Chapter 1

Fluorescent Analog Cytochemistry of Contractile Proteins

YU-LI WANG, J. M. HEIPLE, AND D. LANSING TAYLOR

Cell and Developmental Biology
Harvard University
Cambridge, Massachusetts

I. Introduction

A new approach to investigate the distribution and interaction of cytoskeletal and contractile proteins was introduced in 1978 (Taylor and Wang, 1978) by combining the techniques of microinjection, fluorescence spectroscopy, and image intensification. The general approach, which we now call fluorescent analog cytochemistry (FAC; previously referred to as molecular cytochemistry), has been defined as the incorporation of functional fluorescent analogs of cellular components into or onto living cells.

Fluorescent analog cytochemistry is specifically designed to follow the distribution and interaction of molecular components in single living cells. However, as with any technique, errors in experimental design, execution, or interpretation can effectively limit this potentially powerful approach. We have discussed some of the advantages and practical considerations of fluorescent analog cytochemis-

METHODS IN CELL BIOLOGY, VOLUME 25

ISBN 0-12-564125-7

try in our early papers. This chapter is a set of guidelines and procedures for the optimal utilization of the technique. We will emphasize the most critical factors and point out common pitfalls.

II. Procedures

The technique involves four major steps: (1) purification and fluorescent labeling of cellular components; (2) biochemical and spectroscopic characterization *in vitro;* (3) incorporation into or onto living cells; and (4) image recording and interpretation. The following discussion will be limited to proteinaceous, cytoplasmic components using our results with 5-iodoacetamidofluorescein-labeled actin (5-AF-actin) as illustrations (Taylor and Wang, 1978; Wang and Taylor, 1979, 1980; Taylor *et al.,* 1980). A variety of labeled proteins have been incorporated into various cell types since we first introduced this technique (Feramisco, 1979; Feramisco and Blose, 1980; Kreis *et al.,* 1979; Wehland and Weber, 1980).

A. Purification and Fluorescent Labeling of Cellular Components

Fluorescent labeling of proteins in general has been discussed in several previous reviews (Dandliker and Portmann, 1971; Fairclough and Cantor, 1978; Stryer, 1978). The fluorescent conjugate (hereafter referred to as the "fluorescent analog" of a cellular component) can be prepared either by (a) labeling the purified protein subunits or by (b) labeling a supramolecular complex that contains the target protein, followed by fractionation of the labeled conjugates. This latter approach has the advantage that active sites for structure formation are more likely to be protected from modification.

The fluorescent-labeling reagents should be chosen based on several criteria. The fluorophore should absorb in the visible range, optimally 450–650 nm in wavelength, so that radiation damage to living cells and interference from autofluorescence can be minimized. Furthermore, the fluorophore should be stable under recording conditions. Optimally, fluorophores with high quantum yields and high extinction coefficients should be chosen, in order to maximize the signal from single cells. The reactive group should create a covalent bond that is stable inside the cell, without affecting the biochemical functions of the protein. The optimal reactive group will be determined to a large extent by the properties of the specific protein. For some well-characterized proteins such as actin, it is possible to choose site-specific reagents directed toward an apparently nonessential site (e.g., sulfhydryl reagents react predominantly with cys-373 in F-actin; Elzinga and Collins, 1975; Wang and Taylor, 1980). If nonspecific reagents are

used, it is important either to isolate a functional, labeled fraction after reaction or to protect the active sites furing the reaction.

To date, several fluorescent reagents have been used for fluorescent analog cytochemistry. 5-Iodoacetamidofluorescein, rhodamine isothiocyanate (RITC), and dichlorotriazinyl aminofluorescein (DTAF) (Wang and Taylor, 1980; Feramisco, 1979; Keith *et al.*, 1980). Many other fluorescent reagents such as 7-chloro-4-nitrobenzo-2-oxa diazole (NBD-Cl), eosin isothiocyanate, and cyanine dyes fit into the above criteria and remain to be explored (Ghosh and Whitehouse, 1968; Cherry *et al.*, 1976; Waggoner, 1979). Of particular interest is the "piggyback" labeling techniques using Rhod$_7$-α-lactalbumin introduced by Shechter *et al.* (1978). This technique has great potential for preparing fluorescent analogs of minor cellular components and yielding high fluorescence intensity from a very small number of labeled molecules.

