Structural transitions of polyadenylic acid due to protonation: the influence of the length of single strands on the polarographic behaviour of the double-helical form

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ABSTRACT

Transition of single-stranded poly(A) into its double-helical protonated form was followed by means of derivative pulse polarography, spectrophotometry, and other methods. It was found that properties of protonated poly(A) depended on the length of single strands from which the protonated double helix was formed. In contrary to longer poly(A) transition of short single-stranded molecules ($s_{20,w}$ lower than about 3) caused practically no decrease in the pulse-polarographic current. It was concluded that the formation of the protonated double helix of poly(A) did not result in the inaccessibility of the reduction sites (located in the vicinity of the surface of the molecule) for the electrode process, as it was in DNA-like double-helical polynucleotides. The current changes observed in the course of transition of longer poly(A) were explained as due to slower transport of long double-stranded molecules to the electrode.

INTRODUCTION

The relation between structure of single-stranded and double-helical non-protonated polynucleotides and their polarographic behaviour was studied in our preceding communications (1-3). Recently we have investigated the polarographic behaviour of protonated homopolynucleotides (4,5). Formation of protonated double-helical poly(C) caused a marked decrease of polarographic current and an appearance of a new pulse-polarographic peak at more positive potentials (4), i.e. changes which were to a certain extent analogous to those accompanying formation of the non-protonated double-helices (e.g. poly(A)·poly(U)). On the contrary, formation of protonated double-helical poly(A) was accompanied only by a relatively low decrease of current (4,5) and no new pulse-polarographic peak appeared. Janík, Sommer and Bobst (6) have recently studied poly(A) protonation by means of d.c. and normal pulse polarography. However, these authors

observed a marked decrease of the polarographic current in the transition region. They explained this decrease by hiding of reduction sites inside of the double helix of protonated poly(A). To elucidate the difference between their (6) and our (4,5) data we investigated the polarographic behaviour of protonated poly(A) in greater detail and we ascertained that a connection exists between the length of molecule of single-stranded poly(A) and the polarographic behaviour of double-helical poly(A).

MATERIALS AND METHODS

Samples of poly(A) were purchased from various firms: Samples 2 and 5 from the firm Schwarz, Orangeburg, New York, sample 3 from Miles Laboratories, Elkhart, Indiana, sample 4 from the firm Reanal, Budapest. Sample 1 was obtained by courtesy of dr. W. Guschlbauer. Poly(C) was from the firm Schwarz, Orangeburg, New York. Derivative pulse-polarograms were measured on a A 3100 Southern-Harwell pulse polarograph with dropping mercury electrode. The details were published earlier (4). Background electrolytes contained EDTA in concentration 1×10^{-3} M if not otherwise stated. Polynucleotide concentration related to monomer content was estimated spectrophotometrically. Spectrophotometric measurements were performed on apparatuses VSU-2 or Specord (Zeiss, Jena). Sedimentation constants were measured on a Spinco ultracentrifuge model E under conditions given in Tab. I.

RESULTS

A comparison of optical, sedimentation, and polarographic measurements for five samples of poly(A) showed that the ratio of the derivative pulse-polarographic peak heights of single- and double-stranded poly(A) grew with increasing $a_{20,w}$ of the single-stranded form (Tab. I), whereas the ratio of optical densities (at $\lambda = 257$ nm) was nearly constant for all samples. To check whether the above mentioned relation is not fortuitous, we carried out an alkaline degradation of sample of poly(A) with relatively high sedimentation coefficient (Table I, sample L). A solution of 1×10^{-4} M poly(A) in 0.1 NaOH was incubated

TABLE I

POLAROGRAPHIC REDUCIBILITY AND SEDIMENTATION COEFFICIENTS
 $s_{20,w}$ OF DIFFERENT SAMPLES OF POLYADENYLIC ACID

Sample	Ratio of the height of pulse-polarographic peaks of single- to double-stranded poly(A)	$s_{20,w}$ of poly(A)	
		single-stranded	double-stranded
1	2.8	6.4	
2 (L)	2.4	5.9	15.1
3	2.3	5.8	
4	1.5	3.6	
5 (S)	1.1	2.6	4.2

The measurements were carried out in 0.1 M NaCl - citrate with 1×10^{-3} M EDTA at pH 5.8 for the single-stranded form and at pH 4.9 for the double-stranded form at a poly(A) concentration of 1×10^{-4} M.

