Hartmann, G., and Coy, U. (1961), *Biochim. Biophys. Acta 47*, 612.
Jacobson, K. B., and Nishimura, S. (1964), *J. Chromatog. 14*, 46.
Kelmers, A. D. (1966a), *J. Biol. Chem. 241*, 3540.
Kelmers, A. D. (1966b), *Biochem. Biophys. Res. Commun. 25*, 562.
Kelmers, A. D., Novelli, G. D., and Stulberg, M. P. (1965), *J. Biol. Chem. 240*, 3979.
Khym, J. X. (1966), *J. Biol. Chem. 241*, 4529.
Kirby, K. S. (1960), *Biochim. Biophys. Acta 41*, 338.
Kirby, K. S. (1962), *Biochim. Biophys. Acta 61*, 506.
Lindahl, T., Adams, A., and Fresco, J. R. (1966), *Proc. Natl. Acad. Sci. U. S. 55*, 941.
Mehler, A. H., and Bank, A. (1963), *J. Biol. Chem. 238*, PC2888.
Millar, D. B., and Steiner, R. F. (1966), *Biochemistry 5*, 2289.
Muench, K. H., and Berg, P. (1966a), *Biochemistry 5*, 970.
Muench, K. H., and Berg, P. (1966b), *Biochemistry 5*, 982.
Nathenson, S. G., Dohan, F. C., Richards, H. H., and Cantoni, G. L. (1965), *Biochemistry 4*, 2412.
Ofengand, E. J., Dieckmann, M., and Berg, P. (1961), *J. Biol. Chem. 236*, 1741.
Okomoto, T., and Kawade, Y. (1963), *Biochem. Biophys. Res. Commun. 4*, 324.
Pearson, R. L., and Kelmers, A. D. (1966), *J. Biol. Chem. 241*, 767.
Portatius, H. von, Doty, P., and Stephenson, M. L. (1961), *J. Am. Chem. Soc. 83*, 3351.
Rubin, I. B., Kelmers, A. D., and Goldstein, G. (1967), *Anal. Biochem.* (in press).
Simon, S., Littauer, U. Z., and Katchalski, E. (1964), *Biochim. Biophys. Acta 80*, 169.
Smith, D. W. E. (1966), *J. Biol. Chem. 241*, 4487.
Stephenson, M. L., and Zamecnik, P. G. (1961), *Proc. Natl. Acad. Sci. U. S. 47*, 1627.
Sueoka, N., and Yamane, T. (1962), *Proc. Natl. Acad. Sci. U. S. 48*, 1454.
Tada, M., Schweiger, M., and Zachau, H. G. (1962), *Z. Physiol. Chem. 328*, 85.
Tanaka, K., Richards, H. H., and Cantoni, G. L. (1962), *Biochim. Biophys. Acta 61*, 846.
Wiesmeyer, H., Kjellen, K., and Bowman, H. G. (1962), *Biochim. Biophys. Acta 61*, 625.
Zachau, H. G. (1965), *Z. Physiol. Chem. 342*, 98.
Zamecnik, P. G., Stephenson, M. L., and Scott, J. E. (1960), *Proc. Natl. Acad. Sci. U. S. 46*, 811.
Zubay, G. (1962), *J. Mol. Biol. 4*, 347.

Titration Properties of Some Dinucleotides*

H. Simpkins and E. G. Richards

ABSTRACT: The absorptivity changes produced by titrating all four dinucleotides containing adenine and uracil derivatives (ApA, ApU, UpA, and UpU) with acid and with alkali suggest that the ionized bases are unstacked. Using this result the titration curves are analyzed and the stacking association constants at pH 7 at 20° are calculated. The stacking association constants increase with the magnitude of the thermal hypochromicities in the order UpU, UpA, ApU, and ApA. The standard free energy of stacking of ApA at 20° is calculated to be −0.99 kcal/mole, a value which is similar to that obtained from the dependence of optical properties on temperature as reported in the literature.

Stacking interactions between adjacent bases in poly- and oligonucleotide chains have been demonstrated for poly- and oligoadenylic acids (Leng and Felsenfeld, 1966; Brahms *et al.*, 1966; Poland *et al.*, 1966), cytidylic acids (Brahms *et al.*, 1967a), and a variety of dinucleotides (Brahms *et al.*, 1967b). All the evidence supports the hypothesis that coooperative effects are minimal in that the interactions between adjacent bases are independent of one another; thus the thermodynamic parameters describing the stacking interactions in dinucleotides are nearly the same as those for a polymer.

A knowledge of the enthalpy and entropy of stacking for all 16 dinucleotides would thus be of considerable interest in the interpretation of the melting curves of RNA.

Several attempts have been made to determine these parameters for several of the dinucleotides (Leng and Felsenfeld, 1966; Brahms *et al.*, 1966, 1967a,b). These attempts have been based on measurements

* From the Department of Biophysics and Medical Research Council Biophysics Research Unit, King's College, London W.C.2., England. *Received March 27, 1967.*

of the changes in optical properties (absorbance, optical rotation, and circular dichroism) which occur as a result of temperature changes.

