

SUBMILLISECOND ROTATIONAL DYNAMICS OF SPIN-LABELED MYOSIN HEADS IN MYOFIBRILS

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ABSTRACT The rotational motion of crossbridges, formed when myosin heads bind to actin, is an essential element of most molecular models of muscle contraction. To obtain direct information about this molecular motion, we have performed saturation transfer EPR experiments in which spin labels were selectively and rigidly attached to myosin heads in purified myosin and in glycerinated myofibrils. In synthetic myosin filaments, in the absence of actin, the spectra indicated rapid rotational motion of heads characterized by an effective correlation time of 10 μ s. By contrast, little or no submillisecond rotational motion was observed when isolated myosin heads (subfragment-1) were attached to glass beads or to F-actin, indicating that the bond between the myosin head and actin is quite rigid on this time scale. A similar immobilization of heads was observed in spin-labeled myofibrils in rigor. Therefore, we conclude that virtually all of the myosin heads in a rigor myofibril are immobilized, apparently owing to attachment of heads to actin. Addition of ATP to myofibrils, either in the presence or absence of 0.1 mM Ca^{2+} , produced spectra similar to those observed for myosin filaments in the absence of actin, indicating rapid submillisecond rotational motion. These results indicate that either (a) most of the myosin heads are detached at any instant in relaxed or activated myofibrils or (b) attached heads bearing the products of ATP hydrolysis rotate as rapidly as detached heads.

INTRODUCTION

A central goal of muscle biophysics is to explain the large-scale motion of sliding filaments during muscle contraction in terms of more elementary ATP-dependent motions at the molecular level. Increasingly detailed models have been proposed to provide this explanation, most of which involve the crossbridges that connect the thick and thin filaments (A. Huxley, 1957; reviewed by Tregear and Marston, 1979). The crossbridge is usually defined as consisting of the rod-like S-2 (subfragment-2) region of myosin, thought to be connected by a flexible hinge to the thick filament backbone, and the two globular S-1 (subfragment-1) regions (myosin heads), thought to be connected by another hinge to S-2 (H. Huxley, 1969). Attention is usually focused on the heads, which contain the ATPase and actin-binding sites. The basic structural features of the thick and thin filaments have been established by electron microscopy and x-ray diffraction and studies of biochemical and mechanical kinetics have supplied us with essential clues about the underlying molecular dynamics. It remains for

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spectroscopic molecular probes to monitor the crossbridges selectively and to measure directly the attachment, orientation, and rotational motion underlying the models.

Fluorescent probes attached to myosin heads have been used in fluorescence depolarization experiments to demonstrate that myosin monomers are flexible in solution, permitting submicrosecond rotational motion of myosin heads (Mendelson et al., 1973). However, the formation of myosin filaments restricts the myosin head mobility to correlation times on the order of a microsecond or more, preventing accurate fluorescence measurements in most cases (Mendelson et al., 1973 and 1976).

To obtain greater sensitivity to slower rotational motion, workers in this laboratory spin-labeled myosin heads with an iodoacetamide analog and performed saturation transfer electron paramagnetic resonance (EPR) experiments (Thomas et al., 1975 *a*). The results confirmed the flexibility of myosin monomers and showed that the more restricted myosin head motions in filaments were still rapid on the microsecond time scale. The binding of myosin heads to F-actin slowed these motions considerably, and the same slow motion was detected with a spin label on actin (Thomas et al., 1979), indicating that the myosin head and the actin monomer form a rigid unit in the absence of ATP.

Although these observations of myosin flexibility are consistent with a rotating crossbridge model of contraction, a more direct test of the model requires at least two further steps. First, myosin head motions must be observed in the presence of ATP, to determine whether the motions are coupled to myosin and actomyosin ATPase activity. These measurements were not provided by our earlier work (Thomas et al., 1975 *a* and *b*), because the iodoacetamide spin labels did not remain rigidly immobilized on the myosin head during ATPase activity. The ATP-induced librational motions of the spin label, detected by conventional EPR techniques, were useful in studying conformational changes during myosin ATPase activity (Seidel et al., 1970; Seidel and Gergely, 1971 and 1973), but these rapid, local motions prevented the detection of overall myosin head motion. In the present study, we have solved this problem by using a maleimide spin label that remains completely immobilized with respect to the head during ATPase activity. A second important requirement is to detect myosin head motions in intact myofibrils and fibers. The principal obstacle to these measurements is the difficulty in selectively labeling myosin heads in the presence of the other myofibrillar proteins. We report here how we have achieved this labeling selectivity, with both the iodoacetamide and maleimide spin labels, and used saturation transfer EPR to detect directly the rotational mobility of myosin heads in myofibrils, in the states of rigor, relaxation, and activation.

METHODS

Protein Preparations

All preparations were carried out at 0°–4°C. Myosin was prepared from rabbit muscle as described by Nauss et al. (1969). Synthetic myosin filaments were prepared by dialyzing myosin against the desired low ionic strength solution. Heavy meromyosin (HMM) and S-1 were prepared by digesting myosin with α -chymotrypsin as described by Weeds and Taylor (1975). Spin-labeled HMM and S-1 were prepared by digesting spin-labeled myosin (or by digesting spin-labeled myofibrils, as described below). F-actin was prepared as described previously (Thomas et al., 1979). The molar concentrations of actin,

myosin, HMM, and S-1 were determined spectrophotometrically, as described previously (Thomas et al., 1979).

Myofibrils were prepared from rabbit skeletal muscle. Minced muscle was added to 3 vol of a solution containing 60 mM KCl, 5 mM MgCl₂, 1 mM EGTA, 1 mM NaN₃, 25 mM MOPS, pH 7.0 (rigor buffer), then homogenized in a Waring blender at top speed for four 15-s periods. The homogenate was filtered through a single layer of cheesecloth and centrifuged at 600 g for 15 min, and the top layer of the precipitate was resuspended in the original volume of rigor buffer. This cycle of filtration and centrifugation was repeated three times, and the myofibrils were suspended at a protein concentration of 10–20 mg/ml in 50% rigor buffer plus 50% glycerol, then stored at –20°C for 1 d to 6 mo. Before use, myofibrils were repeatedly sedimented and resuspended in rigor buffer to remove glycerol.

