Optical Properties of Specific Complexes between Complementary Oligoribonucleotides*

Robert B. Gennis† and Charles R. Cantor‡

ABSTRACT: Specific complexes between oligoribonucleotides have been investigated using circular dichroism. The following sets of compounds were prepared enzymatically: G(pU)*n*, (Up)*n*G, C(pA)*n*, (Ap)*n*C, where $n = 5, 6$, and 7. The uridine-containing oligomers were mixed with the adenosine-containing oligomers of equal total chain length under conditions designed to maximize specific complex formation. Both melting curves and circular dichroism difference spectra showed evidence of complex formation but only at relatively high concentrations, about 10^{-2} M in nucleotide residues. Complexes formed from mixtures of antiparallel complements have higher melting temperatures than those formed from mixtures of parallel complements. Ultracentrifuge studies on one oligonucleotide mixture indicate that the complex formed is low molecular weight, consistent with a double- or triple-strand structure. As would be expected, the stability of the complexes formed is strongly concentration and chain-length dependent.

Substantial differences in melting temperature are found when two antiparallel complexes containing the same base composition but different sequences are compared. Several simple tests were devised using circular dichroism difference spectra to investigate the stoichiometry of the complexes formed. These indicate that 1:1 complexes will form between the complementary oligonucleotides that have been studied.

Complexes between oligonucleotides should be good models for codon–anticodon interactions (Uhlenbeck *et al.*, 1970; Clark *et al.*, 1968). They can also yield information about the stability and structure of other short helices postulated to be important structural components of tRNA and 5S RNA (Madison, 1968). The principal aim of the present study is to examine the properties of a set of oligonucleotide complexes. If one can generalize what is known about the properties of polymer–polymer (Blake *et al.*, 1967; Felsenfeld and Miles, 1967) and polymer–oligomer complexes (Michelson and Monny, 1967; Bautz and Bautz, 1964; Lipsett *et al.*, 1961; Cantor and Chin, 1968) to these smaller units, it will facilitate our understanding of the interactions which stabilize nucleic acid structure.

The formation of specific base pairs is one of the major factors which determine the conformation of nucleic acids. This could best be studied if complexes between individual mononucleotides or specific oligonucleotide sequences could be prepared. Evidence for base-pairing interactions between mononucleotides has been found only in nonaqueous solutions (Katz and Penman, 1966; Newmark and Cantor, 1968). When different monomers are mixed in water, base-stacking interactions seem to be completely dominant (Solie and Schellman, 1968; Broom *et al.*, 1967). Oligonucleotide complexes offer a better opportunity for observing base-pairing interactions in aqueous solutions. The difficulty of preparing suitable compounds has limited the amount of work on oligonucleotide interactions that has been accomplished thus far.

Previous studies on oligonucleotide interactions have been plagued by the possibility of aggregate formation. Jaskunas *et al.* (1968), using experimental conditions designed to maximize the stability of base pairs, looked for interactions between sets of complementary trinucleoside diphosphates. They were successful in only one case, mixtures of GpGpC and GpCpC. This system was complicated, however, by the self-aggregation of each oligomer. Felsenfeld and Miles (1967)[1] reported the examination on the interaction of the tetramer compounds, ApApApA and UpUpUpU, and evidence for a complex ApApApA:2UpUpUpU at high concentrations[2] (total base 0.12 M) using infrared spectroscopy. In this case, there is a very good possibility that the actual aggregate consists of overlapping oligomers forming a triple-strand concatomer similar to poly (A):2 poly (U) (see Figure 1).

Earlier studies in this laboratory dealt with the interaction between UpUpUpUpUpU and ApApApApApA. These two oligonucleotides formed a stable complex under substantially the same conditions we are using at present (Cantor and Chin, 1968). The circular dichroism spectrum indicated that the structure is similar to that of poly (A):2 poly (U) triple strand. It is very likely that this system also results in the formation of a concatomer. Other oligonucleotide systems studied have been the acid forms of oligo (A) (Brahms *et al.*, 1966) and oligo (C) (Brahms *et al.*, 1967). These too are likely to form concatomers and, in any case, are not good models for general use in RNA interactions due to the fact that in both cases the bases are protonated.

Some work has also been done on deoxyoligonucleotide

* Contribution from the Departments of Chemistry and Biological Sciences, Columbia University, New York, New York 10027. *Received June 4, 1970.* This work was supported by a grant (GM14825), from the U. S. Public Health Service.

† U. S. Public Health Service predoctoral Fellow 5-501-GM-41,373-02; to whom to address correspondence.

‡ Alfred P. Sloan Fellow.

[1] Unpublished experiments by Miles, Frazier, and Rottman referred to on p 413.

[2] All concentrations are reported per residue.

complexes. Naylor and Gilham (1966) examined the interaction between oligo (dT) and oligo (dA) using hypochromicity. Here again, concatomers are probable.

We have designed our experiments to reduce the possibility of concatomer formation by using heterooligomers. We have enzymatically synthesized four sets of oligomers: $G(pU)_n$, $(Up)_nG$, $C(pA)_n$, and $(Ap)_nC$. We were primarily interested in the size range $n = 5$, 6, and 7. The introduction of the terminal G and C reduces the possibility of finding structures analogous to Figure 1 since that would require noncomplementary bases to be paired.

The adenosine-containing oligomers were mixed with those uridine-containing oligomers of equal total chain length. Conditions were chosen to attempt to maximize complex formation: 1 M NaCl (pH 7.0–7.5)–phosphate buffer (0.04 M), oligonucleotide concentrations always near 0.01 M in nucleoside residues unless otherwise stated.

It is known that mixtures of poly (A) with poly (U) can form both double- and triple-stranded complexes depending on the conditions (Felsenfeld and Miles, 1967; Blake *et al.*, 1967). For this reason, each set of oligomers was mixed in two solutions with the U:A ratio being 1.0 and 2.0, respectively. Complexes were detected and analyzed using circular dichroism. The change in circular dichroism (or optical rotatory dispersion) in going from the single-strand to double-strand form in polymers has been well characterized and is known to be a sensitive indication of complex formation (Cantor *et al.*, 1966; Brahms, 1965).

Experimental Section

Materials. Nucleoside 5′-diphosphates were purchased from Biopolymers, Inc. Dinucleoside phosphates (GpU, CpA) were obtained from Gallard-Schlesinger. Beef pancreatic ribonuclease, *Escherichia coli* alkaline phosphatase (BAPC), and ribonuclease T_1 were all obtained from Worthington. Tritium-labeled ADP, used as a marker in our column, was purchased from Schwarz BioResearch. Partially purified polynucleotide phosphorylase was purchased from P-L Biochemicals; the insolubilized trypsin, Enzite-Try, was obtained from Gallard-Schlesinger. The urea solutions were purified by stirring with a small amount of Whatman DE-23 DEAE-cellulose.

