Proc. Natl. Acad. Sci. USA
Vol. 74, No. 4, pp. 1407–1411, April 1977
Biochemistry

Rotational relaxation of 70S ribosomes by a depolarization method using triplet probes

(correlation time/triplet–triplet absorption)

D. LAVALETTE, B. AMAND, AND F. POCHON

Fondation Curie-Institut du Radium, Section de Biologie, Orsay 91405, France

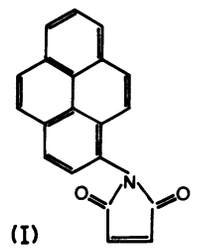
Communicated by Seymour S. Cohen, January 12, 1977

ABSTRACT **Rotational relaxation on the microsecond time scale has been followed by a depolarization technique using the properties of the long-lived triplet state of covalently bound labels. Two triplet probes, which efficiently bind to ribosomal proteins, are described. The rotational correlation time of 70S ribosomes of *Escherichia coli* has been measured. The average hydrodynamic radius of the functionally active 70S particle in solution has been estimated to 147 Å. A concentration dependence of the correlation time has been observed, which may result from an association of the 70S ribosomes to form 100S dimers.**

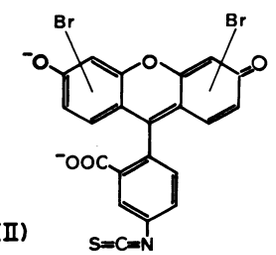
The rotational diffusion of macromolecules in solution is controlled by molecular shape, dimensions, and flexibility. Therefore, it may give information on structural modifications induced by external parameters, such as temperature, pH, and ionic strength, and on the kinetics of association of macromolecular species. Perhaps the most widely used method for investigating the rotational brownian motion is the fluorescence depolarization technique (1). Weber (2) measured rotational correlation times of proteins by using fluorescent probes that were bound covalently to amino-acid residues. In his steady-state method, the fluorescence lifetime of the probe must be determined in a separate experiment. A further improvement was the introduction of the single photon counting technique for recording directly the decay of the fluorescence anisotropy (3). However, because fluorescence lifetimes rarely exceed a few tens of nanoseconds, the method is limited to short correlation times, i.e., to species of molecular weight less than about 2×10^5, immersed in a low viscosity medium. Although long correlation times have been measured in a few exceptional cases (4–6), a general method permitting investigations in the microsecond to millisecond range is of great interest for studying large and complex systems like viruses and ribosomes.

Most fluorescent molecules undergo a radiationless transition to their lowest triplet state, which may last as long as milliseconds in deaerated solutions. Thus, flash photolysis techniques combined with photoselection by linearly polarized light can be used for monitoring the transient triplet-triplet absorption instead of the fluorescence and for following the time course of the depolarization over much longer times. A suitable probe must have a high yield for triplet formation, i.e., a moderate or low fluorescence quantum efficiency. The degree of polarization should be high for the particular combination of exciting and of triplet–triplet absorption wavelengths. When microsecond time resolution is required, excitation with lasers becomes appropriate. This may limit the choice of the chromophore because excitation at fixed discrete wavelengths occasionally results in a poor polarization of the triplet–triplet absorption bands (7). Alternatively, the presence of an intense triplet–triplet absorption can be dispensed with. Indeed, provided the yield of triplet state formation is high, the ground-state absorption will be depleted as long as the probe remains excited in the triplet state. The depleted ground-state absorption in turn becomes dichroic and the induced dichroism reflects the rotational motion of the photoselected molecules.

Starting from these general considerations, we selected two compounds: *N*-(3-pyrene)maleimide (pyrene-Mal) (I) and dibromo-fluorescein isothiocyanate (Br_2FITC) (II), which were found to be adequate triplet probes.



(I)



(II)

In the present communication, we report a study of the rotational relaxation of *Escherichia coli* 70S ribosomes by following the anisotropy decay of the probes excited into their first triplet state by a short laser pulse. Both probes could be bound covalently to functional ribosomes without appreciable loss of biological activity.

