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Received for review Feburary 27, 1975. Accepted September 3, 1975. Michigan Agricultural Experiment Station Journal Article No. 7156. The research reported herein is from an M.S. Thesis that the senior author presented to Michigan State University in partial fulfilment of the requirements for the degree.

Electron Paramagnetic Resonance Studies of Actin-Lipid Interaction in Aqueous Media

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The interaction between fish lipid and fish actin in aqueous media was investigated by the use of spin-label techniques. Three types of spin-labels were used in the study. (a) 12-Doxyl stearic acid was incorporated into lipid–liposomes by sonication. The incorporation of the spin-label took place at a lipid/water ratio of 3 mg of lipid/1 ml of H_2O. Addition of actin to the labeled liposomes showed that both neutral fish lipids and polar fish lipids interact with actin. The neutral lipids showed stronger interaction than polar lipids. Results of (b) maleimide and (c) iodoacetamide spin-labels suggested that all the sulfhydryl groups and the majority of the amino groups, which bind the labels, were on the surface of the actin molecules and exhibited considerable mobility. The results also showed that these sites did not experience any conformational changes, which would restrict the spin-label mobility, during actin–lipid interaction. At the isoelectric precipitation region of action, the mobility at these sites was considerably restricted. Raising the pH to the alkaline side, i.e. pH 10.0, caused expansion of the actin molecules, accompanied by exposure of the hidden amino group sites to the surface of the molecule.

There is an increasing interest in understanding the nature of lipid–protein interaction in biological and nonbiological systems. Lipid–protein interaction in cell membranes, various enzyme reactions, the blood coagulation process, various lipase reactions, and serum lipoproteins were considered by Chapman (1969) as the areas attracting the most interest of researchers at the present time. Much work has been published covering the importance of lipid–protein interactions in other systems such as lipovitellin (Evans et al., 1968a, b), wheat flour and bread-making (Chiu and Pomeranz, 1966; Pomeranz et al., 1968), nutritional quality of foods (Varma, 1967; Rao and Rao, 1972), post-mortem stability of fish myosin (Braddock and Dugan, 1973), interaction of antigen with mylin lipids (Palmer and Dawson, 1969), and binding of botulinum toxin to membrane lipids (Simpson and Rapport, 1971).

The study of lipid–protein interaction has, as well, a valuable interest to the fish technologist. One of the methods used in extracting and concentrating fish proteins for industrial purposes is a direct application from biochemistry for extracting and purifying proteins, i.e. using aqueous buffers. The problem arose in industry of high lipid contents in the extracted proteins; this also could be an unseen problem to the biochemist, since there are usually no tests performed for the contaminating lipids in purified protein preparations. It was found (Shenouda, 1974) that the purified myosin, after Sephadex G-200 column purification, contained a significant amount of contaminating polar and neutral lipids. Taguchi and Ikeda (1968) reported the important role of lecithin in fish actomyosin ATPase activity suggesting the existence of lipid–protein complexes in the living myofibrillar tissues. The interaction of fish myosin and actin with fish lipid in aqueous media was investigated by Shenouda and Pigott (1974, 1975b) using sucrose gradient centrifugational and gel electrophoretical procedures.

The use of synthetic free radicals known as spin-labels has a wide use in probing the structure, motion, and chemical reactions of biological macromolecules. This technique proved to be sensitive to protein conformational changes. Griffith and Waggoner (1969) reviewed the advantages of using spin-labeling in studying biomolecules, in comparison to other existing biochemical–biophysical techniques. Roubal (1972) recommended the use of nitroxide spin-labels for studying lipid–protein interactions. Jost et al. (1971) have grouped spin-labels into three major arbitrary classes: (a) covalently bound spin-labels; (b) noncovalently bound, nonbiological spin-labels; and (c)

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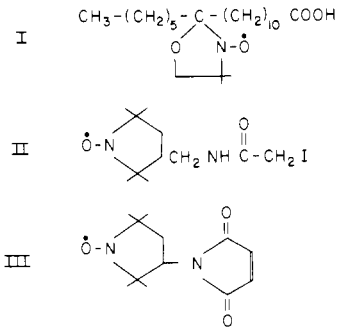

Figure 1. Nitroxide spin-labels used in the study: I, 12-doxyl stearic acid; II, *N*-(1-oxyl-2,2,5,5-tetramethylpyrrolidyl)iodoacetamide; III, *N*-(1-oxyl-2,2,5,5-tetramethylpyrrolidyl)maleimide.

spin labels which are analogues of biological compounds. Quinlivan et al. (1969) applied the sulfhydryl-directed spin-labels to the study of myosin ATPase activity. Burley et al. (1972) stated that there is no loss of actin activity for polymerization or interaction with myosin after labeling the actin covalently with maleimide spin-labels. Berger et al. (1971) used covalently and noncovalently bound spin-labels to study the lipid–protein interaction in human erythrocyte membranes.