TABLE II

HEIGHTS OF PULSE-POLAROGRAPHIC PEAKS AND $s_{20,w}$ OF POLYADENYLIC ACID AT VARIOUS pH VALUES

pH	Sample			
	L_n		S_d	
	non-annealed peak height a)	heat-annealed peak height a)	non-annealed peak height b)	heat-annealed peak height b)
	$s_{20,w}$	$s_{20,w}$	$s_{20,w}$	$s_{20,w}$
5.9	40	41	49	48
5.1	19	20	67	61
4.3	22	6	70	44
		16 ^c		16 ^c

Poly(A) at a concentration of 2.5×10^{-5} M for pulse-polarographic and 8.5×10^{-5} M for sedimentations measurements in 0.5 M NaCl with McIlvaine buffer. Heat-annealing was performed in the way described by Janík et al. (6).

a) amplifier sensitivity 1/32; b) amplifier sensitivity 1/64;
 c) approximate value; diffuse boundary.

Fig. 1. Dependence of the pulse-polarographic peak heights of poly(A) and poly(C) on a degree of their degradation. $\circ$ — $\circ$, single-stranded poly(A); $\bullet$ — $\bullet$, double-stranded protonated poly(A); $\square$ — $\square$, single-stranded poly(C); $\blacksquare$ — $\blacksquare$, double-helical protonated poly(C). A polynucleotide in concentration 1×10^{-4} M was incubated at 23° C in 0.1 M NaOH; samples were withdrawn in time intervals given in the graph and neutralized. Polarograms of polynucleotides at a concentration of 4×10^{-5} M were measured in a medium containing 0.55 M NaCl, 0.1 M sodium citrate at pH 5.7 for the single-stranded form and at pH 5.0 for the double-stranded protonated form. The peak height of a sample of single-stranded polynucleotide withdrawn in time 0 was taken as 100%; derivation 50 mV.

at 23° C; samples withdrawn at different time intervals were neutralized. Pulse-polarographic measurements were then performed in 0.55 M NaCl with 0.1 M sodium citrate at pH 5.0 and 5.7. As it follows from Fig. 1, the ratio of the peak heights of single- to double-stranded poly(A) decreased continuously with time. After 15 min. degradation both forms of poly(A) yielded the peaks of approximately the same height (similarly to sample S in Fig. 2A). After longer degradation the peaks of double-stranded poly(A) were even slightly higher than those of single-stranded form. This result thus confirmed that the differences in polarographic behaviour of different preparations of poly(A) are connected with the length of its molecule. Similarly to poly(A), the degradation was also carried out with poly(C), for which marked changes in pulse-polarographic behaviour were observed earlier in the region of transition into its protonated double-stranded form (4). In agreement with the fact that these changes were basically the same in all studied preparations of poly(C) (4), the degradation of poly(C) influenced relatively little the pulse-polarographic behaviour of single- and double-stranded poly(C) (Fig. 1). The higher currents yielded by both forms of the degraded poly(C) are probably in a connection with more rapid transport of the depolarizer to the electrode.

Transition of poly(A) was investigated in a greater detail in the sample L mentioned above and in the sample S, which had the lowest sedimentation coefficient in the series of preparations studied (Tab. I). For the sample S practically no change in the height of the pulse-polarographic peak (Fig. 2A) was observed in the transition region (Fig. 2B). A small change in the peak height between pH 5.6 and 5.9 took place under conditions, when /according to the spectrophotometric measurements/ poly(A) was in the single-stranded form. On the contrary with the sample L a distinct decrease of the pulse-polarographic peak height was observed in the transition region (Fig. 2A). If the measurements were carried out at a lower ionic strength, the region of transition detected polarographically was shifted to higher pH values in agreement with spectrophotometric measu-

Fig. 2. A/ Dependence of the pulse-polarographic peak heights of the poly(A) samples L and S on pH. $\circ$ — $\circ$, sample S; $\times$ — $\times$, sample L; the measurements were carried out at polynucleotide concentration of 1×10^{-4} M in 0.5 M NaCl with 0.1 M sodium citrate; derivation 10 mV, amplifier sensitivity 1/8. B/ Dependence of optical parameters of poly(A) on pH. $\circ$ — $\circ$, sample S; $\times$ — $\times$, sample L; $A_{\max}$, optical density in absorption maximum; A_{300} , optical density at $\lambda = 300$ nm; $\lambda_{\max}$, wavelength of absorption maximum.

rements.

