STUDY OF POLYMER CHAIN DYNAMICS IN SOLUTION BY TIME-RESOLVED SPECTROFLUOROMETRY

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Dedicated to late Academician Eduard Hála.

The methodology of polarization time-resolved fluorometry and interpretation of its results are outlined at a general level, and the measurement on and use of facilities of the Edinburgh Instruments Model 299T apparatus are discussed in detail. The dynamics of conformational changes in chains of poly(methacrylic acid) and poly(acrylic acid) containing covalently bonded dansyl labels are studied in aqueous solutions at various pH. It is shown that at pH > 6, the shorter effective rotational correlation time $\tau_r < 2$ ns corresponds to the rotation of the free dansyl label about bonds by which it is attached to the polymeric chain; at pH < 4 the longer effective rotational correlation time $\tau_r = 20-26$ ns corresponds to the rotation of the compact spherical formation constituted by a part of the collapsed polymeric chain in which the label is fixed and whose relative molecular mass is approx. 15 000—20 000.

The fluorescence anisotropy measurement technique serves for examining the relaxation of the nonequilibrium inhomogeneous distribution of excited molecules after sample irradiation by a plane-polarized monochromatic light pulse at a suitable wavelength. Measured is the time decay of fluorescence, polarized both parallel with and perpendicular to the excitation polarization ($I_{\parallel}(t)$ and $I_{\perp}(t)$, respectively). The time dependences monitor several processes occurring simultaneously, such as the reorientational Brownian molecular motion, energy relaxation or the reorientational relaxation of the microenvironment. The following quantities are used to characterize these processes:

$$S(t) = I_{\parallel}(t) + 2I_{\perp}(t) \qquad (1)$$

$$r(t) = [I_{\parallel}(t) - I_{\perp}(t)]/[I_{\parallel}(t) + 2I_{\perp}(t)] \,. \qquad (2)$$

The quantity $S(t)$ is proportional to the total population depleting of the excited state, which is independent of the reorientational motion, whereas $r(t)$ is the time-dependent fluorescence polarization anisotropy which, being associated with the

correlation function of the transition moments of the molecule, contains information about the orientational relaxation solely.

The fluorescence anisotropy decay should be analyzed on the basis of a suitable theoretical model of the rotational motion of the molecule involved. This problem has been addressed by a number of authors[1-6], and several models have been devised to account for the reorientation of rigid molecules in isotropic medium, based on various concepts of the effect of microstructure of the solution treated. The rotational diffusion (RD) model is employed most frequently for the interpretation of the experimental data[2,3]. In this model, the molecule is regarded as a rigid ellipsoid immersed in a viscous liquid which is considered as a structureless continuum. The fluorescence anisotropy decay $r(t)$ for the general case of an asymmetric molecule (in this context, molecular symmetry is classified with respect to the diagonal elements of the rotational diffusion tensor D_i) can be expressed in the RD model analytically as the sum of five exponential functions,

$$r(t) = \sum A_i \exp(-t/\tau_{ri}) \tag{3}$$

where τ_{ri}'s are rotational correlation times and A_i's are preexponential factors. Analysis of these quantities reveals that only three rotational correlation times are linearly independent. In cases of a particular symmetry of the diffusion tensor or the orientation of the absorption and emission transition moments (μ_a and μ_e, respectively) of the molecule with respect to the principal diffusion axes, the number of rotational correlation times decreases, and the general relationship simplifies. The rotational relaxation of a symmetric molecule (with two diffusion coefficients identical and different from the third), with the two transition moments parallel with the symmetry axis, or a spherical molecule (with the three diffusion coefficients identical) can be described by a single-exponential function $r(t)$, i.e., a single rotational correlation time exists. The fact that many actual cases can be well fitted by the single-exponential course of $r(t)$ is of importance because contemporary instrumentation enables two, or at most three, rotational correlation times to be discerned within the limits of experimental error. In addition to the rotational correlation times, the RD model also enables the initial anisotropy $r_0 = r(t = 0)$ and residual anisotropy $r_\infty = \lim r(t)\,(t \to \infty)$ to be determined. The following kind of information can be extracted from fluorescence anisotropy measurements:

a) The number of existing rotational correlation times provides information about molecular symmetry, mutual orientation of μ_a and μ_e and, in some cases, their orientation with respect to the symmetry axes, and it may in special cases provide information about the heterogeneity of the microenvironment of the fluorescence labels.