Synthesis of Oligoribonucleotides. The $(Ap)_nC$ series of oligomers was made by enzymatic degradation of poly (A + C). The copolymer was synthesized in a reaction mixture containing ADP (25 mg/ml); CDP (5 mg/ml), 0.35 M NaCl–0.2 M glycine buffer (pH 9.1)–0.01 M $MgCl_2$; 0.4 mM EDTA, and P-L Biochemicals *Micrococcus luteus* PNPase[2] (4 mg/ml) used without further purification. The mixture was incubated for 3.5 hr at 37°. The resulting polymer was purified as follows. The reaction mixture was cooled and two volumes of cold water was added, followed by one-tenth volumes of both 5% (w/v) SDS solution and 10% (w/v) phenol. After shaking for 10 min at room temperature, the solution was centrifuged to separate the layers. To the upper (aqueous) layer, one-fifth volume of phenol was added, and the shaking and centrifugation were repeated. The upper layer was separated and combined with one-fifth volume of acetate buffer (0.01 M, pH 5), three volumes of cold 95% ethanol, and three volumes of cold acetone. This was shaken very vigorously until a flocculent precipitate formed, and then centrifuged. The precipitate was redissolved in a small amount of water and dialyzed to remove additional diphosphates. The product was then lyophilized. Final yields were usually near 20%.

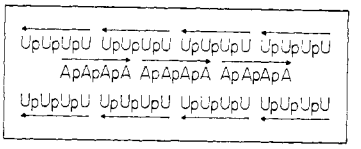

FIGURE 1: One possible structure for the complex $(Ap)_3A$: $2(Up)_3U$. Such structures of overlapping oligomers will be referred to as concatomers.

The polymer degradation was carried out in 1 ml of solution which contained 0.25 M NaCl–0.06 M Tris (pH 8.1), 10 mM MgOAc, and 1 mM EDTA. For each milligram of polymer, about 0.5 μg of pancreatic RNase was added. The solution was incubated for 6 hr after which the pH was adjusted to approximately 9 using 0.5 M NaOH. The 3′-terminal phosphate left by the RNase was removed using *E. coli* alkaline phosphatase. About 10 μg of enzyme was used per mg of RNA. The incubation was then continued for another 2–3 hr. This results in a mixture of oligomers of the form $(Ap)_nC$.

The series $(Up)_nG$ was prepared in virtually the same manner as the $(Ap)_nC$. The poly (U + G) was made by the same procedure. The yields in this case were found to be considerably worse, occasionally no high molecular weight polymer was formed at all. Due to this difficulty, we were not able to get sufficient quantities of $(Up)_7G$ to work with.

The poly (U + G) degradation was performed with T_1 RNase in a reaction mixture containing: 0.05 M Tris (pH 7.7; about 1 ml/10 mg of polymer), 1.0 mM EDTA, and approximately 300 Worthington units of T_1 RNase/mg of polymer. The solution was incubated for 2.5 hr at 37°. The pH was then lowered to between 2 and 3 by addition of HCl and then the solution was incubated for a period of 3 hr at 37° to ensure the cleavage of cyclic phosphates. Following this, the solution was adjusted to pH 8 and alkaline phosphatase was added to remove the terminal phosphate.

Both series $C(pA)_n$ and $G(pU)_n$ oligonucleotides were prepared by the method of Thach and Doty (Thach, 1966; Thach and Doty, 1965). The procedure was scaled up to about 4 mg of dinucleoside phosphate primer with no difficulty. Incubation was carried out for 22 hr at 33°. Our primer-dependent PNPase was prepared by the method of Klee and Singer (1968) as modified by Mr. Joseph Blattner in our laboratory. P-L Biochemicals PNPase (equivalent to stage VI in activity) was chromatographed on a Sephadex G-200 column. The material eluting in the void volume was treated with insolubilized trypsin (Enzite-Try) until a satisfactory degree of primer dependence developed. Our enzyme had a primer dependent:primer independent ratio of about 10:1 in the absence of salt, vastly higher in high salt, and had an activity of approximately 30 phosphorolysis units/mg. We used 0.1 mg of PNPase/ml of reaction mixture.

Separation of Oligonucleotides. Paper chromatographic separations were performed for analytical purposes. Whatman No. 3MM paper was used for descending elution using a 1:1

[2] Abbreviations used are: PNPase, polynucleotide phosphorylase; SDS, sodium dodecyl sulfate.

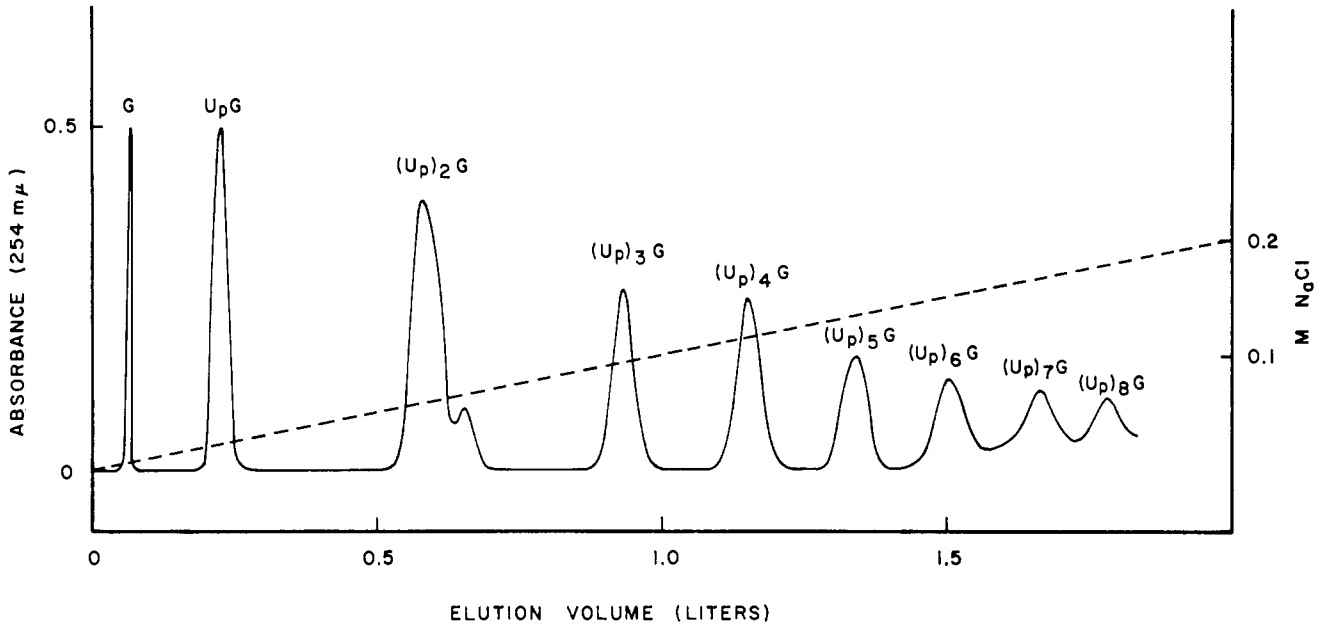

FIGURE 2: Elution pattern from the column chromatography of the set $(Up)_nG$. The column consisted of DEAE-cellulose (Whatman DE-23; chloride form, 1.5 × 30 cm). Elution was effected by a 4-l. linear NaCl gradient (0.0–0.4 M) at pH 7–8, Tris (0.0025 M). Material was collected in 10-ml fractions at a rate of 1 ml/min.

mixture of 95% ethanol and 1 M ammonium acetate. Our chromatographic patterns were similar to those previously reported (Thach, 1966).

