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Myosin Step Size
Estimation From Slow Sliding Movement of Actin Over Low Densities of Heavy Meromyosin

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We have estimated the step size of the myosin cross-bridge (d, displacement of an actin filament per one ATP hydrolysis) in an *in vitro* motility assay system by measuring the velocity of slowly moving actin filaments over low densities of heavy meromyosin on a nitrocellulose surface. In previous studies, only filaments greater than a minimum length were observed to undergo continuous sliding movement. These filaments moved at the maximum speed (v_o), while shorter filaments dissociated from the surface. We have now modified the assay system by including 0·8% methylcellulose in the ATP solution. Under these conditions, filaments shorter than the previous minimum length move, but significantly slower than v_o, as they are propelled by a limited number of myosin heads. These data are consistent with a model that predicts that the sliding velocity (v) of slowly moving filaments is determined by the product of v_o and the fraction of time when at least one myosin head is propelling the filament, that is, $v = v_o \{1-(1-t_s/t_c)^N\}$, where t_s is the time the head is strongly bound to actin, t_c is the cycle time of ATP hydrolysis, and N is the average number of myosin heads that can interact with the filament. Using this equation, the optimum value of t_s/t_c to fit the measured relationship between v and N was calculated to be 0·050. Assuming $d = v_o t_s$, the step size was then calculated to be between 10 nm and 28 nm per ATP hydrolyzed, the latter value representing the upper limit. This range is within that of geometric constraint for conformational change imposed by the size of the myosin head, and therefore is not inconsistent with the swinging cross-bridge model tightly coupled with ATP hydrolysis.

1. Introduction

Muscle contraction results from active sliding between actin filaments and myosin filaments driven by chemical energy liberated by hydrolysis of ATP (Huxley & Niedergerke, 1954; Huxley, 1957). The myosin molecule can be divided into two domains by limited proteolysis. The head domain, called S-1, contains the ATP and actin binding sites of the parent myosin molecule (Toyoshima & Wakabayashi, 1985; Sutoh *et al.*, 1986). Since S-1 has been shown to be sufficient to induce sliding movement of (Toyoshima *et al.*, 1987) and tension on (Kishino & Yanagida, 1988) actin filaments *in vitro*, it is evident that at least a part of the mechanochemical transformation takes place in the complex of actin and the head domain of myosin. However, the molecular mechanism of the energy transformation remains obscure.

The swinging cross-bridge theory (Huxley, 1957, 1969) predicts that the mechanochemical cycle of myosin involves a coupling between the hydrolysis of ATP and a change in angle of the cross-bridge bound to the actin filament and a subsequent release from actin, allowing net displacement. This implies that the step size d (displacement of an actin filament per one stroke of a cross-bridge or one ATP hydrolysis‡) is certainly less than 40 nm, which is twice the ≈20 nm chord length of the myosin head. The response of muscle fibers to quick changes in load (Civan & Podolsky, 1966) or in length (Huxley & Simmons, 1971) indirectly suggested $d \approx 10$ nm per stroke of the cross-bridge cycle (Bagshaw, 1982). However, recent studies have yielded highly divergent results; ≈10 nm (Toyoshima *et al.*, 1990), which was estimated from the ratio of ATP hydrolysis to sliding velocity *in vitro*; ≈20 nm (Taylor,

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‡ Abbreviations used: d, displacement of an actin filament per one stroke of a cross-bridge or one ATP hydrolysis; HMM, heavy meromyosin; RhPh, rhodamine phalloidin; BSA, bovine serum albumin.

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1989), which was estimated from kinetic studies of myofibrils; ≈60 nm (Yanagida *et al.*, 1985), which was estimated from the ratio of ATP hydrolysis to sliding velocity in myofibrils; and larger than 120 nm (Yanagida, 1989; Harada *et al.*, 1990), which was estimated from the ratio of ATP hydrolysis to sliding velocity *in vitro*. If d is larger than 40 nm, as reported by Yanagida and colleagues, then force generation by swinging cross-bridges tightly coupled with ATP hydrolysis would be impossible (Oosawa & Hayashi, 1986). Considering the fundamental importance of determining d, these conflicting results led us to attempt an independent estimate of d by measuring the velocity of slowly moving actin filaments at low densities of heads on the surface in an *in vitro* assay system.

In previous studies using heavy meromyosin (HMM), a two-headed proteolytic fragment of myosin (Toyoshima *et al.*, 1990), or using native myosin (Harada *et al.*, 1990), a minimum length of filaments, as a function of the head density, was observed for continuous movement *in vitro*; filaments longer than the minimum length moved continuously at the maximum speed (v_0), while shorter filaments dissociated from the surface in the presence of ATP. These authors interpreted the result to mean that the filaments have to be held by at least one head undergoing its power stroke almost continuously to prevent dissociation of the filament from the surface. We overcame this limitation by including methylcellulose in the ATP solution. The resulting decrease in Brownian movement keeps free filaments close to the surface so that filaments shorter than the minimum length may move continuously, albeit slower than v_0. In this regime, a filament is predicted to move at a speed determined by the fraction of time that at least one head is propelling or interacting with the filament. Measuring this relationship in our improved *in vitro* movement assay system has enabled us to estimate the ratio of the average number of heads bound to a filament in force-producing states to the total number of heads that can interact with the filament. We have used this ratio to estimate the value of d, the step size.

2. Materials and Methods

(a) *Protein purification*

Myosin was purified from rabbit back muscle by the method of Szent-Gyorgyi as modified by Mommaerts & Parrish (1951) and stored at −20°C in 50% (w/v) glycerol. Chymotryptic heavy meromyosin (HMM) was prepared as described (Toyoshima *et al.*, 1987), and used within 3 days. Contaminating rigor-forming heads were removed by centrifugation with 0·17 mg actin/ml and 2 mM-ATP in assay buffer (AB buffer: 25 mM-imidazole·HCl (pH 7·4), 25 mM-KCl, 4 mM-$MgCl_2$, 1 mM-EGTA and 10 mM-dithiothreitol; modified from Toyoshima *et al.*, 1987) for 10 min at 75,000 revs/min in a Beckman TLA 100.3 rotor, immediately before use. For ATPase experiments in solution, this HMM was resolved from ATP by gel filtration chromatography (Superose 12; Pharmacia LKB, Bromma, Sweden). Actin was purified from rabbit skeletal muscle (Pardee & Spudich, 1982). Rhodamine phalloidin (RhPh)-labeled F-actin was prepared by incubating 50 μg actin/ml in AB buffer with 1·6 μM-RhPh (Molecular Probes, Eugene, OR, U.S.A.) for several hours (Kron & Spudich, 1986). Protein concentrations were determined by a dye-binding assay (Bradford, 1976). The concentration of HMM was calibrated by absorbance at 280 nm using an extinction coefficient of $6{\cdot}0 \times 10^2$ cm^2/g (Margossian & Lowey, 1982).

