POLAROGRAPHIC STUDY OF STRUCTURAL TRANSITION OF HOMOPOLYNUCLEOTIDES CAUSED BY PROTONATION

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The behaviour of polycytidylic and polyadenylic acids was studied by classical polarography, a.c. polarography (after Breyer) and pulse polarography. Derivative pulse polarography enables to determine both substances at low concentrations down to $5 \cdot 10^{-7}$ M (relative to the monomer content). A study of the dependence of the optical density in the ultraviolet region and polarographic currents of both polynucleotides on pH revealed that changes in their polarographic behaviour occurring in a narrow pH range correspond to their structural transition observed spectrophotometrically. With polycytidylic acid the current decreases markedly owing to the formation of a protonated double-helical structure, and as a result of protonation a new peak appears on the pulse-polarographic curve. The suppression of the polarographic reducibility of the protonated polycytidylic acid is attributed to hiding of the reducible groups inside the double helix. The formation of a protonated double helix of polyadenylic acid results in a much less pronounced decrease of the polarographic current.

We showed[1-4] that the single-stranded synthetic polynucleotides, polyadenylic and polycytidylic acids (further: polyA and polyC) are polarographically reducible. In polynucleotide complexes (composed of two complementary strands, *e.g.* polyA and polyU (polyuridylic acid) of a double helical structure similar to the structure of desoxyribonucleic acid (DNA), the reducibility of polyA and polyC is strongly suppressed[1,2,5,6]. The difference in the polarographic behaviour of single-stranded and double-helical unprotonated polynucleotides was made use of in structural analysis of nucleic acids[2,7]. Besides the polynucleotide complexes, polyA and polyC can form double-helical structures composed of two strands of the same polynucleotide stabilized by protons[8,9]. In the double-helical protonated form of polycytidylic acid (further: poly$(C \cdot C)^+$), one proton is bound between two cytosine residues and forms a third hydrogen bond (Fig. 1*a*). On the other hand, in the double-helical protonated form of polyadenylic acid (further: poly$(A \cdot A)^+$), the protons do not participate in the formation of hydrogen bonds but stabilize the structure by their charges (Fig. 1*b*). We attempted to find out how the double-helical protonated structure influences the reducibility of the cytosine or adenine residues. We found earlier[2] a drop in the reducibility of polyC as a result of a small decrease of pH in the region where the single-stranded polyC is converted to the double-helical protonated poly$(C \cdot C)^+$, and pointed out certain differences in the behaviour of poly$(C \cdot C)^+$ and poly$(A \cdot A)^+$. Berg and coworkers[10] studied the thermal denaturation of these substances by a.c. polarography; they found that the changes in the height of the peak formed at a potential close to $-1{\cdot}4$ V reflect changes occurring in the polynucleotides by denaturation. However, the course of the denaturation was not checked by other techniques.

The present work shows on the basis of parallel spectrophotometric and polarographic measurements that the polarographic methods can detect the conversion of single-stranded polynucleotides to their double-helical protonated forms (as a result of pH change) and that they can give valuable information as to the structure and properties of these compounds.

a *b*

FIG. 1

Scheme of Hydrogen Bonds between Bases in Double-Helical Protonated Polynucleotides *a* PolyC (ref.[28]); *b* polyA (ref.[29]). Polarographically reducible double bonds are denoted by circles (broken line); R ribose.

