Ragland, T. E., Kawasaki, T., and Lowenstein, J. M. (1966), *J. Bacteriol. 91*, 236.
Sanwal, B. D., and Stachow, C. S. (1964), *Biochim. Biophys. Acta 96*, 28.
Spackman, D. H., Stein, W. H., and Moore, S. (1958), *Anal. Chem. 30*, 1190.
Stadtman, E. R. (1966), *Advan. Enzymol. 28*, 41.
Sund, H. (1968), *in* Biological Oxidations, Singer, T. P., Ed., New York, N. Y., Interscience, p 641.
Van Holde, K. E., and Baldwin, R. L. (1958), *J. Phys. Chem. 62*, 734.
Wilson, P. W., and Knight, S. G. (1952), Experiments in Bacterial Physiology, Minneapolis, Minn., Burgess, p 53.
Yang, J. T. (1967), *in* Biological Macromolecules, Vol. 1, Fasman, G. D., Ed., New York, N. Y., Marcel-Dekker, Chapter 6.
Yphantis, D. A. (1964), *Biochemistry 3*, 297.

The Mechanistic Significance of Phosphate Labeling of Alkaline Phosphatase*

Ted W. Reid,† Milos Pavlic,‡ Daniel J. Sullivan, and Irwin B. Wilson

ABSTRACT: Alkaline phosphatase reacts with orthophosphate at pH <8 to form a thermodynamically stable phosphoprotein. In order to study this phosphoprotein, a rapid quenching technique in conjunction with scintillation counting was developed which allowed for an accurate determination of amount of phosphoprotein formed. The reaction was stopped in 6 M perchloric acid by forcing 0.5 ml/sec of the solution through a 0.020-i.d. capillary tip into the quenching solution by means of a motor-driven syringe. The covalent labeling was measured from pH 8.0 to 5.0 and at different concentrations of phosphate. In this way the equilibrium constants for the dissociation of the enzyme–phosphate addition complex at 26° (Michaelis complex) were found to be 4.1×10^{-6} at pH 7.0, 6.7×10^{-6} at pH 6.0, and 5.4×10^{-5} at pH 5.5, while the equilibrium constants for the hydrolysis of the covalent phosphoprotein were found to be 1.4×10^{-4} at pH 7.0, 6.0×10^{-6} at pH 6.0, and 1.2×10^{-5} at pH 5.5. Measurements were also made at 3°. These constants are in good agreement with the values of these quantities for the phosphoryl-enzyme intermediate determined previously by kinetic methods. Similarly the calculated kinetic inhibition constants for phosphate at 26°, 3.8×10^{-6} at pH 7.0, 3.1×10^{-6} at pH 6.0, and 1.0×10^{-5} at pH 5.5, agreed with the experimental values. Thus the properties of the kinetically deduced phosphoryl-enzyme intermediate are the same as the properties of the phosphoprotein obtained in labeling experiments so that there is good reason to conclude that the two are the same. The phosphoprotein is stable relative to free enzyme and inorganic phosphate at all pH's studied, yet very little is formed at pH 7.0 and 8.0. The reason for this seeming anomaly is that the addition complex between enzyme and phosphate is even more stable at these pH's. As the pH is lowered, the relationship changes so that at pH 5.5 the covalent phosphoryl-enzyme is much more stable than the enzyme–phosphate complex and labeling is extensive.

Several workers have confirmed and extended the important observation of Engstrom and Agren (1958) that a phosphoprotein is formed when ^{32}P-labeled inorganic phosphate is added to a solution of alkaline phosphatase (Engstrom, 1961, 1962a,b, 1964; Schwartz, 1963; Schwartz and Lipmann, 1961; Milstein, 1964; Pigretti and Milstein, 1965). The phosphate is covalently linked to serine (Schwartz and Lipmann, 1961).

It is not suprising that protein should be phosphorylated in this procedure because alkaline phosphatase in its role as a nonspecific catalyst must catalyze the formation of phosphate esters as well as their hydrolysis and indeed alkaline phosphatase has been used for just this purpose. It is therefore to be expected that all groups that can serve as phosphate acceptors will be phosphorylated to an extent depending upon the thermodynamic stabilities of the resulting phosphate derivatives. In the present instance we have the surprising result that the phosphoprotein that is formed is about one million times more stable than ordinary phosphate esters (Wilson and Dyan, 1965).

