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Studies on Spin-Labeled Actin*

Deborah B. Stone,† Shirley C. Prevost, and Jean Botts

ABSTRACT: Using the spin-labeling technique of McConnell it has been found that a paramagnetic derivative of maleimide is an excellent probe of actin–actin interactions. Incorporation of molar quantities of this spin label into the actin molecule does not impair the polymerizability of this muscle protein. Electron paramagnetic resonance spectra of spin-labeled actin reveal a strong immobilization of the label accompanies polymerization. As with the viscosity change, both the rate and the extent of the spectral change were found to depend on the actin and KCl concentrations employed in the polymerization reaction. Analysis of these data suggests that the spectral change results either from masking of label situated near the polymerization site or from a conformational change within the monomer unit brought about by linkage of monomer to the growing polymer chain. In contrast to actin–actin interactions, spin label on actin partially inhibits interaction with myosin. No spectral changes were observed to accompany the binding of spin-labeled actin to myosin.

The two characteristic properties of the muscle protein actin are (1) conversion from a monomeric state of globular subunits (G-actin) into a fibrous polymer (F-actin) in the presence of neutral salts, and (2) interaction with another muscle protein, myosin A, to provide the structural continuity and elevated ATPase activity requisite for muscle contraction. Although it is now reasonably certain that polymerization of actin is an *in vitro* phenomenon which does not accompany contraction, the finding of increased exchangeability of F-actin-bound nucleotide during the contraction-like process of superprecipitation (Szent-Györgyi and Prior, 1966; Moos *et al.*, 1967) suggests that some loosening of the actin polymer may occur during contraction. It is thus evident that knowledge of actin–myosin as well as actin–actin interactions is central to the understanding of contraction.

In studying these interactions, we have employed the spin-labeling technique of McConnell and coworkers (Stone *et al.*, 1965; Hamilton and McConnell, 1968). In this procedure 1 or more moles of a nitroxide-free radical (spin label, I–III) are covalently bound to a protein. The electron paramagnetic resonance of these compounds is sensitive to molecular motion and the restrictions imposed on the free rotation of the spin label by covalent bonding to a large, relatively motionless protein molecule produce sizable changes in the spectra. Furthermore, small changes in the rotational freedom of appropriately located spin labels such as might result from a conformational change or union of one protein with another are readily detectable in the spectra.

We have found that the maleimide spin label (I) is an excellent probe of actin–actin interaction. This spin label does not interfere with polymerization, but suffers a large immobi-

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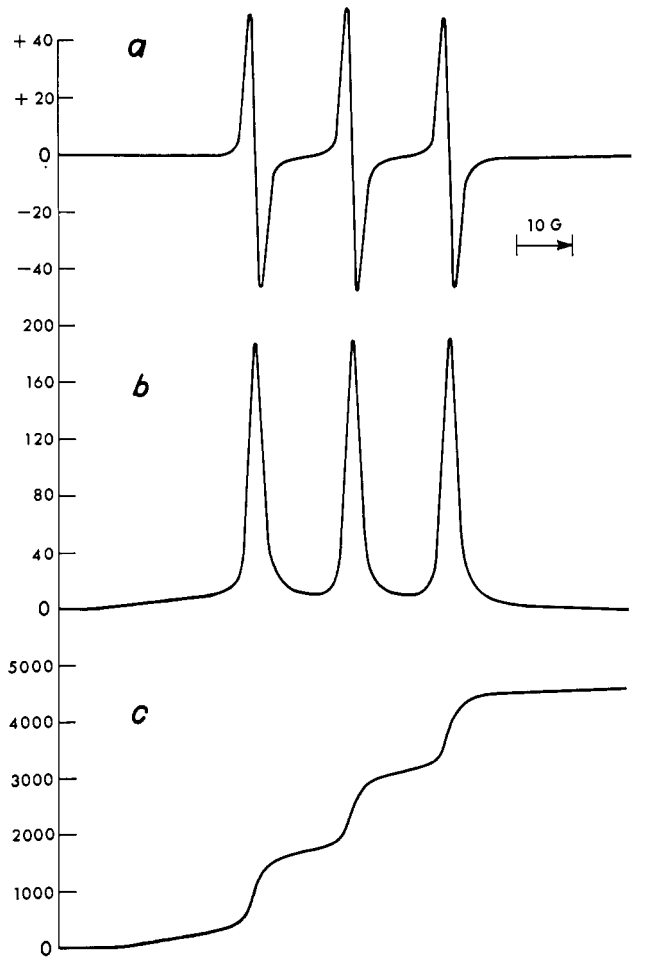
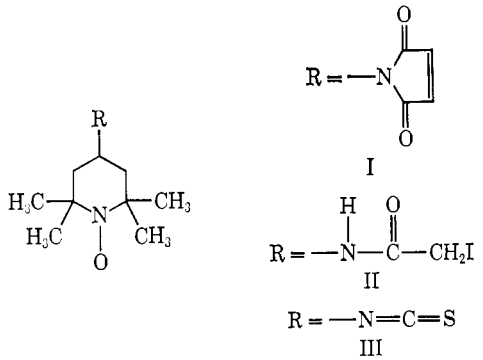


FIGURE 1: Electron paramagnetic resonance spectrum of 5×10^{-5} M maleimide spin label (I) in 10 mM Tris-Cl (pH 7.5) (a) as recorded by a Varian E-3 EPR spectrometer at a gain setting of 2×10^4, (b) after a single integration, and (c) after double integration. Ordinate values are given in arbitrary units.



lization coincident with polymerization. The resulting change in paramagnetic resonance should prove useful in monitoring actin–actin interactions in many experimental situations.

Experimental Section

Preparation of Actin. Acetone-dried muscle powder was prepared by the procedure of Carsten and Mommaerts (1963) with the exception that the final residue was extracted five times with two to three volumes acetone to ensure removal of all lipid material. The air-dried muscle powder was stored in a desiccator at 4° for periods of as long as 6 months without perceptible change in its properties. Actin was extracted from the dried muscle powder at 4° with 0.2 mM ATP (pH 7.5) and purified by repeated polymerization–depolymerization as described by Carsten and Mommaerts (1963). The initial polymerization was brought about by the addition of 0.1 mM $MgCl_2$ and 50 mM KCl; thereafter only 50 mM KCl was added for polymerization. Sodium ascorbate was found to interfere with the spin-label studies (presumably through catalyzing the reduction of the nitroxide group in the spin-label compounds) and was therefore omitted from all actin preparations used in this work. The omission of ascorbate resulted in no perceptible change in the properties of freshly prepared actin, although the lability of these preparations was slightly increased. For studies on G-actin, pellets of F-actin (obtained from a 4-hr centrifugation at 65,000 rpm) were suspended in salt-free medium (0.2 mM ATP, pH 7.5) by homogenization (as described by Carsten and Mommaerts, 1963), dialyzed overnight against 50–100 volumes of solvent to ensure complete depolymerization, and centrifuged to remove denatured protein. A value of 60,000 was assumed for the molecular weight of actin in these studies, although recent investigations have suggested a lower figure (see, for instance, Rees and Young, 1967).

Other Materials. Myosin A was prepared from rabbit skeletal muscle by the procedure of Tonomura *et al.* (1966). The spin labels used in this work were prepared by Varian Associates. *N*-(1-Oxyl-2,2,6,6-tetramethyl-4-piperidinyl)maleimide (I) was kindly donated by Dr. Harden M. McConnell, *N*-(1-oxyl-2,2,6,6-tetramethyl-4-piperidinyl)iodoacetamide (II) was purchased from Varian Associates, and *N*-(1-oxyl-2,2,6,6,-tetramethyl-4-piperidinyl)isothiocyanate (III) was kindly donated by Dr. William C. Landgraf.