EXPERIMENTAL

Apparatus and chemicals. D.c. and a.c. polarographic measurements were made on the apparatus GWP 563 (Akademie Werkstätten für Forschungsbedarf der DAW, Berlin); the a.c. voltage amplitude was 20 mV at 78 c.p.s., other details were given earlier[11]. Pulse-polarographic measurements were made on an A 3100 Southern-Harwell pulse polarograph with the use of a dropping mercury electrode (preliminary measurements were made on a Mark II Southern-Harwell pulse polarograph in the laboratory of Dr M. Branica, Ruder Boskovic Institute, Zagreb). Unless otherwise indicated, the setting of the apparatus was: *1.* 1 V in 15 min, *2.* integration 3, *3.* auto trig, delay time 2 s, *4.* integrator discharge 20 ms and integrator signal gate 20 ms (the time during which the current is integrated before it is recorded), *5.* recorder sensitivity 1/5. The sensitivity of the amplifier was set so as to obtain well-developed curves. The solutions were deaerated with argon or nitrogen. A mercury pool on the bottom of the polarographic cell served as a reference electrode. The capillary used in pulse polarography had a drop time t 5·5 s and that used in d.c. and a.c. polarography 4·0 s, both at a height of mercury column 40 cm. Spectrophotometric measurements were made on the apparatus Zeiss VSU-2, measurements of pH on a pH meter Radelkis OP 205. The samples of polycytidylic acid were products of the firms Miles, Elkhart, Indiana and Schwarz, Orangeburg, New York. The diagrams shown were obtained with a sample from the latter firm; its single-stranded form had a sedimentation coefficient $S_{20,w}$ 4·2 (pH 6) and its double-stranded protonated form $S_{20,w}$ 6·8 (pH 5). Polyadenylic acid was product of the firm Miles, Elkhart, Indiana. The supporting electrolytes were solutions of sodium chloride with a citrate, phosphate or Britton–Robinson buffer in the usual concentration[12] or diluted 1 : 1. Ethylenediaminetetraacetic acid was always present in 0·001M concentration. The chemicals were of analytical grade. The concentration of the polynucleotide relative to that of the mononucleotide was determined spectrophotometrically.

RESULTS

The single-stranded polyA and polyC are converted to their double-stranded protonated forms in a narrow range of pH values. This structural transition is accompanied by a change in optical properties of the solutions of both compounds[9]. We therefore studied their polarographic behaviour at different pH values and measured simultaneously the optical density of their solutions.

Polycytidylic Acid

Classical polarography and a.c. polarography. The single-stranded form of polyC gives a well-developed d.c. polarographic wave (Fig. 2); the dependence of its height on pH in 0·5M-NaCl + citrate buffer shows an abrupt decrease in the pH range where the single-stranded polyC is converted to its double-helical protonated form (Fig. 2). This change in the polarographic behaviour occurs at a pH lower by 0·2 if a base electrolyte 0·1M-NaCl + Britton–Robinson buffer (diluted 1 : 1) of a lower ionic strength is used, in agreement with the shift of the structural transition[13]. In solutions

FIG. 2

D.C. Polarograms of PolyC

$2 \cdot 10^{-4}$M PolyC + 0·5M-NaCl + citrate buffer, *1* pH 5·0 (double-stranded form), *2* pH 5·8 (single-stranded form). Beginning at −1·0 V, 100 mV/absc., full-scale sensitivity 1·5 μA; S.C.E.

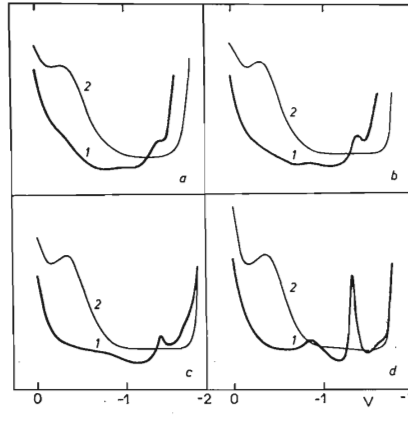

FIG. 3

A.C. Polarograms of PolyC

1 10^{-3}M PolyC + 0·5M-NaCl + citrate buffer; *2* base electrolyte; pH: *a* 5·0; *b* 5·8; *c* 6·2; *d* 7·6. Sensitivity 6 μA; S.C.E.

of 0·1 – 0·3M-NaCl + Britton–Robinson buffer, the curve of poly(C·C)$^+$ shows two waves which, however, are difficult to measure even at a concentration of 0·001M poly-(C·C)$^+$, so their study did not give dependable results.