B. Biochemical and Spectroscopic Characterization *in Vitro*

It is very important to characterize various properties of the fluorescent analog *in vitro* before microinjection into living cells. These assays not only minimize the possibility of artifacts after microinjection, but also provide necessary information for interpreting results from injected cells.

The solution of the fluorescent analog should be free of unbound or noncovalently associated fluorophores; otherwise, a confusing background fluorescence will be observed in the injected cell, and the results will be impossible to interpret. The test can be performed by using either a desalting column preequilibrated with SDS or SDS-gel electrophoresis. A single fluorescent band comigrating with the protein should be detected.

The average number of fluorophores per protein molecule (F/P ratio) must be determined in order to allow proper evaluation of biochemical assays. The concentration of fluorophore can be determined most conveniently by light adsorption (Dandliker and Portmann, 1971). However, it is necessary first to determine the extinction coefficient of the bound fluorophore. A simple method to obtain approximate values has been described by Hartig *et al.* (1977). The method assumes that the extinction coefficient of the fluorophore bound to unfolded protein (in 7.5 *M* urea) is equal to that of the original fluorescent reagent in the same solvent. For those fluorescent reagents that undergo significant changes in electron distribution on reaction, model compounds prepared by reaction with small molecules (i.e., amino acids) should be used.

If a low F/P ratio is obtained, it is necessary to isolate the labeled fraction to obtain a F/P $\geq$ 0.5, in order to allow unambiguous evaluation of biochemical assays. For reactions that alter the net charge of proteins, the fractionation can be carried out by ion-exchange chromatography (Wang and Taylor, 1980).

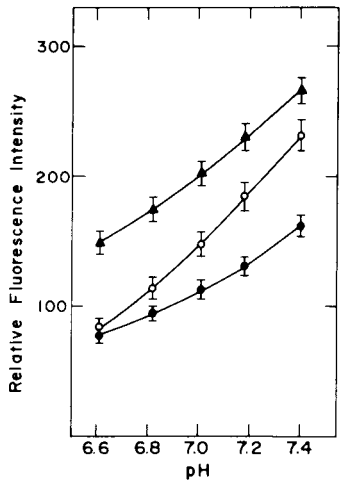

FIG. 1. Peak fluorescence intensities of 5-AF-actin are affected by both pH and the state of actin assembly. The graph indicates that the detection of G-actin (open circles) in the cell will be slightly favored over that of F-actin (solid circles). The binding of HMM (triangles) has a significant effect under saturation conditions, but given the low myosin/actin ratio in nonmuscle cells, the net effect inside cells should be limited. (From Wang and Taylor, 1980.)

Detailed biochemical assays should be carried out to assess the functional activities of the analog. For 5-AF-actin, the assays include viscosity measurement, activation of heavy meromyosin Mg^{2+} ATPase, and formation of Mg^{2+} paracrystals. The results are compared to unlabeled controls and are evaluated in relation to the percentage of protein molecules that are labeled.

Along with assays with purified proteins, additional assays can be performed using crude cell-free extracts. For example, motile extracts of *Dictyostelium discoideum* have been used to test the incorporation of 5-AF-actin into contracting fibrils and pellets (Taylor and Wang, 1978; Wang and Taylor, 1980). The extract system allows assays to be performed at a level between living cells and purified proteins and could cover functions not well characterized with purified proteins.

Spectroscopic characterization is very important for the interpretation of fluorescence signals from injected cells. It is necessary to determine whether the fluorescence intensity of spectra are affected by solvent parameters, such as pH and free Ca^{2+} ion concentration, which could vary in different regions of the cell or during different physiological states of the cell. In addition, the effect of conformational change or structural transformation on fluorescence properties should be investigated, since a particular conformation or structure could be favored at certain sites of the cell and affect the pattern of fluorescence distribution (Fig. 1).