EXTRACTION OF MYOSIN FROM MYOFIBRILS Myofibrils were extracted at a protein concentration of 20 mg/ml in rigor buffer containing 0.3 M KCl and 2 mM ATP for 15 min at 0°C. After 15 min, the suspension was centrifuged at 10,000 g for 30 min, and the supernate diluted with 8 vol of 1 mM EDTA, pH 7.0, to precipitate the myosin. After centrifugation at 2,000 g for 15 min, the pellet was resuspended at a protein concentration of ~20 mg/ml in 0.5 M KCl, 5 mM potassium pyrophosphate (PP_i), 5 mM MgCl₂, 1 mM EGTA, 25 mM MOPS (pH 7.0), and incubated for 15 min. After 15 min, the suspension was centrifuged at 140,000 g for 2 h, and the supernate was dialyzed exhaustively against 0.5 M KCl, 0.1 mM EDTA, 10 mM MOPS (pH 7.0). The gel electrophoretic pattern of this material indicated that the myosin was quite pure; in particular, it was free of actin.

PREPARATION OF SUBFRAGMENT-1 (S-1) FROM MYOFIBRILS Myofibrils were suspended at 10 mg/ml in 0.1 M KCl, 2 mM EDTA, 10 mM MOPS, pH 7.0 at 25°C and digested with 0.05 mg of α -chymotrypsin (Sigma Chemical Co., St. Louis, Mo.) per milliliter. After 10 min, the reaction was stopped by adding 1/500 vol of a 0.1 M solution of phenylmethyl sulfonyl fluoride (Sigma) in ethanol. The suspension was washed twice with rigor buffer, and potassium pyrophosphate was added to a concentration of 5 mM. The suspension was stirred briefly and centrifuged at 30,000 g for 30 min. The supernate was fractionated with (NH₄)₂SO₄, and the fraction precipitating between 45% and 60% saturation was retained and dialyzed against rigor buffer. The gel electrophoretic pattern and ATPase activity of this material was very similar to that of the S-1 we prepared by digesting myosin with α -chymotrypsin (Weeds and Taylor, 1975).

PREPARATION OF IMMOBILIZED S-1 S-1 was immobilized by attaching it covalently to glass beads. Corning controlled pore glass beads coated with "Glycophase G" (5–10 μ m particle size, 550A pore size, Pierce catalogue number 23518; Corning Glass Works, Science Products Div., Corning, N.Y.) were activated by incubation with a solution of 10 mM CNBr at room temperature. The pH was maintained between 7.5 and 8.5 by adding NaOH, and the reaction was allowed to proceed until there was no further pH change. The beads were then washed thoroughly with 50 mM EPPS (4-[2-hydroxyethyl]-1-piperazine propane sulfonic acid, Sigma), pH 8.0, and then incubated with S-1 (prepared either from purified myosin or from myofibrils) in the same buffer for 18 h at 4°C, with gentle shaking. Unreacted protein was removed by washing the beads with the labeling buffer, then 0.1 M sodium acetate (pH 5.5), then 0.5 M KCl (pH 7), then rigor buffer. This preparation will be referred to below as immobilized S-1.

LABELING MYOSIN Spin labels were synthesized and tested for purity as described previously (Seidel et al., 1970; Thomas et al., 1979; Barratt et al., 1971). Unless otherwise noted, myosin was spin-labeled at a concentration of 20 μ M (10 mg/ml) in 50 mM KCl, 0.1 mM EDTA, 1 mM NaN₃, 10 mM morpholinopropane-sulfonic acid (MOPS), pH 7.0 at 0°C. The spin label was dissolved in ethanol, diluted with the above solution, then added to the myosin; the final ethanol concentration was 0.1%. For the preparation of iodoacetamide-spin-labeled myosin (IASL-myosin), *N*-(1-oxyl-2,2,6,6-tetramethyl-4-piperidinyl) iodoacetamide (IASL) was added to a concentration of 50 μ M (2.5 labels/myosin). For the preparation of maleimide-spin-labeled myosin (MSL-myosin), *N*-(1-oxyl-2,2,6,6-tetramethyl-4-piperidinyl) maleimide (MSL) was added to a concentration of 80 μ M (4 mol/mol of myosin). After reacting with either label for 8–12 h, myosin was sedimented at 10,000 g for 15 min, the pellet was resuspended in buffer and centrifuged at 100,000 g for 1 h, and the pellet was dissolved in 0.5 M KCl, 0.1 mM EDTA, 10 mM MOPS, pH 7.0. For IASL-myosin, the preparation was completed by dialyzing

overnight against the same solution. For MSL myosin, 50 mM $K_3Fe(CN)_6$ was added (Graceffa and Seidel, 1980), and the solution was incubated for 6–8 h at 0°C. After overnight dialysis against 50 mM KCl, 0.1 mM EDTA, 1 mM NaN_3 , 10 mM MOPS, pH 7.0, 0°C, the precipitated MSL-myosin was sedimented at 100,000 g for 1 h. The pellet was dissolved and resuspended at a protein concentration of 10–20 mg/ml in 0.5 M KCl, 0.1 mM EDTA, 10 mM MOPS, pH 7.0 and dialyzed overnight against the same solution. Labeled HMM and S-1 were prepared by digesting labeled myosin with α -chymotrypsin (Weeds and Taylor, 1975). Synthetic myosin filaments were prepared by dialyzing myosin against the appropriate low ionic strength solution.

LABELING MYOFIBRILS Unless otherwise noted, the following procedure was used. Myofibrils were suspended at a protein concentration of 10 mg/ml in rigor buffer plus 1 mM PP_i at 0°C. IASL-myofibrils were prepared by incubating myofibrils with 0.5 mM IASL for 15 min. Unreacted label was removed by low-speed centrifugation and washing with rigor buffer + PP_i . The myofibrils were washed two more times with rigor buffer. $K_3Fe(CN)_6$ was then added to a final concentration of 25 mM (Graceffa and Seidel, 1980). After 16 h, the myofibrils were washed repeatedly with rigor buffer to remove $K_3Fe(CN)_6$. Before labeling with MSL, myofibrils were incubated with 15 μ M MalNet (N-ethylmaleimide) for 10 min. In some cases, [^{14}C]MalNet (New England Nuclear, Boston, Mass.) was used, so that the concentration of bound MalNet could be determined by scintillation counting. Then MSL was added to a concentration of 40 μ M, and after 10 more min, the myofibrils were washed and treated with $K_3Fe(CN)_6$ as described above for IASL (except that the treatment lasted 40 h).

