

Adenine and Cytosine: Basic Polarographic Behavior and Its Interpretation *

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Summary

The basic information concerning the behavior on electrochemical reduction in aqueous media at mercury electrodes of cytosine, adenine, model compounds, their nucleosides and nucleotides, and their simpler polymers, is summarized and interpreted with particular reference to the effects of the addition of sugar and sugar-phosphate substituents on the course of the faradaic redox process, intermediate chemical steps in that process, the kinetics and energetics of individual steps, association in solution, and adsorption at the solution|electrode interface and association in the adsorbed state. Since cytosine and adenine are the only two nucleic acid bases, which are normally electrochemically reducible in aqueous media, it is hoped that the discussion will provide the background necessary for the interpretation of the observed behavior on electrochemical reduction (*e.g.*, at dropping and hanging drop mercury electrodes) of polymeric species as in the polarographic studies of DNA and RNA, and synthetic polynucleotides.

Introduction

In recent years, many of the factors in the electrochemical reduction of the purines and pyrimidines in aqueous media have been identified and evaluated.^{1,2} The compounds investigated have included biologically important species ranging in complexity to the nucleic acids. Two areas, in which more information is needed in order better to understand the behavior of the various species involved, are:

- (a) the kinetics of the individual electron-transfer and accompanying chemical steps composing the overall redox process, and the exact sequence of these individual steps, and

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- (b) the effects on the kinetics and energetics of the individual steps of orientation of the compound at the electron-transfer interface, adsorption of the compound and other species in the redox path at that interface, and association in the adsorbed state.

Since the electrochemical behavior of polymeric nucleic acids and synthetic polynucleotides is essentially determined by that of the constituent purine and pyrimidine bases, detailed knowledge of the behavior of the monomeric units will facilitate correct interpretation of results obtained with polymers, as well as permitting estimation of the effects of sugar and sugar-phosphate moieties and polymer secondary structure on the behavior of the parent bases. Although electrochemical reduction in nucleosides and nucleotides occurs primarily in the pyrimidine ring moiety, sugar and sugar-phosphate groups may influence association, conformation, orientation and adsorption, as well as electron density at electroreactive sites, whose effects may then be manifest in the current-potential patterns.

The present paper summarizes basic information concerning the electrochemical behavior and its interpretation for the nucleic acid bases and their simpler nucleoside and nucleotide derivatives; hopefully, this will provide the background necessary for the interpretation of the behavior observed on electrochemical reduction [*e.g.*, at dropping and hanging drop mercury electrodes (D.M.E.; H.M.D.E.)] of polymeric species as in the polarographic studies of DNA and RNA. Since cytosine and adenine are the only two nucleic acid bases, which are normally electrochemically reducible in aqueous media, emphasis is placed on their behavior.

Electrochemical pyrimidine reduction: cytosine species

Pyrimidine

In aqueous media, pyrimidine itself exhibits five reduction waves at mercury electrodes over the available pH range (Fig. 1; Table I).³⁻⁷ In acidic solution, pH-dependent one-electron ($1 e^-$) wave I is seen; at about pH 3, pH-independent $1 e^-$ wave II emerges from background discharge; near pH 5, these merge to form pH-dependent $2 e^-$ wave III. Near pH 7, pH-independent $2 e^-$ wave IV emerges from background and, at about pH 9, merges with wave III to form pH-dependent $4 e^-$ wave V. A detailed exposition of the reduction paths is given in Fig. 2.

Wave I, associated with a $1 e^-$, one-proton addition to the $3,4 N=C$ bond to produce a neutral radical, is the most nearly reversible of the pyrimidine waves; as more of the pyrimidine is protonated, the coupled protonation reaction becomes less critical in determining the rate of the first step (pK_a for protonation of pyrimidine is 1.30). The free radical dimerizes, apparently to the $4,4'$ species.⁶ Wave II ($1 e^-$ reduction of the free radical to a dihydropyrimidine) appears to be the most irre-

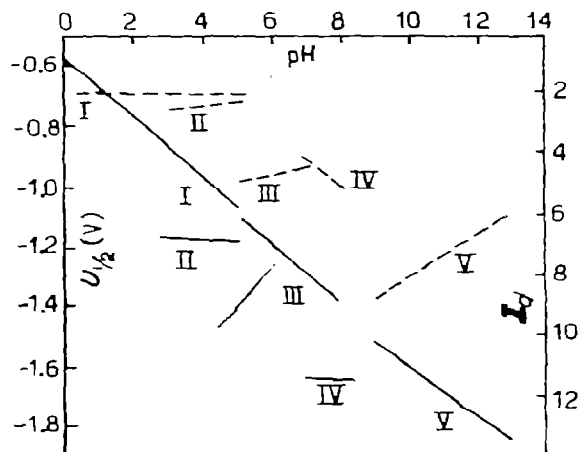


Fig. 1.

Variation with pH of half-wave potentials ($U_{1/2}$: solid lines) and diffusion current constants (I_d : dashed lines) for pyrimidine. Roman numerals refer to the various pyrimidine waves. (From Ref. 2 with permission).

versible pyrimidine wave, as might be expected from the stability of the dihydro species and the difficulty of reoxidizing an aliphatic carbon site; wave II differs most from the other pyrimidine waves in that its first step involves attack on a neutral radical, whereas the initial step for waves I, III and V presumably involves attack on the 3,4 N=C bond and that for wave IV of the 1,2 N=C bond.

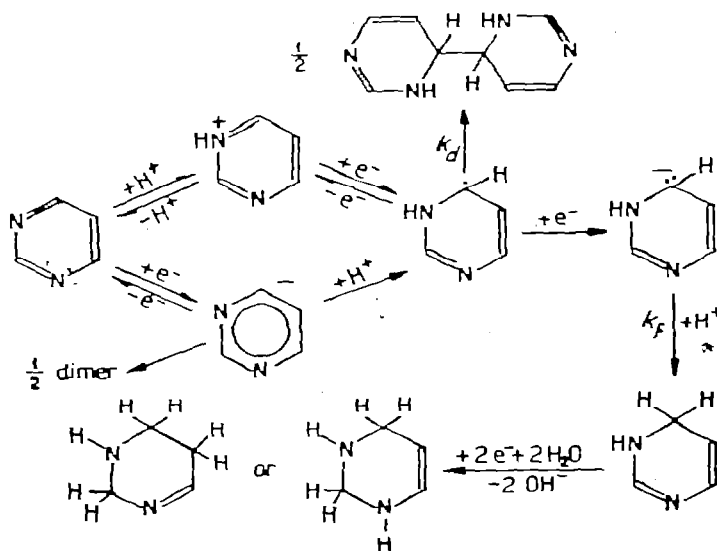


Fig. 2.