C. Incorporation into Living Cells

The optimal target cell is determined to a large extent by the questions under investigation. For example, cultured mammalian cells are particularly amenable to whole-cell image analysis using tv-image intensifiers and computers, because of the short pathlength and distinct cytoskeletal structures. On the other hand, large cells, such as sea urchin eggs, giant amoebas, and large cultured cells (e.g., myotubes and newt lung fibroblasts), can be measured with large measuring spot sizes and yield strong fluorescence signals, and thus are more readily applicable to microspectrofluorimetric measurements.

A number of microinjection techniques, including direct-pressure microinjection, red cell ghost fusion, liposome fusion, and cell permeation, have recently been introduced or refined to deliver macromolecules into living cells (for a review, see Taylor and Wang, 1980). Two important precautions should be followed when applying these techniques.

First, the solvent condition should be compatible with both the target cell and the fluorescent analog. The cytoplasm should be kept from exposure to harmful conditions such as high pH (>7.2) or high free Ca^{2+} ion concentration ($>10^{-5}M$). In addition, appropriate solvent conditions should be chosen to stabilize the analog. For example, G-actin should be kept in the presence of nucleotides and divalent cations. Conditions that favor actin polymerization, such as high ionic strength (>10 mM) and high Mg^{2+} ion concentration (>0.2 mM), should be avoided.

Another consideration is the amount of the fluorescent analog to microinject. The final concentration of the analog in the cell should be sufficiently high to yield detectable signals, but low enough to minimize disturbance of the cell. For proteins with enzymatic activities, this precaution is especially important. We have set an upper limit at ~10% of the concentration of the corresponding endogenous component. Unfortunately, for most microinjection techniques (except direct-pressure microinjection; see Kiehart, Chapter 2, this volume), reliable ways to control the volume of delivery have yet to be developed.

An important question is whether the analog can actually be utilized by the cell after microinjection into the cytoplasm. The incorporation of analogs into cellular structures could be affected by such factors as the rate of turnover, the accessibility of incorporation sites, and the mechanism of structure formation. Using mass-incorporation methods such as ghost fusion, it should be possible to apply biochemical techniques to study the turnover of the injected analog (Rechsteiner, 1979). The question of incorporation can be studied by using model systems in which the behavior of the component has been characterized in detail. For example, the single-cell model of *Chaos carolinensis* has been used to test the incorporation of 5-AF-actin (Taylor and Wang, 1978; Taylor *et al.*, 1980). A significant fraction of 5-AF-actin is found to remain associated with the ghost

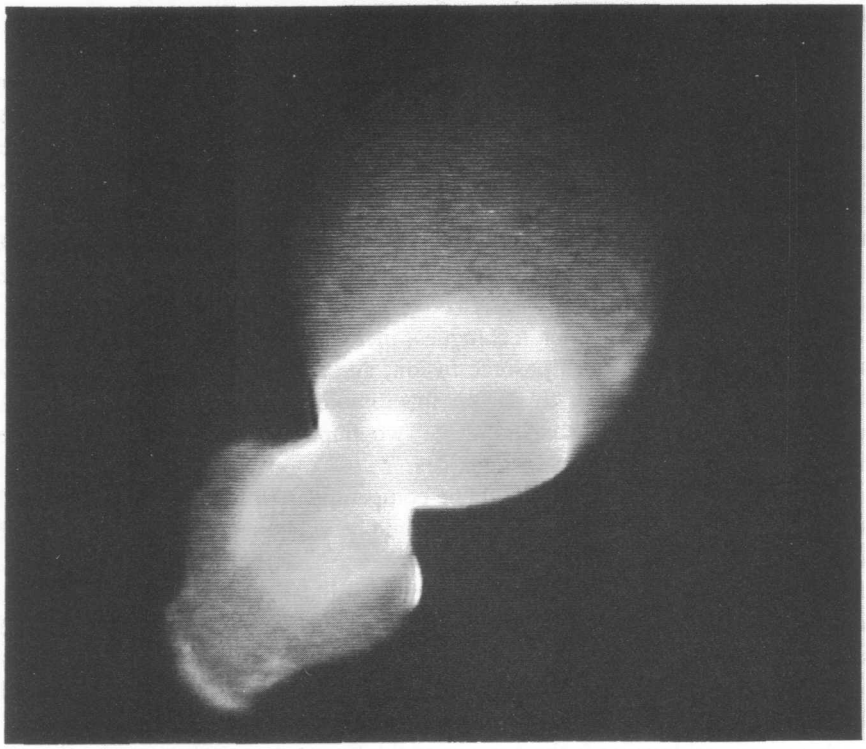

FIG. 2. Phalloidin is injected into a *Chaos carolinensis* that contains 5-AF-actin. The fluorescent fibrils contract irreversibly to one end of the cell and recruit most of the 5-AF-actin. Image recorded using a RCA SIT coupled to a NEC videotape recorder.