(b) *Motility assay*

Movement of RhPh/F-actin over nitrocellulose surfaces coated with HMM, observed by intensified video epifluorescence microscopy, was performed according to the method described by Kron & Spudich (1986) and Toyoshima *et al.* (1987, 1990) with some modifications. In brief, flow cells were freshly constructed from a glass slide and a coverslip, separated by 2 mm wide strips of coverslip. The inner surface of the glass slide was precoated with bovine serum albumin (BSA) and the coverslip with a thin film of nitrocellulose. The nitrocellulose film was made by air-drying coverslips coated with approximately 10 μl of 0·1% (w/v) nitrocellulose solution in isoamyl acetate. HMM in AB buffer was introduced into the flow cell. After 2 min of incubation, the HMM solution was replaced with AB/BSA buffer (0·5 mg BSA/ml in AB buffer) to block the remaining binding sites of the nitrocellulose surface. Electron microscopic observation after negative staining (Y. Toyoshima, unpublished results) or immunogold labeling (T. Uyeda, unpublished results) demonstrated that HMM molecules are randomly distributed over nitrocellulose surfaces prepared in this way.

From 1 to 5 μg RhPh/F-actin/ml in AB/BSA buffer was then introduced and was allowed to bind to the immobilized HMM. After rinsing with AB/BSA buffer, RhPh/F-actin was observed sliding over the surface by perfusing AB/BSA buffer that contained 2 mM-ATP, an oxygen scavenger system (Kishino & Yanagida, 1988), and various concentrations of methylcellulose. Methylcellulose (M-0512; Sigma Chemicals, St Louis, MO, U.S.A.) was dissolved in AB buffer at 1% (w/v) and dialyzed against AB buffer before use.

Flow cells were then placed on the microscope stage and observed under a ×63 oil immersion objective, both of which were thermostatically controlled at 30°C. After 5 min of preincubation to equilibrate the temperature, the sliding movement was recorded using a Panasonic model AG-6300 VHS video recorder, and average sliding velocity and length of individual filaments were measured using a video micrometer with a video frame counter (Sheetz *et al.*, 1986). Average sliding velocity was determined for filaments that moved for at least 15 μm of continuous movement. Filaments shorter than 0·5 μm were not scored because the error of length measurement in our system gradually increases as the filaments become shorter and reaches ≈20% for a 0·5 μm filament (Y. Toyoshima, unpublished results).

(c) *Analysis of Brownian movement of actin filaments in solution*

Fluorescence images of RhPh/F-actin, suspended in AB/BSA buffer that contained the oxygen scavenger system and various concentrations of methylcellulose, were recorded using a Sony model VO-5800H U-matic

video recorder at 30 °C. Selected frame sequences were transferred to an optical memory disc using a Panasonic model TQ-2028F recorder. Centroids of fluorescence images from successive frames were calculated using an image analysis program (JAVA; Jandel Scientific, Sausalito, CA, U.S.A.), and centroid displacements of each filament were calculated by 3-frame (0·1 s) difference intervals.

(d) *Density of HMM*

The number of HMM molecules immobilized on the nitrocellulose surface was estimated by comparing the K-EDTA–ATPase activity in the flow cell to the specific activity of the K-EDTA–ATPase of HMM in solution. It was assumed that the K-EDTA–ATPase activity is the same between immobilized HMM and HMM in the solution. To measure the K-EDTA–ATPase of flow cells to which HMM had been applied, 1 vol. of 0·5 mM-ATP in high salt–EDTA buffer (0·5 M-KCl, 4 mM-EDTA, 1 mM-dithiothreitol, 0·5 mg BSA/ml, 25 mM-imidazole·HCl (pH 7·4)) was introduced into the flow cells after rinsing with high salt-EDTA buffer and equilibrating to 30 °C. The reaction was terminated by perfusing 4 vol. of cold water into the flow cell, and phosphate content of the combined effluent was determined by the Malachite green assay (Kodama *et al.*, 1986). The specific activity of K-EDTA–ATPase of HMM in solution was determined in 0·5 mM-ATP in the high-salt EDTA buffer at 30 °C.

(e) *Estimation of cycle time*

To estimate t_c of the actin-activated Mg-ATPase in the flow cells, 1 vol. of 2 mM-ATP solution in AB/BSA buffer containing 0·3% methylcellulose was introduced into a flow cell that contained immobilized HMM and actin filaments bound to HMM. The reaction mixture was recovered by perfusing with 4 vol. of cold water after appropriate period of incubation, and the phosphate content of the combined effluent was measured as described above. The Mg-ATPase of HMM activated either by phalloidin-labeled or unlabeled actin have identical K_m and V_{max} values in solution (S. Kron, unpublished results).

(f) *Measurement of viscosity*

The absolute viscosity of 0·22 ml samples of solutions of methylcellulose in AB/BSA buffer that contained 2 mM-ATP was measured at 30 °C in a Wells-Brookfield cone/plate viscometer (spindle no. CP-50; Brookfield Engineering, Stoughton, MA, U.S.A.).

3. Results

(a) *Rationale*

(1) Toyoshima *et al.* (1987) observed that actin filaments can slide over nitrocellulose surfaces densely coated with HMM heads at a speed close to that of the shortening of unloaded muscle fibers, v_o. The step size (d) can be expressed as the product of t_s, the duration of power stroke(s) or the time of strongly bound state in one ATP hydrolysis cycle, and v_o:

$$d = v_o \times t_s. \quad (1)$$

Provided that the heads are cycling at V_{max}, equation (1) can be rewritten as a function of f, the proportion of stroking time to the total cycle time (Bagshaw, 1982):

$$d = v_o \times f \times t_c, \quad (2)$$

$$f = t_s/t_c, \quad (3)$$

where t_c is the cycle time of ATP hydrolysis given by $1/V_{max}$. These equations show that d can be estimated by measuring v_o, t_c and f. Among the three parameters, v_o and t_c are readily measurable. Thus, the major goal of the present study is to determine f.

(2) The mass and velocity of actin filaments undergoing sliding movement are so small compared with viscous drag that filaments move randomly in a Brownian fashion when not actively propelled by power strokes (Yanagida *et al.*, 1985). However, the drag force due to surrounding solvent on filaments moving lengthwise is much smaller than the force produced by power strokes (Oosawa, 1977; Kron & Spudich, 1986), so that the action of a single myosin head is sufficient to move a filament at v_o during the period of t_s. Were actin filaments ideally rigid, the expected average velocity of a filament sliding over multiple heads (v_{exp}) would be given by the product of v_o and the probability that the filament is propelled by at least one stroking head at any instant. Noting that f represents the probability that a myosin head interacting with actin is undergoing a power stroke and assuming that all the heads stroke asynchronously, the probability that of N heads that are available to interact with a filament none is undergoing a power stroke at any instant is given by $(1-f)^N$. Consequently, the probability that the filament is propelled by at least one out of the N heads is $1-(1-f)^N$. Therefore:

$$v_{exp} = v_o \times \{1-(1-f)^N\} \quad (4)$$

(Harada *et al.*, 1990).

(3) In reality, actin filaments are not ideally rigid (e.g. see Nagashima & Asakura, 1980; Yanagida *et al.*, 1984) and therefore a local displacement of a filament by a power stroke may not be transmitted effectively to result in movement of the whole filament. Some part of this motion may result in buckling or straightening of the filament. Taking into account this transmission efficiency, equation (4) is rewritten as:

$$v'_{exp} = \eta \times v_o \times \{1-(1-f)^N\}, \quad (5)$$

where η (<1) is the efficiency of transmission. Local movement of a segment of a filament near a stroking head is governed mainly by the activity of that head, and the activity of one stroking head should have little effect beyond adjacent simultaneously stroking heads. It is obvious that the effect of a stroking head is limited by the free filament ends. Therefore, we assume here that η is a function of the average length of filament that each stroking head can exert effects on, which is given by $l/(N \times f+1)$, where l is the length of the filament.