METHODS

Detection of Transient Dichroism. A laser flash photolysis apparatus has been constructed for recording transient dichroism between 200 ns and 200 μs. The samples were excited by the second (530 nm) or the third (353 nm) harmonic of a Q-switched neodymium laser (Quantel). The exciting pulse was polarized and had a duration of 15 ns. The monitoring light was provided by a xenon arc (Osram, 75 W) that was pulsed for 1 ms to about 200 times its nominal brightness in order to achieve the high signal-to-noise ratio required for recording small absorbance changes on a fast time scale. The collimated beam (3 mm in diameter) crossed the sample at right angle to the laser direction. By means of a diaphragm, transient absorption was observed in the sample along 5 mm in the first 3 mm of excited solution. The emerging light entered a grating monochromator (Hilger & Watts, 600 grooves per mm) and was finally split into orthogonal polarization components by means of a Glan Thomson prism. The spatially separated beams were received on two balanced photomultipliers (RCA 1P28) used with five amplification stages only. The output current was about 2.5 mA during the flat portion of the xenon arc pulse that was available for absorption measurements. A Tektronix 549 storage oscilloscope displayed either the difference, $\Delta = A_{\parallel} - A_{\perp}$ (i.e., the dichroism), or the sum, $\Sigma = A_{\parallel} + 2A_{\perp}$, of the transient absorbance changes, polarized in a parallel ($A_{\parallel}$) or perpendicular

Abbreviations: pyrene-Mal, *N*-(3-pyrene)maleimide; Br_2FITC, dibromofluorescein isothiocyanate.

($A_\perp$) direction to the electric vector of the exciting laser pulse.

Theory of Measurement. The expressions for $\Delta(t)$ and $\Sigma(t)$ are derived directly from the corresponding formulas of the theory of fluorescence depolarization (1, 8). For most practical purposes, the approximation of spherical particles is sufficient. The dichroism (Δ) and the absorption sum (Σ) are given by:

$$\Delta = A_\parallel - A_\perp = 3\,A_i \cdot \exp(-t/\tau) \cdot R(t) \quad [1]$$

$$\Sigma = A_\parallel + 2\,A_\perp = 3\,A_i \cdot \exp(-t/\tau) \quad [2]$$

where τ is the triplet lifetime and A_i a constant proportional to the initial population of the excited triplets. Σ is insensitive to the rotational relaxation and simply reflects the spontaneous decay of the excited state. The relevant information is contained in the anisotropy, $R(t)$, which decays according to:

$$R(t) = \Delta/\Sigma = R_0 \cdot \exp(-t/\Phi) \quad [3]$$

Eq. 3 defines the rotational correlation time Φ (9), which is related to the volume of the hydrated sphere V, the solvent viscosity η, the absolute temperature T, and Boltzmann's constant k, by:

$$\Phi = (\eta V/kT) \quad [4]$$

Although a single measurement of Φ at known temperature and viscosity is sufficient to determine V, a greater precision is obtained by plotting Φ against η and using the slope of the regression line.

The value of R_0 in Eq. 3 is a function of the angle between the dipole transition moments at the excitation and observation wavelengths. The theoretical values of +0.4 and −0.2 for parallel and perpendicular transition moments, respectively, are rarely observed, due to transitions overlap and vibronic interactions. R_0 also decreases with increase in the population of the photoselected molecules (10) (in the limiting case of 100% excitation, there would be no photoselection and consequently no dichroism at all). Experimentally, a 10–15% excitation into the triplet state has been found to be a satisfactory compromise.

MATERIALS

Buffers. The following buffers were routinely used: (A) 50 mM Tris·HCl, pH 7.8/50 mM KCl/30 mM $MgCl_2$; (B) 50 mM Tris·HCl, pH 7.8/50 mM KCl/1 mM $MgCl_2$; (C) 50 mM borate, pH 8.5/50 mM KCl/30 mM $MgCl_2$.

Ribosomes. 70S Ribosomes were prepared from *E. coli* MRE 600 according to Kurland (11) and were submitted to a zonal centrifugation (12) in buffer A. The isolated 70S fraction was further used for preparing ribosomal subunits in buffer B, and for the labeling reactions.

Protein Synthesis Assay. The activity of the ribosome-conjugates was assayed with a poly(U)-dependent phenylalanine incorporation system (13) under conditions of limiting ribosomes.