In the pH region 5.5 - 4.3 heights of the pulse-polarographic peak of the sample S did not depend practically on pH (Fig. 2A); in the pH region 3.9 - 3.3 the peak decreased, most probably in a connection with aggregation indicated by changes in optical density (Fig. 2B). For the sample L the decrease of the peak at pH 3.9 was preceded by its increase in the region of pH 4.3. The change of the height as well as of the wavelength of the absorption maximum in the transition region was approximately the same for both samples (Fig. 2B). In the region of pH 3.8 - 5.2 the height of the absorption maximum changed with pH only slightly; the maximum wavelength practically did not change. At low pH values a marked increase of absorbance was observed at wavelengths in the vicinity of 300 nm due to light scattering on aggregated poly(A) (7).

The pulse-polarographic peak height of double-stranded poly(A) was followed in dependence on the polynucleotide concentration in the course of formation of the protonated form. To poly(A) solutions of different concentrations in 0.05 M NaCl with 1×10^{-4} M sodium phosphate at pH 7.6 an equal volume of an acidifying solution was added to final concentration 0.5 M NaCl with 0.1 M sodium citrate (pH 4.9). Concentration of the double-stranded poly(A) was then adjusted to 2×10^{-5} M with 0.5 M NaCl - 0.1 M sodium citrate (pH 4.9) and at this concentration of the polynucleotide the pulse polarogram was measured. Whereas only a slight decrease of the pulse-polarographic peak with increasing concentration of the polynucleotide (from 2×10^{-5} M to 3.15×10^{-3} M) took place for the sample S the decrease was very expressive for the sample L (Fig. 3). The peak height of poly(A) the double-helical structure of which was formed in the highest polynucleotide concentration was nearly by one half lower. On the contrary the height of absorption maximum of both samples did not practically change in dependence on poly(A) concentration at which the double helix was formed. The increase of ionic strength from about 0.5 to 2.0 at constant polynucleotide concentration resulted in a decrease of polarographic peaks of both single- and double-stranded poly(A). This decrease was

Fig. 3. Dependence of the pulse-polarographic peak height on concentration of poly(A) in the course of acidification. x—x, sample S; •—•, sample L. The solutions of poly(A) in concentrations given in the graph in 0.05 M NaCl with 1×10^{-4} M sodium phosphate at pH 7.6 were acidified by addition of the same volume of 1.0 M NaCl with 0.2 M sodium citrate, pH 4.9. Pulse-polarographic measurements were carried out at polynucleotide concentration of 2×10^{-5} M in 0.5 M NaCl - 0.1 M citrate with 1×10^{-3} M EDTA at pH 4.9; derivation 50 mV, amplifier sensitivity 1/16 for the sample S, 1/8 for the sample L.

more marked in the sample L.

The current increase observed around pH 4.4 in the sample L (Fig. 2A) basically agreed with the measurements of Janík et al. (6), who analyzed only poly(A) of high molecular weight ($1.2 - 1.6 \times 10^6$ daltons). This current increase was explained by the above mentioned authors (6) by formation of the "frozen" form of poly(A) with imperfect double helix. However, if poly(A) was heat-annealed (i.e. heated approximately to 80°C and then slowly cooled) or annealed (i.e. transferred slowly, by means of dialysis, from neutral to acid pH), instead of the current increase a further current decrease was observed, which was explained (6) as due to formation of the "tightly packed" conformation of double-stranded poly(A). We attempted to ascertain what would be the effect of the heat-annealing on samples of different molecular weights. Molecular weight of our original sample L was considerably reduced due to longer storage (further it will be denoted as L_d); therefore it was substituted by a new sample L_n having sufficiently high $s_{20,w}$ (Tab. II). The storage decreased also $s_{20,w}$ of the sample S (Tab. II), however its applicability was not influenced, and thus it was further used with a new denotation S_d . After heat-annealing of both samples at pH 4.4 /in the same manner as described by Janík et al. (6)/ a disappearance of the peak of the sample L_n (Tab. II) was observed, while only slight decrease of the peak height took place in the sample S_d . The heat-annealing of the sample L_n was carried out at different temperature and we found that the peak height decreased markedly even upon heating to temperatures lower than the denaturation temperature detected spectrophotometrically (Fig. 4). The peak was reduced also at room temperature, even though more slowly than at 50°C . The heat-annealing carried out at 50°C led to a faster decrease of the peak in a medium of higher ionic strength (0.5 M NaCl) than of lower ionic strength (0.1 M NaCl) (Fig. 5).