For just the reasons discussed above, the labeling of protein in alkaline phosphatase preparations is a matter of thermodynamics and not of mechanism and therefore does not prove that a phosphoryl-enzyme is formed as an intermediate during the catalyzed hydrolysis of phosphate esters. However, other observations of a kinetic nature either suggest or indicate that a phosphoryl-enzyme intermediate occurs (Aldridge *et al.*, 1964; Fernley and Walker, 1966; Fife, 1967; Williams, 1966;

* From the Department of Chemistry, University of Colorado, Boulder, Colorado 80302. *Received March 26, 1969*. This work was supported by Grant NB07156 from the National Institutes of Health and Grant No. GB-7904 from the National Science Foundation.

† National Institutes of Health Postdoctoral Fellow 1 F2 AM-39,-314-01.

‡ National Institutes of Health International Research Fellowship F05 TW 1148. Present address: Institute of Pathophysiology, University of Ljubljana, Ljubljana, Yugoslavia.

Ko and Kézdy, 1967; Barrett *et al.*, 1969). We use the term phosphoprotein to refer to the phosphate ester derivative of the protein that is formed by incubation with inorganic phosphate and whose existence is indicated by the labeling experiments with ^{32}P. We use the term phosphoryl-enzyme intermediate to refer to the phosphate ester derivative of the enzyme which occurs as an intermediate in the hydrolysis of phosphate esters and whose existence is inferred from kinetic experiments. Even though the phosphoprotein is very stable thermodynamically, it could be the phosphoryl-enzyme intermediate, and the question arises whether the two are indeed the same. Support for the identity of the two has come from a kinetic method of evaluating the thermodynamic stability of the phosphoryl-enzyme intermediate which showed that the latter, like the phosphoprotein, was far more thermodynamically stable than ordinary phosphate esters (Levine *et al.*, 1969).

In the kinetic work alluded to above, it was possible to evaluate the free energy of hydrolysis of the phosphoryl-enzyme intermediate and the free energy of dissociation of the enzyme–phosphate complex (Michaelis complex) and also the inhibitory constant for the inhibition of phosphate ester hydrolysis by inorganic phosphate.

The corresponding quantities should be obtainable from labeling studies at different concentrations of inorganic phosphate but this can only be done experimentally if the precision of labeling can be vastly improved. It seemed likely that the lack of precision arises in the quenching procedure. To improve the rapidity of quenching we used a motor-driven syringe to force 0.5 ml of solution/sec through a stainless steel capillary tube (0.020-i.d.) into 6 M perchloric acid. This procedure proved to be satisfactory and this work is reported in this paper.

A close correspondence was found between the quantities measured by kinetic methods and those measured by phosphate labeling.

Experimental Section

Chromatographically purified alkaline phosphatase (Garen and Levinthal, 1960) (orthophosphoric monoester phosphohydrolase, EC 3.1.3.1) (*Escherichia coli*) was obtained as a suspension, 10 mg/ml in saturated ammonium sulfate, from Worthington Biochemical Corp. The $H_3{}^{32}PO_4$ was obtained from New England Nuclear.

Enzyme Assay. The enzyme concentration in each experiment was determined by assaying activity toward *p*-nitrophenyl phosphate in 1 M Tris at 27° and comparing this with the value obtained for crystalline enzyme by Malamy and Horecker (1964). The molecular weight was assumed to be 86,000 (Schlesinger and Barrett, 1965).