Techniques of Spin Labeling and Recording Electron Paramagnetic Resonance Spectra. G-actin, completely depolymerized by dialysis *vs.* 0.2 mM ATP (pH 7.5), was reacted with 1–2 moles of spin label/mole of G-actin for 6–24 hr at 0°; unreacted label was then removed by overnight dialysis *vs.* 50–100 volumes of solvent. F-actin was reacted with 1–2 moles of spin label/mole of monomer subunit for several days at 0°. The unreacted spin label was removed by: (a) dialysis *vs.* 50–100 volumes of solvent (0.2 mM ATP, pH 7.5, and 50 mM KCl) when F-actin was desired or (b) the combined centrifugation–dialysis procedure described above for the depolymerization of actin when monomer actin was desired. Unlabeled samples of the same actin preparations (used as controls for the biochemical determinations) were treated in a similar manner.

The electron paramagnetic resonance spectra of the spin-labeled proteins were recorded with a Varian E-3 EPR spectrometer operating at 9.5 GHz with 100-mW power, a field setting of 3380 G, a field scan rate of 100 G/4 min, a time constant of 1 sec, and a modulation amplitude of 1 G. All measurements were carried out at room temperature using a quartz liquid flat cell. The temperature within the cell was approximately 24°. Spectra were usually recorded in duplicate or triplicate (except in time-course studies) and varied no more than 5% in peak heights. The amount of spin label covalently bound to actin was estimated by

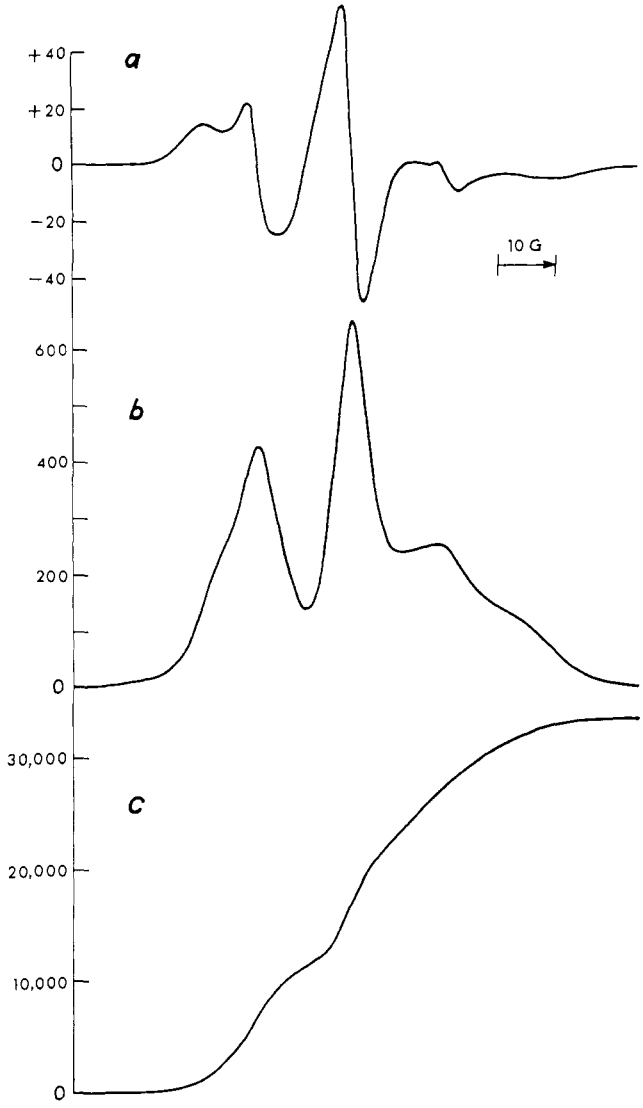


FIGURE 2: Electron paramagnetic resonance spectrum of G-actin (2.32 mg/ml) in 0.2 mM ATP (pH 7.5) labeled in the polymer form with 0.93 mole of maleimide spin label (I)/mole of monomer subunit (a) as recorded by a Varian E-3 EPR spectrometer at a gain setting of 2×10^5, (b) after a single integration, and (c) after double integration. Ordinate values are given in arbitrary units.

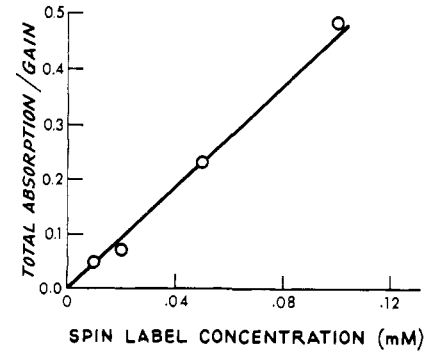


FIGURE 3: Standard curve for calculating spin-label concentrations from electron paramagnetic resonance spectra. The total microwave absorption was calculated from the second integral of the electron paramagnetic spectrum as described in the text and adjusted for the instrument gain setting. Ordinate values are given in arbitrary units.

comparing the total microwave absorption of well-dialyzed spin-labeled samples of monomer actin with that of known quantities of unbound label. Computation of the total microwave absorption was carried out by double integration (using Simpson's rule) of the paramagnetic resonance spectra (which are recorded as the derivative of the absorption with respect to field strength) with the aid of a digital computer. Typical plots of the computer output after the first (the true absorption curve) and second integrations are compared with the original spectra in Figures 1 and 2 for unbound and actin-bound label, respectively. The total microwave absorption (given by the final value of the second integral, curve C in Figures 1 and 2) measured for different concentrations of free label was adjusted for the instrument gain setting used for recording the initial spectrum and plotted against the molar concentration of spin label for a series of samples to produce the standard curve given in Figure 3.

Viscosity Measurements. The viscosity of actin solutions was measured before and during polymerization at 25.0° using an Ostwald viscometer with a solvent outflow time of 66 sec.

Actin-Bound Nucleotide. Free nucleotide was removed from G-actin solutions by treatment with 20 mg/ml of Dowex 1-X8 (200–400 mesh) at room temperature for 5 min (Asakura, 1961). The nucleotide content of actin was determined, after precipitation of actin with 4% perchloric acid, by measuring the absorbancy of the supernatant at 260 and 300 mμ (Bárány *et al.*, 1966).

ATPase Activity. The ability of F-actin to activate myosin A was assayed at 25° in 25 ml of medium containing 50 mM KCl, 50 mM Tris-Cl (pH 8.0), 1 mM $MgCl_2$, 1 mM ATP, 0.1 mg/ml of myosin A, and varying concentrations of control or spin-labeled F-actin. At 50-sec intervals four aliquots (5 ml) were removed from the reaction mixture and assayed for P_i by the colorimetric technique of Fiske and Subbarow (1925) as modified by Morales and Hotta (1960).

Superprecipitation. The ability of F-actin to superprecipitate with myosin A was determined by the procedure of Bárány *et al.* (1966).