In the presence of the double-stranded poly(C·C)$^+$, the a.c. polarographic current at positive potentials (Fig. 3*a*) is lowered less markedly than with the single-stranded polyC (Fig. 3*b*,*c*,*d*). The decrease in adsorbability of the protonated polynucleotide at positive potentials is related probably to a partial neutralization of the negative charges of the phosphate groups by the positive charges of the cytosine residues. At negative potentials, there is a marked difference between the curve of polyC at a pH value so high that this compound is not reducible[3] (Fig. 3*d*) and that obtained at lower pH (Fig. 3*b*,*c*) at which polyC is reducible. However, the transition of the single-stranded polyC into the double-helical poly(C·C)$^+$ is nearly undistinguishable on the a.c. polarographic curves at negative potentials (Fig. 3*a*,*b*).

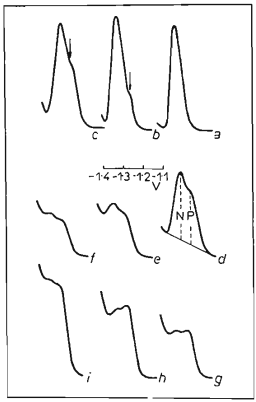

FIG. 4

Derivative Pulse Polarograms of PolyC

$5 . 10^{-5}$M Poly C + 0·5M-NaCl + citrate buffer + 10^{-3}M-EDTA; pH: *a* 6·6; *b* 6·4; *c* 6·0; *d* 5·7; *e* 5·4; *f* 5·1; *g* 4·5; *h* 3·7; *i* 3·5. Beginning at: *a*, *b* —1·2 V; *c*—*g* —1·15 V; *h*, *i* — 1·1 V. 1 V/30 min, amplitude of pulse 50 mV, delay time 2·4 s.

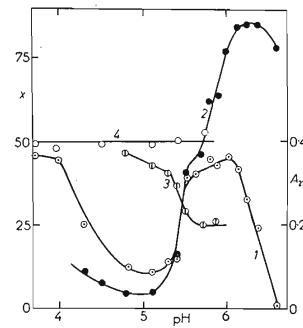

FIG. 5

Dependence of Absorbance and Derivative Pulse-Polarographic Peak Height of PolyC and Cytidine on pH

$5·5 . 10^{-5}$M PolyC + 0·5M-NaCl + citrate buffer. *1* Peak P, *2* peak N; sensitivity 1/16; *3* optical density at 275 nm; *4* $5·5 . 10^{-5}$M cytidine + 0·5M-NaCl + citrate buffer, peak, sensitivity 1/64, *x* peak height (divisions).

Derivative pulse polarography. In agreement with d.c. polarographic results, polyC in various media shows an abrupt decrease in current corresponding to the structural transition. A solution of polyC in 0·5M-NaCl + citrate buffer, pH close to 6·6, gives a simple peak (further: peak N) shown in Fig. 4*a*. With decreasing pH down to pH 6, a linearly increasing inflexion P appears on the positive side of the peak (Figs 4*b*—*d* and 5)*. With a further decrease in pH, both the inflexion P and the peak

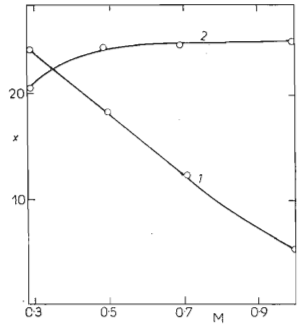

FIG. 6

Dependence of Heights of Derivative Pulse-Polarographic Peak N and Inflexion P on Concentration of NaCl at Constant pH

$5 . 10^{-5}$M PolyC + citrate buffer + 10^{-3}M-EDTA, pH 6·2; concentration of NaCl shown in Table. *1* Inflexion P, *2* peak N; *x* peak height (divisions).