and membrane-free cytoplasm on rupturing a preinjected cell in a stabilizing solution. Furthermore, 5-AF-actin becomes incorporated into cytoplasmic fibrils when a cell with 5-AF-actin is postinjected with phalloidin (Fig. 2). These observations are consistent with the known characteristics of endogenous actin in amoebas (Taylor *et al.*, 1976; Stockem *et al.*, 1978). The use of model systems indicates whether the analog can be utilized by the cell under specified conditions, but does not prove that the analog can be incorporated into all possible cell structures or under all conditions. When interpretating results from injected cells, the possibility of "false negatives" should always be considered.

D. Image Recording and Interpretation

The fluorescence image of injected cells can be recorded using photography, and high-quality records can be obtained from nonmotile processes (under condi-

tions similar to those used for immunofluorescence). However, the relatively long exposure required (>1–2 minutes) is not desirable for motile processes or for probes sensitive to photobleaching.

The technique of image intensification provides a powerful alternative for image recording (Reynolds, 1972; Reynolds and Taylor, 1980; Willingham and Pastan, 1978; Sedlacek *et al.*, 1976). The sensitivity is at least 3–4 orders of magnitude higher than that of photography. As a result, strong excitation light can be avoided and photobleaching can be minimized. The time resolution is 1/30 to 1/60 of a second, so most motile processes can be recorded in real time. In addition, the image can be readily digitized and processed by computers. Although the image quality becomes a limiting factor at very low light intensity, photography under those conditions is very often impossible to carry out.

The detection limit of fluorescence from single cells depends on the specific recording conditions and on the fluorophore employed. It has been demonstrated that small bundles of 5-AF-actin containing no more than ~4000 fluorophore molecules per micron (~10 filaments) can be readily detected using a 60-watt quartz-halogen lamp and a silicon-intensified target (SIT) tv camera (Wang and Taylor, 1980, Fig. 3). The actual detection limit of discrete structures in the cell will probably be more limited, since the analog will be diluted by the endogenous component, and fluorescent structures will probably be surrounded by a certain level of unorganized fluorescence. We estimate that actin bundles containing ~50 filaments should be detectable under the above conditions. However, using more sensitive image intensifiers in conjunction with computer manipulation of video images, the sensitivity can be significantly higher.

Great caution must be exercised when interpreting fluorescence images of injected cells. The two-dimensional distribution of fluorescence intensity is affected by several factors, including the plane of focus, the accessible volume of the analog, local solvent conditions, local fluorophore concentration, and the conformation or structure of the analog. Some of these factors will affect not only the image of the fluorescent analog being studied but also the image of essentially any fluorescent macromolecule (e.g., fluorescently labeled ovalbumin, fluorescent antibodies).

Accessible volume is determined by the thickness of the cell and by the distribution of membrane-bound organelles (Fig. 4). In those regions of the cell including stress fibers, mitotic spindles and hyaline cytoplasm, which are relatively free of membrane-bound organelles, high apparent fluorescence intensity should be interpreted with caution (Fig. 5). Controls for accessible volume can be carried out by incorporating a fluorescently labeled inert macromolecule with similar solubility properties. For example, labeled ovalbumin has been used as a control for 5-AF-actin. Control experiments can be performed in a separate cell (Wang and Taylor, 1979), or, using fluorophores of different excitation and emission wavelengths, performed in the cell that contains the experimental