Since $l/(N \times f + 1)$ is a function of density of heads on the surface (ρ), equation (5) can be rewritten as:

$$v'_{exp} = \eta(\rho) \times v_0 \times \{1 - (1-f)^N\}. \quad (5')$$

In principle, f can be estimated by fitting to data demonstrating the relationship between the actual sliding velocity (v) and N, using this equation. Note that $\eta(\rho)$ can be treated as a constant when fitting to sliding movements over surfaces of the same ρ.

(4) Among the parameters required for fitting in order to estimate f, v for individual filaments is readily measurable but N for individual filaments is not directly measurable. The number of heads that are available to bind to a unit length filament, N/l, is clearly a function of ρ, which can be estimated by measuring the K-EDTA–ATPase activity and surface area of the flow cell. However, the relationship between N/l and ρ could take the form of at least two possible models.

The nearest-neighbor model (Harada *et al.*, 1990) assumes that the front end of a sliding filament moves so as to connect nearest-neighbor heads along its path. This model implies that filaments have a large degree of freedom to change direction *via* a rapid lateral Brownian movement of their front ends. With respect to a single head, the average radius of a circle on which is encountered the nearest-neighboring head on a randomly distributed field of heads (r_0) is given by:

$$r_0 = \sqrt{\ln 2/\pi \times \rho}. \quad (6)$$

Therefore, the nearest-neighbor model predicts:

$$N/l = 1/r_0 = \sqrt{\pi \times \rho/\ln 2}. \quad (7)$$

A second model, the band model, assumes that the filaments do not have a large degree of freedom to move laterally so that the front ends move straight forward without lateral deviation due to Brownian movement. Instead, it assumes random swiveling movement of myosin heads around the points where they are fixed to the surface, and takes into account the effective reach of heads. In this case, heads inside a band of a certain width that extends on both sides of a moving actin filament can interact with the filament, but heads outside the band cannot interact with the filament. The band model predicts:

$$N/l = w \times \rho, \quad (8)$$

where w is the width of the band.

(b) *Experimental*

(i) *Effects of methylcellulose on the sliding movement*

In previous *in vitro* motility assay studies, actin filaments failed to move continuously over nitrocellulose surfaces sparsely coated with HMM and dissociated from the surface into the medium (Toyoshima *et al.*, 1990). We reasoned that increasing the viscosity of the ATP solution might help keep filaments close to the surface and thereby allow continuous movement of filaments over surfaces sparsely coated with HMM. Among several high and low molecular weight viscosity enhancers tested, methylcellulose was most effective in preventing dissociation of filaments from a surface sparsely coated with HMM but did not affect the continuous movement of filaments.

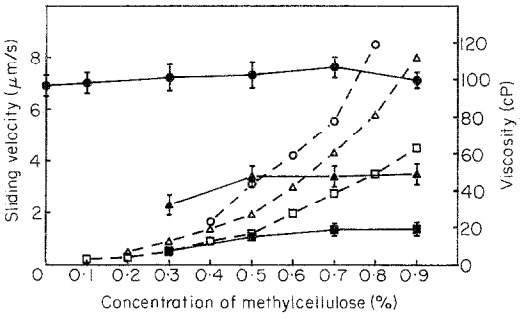

Figure 1. Effect of methylcellulose concentration on sliding velocity and on viscosity of the solution. Sliding velocity of long RhPh/F-actin in 2 mM-ATP in AB/BSA buffer that contained various concentrations of methylcellulose was measured at 30 °C. Flow cells with various densities of HMM on the nitrocellulose surface were prepared by introducing 30 (●), 6·5 (▲) and 2 μg (■) HMM/ml in AB buffer into fresh flow cells, and movement of filaments longer than 6, 7·5 and 10 μm were scored, respectively. Data are shown by average ±S.D. ($n > 20$). Continuous movement was not observed with 0 and 0·1% methylcellulose when flow cells prepared with 6·5 or 2 μg/ml HMM were used. Viscosity of methylcellulose solutions in AB/BSA buffer that contained 2 mM-ATP was measured at 30 °C using a cone/plate viscometer at shear rates of 11·25 (○), 45 (△) and 225/s (□), respectively.

Figure 1 shows the relationship between the concentration of methylcellulose and the sliding velocity at three different HMM densities on the surface at 30 °C. When surfaces that were prepared by applying 6·5 or 2 μg HMM/ml were used, no continuous movement was observed in the absence of methylcellulose, as seen previously (Toyoshima *et al.*, 1990). Addition of 0·1% (w/v) methylcellulose had no effect. However, 0·3% methylcellulose in the ATP solution inhibits dissociation from the surface and permitted continuous movement of relatively long filaments ($\approx > 5$ μm). Under these conditions, filaments stayed close to the surface and moved unidirectionally lengthwise, although the trailing part of the filaments did not exactly trace the path of the leading part.

In the presence of 0·5 to 0·9% methylcellulose, filaments longer than ≈2 μm moved smoothly over surfaces prepared by applying 6·5 or 2 μg HMM/ml. The tail of these filaments traced the path of the front end, as is observed in movement at high head densities (Toyoshima *et al.*, 1987). Concomitantly, the sliding velocity considerably increased compared with that in the presence of 0·3% methylcellulose (Fig. 1). Short filaments on the surface sparsely coated with 2 μg HMM/ml showed a random, lengthwise vibrating motion in the