Preparative separations were made on a 1.5 × 30 cm DEAE-cellulose column using Whatman DE-23 DEAE-cellulose. All column chromatography was done in the presence of 7 M urea (Tomlinson and Tener, 1963). A 0.0–0.4 M sodium chloride gradient at pH 7–8 Tris (0.0025 M) was used in all cases. A typical column profile is shown in Figure 2. (See caption for details.) The center tubes of each peak were combined and all oligomers with chain lengths larger than 4 were extensively dialyzed at 4°. Each was then lyophilized, and stored dry at −20°. Typically, the yields of those compounds of interest were small, varying from $^1/_3$ to $^2/_3$ mg.

Identification of Products. Due to both the small amount of each product and the nature of the material, unambiguous chemical identification was impossible. We have relied upon peak location to identify our oligomers. Ultraviolet spectra (Warshaw and Tinoco, 1965) were used in each case to identify the dinucleoside phosphate (*e.g.*, UpG, GpU, CpA, and ApC) as well as trends in the optical properties as the peak number increased (to make sure no anomalous peaks were present). In some cases circular dichroism spectra were used to help confirm the dinucleoside phosphate identification. Once ADP-C14 was added to the column along with the reaction mixture to confirm the assignment of peak number with total negative charge which had been made. Furthermore, the peak locations were compared to those resulting from a base hydrolysis of poly (A) (Lane and Butler, 1959; Bock, 1967). Paper chromatograms were compared with published patterns (Thach, 1966). The optical properties of the material eluted from the paper chromatograms were compared to those of the materials from our column peaks. All evidence was consistent with our identification. We consider the chances of an error in the identification of the oligomers to be remote.

Mixing Procedure. The extinction coefficients of the oligomers were calculated using the nearest-neighbor approximation (Cantor and Tinoco, 1965) from values for the appropriate nucleosides (Pabst, 1956), dinucleoside phosphate (Warshaw and Tinoco, 1965), oligo (U's) (Simpkins and Richards, 1967) and oligo (A's) (Brahms *et al.*, 1966). It was assumed that the extinction coefficients of the complexes were all 1.0×10^4/residue. This is a sufficiently good approximation in this chain-length region and is consistent with what one would calculate by nearest-neighbor methods (Cantor and Tinoco, 1965). The consistency of any approximations concerning base concentrations was automatically checked by the circular dichroism difference curves at high temperature.

Stock solutions were made for all oligomers containing 0.01 M oligonucleotide residues, 1 M NaCl, and 0.04 M phosphate buffer (pH 7.0–7.5). Only oligomers of equal chain length were mixed. The solutions with a U:A ratio of 1 were made up by combining 5 μl from each stock solution. The 2:1 mixtures were made from 5 μl of the adenosine-containing oligomers and 10 μl of the uridine oligomers. In addition, mixtures with a U:A ratio of 2 were made with the two uridine containing oligomers of equal chain length; for instance, 5 μl of $(Ap)_nC$, 5 μl of $(Up)_nG$, and 5 μl of $G(pU)_n$. Specially accurate (sp) micropipets made by G. Pederson Corp. (Denmark) were used to reduce mixing errors.

Optical Measurements. All ultraviolet spectroscopy was performed on the Cary Model 15 spectrophotometer; circular dichroism was measured using a Cary Model 60 with circular dichroism attachment (6001). All optical measurements on the final mixtures were made in a 0.1-mm path-length circular dichroism cell obtained from Opticell, Inc., Beltsville, Md.

This cell contained two glass-stoppered necks set at right angles to facilitate filling and emptying. The volume of sample needed to fill the cell was only 6 μl. The cell was water jacketed and temperatures were maintained constant to $\pm 0.1°$ with a Lauda Model Super K2R bath and circulator equipped with a platinum resistance thermometer. All ultraviolet measurements were done at room temperature, but all circular dichroism work was temperature controlled. To prevent evaporation from the cell (an initial problem), a drop of paraffin oil was placed over the solutions. This completely solved the problem; the oil was easily removed each time with hexane. However, this made recovery of the 6 μl of oligomer mixture quite difficult. Since it was highly inconvenient to calibrate the temperature inside the 0.1-mm cell, corrections relative to the bath temperature were assumed to be the same as in a similarly constructed 1.0-cm cell. These were found by using a thermistor inserted directly into the 1-cm cell.

For each mixture as well as for each pure component, the circular dichroism spectrum was measured both at high and low temperatures. Spectra were taken from 320 to 220 nm. The melting of the complexes formed was followed by observing the change in molar ellipticity with temperatures at a wavelength near 255 nm.

It should be noted that all our melting curves have been corrected for any changes occurring in the single-strand constituents. We can approximate the total circular dichroism at any wavelength as the sum of the contributions from the single-strand and double-strand components

$$[\theta(T)]_{tot} = \chi_{ss}(T)[\theta(T)]_{ss} + \chi_{ds}(T)[\theta(T)]_{ds}$$

where $[\theta(T)]_{tot}$, $[\theta(T)]_{ss}$, and $[\theta(T)]_{ds}$ are the molar ellipticities of the mixture, of pure single strand, and of pure double strand; and $\chi_{ss}(T)$ and $\chi_{ds}(T)$ are the mole fractions of single and double strand in the mixture. Note that each of them is a function of temperature. Since

$$\chi_{ss}(T) + \chi_{ds}(T) = 1$$

we can rewrite this after rearranging as

$$\chi_{ds}(T) = \frac{[\theta(T)]_{tot} - [\theta(T)]_{ss}}{[\theta(T)]_{ds} - [\theta(T)]_{ss}}$$

What we want to plot as a melting curve is the quantity $\chi_{ds}(T)$ *vs. T*. Since we have no measure of how $[\theta(T)]_{ds}$ changes over the temperature region of interest, and since the circular dichroism changes very little prior to melting, we shall assume that $[\theta(T)]_{ds}$ is a constant which we measure at low temperatures prior to the onset of melting. However, we do know that $[\theta(T)]_{ss}$ can change significantly over this temperature range and, furthermore, it is an easy task to measure this change by observing the circular dichroism change with temperature of each component oligomer separately. Since $[\theta(T)]_{tot}$, $[\theta(T)]_{ss}$, and $[\theta]_{ds}$ are measured one can then calculate $\chi_{ds}(t)$. We were fortunate in half our cases to select a wavelength for monitoring the complex formation where $[\theta(T)]_{ss}$ was a constant over the temperature range. So we can write

$$\chi_{ds}(T) = \frac{[\theta(T)_{tot} - [\theta]_{ss}}{\Delta\theta}$$

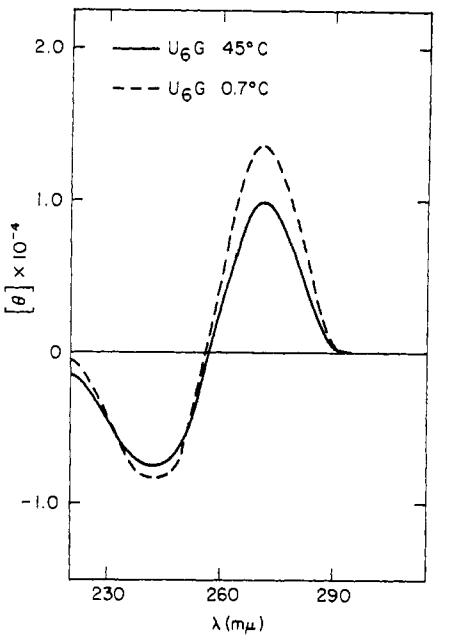

FIGURE 3: High- and low-temperature circular dichroism spectra of $(Up)_6G$.

where $\Delta\theta = ([\theta]_{ds} - [\theta]_{ss})$. Since the two extremes for $[\theta(T)]_{tot}$ are $[\theta]_{ss}$ at high temperatures, and $[\theta]_{ds}$ at low temperatures, we can express $\chi_{ds}(T)$ on a per cent basis, as $\%\Delta\theta$. In some cases, the T_m was low and the melting curve so broad that helix formation was incomplete even at 0°. In these cases, we extrapolated the empirical curve to lower temperature to get a reasonable value for $[\theta]_{ds}$. This is why the melting curves shown in Figures 5 and 6 do not all begin at 100%.