An alternative approach is through a consideration of the titration properties of the dinucleotides and the basic model for such an analysis has been presented by Cox (1966). In the present work we have elaborated this approach and applied it to the titration of all four 3′–5′ dinucleotides containing adenine and uracil residues.

Although polyadenylic acid forms a double helix in acid solution (Fresco and Doty, 1957), diadenylic does not do so above 20° (Brahms *et al.*, 1966; Applequist and Damle, 1965). Thus the present results are not complicated by such effects.

Experimental Section

Materials

Adenylyl-(3′–5′)-adenosine (ApA), adenylyl-(3′–5′)-uridine (ApU), uridylyl-(3′–5′)-adenosine (UpA), and uridylyl-(3′–5′)-uridine (UpU) were obtained from Calbiochem Inc. They were checked for purity by high-voltage paper electrophoresis according to Heppel *et al.* (1956, 1957) and found to run as single spots with mobility ratios as given by these authors.

Methods

Spectrophotometric titrations were carried out as previously described (Simpkins and Richards, 1967) using 0.1 N H_2SO_4 or 0.1 N NaOH. Potentiometric titrations were performed under an atmosphere of washed nitrogen in lucite vessels; the pH was measured using a Jena U combined calomel and glass electrode in conjunction with an E.I.L. Vibron pH meter. Proton binding was calculated from the pH after the addition of known increments of 0.15 N H_2SO_4 and 1 N H_2SO_4 from a microburet, after due allowance for an appropriate blank.

All titrations were carried out in the presence of 0.1 M NaCl at 20°.

Thermal difference spectra were calculated from the difference in absorbance of solutions of identical concentration as measured in thermostated cuvets at 20 and 92° in a Zeiss PMQ II spectrophotometer. They are expressed as mean residue millimolar absorptivity differences ($\Delta\epsilon_t$) by dividing the absorbance difference by the total millimolar base concentration.

Ionization difference spectra were measured as previously described (Simpkins and Richards, 1967). Acid difference spectra are expressed as mean millimolar adenine absorptivity differences ($\epsilon_{pH\ 1} - \epsilon_{pH\ 7}$) by dividing the absorbance difference by the millimolar adenine concentration. Similarly alkaline difference spectra were expressed as mean millimolar uracil absorptivity differences ($\epsilon_{pH\ 12} - \epsilon_{pH\ 7}$).

Concentrations were determined from absorbance measurements at 260 mμ using the molar residue absorptivities given by Warshaw (1966) for ApA, ApU, UpA, and UpU.

Theoretical

It is convenient to distinguish between homodinucleotides such as ApA and UpU in which both base residues are the same and heterodinucleotides such as ApU and UpA in which they are different.

It has been postulated that in neutral solution a dinucleotide can exist in one of two forms (stacked and unstacked) (Brahms *et al.*, 1966; Leng and Felsenfeld, 1966; Poland *et al.*, 1966) and that these two forms are in thermodynamic equilibrium with one another.

In acid the adenine residues of ApA, ApU, and UpA accept protons and become positively charged; similarly in alkaline solution the uracil residues of UpU, UpA, and ApU donate protons and acquire a negative charge. Thus in the case of the homodinucleotides at intermediate pH there are, in general, six species in thermodynamic equilibrium with one another and with protons. These correspond to the un-ionized, singly ionized, and doubly ionized forms, both stacked and unstacked; similarly with the heterodinucleotides there are four such species.

The mean residue absorptivities of all six species in equilibrium are, in general, different so that changes in the pH or temperature leading to changes in the degree of stacking or ionization result in changes in the absorbance of a solution of a dinucleotide.

Consider the mean residue absorptivities, ϵ, of a dinucleotide in the four forms: stacked (s), unstacked (u), un-ionized (n), and completely ionized (i). Then identically

$$(\epsilon_{u,i} - \epsilon_{s,n}) - (\epsilon_{u,n} - \epsilon_{s,n}) = (\epsilon_{u,i} - \epsilon_{u,n}) \qquad (1)$$

The first bracket on the left-hand side would be equal to the change in absorptivity, $\Delta\epsilon_i$, per ionizable residue on ionization of a homodinucleotide (or one-half that of a heterodinucleotide) *if the ionized form is unstacked.*

The second bracket would be equal to the change in mean residue absorptivity, $\Delta\epsilon_t$, produced by heating, *if the high-temperature form is completely unstacked.*

The right-hand side is expected to resemble the change in absorptivity on ionization of the monomer ($\Delta\epsilon_i°$), though this resemblance may not be perfect if there are residual interactions between the two bases in the unstacked form.

Thus if the ionized forms at 20° and the un-ionized form at 92° are both unstacked we would expect that

$$\Delta\epsilon_i - \Delta\epsilon_t = \Delta\epsilon_i° \qquad (2)$$

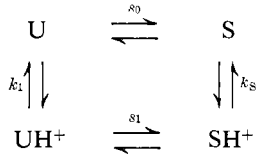
Thus a comparison of the spectra comprising the left- and right-hand sides of eq 2 will provide evidence of the state of stacking of the ionized forms of dinucleotides.