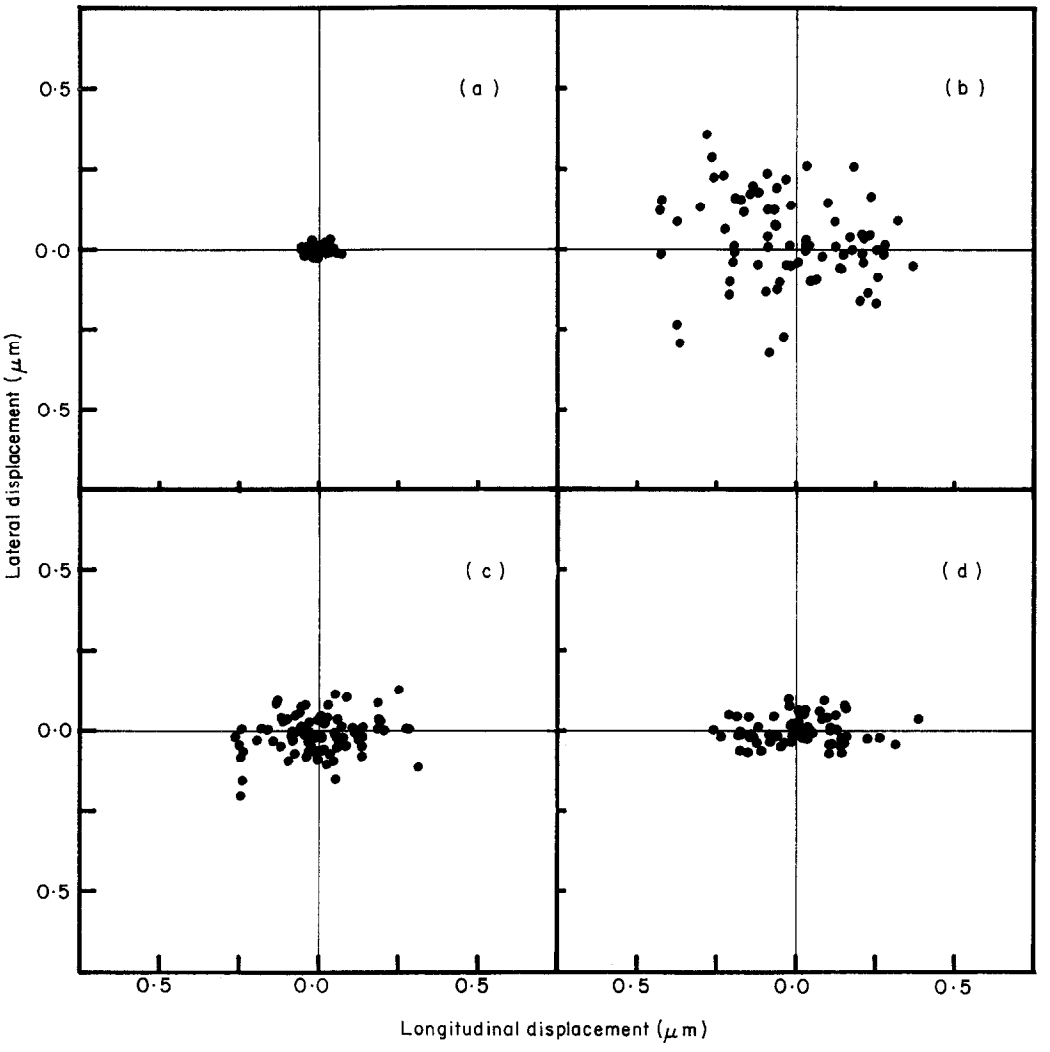

Figure 2. Brownian movement of actin filaments in methylcellulose solution. Fluorescence images of RhPh/F-actin, suspended in AB/BSA buffer that contained: (b) 0·1%, (c) 0·3% and (d) 0·8% methylcellulose, were recorded for 9 s at 30°C. Displacement of the centroids of images were determined every 0·1 s (3-frame interval), and plotted in the 2-dimensional plane. The longitudinal axis of each filament is parallel to the x-axis. To estimate overall noise of the measurement system, filaments were bound by rigor cross-bridges to a nitrocellulose surface coated with HMM, and the centroids of such fixed filaments were analyzed, see (a). The lengths of the filaments were: (a) 4·0, (b) 5·6, (c) 6·6 and (d) 4·9 μm, respectively.

presence of ATP and methylcellulose. The amplitude of the lengthwise vibrating motion was larger for shorter filaments so that very short filaments ($< \approx 2$ μm) on the sparsely coated surface apparently showed only the lengthwise vibrating motion. However, observation over a long time period clearly demonstrated that even those short filaments move unidirectionally at a speed less than 1 μm/s on average. These results suggested that 0·5 to 0·9% methylcellulose effectively kept the filaments close to the surface, so that the probability of interaction between the filament and the heads was increased.

When a nitrocellulose surface to which 30 μg HMM/ml had been applied was used, both long and short ($> \approx 0{\cdot}5$ μm) filaments moved continuously at $6{\cdot}9 \pm 0{\cdot}4$ μm/s in the presence of 2 mM-ATP without methylcellulose. Addition of up to 0·9% methylcellulose to the ATP solution did not inhibit the movement, but rather slightly increased the sliding velocity to 7·2 to $7{\cdot}6(\pm 0{\cdot}4)$ μm/s (Fig. 1). Therefore, 0·8% methylcellulose was added to the ATP solution in all the following *in vitro* motility experiments, and v_0 was taken as $7{\cdot}4(\pm 0{\cdot}4)$ μm/s in the present study.

(ii) *Effect of methylcellulose on Brownian movement of actin filaments in solution*

In order to study the effect of methylcellulose on the movement of actin filaments, we observed the Brownian movement of RhPh/F-actin in AB/BSA buffer, in the presence of increasing concentrations of methylcellulose (Fig. 2). When single filaments were observed in AB/BSA buffer, they showed rapid and random diffusive movement as well as bending motion (Yanagida *et al.*, 1984), so that their image could not be recorded continuously for even one to