Results

Electron Paramagnetic Resonance Spectra of Actin Spin Labeled in the Polymer State. Spectra of actin spin labeled in the polymer or F form, then converted into G-actin are shown in Figure 4 (solid lines). The spectra revealed that the maleimide (I, Figure 4A) and isothiocyanate (III, Figure 4B) labels reacted with at least two different sites on the F-actin molecule. These sites can be differentiated by the degree to which the rotational motion of the spin label is restricted: highly immobilized label gives the outermost lines in the spectrum (labeled 1 and 5 in Figure 4A), weakly immobilized label contributes the next inner lines of the spectrum (2 and 4 in Figure 4A). The iodoacetamide label (II, Figure 4C) reacted almost exclusively with sites which only weakly immobilized the spin label. When the spin-labeled actins were polymerized with 50 mM KCl (Fig-

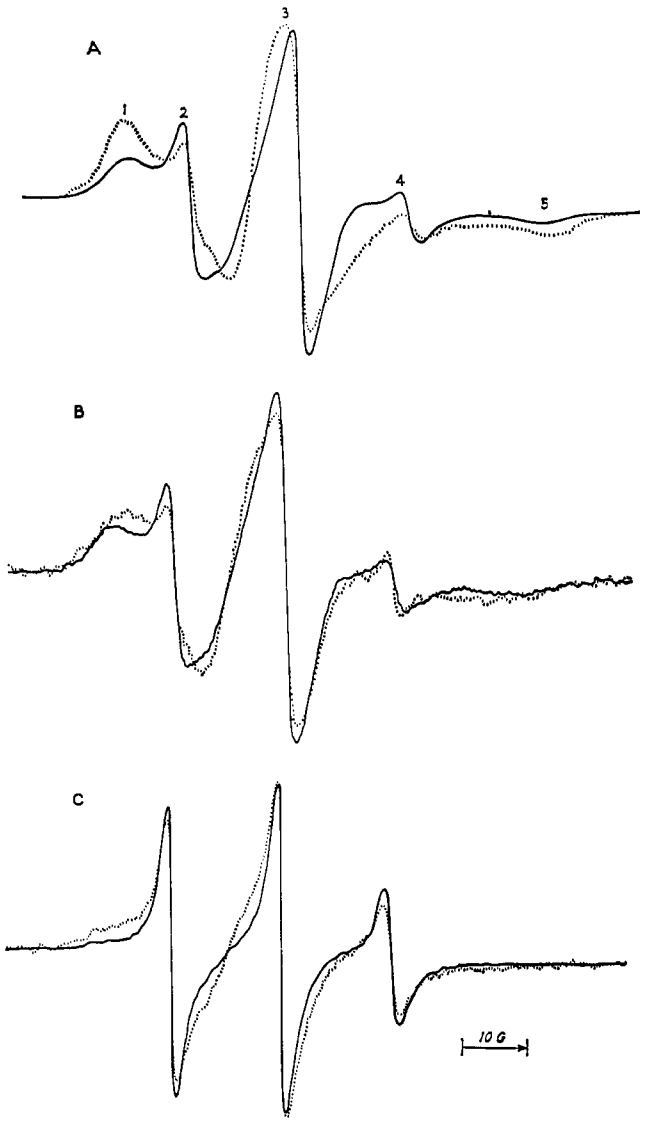


FIGURE 4: Electron paramagnetic resonance spectra of actin (preparation VIIIB) spin labeled in the polymer form with: (A) 1.03 moles of maleimide spin label (I), (B) 0.24 mole of isothiocyanate spin label (III), and (C) 0.18 mole of iodoacetamide spin label (II) per mole of monomer subunit. In each case labeling was carried out for 10 days at 0° in the presence of 1.5 moles of spin label/mole of monomer subunit. Spectra were recorded (at a protein concentration of approximately 5 mg/ml) of G-actin (solid lines) and following repolymerization to F-actin with 50 mM KCl (broken lines).

ure 4, broken lines) there was an increase in the proportion of label attached to strongly immobilizing sites and a proportionate decrease in the amount of label attached to weakly immobilizing sites. The degree of immobilization of the label upon polymerization was large with the maleimide and isothiocyanate labels, but relatively small with the iodoacetamide label.

Since the maleimide spin label gave the best labeling (see legend to Figure 4) and also showed the largest spectral change upon polymerization, further studies were carried out with this label. The results of several experiments are summarized in Table I. A convenient method for reporting changes in electron paramagnetic resonance spectra is in

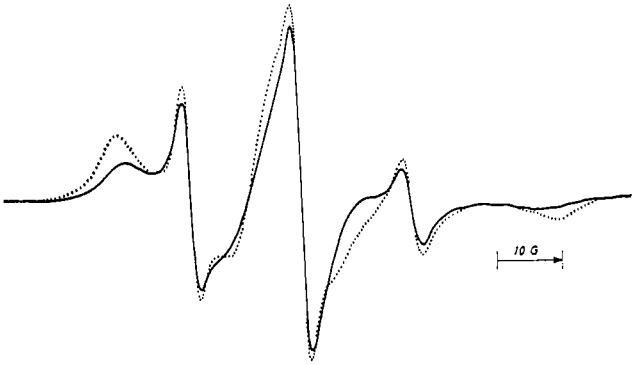


FIGURE 5: Electron paramagnetic resonance spectra of actin (preparation IXA) spin labeled in the monomer form with maleimide spin label (I). Spectra were recorded (at approximate protein concentrations of 5 mg/ml) of G-actin (solid line) and following repolymerization to F-actin with 50 mM KCl (broken line).

terms of the ratio of the heights of the first two peaks of the spectrum (herein denoted $H_1:H_2$). This ratio gives an indication of the degree of mobility of the spin label, an increase in the ratio results from a rise in the population of spin labels which are strongly immobilized and a decrease in the labels which are only weakly immobilized. On the average, a twofold increase in $H_1:H_2$ was observed upon polymerization.

Mention should be made of the variability in the $H_1:H_2$ ratio obtained from different preparations of actin labeled with similar quantities of maleimide spin label. This could arise from contamination of the actin preparations with a variable amount of a second protein reactive to spin label, but having an $H_1:H_2$ ratio different from that of actin. In order to test this hypothesis, pellets of actin spin labeled in the polymer form acording to procedures outlined in the methods section were suspended in and dialyzed against 5×10^{-4} M ATP–5×10^{-4} M 2-mercaptoethanol–2×10^{-4} M $CaCl_2$ and passed through a Sephadex G-200 column according to the procedure of Rees and Young (1967). Only two protein fractions were obtained. The first of these appeared at the void volume, comprised approximately 11% of the recovered protein, and contained no measureable spin label. All of the spin label was found associated with the second, G-actin-containing peak. These results suggest that large molecular weight contaminants (*e.g.*, relaxing protein) present in the actin preparations are not labeled with spin label under the conditions employed here. Inactive aggregates of actin have also been found as contaminants of G-actin preparations (Rees and Young, 1967). These would be expected to have a partially denatured and therefore a more open conformation in which the spin label could rotate more freely; the presence of a small amount of such inactive spin-labeled actin in our preparations would result in a relatively lower $H_1:H_2$ ratio. Since Rees and Young (1967) have shown that the ATP–$CaCl_2$–2-mercaptoethanol solvent reduces the proportion of this inactive fraction, it was interesting to find that suspension of actin (labeled with spin label in the polymer form) as the monomer form in this solvent gave an $H_1:H_2$ ratio of approximately 1, significantly higher than that obtained in the 0.2 mM ATP solvent. These findings are consistent with the presence of small,

TABLE I: Effect of Polymerization on the Electron Paramagnetic Resonance Spectra of Actins Labeled with Maleimide Spin Label.