b) The magnitude of the rotational correlation times provides information about the magnitude of the principal diffusion coefficients D_i, or about the D_i/D_j ratio

(information concerning the microviscosity of the environment, interactions with the environment, geometry of the molecule in the excited state).

c) The initial anisotropy provides information about the mutual orientation of μ_a and μ_e.

d) The residual anisotropy provides a criterion of equivalence of all directions of rotation (anisotropy of medium) and information about the possibility of occurrence of rotational phenomena several orders of magnitude slower than the fluorescence lifetime.

A polymer chain labelled with a fluorescence probe is a flexible dynamic system where internal rotation is possible; thus, this is a considerably more complex case than a rigid molecule in an isotropic medium as discussed above. In this case, time-resolved anisotropy measurements address polymer segments via fluorescence probes that are covalently bonded to them. The rotation of such a probe reflects the overall dynamics of the polymer chain in a rather complex manner. The fluorescence anisotropy of this flexible system monitors several motions simultaneously: rotation of the fluorophor about the bond, rotation of the system as a whole, rotation of the fluorophor together with a part of the macromolecule, etc. These motions largely reflect conformational changes in the polymer chain. When studying the polymer chain dynamics by fluorescence anisotropy measurements, the fact must be taken into account that the conformational changes of the polymer chain which are closely related to the possible internal rotation of segments in the macromolecule are also influenced by the microenvironment of the fluorophor, its anisotropy, viscosity, polarity, and even its chemical composition. In the general case, changes in the microenvironment can affect the number and magnitude of the rotational conformation times, the residual anisotropy as well as the energy relaxation kinetics. Because of the complexity of the problem, no general theory exists for such a system; however, since this method is a promising source of immediate information, special cases have been dealt with at a semiquantitative level, e.g. in refs[7–10]. A combination of anisotropy decay measurements with the classical methods of characterization of the size of macromolecules in solutions (viscometry, light scattering, etc.) offers new possibilities for the study of the dynamics of polymer chains.

The aim of the present work was to examine the possibility of studying the dynamics of conformational changes of chains of polyelectrolytes in aqueous solutions by polarization time-resolved fluorometry.

EXPERIMENTAL

Apparatus

Pulse spectrofluorometry with time-resolved single photon counting was employed for the fluorescence measurement with a nanosecond resolution. The measurements were performed on

an Edinburgh Instruments Model 299T spectrofluorometer in the multiplex mode. A scheme of this apparatus is shown in Fig. 1.

The excitation light pulses are generated by a pulse discharge lamp filled with hydrogen, nitrogen or a noble gas; its electrode spacing being adjustable, supply voltage up to 10 kV and repetition frequency up to 100 kHz. The stability of the discharge is electronically controlled. For routine measurements in the excitation wavelength region of 240—400 nm, hydrogen is used at a pressure of 50 kPa; repetition frequency 40 kHz, voltage 6·5—7 kV, electrode spacing 0·8—1 mm. The pulses have a high stability and their half width is 1·2—1·7 ns.