Computations. All calculations, such as the evaluation of circular dichroism differences curves, were performed on the IBM 360-91. For these purposes the spectra were digitized taking points every 2.5 nm over the entire range scanned.

Results

Detection of Complexes. The principal finding in this work is that specific complexes do form between complementary oligonucleotides. It is unfortunate that quite high total concentrations are required for stability. Evidence for complex formation comes principally from circular dichroism spectra and it is confirmed by ultracentrifugation. The use of circular dichroism data for following complex formation was relied on heavily because the large effects observed permit some of the consistency checks discussed below. It is clear that circular dichroism is a sensitive probe for conformational change in nucleic acids. The change in circular dichroism in going from single- to double-strand RNA is well characterized (Yang and Samejima, 1969). There is an increase in the size of the positive band near 260 mμ, and a substantial blue shift. These changes observed for polymers are also seen in our oligomer mixtures in going from high to low temperatures. However, the circular dichroism of a single-strand oligonucleotide is also strongly temperature dependent. Figure 3 shows the circular dichroism spectra of a typical component oligomer, $(Up)_6G$, at high and low temperatures. Note that both the shape and position

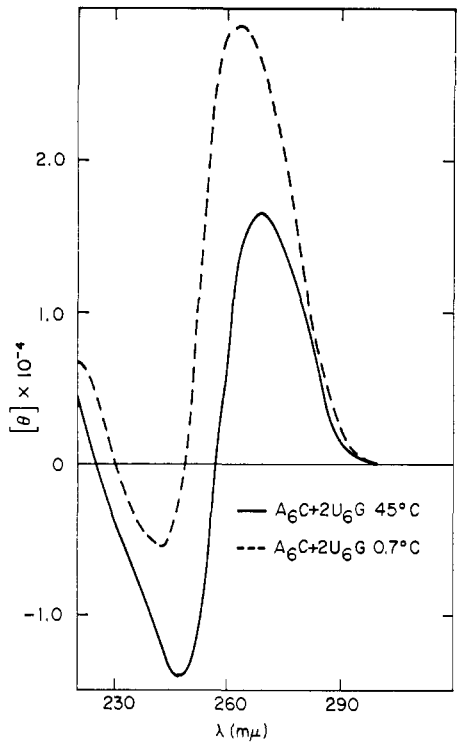

FIGURE 4: High- and low-temperature circular dichroism spectra of the mixture $(Ap)_6C + 2(Up)_6G$.

remain the same. The increase in magnitude in going to low temperature is due to an increased stacking interaction. The same sort of results hold true form the adenosine-containing oligomers. However, in these cases there is a much larger increase in magnitude due to the greater tendency to stack. In contrast to this, Figure 4 shows the circular dichroism spectrum of a typical oligonucleotide mixture, A_6C:2 U_6G. The marked blue shift with decrease in temperature is readily apparent. Monitoring the change in molar ellipticity with

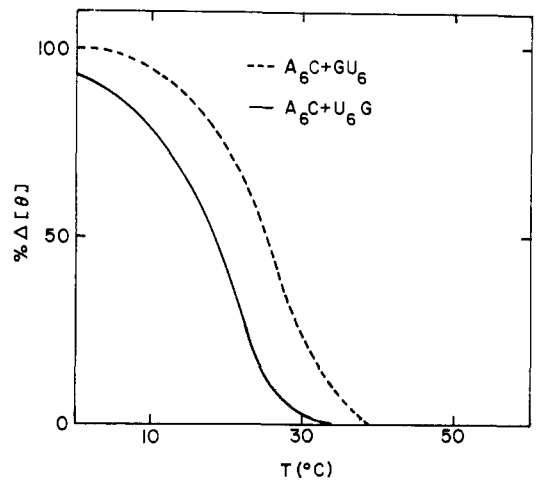

FIGURE 5: Melting curves of antiparallel complementary and parallel complementary (noncomplementary) pairs of heptamers with U:A ratio of 1.0. Ellipticity was measured at 255 mμ. The ordinate, $\%\Delta[\theta]$ is defined in the text.

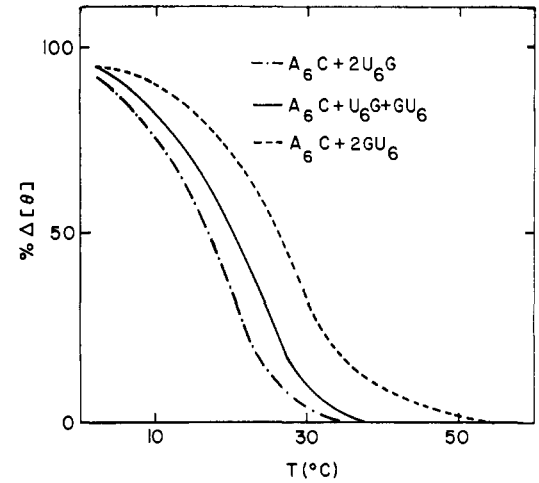

FIGURE 6: Melting curves of the three mixtures with $(Ap)_6C$ where the U:A ratio is 2.0. Ellipticity measured at 255 mμ.

temperature results in a melting curve which follows the change in the degree of intermolecular interaction. Several melting curves are shown in Figures 5 and 6. To further quantify the data, circular dichroism difference curves were used.

At high temperatures, we would expect the circular dichroism spectrum of the mixture to be equal to the weighted linear combination of the circular dichroism spectra of the individual components taken separately. However, at low temperature, if there is complex formation this would not be the case. We have calculated for each mixture the spectrum we would expect to see in the absence of intermolecular interaction at both high and low temperatures by an appropriately weighted sum of the spectra of the components. This calculated spectrum was then subtracted from the observed spectrum for each mixture. The results were consistent and clearcut. At high temperature, the difference spectrum was zero. Thus, the two spectra are identical which demonstrates the absence of any interaction. The negligible circular dichroism difference spectrum also serves as a check on any errors in concentration and allows the data to be corrected for pipetting errors. (In only one case was a substantial correction required. This will be referred to later.) At low temperatures, the difference curves show a significant divergence from zero. A representative set of difference curves is illustrated in Figure 7.

Our final indication of complex formation was ultracentrifugation. Equilibrium ultracentrifugation runs performed by Dr. Gary Felsenfeld were made with the complementary pair $A_7C:GU_7$ in a 1:1 mixture over the concentration range 10–1 mM. Lack of material made an exact determination of the molecular weight impossible. However, the results did indicate that at low temperatures, an aggregate was formed. Further, the aggregate was shown to have a low molecular weight, consistent with two or three (but no more than five) oligomers per complex.