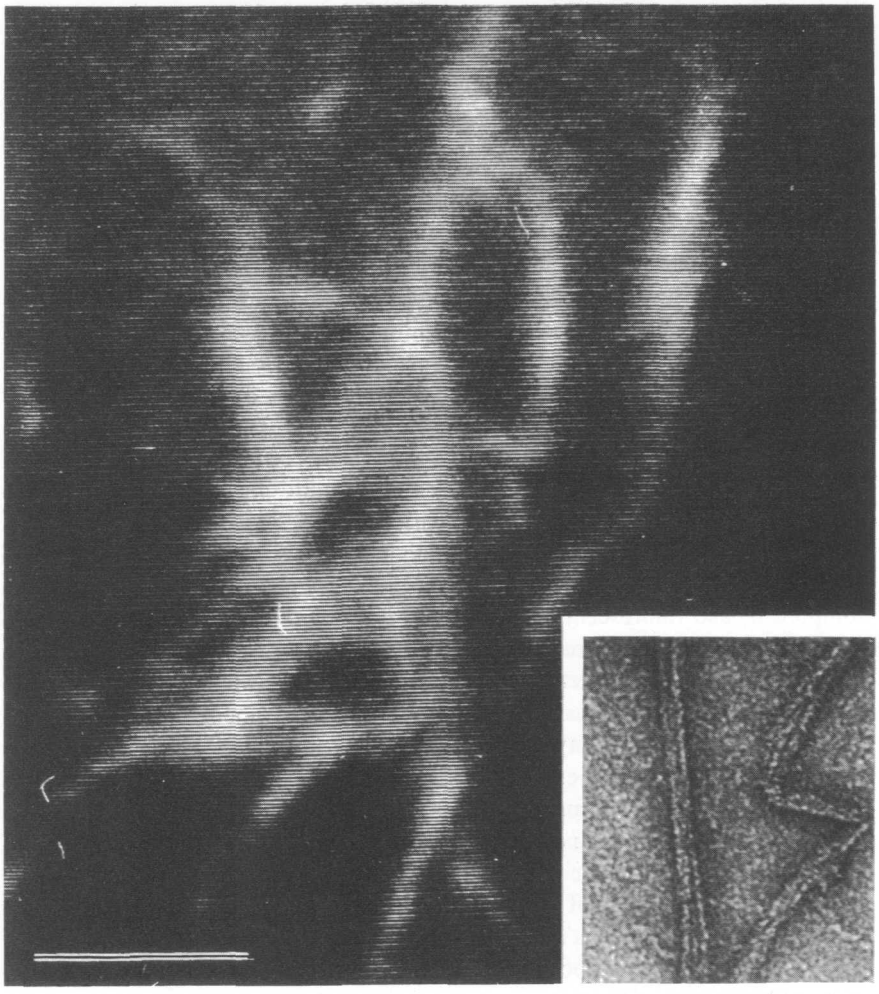

FIG. 3. Ni^{+2} paracrystals of 5-AF-actin, as detected by a fluorescence microscope coupled to a tv-image intensifier. Bar = 5 μm. Inset: electron microscopy of negatively stained Ni^{+2} paracrystals.

analog (Taylor *et al.*, 1980). This latter method is preferable for cells of irregular shape, since images of the analog and the control can be compared directly by shifting the excitation and emission filters. Such comparison can even be performed electronically by computer-aided image analysis.

As discussed previously, the sensitivity of the fluorophore to solvent conditions should be studied *in vitro* before incorporation into living cells. For those fluorophores that are sensitive to solvent conditions, experiments should be

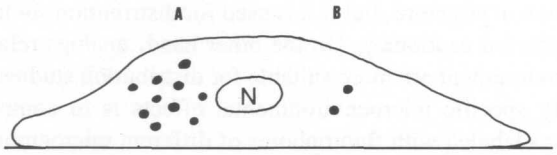

FIG. 4. Simplified diagram of a cell viewed from the side. Injected soluble macromolecules are excluded by the membrane-bound organelles. As a result, region B shows higher fluorescence intensity than region A, even if the injected fluorescent molecules are uniformly distributed.

performed to identify possible solvent effects inside the cell. The easiest approach is to inject a control molecule labeled with the same fluorophore into a separate cell, and to compare the fluorescence distribution with that of the analog. Alternatively, some solvent factors such as pH and free Ca^{2+} ion concentration can be measured by independent methods (Blinks *et al.*, 1979; Caswell, 1979; Heiple and Taylor, 1980).