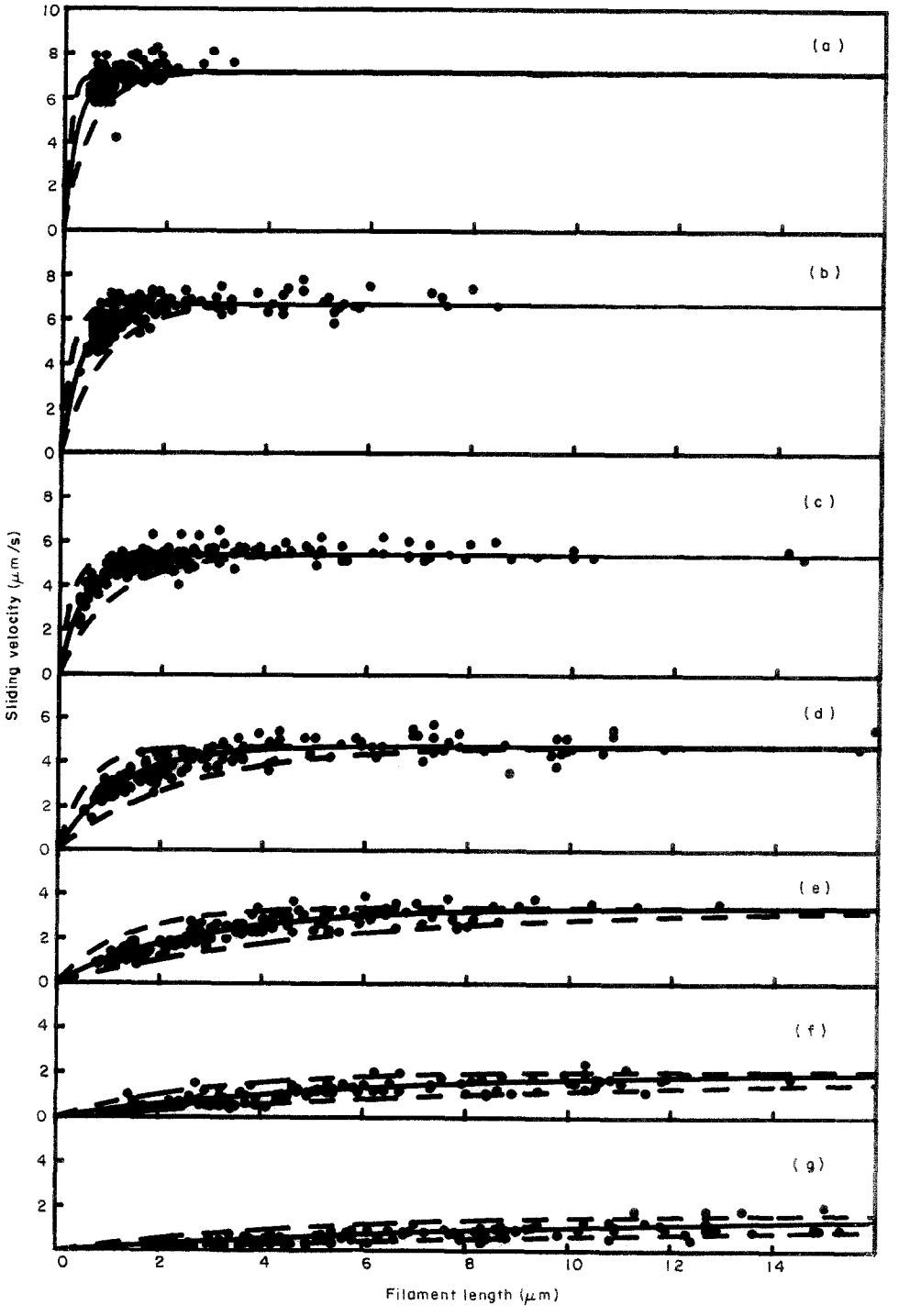

Figure 3. Dependence of sliding velocity on filament length. Flow cells with nitrocellulose surface of various densities of bound HMM were prepared by introducing: (a) 40, (b) 20, (c) 15, (d) 9·8, (e) 4·9, (f) 2·6 and (g) 1·5 μg HMM/ml solutions in AB buffer into fresh flow cells. RhPh/F-actin was allowed to slide over the surface in AB/BSA buffer that contained 2 mM-ATP, 0·8% methylcellulose at 30°C. Each data point represents velocity and length of an individual moving filament. Continuous curves show relationships between velocity and filament length calculated using eqn (5′) with optimum f and $\eta(\rho) \times v_0$ values obtained by fitting, assuming the band model. Two broken lines associated with each continuous curve are the relationships calculated using an f value that is twice the optimum (lower broken line) and half of the optimum (upper broken line). Calculated relationships based on the nearest-neighbor model are virtually superimposable with those based on the band model, and therefore are not shown.

two seconds. When 0·1% methylcellulose was added, filaments only rarely stayed in one focal plane for as long as ten seconds. The centroid of the image of those rare actin filaments underwent a random walk within the plane of focus (Fig. 2(b)). When 0·3 or 0·8% methylcellulose were added, those filaments that stayed in a single focal plane showed a remarkable inhibition of the component of

Brownian movement in the direction perpendicular to the major axis of the filament, while that component parallel to the axis was not significantly damped (Fig. 2(c) and (d)). These observations are consistent with the described characteristics of reptation of fibers through gels or high polymer solutions (de Gennes, 1971; Doi & Edwards, 1978).

(iii) *Relationship between sliding velocity and filament length*

We then determined the relationship between sliding velocity and the length of individual filaments at various HMM densities with the aim of obtaining data for fitting to estimate the value of f (Fig. 3). When a surface densely coated with HMM, which was prepared by incubating with 40 μg HMM/ml, was used, all the filaments longer than ≈ 1 μm moved at a speed close to v_0 (Fig. 3(a)). Filaments longer than ≈ 3 μm were seldom observed after the five minutes of preincubation with ATP, due to the severe fragmentation. It was determined, however, that these longer filaments, initially present during the preincubation period and observed prior to fragmentation, move at a velocity similar to that of shorter filaments (data not shown). Thus, long filaments also move at a speed close to v_0 on a densely coated surface. These observations are consistent with the previous experiments of Toyoshima *et al.* (1990) and Harada *et al.* (1990), which were carried out using no added methylcellulose.

When surfaces moderately coated with HMM; that is, surfaces to which 2·5 to 20 μg HMM/ml had been applied, were used, the sliding velocity was greater for longer filaments, demonstrating a gradual increase to an asymptotic value (Fig. 3(b) to (f)). On the other hand, when a sparsely coated surface, which was prepared with 1·5 μg HMM/ml, was used, sliding velocity was greater for longer filaments, but no apparent saturation was observed up to a filament length of 16 μm (Fig. 3(g)).

To do a meaningful fitting using equation (5′), the velocity–filament length curve must have two phases, the increasing phase and the saturation phase. Therefore, the relationships obtained from surfaces to which 20, 15, 10, 5 and 2·5 μg HMM/ml had been applied were used for the following fitting.

(iv) *Estimation of the number of heads that can interact with a filament*

In a parallel experiment with the above motility assay, the K-EDTA–ATPase activity of flow cells was measured. Assuming that the K-EDTA–ATPase activity of HMM fixed to the surface is the same as that in solution, which is $19(\pm 0{\cdot}2)P_i$/HMM per second at 30 °C, the average density of HMM on the surface was calculated by dividing total K-EDTA–ATPase activity in the flow cell by the surface area and by $19 \pm (0{\cdot}2)P_i$/HMM per second (Table 1).

Recently, Toyoshima *et al.* (1989) formed tracks of oriented HMM heads on the nitrocellulose surface by forming actin–HMM complexes, binding these to the surface and washing the actin filaments away with ATP. They demonstrated that these oriented HMM heads can support sliding movement of actin filaments in either direction along the track which suggests that HMM heads can swivel 180° about the points at which they are fixed to the surface. Therefore, we assumed that all heads that come into contact with an actin filament can interact productively with that filament without regard to the orientations of the HMM head or actin filament. The number of heads that can interact with a unit length filament based on the nearest-neighbor model (eqn (7)) and the band model (eqn (8)) using the above values of HMM densities (Table 1) was calculated.

Table 1

Estimate of the number of HMM molecules that are available to interact with a unit length actin filament

Concentration of HMM (μg/ml)	Density of HMM ($1/\mu m^2$)	Number of HMM per 1μm filament	
		Nearest-neighbor model ($1/\mu m$)	Band model ($1/\mu m$)
40	2400	220	72
20	870	140	27
15	540	100	16
9·8	270	72	8·0
4·9	130	50	4·0
2·6	64	36	1·9
1·5	40	29	1·2

Densities of HMM on the nitrocellulose surfaces, to which various concentrations of HMM in AB buffer had been applied as indicated in the left-hand column, were estimated from K-EDTA–ATPase activities of the flow cells. The number of HMM molecules that are available to interact with a unit length filament was calculated on the basis of the two models described in the text, using these estimated densities of HMM on the surfaces.

The band model involves a new parameter to be determined, w, the width of a band in which heads that can interact with a moving actin filament are fixed to the nitrocellulose surface. Negative staining of actin–HMM complexes bound to nitrocellulose surfaces (Toyoshima *et al.*, 1989) showed that heads interacting with an actin filament are fixed to the surface approximately 15 nm or less from the center of the filament (Y. Toyoshima, personal communication). This provides a reasonable estimate of 30 nm for the width, w, which we have used in the calculations shown in Table 1.

The number of heads that are available to interact with a filament, N, is given by the product of the measured length of the filament, l, and the calculated number of heads that are available to interact with a unit length filament at that density, N/l.

(v) *Calculation of* f

Knowing N allows prediction of the sliding velocity of individual filaments using equation (5′) for a

Table 2
Best fit value of f *and* $\eta(\rho) \times v_0$ *at various densities of HMM*

Concentration of HMM (μg/ml)	$\eta(\rho) \times v_0$ (μm/s)	f	
		Nearest-neighbor model	Band model
20	6·7	0·0080	0·043
15	5·6	0·012	0·059
9·8	4·7	0·0057	0·053
4·9	3·4	0·0033	0·045
2·6	2·1	0·0022	0·048
Average value of $f \pm$ S.D.		0·0060 ± 0·0038	0·050 ± 0·006

The best fit values of f and $\eta(\rho) \times v_0$ are calculated using eqn (5') and the numbers of HMM molecules that are available to interact with a unit length filament (as estimated in Table 1 and based on the two models).