Conditions of Spin Labeling						
Prepn	Polymer State of Actin during Reaction	Moles of Spin Label Added/ 6×10^4 g of Actin	Length of Reaction (Days)	Moles of Spin Label Reacted/ 6×10^4 g of Actin	$H_1:H_2$[a] G-actin	$H_1:H_2$[a] F-actin[b]
VIIIA	F	1	3	0.49	0.57	1.15
VIIIB	F	1.5	10	1.03	0.51	1.39
VIIIC	F	1.5	4	0.93	0.69	1.40
IXA	F	1.5	6	1.06	0.21	0.45
IXB	F	2	2	1.08	0.31	0.51
IXA	G	1.5	0.4	0.53	0.39	0.58
IXB	G	2	1	1.41	0.10	0.14

[a] $H_1:H_2$ = ratio of heights of first two peaks of electron paramagnetic resonance spectrum. [b] All samples were polymerized with 50 mM KCl except preparation VIIIC which was polymerized with 15 mM KCl.

but variable amounts of an inactive form of actin in our standard preparations.

Electron Paramagnetic Resonance Spectra of Actin Spin Labeled in the Monomer State. Spectra of actins spin labeled in the monomer state qualitatively resembled those of actins spin labeled in the polymer state: the maleimide (Figure 5) and isothiocyanate labels were bound to both strongly and weakly immobilizing sites, but the iodoacetamide label reacted almost exclusively with weakly immobilizing sites. A comparison of the spectra of actins labeled with similar quantities of maleimide spin label in the G and F forms reveals a lower $H_1:H_2$ ratio for the actins reacted in the monomer form (Table I). This could be due to the presence of a greater proportion of inactive actin in preparations where the label is attached to the monomer form or, alternatively, to the presence in the monomer form of more weakly immobilizing sites which are reactive for the spin label. In support of the latter suggestion, it was found that treatment of monomer actin with *N*-ethylmaleimide (2 moles/mole of actin) prior to reaction with maleimide spin label results in a more immobilized spectrum ($H_1:H_2$ = 0.39 as opposed to 0.25 for a similar sample treated with maleimide spin label alone).

Actin labeled with maleimide spin label in the monomer state showed an increase in the $H_1:H_2$ ratio of 35–50% when polymerized with 50 mM KCl (Figure 5 and Table I), a change which was significantly less than that observed with actin labeled in the polymer form. The spectral change observed upon polymerization of actins labeled either as monomer or polymer could be reproduced upon depolymerization and repolymerization of the samples. Depolymerization of actin in 0.6 M KI (Nagy and Jencks, 1965) was also found to reverse the spectral change.

Effect of Polymer State on the Electron Paramagnetic Resonance Spectra of Spin-Labeled Actins. F-actin solutions contain polymer in equilibrium with monomer; the ratio of the two forms is determined by the solvent conditions (Kasai *et al.*, 1962a). It was therefore of interest to investigate the relationship between the electron paramagnetic resonance spectrum of spin-labeled actins and the state of polymerization. In the experiment reported in Table II, two F-actin samples (labeled with maleimide spin label in the monomer and polymer forms, respectively) were centrifuged to separate the sedimentable F-actin from nonsedimenting G-actin. Since reestablishment of equilibrium is slow in the absence of ATP (Kasai *et al.*, 1962a) this procedure can be used to effect a relatively good separation of monomer and polymer actin. The resulting pellet showed an increased $H_1:H_2$ ratio, a finding which is consistent with enrichment of the F-actin following removal of G-actin. The supernatant from this centrifugation (which contained 11–14% of the original protein) had an $H_1:H_2$ ratio that was lower than that of the F-actin solution before centrifugation, but closely resembled

TABLE II: Effect of Polymer State on the Electron Paramagnetic Resonance Spectra of Actin Labeled with Maleimide Spin Label.[a]

Exptl Conditions	Polymer State	$H_1:H_2$[b] Labeled as G	$H_1:H_2$[b] Labeled as F
1. 50 mM KCl	F	0.138	0.55
2. Centrifugation[c]			
Pellet	Enriched F	0.153	1.72
Supernatant	G	0.127	0.33
3. Pellet dialyzed *vs.* salt-free medium	G	0.124	0.31

[a] Actin preparation IXB was labeled in both the monomer and polymer forms with maleimide spin label, then dialyzed to remove unreacted label. The monomer-labeled sample was then polymerized in 50 mM KCl. [b] $H_1:H_2$ = ratio of heights of first two peaks of electron paramagnetic resonance spectrum. [c] Centrifugation was carried out at 27,000 rpm for 4 hr in a Spinco 30 rotor.

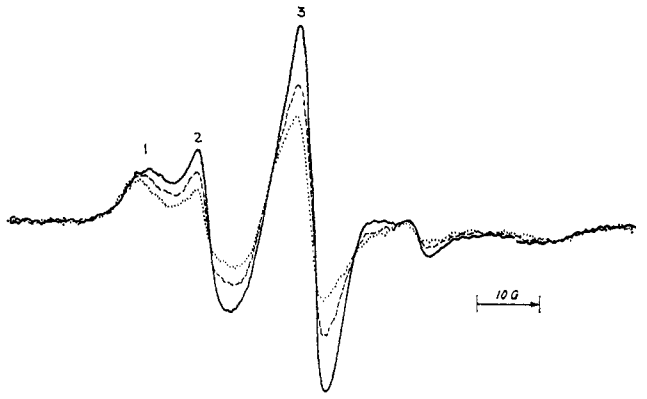


FIGURE 6: Polymerization of spin-labeled actin at 1.54 mg/ml and 15 mM KCl. Actin from preparation VIIIC was labeled with maleimide spin label in the polymer form. Spectra were recorded before polymerization (——) and at 3 (– – – – –) and 39 min (. . . . . .) after the addition of 15 mM KCl.

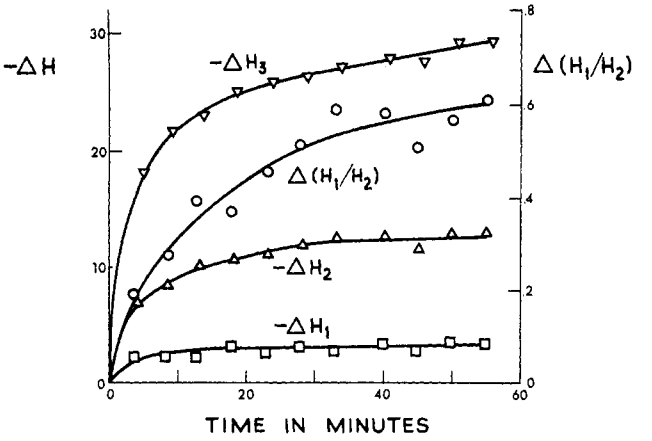


FIGURE 7: Change in several parameters of the electron paramagnetic resonance spectrum during polymerization. Actin sample and polymerization conditions were the same as in Figure 6. Parameters given are the change from zero time in the heights of peaks 1, 2, and 3 (ΔH_1, ΔH_2, and ΔH_3, respectively) and in the ratio of the heights of the first two peaks $\Delta(H_1:H_2)$.

that of pellets which were suspended in and dialyzed exhaustively against salt-free medium. This suggests that the supernatant fraction following centrifugation contained G-actin. This G-actin does not reestablish a G-F equilibrium presumably because the protein concentration is below the critical concentration required for observable polymerization (Kasai *et al.*, 1962b).