Determination of E–P. In order to accurately determine the amount of phosphoprotein present, it is necessary to rapidly lower the pH of the solution. For this purpose a high-speed sampling device was used which would add 0.5 ml of enzyme solution in 1 sec to a mixture of 1 ml of 6 N perchloric acid, 0.5 ml of 3 mg/ml of bovine serum albumin, and 0.1 ml of 1 M inorganic phosphate. The bovine serum albumin was always added to the perchloric acid just prior to the addition of the enzyme. Within 10 sec after addition of the enzyme to perchloric acid solution, 10 ml of H_2O was added which precipitated the protein. The solution was then centrifuged. The precipitate was resuspended in 5% trichloroacetic acid and centrifuged again. After removing the supernatant, the pellet was dissolved in 0.1 ml of 2 N NaOH by heating at 60° for 35–40 min. This solution was washed into a counting vial with 0.4 ml of 2 N NaOH in 95% ethanol followed by 2 ml of Biosolve and 10 ml of scintillation cocktail. The scintillation cocktail consisted of 7 g of 2,5-diphenyloxazole and 0.42 g of 1,4-bis[2-(4-methyl-5-phenyloxazoylyl)]benzene in 1 l. of toluene.

Preparation of the Phosphoprotein. To 40 ml of buffer was added 0.3 ml of 10 mg/ml enzyme suspension, 10 μl of 10 mCi/ml $^{32}PO_4$ solution, and 40 μl of 10^{-6} M inorganic phosphate. (It was found that enzyme added directly from the suspension required about 30–40 min to pick up all of its $^{32}PO_4$, whereas enzyme which was preincubated in solution at pH 6.0, 7.0, or 8.0 for 30 min required less than 1 min to to pick up the $^{32}PO_4$. The same equilibrium values were obtained by either method.) Aliquots (5 ml) of this solution were then added to tubes containing 0.15 ml of solutions of inorganic phosphate which resulted in final concentrations in the range 10^{-4}–10^{-6} M phosphate. At the lowest phosphate concentrations small corrections were subtracted from the amount of phosphate added to obtain free phosphate concentrations. At pH 7.0 and 8.0 the buffer was 0.01 M Tris–1 M NaCl and at pH 5.0, 5.5, and 6.0 it was 0.01 M citrate–1 M NaCl.

Quenching. With two simple high-speed jets 98% mixing is obtained in 3 msec or so. In our case we need only a few per cent mixing to quench the reaction since our quenching solution is 6 N perchloric acid. Probably less than 0.1 msec is required. Since this time is at least two orders of magnitude less than the half-time for dephosphorylation of the enzyme at its fastest rate, we conclude that only negligible dephosphorylation can occur as the enzyme, still undenatured, passes through various pH conditions during the quenching reaction. Similarly only negligible phosphorylation can occur during quenching as evidenced by the fact that the enzyme solution incubated at pH 8.0 and subject to the quenching process yielded less than 0.1% covalently bound phosphate.

Complexes of the addition type, in contrast to the covalent type, will break up upon denaturation because the binding is reversible and depends upon the precise native conformation.

Results and Discussion

The equilibria between inorganic phosphate (P_i) and alkaline phosphatase (E) is represented as follows:

$$E + P_i \underset{k_2}{\overset{k_1}{\rightleftharpoons}} E \cdot P_i \underset{k_4}{\overset{k_3}{\rightleftharpoons}} E\text{–}P + H_2O$$

The equilibrium constant for dissociation of the Michaelis complex ($E \cdot P_i$) is

$$\frac{(E)(P_i)}{(E \cdot P_i)} = \frac{k_2}{k_1} \tag{1}$$

and the equilibrium constant for the hydrolysis of the phosphoryl-enzyme (E–P) to yield enzyme and inorganic phosphate is