S.D., standard deviation.

given set of f and $\eta(\rho) \times v_0$ values. For each set of f and $\eta(\rho) \times v_0$ values, the square of the difference of the actual measured sliding velocity and the predicted sliding velocity was summed for more than 130 filaments of various lengths shown in Figure 3, for each HMM density. Then the optimum values of f and $\eta(\rho) \times v_0$ were determined to minimize the sum of the square of the differences for each HMM density (Table 2). The predicted relationship for each HMM density between sliding velocity and filament length using the optimum f and $\eta(\rho) \times v_0$ values are shown in Figure 3. The relationship predicted by twice the optimum f and that predicted by half of the optimum f value are shown by broken lines.

The optimum values of f calculated using the band model for five intermediate HMM densities were very consistent with each other, yielding $f = 0{\cdot}050 \pm 0{\cdot}006$. On the other hand, the optimum values of f for these HMM densities calculated using the nearest-neighbor model were not consistent with each other, ranging from 0·0022 to 0·012 with an average of $0{\cdot}0060 \pm 0{\cdot}0038$.

(vi) *Rate of actin-activated Mg-ATPase of HMM fixed to the surface*

For the measurement of t_c of actin-activated Mg-ATPase of HMM fixed to the nitrocellulose surface in the flow cell, all the HMM heads must be saturated with actin filaments during the incubation with ATP. To examine whether such a saturation can be achieved in our *in vitro* motility system, flow cells coated with HMM were preincubated with different concentrations of actin filaments and then incubated with ATP. It was found that preincubation with 0·5 mg/ml or higher concentrations of actin filaments for four minutes saturates the subsequent Mg-ATPase reaction (Fig. 4). The average actin-activated Mg-ATPase of flow cells saturated with actin filaments was $1{\cdot}38(\pm 0{\cdot}09)$ times higher than the K-EDTA–ATPase of identical flow cells (Fig. 4). The finding that the initial rate of Mg-ATPase was faster than the average calculated

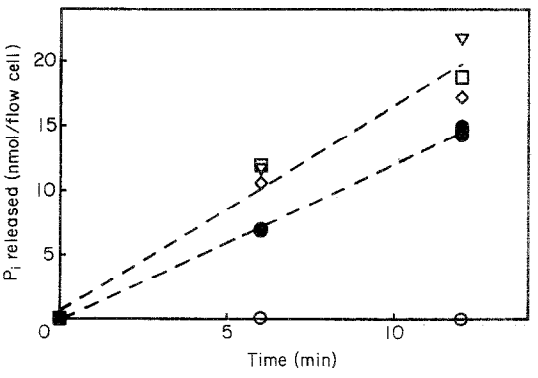

Figure 4. HMM ATPase in the flow cell. Flow cells, to which 40 μg HMM/ml in AB buffer had been applied, were preincubated with 0 (○), 0·5 (◇), 0·625 (□), and 1 mg (▽) F-actin/ml in AB/BSA buffer for 4 min. They were then incubated with 2 mM-ATP in AB/BSA buffer containing 0·3% methylcellulose for 6 or 12 min, and the amount of released phosphate in this actin-activated Mg-ATPase was measured by the Malachite green assay. In a parallel experiment, the K-EDTA–ATPase activity was measured in triplicate (●) in identical flow cells in a buffer that contained 2 mM-ATP, 0·5 M-KCl, 4 mM-EDTA. The background phosphate level of the ATP solutions has been subtracted from all data. The 2 broken lines represent regression lines of the K-EDTA–ATPase and the actin-activated Mg-ATPase of flow cells that had been preincubated with F-actin, respectively. The filled square at the origin represents the superimposition of all the different symbols.

over the full 12 minutes (Fig. 4) suggests that actin filaments gradually dissociated from the surface during the incubation with ATP and that this value can be an underestimate of the Mg-ATPase. If the Mg-ATPase is calculated from the first six minutes of the incubation with ATP, a value of 1·69 times higher than the K-EDTA–ATPase activity is obtained.

In solution, the rate of HMM K-EDTA–ATPase was $19(\pm 0{\cdot}2)P_i$/HMM per second at 30 °C under our experimental conditions. Thus, assuming that the rate of the K-EDTA–ATPase of HMM in solution and that of HMM fixed to the nitrocellulose surface are the same, the lower limit of V_{max} of the actin-activated Mg-ATPase is $19(\pm 0{\cdot}2) \times 1{\cdot}38(\pm 0{\cdot}09) = 26(\pm 2)$ P_i/HMM per second or $13(\pm 1)P_i$/head per second. This yields an upper limit of t_c for the actin-activated Mg-ATPase equal to $77(\pm 5)$ milliseconds.

4. Discussion

(a) *Slow sliding movement over low densities of HMM in the presence of methylcellulose*

We have succeeded in observing slow *in vitro* movement of actin filaments on a nitrocellulose surface sparsely coated with HMM by including 0·8% methylcellulose in the ATP solution. Calculations based on either the nearest-neighbor model or the band model show that shorter filaments on such

sparsely coated surfaces can interact with a relatively small number of heads (Table 1). The limitation of the number of heads that can interact with a filament increases the fraction of time when no heads are interacting with the filament, and results in slower sliding velocity (Fig. 3), as equation (5′) predicts.

Actin filaments suspended in 0·8% methylcellulose solution in AB/BSA buffer undergo a characteristic one-dimensional Brownian motion without a net displacement along the filament axis over long time periods (Fig. 2(d)). On a surface sparsely coated with HMM, filaments shorter than ≈ 2 μm showed these vigorous lengthwise vibrating motions, yet still achieved net displacement, albeit very slowly. Presumably the fraction of time when no heads are interacting with these short filaments is so large at very low head density that lengthwise Brownian movement becomes significant while lateral Brownian movement is suppressed by methylcellulose. The filaments, however, do move slowly unidirectionally propelled by infrequent interaction with the heads. This observation strongly supports our conclusion that we have established a condition under which actin filaments move slowly due to interaction with a limited number of heads.

One criticism of this work is that the slow movement described here might be partly attributable to a drag force due to the high viscosity of methylcellulose. However, this possibility is unlikely. At any of the head densities examined, no significant decrease of sliding velocity was observed when the methylcellulose concentration was increased even though the apparent viscosity of the solutions greatly increased (Fig. 1). At high head densities, the finding that a maximum sliding velocity exists with no load suggests that this speed is determined by characteristics of the power stroke itself. Therefore, the constant sliding velocity close to v_0 at high head density over a wide range of methylcellulose concentrations suggests that the local viscosity around individual stroking heads is not large enough to slow down the power stroke. At lower head densities, each filament is propelled by a very small number of heads, as discussed above. Thus, the constancy of sliding velocity between 0·5 and 0·9% methylcellulose at lower head densities suggests that the drag force due to interaction between methylcellulose and actin filaments is negligible, especially when compared with the force produced by individual power strokes. This is consistent with the finding that the amplitude of longitudinal Brownian movement of actin filaments in methylcellulose solution did not significantly decrease when the methylcellulose concentration was increased from 0·1% to 0·8% (Fig. 2). The effectiveness of the methylcellulose is probably not related to a viscosity effect *per se*, but may result from a change in macromolecular structure of the solvent whereby the filamentous methylcellulose radically inhibits diffusion of actin filaments in directions perpendicular to their long axis while not retarding the motion of filaments along that axis through volume excluded by methylcellulose.