Studies of polymerization as a function of the solvent were carried out under suboptimal polymerizing conditions. This enabled a simultaneous study of the kinetics of the polymerization reaction. The course of this reaction was followed by measuring both the electron paramagnetic resonance spectra and the development of viscosity in duplicate samples of the same spin-labeled protein preparation. Electron paramagnetic resonance spectra from a typical experiment are shown in Figure 6. When recordings of the polymerizing solution were made at a constant gain setting (in Figures 4 and 5 gain settings for polymerized samples were raised to match peak 3 heights with those of unpolymerized samples), the major changes in the electron paramagnetic resonance spectra are a decrease in the heights of peaks 2 and 3 and an increase in the ratio $H_1:H_2$. A plot of the change in these parameters with time is given in Figure 7. The change in height of peak 3, ΔH_3, generally showed less scatter than other spectral parameters and was therefore chosen to demonstrate the effect of polymerization conditions on the spectral change. Figures 8A and 9A show ΔH_3 as a function of time in solutions polymerizing at different concentrations of KCl and actin, respectively; the analogous viscosity data are given in Figures 8B and 9B. In each case, the final value of ΔH_3 or the viscosity depended on the conditions of polymerization. This is the expected result if the equilibrium point of the G–F transformation depends on the solvent condition. The rate of change in both parameters is also dependent on the polymerization conditions, however the form of this dependence is clearly different for the two parameters. This is understandable if the spectral change is related directly to the number of monomers incorporated into polymer (see Discussion) while viscosity depends on a higher order of the average polymer size.

Interaction of Spin-Labeled Actin with Myosin. Experiments were carried out to determine if the spin label attached to F-actin would exhibit altered mobility following interaction with myosin A or during superprecipitation of the reconstituted actomyosin. Addition of myosin A to F-actin (labeled with the maleimide spin label in the polymer form) at low ionic strength (50 mM KCl–5 mM Tris, pH 7.5, with or without 1 mM $MgCl_2$) consistently resulted in a significant increase in $H_1:H_2$. However, for the following reasons, we interpret this change as a shift in the G–F equilibrium rather than a direct result of myosin binding. (1) When myosin was added to "enriched" F-actin under conditions identical to those used above, a significant *decrease* in $H_1:H_2$ was observed. If the spectral change resulted solely from binding of myosin A to actin, then addition of myosin to an equilibrium mixture of the monomer and polymer forms of actin would be expected to give the same type of spectral change as addition to "enriched" F-actin. (An alternative explanation, that binding to monomer and polymer actin produces different shifts in the electron paramagnetic resonance spectrum is considered unlikely.) (2) Attempts to reverse the spectral change by procedures which loosen actin–myosin links (*e.g.*, raising the ionic strength, addition of sodium pyrophosphate) proved ineffective.

Superprecipitation of actomyosin reconstituted from myosin A and F-actin labeled in the polymer form with the maleimide spin label was studied under the conditions used by Bárány *et al.* (1966) as well as in the KCl–Tris medium used for the study of actin–myosin interaction. Superprecipitation was initiated by addition of 1–10 mM ATP. No consistent and reproducible changes in the electron paramagnetic resonance spectrum were observed during superprecipitation (the presence of which was confirmed by visual observation or by measurement of the turbidity).

Effect of Spin Labeling on the Properties of Actin. The polymerizability of actin was not impaired by reaction with spin label in either the monomer or polymer form. Occasionally spin-labeled samples showed an enhanced rate of viscosity development such as that shown in Figure 10 for a preparation labeled in the monomer form. There was

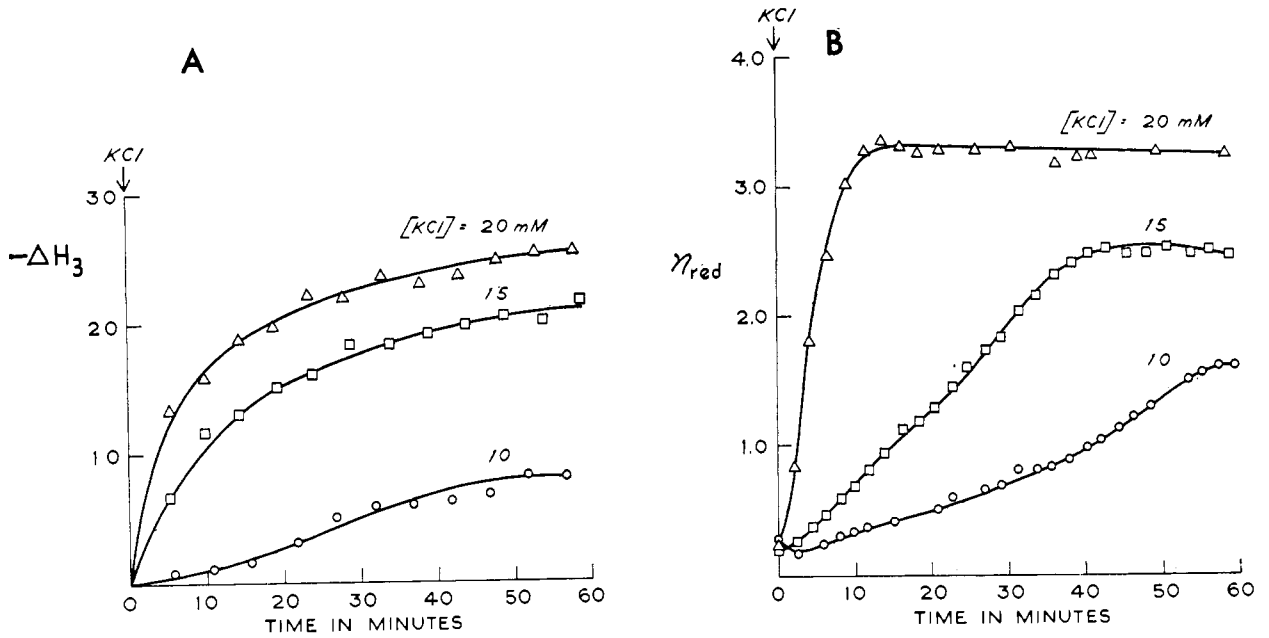


FIGURE 8: Effect of KCl concentration on polymerization of actin. Actin from preparation VIIIB was labeled with maleimide spin label in the polymer form, then converted into G-actin. Polymerization was carried out at an actin concentration of 1.60 mg/ml with 10 (○), 15 (□), or 20 mM (△) KCl. (A) Change in height of peak 3 of electron paramagnetic resonance spectrum (at constant gain) with time. (B) Reduced viscosity (in deciliters per gram) of polymerizing solutions.