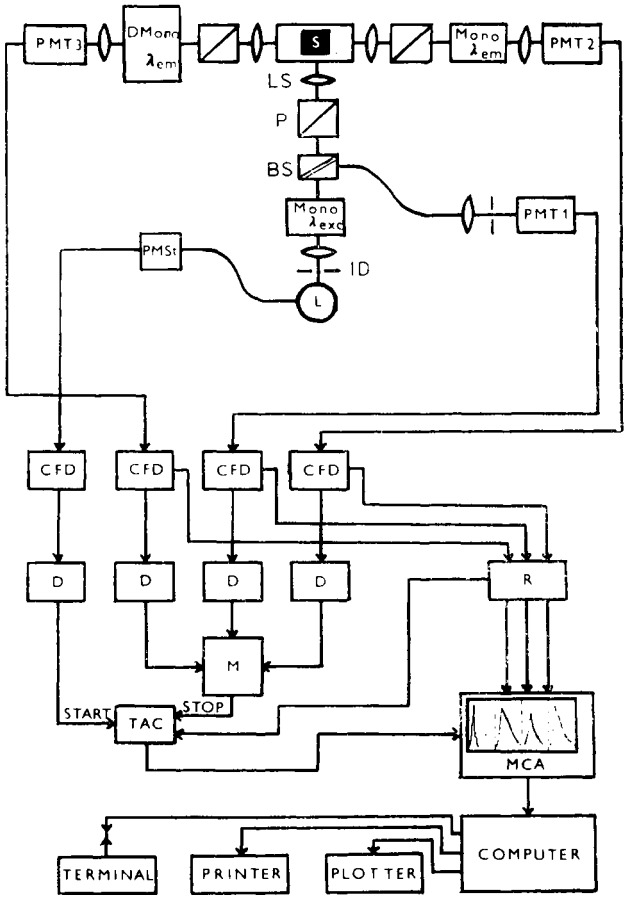

FIG. 1

Layout of the EI 299T spectrofluorometer. L coaxial flashlamp, BS beam splitter, P polarizer, LS lense, ID iris diaphragm, — optical filament, CFD constant fraction discriminator, D delay box, MCA multichannel analyzer, R router, M multiplexer, TAC time-to-amplitude converter, Mono single monochromator, Dmono double monochromator (SPEX), PMT XP 2020 photomultiplier, S sample

A suitable excitation wavelength is selected from the spectrum by a monochromator. The half band width of the beam can be varied over the region of 2·65 to 21·2 nm by input and output slit adjustment. The actual excitation pulse profile after passing the excitation monochromator is recorded during measurement by a special channel (SAFE — Simultaneous Acquisition of Fluorescence and Excitation).

Photons emitted by the sample are detected by two independent channels. When measuring the anisotropy kinetics, a polarizer determining the vertical polarization of the excitation pulses is inserted after the excitation monochromator. One of the detection channels is equipped with a polarizer actuated by a servomotor which, in conjunction with electronic control, makes possible automatic alternating measurement of the parallel and perpendicular components of the fluorescence decay on the same detection channel (in regular intervals from 0·5 to 30 s) during a single experiment. In this manner, systematic error that could arise when measuring by means of two insufficiently matched detection channels is eliminated. This detection channel in 299T apparatus works effectively as a pair of channels; the other independent channel is fitted with a manually operated polarizer.

Detection

Multiplexed time correlated single photon counting (TCSPC) was used for the detection. This method is based on the fact that if the fluorescence intensity is low enough and certain further conditions are satisfied then the distribution of the time intervals between the excitation and the emission of the first photon (in repeated pulse excitation regime) is identical with the fluorescence decay profile (for further details see, e.g. ref.[11]). Pulse excitation with a high frequency of repetition must be used in practice in order to accumulate enough data for statistical treatment to be applicable. For the TCSPC principle to be maintained, the photon counting rate (number of photons detected in a second) at the channels should not be higher than approximately 2% of the frequency of repetition of the pulse source. The time intervals between the excitation and detection of the photons are measured to obtain their distribution for a sufficient number of events (higher than 10^5), which then gives the fluorescence decay profile. This distribution can be usually determined with a high accuracy and within a considerable dynamic range.

In the instrument used, the detection by the TCSPC method is the classical one; a novel element in the EI 299T is the multiplexing, enabling up to four signals (profiles)to be measured effectively while employing a single common control unit of the detection electronics (see later).

The electric signal (pulse) determining the moment of excitation is obtained by means of a starting photomultiplier (PMSt in Fig. 1). The moment of photon detection on a channel is determined by the electric pulse ("STOP") at the output of the XP 2020 channel photomultiplier (Fig. 1). The two pulses, "START" and "STOP", must be first transformed to the standard shape by means of constant fraction discriminator (CFD) amplifiers (EG & G). Evaluation of the time intervals within the "START"–"STOP" pulse pairs proceeds in the common control section of the detecting electronics. Its heart is a time-to-amplitude converter (TAC) (EG & G), generating electric pulses of a constant width and with amplitudes proportional to the time interval between the mutually corresponding "START" and "STOP" pulses (for details see, e.g., refs[12–17]). These pulses are then processed in a multichannel amplitude analyzer (MCA) (EG & G). After evaluation of a sufficient number of events, the frequencies of events in given time intervals between the moment of excitation and the detection of the first emitted photon are accumulated in registers. Under the given conditions, the dependence of the frequency on the length of this interval corresponds exactly to the fluorescence decay profile, as follows from the principle underlying the TCSPC method.