Interpretation of the electrochemical behavior observed for pyrimidine in aqueous media. The lower reaction sequence involving electron addition to unprotonated pyrimidine is that in aprotic media. The reactions involved in the reduction of the 1,2 N=C bond are analogous to those indicated for the 3,4 N=C bond reduction. (From Ref. 7 with permission).

Table 1. Comparative polarographic reduction patterns for cytosine, adenine and related compounds^a.

Compound	pH-Dependence of $U_{\frac{1}{2}}^b$ V	n^c	$U_{\frac{1}{2}}$ at pH 4.2 V
Pyrimidine	I -0.576 - 0.105 pH	1	-1.017
	II -1.142 - 0.011 pH	1	-1.188
	III -0.680 - 0.089 pH	2	^d
	IV -1.600 - 0.005 pH	2	^d
	V -0.805 - 0.079 pH	4	^d
2-Hydroxypyrimidine	-0.530 - 0.078 pH	1	-0.858
4-Aminopyrimidine	I -1.13 at pH 1.2	2 ?	
	II -1.23 at pH 1.2	1 ?	
Cytosine (4-amino-2-hydroxypyrimidine)	-1.125 - 0.075 pH	3	-1.440
Cytidine		3	-1.38
CMP		2 ?	-1.37
CpC	-1.085 - 0.056 pH	4	-1.32
CpA	-1.075 - 0.061 pH	6	-1.33
ApC	-1.055 - 0.064 pH	6	-1.32
Purine	I -0.697 - 0.083 pH	2	-1.046
	II -0.902 - 0.080 pH	2	-1.238
Adenine (6-aminopurine)	-0.975 - 0.084 pH	4	-1.328
Adenosine	-1.040 - 0.070 pH	4	-1.33
AMP	-1.015 - 0.083 pH	4	-1.36
ATP	-1.035 - 0.083 pH	4	-1.38
ApA	-0.980 - 0.070 pH	6 + ?	-1.27
(Ap) ₂ A	-1.060 - 0.053 pH	12	-1.28
(Ap) ₃ A	-1.060 - 0.053 pH	13	-1.28
(Ap) ₅ A	-1.120 - 0.043 pH	13	-1.30

^a Data are for 25 °C in acetate and/or McILVAINE buffers.

^b Roman numbers refer to the wave sequence.

^c The faradaic n values are generally based on a combination of estimation from measured polarographic diffusion current constants and from coulometry on controlled electrode potential electrolysis. A question mark indicates an uncertain magnitude. Details are generally given in the text.

^d Wave is not seen at pH 4.2.

The similar characteristics of waves I and III are attributable to their both involving the same initial step (1 e⁻ attack on the 3,4 N=C bond); wave III is somewhat less reversible than wave I. Waves IV and V, associated with formation of a tetrahydropyrimidine species unstable

under the alkaline conditions of its formation, are irreversible; their similar behavior is probably due to the composite nature of wave V. Waves III and IV both involve a $2e^-$ attack on a double bond.

The variation in behavior of substituted pyrimidines from that described for pyrimidine, as well as the differences between individual pyrimidines, results from the effects of substitution on the electron density at the electrochemical reaction sites and on the kinetics of accompanying chemical reactions. These effects are seen in the reduction path postulated for cytosine.

The standard heterogeneous rate constant, $k_{s,h}$, for the initial electron transfer step in at least the simpler pyrimidine and purine species can be estimated on the basis of cyclic voltammetric and *a.c.* polarographic measurements to exceed 0.1 cm/s in both aqueous and nonaqueous media.^{6,7,9,13}

Cytosine

At pH 3.5 to 6, cytosine (2-hydroxy-4-aminopyrimidine) gives a fairly well-defined polarographic wave, close to background discharge (Table I);^{2,5,8,9} a poorly defined wave is seen at higher pH. Macroscale electrolysis gives a product identical to that obtained on electrolysis of 2-hydroxypyrimidine.

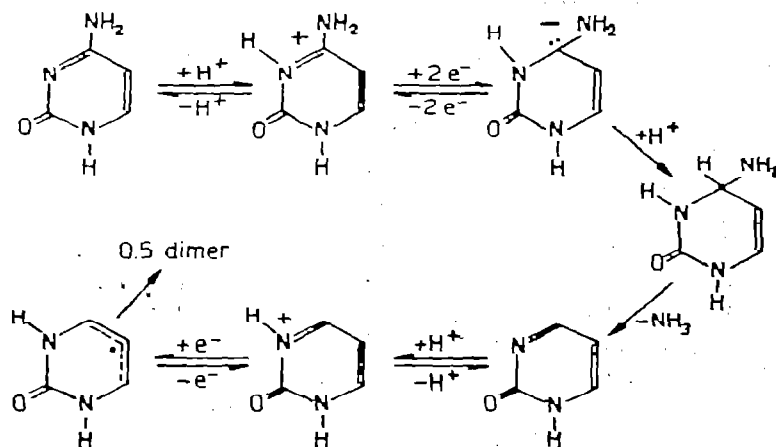


Fig. 3.

Proposed reaction pathway for the reduction of cytosine in aqueous media. (From Ref. 9 with permission).

Cytosine both at the D.M.E. and on exhaustive electrolysis undergoes a $3e^-$ reduction, involving $2e^-$ hydrogenation of the 3,4 N=C bond (corresponds to 1,6 N=C bond in adenine), deamination to form 2-hydroxypyrimidine, and $1e^-$ reduction of the latter to a free radical which dimerizes.^{3,9} Cytosine nucleosides and nucleotides are similarly reduced^{1,2,9-12} with modifications of the types subsequently discussed.

The basis for the detailed mechanism proposed for cytosine reduction (Fig. 3) is evident from consideration of the half-wave potentials ($U_{1/2}$) and faradaic n values tabulated in Table 1. Addition of an amino group at C(4) in pyrimidine [equivalent to C(6) in purine] makes initial 3,4 N=C bond reduction more difficult; it occurs at so negative a potential that the first two reduction steps merge, *e.g.*, compare pyrimidine and 4-aminopyrimidine, and purine and adenine. Hydroxyl group addition at C(2) removes the 1,2 N=C bond due to ketonization, facilitates 1 e^- reduction of the 3,4 N=C bond, and accelerates dimerization of the resulting free radical; it also seems to accelerate deamination in a reduced 3,4 N=C bond having an amino group on C(4), *i.e.*, hydrogenation of such a 3,4 N=C bond produces a *gem*-diamine centered on C(4), resulting in elimination of NH_3 and regeneration of the 3,4 N=C bond, which is reduced as formed since addition of the amino group makes the 3,4 N=C bond more difficult to reduce than in pyrimidine or 2-hydroxypyrimidine. For example, 2-hydroxypyrimidine undergoes only a 1 e^- reduction, the initial 4-aminopyrimidine reduction wave is a 2 e^- process, and cytosine reduction is a 3 e^- process. Unfortunately, 2-hydroxy-6-aminopurine (*iso*-guanine), whose pyrimidine ring configuration is similar to that of cytosine, cannot be used for comparison due to fundamental differences in its reduction mechanism.¹⁴ Data for the carbanion protonation and deamination reactions are given in Table 2.