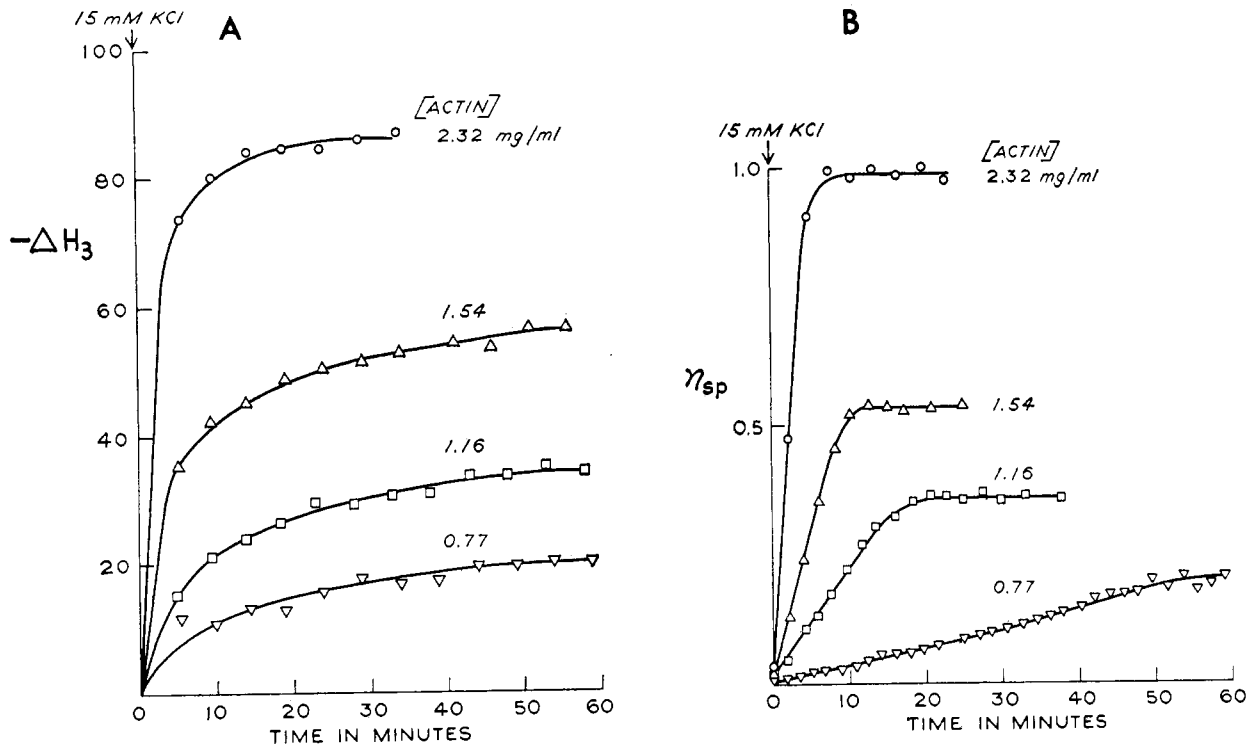


FIGURE 9: Effect of concentration of actin on the polymerization reaction. Actin from preparation VIIIC was labeled with maleimide spin label in the polymer form, then converted into G-actin. Polymerization was carried out at actin concentrations of 0.77 (▽), 1.16 (□), 1.54 (△), and 2.32 mg per ml (○) and was initiated by the addition of 15 mM KCl. (A) Change in height of peak 3 of electron paramagnetic resonance spectrum (at constant gain) with time. (B) Specific viscosity of polymerizing solutions.

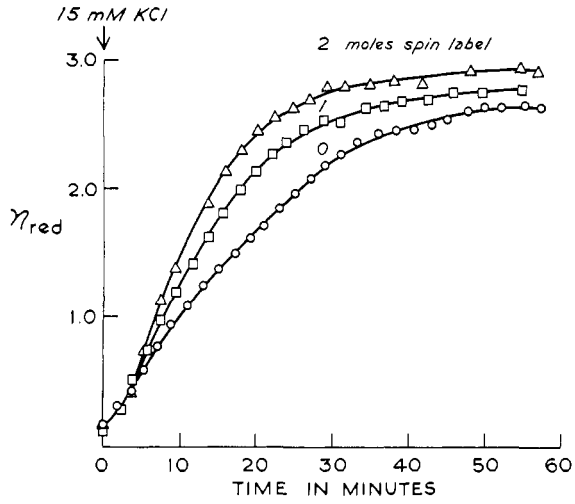


FIGURE 10: Effect of spin labeling on actin polymerizability. Actin was reacted in the monomer form with 0 (○), 1 (□), or 2 (△) moles of maleimide spin label for 6 hr, then dialyzed to remove unreacted label. Polymerization was initiated with 15 mM KCl and followed by viscosity measurements. Reduced viscosity is given in deciliters per gram.

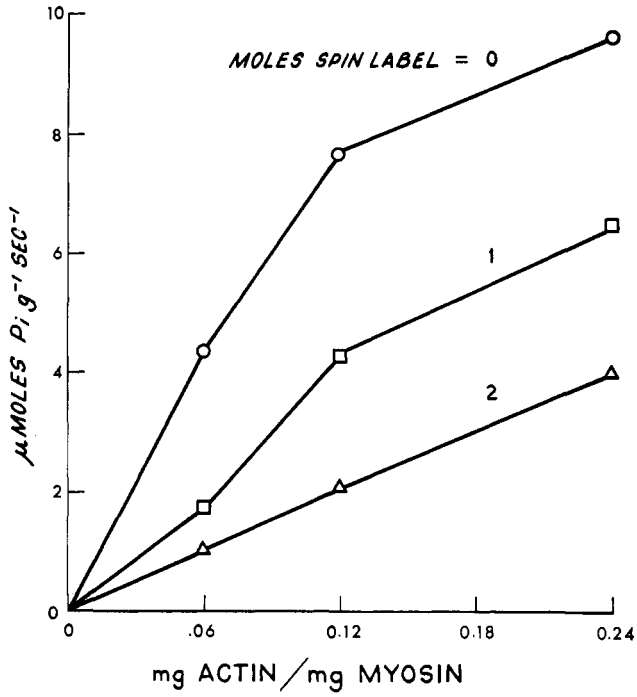


FIGURE 11: Adenosine triphosphatase activity of actomyosin reconstituted from myosin A and spin-labeled actin. Actin was reacted in the polymer form with 0(○), 1 (□), or 2 (△) moles of maleimide spin label for 2 days, then dialyzed to remove unreacted label.

also no marked change in the actin-bound nucleotide following spin labeling. Both unlabeled and labeled samples of actin were found to have close to one mole of adenine nucleotide bound per 6×10^4 g of protein.

The ability of spin-labeled actins to interact with myosin A was tested by both ATPase and superprecipitation measurements. Both types of measurement revealed impairment in actin–myosin interaction following labeling of actin in either the monomer or polymer form. Actin-activated myosin A–ATPase activity was diminished (Figure 11) and both the rate and extent of superprecipitation were decreased (Figure 12). The latter effect was most evident at low actin to myosin ratios (*e.g.*, 1:20).

Discussion

Recent evidence from other laboratories indicates that the actin molecule possesses three surface SH groups (based on a molecular weight of approximately 45,000) which are not directly involved in the polymerization reaction or in the binding of actin to myosin (Martonosi, 1968; Lusty and Fasold, 1969). Of the three groups, one reacts rapidly with alkylating agents (iodoacetamide and *N*-ethylmaleimide) in either the monomer or polymer form, while the other two groups are more slowly reacted in the monomer form and are apparently covered by polymerization. If the spin labels possess the same reactivity as the parent compounds, reaction of monomer or polymer actin with 1 mole of iodoacetamide or maleimide spin label should result in labeling of the fast-reacting SH group. Label bound at this site should be relatively unhindered in its motion and experience little or no immobilization upon polymerization. This is, in effect, the result obtained with the iodoacetamide spin label: the spectra revealed attachment of this spin label to a site which only weakly immobilized the nitroxide group, the specificity of this attachment was independent of the polymer state during reaction, and label attached at this site experienced only slight immobilization upon polymerization.