The multiplex unit allows the central electronics (TAC and MCA) to be engaged for several

detecting channels simultaneously (M in Fig. 1) and the principle of the multiplex regime is as follows: The average counting rate at the individual detecting channels must not exceed 2% with respect to the excitation frequency, for preserving the Poisson distribution (hence, given the 40 kHz repetition frequency of the excitation discharge lamp, the maximum usable counting rate at the individual channels is about 800 s^{-1}). Still, the signals at the channels have a statistical character. The probability of the simultaneous occurrence of usable signals on more than one detecting channels after one excitation pulse is thus lower than 10^{-4}, and such cases can be neglected or electronically suppressed. Hence, it is possible to simply sum up all "STOP" signals from the detection channels in the mutliplex unit and submit this sum to the TAC and MCA equipment for processing, the memory registers of the MCA being divided into four independent groups and the results being stored in one of them according to by which channel the signal has been recorded. This storing is controlled by logic signals generated by the multiplex unit according to the origin of the incoming "STOP" pulses. In the MCA, as many as four curves corresponding to the signals from the four detecting channels are so accumulated "simultaneously". At the same time, the multiplex unit rejects those events where the excitation pulse is followed by signals at more than one detecting channels.

In the apparatus used, the MCA has a memory of 4 kB, which implies that 1 024 channels (points) are available for each curve. In the fastest TAC mode (50 ns) the time resolution thus is 50 ps/channel. Although some standard data handling (e.g. curve smoothing) can be performed on the MCA directly, the experimental data are nearly invariably on-line transmitted from the MCA to the PDP11/23+ computer for processing and evaluation.

Data Handling

Data handling comprises two tasks:

1) The experimental points are fitted by a curve according to the theoretical model used (e.g., a sum of exponentials), and parameters such as the fluorescence decay time, relative amplitudes of the components, etc., are derived from it.

2) If the fluorescence decay rate is comparable to the pulse width, deconvolution of the experimental time dependence of fluorescence with the excitation pulse profile must be carried out to obtain the true decay profile[18,19], i.e. the response of the system to excitation by a δ-pulse. Both procedures are generally necessary if the excitation pulse width cannot be neglected with respect to the fluorescence decay rate.

RESULTS

To verify the possibility of employing time-resolved polarization spectrofluorometry for the study of the dynamics of polymer chains, the decay of fluorescence was measured at various pH for aqueous solutions of poly(methacrylic acid) (PMA) and poly(acrylic acid) (PAA) labelled with dansyl (1-(dimethylamino)-5-naphthalene-sulfonic acid, DNS) as the fluorescence probe. A detailed discussion of polymer chains behaviour of PMA in dilute aqueous solutions is the subject of another paper[20]. In the present work we concentrated on the evaluation of the precision and of the potential of the various procedures for primary data handling; attention was also paid to the relation between changes in the measured quantities and the polymer chain dynamics.

DNS was chosen as the label with regard to its high sensitivity to the polar character of its microenvironment, observed in steady-state fluorescence measurements (e.g. refs[21-24]). With increasing solvent polarity (or more precisely polarity of the label's microenvironment) the emission of DNS exhibits a red shift and its fluorescence lifetime becomes shorter. The excitation wavelength of 340 nm was chosen for the basic form of DNS to be the sole object of excitation. After passing through the double monochromator, the fluorescence decay was detected under the magic angle (54·7°), whereas after passing the single monochromator, the parallel and perpendicular fluorescence decay was detected. The measurement was performed within the 500–540 nm region in accordance with the position of the fluorescence maximum as a function of pH.

