The Reaction of Ribonucleic Acid with Formaldehyde. I. Optical Absorbance Studies*

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ABSTRACT: The effect of temperature and formaldehyde concentration on the extent of reaction of ribonucleic acid (RNA) with formaldehyde was studied by measuring the absorbance at 270 mμ. The reaction is about 85% complete after 10 min at 63° in 1 M formaldehyde. Absorbance–temperature studies of adenosine monophosphate (AMP), cytidine monophosphate (CMP), and guanosine monophosphate (GMP) before and after reaction with formaldehyde show that the extent of reaction decreases reversibly as the temperature is raised. At 250 mμ, however, the average absorbance is almost independent of the extent of reaction. Absorption spectra at 15 and 85°, and absorbance–temperature profiles were measured for *Escherichia coli* and yeast transfer ribonucleic acid (tRNA), R17 phage RNA, and tobacco mosaic virus–ribonucleic acid (TMV–RNA) before and after reaction with formaldehyde. The formaldehyde derivatives have hypochromicities of 0.04–0.06 compared to values of 0.19–0.23 obtained for unreacted RNA. The absorbance–temperature changes are largely reversible and independent of wavelength or counterion concentration. A comparison of the modest hypochromicity observed with the large optical rotatory dispersion that has been reported for tRNA treated with formaldehyde suggests that the single-stranded asymmetric structures present in RNA result from stacking that has only a minor influence on the absorbance. A practical consequence of this observation is that RNA hypochromicity can be attributed largely to the double-stranded hydrogen-bonded regions of the molecule. After correcting for the contribution from single-stranded base stacking, the helical content of RNA can be estimated as 59–73% depending on the RNA.

The double-stranded helical regions of RNA can be partly regenerated after removing excess formaldehyde either by dialysis or passage through a Sephadex G50 column followed by heating to 80° for 10 min. Prolonged exposure to elevated temperatures is required to effect complete reformation.

Ribonucleic acid is believed to be composed of short DNA-like helical segments connected by single-stranded regions. A variety of methods has been applied to study the extent, size, and stability of the helical regions; one of the most extensively used has been the reaction of RNA with formaldehyde (Haselkorn and Doty, 1961; Penniston and Doty, 1963; Marciello and Zubay, 1964). Since formaldehyde reacts with the primary amino group of adenine, guanine, and cytosine which is hydrogen bonded in the helical form, the rate and extent of reaction should distinguish hydrogen-bonded nucleotides from unbonded one. Unfortunately, quantitative estimates of the fraction of RNA nucleotides involved in helical regions based on their reactivity are complicated by the fact that it is necessary to use relatively low concentrations of formaldehyde to prevent it from acting as a denaturing agent (Haselkorn and Doty, 1961), and under these conditions the reaction is incomplete, even with mononucleotides (Grossman *et al.*, 1961). Moreover, the assumption that RNA which had reacted with formaldehyde would be wholly disordered was challenged when Fasman showed that poly C, at neutral pH, had a high optical rotation both before and after reaction with formaldehyde (Fasman *et al.*, 1964). The observed rotation is believed to result from short single-stranded helices formed as a result of base stacking similar to that found in dinucleotides (Warshaw and Tinoco, 1966). If an appreciable fraction of the hypochromicity and optical rotation previously attributed to double-stranded regions of RNA are actually caused by single-stranded stacked bases, all estimates of the fraction of hydrogen-bonded bases in RNA, based on these measurements would be seriously in error.[1] Optical rotatory dispersion (ORD) studies of yeast tRNA before and after reaction with formaldehyde suggested that a large fraction of the observed rotation might result from single-stranded stacked bases (Fasman *et al.*, 1965). This result is in apparent contradiction to earlier optical absorbance studies of the formaldehyde reaction with RNA in which the product exhibited very little hypochromicity (Hall and Doty, 1959); (Haselkorn and Doty, 1961). It seemed of interest, therefore to study the relative absorbance–temperature profile of RNA before and after reaction with formaldehyde and find out if the results of hypochromicity studies would confirm

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[1] In the first estimate of the helical content of RNA, it was assumed that single-stranded bases contributed less than 0.05 of the observed hypochromicity (Doty *et al.*, 1959).

or contradict the results obtained from ORD studies.

Although the extent of reaction of formaldehyde has been studied in detail for mononucleotides at room temperature (Grossman *et al.*, 1961) and for AMP[2] as high as 45° (Stevens and Rosenfeld, 1966) there is little known about the extent of reaction of the bases at higher temperatures. Moreover very little data is available on the extent of reaction of RNA with formaldehyde as a function of temperature and HCHO concentration. To study the optical properties of single-stranded RNA properly, most of the bases should have reacted and remain reacted during the experiment. The following report will present some criterion that should be fulfilled to meet these requirements. In addition we will show that the optical absorbance–temperature studies of RNA before and after reaction with formaldehyde are consistent with single-stranded base stacking that results in relatively little hypochromicity.

Material and Methods

RNA was isolated from TMV (Boedtker, 1960), R17 bacteriophage (Gesteland and Boedtker, 1964), and yeast (Penniston and Doty, 1963) by methods described previously. *Escherichia coli* tRNA was purchased from General Biochemicals, Laboratory Park, Chagrin Falls, Ohio. It was further purified by chromatographing on DEAE-cellulose. The mononucleotides (AMP, CMP, and GMP) were purchased from Sigma Chemical Co., St. Louis, Mo. Formaldehyde (37% reagent grade) was obtained from the Mallinckrodt Chemical Co. and used without purification. Uniformly labeled [^{14}C]-formaldehyde (sp act. 14 mc/ml) was obtained from New England Nuclear Corp., Boston, Mass.

In all but a few experiments, the reaction was carried out in either 0.1 M Na_2HPO_4 (pH 8.5) or in 0.09 M Na_2HPO_4 and 0.01 M NaH_2PO_4 (pH 7.5) (SPB). For comparison with results of earlier work, some reactions were carried out in 0.01 M triethanolamine–hydrochloric acid buffer (pH 7.6) (TEA) and in 0.01 M TEA and 0.001 M $MgCl_2$.