The scheme of equilibria used in the present work to describe the titration properties of dinucleotides is similar to that discussed by Cox (1966). In order to be

able to apply the same equations to both acid and alkaline titrations it is convenient to define the over-all degree of protonation by α and the stacking association constant of a species bearing i protons as s_i. It should be noted that s_i defined in this way bears a different significance according to whether an acid or alkaline titration is under consideration. It is convenient to consider the titration of hetero- and homodinucleotides separately.

Heterodinucleotides

We consider the following set of equilibria



Since we are here concerned only with dilute solutions (10^{-3}–10^{-4} M) it is justifiable to set all activity coefficients equal to unity or absorb them into the equilibrium constants. Then from the law of mass action we may write

$$\frac{[UH^+]}{[U]h} = \frac{1}{k_1}, \frac{[S]}{[U]} = s_0, \text{ and } \frac{[SH^+]}{[UH^+]} = s_1 \qquad (3)$$

where h is the hydrogen ion concentration.

These expressions then allow the concentration of all four species to be expressed in terms of [U] and the equilibrium constants as

$$[S] = [U]s_0, [UH^+] = [U]h/k_1, \text{ and } [SH^+] = h[U]s_1/k_1 \qquad (4)$$

Now the degree of protonation, α, may be written as

$$\alpha = \frac{[UH^+] + [SH^+]}{[U] + [S] + [UH^+] = [SH^+]} \qquad (5)$$

and substitution then gives

$$\frac{1 - \alpha}{\alpha} = \frac{(1 + s_0)k_1}{(1 + s_1)h} \qquad (6)$$

Then by taking logarithms

$$\text{pH} = \text{p}K_1 + \log \frac{1 - \alpha}{\alpha} - \log \frac{1 + s_0}{1 + s_1} \qquad (7)$$

Thus it is to be expected that the titration curve of a heterodinucleotide would follow the Henderson curve but the pK would be displaced toward the acid by an amount $\log (1 + s_0)/(1 + s_1)$ from pK_1.

It should be noted that the validity of eq 7 depends only on the assumptions made concerning activity coefficients and on the validity of the two-state model of a heterodinucleotide.

It is often convenient to attempt to measure the degree of ionization spectrophotometrically, since, as noted above, the ionized forms of a dinucleotide have, in general, a different absorptivity from that of the un-ionized form. Let us denote by ϵ_i the absorptivity of a species i; then the total absorbance of a solution at any pH is given by

$$A = \epsilon_S[S] + \epsilon_U[U] + \epsilon_{SH^+}[SH^+] + \epsilon_{UH^+}[UH^+] \qquad (8)$$

assuming that Beer's law holds for all species. If A_0 is an absorbance at a pH such that the dinucleotide is completely un-ionized and A_∞ the absorbance such that it is completely ionized we may write

$$A_0 = \epsilon_S[S]_0 + \epsilon_U[U]_0 \qquad (9)$$

$$A_\infty = \epsilon_{SH^+}[SH^+]_\infty + \epsilon_{UH^+}[UH^+]_\infty \qquad (10)$$

Substituting the expressions from eq 4 in 8–10 and noting that $[U]_0 = C/(1 + s_1)$, $[U]_\infty = C/(1 + s_1)$, and $[U] = C/(1 + s_0) + (h/K_1)(1 + s_1)$ (where C is the total concentration) it can be shown that

$$\frac{A_\infty - A}{A - A_0} = \frac{k_1(1 + s_0)}{h(1 + s_1)} \qquad (11)$$

so that by eq 6 we have

$$\frac{A_\infty - A}{A - A_0} = \frac{1 - \alpha}{\alpha} \qquad (12)$$

It follows that the titration of a heterodinucleotide may be followed spectrophotometrically at any convenient wavelength at which the absorbance of the solution changes as the titration proceeds; this is so whether this change be associated with the ionization of the base or the unstacking of the dinucleotide.

It remains to discuss the significance of the acid dissociation constant, k_1, of the ionizing base in the unstacked configuration of the dinucleotide. The dinucleotides are asymmetric in that one of the nucleotide residues is linked to the phosphate group through a 3′ phosphodiester bond and the other through a 5′ linkage. Thus the pK of, say, the uridine residues in ApU and UpA would be differently affected by electrostatic interactions with the charged phosphate group. However the difference between the pK's of 3′- and 5′-uridine monophosphates is only about 0.1 (Steiner and Beers, 1961) and the magnitude of the difference is expected to be less for the dinucleotide since the charge on the phosphate group is one-half that on the nucleotide monophosphate. Further at the ionic strength employed in the experiments reported below, electrostatic interactions would be reduced because of the screening effect of the electrical double layer; thus the pK of 2′-(3′)-uridine monophosphate de-