$$\frac{(E)(P_i)}{(E\text{–}P)} = \frac{k_2 k_4}{k_1 k_3} = K_2 \tag{2}$$

TABLE I

pH	K_2	K_i	k_2/k_1	k_3/k_4	(E–P)/(E°) × 100	Temp (°C)	Buffer (M)	Salt[b]
7.0	1.4×10^{-4}	3.8×10^{-6}	4.1×10^{-6}	2.9×10^{-2}	2.7	26	Tris (0.1)	NaCl
	1.2×10^{-4}	3.5×10^{-6}	3.6×10^{-6}	3.0×10^{-2}	2.9	3	Tris (0.01)	NaCl
a	(2.1×10^{-4})	(2.3×10^{-6})	(2.3×10^{-6})	(1.2×10^{-2})	(1.2)	25	Tris (0.1)	NaCl
6.0	6.0×10^{-6}	3.1×10^{-6}	0.67×10^{-5}	1.1	51	26	Citrate (0.01)	NaCl
	9.5×10^{-6}	6.3×10^{-6}	1.90×10^{-5}	2.0	67	3	Citrate (0.01)	NaCl
a	(1.4×10^{-5})	(5.1×10^{-6})	(0.76×10^{-5})	(0.7)	(37)	25	Acetate (0.20)	NaCl
5.5	1.2×10^{-5}	1.0×10^{-5}	0.54×10^{-4}	4.4	81	26	Citrate (0.01)	NaCl, $ZnCl_2$
	1.3×10^{-5}	1.5×10^{-5}		>20	105	3	Citrate (0.01)	NaCl
a	(1.8×10^{-5})	(1.6×10^{-5})	(1.1×10^{-4})	(6.6)	(85)	25	Acetate (0.20)	NaCl
5.0	1.0×10^{-5}	1.0×10^{-5}	5.0×10^{-4}	>20	99	3	Citrate (0.01)	NaCl

[a] Kinetically determined values (Levine *et al.*, 1969). [b] NaCl is 1 M; $ZnCl_2$ is 10^{-5} M.

where k_4 is defined to contain the concentration of water (*i.e.*, water is taken as unit activity). Also, at equilibrium

$$\frac{(E\text{–}P)}{(E \cdot P_i)} = \frac{k_3}{k_4} \tag{3}$$

$$(E^\circ) = (E) + (E\text{–}P) + (E \cdot P_i)$$

From eq 1–3 we obtain

$$\frac{(E^\circ)}{(E\text{–}P)} = 1 + \frac{k_4}{k_3} + \frac{K_2}{(P_i)} \tag{4}$$

In these labeling experiments, E–P is measured at various concentrations of P_i (see Figure 1). If these data are plotted in accord with eq 4, the y intercept is $1 + k_4/k_3$ and the slope is K_2. Thus it is possible to evaluate k_4/k_3, K_2, and then k_2/k_1 from eq 2.

It is also possible from these labeling experiments to evaluate the kinetic inhibition constant, K_i, for the competitive inhibition of phosphate ester hydrolysis by phosphate.

The expression for K_i is

$$K_i = \frac{k_2}{k_1} \Big/ \left(1 + \frac{k_3}{k_4}\right) \tag{5}$$

(Levine *et al.*, 1969).

Since we can evaluate k_2/k_1 and k_3/k_4 as described above we can also evaluate K_i from eq 5 and this value can be compared with the experimentally determined K_i. Alternatively we can transform eq 4 to explicitly contain K_i.

$$\frac{(E^\circ)}{(E\text{–}P)} = \frac{K_2}{K_i} + \frac{K_2}{(P_i)} \tag{6}$$

A plot of $(E^\circ)/(E\text{–}P)$ *vs.* $1/(P_i)$ should yield a straight line whose slope is K_2 and whose intercept is K_2/K_i. A typical plot of this type is given in Figure 2.

Binding studies were carried out in which values for K_2 and K_i were determined at different temperatures and pH. These values were compared with those obtained from kinetic stud-

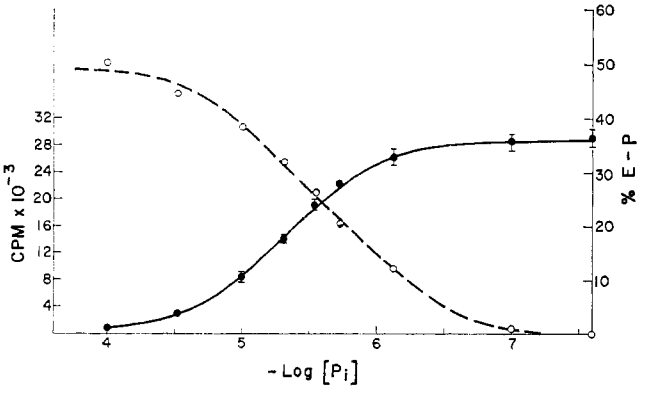

FIGURE 1: Effect of inorganic phosphate on the binding of $^{32}PO_4$ to alkaline phosphatase at pH 6.0, 1 M NaCl–0.01 M citrate. Counts per minute (—●—) from $^{32}PO_4$ bound to 2.1×10^{-4} μmole of enzyme. Per cent phosphoryl-enyzme (—○—) formed at various inorganic phosphate concentrations.