RNA concentrations (before reaction with formaldehyde) were determined from measurements of the absorbance at 260 mμ, using a value of 22.5 mg/ml for the specific absorbance of TMV and R17 RNA[3] and 21.5 mg/ml for that of *E. coli* and yeast tRNA (Lindahl *et al.*, 1965). Measurements were made in phosphate buffers in which the sodium concentration was approximately 0.2 M (see above).

The extent of reaction of RNA with formaldehyde was determined by measuring the moles of [^{14}C]formaldehyde bound per mole of RNA nucleotide. Aliquots (0.1–0.2 ml) of reacted RNA (~1 mg/ml) were precipitated with 5 ml of 5% cold trichloracetic acid. The precipitates were collected and washed with cold trichloracetic acid on Millipore filters (type HA). After drying at room temperature, the precipitates were counted in a Packard Tri-Carb counter, in toluene scintillation fluid. Solutions from which excess formaldehyde had been removed by gel filtration (see below) were counted by adding 0.1 ml of RNA to Bray's solution. Corrections were made for the different efficiency in the two scintillation fluids.

Absorbance measurements were made with a Beckman Model DU spectrophotometer equipped with a thermostated cell housing. Spectra of mononucleotides and RNA were measured at 15 and 85° by reading the absorbance at 5-mμ intervals from 220 to 310 mμ. The high temperature results were corrected for the dilution due to expansion of water. Absorbance–temperature profiles were obtained for at least two wavelengths by raising the temperature about 5° at a time and waiting 15 min before measuring the absorbance at a given wavelength. Because the experiments took 3 to 4 hr, the cells containing both solution and solvent were weighed before and after each experiment to check on loss due to evaporation. Using stoppered fused quartz cells, less than 0.3% loss was observed in any experiment. In measurements above room temperature, the concentration of formaldehyde in the blank was always equal to that in the solution. This is necessary because above 50° the hydrated form is converted to the free aldehyde which has a broad absorption band with a maximum at 295 mμ.

Sephadex columns (1 × 25 cm) were prepared by allowing 20 g of Sephadex G50 (Pharmacia, Uppsala, Sweden) to swell in 100 ml of buffer (0.05 M Na_2HPO_4 and 0.05 M NaH_2PO_4, pH 6.8) overnight. It was then slurried with five volumes of buffer five times, and the column was packed by allowing the gel to settle without added pressure.

Results

The Extent of Reaction with RNA as a Function of Temperature and Formaldehyde Concentration. As first observed by Fraenkel-Conrat (1954), when formaldehyde reacts with RNA the absorbance increases and the maximum shifts to a longer wavelength. An example of such a change is shown in Figure 1A. If one compares the spectrum of RNA treated with formaldehyde (curve III) with the spectrum of RNA at high temperature (curve II) and at low temperature (curve I), it is obvious that formaldehyde addition has two separate effects on the RNA absorbance; one is the result of the denaturation of the secondary structure; the second is the result of a change in the chromophoric group due to formaldehyde addition. Several years ago, Haselkorn pointed out that is was possible to resolve the relative contributions of these two effects (Haselkorn and Doty, 1961). By subtracting the spectrum of the high-temperature or coil form (curve II) from that obtained after reaction with formaldehyde (curve III), the contribution due to the reaction can be obtained; by subtracting the original spectrum (curve I) from that obtained at high

[2] Abbreviations used: AMP, adenosine monophosphate; CMP, cytosine monophosphate; GMP, guanosine monophosphate; SPB, standard phosphate buffer, 0.09 M Na_2HPO_4 plus 0.01 M NaH_2PO_4 (pH 7.5); TEA, triethanolamine hydrochloric acid buffer (pH 7.6).

[3] N. S. Simmons, personal communication.

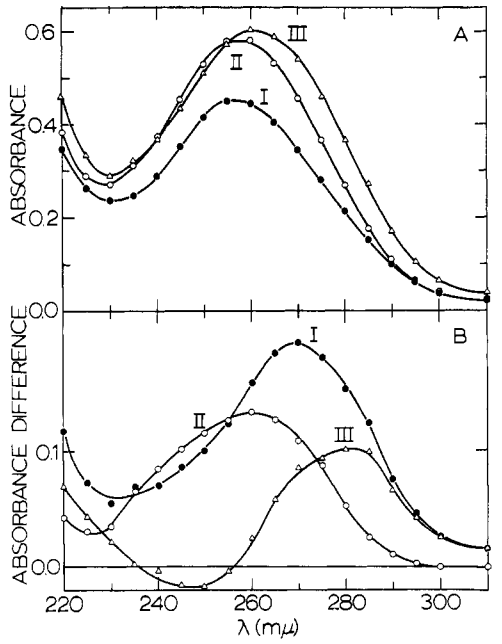

FIGURE 1: Absorption and spectra of TMV-RNA. (A) Curve I: (●) control at 15°; curve II: (○) control at 85°; and curve III: (△) after reaction with 1.2 M HCHO for 15 min at 85°, measured at 15°. All solutions in $^1/_{100}$ SPB. (B) Curve I (III − I): (●) absorbance of RNA treated with HCHO minus absorbance of control, at 15°; curve II (II − I): (○) absorbance of RNA at 85° minus absorbance of control at 15°; and curve III (III − II): (△) absorbance of RNA treated with HCHO (measured at 15°) minus absorbance of RNA at 85°.