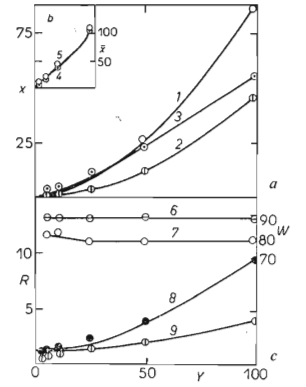

FIG. 7

Dependence of Height and Width of Derivative Pulse-Polarographic Peak of PolyC and Cytidine on Pulse Amplitude

Base electrolyte 0·5M-NaCl + citrate buffer. *a*) Peak height *x* (divisions); *1* $1·4 . 10^{-4}$M polyC, pH 6·2; *2* same as 1 but pH 3·7; *3* $5·5 . 10^{-5}$M cytidine, pH 6·2. Sensitivity 1/128 or higher, the number of divisions recalculated for sensitivity 1/128. *b*) Peak height $\bar{x}$ (divisions); *4* 10^{-4}M polyC, pH 6·2; *5* $5·5 . 10^{-5}$M cytidine, pH 6·2. Sensitivity 1/8 or higher, the number of divisions recalculated for sensitivity 1/8. *c*) Width *w* (mV) in one half of peak height for *6* cytidine, *7* polyC; ratio *R* of peak height obtained with pulse of the same direction as voltage ramp to peak height obtained with pulse of opposite direction: *8* polyC, *9* cytidine.

N decrease first slowly, then beginning from pH 5·5 rapidly (Figs 4*d* – *g* and 5). This rapid decrease corresponds to the transition polyC ⇄ poly(C·C)$^+$ detected spectrophotometrically at 275 nm (Fig. 5). Near to pH 5·0 at which the polynucleotide exists in its protonated double-helical form, the inflexion is observed instead of the peak N. A further decrease in pH causes a change of the inflexion into an increasing peak (Figs 4*g*,*h* and 5) whose growth stops at pH about 4; at pH 3·3 only ill-defined inflexions appear (Fig. 4*i*). In contrast to polyC, monomeric cytidine gives a single peak whose height and width are practically independent of pH (Fig. 5); other peaks or inflexions do not appear.

The results of potentiometric and spectrophotometric titrations[14] suggest that the peak N or inflexion P might be related to protonation of polyC. We therefore studied

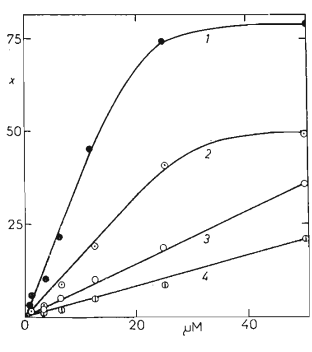

FIG. 8

Dependence of Height of Derivative Pulse-Polarographic Peaks on Concentration of PolyC

Base electrolyte 0·5M-NaCl + citrate buffer + 10^{-3}M-EDTA. *1* pH 5·9, peak N; *2* pH 3·7, peak P; *3* pH 4·8, peak P; *4* pH 4·8, peak N. Pulse amplitude 50 mV, sensitivity 1/64 or higher, number of scale divisions recalculated to sens. 1/64; *x* peak height (divisions).

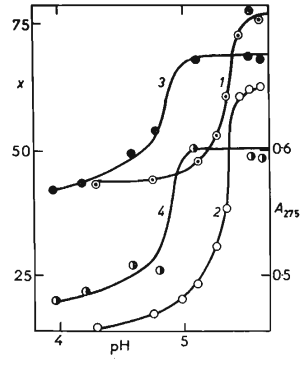

FIG. 9

Dependence of Absorbance and Height of Derivative Pulse-Polarographic Peak for Poly-A on pH

5·5 . 10^{-5}M PolyA + 0·1M-NaCl + citrate buffer: *1* pulse polarography, *2* spectrophotometry. 5·5 . 10^{-5}M polyA + 0·5M-NaCl + citrate buffer: *3* pulse polarography, *4* spectrophotometry. Pulse amplitude 25 mV, sensitivity 1/16; *x* peak height (divisions).