This paper presents the results of studies on the interaction between fish actin and fish lipids (polar and neutral) in aqueous systems, using covalently and noncovalently bound spin-labels. The experiments were designed to: (a) determine the minimal lipid–water ratio required to incorporate the spin-label into lipid liposomes; (b) provide information about the actin–lipid interaction and the behavior of the free amino groups and SH sites in the actin molecules during actin–lipid interaction; and (c) to show the effect of pH on the conformational changes near these sites.

MATERIALS AND METHODS

Fish Actin. Actin was prepared and purified from yellow perch (*Perca flavescens*) according to the method described by Shenouda and Pigott (1975a).

Fish Lipids. Fish lipids were extracted from whole fish by the Bligh and Dyer (1959) method. The lipids were separated into polar and neutral fractions by partition between petroleum ether and 87% ethyl alcohol, as described by Galanos and Kapoulas (1962). The separation was checked by thin-layer chromatography (TLC).

Spin-Labels. Spin-labels used are illustrated in Figure 1. 12-Doxyl stearic acid (probe I) was synthesized according to the method of Waggoner et al. (1969), as modified by Roubal (1974). *N*-(1-Oxyl-2,2,5,5-tetramethylpyrrolidyl)iodoacetamide (probe II), which is known to label SH groups of protein, and *N*-(1-oxyl-2,2,5,5-tetramethylpyrrolidyl)maleimide (probe III), which is known to label SH and NH_2 groups of protein, were purchased from Synvar Associates (Palo Alto, Calif.).

Spin-Labeling the Lipids (Noncovalent Labeling). Probe I in chloroform solution (25 mg of spin-label/100 ml of chloroform) was added to various amounts of neutral or polar fish lipids. The chloroform was evaporated under reduced pressure, and the lipids were dispersed in water by ultrasonication at 4°C for a total period of 3 min. The final concentration of the probe in aqueous dispersion of the lipids was 2×10^{-4} *M*. Various amounts of lyophilized actin were dissolved in the sonicated lipid–water–probe mixture to give the desired lipid/actin ratio. Samples were pipetted into capillary tubes (1.8 o.d. × 90 mm long) for electron paramagnetic resonance (EPR) examination. Spectral changes were followed during (a) increase in lipid/water ratio and (b) increase in actin/lipid ratio.

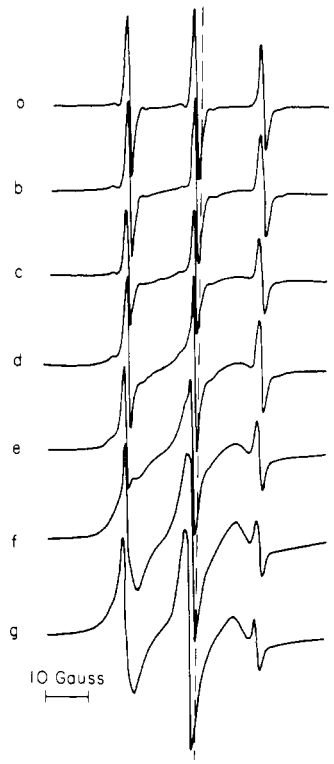

Figure 2. The effect of polar fish lipids/water ratio on the EPR spectra of probe I. All spectra were recorded using 2×10^{-4} *M* spin-label. Here and elsewhere the magnetic field increases to the right. Spectra of the spin label in (a) water, (b) 1.28/1 (mg of lipid/1 ml of H_2O), (c) 1.8/1, (d) 3/1, (e) 4.27/1, (f) 5.19/1, and (g) 9.4/1.