Gel Electrophoresis. The ribosomal proteins were isolated by LiCl/urea treatment (14). Sodium dodecyl sulfate and two-dimensional gel electrophoresis were performed according to Laemmli (15) and to Kaltschmidt and Wittmann (16), respectively. The labeled proteins were identified by UV illumination for Br_2FITC (17) and by determination of radioactivity for pyrene-Mal (18).

Synthesis of Labels. Fluorescein isothiocyanate (isomer I, Sigma) was brominated according to Orndorff and Hemmer (19), except that the amount of the added bromine was halved. The Br_2FITC probe consisted of an isomer mixture of dibromo-derivatives, as shown by elementary analysis and nuclear magnetic resonance spectra. The dye was further used as a 10% adsorbate on Celite (20).

N-(3-Pyrene)maleimide was synthesized as described (21). The radioactive compound (5 mCi/mmol) was obtained by the same procedure using [2,3-^{14}C]maleic anhydride.

Labeling Reactions. The conjugation reactions were routinely performed with 600 mg of 70S ribosomes suspended in appropriate buffers. pyrene-Mal (15 mg in 0.3 ml of dioxane) was added in buffer A and Br_2FITC (2 mg on Celite) in buffer C. The solutions were stirred at 0° for 1 hr, centrifuged at 10,000 × *g* for 20 min, and filtered on Sephadex G-100 equilibrated with buffer. A. The ribosomal fraction was finally submitted to a zonal sucrose gradient centrifugation in buffer A or B.

Preparation of Samples. The combination of ribosome concentration and probe absorbance was determined by the mole ratio, dye/70S ribosome, of the given conjugate. The ribosome concentration was chosen in order to keep the probe absorbance at the laser wavelength below 0.5 per cm of solution. The viscosity was varied by adding known amounts of a stock sucrose solution in buffer A, keeping constant the final ribosome and $MgCl_2$ concentrations. The η values were calculated from available tables (22). The samples were deaerated in the lateral bulb of a 10 × 5 mm optical cuvette by allowing a gentle stream of water-saturated argon to flow over the solution for 10 min with slow magnetic stirring. The temperature was regulated at 15° (equilibration period, 10 min).

RESULTS AND DISCUSSION

Specificity of Probes and Functional Assays. *N*-Ethylmaleimide is a specific reagent of SH groups of ribosomal cysteine residues (23, 24). Such a specificity can be reasonably expected for pyrene-Mal (25). Indeed, the fluorescence emission spectra of the labeled ribosomes were similar to those of standard SH-conjugates (26). A further reaction of the succinimido ring, after initial SH-binding, did not take place because no fluorescence shift corresponding to this secondary reaction was observed (26). Pyrene-Mal reacts to the same extent with the 30S and 50S subunits of the 70S ribosome. Protein analysis of the 30S subunit indicated that S1, S4, S17, and S21 were mainly labeled. The phenylalanine polymerization activity remained unaffected up to a mole ratio of 3 pyrene-Mal/70S ribosome (determined by using an extinction coefficient of 40,000 M^{-1} cm^{-1} at 345 nm for pyrene-Mal). The pyrene-Mal–70S conjugates used for correlation time measurements incorporated 50 mol of phenylalanine per mol of ribosomes.

Fluorescein isothiocyanate reacts preferentially with nonprotonated amino groups of proteins (27). We have tested the conjugation reaction of Br_2FITC over a wide range of pH and, as expected, a greater labeling was found at basic pH. The presence of the bromine atom does not significantly affect the specificity of the reagent towards the 70S ribosomal proteins (17). The 30S subunit is very reactive; the labeled proteins are S1, S2, S3, S4, and S21. More than two labels (determined by using an extinction coefficient of 80,000 M^{-1} cm^{-1} at 515 nm) could not be bound under various experimental conditions to the 50S particle. They were found to be located on L1, L2, L3, and possibly L21. Br_2 FITC had little effect on the amino-acid incorporation activity. Br_2FITC–50S subunits (2 Br_2FITC/50S ribosome), in the presence of intact 30S subunits, exhibited the same activity as the controls, whereas Br_2FITC–30S particles (3 Br_2FITC/30S ribosome) complexed with unlabeled 50S subunits showed 80% of the phenylalanine synthetic activity of the standards. The Br_2FITC–70S conjugates used in the