Cytosine nucleosides and nucleotides show significant trends.^{9,12} Cytidine reduction does not differ significantly from that of cytosine. For CMP, the rate of deamination is slower than for cytosine, but that for carbanion protonation is about the same. Based on a faradaic n of 4, each ring in CpC is likely reduced by the initial 2 e^- process with deamination occurring very slowly or not at all. This is explicable from the following considerations. At 25 °C in neutral or slightly alkaline pH, CpC is in equilibrium between stacked single stranded and disordered conformation.¹⁵ Charge-transfer complexes stabilized by VAN DER WAALS-LONDON forces were observed¹⁶ in frozen solutions (77 K) of cytidine, CpC and poly C at pH around pK_a . Similar complexes, although in much lesser extent and more loosely bound, may exist in solutions above 0 °C. Due to stacking and possible charge-transfer interaction, the reduction sites in CpC may be partially shielded and, consequently, deamination proceeds very slowly. Deamination may also be inhibited by strong adsorption of the dihydrocytosine product.

The behavior of dinucleoside phosphates containing cytosine is subsequently discussed.

Electrochemical purine reduction: adenine species

Although imidazole is relatively inert electrochemically, its presence as part of the purine molecule profoundly affects the latter's behavior due to the altered electron densities in the pyrimidine ring on fusion with the imidazole ring to form purine, *e.g.*, comparison of the

Table 2. Rate constants for pyrimidines and purines in aqueous medium^a.

	Carbanion protonation ^b s ⁻¹	Reduction product deamination ^c s ⁻¹
Pyrimidine (1,2-diazine)	5×10^4	
Cytosine (2-hydroxy-4-aminopyrimidine)	5×10^4	10
CMP	5×10^4	3
CpC	5×10^4	very slow
Purine (first 2 e ⁻ addition)	4.9×10^3	
Purine (second 2 e ⁻ reduction)	1.5×10^4	
Adenine (6-aminopurine)	2×10^5	< 1
ApA	5×10^5	very slow

^a Rate constants for dimerization and protonation of free radicals and radical anions produced on one-electron reduction of pyrimidine and purine derivatives in aqueous and nonaqueous solution are given in Ref. 6, 7, 9 and 13.

^b *Pseudo* first-order rate constants at pH 3.5 to 4.2 for the product of a one-proton, two-electron reduction. Heterogeneous charge-transfer rate-constant, $k_{s,h}$ exceeds 0.1 to 1 cm s⁻¹.

^c Deamination of a dihydro reduction product of a 3,4 N=C bond in pyrimidines and a 1,6 N=C bond in purines, where an amino group is attached to the carbon atom.

calculated ground state electron densities (π charges) at the nine positions in purine¹⁷ and the corresponding positions in pyrimidine (π -deficient N-heterocycle) and imidazole (π -excessive N-heterocycle) indicate an electron flow from the imidazole region to the pyrimidine region of the purine.¹⁷ The increases in base strength of N(1) in purine [comparable to N(3) of pyrimidine] and in acid strength of purine N(9) [comparable to N(1) of imidazole] as a result of the electron shift are evident on comparing the pK_a values for proton acquired (basic pK_a) and for proton lost (acidic pK_a): imidazole, 7.1 and 14.52; for pyrimidine, 1.23; purine, 2.52 and 8.92.

The increased electron density makes the initial electrochemical reduction of purine more difficult than that of pyrimidine, even though the pyrimidine ring is involved in both processes. The potential required for the initial electron addition to purine is sufficiently great, so that the free radical species (or one derived from it by an exceedingly rapid chemical reaction) is immediately reduced, resulting in an initial 2 e⁻ wave. (The easier initial 1 e⁻ pyrimidine reduction may also reflect the free radical dimerization.)

Once the 1,6 and 3,4 N=C sites in purine and pyrimidine, respectively, have been hydrogenated, the situation is altered; 1,6-dihydropurine is more readily reduced than 3,4-dihydropyrimidine, *e.g.*, $U_{\frac{1}{2}}$ values at pH 7 are -1.46 V for $2e^-$ purine wave II and -1.64 V for $2e^-$ pyrimidine wave IV (Table I).

Purine

Purine exhibits two pH-dependent polarographic waves in aqueous media (Table I); the sum of the diffusion current constants, I_d , approximately equals the sum of those for pyrimidine waves III and IV, or that for the single adenine wave.^{3,8,18}

Purine and 6-methylpurine normally undergo two successive $2e^-$ reductions of the 1,6 and 3,2 N=C bonds (the 6-methylpurine waves fuse to a single $4e^-$ wave at pH 6); other 6-substituted purines studied undergo a single $4e^-$ reduction of these two double bonds.^{5,7,14,18,19} At higher pH, reduction is inhibited, *e.g.*, at pH 9 for purine and at pH 5-6 for adenine, presumably because prior protonation [probably at N(1)] is necessary for reduction.^{9,18-20} There is a basic question involving the electrochemical reducibility of the unprotonated purines (neutral form).

The latter question is related to the non-reducibility of purine bases in double stranded polynucleotides in aqueous media,²¹ which has been ascribed to inaccessibility of reducible groups and to existence of the purines in neutral form with non-available potential range for their reduction. Pertinent information about reducibility of the neutral bases has been provided by examination¹³ of purine, adenine, and other 6-substituted purines in non-aqueous media, which makes more explicit the sequence of steps in aqueous reduction.

In non-aqueous media, the purines undergo initial $1e^-$ reduction to radical anions, which dimerize (rate constants of 10^3 to $10^5 M^{-1} s^{-1}$); the dimers are oxidized to the original purines at considerably more positive potential. $U_{\frac{1}{2}}$ for the initial $1e^-$ wave in non-aqueous media (-1.89 V for purine; -2.44 V for adenine) is in marked contrast to $U_{\frac{1}{2}}$ in aqueous media (Table I). On addition of weak proton donors, the protonated radical anion is further reduced at the potential of its formation (E.C.E. process); the reduction reaches the $4e^-$ level at an acid/purine mole ratio of 3.8-4.0. In the presence of strong acid (perchloric), purine and 6-methylpurine exhibit two $2e^-$ waves and the other 6-substituted purines a single $4e^-$ wave. The effect of substitution in the 6-position on ease of reducibility is the same in the neutral purines (non-aqueous media) and in positively charged (protonated) purines (aqueous media).