Spin-Labeled Fish Actin (Covalent Labeling). Lyophilized actin (100 mg) was dissolved in 5 ml of Tris–ATP buffer (2 m*M* Tris–0.2 m*M* ATP, pH 7.5). Five milligrams of either probe II or probe III was added, and labeling was conducted at 4°C, with continuous stirring for 3 days. Unreacted spin-label was removed by exhaustive dialysis at 4°C against the above buffer, followed by passage of the labeled actin through a Sephadex G-200 column (45 × 5 cm). Changes in EPR spectra were recorded before and after the addition of lipids. Spectra were also recorded at different pH values.

All the EPR spectra were recorded at room temperature at 9.5 GHz, using a modulation frequency of 100 kHz and 8-mW incident microwave power with a Varian-E3 X-band spectrometer. Reorientation correlation times (τ_c) were calculated according to Waggoner et al. (1967).

RESULTS

Figure 2 shows the spectra of label I associated with fish polar lipids as the lipid/water ratio was increased from 0.0 mg of lipid/1 ml of H_2O to 9.4 mg of lipid/1 ml of H_2O. A decrease in the reorientation correlation time (τ_c) occurred as the lipid/water ratio reached 3 mg of lipid/1 ml of H_2O (Figure 2d). At points well below this lipid/water ratio, the nitroxide probe tumbled at the rate characteristic of rotation in pure water. From these results the lipid/water ratio of 5–6 mg of lipid/1 ml of H_2O was chosen and used in the remaining studies of actin–lipid interaction. An EPR pattern similar to the spectra of Figure 2 was observed when polar lipids were replaced by neutral lipids.

Figure 3 shows the EPR spectra of probe I recorded in the presence and absence of actin. Figure 3a is the typical spectrum of the probe incorporated into liposomes made of polar or neutral lipids. Figure 3b shows what occurs when actin was added to polar liposomes in a ratio of 1:15

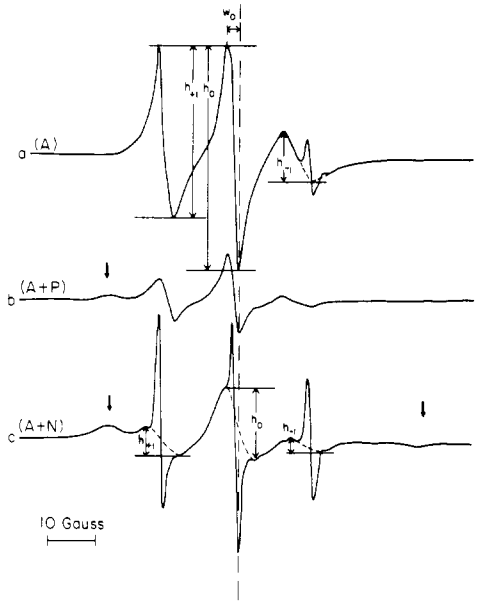

Figure 3. EPR spectra of probe I incorporated in fish lipids-liposome in the presence and absence of fish actin: (a) in the absence of fish actin with a fish lipid (polar or neutral):water ratio of 5 mg/1 ml, spin-label concentration 2×10^{-4} M; (b) actin added to the polar lipids-liposome preparation in a ratio of 15/1 (w/w); (c) actin added to neutral lipids-liposome preparation in a ratio of 14.5/1.

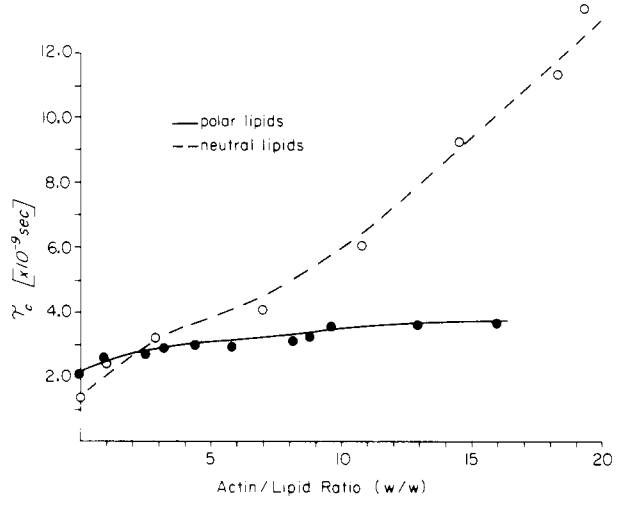

Figure 4. A plot of τ_c calculated from the EPR spectra of polar lipid/water and neutral lipid/water systems containing probe I as a function of added actin.

lipid/actin (w/w). A noticeable decrease in the size of the spectra results, as well as a decrease in the tumbling rate from 2×10^{-9} to 3.6×10^{-9} sec. Makinen and Kon (1971) attributed the decay in signal intensity to a quenching of the electron paramagnetic resonance absorption of the spin-label, which was caused by structural changes in the host specimen. A broad peak, indicated by an arrow in Figure 3b, arose from strongly immobilized attached probe molecules. The quantitative measurement of the broad components was difficult to calculate because of overlap.