Based on experimental data of fluorescence decay, the fluorescence lifetimes were first evaluated. The fluorescence decay curves were approximated by single-exponential and double-exponential descending functions (generally, $I = \sum A_i \exp(-t/\tau_i)$). The curve parameters were sought by nonlinear regression so as to obtain the best statistical correlation parameters (normalized χ^2, autocorrelation function[18]). In the case of the fluorescence label bonded to the polymer chain, it was found that to a first approximation, the fluorescence decay can be fitted by a double-exponential function. The two lifetimes correspond to the different behaviour of the label in the coiled part of the polymer chain and in that expanded where the fluorophor is exposed to the polar effect of solvent molecules to a greater extent. The fluorescence lifetimes of PMA-DNS and PAA-DNS at various pH of the solution are given inTables I and II, respectively; lifetimes calculated from the fluorescence decay data measured under the magic angle ($F_m(t)$) and those derived from the sum $S(t) = F_{\parallel}(t) + 2F_{\perp}(t)$ are included. In both cases, deconvolution was applied. By comparing the two sets of data, the precision of both measurements can be assessed. Taking into account the fact that in a macroscopical ensemble of macromolecules, the attachement of the fluorescence labels to the various sites of the polymeric chains has a statistical character, we attempted to evaluate the fluorescence data based on a method taking into account a certain distribution of the fluorescence lifetimes, arising from different interactions with the environment.

In the case of two sufficiently different fluorescence lifetimes, the procedure is as follows. First, fluorescence lifetime estimates are derived from the double-exponential fit. Small, but systematical departures of the experimental data from the regression curve in the short time region are largely due to the distribution of the shorter lifetimes τ_1, whereas in the longer time region, the distribution of the longer lifetimes τ_2 is the most important source of systematical departures. In the next step, the fit is refined iteratively by varying the parameters of distribution of the short-lifetime component (i.e. the value in the maximum, $\tau_{1,m}$, and the distribution width δ_1) while keeping the τ_2 value constant; the intial τ_1 value is used as the starting approximation for $\tau_{1,m}$. After this refinement, the distribution of $\tau_{2,m}$, δ_2, is sought.

If $\tau_{2,m}$ differs from τ_2 more than by δ_2, the whole cycle must be repeated using $\tau_{1,m}$ and $\tau_{2,m}$ as the input values.

Although this approach does not represent a simultaneous and independent determination of all parameters of the double-peak distribution, reliable data within the scope of the assumed analytical peak shapes are obtained if the $\tau_{1,m}$ and $\tau_{2,m}$ values differ significantly. In this case both parts of the fluorescence decay curve in the regions of short and long times are significantly affected by the distribution of fluorescence lifetimes in corresponding time region only. In the case of a single-peak distribution of the fluorescence lifetimes, the principle of the method remains

TABLE I

Fluorescence lifetimes τ_1, τ_2, their standard deviations $\Delta\tau_1$, $\Delta\tau_2$ and relative amplitude of the shorter component $A_{1,rel}$ for PMA-DNS of a relative molecular mass $M_w = 27\,000$ at an ionic strength $I = 0{\cdot}05$; χ^2 is the normalized weighted average of squares of residuals. The upper data were obtained from measurements under the magic angle, the lower data, from measurements of the perpendicular and parallel components of fluorescence

pH	τ_1 ns	$\Delta\tau_1$ ns	τ_2 ns	$\Delta\tau_2$ ns	$A_{1,rel}$ %	χ^2
1·89	8·86	0·82	22·02	0·06	7	1·11
	8·57	0·79	22·06	0·06	7	1·16
3·39	9·69	0·80	22·12	0·06	7	1·10
	8·53	0·57	22·11	0·04	7	0·98
3·84	11·58	0·59	22·30	0·05	12	1·26
	8·74	0·48	21·99	0·04	7	1·48
4·93	9·09	0·33	21·50	0·04	12	1·25
	8·31	0·36	21·39	0·04	10	1·46
5·03	9·52	0·35	21·36	0·07	15	1·08
	8·44	0·31	21·26	0·05	13	1·08
5·57	4·43	0·10	18·60	0·09	25	1·07
	4·26	0·09	18·87	0·08	26	1·20
6·02	2·68	0·07	7·21	0·14	65	1·19
	2·58	0·06	7·13	0·11	66	1·06
6·51	2·72	0·04	8·47	0·35	85	1·03
	2·41	0·06	5·98	0·14	75	1·06
8·90	2·74	0·02	13·81	2·02	92	1·07
	2·53	0·04	8·54	0·59	89	1·15
9·02	2·49	0·02	7·58	0·24	88	1·16
	2·57	0·02	8·91	0·36	92	1·10

the same, the procedure, however, simplifies considerably. The fluorescence lifetime distribution parameters for PMA-DNS are given in Table III.

