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Critical Review of the Swinging Crossbridge Theory and of the Cardinal Active Role of Water in Muscle Contraction

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ABSTRACT: A critical analysis is presented of the experimental findings that led to the sliding filament model and to its offspring — the swinging (by rotating or tilting) crossbridge theory of muscle contraction (SCBT). Several principles that have been taken for granted implicitly and explicitly by the creators of these dogmas are discussed. The failure of numerous efforts to verify predictions of the SCBT, particularly the idea that the myosin molecules undergo a major conformational change, is critically reviewed. Analysis of various experimental data suggests that water may play an active role in muscular contraction. Examination of both the experiments that do not fulfill the expectations of the SCBT and the measurements of water liberation during the "contractile" process suggests a new outlook according to which tension development and movement are not due to major conformational changes but rather to restructuring of the hydration shells of actin and myosin.

KEY WORDS: hydration forces, muscle contraction, muscle regulation, motility, water.

Abbreviations and Symbols: c.b., crossbridge (c.b.s-crossbridges); **G-actin,** monomeric actin; **F-actin,** filamentous actin; **HMM,** Heavy meromyosin; **m.t.,** microtubule; **n,** number of water molecules liberated per myosin head during the isomerization reaction of acto-S-1; $\mathbf{P_o}$, isometric tension; **S-1,** heavy meromyosin subfragment-1; **S-2,** heavy meromyosin subfragment-2; **SCBT,** swinging crossbridge theory; **s.d.,** sliding (or step) distance; **-SH1, -SH2,** sulfhydryl groups on the myosin head; **V,** velocity of shortening (V_m-maximal velocity); ΔH°, molar enthalpy change; ΔS°, molar entropy change; ΔV°, molar volume change; ΔL_1, thermal step distance as defined by H. E. Huxley; ΔL_2, mechanical step distance as defined by A. F. Huxley et al.; ΔL_3, chemical step distance obtained mainly from *in vitro* motility assays and defined as V/υ; ΔL_4, chemical step distance defined as $V/x\upsilon$ (x-number of active heads interacting with an actin filament); β, mechanical impulse of a myosin head; υ, number of ATP molecules split by a myosin head per second; φ, momentary value of the force generated by the combined action of a myosin head and its associated thin filament; $\bar{\varphi}$, average value of the force generated per head.

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I had never any doubt that muscle contraction is essentially a play of water, the building and destruction of water structures built up by the contractile proteins

Albert Szent-Guyorgyi, 1972

I. CRITICAL REVIEW OF THE SLIDING FILAMENT THEORY

For some time, especially during the 1940s, it was widely believed that muscle shortening was the outcome of the coiling of a continuous actomyosin complex extending from one Z-band (the end a sarcomere, that is the repeating unit in striated muscles) to the other. However, careful examination showed that myosin is confined to the A-band that is located at the center of the sarcomere (Hasselbach and Schneider, 1951). H. E. and A. F. Huxley and their colleagues demonstrated that myosin exists as a set of parallel filaments, whereas two sets of parallel actin filaments extend from the two Z-bands toward the center of the sarcomere, part of which overlaps the A-band (the other part making the I-bands). The lengths of the filaments did not then appear to change in active muscle, and it was concluded that shortening is the outcome of the sliding of the actin past the myosin filaments (Huxley and Niederke, 1954; Huxley and Hanson, 1954). It was noticed (Huxley, 1953) that projections on the surface of the myosin ("thick") filaments cross-link them to the "thin", actin-containing filaments in muscles in the rigor state (i.e., in the absence of ATP). Biochemical studies have shown earlier (cf. Szent-Gyorgyi, 1953) that the myosin molecule can be split by proteolytic enzymes into a fragment that is insoluble under physiological conditions, thus aggregating to form the core of the thick filament, and a soluble fragment (heavy meromyosin, HMM) that can bind both ATP and actin. Therefore, it was concluded that the chemically active portion of the HMM fragment is part of the cross-links ("crossbridges", c.b.s). HMM, which later was shown (Slayter and Lowey, 1967) to contain two globular "heads" (subfragment-1, S-1), each binding one ATP molecule and capable of forming a complex with actin in the absence of ATP. The c.b.s were *believed* to bind firmly to the actin filaments *also* in active muscle because it has been taken for granted that the rigor state must be the last stage in the ATP hydrolysis cycle in active muscles. It was proposed that the binding of the S-1's or, for example, S-1-ADP, is followed by some conformational change in the c.b.s that will cause the translocation of the actin filaments. The *continuous* protein network in an active muscle, composed of the actin and myosin filaments crosslinked by the c.b.s, has been viewed as the hydrocarbon network in a rubber band (in which the macromolecular chains are also chemically crosslinked) that had been stretched and then released, that is, the force responsible for shortening has been considered to be associated with the stretching of an "elastic element" (Huxley and Simmons, 1971).