On the contrary, it is possible that the observed slow movement at low head density is slower than it ideally should be, because the methylcellulose solution may not be sufficient completely to overcome the lateral, Brownian motion of actin filaments, so that the filaments may not stay close to the surface all the time and may not interact with the heads at the maximum rate. However, the fact that the sliding velocities in the presence of 0·5 to 0·9% methylcellulose are the same even though the apparent viscosities of the solutions greatly differ (Fig. 1) suggests that these solutions are sufficient to keep filaments in maximum interaction with the heads.

In conclusion, the addition of 0·5 to 0·9% methylcellulose in the ATP solution does not invalidate the premises introduced in part (2) of Results, section (a). Yet, it is sufficient to keep filaments in maximum contact with the heads on the sparsely coated surfaces and thereby allow heads to cycle at their maximum rate, which is a basic requirement introduced in part (1) of Results, section (a).

(b) *Estimation of* f

It was recently suggested that the head–rod junction of myosin in muscle is very flexible, allowing swiveling of at least 180° (Reedy *et al.*, 1989). Consistent with this observation, Toyoshima *et al.* (1989) have demonstrated that HMM heads fixed initially in one direction on the nitrocellulose surface can support sliding movement of an actin filament not only in one direction, but also in the opposite direction, depending on the polarity of the actin filament. These results suggest that we do not need to consider the orientation of heads on the surface and can postulate that all the heads that come into physical contact with a filament undergo an effective power stroke. We thus calculated the number of heads that can interact with a unit-length filament on the basis of the nearest-neighbor model and the band model, taking into account all the active heads on the surface, the density of which is estimated assuming that the K-EDTA–ATPase activity of immobilized HMM and that of HMM in solution are the same (Table 1).

We estimated the value of f by fitting the dependence of sliding velocity on the filament length to equation (5′) under the condition with a limited number of heads. This yielded f as $0{\cdot}0060 \pm 0{\cdot}0038$, based on the nearest-neighbor model, or $0{\cdot}050 \pm 0{\cdot}006$, based on the band model (Table 2).

In the latter model, we postulated that the width of the band, w, is 30 nm. However, this value can be an underestimate because it is based on observations using preformed actin–HMM complexes (Toyoshima *et al.*, 1989) and we cannot exclude the possibility that heads outside a 30 nm band can interact with the filament. In fact, the extent of activation of Mg-ATPase in a flow cell coated with HMM by a known total length of actin filaments is

consistent with a band of 50 to 70 nm wide (Y. Toyoshima, unpublished results). If 30 nm is an underestimate of w, then f would be smaller than that shown in Table 2. Thus, the 30 nm wide band model may result in an overestimate of f and consequently of d, the step size.

The dependence of velocity on filament length predicted from equation (5′) using the optimum values of $\eta(\rho)\times v_0$ and f fits well with the velocity data over a wide range of filament lengths and HMM densities. This suggests that the assumptions we made to develop equation (5′), especially that η is a function of ρ (part (3) of Results, section (a)), are reasonable and valid. We conclude that the sliding velocity–filament length curves can be approximated by equation (5′).

(c) *The nearest-neighbor model* versus *the band model*

The two models result in significantly different values of f. The band model assumes that the actin filaments do not have a large degree of freedom to move laterally so that the front ends move only straight forward, while the nearest-neighbor model assumes that the filaments have such a large degree of freedom to move laterally that the front ends can rapidly diffuse around to find the next nearest-neighbor head. The nearest-neighbor model predicts that the filaments would show sharp bends along their length during the sliding movement as a result of finding and interacting with the nearest-neighbor heads. In reality, however, moving filaments always showed smooth curves, which suggests only a small degree of freedom of filaments to move laterally. This observation supports the band model. Under the conditions used here, the band model is reasonable because the lateral movement of the filament ends must be greatly suppressed by methylcellulose. In accordance with this speculation, the band model gives relatively consistent estimates of f for different HMM densities, while the nearest-neighbor model gives diverse, inconsistent estimates (Table 2).

It is also possible to decide which model is more plausible from the relationship between sliding velocity and head density. When $N\times f\ll 1$,

$$\begin{aligned} v'_{\mathrm{exp}} &= \eta(\rho)\times v_0\times\{1-(1-f)^N\},\\ &= \eta(\rho)\times v_0\\ &\quad\times\left\{1-\left(1-N\times f+\frac{N\times(N-1)\times f^2}{2\times 1}\right.\right.\\ &\quad\left.\left.-\frac{N\times(N-1)\times(N-2)\times f^3}{3\times 2\times 1}+\cdots\right)\right\},\\ &\simeq \eta(\rho)\times v_0\times f\times N. \end{aligned} \tag{9}$$

If the nearest-neighbor model is true, by substituting

$$N = l\times\sqrt{\pi\times\rho/\ln 2}$$

(eqn (7)) in equation (9):

$$\begin{aligned} &v'_{\mathrm{exp}}\simeq\eta(\rho)\times v_0\times f\times l\times\sqrt{\pi\times\rho/\ln 2},\\ \therefore\quad &\ln v'_{\mathrm{exp}}-\ln(\eta(\rho)\times v_0)\\ &\qquad\simeq\ln(f\times l\times\sqrt{\pi/\ln 2})+0{\cdot}5\times\ln\rho. \end{aligned} \tag{10}$$

On the other hand, if the band model is true, by substituting $N = l\times w\times\rho$ (eqn (8)) in equation (9):

$$\begin{aligned} &v'_{\mathrm{exp}}\simeq\eta(\rho)\times v_0\times f\times l\times w\times\rho,\\ \therefore\quad &\ln v'_{\mathrm{exp}}-\ln(\eta(\rho)\times v_0)\simeq\ln(f\times l\times w)+\ln\rho. \end{aligned} \tag{11}$$

Therefore, when $N\times f\ll 1$, slopes of curves relating $\ln v-\ln(\eta(\rho)\times v_0)$ to $\ln\rho$ for filaments of arbitrary lengths would suggest which of the two models applies under our conditions.

The curves were constructed from data presented in Figure 3 (see Fig. 5), and initial slopes, to satisfy $N\times f\ll 1$, were calculated. The slopes were 0·91, 1·03, 0·89 and 0·47 for filament lengths of $1(\pm 0{\cdot}2)$, $2(\pm 0{\cdot}4)$, $4(\pm 0{\cdot}8)$ and $8(\pm 1)$ μm, respectively. All of the initial slopes except that for $8(\pm 1)$ μm filaments, which may be too long to satisfy $N\times f\ll 1$ even at the lowest HMM density examined, were close to unity. This result is more compatible with the band model than the nearest-neighbor model.

The nearest-neighbor model clearly requires refinement because it considers the heads as fixed points on the surface and ignores the reach of heads. Even if we take into account the reach of the heads, however, the improbability of the nearest-neighbor model remains essentially the same. Therefore, we use the band model with w equal to 30 nm and conclude that the upper limit of f is $0{\cdot}050\pm 0{\cdot}006$.