Tables IV and V give the effective rotational correlation times for the PMA-DNS and PAA-DNS systems, respectively, in dependence on pH. The calculations were based either on the difference $D(t)$ and sum $S(t)$ of the two components of the fluorescence decay, including deconvolution, or on the anisotropy decay obtained from the parallel and perpendicular components of the fluorescence decay without deconvolution. Comparison of the results enables us to assess the applicability of the simpler $R(t)$ evaluation procedure without deconvolution.

TABLE II

Fluorescence lifetimes for PAA-DNS of a relative molecular mass $M_w = 280\,000$; symbols and arrangement as in Table I

pH	τ_1 ns	$\Delta\tau_1$ ns	τ_2 ns	$\Delta\tau_2$ ns	$A_{1,rel}$ %	χ^2
3·88	3·35	0·14	10·15	0·10	34	1·03
	3·38	0·17	10·33	0·16	35	1·10
4·89	2·70	0·08	7·23	0·06	43	1·07
	2·98	0·07	7·71	0·07	52	1·18
5·59	2·82	0·11	6·35	0·14	64	1·06
	2·58	0·13	5·87	0·11	57	1·08
7·03	2·53	0·06	5·40	0·09	70	1·20
	2·17	0·11	4·40	0·05	51	1·29
8·85	2·68	0·04	8·12	0·24	79	1·09
	2·73	0·04	7·98	0·22	81	1·11

TABLE III

Lifetime distribution for PMA-DNS at $M_w = 27\,000$ ($I = 0{\cdot}05$); $\tau_{1,m}$, $\tau_{2,m}$ are the lifetimes in the distribution maxima, δ_1, δ_2 are widths of their distributions, f_1 is the relative abundance of the first component

pH	$\tau_{1,m}$	$\Delta\tau_{1,m}$	δ_1	f_1	$\tau_{2,m}$	$\Delta\tau_{2,m}$	δ_2	χ^2
1·88	11·88	0·87	8·20	10	21·87	0·06	2·41	1·11
5·57	4·40	0·06	2·20	25	18·17	0·06	4·54	1·05

A single fluorescence lifetime, $\tau_f = 3{\cdot}47 \pm 0{\cdot}02$ ns ($\chi^2 = 1{\cdot}01$), was obtained for DNS attached to monomeric methacrylic acid at pH 3·88. The χ^2 value approaching unity indicates that the single-exponential fit is appropriate. The rotational correlation time is $\tau_r = 0{\cdot}80 \pm 0{\cdot}23$ ns ($\chi^2 = 1{\cdot}02$).

DISCUSSION

The experimental data obtained demonstrate that deconvolution is necessary in those cases where the time characteristics of the dynamic phenomena involved are on the nanoseconds scale. The experimental profile of the fluorescence decay is highly

TABLE IV

Effective rotational correlation times τ_r(ns), their standard deviations $\Delta\tau_r$(ns), initial anisotropies r_0, their standard deviations Δr_0, residual anisotropies r_∞ and their standard deviations Δr_∞ for PMA-DNS at $M_w = 27\,000$; $I = 0{\cdot}05$. The upper values were obtained by deconvolution from measurements of the perpendicular and parallel fluorescence components, the lower values were obtained without deconvolution

pH	τ_r	$\Delta\tau_r$	r_0	Δr_0	r_∞	Δr_∞	χ^2
1·89	21·76	1·30	0·272	0·003	0·178	0·002	1·10
	23·48	1·17			0·090	0·002	0·95
3·39	25·15	1·42	0·268	0·003	0·090	0·001	1·13
	26·74	1·29			0·087	0·002	1·06
3·84	26·28	1·11	0·265	0·002	0·082	0·001	1·22
	26·67	0·96			0·080	0·001	1·06
4·93	18·80	0·55	0·261	0·002	0·058	0·001	1·20
	20·02	0·57			0·055	0·001	1·05
5·03	17·09	0·59	0·263	0·002	0·052	0·001	0·99
	19·62	0·58			0·053	0·001	0·97
5·57	12·99	1·33	0·201	0·005	0·025	0·003	1·01
	13·61	0·93			0·023	0·002	0·97
6·02	1·04	0·59	0·205	0·009	0·045	0·003	1·08
	3·45	0·43			0·034	0·004	1·04
6·51	1·24	0·39	0·186	0·009	0·030	0·003	1·15
	3·17	0·35			0·026	0·004	0·99
8·90	1·45	0·52	0·179	0·012	0·018	0·004	1·16
	3·43	0·46			0·020	0·005	1·13
9·02	0·69	0·18	0·249	0·009	0·011	0·003	1·08
	2·41	0·19			0·002	0·003	1·08