In order to account for the observation made by Ramsey and Street (1940) that the isometric force developed by a tetanically stimulated muscle decreases (linearly, as shown by Gordon et al., 1966) with length when stretched beyond rest length, it has been concluded that the "force generators" (i.e., the c.b.s that lie at equal intervals along a thick filament) are independent of each other. Surprisingly enough, the idea of "independent force generators" was proclaimed to be a basic *postulate* of the theory rather than a *conclusion* from the experimental finding. Actually, to say the least, Huxley's idea of "force generators" operating cyclically did not add much to Albert Szent-Gyorgyi's (1964) and *any* enzymologist's conclusion that tension generation *must* be

due to a *cyclic* interaction of the enzymatically active part of myosin with actin in intimate conjunction with the ATP hydrolysis cycle. After all, *all* enzymes act cyclically and there is no reason for the enzymic breakdown of ATP by actomyosin to be different. Unfortunately, to be frank, we still do not know much more than this about the mechanism of muscular contraction. Because each c.b. contains several myosin molecules and as myosin is double-headed, it is not yet clear whether all the heads in a c.b. are equal in their performance that is, whether they *also* are independent (of each other in the same myosin molecule and of those of neighboring myosin molecules) force generators. After all, it is the *heads* that initiate the generation of tension, even after cutting them from the rest of the myosin molecule, that is, as *free* HMM or S-1 (Oplatka et al., 1974; Borejdo and Oplatka, 1976; Tirosh et al., 1990). The rest of the myosin head, particularly the S-2 subfragment that connects S-1 to the myosin filament shaft, cannot *by itself,* that is, in the absence of S-1, give rise to the development of force; it can only modify (and even amplify) the force associated with the interaction of S-1 with actin (Oplatka, 1991c). We do not know yet if the heads in a given c.b. act synchronously or cooperatively and whether in active muscle the two heads of a given myosin molecule interact with the same actin filament or also with two neighboring ones (Borejdo and Oplatka, 1981).

Because the force per c.b. appears to be the same for different degrees of overlap (in a stretched muscle), when the spacing between the filaments must vary, it was concluded that the "independent force generators" are also independent of spacing. Therefore, it was quite surprising to observe that if the spacing is decreased by increasing the osmotic pressure of the medium surrounding a muscle fiber (e.g., by the addition of a polymeric substance), rather than by stretching the muscle, the isometric tension changed and could initially increase and then decrease after increasing the polymer concentration (cf. Ford et al., 1991). Furthermore, the force-length relationship changed when the osmotic pressure of the medium was either lowered or increased relative to the "physiological" one so that the maximal force was not observed at the point of maximal overlap but rather at a longer or at a shorter length, respectively (Bagni et al., 1990). Unfortunately, no attention has been paid to the fact that the decrease in spacing following the addition of, for example, a polymer, was the *outcome* of the lowering of the chemical potential (or thermodynamic activity) of the *water* component, which does not occur when the spacing is decreased by stretching the muscle under physiological conditions. Thus, it appears that the different force generators can be affected differently by a change in the properties of water to such an extent as to obscure the proportionality between tension and the number of operating crossbridges. It then makes sense to believe that their performance might depend in some way on water in their close vicinity (i.e., structure and capability to change in conjunction with the force-developing event) *also* in the absence of a polymer (or added sucrose) that is, in *living* muscle, at both the desending and the ascending part of the force-length relationship and near the point of no overlap (Oplatka et al., 1974). Tension can also be altered by the addition of an organic solvent (Endo et al., 1979) or by changing the ionic strength (Piazzesi et al., 1994). All these should cause a diminution of water activity and, *in addition*, to affect the electrostatic and hydration forces between actin and myosin. Needless to say that on first sight these observations merely suggest that water plays a *modulatory* role. In the following we shall see that several experimental results reflect on an indispensable *active* role of water.