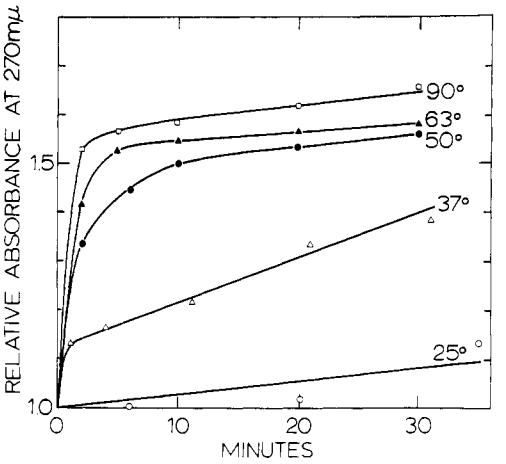

FIGURE 2: Effect of temperature on the extent of reaction of 1.1 M HCHO with *E. coli* tRNA (36 μg/ml). Reaction at 90 (□), 63 (▲), 50 (●), 37 (△), and 25 (○). Absorbance measured at 25° in 0.1 M Na_2HPO_4 (pH 8.5).

temperature (curve II), the contribution due to denaturation is obtained. Such a "difference spectrum" was obtained for TMV-RNA and the result is shown in Figure 1B. It is clear that the maximum absorbance change at 270 mμ is the result of almost equal contributions of denaturation and reaction. Although it is possible in principle to follow the two aspects of formaldehyde addition separately by appropriate choice of wavelength, the absorption at 295 mμ, which reflects only formaldehyde reaction, is too small to permit accurate determination of the extent of reaction. For this reason the extent of reaction was followed at 270 mμ where the maximum total change in absorbance occurs.

The effect of temperature on the extent of reaction of *E. coli* tRNA with 1.1 M formaldehyde is shown in Figure 2. RNA solutions were incubated at the indicated temperature for various times, fast cooled in crushed ice, and the absorbance was measured at 25°. Although there was a small increase in absorbance as a function of time at 90°, this probably reflects the increase in the effective formaldehyde concentration resulting from the conversion of the dihydrate to the free aldehyde. As it was planned to study the absorbance of reacted mononucleotides and RNA at 85°, it was obviously desirable to avoid slow changes in absorbance due to additional reaction as the effective formaldehyde concentration increases at high temperature. For this reason solutions were allowed to react for 15 min at 85° in most of the subsequent experiments. Nevertheless the reaction is nearly complete after 10 min at 63° and these are the preferred conditions whenever the maintenance of an intact polynucleotide is essential.

The double-helical regions of most RNA molecules are unstable above 60°. Therefore it is possible to study the effect of formaldehyde concentration on the extent of reaction of "denatured" RNA. RNA was treated with various concentrations of formaldehyde at 63°,

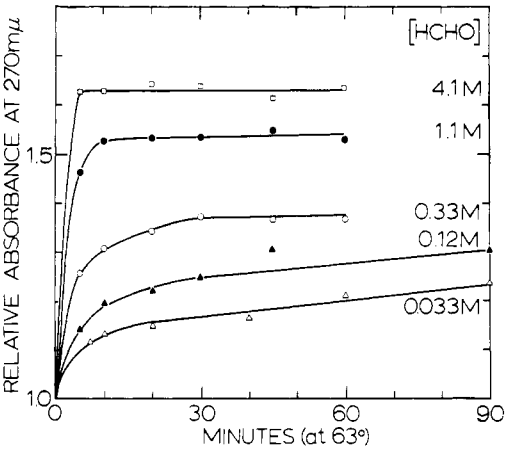

FIGURE 3: Effect of HCHO concentration on the extent of reaction with yeast tRNA (30 μg/ml). Reaction at 63° in 4.1 (□), 1.1 (●), 0.33 (○), 0.12 (▲), and 0.033 M HCHO (△). Absorbance measured at 25° in 0.1 M Na_2HPO_4 (pH 8.5).

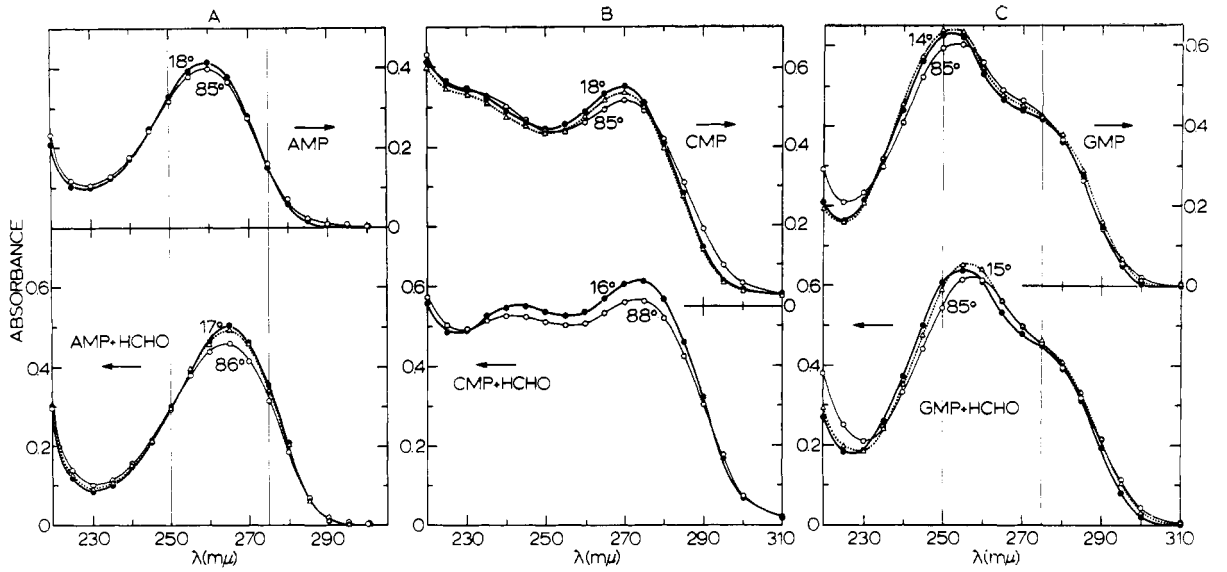

FIGURE 4: Absorption spectra of mononucleotides at low and high temperatures before and after reaction with 1.2 M HCHO for 15 min at 85° in SPB. (●) Original spectrum at low temperatures; (○) spectrum at 85–88°; and (△) spectrum at low temperatures after heating to 85–88°. (A) AMP, (B) CMP, and (C) GMP.