On the other hand, the maleimide spin label did not give the expected result. This label reacted with sites which were significantly immobilizing in the monomer form, experienced a great immobilization upon polymerization, and may have experienced some differences in reactivity between monomer and polymer forms. These properties could result either from the increased length of the maleimide spin label relative to the iodoacetamide spin label or from attachment of this label to residues other than the fast-reacting cysteine. Other possible sites of attachment of the maleimide spin label are the two slowly reacting surface SH groups which become covered during polymerization or an ϵ-NH_2 group of a lysine residue. Reaction of the maleimide spin label with a lysine residue in actin is supported by several lines of evidence. Griffith and McConnell (1966) have reported labeling of lysine residues with this spin label. Secondly, the spectra of maleimide spin-labeled actin are markedly similar to those of actin labeled with the isothiocyanate analog. This latter compound should react with amine functions, but not with SH groups. And, finally, modification of a single lysine residue to actin with another reagent, trinitrobenzenesulfonate, does not interfere with polymerization, but does hinder actin–myosin interaction (Tonomura *et al.*, 1962; Mühlrad, 1968), a finding which is consistent with the effects of the maleimide spin label on these properties.

The change in the electron paramagnetic resonance spectrum observed upon addition of KCl to actin labeled with the maleimide spin label is clearly associated with the monomer–polymer transition. Although actin preparations used in most of the work reported above may contain small

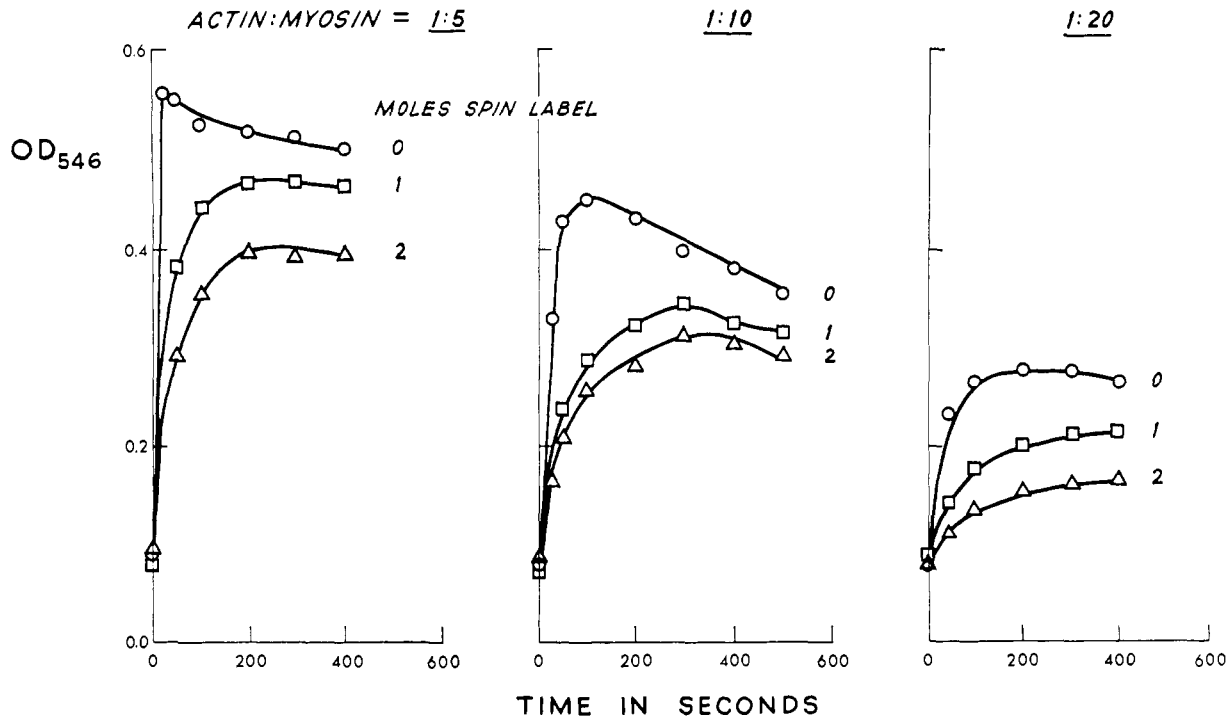


FIGURE 12: Superprecipitation of actomyosin reconstituted from myosin A and spin-labeled actin. See legend to Figure 11 for details of labeling.

amounts of impurities, only the actin in these preparations was labeled with spin label, and removal of the impurities had little effect on the spectral change. The polymerizability of actin (measured by viscosity) was not impaired by reaction with spin label. The spectral change occurred over approximately the same time span as the change in viscosity and the rate and extent of both of these parameters were found to be functions of the actin and KCl concentrations employed in the polymerization reaction. The spectral change could be reversed by dialysis against salt-free medium or by addition of 0.6 M KI. All of these findings indicate a close coupling of the spectral change to the polymerization process.

The actual cause of the spectral change is difficult to assess without more detailed knowledge of the structure of actin than is now available. A nonspecific immobilization of the spin label by the viscosity increases which accompany polymerization can be ruled out. This is evident from the finding that the three spin labels employed in this work exhibited different degrees of immobilization upon polymerization despite the fact that the same large increase in viscosity accompanied polymerization. Furthermore, concentrated F-actin solutions containing unreacted spin label always showed free spectra which were indistinguishable from those of aqueous solutions of the spin labels. Thus immobilization was a property observed only for bound spin label. An immobilization of label due merely to changes in rotation of the protein is also unlikely since the rotational correlation time for actin ranges from 8×10^{-8} sec for the monomer to $40\text{–}80 \times 10^{-8}$ sec for the polymer (Martonosi and Teale, 1965) while correlation times for the actin-bound nitroxide labels are probably of the order of $1\text{–}4 \times 10^{-8}$ sec (see Hamilton and McConnell, 1968). Clearly the immobilization observed must be due to a change in the rotational correlation time of the nitroxide label.

Molecular changes which might cause the observed spectral change fall into two categories. The first of these is a salt-induced change in the conformation of actin monomers occurring prior to but necessary for polymerization. This may be represented schematically by eq 1, where G and G′

$$G \longrightarrow G' \longrightarrow F' \qquad (1)$$

represent monomer units having partially immobilized and strongly immobilized spin label, respectively, and F′ represents polymer actin having strongly immobilized spin label. The type of conformational change which could result in the observed immobilization of the spin label is a folding inward of groups previously situated on the exterior of the molecule. These affected groups would necessarily have to be in the vicinity of the bound spin label. A second scheme may be represented by

$$G \longrightarrow F' \qquad (2)$$

Here the immobilization of the spin label occurs coincident with the formation of linkages in the growing polymer. Immobilization of the label could result from masking of label situated near the polymerization site by monomer–monomer union or from a conformational change within the monomer unit brought about by monomer–monomer union.