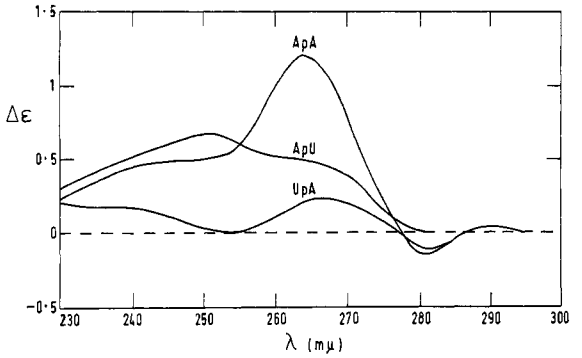
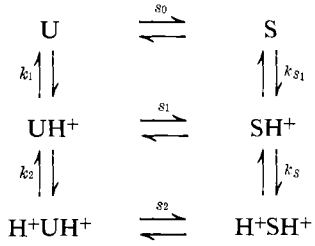


FIGURE 1: Thermal denaturation difference spectra of ApA, ApU, and UpA in 0.1 M NaCl–0.01 M phosphate (pH 7.0). $\Delta\epsilon$ is the difference between the mean millimolar residue absorbtivities and 20 and 92°.

creases as the ionic strength is increased to 0.1 M but above that is constant. Thus it is justified to assume that the pK values of the uridine residues in unstacked ApU and UpA are essentially the same (pK_u), and similarly those of the adenine residues (pK_a).

Homonucleotides

For the homonucleotides we consider the more complicated set of equilibria



In writing out this scheme we have implicitly assumed that the dinucleotide is essentially symmetrical: that the ionization constant of a nucleoside is independent of whether it is 3′ or 5′ substituted. The justification of this assumption was presented in the previous section.

We now make a further assumption: that the two bases titrate independently and that electrostatic interactions between them are negligible. This will always be the case at a sufficiently high ionic strength on account of the screening effect of the double layer. Further justification is presented in the discussion. With this assumption in mind we may put $k_1 = 2k$ and $k_2 = k/2$ (see Morawetz, 1965). The factor of 2 arises from the essential symmetry of the dinucleotides. k is then the acid dissociation constant of the independently titrating nucleoside in the environment of the dinucleotide and as such may be put equal to k_a or k_u for the titration of the adenine groups of unstacked ApA or the uracil group of unstacked UpU, respectively.

In terms of these assumptions, and proceeding in a manner analogous to that employed in the case of the heterodinucleotides, it is easy to show that

$$\mathrm{pH} = \mathrm{p}K + \log\frac{1-\alpha}{\alpha} - \log\frac{h(1+s_1)+k(1+s_0)}{k(1+s_1)+h(1+s_2)} \quad (13)$$

where pK = $-\log k$ and, as before, h is the hydrogen ion concentration.

It is immediately apparent that the titration curve of the dinucleotide should not follow the shape of the Henderson equation. It will be sharper if the dinucleotide is less stacked in the ionized form than in the un-ionized. In order to allow a precise comparison of theory and experiment it is convenient to calculate the value of the pH and the slope of the titration curve (dα/dpH) at half-ionization. The former is calculated by putting $\alpha = 0.5$ in eq 13 and simplifying to give

$$(\mathrm{pH})_{\alpha=1/2} = \mathrm{p}K - \frac{1}{2}\log\frac{1+s_0}{1+s_2} \quad (14)$$

and the former is calculated by differentiating eq 13 and then substituting $\alpha = {}^1/_2$ to give

$$(\mathrm{d}\alpha/\mathrm{dpH})_{\alpha=1/2} = \frac{1.15}{1+\dfrac{1+s_1}{\sqrt{[(1+s_0)(1+s_2)]}}} \quad (15)$$

It may be noted that the Henderson equation gives a corresponding slope of 0.575.

The titration of the homonucleotides cannot be followed spectrophotometrically unless a wavelength at which the absorptivity is independent of the degree of stacking (thermal isosbestic wavelength) can be located. It can then be shown that eq 12 holds for absorbances measured at this wavelength.

Results

Figure 1 shows thermal difference spectra of ApA, ApU, and UpA at pH 7.0 in 0.1 M NaCl. The curve for ApA is similar to that given by Massoulié and Michelson (1964). The spectra of UpU at 20 and 90° were identical within experimental error. Attempts to measure thermal difference spectra at extreme pH were vitiated by time-dependent degradation effects.

Figure 2a shows acid difference spectra of ApA, ApU, and UpA while Figure 3a shows alkaline difference spectra of UpU, UpA, and ApU.