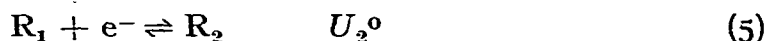
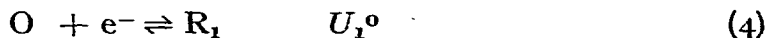
The general mechanistic pattern for purines in aqueous media^{7,14,18} can be represented as



where RH_2 and RH_4 represent dihydro- and tetrahydropurines. Purine and 6-methylpurine exhibit two separate $2 e^-$ waves due to RH_2 having a higher electron density at the 3,2 N=C bond than protonated RH^+ at the 1,6 N=C bond, which makes the 3,2 N=C reduction more difficult. When the presence of electron-releasing groups such as amino, methylamino or methoxy on C(6) causes the initial 1,6 N=C bond reduction to be more difficult than that of the 3,2 N=C bond in the 1,6-dihydropurine species, both reactions 2 and 3 occur at the same potential.

Thus, the mechanistic and potential differences in aqueous and non-aqueous media are due to protonation. Consequently, it can be concluded that neutral purines are reducible, but that the potentials required are beyond realization in aqueous media due to prior reduction of hydrogen ion, background electrolyte cation or water itself. This is probably the reason for non-reducibility of the purine bases in double stranded polynucleotides;²¹ studies in non-aqueous solvents could provide more definite information on their reducibility. However, hydrogen bonding present in the double stranded configuration may sufficiently stabilize the bases as to prevent their reduction within the available potential range. The mutual polynucleotide hydrogen bonding in non-aqueous media would be expected to be more stable than in aqueous solution due to the decreased tendency for hydrogen-bonding with the solvent.

Equation 2 is a simplification of the more general mechanism :



Observation of only one $2 e^-$ cathodic wave for each purine bond reduction indicates that U_1^0 is equal to or more negative than U_2^0 , *i.e.*, the initially produced free radical, R_1 , is as readily or more readily reduced than the protonated purine, O. An exceedingly rapid change in the nature of species R_1 may possibly occur between reactions 4 and 5.

Combination of all available data suggests for the initial purine reduction step (wave I) the mechanism outlined in Fig. 4.7 Reduction of the 3,2 N=C bond, which produces the purine wave II, apparently follows a path similar to that of the initial 1,6 N=C reduction; the final reduction product can hydrolyze to cleave the tetrahydropyrimidine ring.¹² The electron-transfer processes for both reduction steps are very rapid with $k_{s,h}$ values probably exceeding 1 cm s^{-1} .

In addition to a normal $4 e^-$ reduction wave, some 6-amino- and 6-alkylaminopurines, including the adenine nucleotides, yield under some conditions a more negative wave of anomalous behavior, which is primarily due to an enhanced supply of purine as a result of onset of streaming of the solution over the electrode surface.¹⁹ Although data for the normal wave obtained in the presence and absence of the abnormal wave are essen-

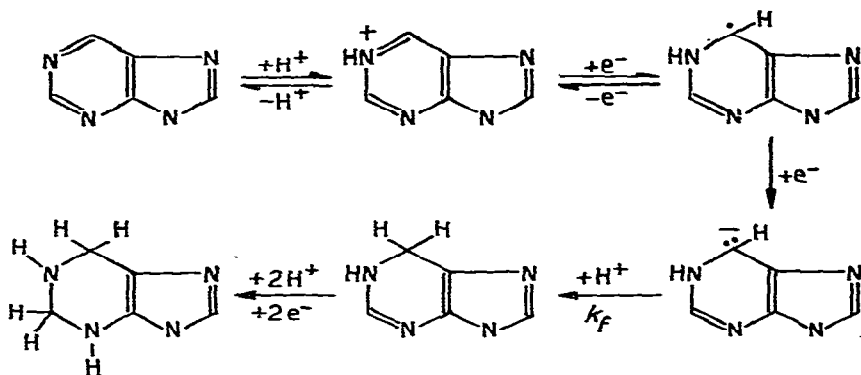


Fig. 4.

Interpretation of the electrochemical behavior observed for purine in aqueous media. The reactions involved in the reduction of the 3,2 N=C bond are similar to those indicated for the 1,6 N=C reduction. (From Ref. 7 with permission).

tially identical, the proximity of the waves makes normal wave data obtained in the absence of the abnormal wave more accurate. Consequently, it is recommended that, in studies of 6-aminopurines, the abnormal wave be avoided by the use of low ionic strength solutions and low temperature, *e.g.*, 0.1 *M* and below 5 °C. Alternatively, if 0.5 *M* ionic strength solutions at 25 °C or the equivalent are to be used, concentration of the electroactive species should not exceed 0.1 mM. Thus, the abnormal wave was not seen with adenine oligo- and dinucleotides at low concentrations (*ca.* 0.05 mM).^{9,12}

Due to reduction product accumulation at the electrode surface, catalytic hydrogen evolution is a participant in the single voltammetric reduction wave obtained on the hanging mercury drop electrode (H.M.D.E.) with some adenine derivatives.

Adenine

Adenine (6-aminopurine) produces a single cathodic wave at the D.M.E. and on cyclic voltammetry at the H.M.D.E. between pH 2 and 6.^{5,8,12,18} In ammonia buffer, it gives an inflection close to the background discharge; point-by-point subtraction of the residual current yields a more or less well defined wave.⁸ Background discharge occurs at less negative potentials in the presence of adenine.

Adenine is initially reduced at the 1, 6 N=C bond at a potential sufficient also to reduce the 3,2 N=C bond (*cf.* Table I). Consequently, a 4 e⁻ reduction is the favored initial step in proton-containing media *e.g.*, aqueous solution of pH 2 to 6; this is explicable on the basis of 1,2-dihydroadenine being reducible at the potential necessary to initiate reduction of protonated adenine and of deamination of the reduced adenine

(*cf.* next paragraph) being relatively slow on the time scale of D.M.E. polarography and cyclic voltammetry^{13,14,18,20} (*cf.* Table 2).