The spectral change in eq 1 is first order with respect to [G] while that in eq 2 should display the same dependence on monomer concentration as the polymerization reaction. From the viscosity increase and phosphate liberation Kasai *et al.* (1962b) have determined that the initial rate

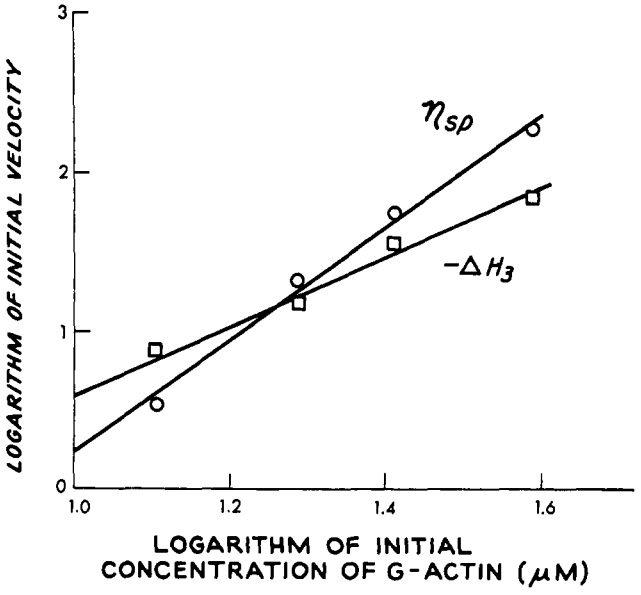


FIGURE 13: Dependence of initial velocity of polymerization on G-actin concentration. Data are taken from Figure 9. The initial velocity of the spectral change (□) was estimated from the value of ΔH_3 at 5 min. The initial velocity of the viscosity change (○) was calculated from the initial linear portion of the curves in Figure 9B and is expressed as $\Delta\eta_{sp}/10^3$ min.

of polymerization increases in proportion to the third or fourth power of the monomer concentration. Accordingly, a plot of the initial velocity of the spectral change against the initial monomer concentration should distinguish between these two equations. Figure 13 gives a logarithmic plot of these parameters taken from the data of Figure 9A. The initial velocities were estimated from the value of ΔH_3 at 5 min (close to the first data point) and are therefore minimal values. The order of the dependence of the initial velocity on the monomer concentration can be determined from the slope of the curve in Figure 13. This value is found to be 2.2 for the spectral change, a finding which clearly eliminates eq 1. Also presented in Figure 13 is the initial velocity of the viscosity change (data from Figure 9B). The slope of this curve is 3.6, a finding which is in good agreement with the results of Kasai *et al.* (1962b). Since the estimates of initial velocity for the spectral change are lowest for the higher velocities, the value of 2.2 for the dependence on monomer concentration is clearly a minimal one and is therefore not necessarily inconsistent with the viscosity data.

Since the spectral change as depicted in eq 2 may result from either an intramolecular conformational change or the masking of spin-labeled residues by monomer–monomer association, further experimentation is required before the exact mechanism of the spectral change is known. Higashi and Oosawa (1965) reported a change in the ultraviolet absorption of tryptophan and tyrosine residues upon polymerization of actin. From an analysis of the spectral change these authors concluded that aromatic residues previously exposed on the exterior of the actin monomer are folded inside the molecule during polymerization. However these authors were also unable to exclude an effect due to masking of aromatic residues at the site of polymerization as a consequence of intermolecular binding. This latter interpretation is supported by the more recent experiments of Gerber and Ooi (1968) which indicate the presence of one tyrosyl residue at the polymerization site.

The fact that the maleimide spin label is extremely sensitive to actin–actin interactions, yet is apparently unaffected by actin–myosin binding, makes this label an effective probe of the actin polymer state in reconstituted muscle systems. The findings reported in the present communication regarding the effect of myosin on spin-labeled actin are consistent with previous reports that, under appropriate conditions, myosin not only shifts the polymerization equilibrium, but also accelerates the attainment of equilibrium (Kikuchi *et al.*, 1969; Tawada and Oosawa, 1969). It had been hoped that this technique might provide evidence for weakening of actin–actin linkages during actin–myosin–ATP interaction. However, the negative results obtained in these experiments do not argue against such an effect, but only limit its magnitude to the 5% level of detection of the present studies. Since Moos *et al.* (1967) have reported that a 50% exchange of nucleotide (during actin–myosin–ATP interaction) requires 1 hr, this conclusion seems within reason.

Acknowledgments

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Characteristics of *in Vitro* Ribonucleic Acid Synthesis by Macronuclei of *Tetrahymena pyriformis**

Young C. Lee and John E. Byfield†

ABSTRACT: The objective in this work was to establish an *in vitro* system for the study of RNA synthesis in intact macronuclei isolated from normal and synchronized cultures of *Tetrahymena pyriformis* GL. A nonionic detergent, Triton X-100, was used to isolate macronuclei. Examination of these macronuclei by electron microscopy showed no cytoplasmic contamination. DNA-dependent RNA polymerase activity was retained in these preparations; the enzyme required all four ribonucleoside triphosphates, Mg^{2+} or Mn^{2+} ions, and intact DNA. No enzyme activity was observed when DNA was destroyed with deoxyribonuclease or when transcription was blocked with actinomycin D. Various factors influencing the rate of RNA synthesis in the isolated macronuclei were studied. In the presence of Mg^{2+} and KCl, it was demonstrated that the macronuclear system of *Tetrahymena* prepared from exponentially growing cultures incorporated about 5 mμmoles of labeled UTP/mg of nuclear DNA. A stimulation of activity occurs when Mn^{2+} and 200 mM ammonium sulfate are substituted for Mg^{2+} and KCl. However, analysis of base composition and nearest neighbor base frequencies indicated that the product of the RNA polymerase reaction is like DNA under both conditions, and does not resemble *Tetrahymena* ribosomal or whole cell RNA. Studies on the kinetics of RNA synthesis in isolated macronuclei showed that the newly synthesized RNA has a high turnover rate (rapid synthesis and breakdown), and that the hydrolysis of the newly synthesized RNA occurred in direct proportion to temperature and reaction time.

A ciliated protozoan, *Tetrahymena*, has several useful biochemical and morphologic properties. Like other ciliates, it is capable of exchanging nuclear materials as well as undergoing internal genetic reorganization (*e.g.*, autogamy) under defined circumstances (Beale, 1954). Its phenotypic expression can also be reversibly altered by physical factors (Lwoff, 1950). In addition, many strains can be induced to divide synchronously by physical or chemical means (Scherbaum and Zeuthen, 1954; Stone, 1968). Its evolutionary development places it close to the divergence between plant and animal forms (Holz, 1966). The cytoplasmic RNAs of *Tetrahymena pyriformis* are suggestive of a very primitive animal cell (Kumar, 1969). These various factors predict a useful role for these organisms in the biochemical analysis of gene expression and cytokinesis in eukaryotes.

We have recently been interested in RNA metabolism during the induction of division synchrony in *Tetrahymena* cultures. A variety of experiments have suggested that temperature effects on mRNA synthesis or turnover are involved (Byfield and Lee, 1970), but kinetic analysis using labeled extracellular ribonucleosides is fraught with interpretative difficulties (Nierlich, 1967). We have therefore developed a procedure to isolate substantial numbers of highly purified macronuclei in order to study the properties of the DNA-dependent RNA polymerase. The experiments reported here indicate that the enzyme from *Tetrahymena* appears similar but not identical with the equivalent enzyme activity existing in various bacterial and mammalian systems (Weiss, 1960; Hurwitz *et al.*, 1962; Chamberlin and Berg, 1962; Tsukada and Lieberman, 1964; Widnell and Tata, 1966; Nair *et al.*, 1967). In addition, some properties of the *in vitro* product, including base composition, nearest neighbor base frequency, and temperature stability *in vitro*, are described.

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Materials and Methods

Culture of Organism. T. pyriformis, strain GL, was grown axenically in a medium containing 2.0% proteose peptone (Difco), and 0.1% each of bactodextrose (Difco), sodium acetate, and dibasic potassium phosphate. Stock cultures