The sliding filament model, as well as the SCBT to which it led, have taken it *for granted*, like many other theories of muscle contraction, that the sole actors are the backbones of the proteins — actually one "protein" only, that is, the c.b.s. Actin has been considered for a long time to play only a secondary, passive, role — that of a "rope" for the myosin to pull.

The words "the structural basis of muscular contraction" have been used quite frequently for many years. They referred, particularly, to the length–"tension" relationship, that is actually a length-*force* relationship because the force measured at each length was not divided by the cross-sectional area at *that* length but rather at rest length. This gave a clear answer to the question of what lies behind the stratification, of striated muscles into constant-width A-bands and varying-width I-bands. However, surprisingly enough, smooth muscles, which are named so because they do not exhibit any stratification *also* give a bell-shaped length-force curve, which is very similar to that of striated muscles (cf. Gordon and Siegman, 1971). If the basis for muscular contraction is "structural" then, for consistency reasons, smooth muscles should not be "entitled" to behave like striated muscles with respect to the force-length relationship that the theory was so proud to "explain". Nevertheless, this problem has simply been "swept under the carpet", the reason probably being that it could cause trouble by hereticts and iconoclasts. People believed that someday we shall "uncover" (with proper sophisticated omnipotent equipment) a hidden striated muscle-like structure. Thus, it seems that the structure formed by the assembly of filaments is important, but it is *not* the only governing factor. Furthermore, the fact that HMM and S-1 (Tirosh et al., 1990; Borejdo and Oplatka, 1976; Oplatka et al., 1974), as well as the single-headed, tail-less, myosin-I present in various organisms (cf. Pollard et al., 1993), that cannot form filaments and are still active mechanochemically, is another challenge to the sliding filament theory because in these systems there exists only one kind of filament, namely, actin and therefore there is no room for the *sliding* past each other of *two* sets of filaments. Moreover, the absence of tails does not enable the formation of a continuous three-dimensional protein *network*. Thus, the latter is *not* essential for the mechanochemical reactivity of actomyosin systems. The value of a theory is judged by its generality and a scientist who respects himself (not to say others) should feel uneasy if a beautiful and elegant theory can be applied only to *one* particular type of actomyosin system (e.g., the frog sartorius muscle), even if this looks nicer then the large variety of unstriated actomyosin engines in smooth muscles and in non-muscle cells where they fulfill many different tasks that in *all probability* are performed on the basis of the *same* molecular mechanochemical transduction mechanism.

II. THE SWINGING CROSSBRIDGE DOGMA: ITS AXIOMS AND PREDICTIONS

To my mind, this theory originated from the sliding filament theory on the basis of miscalculation, two observations and one speculation.