Hydrogenation of the 1,6 N=C bond produces a *gem*-diamine centered on C(6), which results in elimination of NH₃ and regeneration of the 1,6 N=C bond to produce 3,4-dihydropurine, which is reduced in a 2 e⁻ process as formed. Consequently, electrolytic reduction of adenine is a 6 e⁻ process under prolonged time-scale conditions, *e.g.*, on controlled potential electrolysis. The 6 e⁻ product can hydrolytically cleave at the 2,3 position to produce the same product as the 4 e⁻ reduction of purine.¹⁸

Reduction of adenyne and cytosine nucleosides and nucleotides

The electrode reduction processes for adenine nucleosides and nucleotides are essentially the same as for adenine itself, centered in the pyrimidine ring but modified by such factors as the changes in electron density and other characteristics of the two pyrimidine ring N=C bonds, association in solution, and orientation and adsorption at the interface.^{1,9,12,20} The need for protonation prior to reduction, *i.e.*, presence of the conjugate acid of the purine as the electrochemically reducible species, has been discussed. Although each adenine ring is only mono-protonated between pH 1.5 and 4.0 with N(1) as the most likely protonation site,^{22,23} monoprotinated species with the proton at N(1), N(3), or N(7) are possible.^{24,25}

Nucleosides and nucleotides

Attachment of a sugar or sugar-phosphate moiety decreases the ease of adenine reducibility except above pH 5, where the waves show kinetic control due to protonation equilibria. The order of observed ease of reducibility may vary somewhat due to the effect of concentration on $U_{\frac{1}{2}}$.²⁰

The net effect of sugar and sugar-phosphate addition is more complex than for such simple substituents as alkyl, amino, alkylamino and arylamino, which decrease the ease of reducibility of the parent pyrimidine or purine;^{10,14,18,20,26} substitution by ribose or ribosophosphate, which involves an electron-withdrawing effect,²⁷⁻²⁹ favors reducibility of the purine ring, but adsorption^{30,31} and intermolecular association^{30,32,33} can decrease the reducibility (*cf.* subsequent discussion). These opposing effects account for the relatively small differences in $U_{\frac{1}{2}}$ in the adenine series (bases, nucleosides and nucleoside monophosphates), which do not exceed 50 mV, compared to *ca.* 120 mV in the cytosine series.

In the cytosine series, the electron-withdrawing effect of the ribose ring would be expected to predominate due to relative proximity of sugar and reduction site, and the decreased tendency for adsorption¹⁸ and intermolecular association compared to the adenine series; the ease of reducibility does increase as expected.¹⁰

The tendency of adenine nucleosides and nucleotides to become more easily reducible than adenine at higher pH, *i.e.*, as the extent of protonation decreases, may indicate that the electron-withdrawing effect of the sugar is weakened on protonation due to the competitive attraction of electrons by the positive charge.

Negatively charged phosphate groups can favor electrostatic repulsion of the compound from the similarly charged D.M.E. surface and can weaken electron-withdrawal by ribose, thus decreasing reducibility; this factor is negligible at low pH where the phosphate group is screened by protons.¹⁵

Di- and oligonucleoside phosphates

Dinucleoside phosphates, which are the lowest-chain-length derivatives capable of base-base intramolecular interactions, *e.g.*, stacking, similar to those operative in polynucleotides and nucleic acids, are the most suitable models for studying the effects of incorporation of a base in a polynucleotide chain on its electrochemical behavior. Their behavior can bridge the gap between the extensive electrochemical data on purines, pyrimidines, and their nucleosides and nucleotides^{1,2} on the one hand and those on polynucleotides and nucleic acids^{21,34,35} on the other.

The reducible di- or oligonucleotide is the protonated form;^{9,12} N(1) in adenine and N(3) in cytosine are the initial reduction sites, as well as the likely protonation sites;¹³ however, equilibria involving non-protonated, monoprotonated and polyprotonated forms may exist.³⁶ Reduction is essentially diffusion controlled with possible kinetic control at higher pH.

The dimer and trimer n values (*e.g.*, ApA, 6.0, (Ap)₂A, 12.0; ApU, 3.6; UpA, 4.2; ApC, 6.2; CpA 6.2; CpC 3.8), although not exact due to approximations made and other influences on the I_d values (*e.g.*, adsorption; association; experimental error), are in good agreement with the respective 4 e⁻ and 2 e⁻ reduction of each adenine and cytosine moiety. Low n values for ApA may be due to uncertainty in extrapolating the I_d - c plot to zero concentration; an n of 6.7 was obtained at the 0.1 mM level. I_d for (Ap)₂A is about three times that of ApU, indicating reduction of all three adenine rings. The similar n values of 12.0, 12.7 and 13.0 for (Ap)₂A, (Ap)₃A and (Ap)₅A, respectively, may indicate that a maximum of three adenine rings are reduced per oligomer. The degree to which intramolecular association and adsorption affect I_d is, at present, uncertain; however, $U_{1/2}$ and the extent of adsorption also show a leveling effect with increasing chain length.

The original paper¹⁷ should be consulted for the effects of the following features of the primary structure on reduction: chemical identity of the heterocyclic bases, chain length, and sequence order.

An important consideration, especially in connection with cytosine derivatives, is the decrease in rate of deamination of the reduced compound as the complexity of the derivatives increases (Table 2). Thus, whereas cytosine gives an apparent faradaic n of between 2 and 3 at the

D.M.E., each cytosine in CpC gives an n of 2; there is, naturally, also the effect of change in diffusion coefficient with increasing size on the observed D.M.E. current.

Adsorption of cytosine and adenine species at aqueous solution|mercury interfaces

The following, largely qualitative discussion is only intended to provide a general background. The increasing interest in studying the adsorption of nucleic acid species, *e.g.*, recent studies of adenine and its nucleosides, nucleotides and higher polymers (*e.g.*, Ref. 37 and 38) should soon result in a sufficiently extensive literature to allow more exact and more conclusive statements to be made concerning adsorption at solution|electrode interfaces. Stress is placed in the present discussion on the adsorption of cytosine species.^{9,12,20}

Among the factors that should be considered in explaining the surface-activity of nucleosides and nucleotides, as well as of higher polymeric species, besides specific adsorption, intermolecular association and reorientation of the molecules on the electrode surface, is multilayer association film formation.

An aspect of adsorption studies, which could be of general interest, involves measurement of the rates at which adsorption equilibrium is established for various types of compounds and experimental conditions.

Adsorption sites and mechanisms

Adsorption at mercury and other electrodes can be due to a variety of causes and potential adsorption sites in the compounds, *e.g.*, π -bonding involving aromatic rings, specific bonding connected to individual nitrogen atoms, C=N double bonds and oxygenated sites, and chemical binding involving negatively charged phosphate groups and metal complex formation. For example, purine-based compounds, with the exception of phosphate derivatives, are adsorbed in the region of the electrocapillary maximum (E.C.M.); phosphate derivatives appear to be more strongly adsorbed on the positive potential side of the E.C.M., suggesting adsorption of a negatively charged species, *e.g.*, one formed by phosphate group dissociation. Adenine possesses the steric configuration necessary for metal chelation; N(7) and the extracyclic nitrogen attached to C(6) provide favorable sites for five-membered ring formation, *e.g.*; divalent metals (copper, zinc and nickel) form metal complexes with purines^{40,41} and mercury(II) reacts with purines, including adenine.⁴²

Purines and pyrimidines

The order of uncharged site-controlled adsorbability observed by total *a.c.* polarography for 6-substituted purines¹⁴ (methoxy- < methylamino-, amino < methyl- < (*iso*-guanine) < dimethylamino- < phenylamino- < benzylamino < hexylamino) indicates that purines with

more bulky substituents are more extensively adsorbed and/or cause greater changes in the double layer (DL) capacitance, probably due to decreased solubility; the latter three derivatives, which exhibit the greatest adsorbability, are quite insoluble in water, *i.e.*, their saturated solutions are *ca.* 0.5 mM.

