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Fluorescence studies on soluble ribonucleic acid labelled with acriflavine

The polarization of fluorescence theory first described by PERRIN has been extended to the study of protein molecules labelled with fluorescent dyes of known lifetime of excitation[1–3] and has allowed the determination of the relaxation time and molar volume of native and modified proteins.

This paper reports results of polarization of fluorescence studies designed to estimate the relaxation time of s-RNA labelled with acriflavine dye.

The lifetime of the excited state of the fluorescent oscillator was determined by the indirect method based on PERRIN's equation.

Soluble yeast RNA prepared by the phenol method[4] was oxidized by $NaIO_4$ according to the method of ZAMECNICK[5]. Excess of IO_4^- was eliminated by dialysis against several changes of 0.1 M acetate buffer (pH 5). To 50 mg of the oxidized RNA in 0.1 M acetate buffer (pH 5) was added 2 mg of acriflavine dissolved in water and the solution was left at 37° for 4 h. The RNA–dye conjugate was precipitated from the reaction mixture with two volumes of ethanol and the precipitate was resuspended in 0.1 M NaCl and purified by further ethanol precipitation. The last step was repeated five times and the resulting RNA–acriflavine complex was run through Sephadex G-50 before the polarization measurements.

A control acriflavine derivative, used for the estimation of the lifetime of the excited state (τ), was prepared by reacting propionaldehyde with acriflavine according to published method[6].

Measurements of the polarization of fluorescence were made with an Aminco Bowman spectrophotofluorometer using unpolarized exciting light from a low pressure mercury arc and a Glan Thompson prism as analyzer. A thermostatted cell was used throughout the experiments.

Sedimentation-velocity experiments were performed at 56000 rev./min in the Spinco model E ultracentrifuge, equipped with a rotor-temperature-indicator control. Runs were conducted at room temperature.

If the relaxation time and molar volume are to be obtained by polarization of fluorescence, the RNA–dye conjugate must meet two requirements: (a) it must be stable; (b) the lifetime of the excited state of the fluorescent oscillator must be known.

The following evidence supports the contention that the dye was labelled to RNA through a stable covalent bond, and that the first of this requirement thus is satisfied: (1) no stable RNA–dye complex could be formed if the periodate oxidation step was omitted; (2) the RNA–dye complex is stable after several precipitations with

TABLE I

POLARIZATION OF FLUORESCENCE IN GLYCEROL–WATER SOLUTIONS

p_A: polarization of fluorescence of acriflavine–propionaldehyde;
p_F: polarization of fluorescein–isothiocyanate.

p_A	p_F	T/η
0.250	0.250	0.20
0.170	0.180	8
0.100	0.100	20

ethanol, and no free dye was detected by paper chromatography (0.1 M acetate buffer; ethanol, 1:1, pH 4.5); (3) dialysis against buffers of different pH in the range of 3 to 8.5, caused no liberation of free dye; (4) absorption spectra of the purified RNA–acriflavine complex revealed two bands, at 470 mμ and 380 mμ, respectively. The propionaldehyde–dye conjugate showed a band at 460 mμ and acriflavine at 450 mμ.

In order to satisfy the second requirement, the lifetime of the excited state of the acriflavin–propionaldehyde was determined in the following manner.

The polarization of fluorescence was measured at different viscosities obtained by stepwise addition of water to glycerol. Simultaneously the degree of polarization of fluorescein isothiocyanate (τ $5 \cdot 10^{-9}$ sec) was recorded (Table I) and it was found that both samples have the same limiting polarization (0.250) upon excitation with natural light of 440 mμ.

From PERRIN's equation:

$$\frac{1}{p} + \frac{1}{3} = \left(\frac{1}{p_0} + \frac{1}{3}\right)\left(1 + \frac{RT\tau}{\eta V}\right) \tag{1}$$

which relates the degree of polarization p to the following parameters: τ, lifetime of the excited state; V, molar volume; η, viscosity; T, absolute temperature and R, gas constant; the simple relationship for two dyes applies:

$$\frac{\left(\frac{1}{p} - \frac{1}{p_0}\right)_F}{\left(\frac{1}{p} - \frac{1}{p_0}\right)_A} = \frac{\tau_F}{\tau_A} \tag{2}$$

when

$$\left(\frac{1}{p_0}\right)_A = \left(\frac{1}{p_0}\right)_F \quad \text{and} \quad V_A = V_F$$

(A: acriflavine–propionaldehyde; F: fluorescein–isothiocyanate).