affected by the finite duration of the excitation pulse (whose half width is about 1·5 – 1·7 ns), and data evaluation, without deconvolution, provides only the upper estimates of the parameters sought (the fluorescence lifetimes and rotational correlation times). The differences between the parameters obtained by applying deconvolution and without deconvolution are reasonably low, i.e. lower than 10% and so comparable to other possible errors of measurement, if the lifetimes are at least 7 to 10 times longer than the excitation pulse half width.

For obtaining accurate results, stability of the excitation pulse must be ensured over the entire time of measurement (which may be longer than 8 h) or else the variations in the intensity and the time profile of excitation must be known. Hydrogen discharge lamps feature a high pulse stability; still, minor fluctuations during an 8 h period may occur. Errors that might arise if the independent consecutive measurement of all parameters were performed are automatically eliminated in the multiplex mode. Moreover, the time of measurement in the multiplex mode is only about a quarter with respect to that spent in the simplex mode.

Dansyl, which exhibits single-exponential fluorescence decay and whose lifetime is dependent on the polarity of the microenvironment, appeared to be a good choice as a label for the indication of dynamic changes of chains of polyelectrolytes in aqueous solutions. The two different fluorescence lifetimes of DNS bonded to the chain of PMA indicate that in a large assembly of macromolecules, the fluorophors (bonded to different chains) occur in two microenvironments differing in their polarity. This is consistent with the results of study of conformations of PMA by other

TABLE V

Effective rotational correlation times for PAA-DNS at $M_w = 286\,000$; $I = 0{\cdot}05$. Symbols and arrangement as in Table IV

pH	τ_r	$\Delta\tau_r$	r_0	Δr_0	r_∞	Δr_∞	χ^2
3·88	4·79	0·86	0·238	0·008	0·056	0·004	1·06
	5·79	0·63			0·049	0·004	0·95
4·89	1·82	0·24	0·203	0·005	0·023	0·002	1·05
	3·44	0·17			0·015	0·002	0·98
5·59	1·29	0·25	0·206	0·008	0·011	0·003	0·88
	3·39	0·29			0·024	0·003	0·85
7·03	1·29	0·22	0·172	0·005	0·017	0·002	0·98
	2·51	0·16			0·010	0·002	1·00
8·85	0·74	0·27	0·193	0·010	0·014	0·003	1·02
	2·49	0·21			0·009	0·003	0·98

techniques such as steady-state fluorometry[24]; detailed discussion of the conformational changes and of the effect of pH, ionic strength, etc., on the conformational states as well as on the fluorescence characteristics is the object of paper[20]. In the present work, we only concentrate on the effect of conformation of the part of the chain immediately surrounding the probe on the fluorescence lifetimes and rotational correlation times. The longer lifetimes correspond to dansyl labels trapped in the nonpolar medium of methyl groups in intramolecularly collapsed or supercoiled parts of the chain (see, e.g., ref.[25]), whereas the shorter lifetimes can be attributed to probes occurring in a strongly polar microenvironment of the uncoiled and expanded chain, whose fluorescence decays strongly.

The lifetimes of this label bonded to PAA chains were also measured for a comparison. In this polymer, no total collapse or chain coiling takes place even at low pH values because no hydrophobic methyl groups are present. The individual chains form coils with rather low segment density which decreases monotonically from the centre towards the surface. The DNS labels interact rather strongly with the polar molecules of the solvent. The two different lifetimes that were observed also in this case probably mirror the different degree of ordering of water molecules in the environment of the various segments of the polymeric chain. The value of the longer lifetime of PAA-DNS is considerably shorter than the corresponding value of PMA--DNS (for details see ref.[20]).