Differential capacitance data³¹ for 1 M NaCl solutions of the common DNA bases indicate that the surface-activity increases in the order: cytosine < thymine < adenine < guanine. Generally, the order of increasing surface activity of purine and pyrimidine bases can be qualitatively correlated with their decreasing dipole moment, which is also related to the increasingly hydrophobic nature of the species.

Other studies of the adsorption of purine and pyrimidine bases at electrodes, employing bridge impedance and *a.c.* polarographic technics, include Ref. 1,4,5,9,12,31,43-46.

Cytosine species

Although the limiting current is essentially diffusion-controlled for the cytosine derivatives to be discussed,²¹ the data strongly support involvement of adsorption phenomena in their electrode processes. The extent of adsorption and nature of the adsorbing molecule depends upon the species involved and its concentration, pH, and the electrode potential. Furthermore, the data indicate that ribose and ribosophosphate groups strongly influence the adsorption through production of additional adsorption sites in the molecule as a result of the added moiety and/or through the effect of the ribose group on adsorption sites in the cytosine ring.

Dinucleoside phosphates (CpC, CpU and CpG) adsorb much more strongly than cytosine, cytidine and CMP (Fig. 5 to 7), presumably due to the presence of more adsorption sites and possible ring interactions when the dinucleoside phosphate is in the stacked configuration.⁴⁷

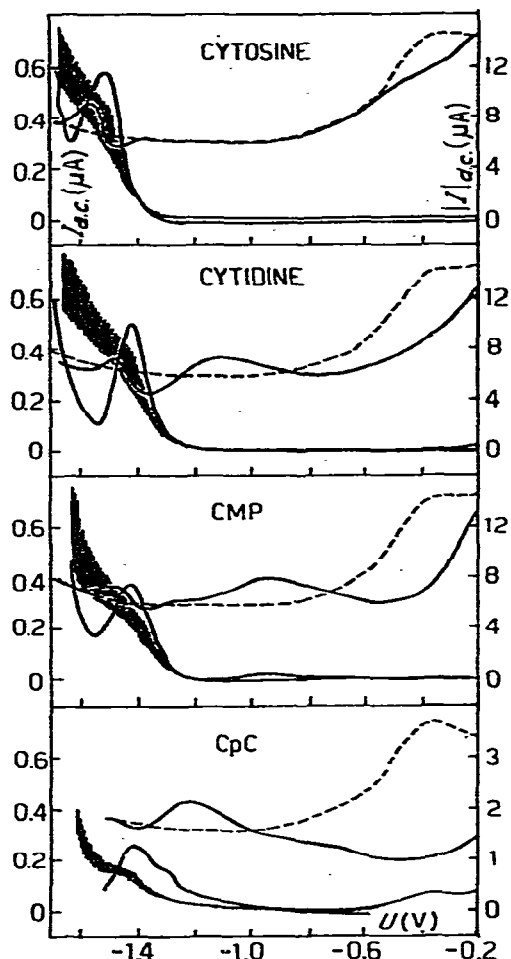
The greater changes in the double-layer capacitance for the dinucleoside phosphates probably also indicate more complex changes and orientations at the interface than for cytosine, cytidine, or CMP, *e.g.*, the smaller peaks seen inside the first depression for the dinucleoside phosphates, especially at higher pH (Fig. 6 and 7), suggest that, over certain pH ranges, two different forms of the compound are adsorbed, which may require its orientation in different ways on the electrode. Since equilibria between species with zero, one or two charges are possible (the species may be zwitterionic or charged positively or negatively),^{36,48} simultaneous adsorption of more than one form is likely with one form, however, probably being predominant, *e.g.*, positively charged and uncharged forms below pH 4 to 5, and zwitterionic and negatively charged forms above pH 5 to 6.

While cytosine, cytidine and CMP produce only a single polarographic reduction wave, CpC and, possibly, CpU and CpG produce two waves (Fig. 5) with only the more positive wave I appearing at very low concentration, *e.g.*, below 0.02 mM. With increasing concentration,

wave I grows in height and levels off at *ca.* 0.2 or 0.3 mM, whereas wave II continues to grow (Fig. 8). This pattern is the consequence of adsorption of the dinucleotide and its reduction product with the prewave (wave I) being assigned to reduction to an adsorbed product.

Fig. 5.

d.c. and *a.c.* polarograms for cytosine, cytidine, CMP and CpC. The upper pair of curves in each panel represents the quadrature *a.c.* component (dashed line is for background solution alone); the lower single line is for the in-phase *a.c.* component. Conditions are 1 mM cytosine derivative, pH 4.2 acetate buffer, 25 °C and 50 Hz, except for CpC, where the conditions are 0.09 mM, pH 5.0 McILVAINE buffer and 0.5 °C. The sensitivity of the in-phase current scale for CpC is four times that shown, *i.e.*, a scale reading of 0.2 μA corresponds to an actual current of 0.05 μA . (From Ref. 9 with permission).



The presence of a small depression for CpC in the quadrature current component just after the potential where the depolarizer is reduced to an adsorbed product (Fig. 5 and 6) suggests a slow adsorption process. A slow process would also explain the presence of wave II at concentrations before the wave I height becomes constant, as the result of competition between the processes producing adsorbed and unadsorbed product.

Association in adsorbed state

Concomitant with adsorption is film formation or association of the adsorbed molecules. Since this occurs at the electrode surface (as opposed to association in solution), it is potential-dependent; as the film forms and then breaks with changing potential, marked changes occur in the double-layer capacitance, *e.g.*, pits or wells appear in the *a.c.* quadrature current component (Fig. 6 and 7). Purine nucleosides generally show a greater tendency to associate than pyrimidine nucleosides. For