ATPase Assays

ATPase activities were measured at 25°C by determining the rate of release of inorganic phosphate (P_i). All solutions contained 25 mM MOPS (pH 7.0). Myofibrils were assayed under two sets of conditions: EGTA-Mg (1 mM EGTA, 5 mM $MgCl_2$, 60 mM KCl) and Ca-Mg (0.1 mM $CaCl_2$, 5 mM $MgCl_2$, 60 mM KCl). Myosin was assayed under three sets of conditions, EGTA-Mg (see above); actin-Mg (same as EGTA-Mg, but containing F-actin); and EDTA-K (1 mM EDTA, 0.6 M KCl). In the case of actin-Mg, myosin and actin were first mixed in the presence of 0.6 M KCl, then dialyzed into the EGTA-Mg assay buffer. The reaction was started by the addition of 5 mM ATP and aliquots were quenched by pipetting into the acidic P_i -determining solution used by Lanzetta et al. (1977). Protein concentrations were chosen so that 1-, 2-, 4-, and 8-min time points produced measurable absorbances that were linear with time. The activity was determined by averaging the values at these four points.

EPR Experiments

Both conventional (V_1) and saturation transfer (V_2') EPR experiments were performed as described previously (Thomas et al., 1979). For a detailed discussion of the techniques and theory, see Thomas et al. (1976). Samples were contained in a quartz flat cell, and the temperature was maintained at $20^\circ \pm 0.5^\circ$ by flowing nitrogen through the radiation slits in the front of the Varian E231 cavity. In conventional (V_1) experiments, the incident microwave power was 20 mW, the field modulation amplitude was 2 G, and the first harmonic response to the 100 kHz modulation field was detected. In saturation transfer (V_2') experiments, the incident microwave power was 84 mW, corresponding to a field strength of 0.25 G, as calibrated with peroxyamine disulfonate (Thomas et al., 1976). Note that this power setting is higher than that required to produce the same field strength in earlier work, which involved a different instrument and sample cell (Thomas et al., 1976). The modulation amplitude was 6.3 G (approximately the same value used to record hemoglobin reference spectra; Thomas et al., 1976), and the phase-sensitive detection was out-of-phase with respect to the second harmonic of the 50 kHz modulation field. V_1 spectra were typically obtained in 4 min, V_2' spectra in 30 min. Spin label concentrations were determined by double integration of EPR spectra using a Nicolet 1070 computer (Nicolet Instrument Corp., Madison, Wisc.) interfaced to the spectrometer. Effective rotational correlation times (τ_2) were determined by comparing spectra with reference spectra obtained from theoretical calculations and model system experiments, using the parameters shown in Fig. 1.

RESULTS

Selective Spin-labeling of Myosin Heads in Myofibrils

For meaningful saturation transfer experiments on spin-labeled myofibrils, several goals are desirable: (a) the spin labels should be selectively attached to the myosin head region, (b) the labels should be rigidly immobilized on the myosin heads even in the presence of ATP so that the observed motion is that of the myosin head, not motion of the label relative to the binding site, (c) the actin-activated ATPase activity of myosin should be preserved, and (d) most of the myosin heads in the preparation should be labeled (so that measured ATPase activities are representative of labeled heads). Our earlier studies with conventional (V_i) spectra showed considerable changes in the mobility of IASL attached to myosin upon addition of ATP. In the present work, we have concentrated on the use of MSL, since previous work suggested a lack of response of MSL to ATP.

The conditions which we developed to selectively spin-label the head region of myosin in myofibrils were based on observations of Duke et al. (1976), showing that dissociation of actin and myosin decreases the reactivity of actin's thiol group (Cys-373) and increases the reactivity of myosin's SH-1 group. Borejdo and Putnam (1976) achieved selective labeling of myosin in glycerinated fibers with a fluorescent iodoacetamide derivative, by carrying out the labeling in relaxing solution (containing ATP and EGTA). In agreement with this result, we have found that the presence of ATP and EGTA greatly enhances the selectivity of labeling myosin heads in myofibrils. However, reaction of myosin with maleimide derivatives in the presence of MgATP tends to block both of the SH-1 and SH-2 groups, thus destroying all ATPase activity (Sekine and Yamaguchi, 1963; Yamaguchi and Sekine, 1966; Seidel, 1972). Since Mg pyrophosphate ($MgPP_i$) decreases the affinity of myosin for actin, and has been reported to increase the reactivity of the SH-1 thiol group for maleimide derivatives, without significantly increasing the reactivity of SH-2 (Burke and Reisler, 1977), we substituted PP_i for ATP during the spin-labeling reaction. We found that the specificity of labeling myosin heads in PP_i was as good as in ATP. As shown in Fig. 2 (second row), the presence of PP_i during labeling (as described in Methods) results in a decrease of the spectral component corresponding to weakly immobilized IASL and MSL, relative to that corresponding to strongly immobilized labels (Fig. 1). For both labels, treatment with $K_3Fe(CN)_6$, which selectively destroys the weakly immobilized component of the spectrum of labeled myosin (Graceffa and Seidel, 1980), removes all traces of the weakly immobilized component (Fig. 2, third row). With MSL, this is facilitated by a mild treatment, before spin-labeling, with MalNEt (as described in Methods) which blocks some fast-reacting thiol groups. The final V_i spectra of IASL-myofibrils and MSL-myofibrils (Fig. 2, bottom) are virtually identical to each other.

When purified myosin is labeled with IASL as described in Methods, the spectrum is virtually identical to those of myofibrils (bottom of Fig. 2), without the need for treatment with $K_3Fe(CN)_6$ (not shown). Even when the concentration of spin label is doubled (to 5 mol IASL/mol myosin), the reaction saturates at 2.0 mol bound IASL/mol myosin, and no weakly immobilized component is observed. This specificity is not achieved in the presence of 0.5 M KCl at pH 8, where >2.0 mol reacts and some weakly immobilized label is observed. The reaction appears to be quite selective for the SH-1 thiol groups, since the EDTA,