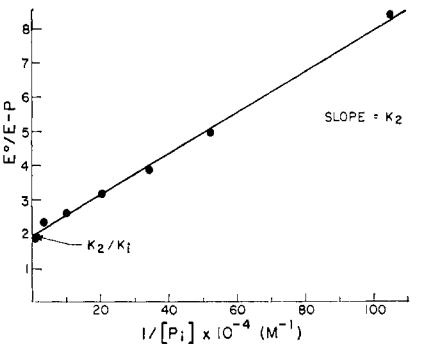

FIGURE 2: Reciprocal of per cent phosphoryl-enzyme *vs.* reciprocal of inorganic phosphate concentration at pH 6.0, 1 M NaCl–0.01 M citrate.

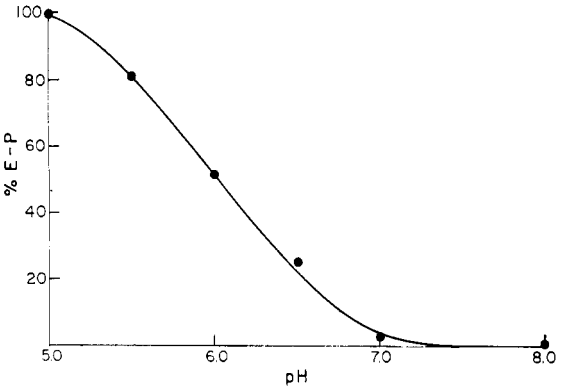

FIGURE 3: pH dependence of the formation of the phosphoryl-enzyme.

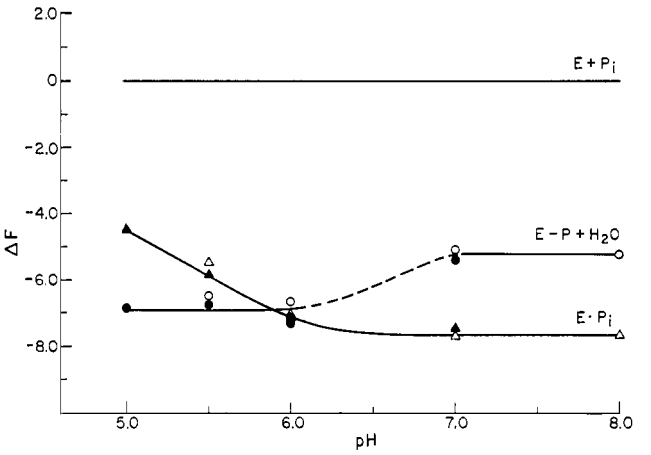

FIGURE 4: pH dependenee of the free energy of the phosphoryl-enzyme (E–P) and enzyme–phosphate addition complex ($E \cdot P_i$) compared to $E + P_i$ which is set at zero. Equilibrium-labeling data (▲, ●) and kinetic data (△, ○) (Levine *et al.*, 1969) are used.

ies (Table I). In view of the errors to be expected in both methods the agreement is very good.

We can tell the maximal number of labeled sites at each pH value. Thus at pH 5.5 somewhat less than one site per molecule is labeled. We cannot however tell from the labeling alone how many active sites there are in each molecule because sites are also occupied by noncovalently bound phosphate as indicated by the symbol $E \cdot P_i$. However, in conjunction with the values obtained by the kinetic method (Levine *et al.*, 1969) which gives the fraction of active sites occupied by covalently bound phosphate we could conclude how many active sites are present per molecule if our results were sufficiently precise. Our results at pH 6.0 are consistent with either one or two active sites per molecule of alkaline phosphatase but our results at pH 5.5 are consistent with only one site. This is consistent with presteady-state kinetic experiments which find only one active site per molecule of protein (Ko and Kézdy, 1967; Gutfreund, 1967; Fernley and Walker, 1969). Equilibrium dialysis at pH 8.0 also indicated only one binding site for phosphate (Reynolds and Schlesinger, 1969). Although alkaline phosphatase is believed to be composed of two identical subunits, they appear to be nonequivalent in the enzyme conformation.