Concentration Dependence. As expected, there is a marked dependence of the stability of the complexes on concentration. At 0.01 M, the heptamer pairs (*e.g.*, $(Ap)_6C:G(pU)_6$) all formed stable complexes at low temperatures with T_m's near

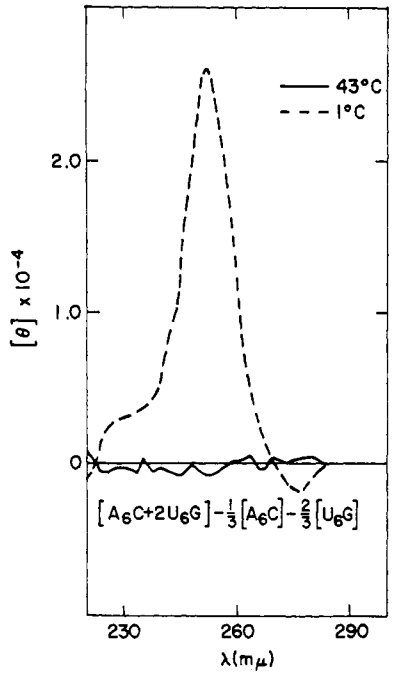

FIGURE 7: Circular dichroism difference spectra of a typical oligonucleotide mixture. A weighted sum of the spectra of the components is subtracted from the spectrum of the mixture.

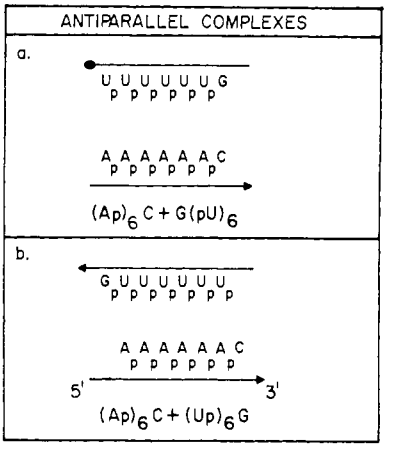

FIGURE 8: (a) Antiparallel complementary heptanucleotide pair. (b) Parallel complementary (noncomplementary) heptanucleotide pair.

15–25°. However, a tenfold dilution eliminated any evidence of complexes.

The octamers show a similar dependence. At 10 mM the pair $(Ap)_7C:G(pU)_7$ melts at 30.5°. At 2 mM its T_m is 22.5°, and at 1 mM it is 21°.

Chain-Length Dependence. The stability of the complexes formed increases significantly with chain length. This is to be expected. Table I shows the T_m's for all mixtures. At the concentrations we used there is a very sharp difference in the stabilities of the hexamer and the heptamer. Whereas at 10 mM quite stable complexes formed between heptamers, under the same conditions there was absolutely no evidence of complex formation between hexamers (*e.g.*, $A_5C:GU_5$). This is not to say that there is something especially significant about the length seven. We would fully expect, at higher concentrations, to see specific complexes forming between the hexamers or even oligomers of lower chain length. This is supported by the fact that the pair $A_4:U_4$ will form stable low-temperature complexes at concentrations near 0.12 M (Felsenfeld and Miles, 1967) but not at 0.01 M (Jaskunas *et al.*, 1966). As will be pointed out later, we feel that these homooligomers are probably concatomers rather than specific double- or triple-strand complexes of short length (see Figure 1). It should be noted that peculiarly the length seven has arisen several times as a cut-off under the various conditions others have used to study the oligomer system. Brahms *et al.* found that heptamers were the shortest segments needed to form stable complexes in the acid oligo (A) (Brahms *et al.*, 1966) as well as the acid oligo (C) (Brahms *et al.*, 1967) systems. However, these studies were done at 10^{-4} M and we feel these are not the most appropriate from which to draw conclusions concerning general RNA interactions at neutral pH. Past studies of deoxyoligomers between oligo (dA) and oligo (dT), also at 10^{-4} M have also shown a requirement for at least a heptamer (Naylor and Gilham, 1966). We feel that in all these cases raising the concentration would serve to stabilize complexes between shorter oligomers.

Complementarity. Perhaps the most important conclusion of this work is that complementarity in the usual antiparallel Watson–Crick sense seems to be operative at the level of short oligonucleotides. The relative order of the melting temperatures is quite consistent with such structures. The pair $(Ap)_6C:G(pU)_6$ is complementary in all base pairs when drawn as in Figure 8a. We will refer to this as an antiparallel complementary pair. The pair $(Ap)_6C:(Up)_6G$ drawn similarly (Figure 8b) is not complementary at either end and will be referred to as being noncomplementary (or parallel complementary). In the 1:1 mixtures, the antiparallel complementary pairs always melted higher than the noncomplementary mixtures. Furthermore, this holds true for the 2:1 mixtures. Three 2:1 mixtures with each adenosine-containing oligomer were prepared. One contained 2 equiv of the complementary uracil oligomer, another had 2 equiv of the noncomplementary oligomer, and the third had 1 equiv each per mole of the adenosine oligomer. The melting temperatures were always ordered such that the complements melted

TABLE I: Melting Temperatures (°C) for All Complexes at 10 mM.[a]

	$C(pA)_6$	$(Ap)_6C$	$C(pA)_7$	$(Ap)_7C$
Complementary				
1:1	19	25		31.5
2:1	18	25		30
Mixed				
$(Up)_6G + G(pU)_6$	15.5	20.5		
Noncomplementary				
1:1	13.5	18.5	23	
2:1	13	17	22	

[a] The T_m value is taken at 50% complex formation.

highest, the noncomplements lowest, with the third mixture having a melting temperature in between the extremes. This is the order one would predict if the complexes were antiparallel. Figures 5 and 6 show a representative selection of these melting curves. Other data are summarized in Table I.

Such qualitative evidence hardly constitutes solid proof of the detailed structure of the oligonucleotide complexes. Several pieces of related data make it seem probable. First of all, the ultracentrifuge data rules out any large aggregates being formed and is consistent with a two- or three-oligomer complex. We also know that the optical changes accompanying the complexes are similar to those which occur in polymers where the nature of the complexes are more well defined. Furthermore, it has been shown that the optical properties of the complexes between (1) oligo (A) and poly (U), and (2) A_6 and U_6, and even (3) adenosine and poly (U) are similar to poly (A) and poly (U) (Cantor and Chin, 1968). This indicates that the structure of the final (2:1) complexes are virtually identical. This, as well as data for the poly (A) and oligo (dT) system (Naylor and Gilham, 1966) shows that intact polymers are not necessary for double- or triple-strand helices. It has also been demonstrated that deoxyoligomers form specific antiparallel-type structures. One indication of this is the ability of complementary pairs of deoxyoligomers to act as a primer for DNA replicase. Noncomplementary sets cannot form stable double-strand complexes and therefore cannot serve as a primer (Wells *et al.*, 1967). Further, Khorana and his coworkers have recently shown that complementary deoxyoligomers form stable complexes at low temperatures (Gupta *et al.*, 1968).