aliquots were removed at regular intervals, fast cooled, and their absorbance was measured at 25°. The results are shown in Figure 3. It is clear that the extent of reaction, as measured by the absorbance changes, varies significantly with formaldehyde concentration. This result was predicted from the equilibrium constants of the reacted mononucleotides measured by Grossman *et al.* (1961). The average equilibrium constant weighted by the base composition of yeast tRNA is 11.0. Using this value, one can compare the predicted with the observed extent of reaction as a function of formaldehyde concentration. The results, shown in Table I, were calculated by assuming the reaction is complete in 12 M formaldehyde. For comparison, the fraction of amino groups reacted with [^{14}C]formaldehyde was measured at three formaldehyde concentrations. Although the latter results agree nicely with those predicted from the nucleotide equilibrium constants, the agreement with the results ob-

TABLE I: Effect of Formaldehyde Concentration on the Extent of Reaction with Yeast tRNA.[a]

Formaldehyde Concn (moles/l.)	Fraction of Amino Groups Reacted after 30 min at 63°		
	Predicted ("K" = 11.0)	Observed	
		270 mμ	^{14}C[b]
4.1	0.98	1.00	
2.1	0.96	0.96	
1.1	0.92	0.84	0.87
0.33	0.78	0.58	0.75
0.12	0.57	0.39	0.52

[a] RNA concentration was 28–32 μg/ml in absorbance studies and 2 mg/ml in ^{14}C measurement. [b] Calculated assuming 0.77 mole of amino groups/mole of yeast tRNA.

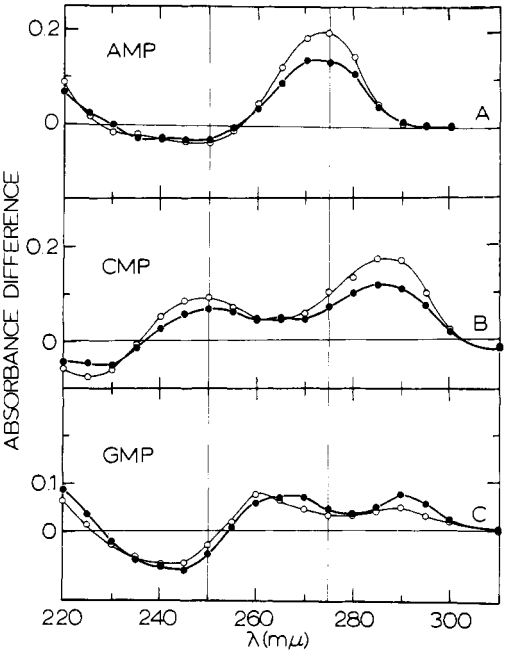

FIGURE 5: Difference spectra of mononucleotides treated with 1.2 M HCHO. Absorbance of nucleotide treated with HCHO minus absorbance of nucleotide: (○) measured at 15°; and (●) measured at 85°.

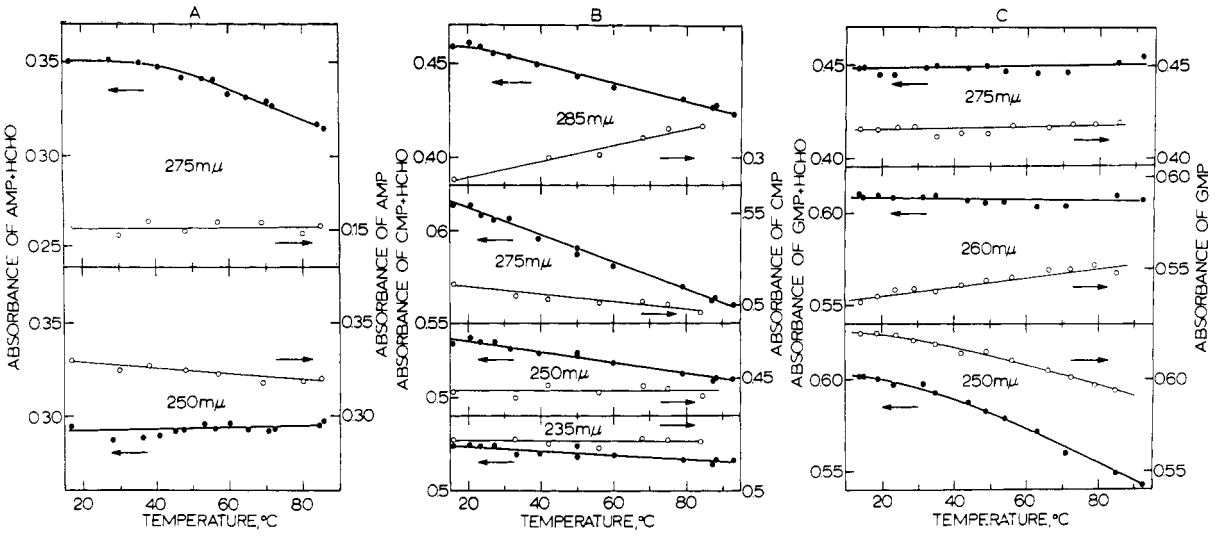

FIGURE 6: Absorbance as a function of temperature of mononucleotides before and after reaction with 1.2 M HCHO for 15 min at 85° in SPB. (●) Nucleotide and HCHO; and (○) nucleotide. (A) AMP, (B) CMP, and (C) GMP.

tained from absorbance measurements is not very good. Since the discrepancy increases with decreasing extent of reaction, it is possible that it reflects a renaturation of the partially reacted RNA after cooling to 25°. Thus the extent of reaction appears too low because the absorbance change is smaller than expected due to a decrease in absorbance that accompanies reformation of double-stranded regions of RNA. It is important to note that the discrepancy becomes significant when the formaldehyde concentration falls below 1 M (3%). Hence it is advisable to use this concentration to prevent partial reformation of helical regions upon cooling.

