	D_0 (erg/mm²)	Volume of Soln (ml)	Depth of Soln (cm)	A_{1cm}[b]	A_d	% T_d	f_{cor}	D_{av} (erg/mm²)
TpψpCpGp Ala-s-RNA (expt 1)	B	392	1.5	2.12×10^6	14.5	0.23	1.2	0.276	52	0.73	1.55×10^6
Ala-s-RNA (expt 2)	A	36.5[c]	3.5	4.58×10^5	10	0.16	4	0.64	23	0.52	2.38×10^5
Ala-s-RNA (expt 3)	A	36.5[c]	3.5	4.58×10^5	10	0.16	0.94	0.15	71	0.85	3.89×10^5
Ala-s-RNA (Figure 6)	A	36.5[c]	6.5	8.50×10^5	10	0.16	0.47	0.07	85	0.92	7.82×10^5
Ala-s-RNA (Figure 6)	B	392	1.5	2.12×10^6	15	0.24	0.40	0.096	80	0.90	1.91×10^6
Ala-s-RNA (Figure 8)	B	392	1	1.42×10^6	15	0.24	1.0	0.24	57.5	0.76	1.08×10^6

[a] I_0 = incident light intensity, $D_0 = I_0 \times$ time of irradiation, $A_d = A_{1cm} \times$ depth of solution, T_d = % transmission, f_{cor} = a correction factor for converting D_0 to D_{av} (Morowitz, 1950), $D_{av} = f_{cor} \times D_0$. See Methods for further discussion. [b] The absorbance was inadvertently measured at 260 mμ instead of 253.7 mμ. The difference between absorbance at these two wavelengths for alanine-s-RNA and for TpψpCpGp is negligible (*e.g.*, see Figure 1). [c] This value was calculated by multiplying I_0 by a geometrical correction factor, 0.42. This correction was necessary because the surface area of the solution (7084 mm²) was greater than the effective area of the lamp (3000 mm²).

5-Formyluracil was detected on paper chromatograms and electrophorograms both by its ultraviolet absorbance and by its characteristic yellow color with an *o*-dianisidine spray (0.5% solution of *o*-dianisidine

in glacial acetic acid) (Feigl, 1954). Inorganic phosphate was measured quantitatively by the method of King (1932). Absorbance measurements were made in a Beckman DU spectrophotometer. Complete spectra were obtained using a Beckman DK-2 instrument.

Alkaline hydrolysis of nucleotide derivatives was carried out in 0.3 M KOH for 18 hr at room temperature. The hydrolysate was neutralized with Dowex 50W-H^+ (50–100 mesh). The resin was removed by filtration and washed with several portions of water. The hydrolysis products were separated by paper chromatography in solvent C and assayed quantitatively as described above. Ribonuclease-catalyzed hydrolysis of pCpGp (6 hr at 37°) was carried out on 1.5 A^7_{260} units of the dinucleoside phosphate in 12 μl of 0.005 M sodium phosphate buffer (pH 7.0) containing 0.02 mg of RNA-ase.

Ultraviolet irradiation (see Table IV) was carried out initially (method A) as described previously (Tomasz and Chambers, 1964). Later experiments (method B) were performed as follows. A dilute, aqueous solution (10–20 ml) of the nucleotide (0.3–1.2 A^7_{260} units/ml) was stirred magnetically in a 9.6-cm diameter Petri dish and irradiated with a low-pressure mercury-resonance lamp (Mineralite, Model V-R 51) that emits at least 95% of its total output at 253.7 mμ. A 2 mm thick piece of Vycor glass (No. 7910) placed between the lamp and the solution served both as a filter to remove all light below 220 mμ and as a cover for the Petri dish. The lamp was placed at a distance of 3.5 cm from the surface of the solution. An electric fan prevented the mixture from warming above room temperature.

The irradiation dose was calculated as follows. In method B (above), the intensity (I_0) of the light source was measured by uranyl oxalate actinometry (McLaren and Shugar, 1964b). The value obtained for I_0 was checked by measuring the quantum yield of the photohydration of uridylic acid (McLaren and Shugar, 1964c). In method A, I_0 was measured directly with a Latarjet dosimeter (Latarjet *et al.*, 1953). The average dose (D_{av}), which assumes that each point within the solution receives the same irradiation, was calculated from the incident dose ($D_0 = I_0 \times$ irradiation time) using the table given by Morowitz (1950). It should be pointed out that the values in this table were calculated from an equation which assumes that the absorbance of the sample remains constant throughout the irradiation. This was not strictly true in the experiments reported here, but D_{av} is used because it represents a convenient way to express the data and because the change in absorbance was small.

Alanine acceptor activity was assayed by a modification of the procedure described by Berg *et al.* (1961). The reaction mixture (1.0 ml) contained 25 μmoles of Tris buffer, pH 7.5, 30 μmoles of $MgCl_2$, 2.5 μmoles of ATP, 0.5 μmole of EDTA, 0.01 μmole of L[^{14}C]-alanine, 0.1–1 A_{260} unit of yeast alanine-s-RNA, and 0.4–0.5 A_{280} unit of activating enzyme. Each reaction mixture was preincubated for 3 min at 37° before addition of the enzyme. After a further 10 min of incubation, the mixture was cooled for 3 min in ice. Carrier yeast RNA (1.2 mg; Schwarz Bioresearch, Orangeburg, N. Y.) was added. The precipitation and washing procedure was as described by Berg *et al.* (1961). The final precipitate was dissolved in 1.2 ml of water. Aliquots (1.0 ml) were plated and dried on 1.25-in. diameter planchets and counted with a thin-window, gas-flow counter.

The activating enzyme was prepared by grinding 50 g of fresh baker's yeast with 200 g of glass beads in 75 ml of cold 0.01 M Tris buffer, pH 7.8, containing 0.01 M $Mg(OAc)_2$, 0.06 M KCl, and 0.006 M mercaptoethanol for two 4-min periods with a 3-min cooling period in between, using a Servall Omnimix. The beads were allowed to settle by gravity and the supernatant was decanted. The beads were washed several times with a total of 200 ml of cold buffer and the combined supernatants were centrifuged at 16,000g for 15 min. The supernatant was then centrifuged at 100,000g for 150 min in a Spinco Model L centrifuge using 12 cups (13.5-ml capacity). The excess 16,000g supernatant was discarded. The clear 100,000g supernatant was adjusted to pH 7.8 with 1 N KOH and put onto a 2 × 19 cm column of DEAE-cellulose which had been previously equilibrated with the buffer described above but without the KCl. Elution was continued with the same buffer (no KCl). The fractions with an $A_{280}/A_{260} > 0.9$ (120 ml total) were pooled, redistributed in 1-ml fractions ($\sim$ 9mg of protein/ml; $A_{280}/A_{260} = 1.0$), and frozen. Just before use 1.0 ml of the enzyme solution was thawed and passed through a 1 × 17 cm column of Sephadex G-25. Fractions (1 ml) were collected and those fractions having an $A_{280}/A_{260} > 1.5$ were combined and used directly for the assay described above. The preparation of the enzyme and the assays were carried out by Miss Irene Taylor.

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