Figure 3c shows the results of actin added to neutral lipid liposomes in a similar ratio as Figure 3b, 1:14.5 lipid/actin (w/w). No change in the size of the EPR spectrum (i.e. signal intensity) occurred when actin was added to neutral lipids, as compared to the decrease when actin was added to polar lipids. The figure shows two other sets of components; one is moderately immobilized, with a corresponding τ_c equal to 9.3×10^{-9} sec, while the other

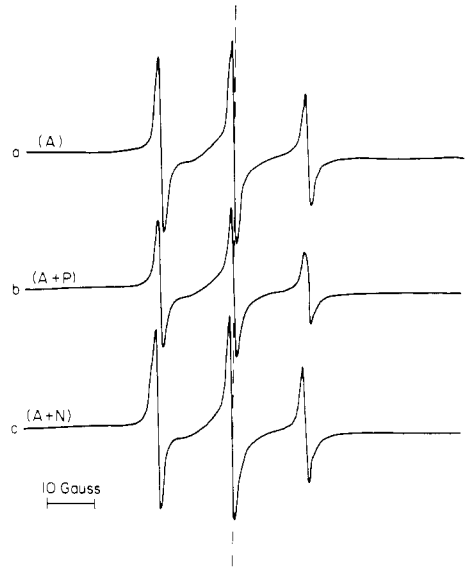

Figure 5. EPR spectra of fish actin labeled with probe II: (a) labeled actin in Tris-ATP buffer (pH 7.5) (20 mg of actin/ml); (b) polar lipids added to the labeled actin in a ratio of 1:1 (w/w); (c) neutral lipids added to the labeled actin in a ratio of 1:1.

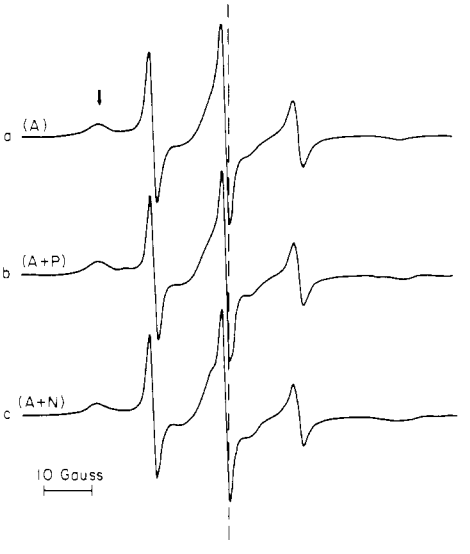

Figure 6. EPR spectra of fish actin labeled with probe III. Vertical arrow indicates bands arising from "strongly immobilized" spin-label: (a) labeled actin in Tris-ATP buffer (pH 7.5) (20 mg of actin/ml); (b) polar lipids added to the labeled actin in a ratio of 1:1 (w/w); (c) neutral lipids added to the labeled actin in a ratio of 1:1.

is strongly immobilized (indicated by arrows in Figure 3c).

Figure 4 shows a plot of τ_c calculated from the EPR spectra of polar and neutral lipid–water systems, containing probe I as a function of added actin. The figure indicates that neutral lipids bind to actin more strongly than the polar lipid liposomes.

Figure 5a represents the spectrum of probe II, which is covalently bound to actin. The spectrum indicates the existence of the probe in a higher mobilized state. Figures 5b and 5c show that there is no difference in the spectra on addition of polar or neutral lipids to the actin (1:1 w/w ratio) which is covalently labeled at the sulfhydryl groups.

The EPR spectra of the labeled actin with probe III are shown in Figure 6. Figure 6a reveals the attachment of the label to sites which strongly immobilized the nitroxide group and to sites which weakly immobilized the group.