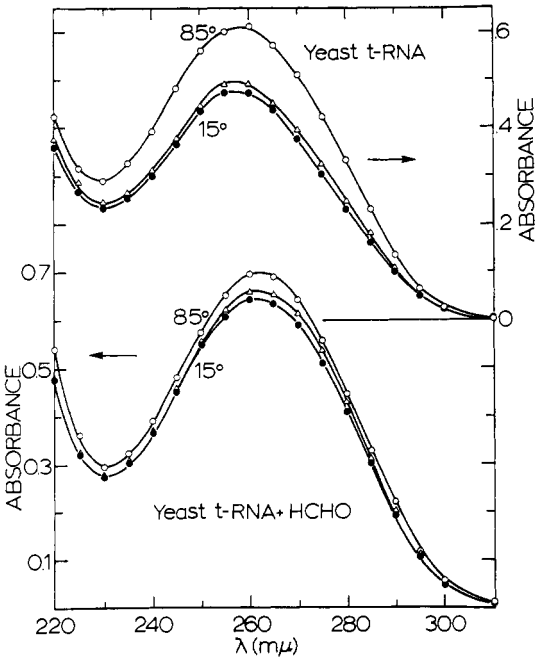

FIGURE 7: Absorption spectra of yeast tRNA before and after reaction with 1.2 M HCHO for 15 min at 85° in SPB. (●) Original spectrum at 15°; (○) spectrum at 85°; and (Δ) spectrum at 15° after heating to 85°.

Absorbance–Temperature Studies of AMP, CMP, and GMP. Prior to measuring the absorbance–temperature profiles of RNA treated with formaldehyde, it was decided to study the effect of temperature on mononucleotides. The spectra of both the unreacted and reacted nucleotides were measured at about 15 and 85°. The results are shown in Figure 4. Although there is a decrease in absorption at the maximum in all cases, the change is less for the unreacted bases than for the bases treated with formaldehyde. The former can be interpreted in terms of a slight broadening of the spectrum in the case of AMP and CMP, and as a shift to longer wavelengths in the case of GMP. Since GMP treated with formaldehyde shows a similar red shift, this effect is probably not due to formaldehyde addition. Although its origin has not been determined, it may simply reflect the fact that both GMP and its formaldehyde derivative are aggregated at low temperatures. The decrease in absorption and blue shift observed for AMP and CMP which have reacted with formaldehyde can only be explained as a decreased extent of reaction at the high temperature. This can be seen more clearly in the difference spectra shown in Figure 5. The changes are largely reversible on cooling as shown by the dashed lines in Figure 4.

The difference spectra of the mononucleotides (Figure 5) enable one to determine the wavelength at which the effect of the reaction with formaldehyde is maximal and the one at which it is minimal. Unfortunately there is no wavelength common to all three nucleotides which reflects either effect. Consequently absorbance–temperature measurements were made at several wavelengths so

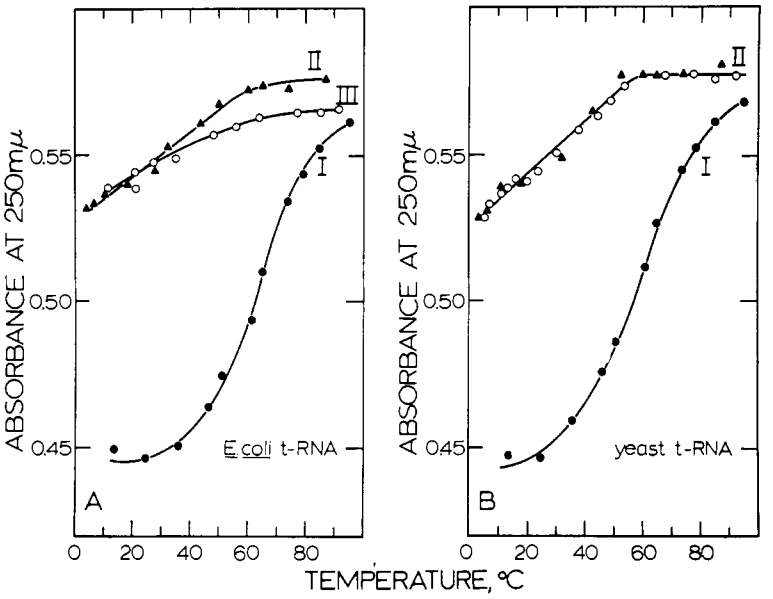

FIGURE 8: Absorbance as a function of temperature of tRNA before and after reaction with 1.2 M HCHO for 15 min at 85° in SPB. (A) *E. coli* tRNA at 250 mμ in SPB. Curve I: (●) control; curve II: (▲) RNA treated with HCHO; and curve III: (○) RNA treated with HCHO, heated a second time. (B) Yeast tRNA at 250 mμ in SPB. Curve I: (●) control; and curve II: (▲, ○) RNA treated with HCHO, two independent experiments.

that one could be found at which the absorbance would be independent of temperature for the three nucleotides. The observed temperature dependence of the absorbance of AMP, CMP, and GMP and of their formaldehyde derivatives is given in Figure 6. These results clearly show that the extent of reaction of AMP and CMP with formaldehyde decreases as the temperature is raised. This can best be seen in the absorbance–temperature curves of the formaldehyde derivative of AMP at 275 mμ (Figure 6A) and of CMP at 285 mμ (Figure 6B). The GMP derivative is evidently more stable because there is no change in absorbance at 260 mμ (Figure 6C). The decrease in absorbance of both GMP and its formaldehyde derivative observed at 250 mμ is not the result of the formaldehyde reaction but of the red shift mentioned above.