Since the absorbance of UpU was independent of temperature it may be presumed that any stacking present does not result in hypochromism and hence it is allowable to follow its titration spectrophotometrically. Figure 4 shows an alkaline titration curve of UpU determined from absorbance measurements at 260 mμ at a concentration of 0.1 mM. The solid line indicates the theoretical Henderson curve corresponding to a pK value of 9.33. The slope of the experimental curve at

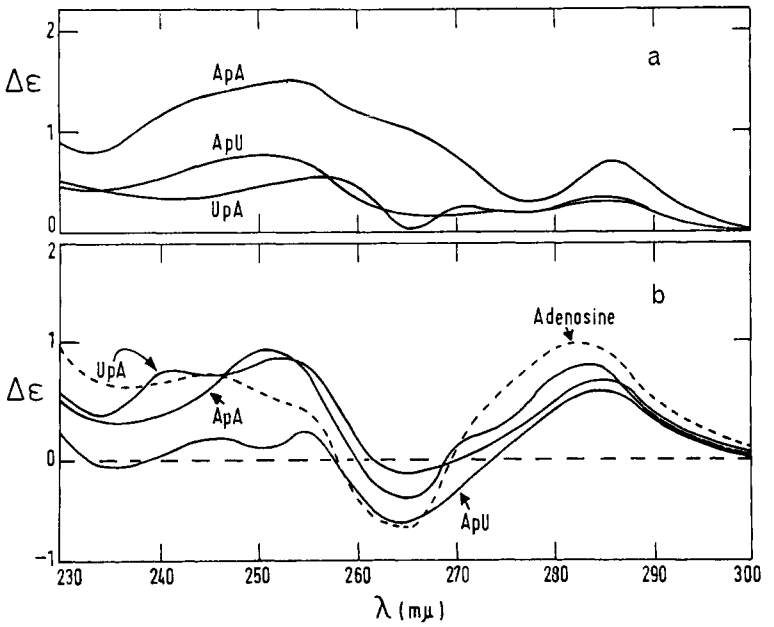


FIGURE 2: Acid difference spectra of ApA, ApU, and UpA in 0.1 M NaCl at 20°. (a) $\Delta\epsilon$ is the difference between the mean millimolar adenine absorptivities at pH 7.0 and 1.0. (b) $\Delta\epsilon$ is the value from Figure 2a less the absorbtivity change on thermal denaturation as taken from Figure 1. (- - - -) Acid difference spectra of adenosine.

TABLE I: pH Values and Slopes of the Titration Curves, $d\alpha/dpH$, at Half Ionization of Various Dinucleotides.

	Alkaline Titration		Acid Titration	
Compd	pH	$d\alpha/dpH$	pH	$d\alpha/dpH$
UpU	9.33 ± 0.02	0.56 ± 0.02		
UpA	9.35 ± 0.02	0.57 ± 0.02	3.85 ± 0.05	0.60 ± 0.05
ApU	9.48 ± 0.02	0.57 ± 0.02	3.70 ± 0.05	0.60 ± 0.05
ApA			3.50 ± 0.02	0.83 ± 0.02

$\alpha = 1/2$ is 0.56 ± 0.02 which is not significantly different from the theoretical value of 0.575.

Similar experiments were carried out with the heterodinucleotides UpA and ApU; in each case the curve closely followed the Henderson equation and the slopes and pH values at $\alpha = 1/2$ are given in Table I.

Acid titration curves of ApU and UpA were determined in a similar manner. The curves showed greater scatter on account of the small absorbance differences involved but again followed the Henderson curve within experimental error. The slopes and pK values obtained are given in Table I.

The acid titration of the homodinucleotide ApA cannot be followed spectrophotometrically except at the thermal isosbestic point which is difficult to locate with sufficient precision. Hence this compound was titrated potentiometrically and the resulting curve is shown in Figure 5; the slope and pK value at $\alpha = 1/2$ are also given in Table I. It should be noted that the points corresponding to low pH are subject to large experimental error.

An attempt was made to follow the same titration spectrophotometrically from measurements at 277.5 mμ, the thermal isosbestic point (see Figure 1), and although less reliance should be placed upon these results, they are, as shown in Figure 5, in reasonable agreement with the results of the potentiometric experiment.

Discussion

It is most probable that UpU is almost, if not completely, unstacked at 20°; thus both the absorptivity (Richards *et al.*, 1963) and the magnitude of the Cotton effect (Sarkar and Yang, 1965) of polyuridylic acid do not change appreciably on heating from 20 to 90°. Also, the magnitude of the Cotton effects of oligouridylic acids is independent of the degree of polymerization (H. Simpkins and E. G. Richards, manuscript in prep-