Due to the statistical character of the attachment of the dansyl labels to the polymer chains and of the dynamic behaviour of the chains in solution, a distribution of the polar or nonpolar types of interaction of the fluorophor with the environment establishes. Thus, the experimental lifetimes or rotational correlation times are actually effective values, averaged over the entire set of macromolecules present. This assumption was confirmed by the fluorescence decay profiles evaluation using the lifetime distribution (see Table III).

Carrying direct information about the rotational velocity and degree of freedom, or about hindrance to the rotational diffusion motion of the probe, and thus, indirect information about the compactness and flexibility of the polymer chain or its segments, the rotational correlation time of the probe bonded to the chain appeared to be the most sensitive parameter, reflecting various conformational changes in the polyelectrolytes in aqueous solutions.

The assumption of two types of differently collapsed arrangements of segments of the polymer chains implies the occurrence of two different rotational correlation times. For illustration, we present the rotational correlation times calculated on the basis of this assumption for the PMA-DNS system (relative molecular mass $M_w = 27\,000$) at two different pH values and ionic strength $I = 0{\cdot}05$. In both cases, two rotational correlation times were obtained from the statistically best data fit (in that case our data were of superior quality, obtained on our instrument under the optimum conditions, particularly at a sufficiently high fluorescence intensity, a suffi-

ciently high difference between the two times and sufficiently high statistical abundances of both different forms of the fluorophor). At pH 3·84 and 5·57, the respective values are $\tau_{r1} = 2{\cdot}04 \pm 0{\cdot}56$ and $0{\cdot}62 \pm 0{\cdot}22$ ns ($A_1 = 0{\cdot}06$ and $0{\cdot}15$) and $\tau_{r2} = 36{\cdot}50 \pm 2{\cdot}11$ and $19{\cdot}73 \pm 2{\cdot}32$ ns ($A_2 = 0{\cdot}18$ and $0{\cdot}16$); the χ^2 parameter is 1·10 and 0·98, respectively. Although both forms are present at either of the pH values, the supercoiled structures predominate at the lower pH, whereas uncoiling and expansion of the chains is induced by the dissociation of the carboxylic groups and their electrostatic repulsion at higher pH. Similarly to the case of two different lifetimes, the shorter rotational correlation time can be attributed to probes which in the aqueous medium are in the vicinity of the uncoiled chain part, whose rotation is sterically nearly unhindered. This τ_{r1} value approaches that observed for the free probe in a low molar mass solvent ($\tau_r = 0{\cdot}55 \pm 0{\cdot}12$ ns in methanol; $\chi^2 = 1{\cdot}18$) or for the probe attached to monomeric methacrylic acid dissolved in a low molecular mass solvent ($\tau_r = 0{\cdot}80 \pm 0{\cdot}23$ ns in aqueous solution at pH 3·88; $\chi^2 = 1{\cdot}02$). The longer time τ_{r2} corresponds to the reorientational relaxation of the labels fixed in the compact collapsed part of the polymer chains. Very high quality experimental data (requiring long times of measurement) are prerequisite for a reliable evaluation of the fluorescence anisotropy based on the two rotational correlation times. Since for PMA-DNS, always a single type of chain structure prevails (except in the narrow range of pH 3·5–5·5), single-exponential fit of anisotropy, giving a single effective rotational correlation time, provides appropriate description of the average compactness of the polymer chains.

The experiments gave evidence that time-resolved fluorometry is capable of providing not only valuable information concerning the dynamics of polymer chains in solutions and their possible conformations but also a new view upon the nature and average contents of the various submolecular structure arrangements in various solvents.

Our measurements also document that experimental data can only be successfully interpreted if a suitable concept of the mechanism of the involved phenomena at the molecular level exists. It can be inferred from data of the system examined[20–24] that the short time is related predominantly with the rotation of the label about the single bonds attaching it to the polymer chain whereas the long time is associated with the rotation of the collapsed part of the chain. Hence, the two times are not accounted for by the rotation of the probe in two different microenvironments differing principally in their microviscosity; instead, rotation of two different structures is involved. Also, the experimental data associated with the fluorescence anisotropy are in no simple relationship to the mobility of the segments of the polymer chain.

Of practical importance is the fact that if one of chain structures predominates, the behaviour of the polymer chains in solution can be reasonably well described in terms of the simplified model in which a single effective rotational correlation time and double-exponential fluorescence decay are considered.

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