The results of absorbance studies of mononucleotides indicate that measurements at 250 mμ are only slightly sensitive to the extent of reaction with formaldehyde and hence changes observed at this wavelength should provide a good index of changes in single-stranded base stacking in RNA. Additional support of this choice of wavelength can be seen in Figure 1 which shows the spectra of TMV–RNA at both 85 and 15° before and after formaldehyde addition. It is clear that the spectral changes observed between 235 and 255 mμ are the result of denaturation and not of formaldehyde addition.

Absorbance–Temperature Studies of RNA. The effect of formaldehyde addition on the spectrum of RNA was illustrated in Figure 1. We now want to study the effect of temperature on the spectra of formaldehyde derivatives of RNA. A typical result is shown in Figure 7. The upper part shows the spectra of unreacted yeast tRNA at 15 and 85°, and the lower half shows that of the same RNA previously treated with 1.2 M formaldehyde, fast cooled, and stored at 4° before measuring the spectra at the indicated temperature. The increase in absorbance on heating observed in both cases is evidence that RNA has some ordered structure at low temperatures both before and after formaldehyde addition. Unreacted RNA is clearly more hypochromic than its formaldehyde derivative, however.

After cooling to 15°, the original spectra were largely restored in both cases. Differences between the spectra obtained before and after heating were less than 4%. However, in the case of RNA treated with formaldehyde, the absorbance differences were greater at 270 mμ and indicated a slight increase in the extent of formaldehyde addition. The increase in reaction is probably the result of the previously mentioned increase in the effective formaldehyde concentration as the hydrated form is converted to the free aldehyde at high temperatures.

Absorbance–temperature studies were carried out at 250 mμ on RNA before and after reaction with formaldehyde. Results obtained with *E. coli* and yeast tRNA are shown in Figure 8. Both tRNAs have the typical sigmoid melting curve before reaction; their formaldehyde derivatives exhibit linear dependence on temperature characteristic of noncooperative processes and of single-stranded stacked bases. The curves level off at about 60°, a result also observed for poly A after reaction with formaldehyde (Stevens and Rosenfeld, 1966).

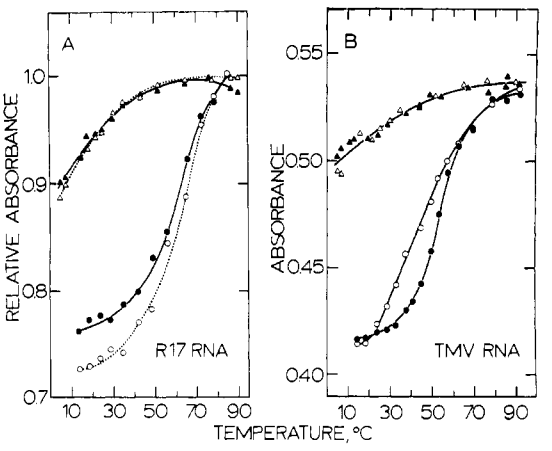

FIGURE 9: Absorbance as a function of temperature of viral RNA before and after reaction with 1.2 M HCHO for 15 min at 85°. (A) R17 RNA in SPB. Control at 250 mμ (●), at 275 mμ (○), RNA treated with HCHO at 250 mμ (▲), and at 275 mμ (△). (B) TMV–RNA at 250 mμ. Control in SPB (●), in $^1/_{100}$ SPB (○), RNA treated with HCHO in SPB (▲), and in $^1/_{100}$ SPB, (△).

This result would seem to indicate that stacked bases are not stable at higher temperatures after formaldehyde addition.

The reproducibility of absorbance–temperature profiles was examined by successive heating cycles on the same sample. The results obtained on *E. coli* tRNA are shown in Figure 8A. Although there is a difference in the absorbance at high temperature, it is only 2%. Normally this extent of agreement would be considered satisfactory. Unfortunately, the difference between curves II and III represents a 20% decrease in the amount of hypochromicity. The problems this creates are discussed later. It should be noted that whether the data from the second heating cycle (curve III) lies above or below the original data (curve II) depends on the wavelength studied and to some extent on the RNA sample. But a smaller change in absorbance with temperature upon reheating was observed in all cases except TMV–RNA. The latter, however, had the smallest initial hypochromicity of the four formaldehyde-treated RNAs studied. For this reason, and because of the change in spectrum observed after the first heating cycle for all but TMV–RNA (for example, see Figure 7), it is assumed that the initial larger hypochromicities results in part from an additional reaction with formaldehyde.

An estimate of the precision of absorbance–temperature measurements and hence of the significance of the very small changes described above, can be made from two different experiments carried out under identical conditions with the same RNA. Figure 8B shows the data obtained from two separate experiments with yeast tRNA. The heating curves of RNA after reaction with formaldehyde are superimposable. This agreement supports the suggestion that the relatively small changes described above are significant.

The effect of formaldehyde addition on the absorbance–temperature profiles of high molecular weight RNA are shown in Figure 9. The results are similar to those obtained for tRNA although the absorbance appears to level off at lower temperatures. It should be noted that the absorbance of unreacted RNA has only slightly increased at 40° while that of its formaldehyde derivative has almost completed the transition at this temperature.

Because the absorbance at 275 mμ is more sensitive to the extent of formaldehyde addition than that at 250 mμ, all RNA heating curves were measured at both wavelengths in the expectation that one might discriminate between the extent of denaturation and the extent of formaldehyde addition. Results obtained with R17 RNA are shown in Figure 9A. Neither the shape of the curve nor the amount of hypochromicity of the formaldehyde adduct appears to depend on wavelength. Apparently changes in the extent of reaction are too small to be detected in this way.

The stability of the double-helical regions of RNA is known to be quite dependent on counterion concentration while that of single-stranded helices is not. Figure 9B shows the effect of a 100-fold dilution of the counterion concentration on the absorbance–temperature profile of TMV–RNA and its formaldehyde derivative. The structure of reacted RNA is independent of counterion concentration, consistent with the hypothesis that this structure is the result of single-stranded stacked nucleotides.