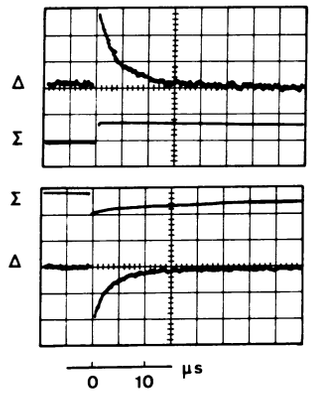


FIG. 1. Oscilloscope traces showing the rotational relaxation of 70S ribosomes in buffer A at 15°, 1.3 centipoise (Δ and Σ defined in the *text*). (*Upper*) Pyrene-Mal triplet–triplet absorption observed at 421 nm. [70S] = 10 μM, pyrene-Mal /70S ribosome = 3; Δ: 0.004 absorbance unit/division; Σ: 0.22 absorbance unit/division. (*Lower*) Br_2FITC ground-state depletion observed at 515 nm. [70S] = 2.7 μM, Br_2FITC/70S ribosome = 1.6; Δ: 0.011 absorbance unit/division; Σ: 0.22 absorbance unit/division.

following experiments had a number of labels, ranging from 1 to 3, and incorporated 50 mol of phenylalanine per mol of ribosomes.

Spectroscopic Properties of Conjugates. The choice of the probes resulted from preliminary investigations that will be reported briefly. Pyrene-Mal has been proposed earlier as a fluorescent probe (21). We found that good triplet–triplet absorption signals could be obtained with pyrene-Mal conjugates upon 353 nm excitation. The triplet–triplet spectrum resembles that of pyrene and has a maximum at 421 nm.

Fluorescein isothiocyanate is a well-known fluorescent probe, but it cannot be excited by the 530 nm harmonic of the neodymium laser. On the other hand, the triplet–triplet spectrum of fluorescein derivatives is rather weak (28). Partial bromination had the result of enhancing the triplet formation efficiency and of shifting the ground-state absorption to a more favorable region of the spectrum. The absorption maximum of free Br_2FITC at 503 nm is further shifted to 515 nm upon binding. The high extinction coefficient of the dye (ϵ = 80,000 M^{-1} cm^{-1}) is quite suitable for detecting the ground-state depletion. Furthermore, the absorbance at the laser wavelength is only one-third that at the maximum. Therefore, a high absorbance at the observation wavelength could be combined with the excitation of an optically thin sample. The latter condition was necessary in order to ensure a homogeneous excitation of the sample.

The particular combinations of excitation and observation wavelengths used (353–421 nm for pyrene-Mal; 530–515 nm for Br_2FITC) fall well outside the absorption range of the ribosomes.

Rotational Relaxation. A typical oscillogram showing the rotational relaxation of pyrene-Mal–70S ribosome conjugates is presented in Fig. 1 *upper*. The dichroism Δ was measured at the peak of the triplet–triplet absorption (421 nm). Since the absorption sum Σ remains constant over the duration of the experiment (due to the long triplet-state lifetime), the time dependence of the dichroism is entirely due to the brownian motion of the ribosomes. Δ decays to zero as isotropy is restored by rotational relaxation. There was no evidence of a free motion of the probe on this time scale; indeed, a constant dichroism was observed with ribosomes coupled to a heavy polymer (CNBr-activated Sepharose 4B) in order to increase the correlation time by several orders of magnitude.

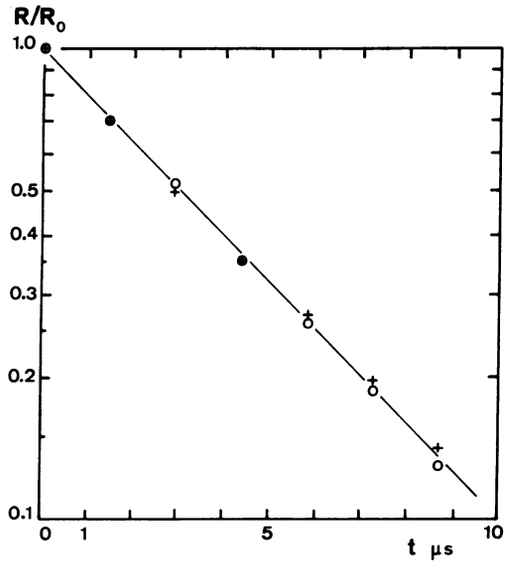


FIG. 2. Relative anisotropy decays, plotted on a logarithmic scale according to Eq. 3. Samples and conditions are identical to those of Fig. 1. (+) Pyrene-Mal conjugates; (○) Br_2FITC conjugates.