* The mode of determining the height of the peak N and inflexion P is showed by the broken line in Fig. 4*d*. The results of measurement of the inflexion depend on the mode of measurement and are subject to a larger error than usual in pulse polarography; however, this does not affect principally the results.

the dependence of the height of the inflexion P on concentration of NaCl at constant pH. The protonation of polyC is suppressed with increasing concentration of sodium chloride[13]. The height of the inflexion P decreases with increasing ionic strength (Fig. 6); similar results were obtained with a pulse amplitude of 10 mV. Hence, it is probable that the more positive peak P corresponds to reduction of cytosine residues that are protonated in the bulk of the solution, whereas the more negative peak N corresponds to reduction of the unprotonated ones.

The peak height of polyC depends linearly on the pulse amplitude only in the range 2–10 mV (Fig. 7); at 25 mV there is a small deviation from linearity and the peaks obtained with an amplitude of 50–100 mV are much higher than would correspond to a linear dependence. If the pulse is applied in a direction opposite to that in which the voltage ramp is increased, the peak height depends linearly on the pulse amplitude up to 25 mV and nearly equals that measured with the usual pulse direction (Fig. 7*c*) With an amplitude of the oppositely directed pulse of 50–100 mV, the peak height is somewhat smaller than would correspond to a linear dependence and smaller by the factor of about 1/4–1/10 than that obtained with the usual direction of the pulse. The peak height of the monomeric cytidine depends on the pulse amplitude similarly as that of polyC (Fig. 7) except that the deviation from linearity is much smaller. The cytidine peak width in one half of its height does not change with the pulse amplitude, with polyC only small changes were observed at small amplitudes. Fig. 8 shows the dependence of the peak height on concentration of polyC at an amplitude of 50 mV. The sensitivity of the determination of polyC is about $5 \cdot 10^{-7}$M at a pulse amplitude of 100 mV and pH 6·2. (We used much higher concentrations of the polynucleotides because of the lower sensitivity of the spectrophotometric measurements made with the same solutions.)

Normal pulse polarography. In accord with the derivative pulse and d.c. polarography (Figs 5 and 2), the height of the normal pulse-polarographic curve decreases as a result of the formation of poly$(C{\cdot}C)^+$. Normal polarograms of polyC are peak-shaped and their height depends on the starting potential. For example, with a solution of 10^{-4}M polyC + 0·5M-NaCl + citrate buffer, pH 6·2, for starting potential values 0·0, −0·3, −0·6, −0·9 and −1·3 V (*vs* mercury pool) the respective wave heights were 68, 63, 65, 64 and 59 scale divisions. When the pulse direction was reversed, no wave of polyC was obtained at pH 5·1. At pH 6·2 its wave was about 1/70 of that corresponding to the usual pulse direction; the cytidine wave was ill-developed and lower by a factor of about 1/10. The sensitivity of determination of the polynucleotide is lower than with the derivative method (amplitude 100 mV).

Polyadenylic Acid

The conversion of the single-stranded poly A into its double-helical protonated form was studied by d.c. and derivative pulse polarography. Both methods showed a decrease of the polarographic current due to the formation of poly$(A{\cdot}A)^+$; however, the drop of current was small compared with polyC (Fig. 9). The pH range in which this drop occurs depends on the ionic strength in accord with the shift of the transition

polyA $\rightleftarrows$ poly$(A{\cdot}A)^+$ determined spectrophotometrically. In contrast to polyC, the derivative pulse-polarographic peaks of polyA are simple and changing pH does not result in the formation of new peaks or inflexions neither in changes of the peak width. The experiments with polyA have not yet been finished and further details will be published elsewhere.