The fluorescence distribution could also be affected by the conformation or the supramolecular structure of the analog. This effect will be specific for the analog and cannot be identified using a different control protein. For example, actin labeled with NBD-Cl shows dramatic increases in fluorescence intensity during polymerization (Detmers *et al.*, in 1981). Therefore, images of injected cells would be biased toward regions enriched in F-actin. This kind of analog with high microenvironmental sensitivity will be useful for studying intracellular pro-

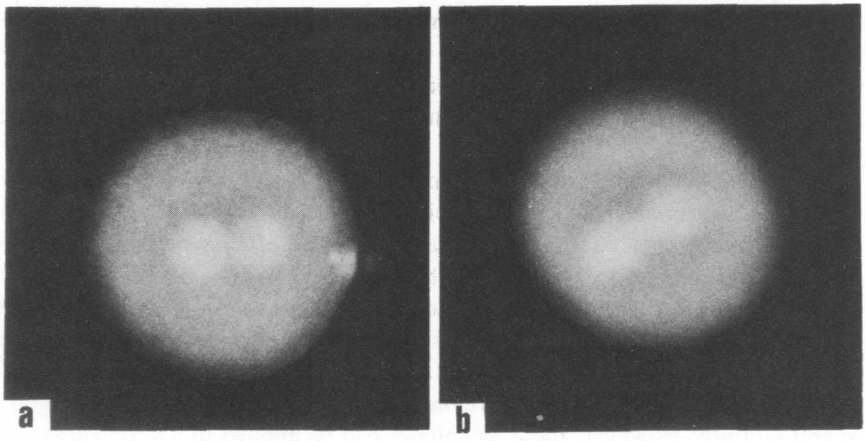

FIG. 5. Two separate sea urchin eggs during mitosis, injected with 5-AF-actin (a), and FTC-ovalbumin (b), respectively. The mitotic spindles in both cells are distinctly fluorescent, most likely because of their large accessible volume. It is impossible to tell, based on qualitative observations, whether 5-AF-actin is more concentrated in the spindle than elsewhere. (From Wang and Taylor, 1979.)

tein conformation or structure, but if it is used for distribution studies, the images should be interpreted cautiously. On the other hand, analogs relatively insensitive to microenvironment are more suitable for distribution studies. One possible way to identify specific microenvironmental effects is to compare images of different analogs labeled with fluorophores of different microenvironmental sensitivities.

III. Conclusion and Prospectus

Three levels of analysis with fluorescent analog cytochemistry are possible: (1) qualitative studies on the distribution of cellular organelles or molecules; (2) quantitative studies on the distribution of cellular organelles or molecules; and (3) microspectrofluorometric analysis of the interaction of organelles or molecules molecules.

In any given experiment, both the nature of the biological question asked and the cell type employed will dictate the level of resolution and quantitation at which this technique can be applied. To date, fluorescent analog cytochemistry has been limited to qualitative studies on the distribution of specific molecules. This approach should continue to be valuable in answering a wide variety of cellular questions in many different cell types.

Quantitative analyses of the distribution of specific proteins will be particularly important in cells with irregular shape and during dynamic cellular processes such as amoeboid movement, karyo- and cytokinesis. Computer manipulation of digitized images will enable a variety of quantitative studies to be performed, including normalization of pathlength and accessible volumes, enhancement of images, and real-time mapping of the distribution of specific molecules.

One of the most important applications of fluorescent analog cytochemistry will be the measurement of spectroscopic properties of the labeled molecules *in vivo*. Fluorescence photobleaching, polarization, and lifetime measurements, as well as resonance energy transfer, can be quantitated in living cells (see Taylor and Wang, 1980, for a review). Such data may yield information on diffusion coefficients, specific binding activities, and supramolecular structures of the labeled protein or proteins.

We have emphasized the use of fluorescent analog cytochemistry in the study of cell motility. However, these techniques can be applied to a wide variety of organelles and cellular constituents (Taylor and Wang, 1980). Careful planning and execution of the experiments and controls should make this a powerful tool in many different fields.