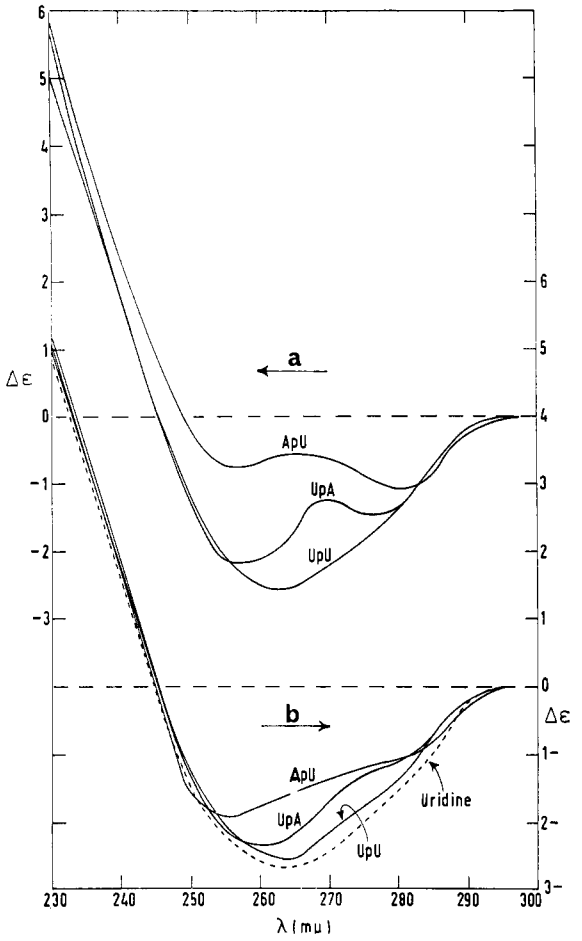


FIGURE 3: Alkaline difference spectra of ApU, UpA, and UpU in 0.1 M NaCl at 20°. (a) $\Delta\epsilon$ is the difference between the mean millimolar uracil absorptivities at pH 7.0 and 12.0. (b) $\Delta\epsilon$ is the values from Figure 3a less the absorbtivity change on thermal denaturation as taken from Figure 1. (- - - -) Alkaline difference spectra of uridine.

aration). This matter is further discussed by Simpkins and Richards (1967).

It thus seems reasonable to suppose that the stacking association constant of the un-ionized form of UpU is essentially zero.

It is unlikely that the ionized forms of UpU are more stacked than the un-ionized forms so that it is reasonable to put $s_0 = s_1 = s_2 = 0$ for the alkaline titration of UpU. This assumption is consistent with the close similarity of the ionization difference spectra of UpU and uridine as shown in Figure 3b.

The alkaline titration curve of UpU closely follows the Henderson equation as shown in Figure 4. This is consistent with eq 15 if $s_0 = s_1 = s_2$ and moreover implies that the ionization of the two uracil residues proceeds independently and that there is no significant electrostatic interaction between them.

If $s_0 = s_2$, eq 14 requires that the pK of UpU at half-

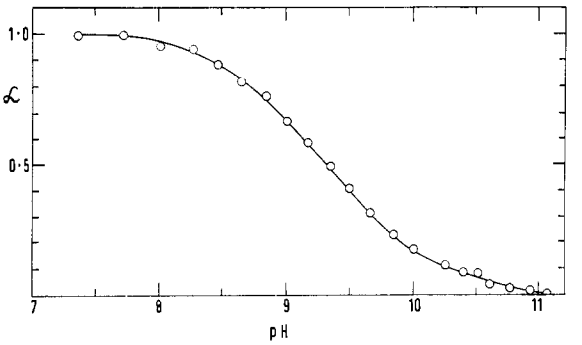


FIGURE 4: Plot of degree of protonation (α) against pH for UpU in 0.1 M NaCl at 20°. (○) Experimental points obtained from spectrophotometric titration. (———) Theoretical curve calculated from Henderson equation.

ionization (9.33) be put equal to pK_u, the pK of an unstacked uracil residue in the environment of a dinucleotide.

ApA, ApU, and UpA all exhibit temperature-dependent absorption spectra as is shown in Figure 1. This implies that these dinucleotides are all to some extent stacked at 20° at pH 7. This is further substantiated by the data in Figures 2a and 3a which show that their alkaline and acid ionization difference spectra differ in shape from those of uridine or adenosine, respectively. We can equate the ionization difference spectra to $\Delta\epsilon_i$ in eq 2. Similarly we may equate the thermal difference spectra of Figure 1 to $\Delta\epsilon_t$. Using eq 2 the difference spectra ($\Delta\epsilon_t°$) were calculated and these are shown in Figures 2b and 3b. It is seen that the curves in Figure 3b for UPA and ApU are closer to that of uridine than those in Figure 3a ($\Delta\epsilon_i$). Similarly the curves in Figure 2b for ApA, ApU, and UpA are much more similar to

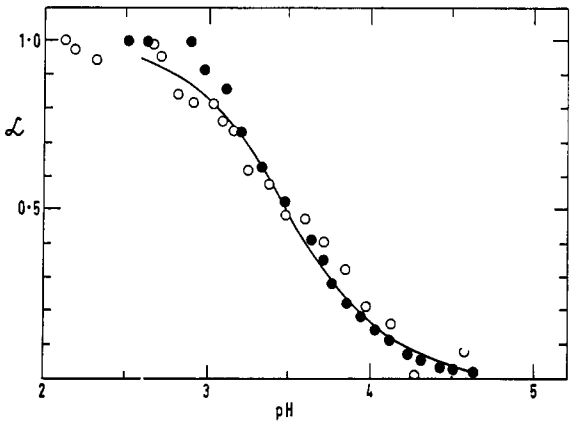


FIGURE 5: Plot of degree of protonation (α) against pH for ApA in 0.1 M NaCl at 20°. (●) Experimental points from potentiometric titration. (○) Experimental points from spectrophotometric titration at 277.5 mμ. (———) Theoretical curve calculated from eq 3 and assuming $s_1 = s_2 = 0$, $s = 4.25$, and p$K_a = 3.86$.