Similar results were obtained with Br_2FITC-70S conjugates (Fig. 1 *lower*). The signals have an opposite polarity because ground-state depletion was observed in this case and corresponds to a decrease in absorbance. The triplet decay (Σ) is appreciable with Br_2FITC, due to the internal heavy atom effect of the bromine. The dichroism (Δ) has to be corrected accordingly (by Eq. 3).

Under identical conditions, the anisotropy decay curves obtained with pyrene-Mal and Br_2FITC conjugates were good exponentials and gave the same correlation time within experimental errors (Fig. 2). The average Φ value measured in buffer A at 15° [η = 1.3 centipoise (1.3×10^{-3} Pa·s)] was: 4.4 ± 0.5 μs.

For a departure from an overall spherical symmetry, a multiexponential decay of the anisotropy is predicted theoretically (4, 29). In practice, however, a curvature of the logarithmic plot would be noticeable only at very long times, when experimental scatter generally prevents accurate measurements from being made. The apparent exponential decay corresponds to the harmonic means of the principal correlation times of the particle, provided there are no preferential orientations of the probes (2). Thus, a random orientation of the probes is desirable principally when experiments involving several probes must be compared (for instance, in acidic media, bleaching prevents the use of the fluorescein derivatives, whereas pyrene-Mal conjugates remain unaffected). The condition of a random orientation of the probes was satisfied for Br_2FITC and pyrene-Mal-70S ribosome conjugates, since both showed an identical anisotropy decay. The large number of ribosomal proteins labeled also supports this assumption.

As expected, the correlation time was proportional to the solvent viscosity (Fig. 3). The concentration dependence was also investigated. For each particular ribosome concentration, the correlation time was measured as a function of the solvent viscosity. Good linear fits, similar to those shown in Fig. 3, were obtained, and the slope was plotted against the ribosome concentration (Fig. 4). The correlation times were identical at 2.7 μM and 10 μM (Figs. 3 and 4). This may be taken as evidence of the absence of aggregation at these concentrations. Since the ribosome preparations have been shown to be in the fully associated (70S) state at [Mg^{2+}] = 30 mM (30), we may consider

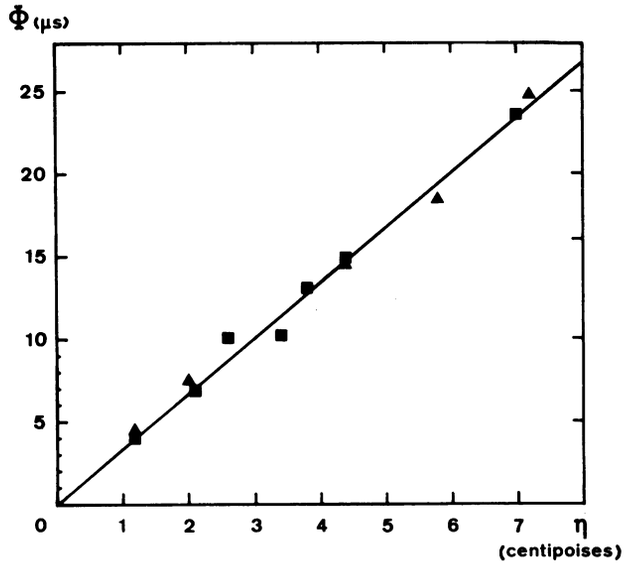


FIG. 3. Viscosity dependence of the correlation time of Br_2FITC-70S ribosome conjugates. Br_2FITC/70S ribosomes = 1.6; buffer (A); (■) [70S ribosome] = 2.7 μM; (▲) [70S ribosome] = 10 μM. (1 centipoise = 1 × 10^{-3} Pa·s.)

that the value of the slope of the regression line obtained at low ribosome concentration (i.e., 3.3 μs/centipoise) corresponds to the monomeric particle in solution. From Eq. 4, we find a value r_h = 147 Å for the radius of the equivalent hydrodynamic sphere. This has to be compared with the value of 87 Å for the dry particle, as computed from the molecular weight and the apparent specific volume (0.64 cm^3/g, ref. 31). In the spherical model, the ratio of the hydrated to the dry volume would be therefore about 5.