Sequence Dependence. When the T_m data presented in Table I are examined closely, an interesting strong dependence on the location of the G:C pair is apparent. One might have expected that such pairs as $(Ap)_6C$:$G(pU)_6$ and $C(pA)_6$:$(Up)_6G$ would melt at the same temperature. This does not seem to be the case. In all four comparisons among the heptamers (see Table I) the complex involving $(Ap)_6C$ melted higher than that with $C(pA)_6$. This is true for both the complementary and noncomplementary combinations. We have no convincing arguments as to why this should be true. It is unfortunate that one oligomer set was not complete. The lack of $(Up)_7G$ prevents confirmation of this observation in another set. We have considered the possibility that one series of oligomers had the chain lengths incorrectly assigned. However, we feel this is a most unlikely possibility. Furthermore, the chromatographic data (peak locations) are most convincing for the adenosine series. Peculiar "end effects" have been observed previously in the oligomer–polymer systems (Lipsett *et al.*, 1961). Perhaps the most notable example is the work of Uhlenbeck *et al.* (1968) where it was found that a 2:1 (U:A) mixture of poly (U) with $(Ap)_4C$ melted significantly lower than that with $C(pA)_4$. More work needs to be done to clarify these differences and explain their causes. The importance of specific sequence on the stability of double-stranded RNA has been well known for several years. For instance, the homopolymer pair poly (rA):poly (rU) has a T_m of 56.8° whereas the alternating copolymer, poly (rAU), melts at 66° under similar conditions (Chamberlin, 1965). Should small sequence changes be as important as our data indicate, it will make calculations of RNA melting curves such as those by Kallenbach (1968) exceedingly difficult.

Stoichiometry. Circular dichroism spectra were used for determining the stoichiometry of the oligonucleotide complexes. As described earlier, equilibrium ultracentrifugation studies were performed for us by Dr. Gary Felsenfeld. Because of the high concentrations needed for complex formation, the oligomers were monitored at a wavelength far from the optical λ_{max}. Lack of material made it impossible to determine specific molecular weight, and could not distinguish between a 1:1 and 2:1 (U:A) complex. For this same reason, we were not able to perform any mixing curves to obtain the constituency of the complex. We have thus used circular dichroism difference curves in an attempt to substitute for mixing curves. The conclusions from such a technique do not constitute hard proof but only indicate that the data are consistent with the assumptions used in treating the data.

The following assumptions were made: (1) that the only complexes that can form in our solutions were 1:1 and 2:1 (U:A) to the exclusion of all others; (2) that there was only one complex in each low-temperature mixture and; (3) that that complex was formed maximally at the temperatures at which our low-temperature spectra were taken. For each pair of oligomers, we have data from the solutions where the U:A ratio is 1.0 and 2.0. Our assumptions leave us only four possibilities for these two solutions. (1) The complex formed in both solutions has a U:A of 1:1 regardless of the U:A ratio in solution. (2) The complex formed in both solutions has a U:A of 2:1 regardless of the U:A ratio in solution. (3) The solution which has a U:A ratio of 1 forms a 1:1 complex; the solution which has a U:A ratio of 2 forms a 2:1 complex. (4) The solution which has a U:A ratio of 1 forms a 2:1 complex; the solution with a U:A ratio of 2 forms a 1:1 complex.

We consider the fourth possibility to be absurd. It violates mass action and we shall not consider it further. We have devised two simple tests using circular dichroism difference curves to test for the validity of the first two alternatives.

Let us say that both solutions have formed 1:1 complexes. In the case of the solution which has a U:A ratio of 1.0, the measured spectrum represents the spectrum of this complex. In the case of the mixture where U:A is 2.0, we have present in solution not only the 1:1 complex but also the additional uridine-containing oligomer. We know that we can calculate the circular dichroism spectrum of a mixture by a weighted linear combination of the circular dichroism spectra of the components. If our assumptions are correct, we already have the spectrum of the 1:1 complex from the first solution as well as the spectra of the separate oligomers under the same conditions. Using this, we calculated the expected spectrum of the 2:1 mixture. The following expression was used

$$[\theta(A_6C + 2GU_6)]_{calcd} = \frac{2}{3}[\theta(A_6C + GU_6)]_{obsd} + \frac{1}{3}[\theta(GU_6)]_{obsd} \quad (1)$$

Note that this is a calculation of the spectrum expected for a solution with an input U:A ratio of 2:0 where a 1:1 complex is forming.

We then subtracted this calculated spectrum from that which is actually observed. If the two are equivalent and the resulting difference curve is zero, we can say that at least the data is consistent with all the assumptions, namely both solutions contain 1:1 complexes. If not, then one or more assumptions concerning the behavior of the system is wrong.

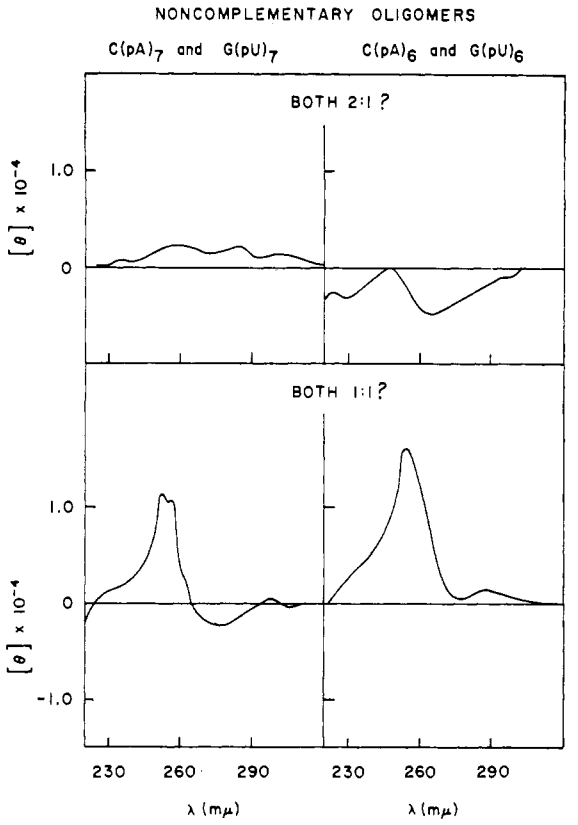

FIGURE 9: Circular dichroism difference spectral tests of complex stoichiometry: mixtures of two noncomplementary pairs. A nonzero difference spectrum indicates that the hypothesis tested is incorrect. (See text for details.)

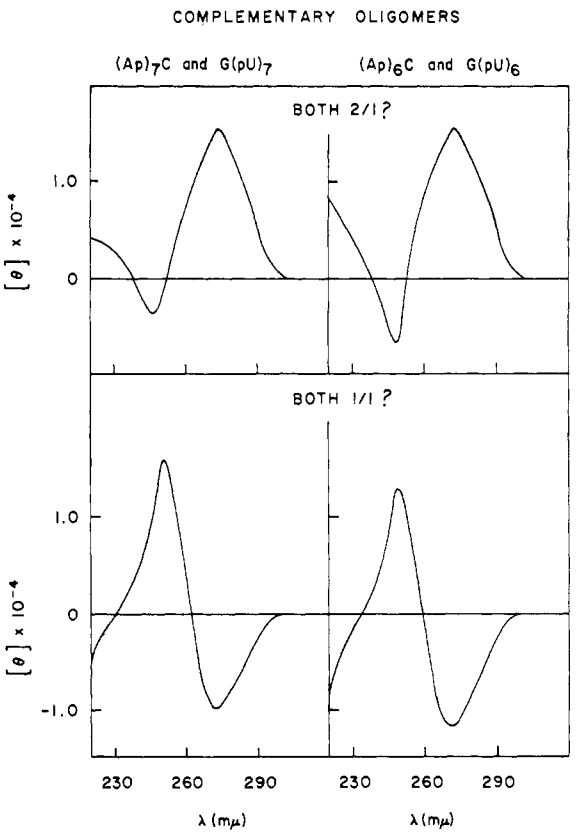

FIGURE 10: Circular dichroism difference spectral tests of complex stoichiometry: mixtures of two complementary pairs. A nonzero difference spectrum indicates that the hypothesis tested in incorrect. (See text for details.)