that of adenosine. In terms of the argument set out in the theoretical section, it is concluded that ionized forms of ApU and UpA both in alkali and in acid are essentially unstacked. Similarly we conclude that the doubly ionized form of ApA in acid is unstacked. In comparing the difference spectra of ApU and UpA in Figure 2b with the ionization spectra of adenosine it must be borne in mind that the experimental errors are relatively large (0.3 in $\Delta\epsilon$) and that the dinucleotides may not be completely unstacked at 92°. These factors may also account for the discrepancies between the difference spectra of UpA and ApU and that of uridine in Figure 3b. It should be noted that, since the absorbance changes produced on heating and on ionization are of opposite sign, the discrepancies in Figure 3b would be even larger if the UpA and ApU were not unstacked in alkaline solution.

In summary the ionization and thermal difference spectra suggest that the ionized forms in both acid and alkali of all four dinucleotides are essentially unstacked and that the un-ionized forms at pH 7 are partially stacked to varying degree. This conclusion is consistent with the classification of these dinucleotides in their various ionization states as stacked or unstacked as given by Warshaw and Tinoco (1965).

It has been remarked above that the titration curves of the heterodinucleotides in both acid and alkaline solution closely follow the Henderson equation. This is consistent with eq 7.

Considering first the alkaline pK values given for ApU and UpA (see Table I), we equate their deviations from 9.33, the value deduced for pK_u, to $\log(1 + s_0)/(1 + s_1)$ as is required by eq 7. Then given that the ionized forms of both dinucleotides are unstacked we may in each case put $s_0 = 0$ and so calculate s_1. The resulting values are given in Table II.

TABLE II: Stacking Association Equilibrium Constants (s) at 20° in 0.1 M NaCl at pH 7.0 of Certain Dinucleotides.[a]

Compd	s	β	$\Delta F°$ (kcal)
UpU	0	0	
UpA	0.05 ± 0.07	0.05 ± 0.07	(+6.0)
ApU	0.41 ± 0.10	0.29 ± 0.05	+0.52 ± 0.08
ApA	5.38 ± 0.26	0.84 ± 0.01	−0.99 ± 0.05

[a] β is the fraction of dinucleotides in the stacked configuration corresponding to these conditions and $\Delta F°$ is the standard free energy of stacking per nucleotide pair as calculated from the values of s at 20°.

Now eq 7 also demands that the deviation of the acid pK values of ApU and UpA from pK_a be equal to $\log(1 + s_0)/(1 + s_1)$. Thus, since we have concluded that both dinucleotides are unstacked in acid solution also, we may put $s_1 = 0$ in each case and hence calculate two independent values of pK_a from the values given in Table I. The resulting values of pK_a are 3.85 and 3.87. The agreement is excellent and supports the validity of this approach.

Given that pK_a = 3.86, it is possible to deduce from eq 14 that $\log(1 + s_0)/(1 + s_2) = 0.72 \pm 0.06$ for ApA. Then since the acid form of ApA is unstacked so that $s_2 = 0$, we finally calculate that s_0, the stacking equilibrium constant for ApA at pH 7, is 4.25 ± 0.7.

The titration curve for ApA is sharper than that given by the Henderson equation, as is seen in Figure 5. This is consistent with eq 15. The slope at half-ionization is 0.83 ± 0.02, so that by taking a value of 4.25 for s_0 and 0 for s_2, $1 + s_1$ is calculated from eq 15 to be 0.90 ± 0.15. This is, within the limits of experimental error, equal to unity, the lowest possible value. Thus it may be concluded that $s_1 = 0$ also, and that the singly ionized form of ApA is unstacked.

Alternatively, we may assume that $s_1 = 0$ and then calculate a second value of s_0 from the slope of the titration curve and eq 15. The result is $s_0 = 5.54 \pm 0.28$. The difference between the two estimates of s_0 can probably be attributed to experimental error and the weighted mean is 5.38 ± 0.26, as given in Table II.

From this value of the stacking association constant of ApA at pH 7, it is possible to calculate a value of the standard free energy of stacking at 20° of −0.99 ± 0.05 kcal/mole of dinucleotide. This value may be compared with the corresponding value of 0.2 ± 1.6 kcal as calculated from the $\Delta H°$ and $\Delta S°$ values given by Brahms *et al.* (1966). The agreement is well within the experimental error quoted by these authors.

It is worth noting that as the stacking association constants of the different dinucleotides displayed in Table II increases, so does the area under the thermal difference spectra shown in Figure 1. Thus the latter may perhaps serve as a rough guide to the former.

It may be concluded then that if the two-state model is valid for the stacking of dinucleotides and if the assumptions made in the present work are correct, then the present approach provides a method for measuring the free energy of stacking of considerably greater precision than previous methods. Further it is easy to see that the method can be extended to yield values of the enthalpy and entropy of stacking and that no assumptions need be made about the properties of the dinucleotides in real or hypothetical states in which they are completely stacked or unstacked.