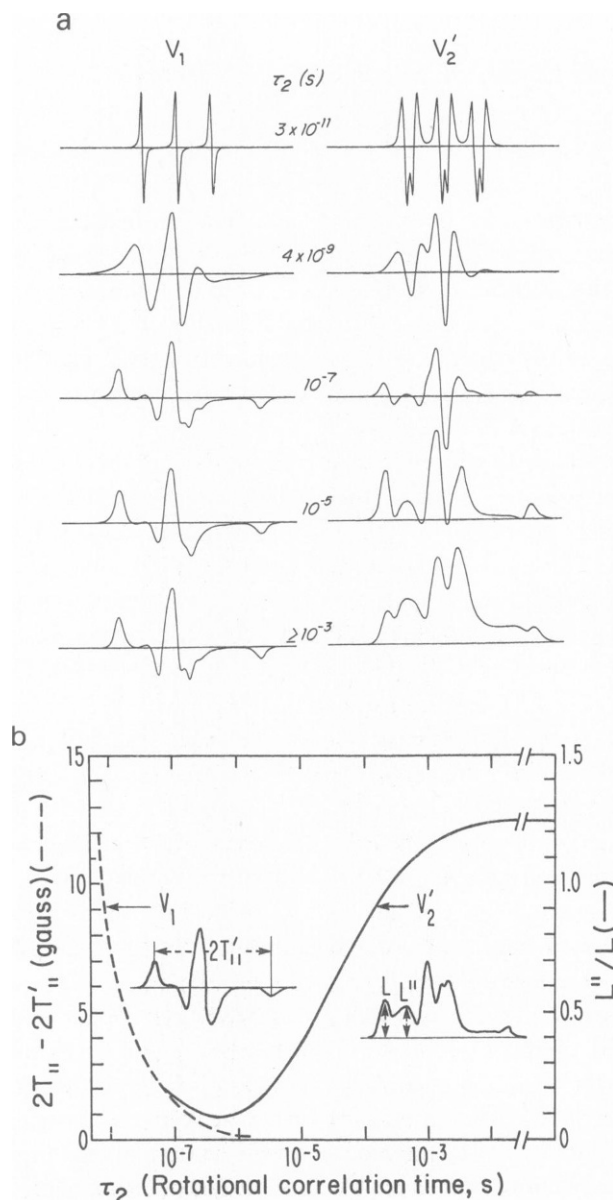


FIGURE 1 Effects of rotational motion, characterized by rotational correlation time τ_2 , on EPR spectra. (a) experimental spectra from model systems undergoing isotropic Brownian rotational diffusion. (b) plots of spectral parameters vs. τ_2 , used to determine effective τ_2 values. Conventional spectra are designated V_1 ; saturation transfer spectra are designated V_2' (from Thomas, 1978).

K-ATPase activity is at least 90% inhibited. The reaction of myosin with MSL is somewhat less specific, and treatment with $K_3Fe(CN)_6$ is required to eliminate the weakly immobilized component, yielding a spectrum virtually identical to that observed for MSL-myofibrils (Fig. 2, bottom).

To further characterize the results of spin-labeling, S-1 was prepared from labeled

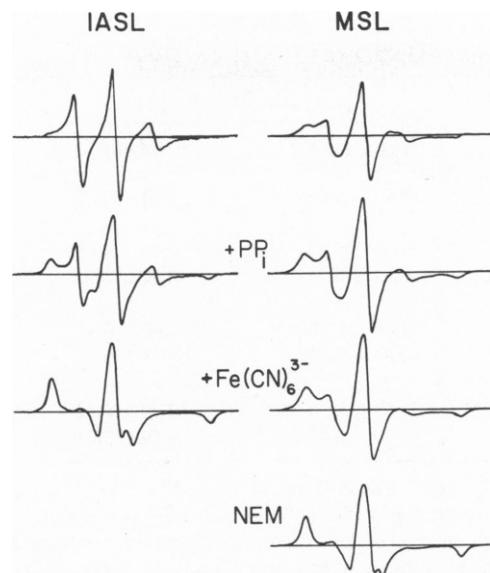


FIGURE 2 Conventional (V_1) EPR spectra of spin-labeled myofibrils. During the recording of spectra, the samples contained 20–30 mg of protein/ml in rigor buffer (60 mM KCl, 5 mM $MgCl_2$, 1 mM EGTA, 1 mM NaN_3 , and 25 mM MOPS, pH 7.0), at 20°C. (Top row) Myofibrils labeled for 15 min at 0°C in rigor buffer at 10 mg protein/ml. The spin label concentrations were 0.5 mM (IASL) and 50 μ M (MSL). Unreacted label was removed by low-speed centrifugation and washing with rigor buffer, repeating three times. (Second row) + PP_i : Myofibrils labeled as above, except that 1 mM pyrophosphate was included during labeling. (Third row) + $Fe(CN)_6^{3-}$: The same myofibrils (+ PP_i) after treatment with 25 mM $K_3Fe(CN)_6$ for 40 h in rigor buffer. $K_3Fe(CN)_6$ was removed by washing. The IASL-myofibrils (left) were used for further experiments. (This is the preparation described in Methods.) (Bottom right) NEM: MSL-myofibrils treated with NEM, then spin-labeled and treated with $K_3Fe(CN)_6$. This preparation was used for further experiments. (This is the preparation described in Methods.)

myofibrils and myosin as described in Methods, and the fraction of spin labels attached to S-1 was determined (Table I). In the preparations used for further experiments (bottom spectra in Fig. 2), ~90–95% of the spin labels remaining in myofibrils are bound to myosin heads for both IASL and MSL (Table I). However, not all of the myosin heads contribute to the final spectrum (no more than 46% for IASL, 19% for MSL). Attempts to increase the percentage of heads that carry a probe, either by reacting with more spin label or by decreasing the extent of treatment with MalNet or $K_3Fe(CN)_6$, resulted in unacceptably large spectral contributions from weakly immobilized labels and from labeled components other than myosin heads.