DISCUSSION

According to our results, the polarographic methods indicate the transition of the single-stranded unprotonated to the double-helical protonated polynucleotide by a change of the polarographic current. With polyC, this conversion is accompanied by a change in the reduction potential. The dependence of the polarographic current of the polynucleotide on pH is different from the apparent polarographic dissociation curves observed with numerous organic compounds[15,16]: the polarographic current of the protonated form of the polynucleotide is smaller than with the unprotonated one and the pK values of the equilibrium obtained polarographically are in accord with the spectrophotometric ones (Figs 5, 9). It seems therefore that the changes in polarographic current in the transition region are not directly related to protonation but result from changes in the structure and properties of the polynucleotide caused by protonation.

The cytosine residues are in the single-stranded polyC reduced analogously as in monomeric units[4]. Cytosine in its monomeric form is reduced only after protonation[17], which can take place either in the bulk of the solution or at the electrode. The single-stranded polyC is reduced probably also in the protonated state[3]; protonation at the electrode, however, does not cause the formation of the double-helical structure. If the latter were formed secondarily at the electrode, the marked changes in the polarographic behaviour of polyC during its structural transition in the bulk of the solution (Figs 4, 5) would be unlikely to happen. Probably the protonation and electroreduction are much more rapid than the formation of the double helix.

The comparison of the heights of the normal pulse-polarographic curves during rise of the potential either to more negative or to more positive values (sweep forward and sweep reverse) suggests, as expected, an irreversible reduction of polyC and cytidine (in the case of a reversible reduction, both curves would be equal in height[17]). The height of the normal pulse-polarographic wave is influenced by the starting potential probably because polyC is accumulated on the electrode surface during the time for which the starting potential is maintained. Since the adsorbability of polyC depends on potential (Fig. 3*c*), the chosen starting potential influences the amount of polyC accumulated on the electrode surface before applying the pulse. It was shown[3] that accumulation of a polynucleotide on the electrode influences also the depth of the indentation on the oscillopolarographic "first curve"[18] $\mathrm{d}E/\mathrm{d}t = \mathrm{f}(E)$. The maximum on the normal pulse-polarographic curve is probably also related to adsorption of the depolarizer[19,20]. The derivative method is best suited for analytical purposes thanks to its sensitivity, resolving power and independence of the starting potential. The derivative peak height of polyC depends linearly on the pulse amplitude (Fig. 7) in agreement with the theory[21] if the pulse height does not exceed

10 mV; otherwise the peak increases more than would correspond to a linear dependence, possibly owing to the closeness of the potentials of reduction and desorption of polyC (Figs 2, 3). In derivative pulse polarography, the voltage applied to the electrode rises slowly and one voltage pulse is applied to every mercury drop at a chosen moment. With a pulse of an amplitude of 100 mV whose potential, E_p, reaches the potential of the peak, E_s, polyC is strongly adsorbed on the electrode before the pulse is started (Fig. 3*b*) and is not reduced. This adsorbed portion is then reduced during the pulse duration together with polyC transported by diffusion. On the other hand, if the pulse amplitude is small, *e.g.* 10 mV, the electrode potential before starting the pulse (with $E_p = E_s$) is such that the polynucleotide is reduced and partially desorbed (Figs 2*b*, 3*b*), hence its accumulation on the electrode is prevented. The dependence of the peak height on the amplitude of the pulse whose direction is opposed to the voltage ramp (Fig. 7*c*) is in accord with the mentioned explanation; in this case the accumulation of the oxidized form of the depolarizer on the electrode before the pulse is applied cannot take place. The increased sensitivity in derivative pulse polarography caused by adsorption of an inorganic depolarizer was observed by Barker and Bolzan[20] who did not study the dependence of the peak height on the pulse amplitude.