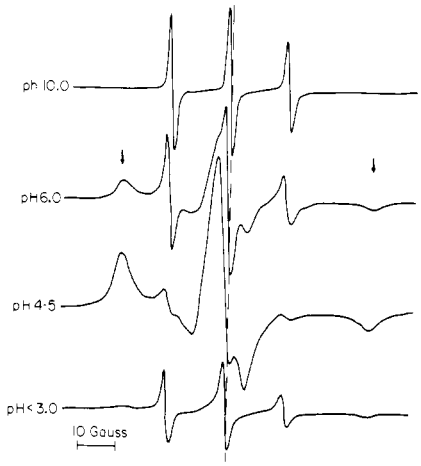

Figure 7. pH effect on the EPR spectra of actin labeled with spin-label III. The arrows indicate bands arising from strongly immobilized spin-label at neutral or acidic pH values. These immobilized bands completely disappeared at higher pH values (pH 10.0).

Table I. Values of the Rotational Correlation Time (τ_c)[a] of the Narrow Components of Probe III Attached to Fish Actin in the Presence or Absence of Fish Lipids at Different pH Values

	pH		
	3.0	7.5	10.0
Actin	7.2	7.6	4.3
Actin + polar lipids (1:1.5)	7.7	7.8	4.3
Actin + neutral lipids (1:1.5)	7.2	7.3	4.8

[a] τ_c expressed as seconds $\times 10^{10}$.

No difference was observed in the spectra when polar or neutral lipids were added to the system in the ratio 1:1 lipid/actin, w/w (Figures 6b and 6c).

Figure 7 shows that a conformational change limiting the mobility of probe III occurred when the pH was lowered from 7.5 to the range of the isoelectric precipitation of actin. There was a significant increase in a_0, indicating exposure of the nitroxide groups to more polar microenvironments. A further decrease in the pH to 3.0 caused the probe to retain the original spectrum of pH 7.5. On the other hand, raising the pH to 10.0 resulted in three sharp hyperfine splitting absorption peaks, accompanied by complete disappearance of the immobilized component (monitored by arrows in Figure 7).

Table I presents the values of τ_c of probe III attached to actin under different treatments. The data show that no change occurs in τ_c ($7.4 \pm 0.3 \times 10^{-10}$ sec) for actin at pH 3.0 or 7.5, with or without the presence of polar or neutral lipids. However, raising the pH to 10.0 increased significantly the tumbling rate to $4.5 \pm 0.3 \times 10^{-10}$ sec.

DISCUSSION

Incorporation of Spin-Label in Liposomes. The typical sharp three-line hyperfine pattern arising from 12-nitroxide stearic acid in water indicates a considerable mobility or rotational freedom and flexibility of the side chain to which the nitroxide moiety is attached. As the lipid/water ratio increased (3 mg of lipid/1 ml of H_2O) a noticeable decrease in τ_c occurred caused by a restriction in the rate of tumbling of the probe in the lipid liposomes. This indicates that the formation of liposomes (lamellar or micellar) starts to take place at this lipid/water ratio. Waggoner et al. (1967) attributed this shift in τ_c (using sodium dodecyl sulfate–water systems) to the critical micelle concentration phenomenon. Jost et al. (1971) pointed out that the a_0 and g_0 of the nitroxide spin-labels are solvent dependent, and the value of a_0 decreased and g_0 increased as the solvent polarity decreased. Figure 2 shows a decrease in the a_0 indicating that the probe was experiencing a different polarity in its microenvironment when incorporated into the lipid liposomes. Waggoner et al. (1967) suggested a dynamic association of the spin-label with liposomes in which the probe preserves a random spatial orientation and experiences a relatively polar–time average environment. Waggoner et al. (1969) showed that temperature (5–90°C) has no effect on the incorporation of spin-labels (nitroxide stearic acids) in micelles.

The spectrum in Figure 3 reveals that the label (probe I) experiences at least three different tumbling rates for actin–lipid interaction. The first state (nitroxide group tumbled freely in the order of $1–2 \times 10^{-9}$ sec) represented the typical tumbling rate of the probe when incorporated in liposomes. The second state occurred where the nitroxide showed a moderate degree of immobilization (τ_c of the order of 3×10^{-9} to 3×10^{-8} sec), and the third state, with the probe showing distinctive anisotropic splitting, reflected a strong immobilization. These differences in tumbling rates are a reflection of conformational changes which result in an opening or closing of the structure around the spin-label. Barratt and Leslie (1971) reported that conditions which favor protein aggregation cause a marked accentuation of the strongly broad signals.