ATPase Activity of Spin-labeled Myofibrils

To assess the relevance of EPR data from MSL-myofibrils in the presence of ATP (reported below), we measured ATPase activities of the myofibrils and of myosin extracted from them. Table II shows data from a typical preparation. The myofibril ATPase in the absence of Ca^{2+} (first column) was increased by labeling, and the activity in the presence of 0.1 mM Ca^{2+} (second column) was slightly inhibited. For seven different preparations, the average inhibition was $6 \pm 3\%$ (SEM). We measured analogous activities of myosin extracted from myofibrils. The activity in the presence of Mg^{2+} and the absence of actin (third column), like

TABLE I
SPIN-LABELING STOICHIOMETRY

	[Spin labels]/[Myosin heads]		
	Intact system	Extracted S-1	Fraction on heads
IASL-myosin	0.95 ± 0.05	0.91 ± 0.05	0.95 ± 0.10
IASL-myofibrils			
before K ₃ Fe(CN) ₆	1.35 ± 0.15	0.85 ± 0.05	0.62 ± 0.10
after K ₃ Fe(CN) ₆	0.51 ± 0.05	0.46 ± 0.03	0.90 ± 0.15
MSL-myosin			
before K ₃ Fe(CN) ₆	1.85 ± 0.10	1.60 ± 0.10	0.86 ± 0.10
after K ₃ Fe(CN) ₆	0.85 ± 0.05	0.78 ± 0.05	0.92 ± 0.10
MSL-myofibrils			
before K ₃ Fe(CN) ₆	0.55 ± 0.06	0.29 ± 0.02	0.53 ± 0.08
after K ₃ Fe(CN) ₆	0.20 ± 0.02	0.19 ± 0.01	0.95 ± 0.16

Myosin and myofibrils were spin-labeled as described in Methods. The two columns on the left show the ratio of spin labels to heads, in intact myosin or myofibrils (first column) and in S-1 isolated after chymotryptic digestion (second column). The third column shows the ratio of the second to the first. Molar concentrations of spin labels and myosin heads were determined as described in Methods, assuming 2.2 heads/10⁶ daltons in myofibrils. Since the spin label concentration was determined by integrating EPR spectra, those labels whose spin has been destroyed by K₃Fe(CN)₆ do not contribute. For MSL-myofibrils, radioactive counts (¹⁴C) indicated that [MalNEt]/[heads] was 0.53 ± 0.04 in myofibrils and 0.22 ± 0.02 in extracted S-1.

the ATPase of myofibrils in the absence of Ca²⁺ (first column), is enhanced by labeling. In the presence of Mg²⁺, a ratio of 40 mol actin/mol myosin yielded maximal activation, for both labeled and unlabeled myosin. As shown in Table II (fourth column), labeling does not significantly perturb the activity. The activity at high [K⁺] in the absence of divalent cations (last column) decreases by ~40% upon labeling. The fractional inhibition of this activity has been shown to be a good indicator of the fraction of SH-1 groups blocked by maleimide derivatives (Sekine and Kielley, 1964). Table I indicates that an average of 0.51 ± 0.4 maleimide derivatives (0.29 MSL plus 0.22 MalNEt) were attached to each head, so the EDTA-K ATPase results (last column of Table II) suggest that most of these probes were attached to SH-1. Thus, although nearly half of the heads in MSL-myofibrils appear to have their SH-1 groups blocked, the interaction between myosin and actin is not seriously perturbed. In fact, the actin-activated ATPase of the extracted myosin is not inhibited at all, suggesting that the slight inhibition of the myofibrillar Ca-Mg-ATPase is due to labels on

TABLE II
ATPase ACTIVITIES

	Myofibrils		Extracted myosin		
	EGTA-Mg	Ca-Mg	Mg	Mg-actin	K-EDTA
Unlabeled	0.021 ± 0.005	0.45 ± 0.04	0.010 ± 0.002	0.51 ± 0.07	2.28 ± 0.1
MSL	0.028 ± 0.010	0.42 ± 0.04	0.016 ± 0.005	0.55 ± 0.08	1.36 ± 0.08

ATPase activities (μmol P_i liberated/mg protein per min) for MSL-myofibrils and for unlabeled myofibrils subjected to the same manipulations. Assays were performed at 25°C as described in Methods. The rates of phosphate release were linear, as judged from time points at 0, 1, 2, 4, and 8 min. For Mg-actin, the molar ratio of actin to myosin was 40.

components other than myosin heads. However, labeling actin with MSL does not affect actomyosin ATPase either (Stone et al., 1970; Thomas *et al.*, 1979). IASL-myofibrils gave results similar to MSL-myofibrils. Myosin labeled after purification gave essentially the same results as myosin extracted from labeled myofibrils.

In summary, we have achieved most of the labeling goals listed at the beginning of Results. Therefore, the saturation transfer spectra of spin-labeled myosin and myofibrils should provide useful data on the rotational motion of myosin heads in the microsecond time range, in the presence and absence of ATP.

EPR Spectra

COMPARISON OF RIGOR MYOFIBRILS WITH IMMOBILIZED MYOSIN HEADS The V_2' spectra of both IASL-myofibrils and MSL-myofibrils in rigor show very strongly immobilized spin labels (Fig. 3, bottom). These spectra are quite similar to that of precipitated MSL-Hb (Fig. 1 *A*, bottom right), and to those of labeled S-1 bound either to glass beads (Fig. 3, immobilized S-1) or to F-actin (Fig. 3). There is some uncertainty in the theoretical value of L''/L (Fig. 1 *B*) corresponding to the rigid limit, i.e., corresponding to no detectable rotational motion ($\tau_2 \geq 1$ ms); this value falls between 1.15 and 1.35 (Thomas, 1978; Thomas and Hidalgo, 1978). Since preparations of labeled myofibrils consistently displayed L''/L values of 1.1–1.25, these probes are undergoing little or no submillisecond rotational motion. By

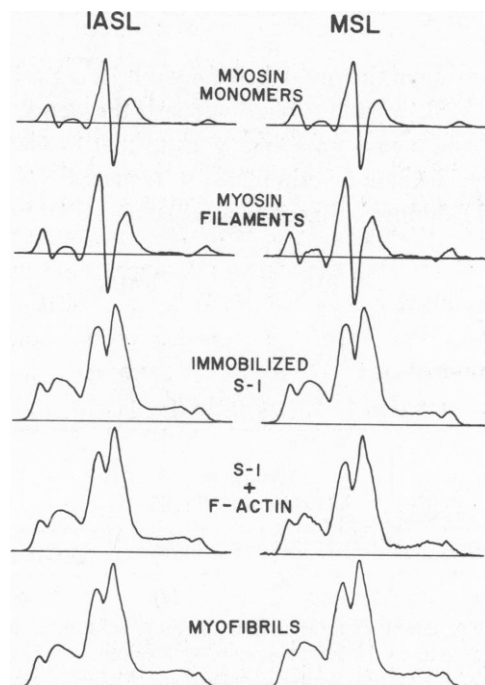


FIGURE 3 Saturation transfer EPR spectra (V_2') of IASL (left) and MSL (right) attached to myosin heads, at 20°. The protein concentrations were 10–20 mg/ml. The solutions contained rigor buffer (60 mM KCl, 5 mM $MgCl_2$, 1 mM EGTA, 1 mM NaN_3 , 25 mM MOPS, pH 7.0), except for myosin monomers, in which the KCl concentration was 0.5 M. In S-1 + F-actin there were 4 mol actin/mol S-1.

contrast, myosin monomers and myosin filaments exhibited rapid submillisecond rotational motion (Fig. 3), with effective correlation times of 0.4 and 10 μ s, respectively.