Substituting in Eqn. 2, the polarization of fluorescence values obtained at $T/\eta = 8$ (Table I) a lifetime of excitation of $4.4 \cdot 10^{-9}$ sec was obtained for acriflavine.

Taking into account the experimental error of the indirect method, there is good agreement between this data and the value ($4 \cdot 10^{-9}$ sec)[7] determined with a fluorometer.

The polarization of fluorescence data for RNA–dye conjugate are given in Fig. 1. Using PERRIN's equation (2) a relaxation time of $0.28 \cdot 10^{-7}$ sec is derived.

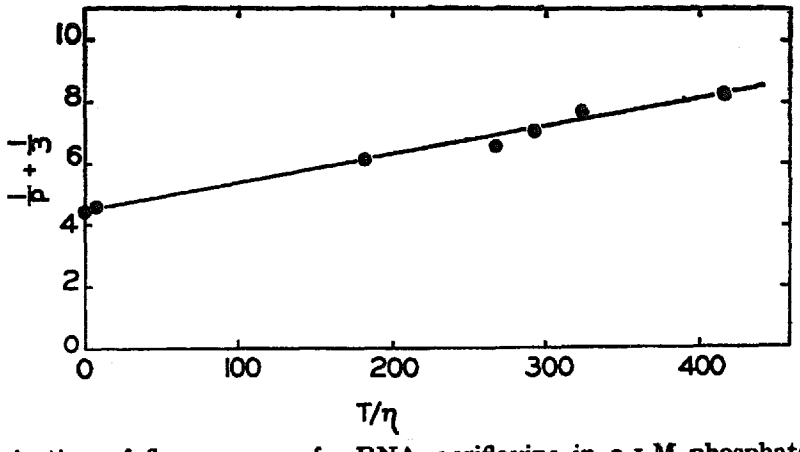

Fig. 1. Polarization of fluorescence of s-RNA–acriflavine in 0.1 M phosphate buffer (pH 7.2). The conjugate has a composition of 0.8 mole dye per mole of s-RNA and the molar extinction coefficient of $4.4 \cdot 10^4$ for acriflavine in solution at 440 mμ was used for the calculation. Experiments were conducted at a s-RNA concentration between 0.1 and 1 mg/ml.

Recently it has been shown that s-RNA is a compact macromolecule of 100 Å length and 20 Å diameter[8,9]. The shape is represented by an ellipsoid of axial ratio 6:1 (ref. 9) and the volume is of the order of 30000 Å^3. For a spherical and unhydrated macromolecule of 30000 Å^3, one can estimate a relaxation time (ρ_h) equal to $0.22 \cdot 10^{-7}$ sec at 20° by Formula 3.

$$\rho_0 = \frac{3\eta V}{kT} \tag{3}$$

The difference between that value ($0.22 \cdot 10^{-7}$ sec) and the experimentally found one ($0.28 \cdot 10^{-7}$ sec) indicates a departure from the spherical symmetry which should be expected for an ellipsoid of revolution of axial ratio 6:1. On the other hand hydration of RNA in solution which is not taken into account by Formula 3 will also tend to increase the relaxation time.

The fact that the sedimentation behavior of RNA–dye complex ($s^0_{20,w} = 3.6$ S) was very similar to that of s-RNA ($s^0_{20,w} = 3.65$ S), indicates that little or no structural change was associated with the incorporation of the dye.

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A simplified apparatus for vertical gel electrophoresis

Zone electrophoresis has been widely used for investigation of biologic compounds, in particular proteins, by virtue of its adaptability—for example, a variety of media can be used to stabilize the moving boundaries of the convection currents of the electrolyte solutions. SMITHIES[1] described a technique of zone electrophoresis in starch gel that combines advantages of low adsorption characteristics of starch, gel filtration, and the additional convenience of detection of substances by a number of procedures. A new synthetic medium for zone electrophoresis, polyacrylamide gel, was reported to be effective in resolving components of serum proteins and to have certain advantages over other media[2,3]. Although the technique of preparation of the polyacrylamide gel is relatively easy, attention to detail must be maintained, since variations in amount of catalyst, pH, and ionic strength of buffers greatly affect the formation of the gel. Further, the apparatus should be air-tight and the gel in close contact