Further work is in progress in this laboratory to extend this work to other dinucleotides and to measurements as a function of temperature.

Acknowledgments

We are grateful to Dr. W. B. Gratzer for helpful discussion, to Professor M. H. F. Wilkins for encouragement, and to Professor Sir John Randall for providing the facilities for this work to be carried out. One of us (H. S.) is indebted to the Science Research Council for financial support.

References

Applequist, J., and Damle, V. (1965), *J. Am. Chem. Soc. 87*, 1450.
Brahms, J., Maurizot, J., and Michelson, A. M. (1967a), *J. Mol. Biol. 25*, 465.
Brahms, J., Maurizot, J., and Michelson, A. M. (1967b), *J. Mol. Biol. 25*, 481.
Brahms, J., Michelson, A. M., and Van Holde, K. E. (1966), *J. Mol. Biol. 15*, 467.
Cox, R. A. (1966), *Biochem. J. 100*, 146.
Fresco, J. R., and Doty, P. (1957), *J. Am. Chem. Soc. 79*, 3928.
Heppel, L. A., Oritz, P. J., and Ochoa, S. (1956), *Science 123*, 415.
Heppel, L. A., Oritz, P. J., and Ochoa, S. (1957), *J. Biol. Chem. 229*, 679.
Leng, M., and Felsenfeld, G. (1966), *J. Mol. Biol. 15*, 455.
Massoulié, J., and Michelson, A. M. (1964), *Compt. Rend. 259*, 2923.
Morawetz, H. (1965), Macromolecules in Solution, Interscience, New York, N. Y., p 349.
Poland, D., Vournakis, J. N., and Scheraga, H. A. (1966), *Biopolymers 4*, 223.
Richards, E. G., Flessel, C. P., and Fresco, J. R. (1963), *Biopolymers 1*, 431.
Sarkar, P. K., and Yang, J. T. (1965), *J. Biol. Chem. 240*, 2088.
Simpkins, H., and Richards, E. G. (1967), *Biopolymers* (in press).
Steiner, R. F., and Beers, R. F. (1961), Polynucleotides, London, Elsevier, p 25.
Warshaw, M. M. (1966), Ph.D. Thesis, University of California, Berkeley, Calif.
Warshaw, M. M., and Tinoco, I. (1965), *J. Mol. Biol. 13*, 54.

Structure Stabilization in *Escherichia coli* Transfer Ribonucleic Acid*

David B. Millar and Maxime MacKenzie

ABSTRACT: Formaldehyde treatment of *Escherichia coli* B transfer ribonucleic acid (tRNA) appreciably changes hydrodynamic and optical properties. The increase in intrinsic viscosity, decrease in sedimentation coefficient coupled with the change in absorbance melting curves, and polarization of fluorescence thermal profiles indicate that (1) Watson–Crick hydrogen bonding is a determinant in the configuration and helix–coil transition of native *E. coli* tRNA and (2) base stacking contributes to the helical rigidity in native tRNA.

Three types of forces are now believed to determine the stability and configuration of polyribonucleotides. In order of chronological recognition they are: hydrogen bonding, base stacking, and the interaction of the C-2 hydroxyl group of ribose with either a phosphate or nucleotide (Spencer *et al.*, 1962; Langridge and Gamatos, 1963; Sato *et al.*, 1966; Ts'o *et al.*, 1966). Since it appears that some polyribonucleotides can exhibit ordered structure in the absence of hydrogen bonding (Fasman *et al.*, 1964, 1965; Poland *et al.*, 1966; Brahms *et al.*, 1966; Leng and Felsenfeld, 1966; Stevens and Rosenfeld, 1966), the contribution of Watson–Crick base pairing to the secondary structure and configuration of ribonucleic acid deserves reassessment.

Formaldehyde treatment reduces or blocks completely polynucleotide interactions which are known to be explicitly dependent upon base pair hydrogen-bond formation (Steiner and Beers, 1959; Grossman *et al.*, 1961; Stollar and Grossman, 1962; Haselkorn and Doty, 1961), by blocking exocyclic amino groups serving as proton donors. Past investigation of the reactivity of tRNA with formaldehyde has shown that agents which promote secondary structure decidedly decelerate the rate of reaction (Penniston and Doty, 1963).

Thus it appears reasonable to assume that by measuring various physical and optical properties of tRNA after reaction with formaldehyde (under reaction conditions in which secondary structure is minimal), at least the qualitative importance of hydrogen bonding in this molecule might be estimated.

* From the Section on Biological Macromolecules, Laboratory of Physical Biochemistry, Naval Medical Research Institute, Bethesda, Maryland, and the Bureau of Medicine and Surgery, Navy Department, Research task MR005.06.0001. *Received April 16, 1967.* The opinions in this paper are those of the authors and do not necessarily reflect the views of the Navy Department or the naval service at large.