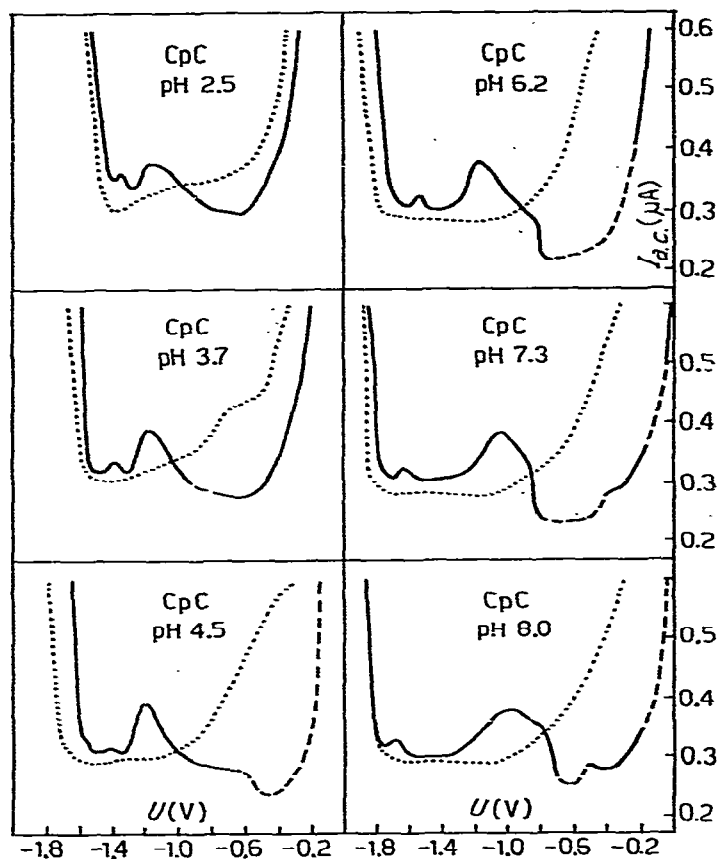
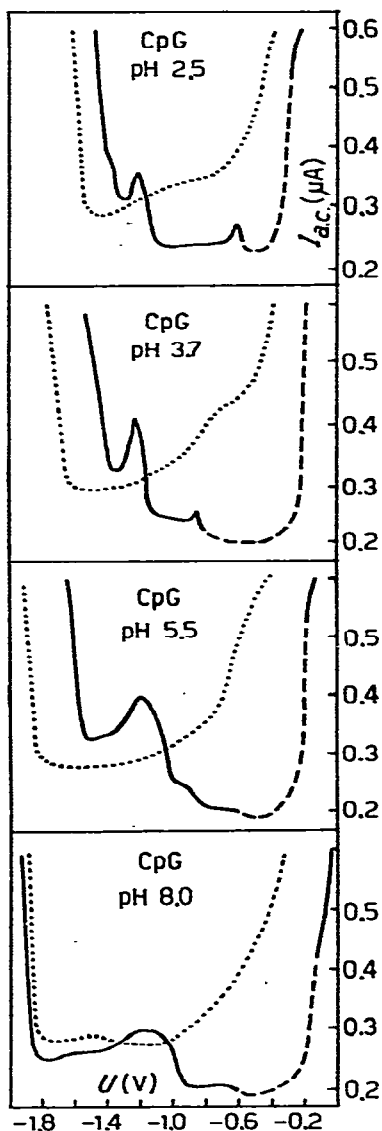


Fig. 6.

Total *a.c.* polarograms for *ca.* 0.05 mM CpC in McILVAINE buffer (pH indicated) at 25°. Dashed lines denote distorted current oscillations; dotted line is for background solution alone. (From Ref. 9 with permission).

example, although cytosine-containing dinucleoside phosphates associate much more than the cytosine nucleoside and nucleotide, *e.g.*, CpC associates even at a solution concentration below 0.05 mM at pH 7.3 (Fig. 6), dinucleoside phosphates containing adenine associate to a greater extent, *e.g.*, at low temperature and pH 5, CpC does not associate at bulk concentrations up to 0.9 mM while ApA associates at concentrations as low as 0.05 mM.¹²

The potential range covered by the pit can also be used to evaluate the extent of association at the interface. For the cytosine dimers studied,¹² this range increases in an order (CpC < CpU < CpA < ApC, CpG) which parallels that for association in solution, *i.e.*, pyrimidine-pyrimidine < purine-pyrimidine < purine-purine,⁴⁹ and, consequently, may indicate similar modes of association at the interface and in solution, *i.e.*,



vertical overlapping or stacking of the bases. Since the principal mode of self-association in the solid state is also base stacking,⁵⁰ it appears reasonable that the adsorbed molecules undergo similar modes of interaction.

In summary, di- and oligonucleoside phosphates are very strongly adsorbed, suggesting the presence of more adsorption sites and possible ring interactions in the stacked configuration. They seem to be adsorbed with rings planar to the electrode surface; in the case of the adenine oligomers, a maximum of four rings can be thus oriented. Most exhibit pH- and concentration-dependent self-association in the adsorbed state, even from very dilute solution; the principal mode of association is probably that of base stacking.

Fig. 7.

Total *a.c.* polarograms for *ca.* 0.05 mM CpG in McILVAINE buffer (pH indicated) at 25 °C. Dashed lines denote distorted current oscillations; dotted line is for background solution alone. (From Ref. 9 with permission).

Association in solution

Association of electroactive species in solution may be related to the changes in I_d and $U_{1/2}$ with concentration. The generally observed decreasing reversibility of the electrode process with increasing concentration of the nucleic acid bases and their ribosophosphate derivatives is in agreement with additional energy being involved, *i.e.*, association increases the activation energy for charge-transfer. Correspondingly, I_d decreases with increasing concentration due to the smaller D for associated molecules.

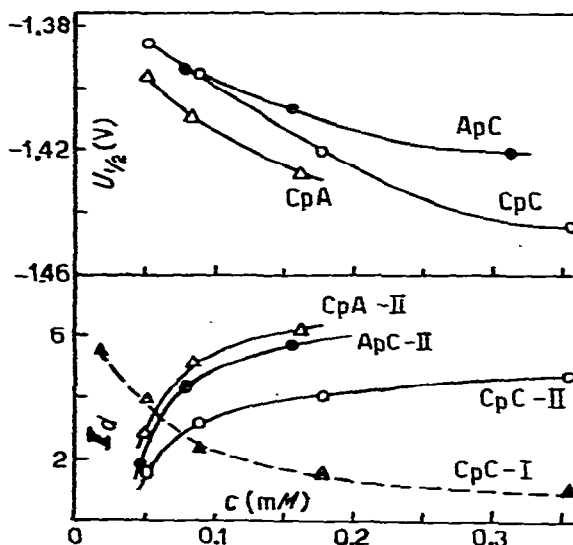


Fig. 8.

Variation of $U_{1/2}$ and diffusion current constant (I_d) with concentration of dinucleoside monophosphates in pH 5.0 McILVAINE buffer (ionic strength: 0.13 M) at 0.5 °C. Roman numerals refer to the waves of each compound. (From Ref. 12 with permission).