These same arguments apply equally well to the second alternative, that the complex formed is 2:1 regardless of the input U:A ratio in solution. In that case we calculate the spectrum to be expected from the solution which has U/A of 1.0 by adding up the spectra in the following way

$$[\theta(A_6C + U_6G)]_{calcd} = \frac{3}{4}[\theta(A_6C + 2U_6G)]_{obsd} + \frac{1}{4}[\theta(A_6C)]_{obsd} \quad (2)$$

Once again this is subtracted from the measured spectrum. If the difference is negligible, both solutions have 2:1 complexes. Both these tests were applied for each of the six pairs of oligomers we looked at. Some of the results are shown in Figures 9 and 10. All three of the noncomplementary (parallel complementary) pairs gave strong indication that there were 2:1 complexes in both solutions. See, for example, the sets of results summarized in Figure 9. The results of the 1:1 test were far from zero in each case, and the final difference curves in each case were very similar. In all cases the 2:1 test gave difference curves which were close to zero.

However, the antiparallel complementary oligomers reveal a different story. One pair of oligomers, $C(pA)_6$:$(Up)_6G$, gave anomalous results. This same pair was the only one requiring a significant correction for the concentration of its components. Most likely the stoichiometry was inexact in this mixture. The other two antiparallel complementary pairs gave quite similarly shaped difference spectra for the two tests. Both tests gave quite large difference circular dichroism spectra. See the results shown in Figure 10. The third (anomalous pair) also gave nonzero difference spectra, but the shapes were not the same. In all cases, the difference spectra were not similar to those derived from the noncomplementary oligomers. Assuming the initial three assumptions which premised these tests are correct, we are led to the conclusion that both alternatives 1 and 2 are false for the complementary oligomers. Since we are ignoring the fourth choice for being chemically absurd, we must conclude that the complex formed varies with the U:A ratio in solution under these conditions and is 1:1 in a 1:1 solution, 2:1 in a 2:1 solution. It is also a possibility that some complex is formed which is neither 1:1 or 2:1 but is some low molecular weight aggregate with perhaps four or five oligomers. We do not have enough data to rule this out. Considering the rather clear way the noncomplementary oligomer data worked out, we feel that there is every indication that we are getting specific double- or triple-stranded complexes among our complementary pairs as well.

Discussion

The results described thus far demonstrate that specific complexes can be formed between complementary systems. There does not seem to be any difference in the nature of the inter-

actions from those found in polymer systems. This gives considerable encouragement for the types of tertiary structures postulated for such molecules as tRNA and 5S RNA. Though our results are not completely definitive, we feel that the melting temperatures of our complexes clearly indicate that both double and triple strands between oligomers must contain antiparallel chains. This has important implications for possible tRNA structures. For instance, the model proposed by Cramer *et al.* (1968) for yeast Phe tRNA contains base pairs between parallel strands of the pseudo-U loop and the dihydro-U loop. Similar interactions between parallel segments are postulated by Levitt (1969). This is not the case for the model proposed by Connors *et al.* (1969). Our data does not completely rule out the possibility of such interactions. However, since strong evidence for interactions between parallel strands in both long and short strands of RNA has failed to materialize in what are so far the only model systems available, it would be worthwhile indeed to investigate whether such interactions are stereochemically possible.

Sequence dependence of the stability of RNA complexes is already well known. Sequence isomers are known which have greatly varying T_m's. It is provocative that the relative order of stabilities depends on the $(Ap)_nC$ and $C(pA)_n$ whether they are complexed with $(Up)_nG$ or $G(pU)_n$. It is conceivable that there may be a few simple rules one could apply to determine *a priori* the relative stability of one double-strand sequence over another. Both the phenomena of significant end effects and the sequence dependence of stability could make the statistical mechanical calculations for such molecules as tRNA much more difficult.

Recently Dr. Olke Uhlenbeck at the University of California, Berkeley, has compared the results shown here with his studies on the T_m of complexes between oligonucleotides in the series $(Ap)_nC(pU)_m$ and $(Ap)_mG(pU)_n$. He finds that the T_m values of our oligonucleotide complexes are in surprisingly close agreement with his results when extrapolations are made to identical experimental conditions. This argues strongly that the effect of a G:C pair on the stability of an oligonucleotide complex is not very different if the pair is located at an end, or in an internal position.

We have not yet carried out a detailed thermodynamic analysis of our data. A preliminary analysis of the data showed that Van't Hoff plots were extremely nonlinear indicating either the possibility of a large ΔCp or the incorrectness of the simplified model we were using. Lack of adequate data at present makes it impossible to carry the analysis further to distinguish between these alternatives.

The oligomer–oligomer system does form a simple well-defined model with which to study RNA interactions. However, both formidable synthetic barriers and the inherent instability of the complexes formed make these systems experimentally difficult to work with. An interesting line of future work will probably be to study the interactions of specific oligonucleotides isolated from partial digests of tRNAs. Other future prospects are examinations of the effects of particular mispairing and the effects of many sequence differences in the strength of oligomer interactions. These will hopefully serve to encourage improvements in the present state of the art of oligoribonucleotide synthesis. This is clearly needed if interesting oligonucleotide complexes are to be studied by an array of physical techniques.

Acknowledgments

The authors are very grateful to Dr. Gary Felsenfeld for performing the equilibrium ultracentrifugation experiment described in the text. Very helpful discussions with Dr. Olke Uhlenbeck provided considerable stimulation. Mr. Joseph Blattner and Mr. Avi Snyder kindly provided primer dependent samples of *M. Luteus* PNPase and Mr. Frederick Schachat assisted with some of the oligonucleotide syntheses.

References

Bautz, E. K. F., and Bautz, F. A. (1964), *Proc. Nat. Acad. Sci. U. S. 52*, 1479.

Blake, R. D., Massoulie, J., and Fresco, J. R. (1967), *J. Mol. Biol. 30*, 291.

Bock, R. M. (1967), *Methods Enzymol. 12A*, 218.

Brahms, J. (1965), *J. Mol. Biol. 11*, 785.

Brahms, J., Maurizot, J. C., and Michelson, A. M. (1967), *J. Mol. Biol. 25*, 465.

Brahms, J., Michelson, A. M., and Van Holde, K. E. (1966), *J. Mol. Biol. 15*, 467.

Broom, A. D., Schweizer, M. P., and Ts'o, P. O. P. (1967), *J. Amer. Chem. Soc. 89*, 3612.

Cantor, C. R., and Chin, W. W. (1968), *Biopolymers 6*, 1745.

Cantor, C. R., Jaskunas, S. R., and Tinoco, I., Jr. (1966), *J. Mol. Biol. 25*, 465.