The validity of comparing the stability of RNA conformation in aqueous medium with that in 1 M formaldehyde may well be questioned because formaldehyde is known to act a general denaturing agent lowering the melting points of helical polynucleotides with which it cannot react. To attempt to evaluate this, a more concentrated solution of TMV–RNA was allowed to react with 1.1 M formaldehyde for 30 min at 63°; it was then diluted sevenfold with buffer resulting in a final formaldehyde concentration of 0.16 M. No obvious difference in the character of the temperature dependence of the absorbance was observed when reacted TMV–RNA was measured in 0.16 M formaldehyde. Yet the melting temperature of helical poly I would be decreased some 50° by a comparable reduction in formaldehyde concentration (Haselkorn and Doty, 1961).

The absorbance results just presented can be summarized by stating that both the gradual change with temperature and the lack of ionic strength dependence are good evidence that the observed hypochromicity is due to single-stranded base stacking. Although the absorbance at 250 mμ should be independent of the extent of reaction, complete reversibility was not observed in the first heating cycle even at this temperature except for TMV–RNA. As subsequent heating cycles did not result in further changes, the hypochromicity was calculated from the second cycle and the results are given in Table II. The calculations were made from data obtained at 255 mμ rather than 250 mμ because the hy-

TABLE II: Hypochromicity[a] of RNA before and after Reaction with Formaldehyde.

RNA	Hypochromicity	
	Unreacted	After Reaction with 1.2 M Formaldehyde
E. coli tRNA	0.195	0.045
Yeast tRNA	0.21	0.06
R17 RNA	0.23	0.045
TMV RNA	0.21	0.04

[a] Hypochromicity $= \left[1 - \frac{\text{absorbance at } 15°}{\text{absorbance at } 85°}\right]_{255\ m\mu}$.

pochromicity of unreacted RNA is independent of the base composition of the double-helical regions at this wavelength (see Discussion).

Reversibility of Formaldehyde Addition to RNA. To determine whether or not the formaldehyde reaction can be readily reversed, an attempt was made to regenerate the original RNA. Formaldehyde was removed either by dialyzing against 1000 volumes of Tris buffer (pH 7.6) for 24 hr at 4°, or by passing over a Sephadex G-50 column. Although excess formaldehyde was removed by this procedure, more than four-fifths of the bases which had reacted with formaldehyde remained reacted. This was shown quantitatively by using ^{14}C-labeled formaldehyde and the results are summarized in Table III. It is clear that the formaldehyde adduct was equally stable regardless of whether it had been formed at high or low temperature, or in the presence or absence of Mg^{2+}.

TABLE III: Recovery of Formaldehyde Derivative of Yeast tRNA after Gel Filtration.

Reaction Conditions	Cpm/OD Unit		% Recov
	Before	After	
Formaldehyde (0.1 M), 30 min, 63°, Na_2HPO_4 (0.1 M)	12.7	10.2	80
Formaldehyde (0.1 M), 72 hr, 25°, TEA (0.01 M)	94	73.5	78
Formaldehyde (0.1 M), 72 hr, 25°, TEA (0.01 M) + $MgCl_2$ (0.001 M)	33 24	27 20	82 83

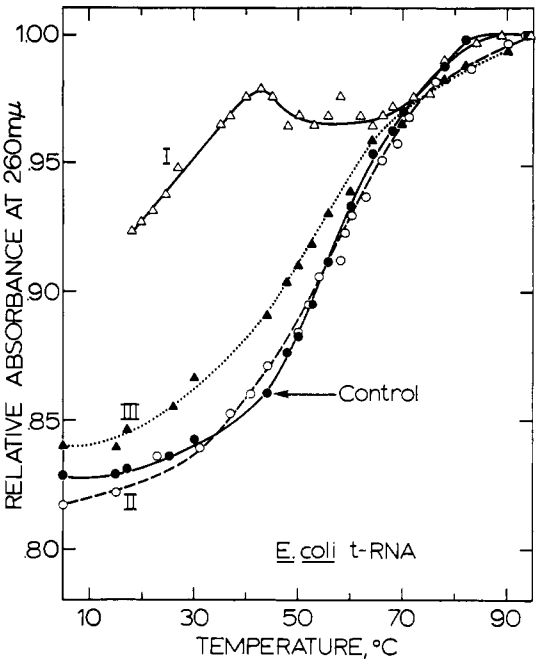

FIGURE 10: Relative absorbance as a function of temperature of *E. coli* tRNA after reaction with 1.1 M HCHO and passage through Sephadex G-50 column. Absorbance measured in 0.1 M Na_2HPO_4; (●) control. Curve I: (△) first heating cycle; curve II: (○) second heating cycle; and curve III: (▲) preheated to 85° for 10 min prior to first heating cycle.

The formaldehyde adduct can only be removed and the bihelical regions of RNA regenerated only after rather extensive heating. The absorbance–temperature profile of a sample of *E. coli* tRNA which was treated with 1.2 M formaldehyde and then passed through a Sephadex G-50 column to remove excess formaldehyde is shown in Figure 10. The first heating cycle (curve I) looks remarkably like that of reacted RNA measured in 1 M formaldehyde. After cooling, however, the absorbance–temperature profile obtained in the second heating cycle (curve II) is almost that of the control.

A briefer exposure of reacted and "Sephadexed" RNA to high temperature (10 min at 80°) resulted in restoring most but not all of the original hypochromicity. The double-stranded helical segments of RNA, reflected in its appreciable hypochromicity, are apparently prevented from fully reforming when only a small fraction of the bases that normally form hydrogen bonds are prevented from doing so.