A Stokes radius of 126 Å was determined by Koppel (32) from translational diffusion measurements using intensity fluctuation spectroscopy. The difference is only 16% and may well be explained by the spherical model approximation. For a particle of irregular shape, the radii obtained from rotational and translational diffusion will be, in general, different. The values should agree only in the case of a sphere. However, the anisotropy decays are also dependent on the shape of the particle. The correlation time for a prolate ellipsoid is always longer than for the sphere of equal volume. If some asymmetry is introduced in the model by assuming an axial ratio of 2, the correlation time of the equivalent sphere must be divided by 1.15, according to published data (9). This would lead to a radius of 140 Å. The dimensions of the ellipsoid would be 220 × 220 × 440 Å and the ratio of the hydrated to the dry volume would reduce to about 4. This is a tentative model which, however, compares reasonably well with the dimensions 135 × 200 × 400 Å found by Hill *et al.* (33) for 70S ribosomes in solution using x-ray scattering. As noted by Koppel (32) and Hill *et al.* (33), dry specimens examined by electron microscopy generally give smaller dimensions, of approximately 160 × 180 × 200 Å (34, 35).

Since correlation times are proportional to the molecular volume (Eq. 4), the increase observed at concentrations higher than 10 μM (Fig. 4) suggests dimer or polymer formation. Upon 2-fold dilution of a 20 μM suspension, the original correlation time was restored, thus excluding irreversible or denaturing associations. As known from electron microscopy and sedimentation studies (31, 35, 36), 70S ribosomes may associate to form 100S dimers. Partial dimerization is likely to occur in the

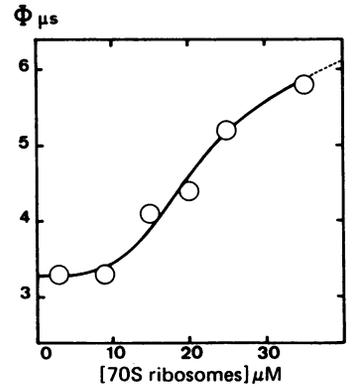


FIG. 4. Dependence of the correlation time on the total concentration of 70S ribosomes. All Φ values reduced to η = 1 centipoise (1 × 10^{-3} Pa·s).

concentrated solutions and might be responsible for the observed effect.

CONCLUSION

One of the advantages of rotational relaxation studies for obtaining structural information about biological macromolecules is the preservation of the functional activity under the experimental conditions. Triplet probes have been shown to be a valuable tool for extending the depolarization methods to such complex macromolecular species as ribosomes. In particular, we are presently investigating the Mg^{2+}-dependent association between 50S and 30S subunits, as well as the role of polyamines.

While this work was in progress, other authors (37, 38) also explored the possibility of using triplet probes, especially for study of the mobility of proteins in membranes. Their work and ours show complementary aspects of the potential applications of triplet states for probing the slow rotational relaxation either of large particles at low viscosity or of smaller units embedded in a highly viscous environment.

We thank Mr. J.-M. Lentz for his technical assistance in electronics. This work was partly supported by a grant from the Ligue Nationale Française contre le Cancer to B.A.