ATP EFFECTS ATP has a striking effect on the V_2' spectrum of MSL-myofibrils. Whereas the spectrum in rigor indicates little or no submillisecond rotational motion (Fig. 4, top left), a spectrum recorded during steady-state ATPase activity in the absence of Ca^{2+} (Fig. 4, top right), corresponding to relaxation, indicates rotational motion comparable to that observed for synthetic myosin filaments in the same buffer without ATP (Fig. 4, bottom left). The peak ratio L''/L decreases from 1.2 to 0.4, corresponding to an effective rotational correlation time (τ_2) of 10 μ s (Fig. 1). By contrast, the spectrum of myosin filaments was insensitive to ATP (Fig. 4, bottom). In the presence of 0.1 mM Ca^{2+} , the ATPase activity of MSL-myofibrils was so high that we were not able to record a complete spectrum before the creatine phosphate and ATP were depleted. Nevertheless, there was sufficient time to monitor the motion by measuring L''/L . Fig. 5 shows the time-dependence of L''/L in detail. For myofibrils in the absence of Ca^{2+} , corresponding to relaxation (open circles), L''/L remains constant between 0.4 and 0.5 (indicating rapid motion) for ~ 1 h after the addition of ATP. This period of steady-state hydrolysis is approximately as long as expected, based on the measured ATPase activity (Table II). L''/L then increases gradually toward the initial (rigor) value ($L''/L > 1$), as the creatine phosphate and ATP are depleted. In the presence of 0.1 mM Ca^{2+} (closed circles, Fig. 5), the addition of ATP quickly increases rotational motion almost the same degree as in the absence of Ca^{2+} . The main apparent effect of Ca^{2+} is to accelerate the ATP hydrolysis, returning the spin-labeled heads more rapidly to the immobilization of rigor.

Comparison of the spectrum of the spin-labeled myofibrils in the presence of ATP with that of filaments formed from MSL-myosin strongly supports the view that during the time ATP is available most of the myosin is in a state analogous to that in filaments alone. ATP has no significant effect on the spectrum of myosin filaments (squares, Fig. 5) in which the heads are already mobile before ATP addition. Since the glass beads experiment shows little or no local

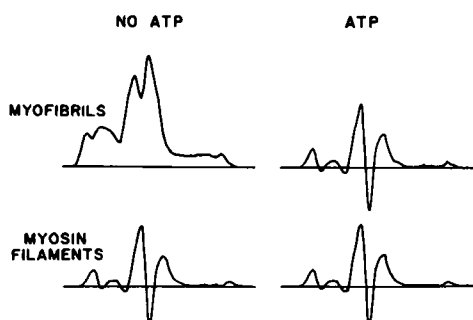


FIGURE 4 Effect of ATP on saturation transfer EPR spectra of MSL-myofibrils (top) and MSL-myosin filaments (bottom). The spectra on the left were obtained from samples containing rigor buffer plus 50 mM creatine phosphate and 0.5 mg/ml creatine phosphokinase. The protein concentration was 30 mg/ml for myofibrils, 20 mg/ml for myosin. The spectra on the right were obtained under relaxing conditions, i.e., after the addition of ATP to a final concentration of 5 mM. Spectra were recorded within 30 min after ATP addition, a sufficiently short time to ensure that the creatine phosphate and ATP was not yet depleted, based on the measured activities (Table II). Immediately after completing each spectrum, the low-field region was scanned again to verify that the spectrum had not changed during the first scan.

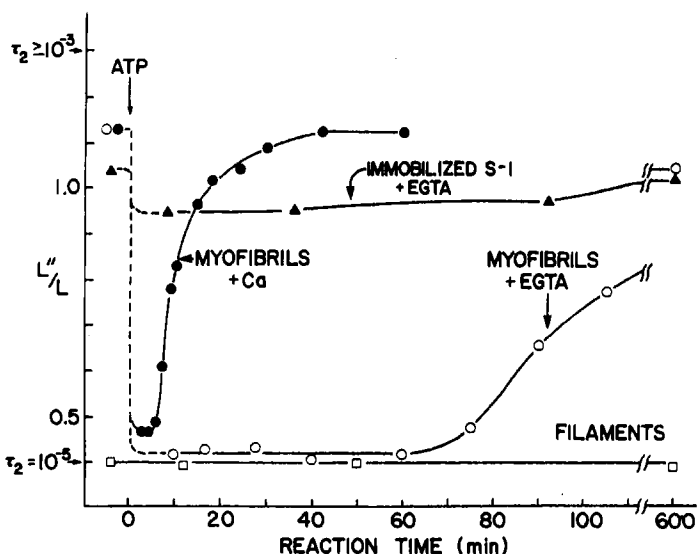


FIGURE 5 L''/L , from saturation transfer EPR spectra (V_1), plotted as a function of time after adding ATP to myosin filaments (\square), myofibrils (\circ, \bullet) and immobilized S-1 (Δ). The reaction mixtures contained 20–30 mg protein/ml in rigor buffer plus 50 mM creatine phosphate, 0.5 mg creatine phosphokinase, and 5 mM ATP, except for the closed circles, where 0.1 mM CaCl_2 was substituted for EGTA.

motion of the label without ATP, ATP was added to S-1 bound to glass beads (immobilized S-1) to test whether the motion observed in myofibrils during ATPase activity is induced within the myosin head. In this experiment we observed very little effect of ATP on L''/L (Fig. 5). The heads are not greatly perturbed by immobilization on beads since (a) the Mg-ATPase activities of S-1, MSL-S-1, and IASL-S-1 are not significantly inhibited by conjugation to the beads, and (b) IASL-S-1 immobilized by the same procedure shows the same kind of large V_1 spectral change upon ATP addition (indicating submicrosecond motion of the probe relative to S-1) that is observed in soluble IASL-myosin. Clearly, this kind of internal motion is not inhibited by attachment to the beads. The spectra of myosin filaments and immobilized S-1, either in the presence or absence of ATP, are not affected by 0.1 mM CaCl_2 .