Degree of Lipid–Protein Binding. EPR and isotope studies showed that native fish myosin weakly interacts with both polar and neutral fish lipids (Shenouda and Pigott, 1974). On the other hand, lysolecithin showed stronger binding over other membrane lipids to human erythrocyte membrane apoproteins as stated by spin-labeling studies by Berger et al. (1971). Shenouda and Pigott (1975b), using ^{14}C labeling, reported that native actin bound more polar lipids than neutral lipids. The same researchers also stated that several factors (such as heating, agitation, and polymerization in the presence of divalent cations) probably cause exposure of hydrophobic regions on the actin molecules and consequently increase the amount of bound neutral lipids.

Figure 4 indicates that the hydrophobic interaction between actin and fish lipids (as represented by actin neutral–lipid binding) is stronger than the electrostatic interactions (as partially represented by polar–lipid actin interaction). Hence, during aqueous extraction of fish proteins, difficulties in separating neutral lipids from actin will not arise only with the high amount of bound neutral lipids, but also with the degree of their binding. In other words, the strong hydrophobic interaction between the fish lipids and fish actin presents the problem in removal of residual lipids from F.P.C., extracted by aqueous procedures. Simpkins et al. (1971), studying membrane structure and phospholipid–protein interaction, likewise concluded that the phospholipids rely very little on ionic interaction of their charged head groups for the stability of an ordered membrane structure.

Amino and Sulfhydryl Group Sites on Actin Molecules. Maleimide-type spin-labels such as probe III are known to react covalently with SH, primary and secondary amino groups, while iodoacetamide spin-labels such as probe II serve as a general reagent for SH groups only (Barratt et al., 1968; Quinlivan et al., 1969).

The free mobility of probe II and the majority of probe III (Figures 5 and 6) suggests that all the labeled SH groups and the majority of the amino groups are located on the surface of the actin molecules, and the environment around these sites is quite fluid. The absence of EPR spectral changes on the addition of lipids (polar or neutral)

should at least indicate that labeled sites have retained their structural integrity during lipid–protein interaction and that no conformational changes, which affect the mobility of the spin-labels, occurred near these sites. Schneider and Smith (1970) also concluded that the structure of some of the membrane proteins is not highly dependent on the presence of lipids.

Lowering the pH of actin near the isoelectric precipitation apparently caused conformational changes near the primarily ε-amino and sulfhydryl groups. The new conformation restricted greatly the mobility of the probe attached to these sites (Figure 7). The resultant anisotropic spectrum at this pH is probably due to deep insertion of the nitroxide groups inside the actin molecules and/or actin–actin interaction. Lowering the pH to 3.0 resolubilized the actin and was probably accompanied by an expansion of the protein molecules, thereby relieving restriction on nitroxide motion. Stone et al. (1965) reported that changes in pH caused expansion of bovine serum albumin molecules, with consequent changes in the EPR spectra. Hsia et al. (1965) reported that the unfolding of chymotrypsin molecules, by urea, increased the tumbling rate of the attached spin-labels. Moreover, Schneider and Smith (1970) cited that the factors which showed larger changes in the EPR spectra of membrane proteins, such as extremes of pH, also caused changes in optical rotary dispersion and enzymatic and immunochemical activities. This indicated that significant conformational changes were occurring and thus possibly changes in the lipid–protein interaction.

Shenouda and Pigott (1975b) reported that at higher pH (pH 11.0) the actin was less dense on the sucrose gradient. The increased mobility of probe III at pH 10.0 (Figure 7 and Table I) can be attributed to a greater expansion of the actin molecules, where unfolding would expose sites containing hidden amino groups attached to the probe.

In conclusion, fish lipids bind with actin in aqueous environment, primarily via hydrophobic interaction. This probably causes problems in delipidation of protein isolates when using aqueous solvents only. The lipid–protein interaction did not affect the mobility of the sulfhydryl and amino group sites on the actin molecules. Also, the structural conformations around these sites at different pH values were apparently the same in the presence or absence of fish lipids. Beside the known effect of pH on the charge conditions of the actin molecules, it also affected the structure around these sites and the size of the actin molecules. At high pH, the actin molecules expanded and exposed the immobilized amino group sites to become freely tumbling groups.

ACKNOWLEDGMENT

The authors are indebted to W. T. Roubal, National Marine Fisheries Service, Seattle, Wash., for his assistance and for placing the EPR facilities at their disposal.

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Received for review May 22, 1975. Accepted October 2, 1975. This work was supported by the National Sea Grant program.