With both polynucleotides under study, a drop of the polarographic current was observed in the pH region where the single-stranded form is changed to the double-helical protonated one (Figs 4, 9). With polyC, this drop is considerable and accompanied by the appearance of the peak P due to protonation (Figs 4, 5), whereas with polyA the current drop is small (Fig. 9) and no other peak is formed. The drop of the polarographic current could be caused by a diminution of the diffusion coefficient of the depolarizer on one hand and by changes of its structure and reducibility on the other hand (*e.g.* steric hindrance of the electroactive group). With polyC and polyA a large decrease of the diffusion coefficient due to formation of their protonated double-helical forms cannot be expected (the diffusion coefficients calculated from the Svedberg[22] or Scheraga–Mandelkern equation[23] can be for double-helical structures even somewhat larger than for single ones as follows from the relation between sedimentation coefficients, relative molecular masses[24] and intrinsic viscosities of the polynucleotides). The polarographic current could be influenced also by aggregation of the double-helical molecules. Their lateral aggregation under similar conditions as ours was observed with polyA[26] but not with polyC[9,25]. Finally, it follows from the a.c. polarographic measurements that the transition of polyC to poly(C·C)$^+$ is not accompanied by marked changes of its adsorbability at negative potentials (Fig. 3*a*,*b*), which could change the height of the polarographic curve. The mentioned facts together with the presence of two peaks at different potentials (Fig. 4) due to reduction of protonated and unprotonated (in the bulk) cytosine residues in polyC suggest that the observed current drop can be attributed to a decreased reducibility of the cytosine residues in poly(C·C)$^+$. The decrease of the pulse-polarographic peak with decreasing pH proceeds in two stages (Fig. 5): in the first stage the height of the peak N decreases (while peak P changes only little) and the optical density at 275 nm is constant, in the second both peaks decrease simultaneously with changes in optical density.

The first stage corresponds to the pH region where protonation of a relatively small number of cytosine residues takes place. The possibility of structural changes in this stage was pointed out by Guschlbauer[27] on the basis of optical rotary dispersion measurements (previous measurements by different methods[14,25] did not reveal their existence) and he concluded that polyC does not have any secondary structure in a narrow pH region slightly above pK of the transition. It seems little probable that the observed decrease of the polarographic current in that pH region could be due to a process in which polyC would lose its ordered structure.

After attainment of the minimum, both peaks P and N increase (Figs 4, 5) probably as a result of structural changes of poly$(C{\cdot}C)^+$ enabling better reducibility of cytosine residues. Structural changes of poly$(C{\cdot}C)^+$ depending on pH at room temperature in the considered pH range have not yet been observed. However, the fact that lowering of pH close to 4 (proton destabilization zone) causes a decrease of the thermal stability of the double helix[25,27] is an evidence for destabilization of the double helix of poly$(C{\cdot}C)^+$ by a further addition of protons. In the case of polyA, the reason for the current decrease during transition of the single-stranded to the double-stranded form (Fig. 9) remains unclear. The arrangement of the adenine residues in the double-helical structure may have a small or no influence on their reducibility (as compared with their arrangement in the single-stranded form) and the observed decrease of current may be related mainly to the degree of aggregation and length of the double-helical molecule. The small influence of the double-helical structure on the reducibility of poly$(A{\cdot}A)^+$ could be attributed to the fact that the reducible double bonds between C-6 and N-1 are not a part of the hydrogen bonds (between 6-NH_2 and N-1). (Fig. 1*b*) and are placed near the surface of the poly$(A{\cdot}A)^+$ molecule. Thus, their localization differs from reduction sites in the poly(A)·(U) complex or in the protonated poly$(C{\cdot}C)^+$ (Fig. 1*a*), which are hidden inside the double helix.

It can be concluded that the polarographic methods reflect sensitively the changes in structure of homopolynucleotides proceeding in the bulk of solution by protonation. This finding is remarkable mainly because both polyA and polyC are reduced in the adsorbed state[3]; moreover, the reduction of polynucleotides unprotonated in the bulk of the solution is probably preceded by their protonation at the electrode. Nevertheless, the resulting polarographic signal of polyC is influenced by its conformation (and protonation) in the bulk of solution. It seems that the initial stage of adsorption of the polynucleotide is controlled by its conformation in solution and that the electron transfer follows immediately after anchoring of poly C on the electrode. Structural changes, if any, of the adsorbed polynucleotide are doubtless much slower than the electroreduction and have practically no influence on the polarographic current.

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