DISCUSSION

Immobilization of Myosin Heads in Rigor

The saturation transfer EPR spectra of both IASL and MSL attached selectively to myosin heads in rigor myofibrils (Fig. 3, bottom) show that the probes undergo little or no submillisecond rotational motion. Since the observed effective correlation time of the probe should be a lower bound for that of the head, we conclude that essentially all of the myosin heads in a myofibril in rigor are rotationally immobile in the submillisecond time range.

The EPR spectrum is primarily sensitive to the reorientation of the probe's principal axis, and an anisotropic rotation of the head that did not reorient the probe's principal axis might not be detected. Our previous work (Thomas et al., 1975 a) indicated that the principal axis of

IASL is aligned approximately with the long axis of the head; therefore, the present spectra of IASL-myofibrils indicate that the long axis of the myosin head is immobile in rigor. The two spin labels (IASL and MSL) are oriented differently with respect to the fiber axis (Thomas and Cooke, 1980), and presumably also with respect to the head, so a submillisecond rotational motion not reported by one probe would probably be reported by the other. In a strict sense, our conclusion of immobilization applies only to the region in which the spin labels are bound, and we can not rule out the possibility of flexibility within other regions of the crossbridge. Because no more than one-fifth of the heads in MSL-myofibrils (and one-half in IASL-myofibrils) contribute to the spectrum, it could be argued that we have selectively labeled those heads that are immobile (i.e., bound to actin). This possibility can be discounted, because binding to actin decreases the reactivity of the SH groups of myosin heads (Duke et al., 1976; Borejdo and Putnam, 1977).

The most obvious interpretation of the observed immobilization is that essentially all myosin heads are attached to actin in rigor. This conclusion follows from the observation that the spectra of unattached heads (in myosin filaments or relaxed myofibrils; see Fig. 4) indicate rapid submillisecond rotational motion, in contrast to the immobilization observed when labeled S-1 or myosin is attached to actin (Fig. 3 and Thomas et al., 1975 *a*, and *b*, 1979). Let us assume that the saturation transfer spectrum of myofibrils (Fig. 3, bottom) is composed of two components, one arising from detached heads and having the shape of the spectrum from myosin filaments (Fig. 3, second row), and the other arising from attached heads and having the shape of the spectrum from S-1 plus F-actin (Fig. 3, fourth row). We have simulated graphically this two-component synthesis for each spin label and have found that satisfactory fits to the rigor spectra are obtained only if the unattached component is assumed to be <10% of the total. On the other hand, if the spectrum of detached heads in native thick filaments showed less motion than in synthetic filaments, the possibility would remain that >10% might be detached in rigor myofibrils.

The present results do not rule out the possibility that although all crossbridges are attached in rigor, only one head of each myosin molecule is attached and its partner is immobilized by some interaction between the two. However, previous EPR (Thomas et al, 1975 *a* and *b*) and fluorescence (Mendelson et al. 1973; Mendelson and Cheung, 1978) experiments suggest that the two heads of myosin rotate independently in solutions of monomeric myosin.

The rigor state in labeled myofibrils probably corresponds closely to rigor in an intact muscle fiber. There is no evidence to suggest that moderate sulfhydryl blocking affects the interaction of actin and myosin in the absence of ATP; for example, the affinities of blocked and unblocked myosin heads (S-1) for actin are not significantly different (Marston and Weber, 1975; Margossian and Lowey, 1978; Mulhern and Eisenberg, 1978; Highsmith et al., 1976).

The conclusion that virtually all crossbridges (and probably all myosin heads) in a rest-length myofibril are attached to actin in rigor has interesting implications for the structure of the filament lattice. There is considerable controversy over the fraction of heads attached in rigor—estimates ranging from 33 to 100% have been made on the basis of x-ray diffraction data and model building (Offer and Elliot, 1978). Because of the different pitches and repeats of the actin and myosin helices, the present results suggest that there must be considerable flexibility in the crossbridges—possibly even permitting the two heads to bind to

different actin filaments (Offer and Elliott, 1978). Flexibility in the thin filaments (Ishiwata and Fujimu, 1973; Thomas et al., 1979) might also facilitate the attachment of heads.

Rotational Mobility of Heads Detached from Actin

Reasoning that the apparent motion of the probe cannot be slower than the motion of the head, we have argued that heads in rigor myofibrils are immobilized. Based on the rapid motion observed in myosin filaments and relaxed myofibrils, but not in acto-S-1 or rigor myofibrils, we have concluded that essentially all heads (or at least all crossbridges) are attached to actin in rigor. However, the observation of rapid probe motion does not necessarily imply that detached heads rotate rapidly. To conclude that heads of myosin filaments or relaxed myofibrils are mobile, we must show that the probes are rigidly bound in detached as well as in attached heads, and in the presence as well as the absence of ATP.

NMR studies indicate the presence of internal motion in S-1 that is suppressed by actin (Highsmith et al., 1979). Even though this motion is on a much faster time scale than observed for spin labels, even in myosin monomers, it might be argued that a similar but slower internal motion (on the microsecond time scale) could be responsible for the apparent motion of myosin heads in myosin filaments and relaxed myofibrils (Fig. 4). However, this interpretation seems unlikely, considering the immobilization observed when S-1 is bound to glass beads. It seems highly unlikely that the reaction of S-1 with cyanogen bromide-activated beads, which must occur randomly over the surface of S-1, would suppress local motion of the label in the same way as would the binding of actin to a specific site on S-1. Similarly, it seems unlikely that attaching S-1 to beads, which has no effect on ATPase activity nor on the ATP-dependent mobility of IASL, would suppress ATP-dependent motion of MSL relative to the head. ATP has little or no effect on the immobilized spectrum of MSL-S-1 attached to glass beads (Fig. 5), indicating that the probe remains rigid even during ATPase activity.

Thus, our results indicate that detached myosin heads in filaments of purified myosin or in relaxed myofibrils undergo rapid (submillisecond) rotational motion. Heads attached to actin are substantially immobilized and, according to our earlier observations, appear to rotate at a barely detectable rate determined by an internal rotational mode of the actin filament (Thomas et al., 1979). Since the spectrum of relaxed myofibrils is so similar to that of free myosin filaments and so different from that of rigor myofibrils, it appears that no significant fraction of heads (no more than 10%) is attached at any instant in relaxation.