ACKNOWLEDGMENTS

The authors would like to thank J. Lupo, M. Rockwell, L. Meszolay, and L. Houck for excellent technical assistance. The research described in this report was supported by grants from the NIH (AM 18111) and NSF (PCM-7822499).

REFERENCES

Blinks, J. R., Mattingly, P. H., Jewell, B. R., Leeuwen, M., Harrer, G. C., and Allen, D. G. (1979). *In* "Methods in Enzymology" (W. B. Jakoby and I. H. Pastan, eds.), Vol. 58, pp. 292-328. Academic Press, New York.

Caswell, A. H. (1979). *Int. Rev. Cytol.* **56,** 145-181.

Cherry, R. J., Cogoli, A., Oppliger, M., Schneider, G., and Semenza, G. (1976). *Biochemistry* **15,** 3653-3656.

Dandliker, W. B., and Portmann, A. J. (1971). *In* "Excited States of Proteins and Nucleic Acids" (R. F. Steiner and I. Weinryb, eds.), pp. 199-275. Plenum, New York.

Detmers, P., Weber, A., Elzinga, M., and Stephens, R. (1981). In press.

Elzinga, M., and Collins, J. H. (1975). *J. Biol. Chem.* **250,** 5897-5905.

Fairclough, R. H., and Cantor, C. R. (1978). *In* "Methods in Enzymology" (S. N. Timasheff and C. H. W. Hirs, eds.). Vol. 48, pp. 347-379. Academic Press, New York.

Feramisco, J. R. (1979). *Proc. Natl. Acad. Sci. U.S.A.* **76,** 3967-3971.

Feramisco, J. R., and Blose, S. H. (1980). *J. Cell Biol.* **86,** 608-615.

Ghosh, P. B., and Whitehouse, M. W. (1968). *Biochem. J.* **108,** 155-156.

Hartig, P. R., Bertrand, N. J., and Sauer, K. (1977). *Biochemistry* **16,** 4275-4282.

Heiple, J. M., and Taylor, D. L. (1980). *J. Cell Biol.* **86,** 885-890.

Keith, C. H., Feramisco, J. R., and Shelanski, M. L. (1980). *J. Cell Biol.* **88,** 234-240.

Kreis, T. E., Winterhalter, K. H., and Birchmeier, W. (1979). *Proc. Natl. Acad. Sci. U.S.A.* **76,** 3814-3818.

Rechsteiner, M. (1979). *In* "Transfer of Cell Constituents into Eukaryotic Cells" (J. E. Celis, A. Graessmann, and A. Loyter, eds.), pp. 113-141. Plenum, New York.

Reynolds, G. T. (1972). *Q. Rev. Biophys.* **5,** 295-347.

Reynolds, G. T., and Taylor, D. L. (1980). *BioScience* **30,** 586-592.

Sedlacek, H., Gundlach, H., and Ax, W. (1976). *Behring Inst. Mitt.* **59,** 64-70.

Shechter, Y., Schlessinger, J., Jacobs, S. Chang, K.-J., and Cuatrescasas, P. (1978). *Proc. Natl. Acad. Sci. U.S.A.* **75,** 2135-2139.

Stockem, W., Weber, K., and Wehland, J. (1978). *Cytobiologic* **18,** 114-131.

Stryer, L. (1978). *Annu. Rev. Biochem.* **47,** 819-846.

Taylor, D. L., and Wang, Y.-L. (1978). *Proc. Natl. Acad. Sci. U.S.A.* **75,** 857-861.

Taylor, D. L., and Wang, Y.-L. (1980). *Nature (London)* **284,** 405-410.

Taylor, D. L., Rhodes, J. A., and Hammond, S. A. (1976). *J. Cell Biol.* **70,** 123-143.

Waggoner, A. S. (1979). *Annu. Rev. Biophys. Bioeng.* **8,** 47-68.

Wang, Y.-L., and Taylor, D. L. (1979). *J. Cell Biol.* **81,** 672-679.

Wang, Y.-L., and Taylor, D. L. (1980). *J. Histochem. Cytochem.* **28,** 1198-1206.

Wehland, J., and Weber, K. (1980). *Exp. Cell Res.* **127,** 397-408.

Willingham, M., and Pastan, I. (1978). *Cell* **13,** 501-507.