As shown in Figure 3, the phosphoprotein, E–P, is remarkably stable with respect to free enzyme and P_i at all pH's between 7.0 and 5.0 (and also at pH 8.0 by the kinetic method). The reason that complete labeling does not occur is because the phosphate–enzyme complex $E \cdot P_i$ is also very stable and is in fact far more stable than E–P at pH 7.0 (and 8.0); at pH 6.0 stabilities are comparable but at pH 5.5 and 5.0 E–P is more stable than $E \cdot P_i$. This is readily seen from the plot of the free energy of $E \cdot P_i$ and E–P *vs.* pH in Figure 4, compared with $E + P_i$ which was set at zero. The plot shows the data from both kinetic and equilibrium-labeling experiments.

The labeled phosphoproteins obtained at pH 6.0, 5.5, and 5.0 correspond to metastable conformations. The evidence for this is that the amount of labeling decreases with increasing preincubation of the enzyme at pH 5.5 until after 2 days no labeling occurs. At the same time there is a corresponding decrease in enzyme activity measured at these pH's. However, the enzyme is active if measured at pH 8.0 but there is a lag of less than 1 min in attaining activity. Similarly if the enzyme is labeled quickly at pH 5.5 and allowed to remain at pH 5.5, the amount of label continually decreases until after 2 days no label remains and again the enzyme is inactive at the pH but active at pH 8.0. Similar observations but quantitatively different were made at pH 6.0 and 5.0. It would appear that the stable conformations at the lower pH are not active enzyme forms but forms which can quickly transform to the active enzyme form when brought to pH 7.0 and 8.0. The metastable forms would seem to be the enzymically active forms in protonic equilibrium at pH 6.0, 5.5, and 5.0 but not at conformational equilibrium.

These forms appear to be rather different from the forms described by Reynolds and Schlesinger (1969).

References

Aldridge, W. N., Barman, T. E., and Gutfreund, H. (1964), *Biochem. J. 92*, 23C.

Barrett, H. W., Butler, R., and Wilson, I. B. (1969), *Biochemistry 8*, 1042.

Engstrom, L. (1961), *Biochim. Biophys. Acta 52*, 49.

Engstrom, L. (1962a), *Biochim. Biophys. Acta 56*, 606.

Engstrom, L. (1962b), *Arkiv Kemi 19*, 129.

Engstrom, L. (1964), *Biochim. Biophys. Acta 92*, 71, 79.

Engstrom, L., and Agren, G. (1958), *Acta Chem. Scand. 12*, 357.

Fernley, H. N., and Walker, P. G. (1966), *Nature 212*, 1435.

Fernley, H. N., and Walker, P. G. (1969), *Biochem. J. 111*, 187.

Fife, W. K. (1967) *Biochem. Biophys. Res. Commun. 28*, 309.

Garen, A., and Levinthal, C. (1960), *Biochim. Biophys. Acta 38*, 470.

Gutfreund, H. (1967), Fast Reaction and Primary Processes in Chemical Kinetics, Claesson, S., Ed., New York, N. Y., Interscience, p 429.

Ko, S. H. D., and Kézdy, F. J. (1967), *J. Amer. Chem. Soc. 89*, 7139.

Levine, D., Reid, T., and Wilson, I. B. (1969), *Biochemistry 8*, 2374.

Malamy, M. H., and Horecker, B. L. (1964), *Biochemistry 3*, 1893.

Milstein, C. (1964), *Biochem. J. 92*, 410.
Pigretti, M. M., and Milstein, C. (1965), *Biochem. J. 94*, 106.
Reynolds, J. A., and Schlesinger, M. J. (1969), *Biochemistry 8*, 588.
Schlesinger, M. J., and Barrett, K. (1965), *J. Biol. Chem. 240*, 4284.
Schwartz, J. H. (1963), *Proc. Natl. Acad. Sci. U. S. 49*, 871.
Schwartz, J. H., and Lipmann, F. (1961), *Proc. Natl. Acad. Sci. U. S., 47*, 1996.
Williams, A. (1966), *Chem. Commun.*, 676.
Wilson, I. B., and Dyan, J. (1965), *Biochemistry 4*, 645.