Ishiwata et al. (manuscript submitted for publication) have studied the effects of PP_i and AMPPNP on spin-labeled myofibrils prepared as in the present study. They have compared these spectra with those of spin-labeled S-1 or HMM diffused into unlabeled myofibrils, and have determined by centrifugation the fraction of S-1 or HMM molecules bound. Their results add to the evidence that the spectral contribution from each molecule (S-1 or HMM) is determined almost exclusively by whether it is attached to actin, with both heads immobilized in attached HMM, indicating that saturation transfer spectroscopy can be used to estimate the fraction of attached crossbridges under a variety of conditions.

The effective rotational correlation time determined from the spectra of MSL attached to myosin filaments and relaxed myofibrils (Fig. 4), using Fig. 1, is 10 μ s. This result is consistent with those of Mendelson and coworkers (1973, 1976, and 1978), who have reported that, at physiological ionic strength and pH, fluorescent probes attached to myosin heads in

either synthetic myosin filaments or relaxed myofibrils have effective correlation times on the order of 1 μ s or more. However, the poor sensitivity of the fluorescence method to motions in the microsecond range prevented them from detecting the large difference between rigor and relaxation that is evident in Fig. 4 of the present study (Mendelson and Cheung, 1976). The effective correlation time depends on the actual time scale and on the amplitude of the rotations. When motion is restricted to a small angular amplitude, the observed effective correlation time is greater than the actual time, for both EPR (Thomas, 1978) and fluorescence (Mendelson and Cheung, 1978). Mendelson and Cheung (1976) estimated a lower bound of 1.6 μ s for the effective correlation time of fluorophores in relaxed myofibrils. Assuming a value of 3 μ s for this time, and assuming that the actual rotations took place in the nanosecond time range (as in monomeric myosin), they concluded that their data were consistent with motion restricted to a 25° angular range (Mendelson and Cheung, 1978). Standing alone, the EPR data in the present study are consistent with this model. However, Thomas and Cooke (1980) have found that the orientational spread of maleimide spin labels on heads in glycerinated fibers changed from 15° or less in rigor to at least 90° in relaxation (or after stretching to eliminate actin-myosin interactions). This suggests that rotational motion occurs over a wide angular range, making the effective correlation time (10 μ s) an accurate estimate of the actual time. At present, until saturation transfer experiments have been performed on fibers, there remains some uncertainty in the angular range of head motion, so some caution is required in discussing absolute value of τ_2 .

Effects of Labeling on Function

The relevance of the observed ATP-induced motion in MSL-myofibrils depends on the effects of labeling on the ATPase activities. As discussed in Results, the data in Table II indicate that about half of the heads have their SH-1 groups blocked, but that the myofibrillar ATPase activities are only slightly perturbed. The most significant effect on the myofibrils is an increased activity in the absence of Ca^{2+} , suggesting that the EPR data on relaxed myofibrils should be assessed with some caution. However, neither the myofibrillar ATPase in the presence of Ca^{2+} nor the actin-activated ATPase of the extracted myosin is seriously perturbed by labeling, in agreement with previous reports on SH-1-blocked myosin (Seidel, 1973; Mendelson et al., 1975; Lin and Morales, 1977). Mulhern and Eisenberg (1978) did observe inhibition of actin-activated ATPase due to spin-labeling, but their preparations differed from ours in at least two important respects. First, they labeled at a higher pH and ionic strength, where the reaction is not as specific for SH-1 groups, as discussed in Results. Second, they measured the actin-activated ATPase of HMM, not intact myosin. We have found that this activity of HMM (or S-1) can be inhibited by labeling, even when myosin's activity is not.

We can not rule out the possibility that labeling affects some of the kinetic constants more than is apparent from the measured steady-state rates. Such changes could affect the concentration of various steady-state intermediates, e.g., the number of attached heads. Thus a detailed understanding of the effects of labeling on enzymatic rate constants is required if the present results are to be extrapolated in a quantitative way to unlabeled myosin and myofibrils.

Rotational Mobility of Heads in Activated Myofibrils

The mobility of heads in myofibrils during ATPase activity in the presence of Ca^{++} , as indicated by the parameter L''/L (Fig. 5), is nearly as great as in the absence of Ca^{++} . The simplest interpretation is that only a very small fraction ($<20\%$) of heads is attached to actin at any instant in these activated myofibrils. Alternatively, a larger fraction might be attached and rotating rapidly in the submillisecond time range. Since we have no independent measure of the fraction of attached heads, we can not determine whether some of the observed motion arises from attached heads.

It is likely that submillisecond rotational motions of attached heads do occur, since changes in sarcomere length (Barden and Mason, 1978) and in tension (Ford et al., 1977) have been observed on this time scale in muscle fibers. However, since our experiments were performed on free contracting myofibrils, the results cannot be directly extrapolated to muscle fibers under tension.

Future saturation transfer experiments on spin-labeled glycerinated fibers should be useful in clarifying these points. It will be interesting, for example, to compare direct EPR measurements on heads in fibers with stiffness measurements, which are often used to estimate the function of attached crossbridges (Guth and Kuhn, 1978). It will also be interesting to compare EPR results with those obtained by x-ray diffraction. For example, the order indicated by the layer lines and meridional reflections in the x-ray diffraction pattern of relaxed muscle (Huxley and Brown, 1967) might be interpreted to suggest that crossbridges are immobile in relaxation. The present results on myofibrils suggest that the Brownian rotation of relaxed crossbridges is much more extensive than previously thought. In general, direct EPR probing of myosin heads under conditions comparable to x-ray and mechanical measurements should prove extremely valuable in testing and refining the crossbridge model of muscle contraction.

We thank Aida Carlos and Vincent Barnett for excellent technical assistance, Susan Karplus for helpful discussions concerning actomyosin ATPase measurements, and Philip Graceffa for advice on the use of $\text{K}_3\text{Fe}(\text{CN})_6$.

This research was supported by National Institutes of Health grants HL-5949, HL-15391, HL-5811, RR 05711, and GM 27906, National Science Foundation grants PCM78-18530 and PCM 80-04512, the Muscular Dystrophy Association, and the Minnesota Medical Foundation.

Received for publication 11 June 1980 and in revised form 13 August 1980.

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