1. Perrin, F. (1929) *Ann. Phys. (Paris)* **12**, 169–240.
2. Weber, G. (1952) *Biochem. J.* **51**, 145–167.
3. Wahl, P. (1966) *C. R. Hebd. Seances Acad. Sci. Ser. D* **263**, 1525–1528.
4. Lombardi, J. R., Raymonda, J. W. & Albrecht, C. A. (1964) *J. Chem. Phys.* **40**, 1148–1156.
5. Cone, R. A. (1972) *Nature New Biol.* **236**, 39–43.
6. Junge, W. & Eckhof, A. (1973) *FEBS Lett.* **36**, 207–212.
7. Langelaar, J., Wegdam-Van Beek, J., Van Voorst, J. D. W. & Lavalette, D. (1970) *Chem. Phys. Lett.* **6**, 460–464.
8. Jablonski, A. (1961) *Z. Naturforsch. Teil A* **16**, 1–4.
9. Yguerabide, J. (1972) in *Methods in Enzymology*, eds. Hirs, C. H. W. & Timasheff, S. N. (Academic Press, New York), Vol. **26**, pp. 498–578.
10. Stepanov, B. I. & Gribkovskii, V. P. (1960) *Opt. Spectros.* **8**, 114–118.
11. Kurland, C. G. (1966) *J. Mol. Biol.* **18**, 90–108.
12. Eikenberry, E. F., Bickle, T. A., Trant, R. R. & Price, C. A. (1970) *Eur. J. Biochem.* **12**, 113–116.
13. Nirenberg, M. W. (1963) in *Methods in Enzymology*, eds. Colowick, S. P. & Kaplan, N. O. (Academic Press, New York), Vol. **6**, pp. 17–23.
14. Traut, P., Mizushima, S., Lowry, C. V. & Nomura, M. (1971) in *Methods in Enzymology*, eds. Moldave, K. & Grossman, L. (Academic Press, New York), Vol. **20**, pp. 391–407.

15. Laemmli, U. K. (1970) *Nature* **227**, 680–685.
16. Kaltschmidt, E. & Wittmann, H. G. (1970) *Anal. Biochem.* **36**, 401–412.
17. Kuei-Huang Huang & Cantor, C. R. (1972) *J. Mol. Biol.* **67**, 265–275.
18. Ginzburg, I. & Zamir, A. (1975) *J. Mol. Biol.* **93**, 465–476.
19. Orndorff, W. R. & Hemmer, A. J. (1927) *J. Am. Chem. Soc.* **49**, 1272–1280.
20. Rinderknecht, H. (1962) *Nature* **193**, 167–169.
21. Weltman, J. K., Szaro, R. P., Frachelton, A. R., Dowben, R., Bunting, J. R. & Cathou, R. E. (1973) *J. Biol. Chem.* **248**, 3173–3177.
22. Sober, H. A., ed. (1970) in *Handbook of Biochemistry* (Chemical Rubber Co., Cleveland, OH), 2nd ed., pp. J288–J291.
23. Moore, P. B. (1971) *J. Mol. Biol.* **60**, 169–184.
24. Ginzburg, I. & Zamir, A. (1976) *J. Mol. Biol.* **100**, 387–398.
25. Schechter, N., Elson, D. & Spitnik-Elson, P. (1975) *FEBS Lett.* **57**, 149–152.
26. Cheng-Wen Wu, Yarbrough, L. R. & Ying-Hsiueh Wu, F. (1976) *Biochemistry* **15**, 2863–2868.
27. Edman, P. (1956) *Acta Chem. Scand.* **10**, 761–768.
28. Kashe, V. & Lindqvist, L. (1965) *Photochem. Photobiol.* **4**, 923–933.
29. Ehrenberg, M. & Rigler, R. (1972) *Chem. Phys. Lett.* **14**, 539–544.
30. Favaudon, V. & Pochon, F. (1976) *Biochemistry* **15**, 3903–3912.
31. Tissières, A., Watson, J. D., Schlessinger, D. & Hollingworth, B. R. (1959) *J. Mol. Biol.* **1**, 221–233.
32. Koppel, D. E. (1974) *Biochemistry* **13**, 2712–2719.
33. Hill, W. E., Thompson, J. D. & Anderegg, J. W. (1969) *J. Mol. Biol.* **44**, 89–102.
34. Hall, C. E. & Slayter, H. S. (1959) *J. Mol. Biol.* **1**, 329–332.
35. Huxley, H. E. & Zubay, G. (1960) *J. Mol. Biol.* **2**, 10–18.
36. Cohen, S. S. & Lichtenstein, J. (1960) *J.. Biol. Chem.* **235**, 2112–2116.
37. Razi Naqvi, K., Gonzalez-Rodriguez, J., Cherry, R. J. & Chapman, D. (1973) *Nature New Biol.* **245**, 249–251.
38. Cherry, R. J., & Schneider, G. (1976) *Biochemistry* **15**, 3657–3661.