Discussion

The optical properties of RNA, in particular ORD and hypochromicity, are direct indications of ordered regions of the polyribonucleotide chain. If there were

but one type of ordered conformation present in natural RNA, the magnitude of an optical effect could readily be related to the amount of ordered structure. Such calculations have been made in the past (Doty *et al.*, 1959; Haselkorn and Doty, 1961; Fresco, 1963) and would suggest that approximately two-thirds of the bases in RNA is in double-stranded helical segments. These calculations made the assumption that the remaining third of the bases made no contribution to the optical properties. However results of numerous studies of dinucleotides and polynucleotides (Van Holde *et al.*, 1965; Warshaw and Tinoco, 1966; Cantor *et al.*, 1966; Fasman *et al.*, 1964; Stevens and Rosenfeld, 1966) demonstrate that single-stranded sequences make a significant contribution to these optical properties. By studying an RNA in which only single-stranded helices can form, such as RNA which has been treated with formaldehyde, it should be possible to obtain an estimate of the *maximum* contribution of a single-stranded ordered structure to the optical properties.

The results just presented demonstrate that at least two-thirds of the hypochromic effect observed in RNA is lost when hydrogen bonding is blocked as a result of formaldehyde addition. By maximizing the extent of reaction and making measurements at the wavelength most sensitive to ordered structures, we observed a hypochromicity of 0.04–0.06 (as compared to 0.19–0.23 for unreacted RNA). It should be noted that this reduced value constitutes the contribution of all the bases including those normally in hydrogen-bonded double-stranded regions of RNA, and hence the hypochromicity which can actually be attributed to single-stranded base stacking in a mixture of single- and double-stranded regions must be considerably less.

The apparent discrepancy between the results presented here and those obtained from ORD studies of tRNA (Fasman *et al.*, 1965; Kay and Oikawa, 1966) requires some explanation. The latter report that RNA treated with formaldehyde has rotations about two-thirds the magnitude of those of unreacted RNA. Even though the magnitude of the rotation cannot properly be compared because the chromophore has been altered, the existence of a large rotation can only occur if the mean orientation of adjacent bases is favorable. The ORD results have been confirmed and a similar discrepancy between ORD and hypochromicity has been observed for alkaline RNA (pH 12.2) (Gratzer, 1966). In addition there are several examples of differences between ORD and hypochromicity in the case of dinucleotides (Warshaw and Tinoco, 1966) and polynucleotides (Ts'o *et al.*, 1966). Hence the existence of structures characterized by high optical rotation and low hypochromicity cannot be disputed. Apparently RNA which has reacted with formaldehyde is an example of such a structure.

One might question if the formaldehyde derivative of RNA is a proper model for single-stranded RNA. It is possible that the geometry of the stacked bases is altered after formaldehyde addition and hence the low hypochromicity observed for RNA treated with formaldehyde is not characteristic of stacked bases in unreacted RNA. Results obtained with poly A, poly C, and dinucleotides suggest that this is unlikely. After reaction with formaldehyde both poly A and poly C have hypochromicities and rotations almost identical with those obtained for the unreacted polynucleotide (Stevens and Rosenfeld, 1966; Fasman *et al.*, 1964). Hence formaldehyde addition *per se* does not change a single-stranded helix from a hypochromic to a nonhypochromic one. Moreover, the small hypochromicity observed for RNA reacted with formaldehyde is comparable to the average value obtained for dinucleotides (Warshaw and Tinoco, 1966).

If the hypochromicity of RNA after reaction with formaldehyde can be assumed to be a reasonably reliable estimate of the contribution of single-stranded base stacking in RNA, then the results reported here can be used to calculate the double-helical content of RNA. The total hypochromicity (h_T) observed at any wavelength must be a sum of the contribution of single- and double-stranded helices. Thus we can write $h_T = (1 - x)h_{ss} + xh_{ds}$, where h_{ss} and h_{ds} are the hypochromicities of completely single- and completely double-stranded RNA, and x is the fraction of nucleotides in double-stranded helical regions. Although h_T and h_{ss} are determined experimentally from high and low-temperature spectra h_{ds} must be estimated from data on helical complexes of poly A + poly U, and poly G + poly C. In general, the contribution of the AU and GC base pairs is very wavelength dependent, and hence h_{ds} will depend both on the wavelength and on the base composition (Fresco, 1963). However at both 255 and 270 mμ, the base pairs contribute equally and hence h_{ds} will be independent of the base composition of a particular RNA at these wavelengths.

The hypochromicity displayed by the base pairs at 255 mμ is about 85% of the value each has at its maximum, or about 0.30. This is similar to the hypochromicity of native DNA and only slightly larger than that reported for double-stranded RNA (Weissmann and Ochoa, 1967). Using 0.3 for h_{ds}, the double-helical content of RNA was calculated and the results are shown in Table IV. Correcting for single-stranded base stacking reduces the estimated helical content 10% for tRNAs, and about 5% for viral RNAs. Unfortunately these results are very sensitive to small errors in absorbance measurements and to the value of the hypochromicity of 100% double-stranded RNA. In the case of *E. coli* tRNA, a 2% error in the absorbance at 85° could change the hypochromicity due to single-stranded helices from 0.045 to 0.060 and result in a decrease in the double-helical content of 5%. Or, if the value of the hypochromicity of double-stranded RNA should have been 10% lower than that used in these calculations, a reduction that might occur when helical regions become very short, the estimated helical content would be increased by 14%. Hence the helical contents reported here should be considered a *minimum* estimate. Although it is clearly not possible to report a precise value of the helical content from the data now available, it has been possible to determine the minimum value to within about 5% and to show that of the contribution of single-stranded

TABLE IV: Double-Helical Content of RNA.

RNA	Fraction of Bases in Double-Stranded Helices[a]		
	Cor for Effect of Single-Stranded Helices	Uncor	% Change
E. coli tRNA	0.59	0.65	10
Yeast tRNA	0.63	0.70	11
R17 RNA	0.73	0.77	5
TMV–RNA	0.66	0.70	6

[a] Calculated from absorbance measurements at 15° in 0.09 M Na_2HPO_4 and 0.01 M NaH_2PO_4 (pH 7.5) and assuming that the hypochromicity of 100% helical RNA is 0.3 at 255 mμ.

base stacking to the hypochromicity of RNA will not decrease the estimate of the fraction of bases in hydrogen-bonded double-helical regions by more than 10%.

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