The difference in the peak heights of the annealed and non-annealed double-stranded samples (L_n) could be observed in a broad concentration range (Fig. 6A). If the dependences of the peak height on concentration of poly(A) samples L_n and L_d

Fig. 4. Dependence of the pulse-polarographic peak height of poly(A) (sample L_N) on temperature and duration of annealing. x—x, temperature dependence. 2.5×10^{-5} M poly(A) in 0.5 M NaCl with Britton-Robinson buffer (2), pH 4.3 was heated at the temperature given in the graph for 10 minutes and then slowly cooled (within 30 minutes) to room temperature; amplifier sensitivity 1/16, derivation 50 mV. o—o, time dependence. Poly(A) was incubated at room temperature and samples were withdrawn and measured in the time intervals indicated in the figure; other conditions were the same as above. The peak height of a sample withdrawn in time 0, respectively at temperature 0° C, was taken as 100%.

Fig. 5. Dependence of the pulse-polarographic peak height on the duration of heat-annealing. Poly(A) was incubated at 50° C; samples were withdrawn in the time intervals indicated in the figure, cooled to room temperature and measured. Other conditions were the same as in the Fig. 5. o—o, 0.1 M NaCl with Britton-Robinson buffer (2), pH 4.3; x—x, 0.5 M NaCl with Britton-Robinson buffer, pH 4.3.

Fig. 6. A/ Dependence of the peak height of the sample L_n on concentration of poly(A). $\bullet$ — $\bullet$, non-annealed sample, $\square$ — $\square$, heat-annealed sample. 0.5 M NaCl with 0.1 M citrate buffer and 5×10^{-4} M EDTA at pH 4.4. Sensitivity 1/32, derivation 50 mV. B/ Dependence of the peak height of the (non-annealed) samples L_d and L_n on concentration of poly(A). $\blacksquare$ — $\blacksquare$, sample L_n , pH 5.0; $\square$ — $\square$, sample L_d , pH 5.0; $\bullet$ — $\bullet$, sample L_n , pH 5.8; $\circ$ — $\circ$, sample L_d , pH 5.8. 0.5 M NaCl with 0.1 M citrate buffer and 5×10^{-4} M EDTA. Sensitivity 1/32, derivation 50 mV.

were followed at pH 5.8 and 5.0, the ratio of the peak heights of single- and double-stranded poly(A) decreased with increasing concentration (Fig. 6B). This ratio differed only little from unity for high concentrations of the polynucleotides, when the peak height did not depend on poly(A) concentration any more. We also followed how $s_{20,w}$ and ultraviolet absorption spectra were changed as a consequence of the heat-annealing. We found that the values of sedimentation coefficient increased after the heat-annealing; for the sample L_n a decrease of absorption maximum was observed together with an increase of optical density at $\lambda = 300$ nm. Absorption spectrum of a sample heat-annealed at pH 4.1 was very similar to that of a non-annealed sample at pH 3.5.

DISCUSSION

The hitherto obtained results of studies of natural and synthetic polynucleotides by means of the derivative pulse-polarographic method (1-5) showed that each structural transition detectable spectrophotometrically manifested itself by change in the polarographic behaviour (formation of the double helix was, as a rule, accompanied by a disappearance or by a marked decrease of the peak of single-stranded polynucleotide, and by an appearance of a small peak at more positive potentials). In the present paper we report for the first time a case, when formation of the double-helical structure (in the sample S) is not accompanied by a marked decrease of the pulse-polarographic peak. An explanation of this fact could be sought in the arrangement of bases in the protonated double-helix of poly(A) (8), which differs from the arrangement of bases in DNA, double-stranded RNA, and synthetic polynucleotides of the type poly(A)·poly(U) or protonated poly(C). In the latter polynucleotides the reducible double bonds between N-1 and C-6 are a part of the system of hydrogen bonds and are not accessible for the electrode reaction. In double-stranded poly(A) the proton located on N-1 does not participate in formation of hydrogen bond and the double bond between N-1 and C-6 is closer to surface of the macromolecule so that it might be polarographically