Purine derivatives usually associate to a greater extent than pyrimidine species. The predominant mode of association in purine derivatives is vertical stacking due to hydrophobic interaction of bases.^{43,51,52} Since the standard free energy for such association is of the order of the thermal energy, the stacks break and reform rapidly with increased stability being expected at lower temperatures, *e.g.*, the relative decrease in I_d for the adenine nucleoside-nucleotide sequence with increasing concentration is about 2 to 3 times greater at 1.5 than at 25 °C.²⁰

Unfortunately, adsorption and subsequent film formation at the electrode cause similar changes in I_d and $U_{1/2}$ with increasing concentration, making it difficult to interpret the polarographic data solely in terms of association in solution; however, with reasonable assumptions, comparisons are possible, *e.g.*, in respect to the concomitant occurrence of adsorption and solution association in the cytosine series.⁹ However, even after maximum coverage of the electrode with the adsorbed reduced species has been reached (*ca.* 0.3 mM), $U_{1/2}$ for the CpC reduction continues to become more negative with increasing concentration (Fig. 8). This may be due to the presence of intermolecular interactions (*cf.* next paragraph).

Extrapolation of $U_{1/2}$ -concentration curves for CpC (Fig. 8), and ApC and CpA,¹² to zero concentration (and, thus, to zero degree of association) results in a more or less common $U_{1/2}$ at about -1.37 V, compared to $U_{1/2}$ values for adenosine and cytidine of -1.38 V at the pH involved. The increasing $U_{1/2}$ differences between the three dimers with increasing concentration can be explained in terms of increasing differences in the degree of interaction, *e.g.*, association, with the latter being greatest for CpA, at least at concentrations below 0.2 mM.

Di- and oligonucleotides generally tend to show strong intramolecular as well as intermolecular association, *e.g.*, Ref. 15, 25 and 47. The mode of association of dinucleotides is similar to that of the nucleotides.

Diffusion of vertical stacks to the electrode should be compatible with the model proposed for monomers,²⁰ *i.e.*, with the purine ring plane perpendicular to the electrode. Although the arrangement of individual molecules in the stacks is still a matter for speculation, in some models favored for purine nucleosides, pyrimidine ring faces pyrimidine ring with the ribose moieties being opposite to one another in the stack;^{32a,53} the presence of protonated N(1) tends to favor alternate stacking (pyrimidine facing imidazole) due to the reduced electrostatic repulsion. Alternate stacking also better explains a relatively strong effect of even low concentrations on the diffusion coefficient; blockage of reduction sites might occur in stacked molecules that approach the interface with alternate pyrimidine rings oriented away from the surface.

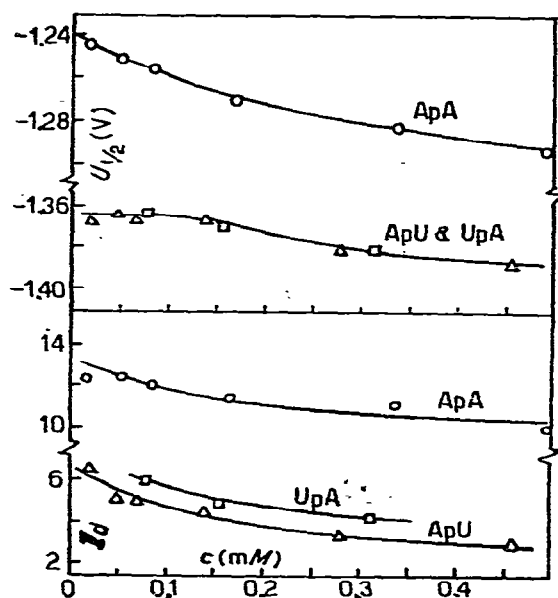


Fig. 9.

Variation of $U_{1/2}$ and diffusion current constant (I_d) with concentration of dinucleoside monophosphates at 0.5 °C. ApU and UpA in pH 5.0 McILVAINE buffer (ionic strength: 0.13 M). ApA in pH 3.5 McILVAINE buffer (ionic strength: 0.1 M). (From Ref. 12 with permission).

Thus, in the case of di- and oligonucleoside phosphates^{9,12} maximum coverage of the interface by the adsorbed reduced layer is presumably reached above 0.1 mM solution concentration (*cf.* variation of I_d with c in Fig. 8 and 9). Above this concentration, however, $U_{1/2}$ continues to become more negative with increasing concentration for adenine-containing dimers and oligomers (Fig. 8 and 10). In addition, $U_{1/2}$ - c plot extrapolation for the adenine oligomers (Fig. 10) to zero concentration (and, thus, to zero degree of association) results in a more or less common $U_{1/2}$ at -1.23 V; similar extrapolation for adenine results in

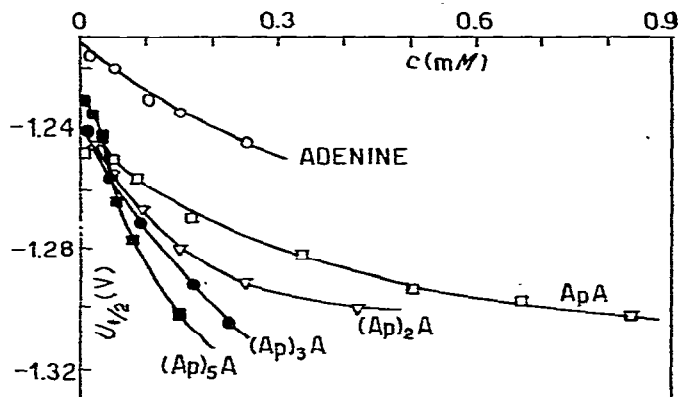


Fig. 10.

Variation of $U_{1/2}$ with concentration of adenine and oligonucleotides in pH 3.5 McILVAINE buffer (ionic strength: 0.1 M) at 0.5°. (From Ref. 12 with permission).

an $\bar{U}_{1/2}$ of about -1.21 V. The increasing differences in $U_{1/2}$ between adenine oligomers with increasing concentration can then be explained in terms of increasing differences in degree of interaction, *e.g.*, association and adsorption, with the latter being greatest for $(Ap)_5A$; the $U_{1/2}$ increment becomes less for each adenine moiety added, indicating possible levelling off at some higher chain length. The differences in extrapolated $U_{1/2}$ for adenine and its oligomers may be related to the stacked configuration for dimer and oligomers, indicating the effect one ring has upon the other when the molecule is reduced.

Even though it is at present not possible to evaluate stacking quantitatively on the basis of polarographically measured diffusion coefficients, the relative variation of the latter with concentration would seem to provide a means of identifying the existence of stacking at concentrations below 1 mM. Although NMR studies provide detailed information concerning the specific mechanisms involved in association phenomena, they have not generally provided information at the millimolar level nor have they furnished an overall index to the extent of association.

It is apparent that quantitative — and even reliable qualitative — interpretation of the polarographic data seen for DNA, RNA, and their large fragments in aqueous media will have to take into account the effects of association in solution upon the observed behavior.

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