(d) *Estimation of step size*

Assuming that the K-EDTA–ATPase activity of HMM in solution is the same as that of HMM fixed to the nitrocellulose surface and that the actin-activated Mg-ATPase is $1{\cdot}38(\pm 0{\cdot}09)$ times higher than the K-EDTA–ATPase, the upper limit of t_c was estimated to be $77(\pm 5)$ ms/ATP. Therefore, by substituting t_c and f in equation (2), we can calculate the upper limit of d:

$$\begin{aligned} d &= v_0\times f\times t_c,\\ &= (7{\cdot}4(\pm 0{\cdot}4)\ \mu\mathrm{m/s})\times(0{\cdot}050\pm 0{\cdot}006)\\ &\quad\times(77(\pm 5)\ \mathrm{ms/ATP}),\\ &= 28(\pm 4)\ \mathrm{nm/ATP}. \end{aligned}$$

Hayashi *et al.* (1989) recently reported that the specific activity of K-EDTA–ATPase of HMM fixed to a nitrocellulose surface is approximately half of that in solution. This result may suggest that half of the HMM heads fixed to the surface are completely inactive, probably due to the wrong orientation of binding, while the rest of the heads are fully active. If this is the case, the whole argument described above is still valid because heads inactive in terms of K-EDTA–ATPase activity should also be inactive in terms of actin-activated Mg-ATPase so that we can simply ignore those inactive heads. Alternatively, it is possible that all the heads fixed

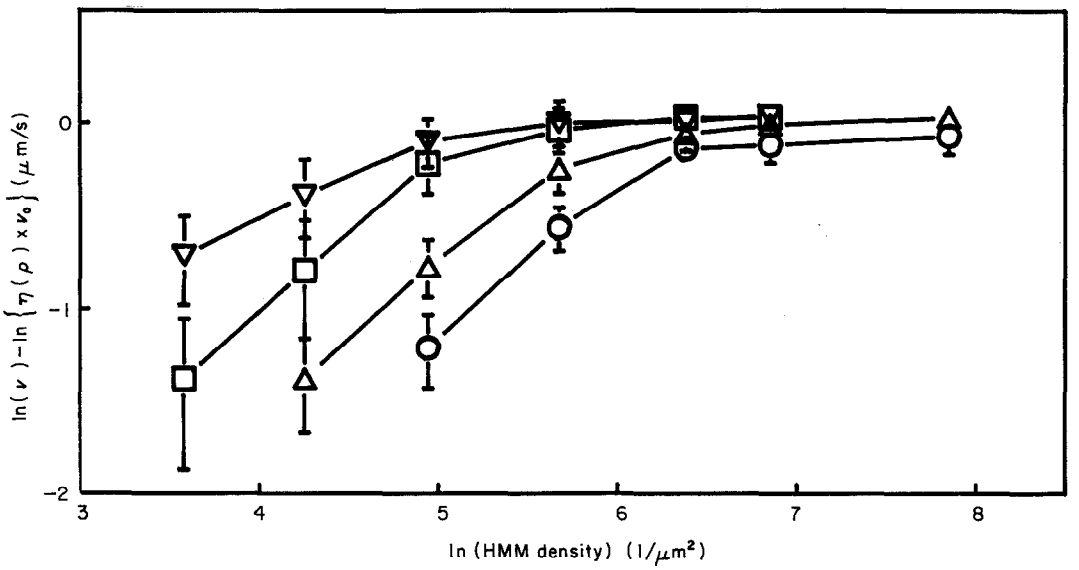

Figure 5. Relationship between sliding velocity and density of HMM on the surface. The average sliding velocity of filaments of length 1(±0·2) (○), 2(±0·4) (△), 4(±0·8) (□) and 8(±1) μm (▽) was calculated for each density from data presented in Fig. 3.

to the surface are equally only half active. If this is the case, some revision of the argument is necessary. In this case, the values for ρ, which were estimated from the K-EDTA–ATPase activity of the flow cell, would become double the present values. Assuming the band model, this would make N double. Fitting using this doubled N makes f roughly half (not shown; but note that eqn (5′) can be approximated as:

$$v'_{\text{exp}} = \eta(\rho) \times v_0 \times f \times N$$

when $N \times f \ll 1$ (eqn 9)). On the other hand, if all heads in the flow cell are only half active, t_c would be double the present value. Therefore, even if all the HMM heads fixed to the surface are only half active, d, which is a product of f and t_c, is not affected. Our result is therefore valid, independently of the assumption that the K-EDTA–ATPase activity of HMM in solution is the same as that of HMM fixed to the nitrocellulose surface.

(e) *Comparison with previous estimates*

Other approaches have been used to estimate the unit step of the myosin head. These studies have yielded results that fall into two classes. The results of the first class, those with relatively short steps close to 10 nm (Huxley & Simmons, 1971; Toyoshima *et al.*, 1990) or 20 nm (Taylor, 1989), are somewhat smaller than the upper limit for the step size reported here. If the step size is derived from the alternate values discussed above of 1·69 rather than 1·38 times higher than the K-EDTA–ATPase for the actin-activated Mg-ATPase and of 60 rather than 30 nm for the width of the band, then d would equal 11 nm per ATP hydrolyzed. Thus, the data presented here are consistent with a shorter step size, probably larger than 10 nm but smaller than 30 nm.

The recent estimates of the step size reported by Yanagida and colleagues (Yanagida *et al.*, 1985; Yanagida, 1989; Harada *et al.*, 1990) are larger than 50 nm per ATP hydrolyzed and fall into a second class: those step sizes greater than twice the chord length of the myosin head. However, this study fails to confirm such large values for the step size. There are some obvious differences in experimental conditions: Yanagida's group used freshly prepared, intact myosin fixed to siliconized glass surfaces and observations were made at pH 7·8 (Yanagida, 1989; Harada *et al.*, 1990), whereas we used HMM prepared from myosin stored with glycerol at −20 °C and fixed to nitrocellulose surfaces and observations were made at pH 7·4 (Toyoshima *et al.*, 1990; the present study). Another major difference is the density of heads on the surfaces. Kinetic arguments (Toyoshima *et al.*, 1990) suggest that the range of step sizes that can be measured is limited to less than the average spacing of heads. While we have used surfaces coated with sparse or moderate densities of heads (Toyoshima *et al.*, 1990; the present study), Yanagida's group has used very densely coated surfaces so that the average nearest-neighbor distance of heads is 11 nm (Harada *et al.*, 1990), perhaps invalidating their measurements. However, we do not know whether these differences in experimental conditions can really explain the large discrepancy.

(f) *Comparison with kinesin*

Using a similar *in vitro* motility assay system, Howard *et al.* (1989) recently reported movement of microtubules with a limited number of kinesin molecules on the surface. Their results are quite distinct from ours using HMM in that a single, two-headed kinesin can move microtubules at a speed close to

the maximum, without the aid of any viscous material. This discrepancy can be explained by a difference in the value of f for the two motor molecules. Probably, unlike HMM heads, the value of f for kinesin heads is close to unity, so that at least one of the two kinesin heads stay associated with microtubules during most of the cycle time of ATP hydrolysis.

(g) *Conclusion*

We have used a novel approach to determine d, the unit displacement of the actin filament associated with the hydrolysis of a single ATP by an HMM head. We have calculated a value for the upper limit of the step size consistent with our data, $28(\pm 4)$ nm, and our data do not exclude a step size of the order of 10 nm. Even our upper limit is well within the range of geometric constraint for conformational change imposed by the size of the myosin head, and therefore is not inconsistent with the swinging cross-bridge model tightly coupled with ATP hydrolysis.

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