reducible. If this interpretation is correct, it is necessary to explain the decrease of the polarographic current which was observed in connection with the formation of double helix in longer molecules of poly(A) by Janík et al. (6) as well as by us. This decrease could be caused by: (a) a reduction of the transport rate of poly(A) to the electrode conditioned by a relatively great length of poly(A) molecules and/or by their aggregation; (b) an inaccessibility of a part of reduction sites for the electrode process as a consequence of their hiding either (i) inside the aggregates, or (ii) inside the poly(A) double helix, the structural form of which then had to differ from the form of short molecules of poly(A) (e.g. the sample S). However, the difference in structural forms of short and long molecules of double-stranded poly(A) is rather improbable.

Using the values of sedimentation coefficient we attempted to estimate roughly diffusion coefficient D characterizing the rate of transport of poly(A) acid to the electrode. Diffusion coefficients were calculated from sedimentation measurements (Tab. I) (9,10). Transition of single-stranded poly(A) into its protonated form caused an approximately twofold increase of diffusion coefficient for the sample S (from the value $3.5 \times 10^{-7} \text{ cm}^2 \text{ s}^{-1}$ to $6.9 \times 10^{-7} \text{ cm}^2 \text{ s}^{-1}$) in contrast to an approximately twofold decrease for the sample L (from the value $1.5 \times 10^{-7} \text{ cm}^2 \text{ s}^{-1}$ to $0.64 \times 10^{-7} \text{ cm}^2 \text{ s}^{-1}$). Even though the calculated values can be subject to a considerable error, they indicate that the change of D upon poly(A) transition can contribute to the current decrease in the case of longer molecules of poly(A). On the contrary, the increase of D due to formation of the double helix from shorter molecules of poly(A) could explain the increase of current observed for the sample of poly(A) strongly degraded by alkali (Fig. 1).

The dependence of the polarographic current on ionic strength as well as the results of the experiment in which the influence of poly(A) concentration in the course of the double helix formation on the peak height was followed (Fig. 3) give evidence for a higher tendency to aggregation and/or to elongation of molecule in the sample L. It follows from the earlier

papers (9,11) that the length of the molecule of newly formed double-stranded poly(A) grows also with increasing concentration of poly(A). The difference in the peak heights of single- and double-stranded samples L_n and L_d disappeared partially or completely if the polarographic measurement was carried out at high polynucleotide concentration, when the peak height ceased to be dependent on the rate of depolarizer transport to the electrode (Fig. 6B). This fact gives evidence that the current decrease observed in connection with formation of the poly(A) double helix in the vicinity of pH 5 is conditioned by a lower rate of transport of poly(A) to the electrode rather than by the inaccessibility of reduction sites for the electrode process, as it was suggested by Janík et al. (6). The transient increase of the polarographic current in the region of pH 4 - 5 observed by Janík et al. (6) and by us (Fig. 2A) for the samples of poly(A) with longer chains could be connected with formation of the "frozen" form (6) containing mismatched strands; in this form also a smaller tendency can be expected to formation of long double-helical molecules occurring by chain overlap.

Our results indicate that the heat-annealing cause primarily aggregation. An evidence for it is given by the marked increase of $s_{20,w}$ due to the heat-annealing (Tab. II), by the changes in the absorption spectra, by the fact that the peak decreases with time faster in higher ionic strength (Fig. 5) (which destabilizes the double helix of poly(A) and stimulates the aggregation (7,12,13), as well as by the fact that the heat-annealing caused the decrease of the polarographic current even then, if the denaturation temperature of double-stranded poly(A) was not reached. It seems probable that in the case of "annealing" as it was performed by Janík et al. (6) above all, the long duration of dialysis was important for the resulting effect, since at lower temperatures the polarographic current decreases slowly even without dialysis (Fig. 4). Groups in the vicinity of C-6 of adenine residues /capable of participation in polarographic reduction (1) as well as in reaction with HCHO (6)/ are hidden in the form of poly(A) which arises owing to the heat-annealing. Hiding of a considerable fraction of these

groups can be realized on the basis of lateral aggregation of poly(A) molecules. It is not excluded that certain changes in the secondary structure of poly(A) also take place as a result of the heat-annealing; it seems, however, that they cannot be reliably studied by methods as polarography, absorption spectrophotometry, and others, the results of which are influenced by aggregation.

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