Spin-Label Studies of Glycerinated Muscle Fibers*

Roger Cooke† and Manuel F. Morales‡

ABSTRACT: Glycerinated rabbit psoas fibers were labeled with nitroxide spin labels having the following reactive groups: maleimide, isothiocyanate, and iodoacetamide. The electron paramagnetic resonance spectrum of the maleimide-spin-labeled fibers was anisotropic with respect to the fiber axis, and changes in the spectrum indicated that the rotational freedom of the labels was restricted when the fibers were adenosine triphosphate shortened. However, the spectrum did not change when the fibers were extended to lengths greater than l_0, where l_0 was the length which the fiber had when it was labeled. Neither iodoacetamide spin labels nor isothiocyanate spin labels showed any changes in spectrum when the fibers shortened. At any length, isometric addition of adenosine triphosphate or adenosine triphosphate + ethylene glycol bis(β-aminoethyl ether)-*N*,*N*′-tetraacetic acid did not change the spectrum of any of the spin labels. Proteins were extracted from maleimide-spin-labeled fibers and studied in solution. The spin labels on myosin and actin were not sensitive to myosin–actin–adenosine triphosphate interactions. The spin label on actin showed a restriction of the rotational freedom of the label when the actin polymerized. In addition, these studies indicate that myosin A extracted from glycerinated fibers had different properties from myosin A extracted from living fibers.

We conclude that the environment of maleimide spin labels changes on shortening of labeled fibers, yet these labels are not sensitive to actin–myosin–adenosine triphosphate interactions. Thus the labels indicate the existence of some as yet uncharacterized interactions which are dependent upon the length of the fiber.

The sliding filament model of muscular contraction has gained wide acceptance in recent years. However, the molecular events which actually produce the forces that cause one set of filaments to slide past another are still unknown. In this study we have used spin labels as probes to monitor changes that may occur in the conformation of the contractile proteins as the muscle fiber contracts, relaxes, etc.

A spin label is a stable organic free radical which can attach to the molecule of interest and report changes in conformation. We will not go into the details of spin-label spectra and techniques since there is an excellent recent review of this field (Hamilton and McConnell, 1968).

The electron paramagnetic resonance spectra are sensitive to the rotational freedom of the spin labels, and it is this property which allows the spin labels to be used as probes. When the spin is freely rotating in solution, the hyperfine interaction between the unpaired electron and the ^{14}N nucleus splits the spectrum into three sharp lines. When the spin label is completely immobilized the anisotropic spin orbit and hyperfine interactions further split the spectrum into three broad peaks. If the rotational freedom of the spin label is only partly restricted, the spectrum is complex and not amenable to simple theoretical analysis. In Figure 2, I_1 and I_5 are due to "strongly immobilized" spin labels while I_2 and I_4 are due to spin labels with a greater degree of rotational freedom, "weakly immobilized." I_3 is due to both strongly immobilized and weakly immobilized spin label. The two low-field peaks are well defined and clearly separated, and thus give the clearest indication of any change that occurs in the rotational freedom of the spin label. We define R as the ratio of the heights of these two peaks: $R = I_1/I_2$. The use of R to describe a spectrum neglects changes in line shape and position which could be caused by relaxation effects due to paramagnetic ions, etc. However, R expresses much of the information which a spectrum contains about the rotational freedom of the spin label, and we will use it as a "practical yard stick" to describe many of the spectra.

The spin labels are attached to the protein, and the shape of the protein neighborhood determines the degree to which the rotational freedom of the spin label is inhibited. Since the electron paramagnetic resonance spectrum is sensitive to the rotational freedom of the spin labels, any change of the conformation of the protein which affects the rotational freedom of the spin labels will be detected.

* From the Cardiovascular Research Institute, University of California, San Francisco Medical Center, San Francisco, California 94122. *Received March 3, 1969*. Research supported by grants from the American Heart Association (CI 8), National Science Foundation (GB 7531), and U. S. Public Health Service (GM 14076).

† Postdoctoral fellow of the U. S. Public Health Service (2-FO2-GM-36).

‡ Career investigator of the American Heart Association.