Cantor, C. R., and Tinoco, I., Jr. (1965), *J. Mol. Biol. 13*, 65.

Chamberlin, M. J. (1965), *Fed. Proc., Fed. Amer. Soc. Exp. Biol. 24*, 1446.

Clark, B. F. C., Dube, S. K., and Marcker, K. A. (1968), *Nature (London) 219*, 484.

Connors, P. G., Labanauskas, M., and Beeman, W. W. (1969), *Science 166*, 1528.

Cramer, F., Doepner, H., v. d. Haar, F., Schlimme, E., and Seidel, H. (1968), *Proc. Nat. Acad. Sci. U. S. 61*, 1384.

Felsenfeld, G., and Miles, H. T. (1967), *Annu. Rev. Biochem. 36II*, 407.

Gupta, N. K., Ohtsuka, E., Sgaramella, V., Buchi, H., Kumar, A., Weber, H., and Khorana, H. G. (1968), *Proc. Nat. Acad. Sci. U. S. 60*, 1338.

Jaskunas, S. R., Cantor, C. R., and Tinoco, I., Jr. (1968), *Biochemistry 7*, 3164.

Kallenbach, N. R. (1968), *J. Mol. Biol. 37*, 445.

Katz, L., and Penman, S. (1966), *J. Mol. Biol. 15*, 220.

Klee, C. B., and Singer, M. F. (1968), *J. Biol. Chem. 243*, 5094.

Lane, B. G., and Butler, G. C. (1959), *Biochim. Biophys. Acta 33*, 281.

Levitt, M. (1969), *Nature (London) 224*, 759.

Lipsett, M. N., Heppel, L. A., and Bradley, D. F. (1961), *J. Biol. Chem. 236*, 857.

Madison, J. T. (1968), *Annu. Rev. Biochem. 37*, 131.

Michelson, A. M., and Monny, C. (1967), *Biochim. Biophys. Acta 149*, 107.

Naylor, R., and Gilham, P. T. (1966), *Biochemistry 5*, 2722.

Newmark, R. A., and Cantor, C. R. (1968), *J. Amer. Chem. Soc. 90*, 5010.

Pabst Laboratories (1956), Circular OR-10.

Simpkins, H., and Richards, E. G. (1967), *J. Mol. Biol. 29*, 349.

Solie, T. N., and Schellman, J. A. (1968), *J. Mol. Biol. 33*, 61.

Thach, R. E. (1966), *in* Procedures in Nucleic Acid Research, Cantoni, G. L., and Davies, D. R., Ed., New York, N. Y. Harper and Row, p 520.
Thach, R. E., and Doty, P. (1965), *Science 148*, No. 3670, 632.
Tomlinson, R. V., and Tener, G. M. (1963), *Biochemistry 2*, 697.
Uhlenbeck, O. C., Baller, J., and Doty, P. (1970), *Nature (London) 225*, 508.
Uhlenbeck, O., Harrison, R., and Doty, P. (1968), *in* Molecular Associations in Biology, Pullman, B., Ed., New York, N. Y., Academic, p 107.
Warshaw, M. M., and Tinoco, I., Jr. (1965), *J. Mol. Biol. 13*, 54.
Wells, R. D., Jacob, T. M., Narang, S. A., and Khorana, H. G. (1967), *J. Mol. Biol. 27*, 237.
Yang, J. T., and Samejima, T. (1969), *Progr. Nucl. Acid Res. 9*, 224.

Fluorescence Studies of *Aplysia* and Sperm Whale Apomyoglobins*

Sonia R. Anderson,† Maurizio Brunori, and Gregorio Weber

ABSTRACT: We have investigated some of the molecular properties of the globins prepared from *Aplysia* myoglobin, sperm whale myoglobin, and human hemoglobin using fluorescence and fluorescence polarization methods. The study of the intrinsic fluorescence of the apomyoglobins shows definite differences between the two proteins. The fluorescence spectrum of *Aplysia* apomyoglobin clearly reveals two different tryptophan residues with emission maxima at *ca.* 330 and 355 nm. These wavelengths correspond, respectively, to the maxima of tryptophan in nonpolar and in aqueous solvents. The fluorescence polarization spectrum of *Aplysia* apomyoglobin demonstrates localized rotational freedom expected for a mobile tryptophan side chain in contact with the water. In contrast, the two tryptophan side chains of sperm whale apomyoglobin are rigidly bound in similar nonpolar regions within the protein matrix. The average lifetime of the excited state is in the range 2.8–2.9 nsec for both apomyoglobins. The fluorescence emission maximum of adsorbed 1-anilino-8-naphthalenesulfonate occurs at 478 nm in *Aplysia* apomyoglobin and at 455 nm in sperm whale apomyoglobin. This difference suggests that the specific dye binding site, believed to be the site normally occupied by the heme moiety of myoglobin, is more polar in *Aplysia* apomyoglobin. The rotational relaxation time obtained from measurements on conjugates with 1-dimethylaminonaphthalene-5-sulfonyl chloride is 30 nsecs at 15° for each of the apomyoglobins. This value indicates approximately spherical symmetry of the apomyoglobin molecule.

Sperm whale and *Aplysia* myoglobins have in common some of the most important structural and functional features such as molecular weight and ligand-binding properties (Rossi-Fanelli *et al.*, 1958b; Wittenberg *et al.*, 1965). On the other hand, they differ markedly in amino acid composition. Of particular interest in *Aplysia* myoglobin is the absence of tyrosine and the presence of only one histidine residue per molecule (Rossi-Fanelli *et al.*, 1958b; Tentori *et al.*, 1968). It is known that the amino acid sequence of the peptide containing the only histidine is considerably different from that of the peptides containing the proximal or distal histidine residues of sperm whale myoglobin (Tentori *et al.*, 1968). Finally, *Aplysia* myoglobin undergoes a reversible thermal denaturation involving the heme–protein interactions (Brunori *et al.*, 1968a).

The work reported here deals with some of the molecular properties of the apoproteins from *Aplysia* and sperm whale myoglobins as revealed by a study of the fluorescence and fluorescence polarization of the globins and of their conjugates with 1-dimethylaminonaphthalene-5-sulfonyl chloride and adsorbates with 1-anilino-8-naphthalenesulfonate.

Experimental Section

Materials. Sperm whale myoglobin was purchased from Seravac Chemical Co. (England). *Aplysia* myoglobin was prepared from the buccal muscles by the method of Rossi-Fanelli and Antonini (1957). Human hemoglobin was prepared by the toluene method (Drabkin, 1946).

The corresponding globins were obtained by acid acetone splitting according to Rossi-Fanelli *et al.* (1958a). The concentration of the globin was obtained spectrophotometrically using the following values for the molar extinction coefficients:

* From Department of Biochemistry and Biophysics, Oregon State University, Corvallis, Oregon (S. R. A.), and the Institute of Biochemistry and C.N.R. Center for Molecular Biology, University of Rome, and Regina Elena Institute for Cancer Research, Rome, Italy (M. B.), and the Biochemistry Division, Department of Chemistry and Chemical Engineering, University of Illinois, Urbana, Illinois (G. W.). *Received July 13, 1970.*
† Career Development Awardee 1 K04GM24134-01 of the National Institutes of Health. Address correspondence to this author or to M. B.