A. The Miscalculation

The term "step (or sliding) distance" (s.d., ΔL) was coined by Huxley (1960) to denote the distance covered by the actin, moving past the myosin, filaments when one ATP molecule was split at *each* c.b. Alhough it was not mentioned, the muscle in mind must have been an unloaded muscle because the value of the step distance must somehow depend on the load because it *should* be zero for an isometrically con-

tracting muscle when no movement occurs despite of the expenditure of ATP. The value of the s.d. was calculated by Huxley as follows: from X-ray and electron microscopic observations, the number of c.b.s for one half sarcomere ($\approx 1.1 \times 10^{-4}$ cm long at rest length) with a cross-sectional area of 1 cm^2 was calculated and found to be 6.5×10^{12}. If this segment was generating a maximal isometric tension of $P_o = 1.5$ kg/cm^2 and had a heat of shortening coefficient, α, of 350 g/cm per centimeter, then the *extra* energy (i.e., that on top of the heat produced by the isometrically contracting muscle) released after shortening by ΔL is $P_o\Delta L + \alpha\Delta L = (P_o + \alpha)\,\Delta L = 1.181 \times 10^6\,\Delta L$ ergs. Dividing this by the number of c.b.s, we get $2.8 \times 10^{-7}\,\Delta L$ ergs per c.b. If the enthalpy liberated by the splitting of one mole of ATP is 10 kcalories, then the energy per ATP molecule is 2.8×10^{-13} erg. Hence, $\Delta L = 10$ nm. This calculation is erroneous for several reasons.

1. The energy per ATP (obtained by the division of 10 kcals by Avogadro's number) is *not* $2.8.10^{-13}$ but 6.7×10^{-13}. Hence either the s.d. is equal to 10(6.7/2.8) = 24 nm or only about 40% of the c.b.s split ATP. Taking into consideration that each c.b. contains several double-headed myosin molecules (cf. Lovell et al., 1981) then the fraction of heads that split ATP to give rise to a s.d. whose value is 10 nm becomes 5 to 10% of the total number of heads. Hence, we have lost contact with the original idea that the s.d. represents the distance covered when *all* the c.b.s (which must contain quite many heads) split one ATP molecule each and the value of 10 nm becoes meaningless
2. The rate of heat production to be considered should have been the *total* change, that is, maintenance (during isometric contraction) plus shortening heat. *Both*, at least largely, should be due to the hydrolysis of ATP. All investigators of heat phenomena in muscle have taken this for granted; otherwise, all their work, which has been believed to be of utmost importance for the elucidation of the molecular mechanism of muscle contraction, would be futile
3. It has been demonstrated (cf. Holroyd and Gibbs, 1993) that the heat of shortening that was originally measured in amphibian skeletal muscles and served as one of the pillars on which the mathematical formulation of sliding filaments theory was based (Huxley, 1957) is not universal, as it was not observed in both amphibian and mammalian cardiac muscles, as well as in some skeletal muscles (all of which are striated) (cf. Oplatka, 1994d)
4. It was taken for granted that the contractile force was the isometric force of 1.5 kg/cm^2 even though an unloaded shortening muscle, which does not perform any work, was considered in the calculation of ΔL
5. The "enthalpy change" (heat plus work) was found not to be equal to that expected from the measured chemical expenditure in an unloaded shortening muscle (Kushmerick and Davies, 1969) and part of the ATP splitting occured during *relaxation* after shortening, which suggests that the s.d. is much longer than 10 nm (Oplatka, 1990; 1991a,b).

Making the necessary corrections we obtain:

$$E = 0.16 \times P_o\Delta L + \frac{ab.\Delta L}{V_m}$$

$$= 0.16P_o\Delta L + \frac{P_o}{4}\frac{V_m}{4}\frac{\Delta L}{V_m}$$

$$= 0.16P_o\Delta L + 0.06P_o\Delta L = 0.22P_o\Delta L$$

where E represents the heat released after shortening by ΔL under zero load when the velocity (V_m) is maximal and $a(\sim P_o/4)$ and $b(\sim V_m/4)$ are, respectively, the constants in A.V. Hill's force-velocity relationship (Hill, 1938) (Hill claimed [1965] that the maintenance heat rate equals a.b).

The myosin concentration in muscle has been evaluated (see Squire, 1981) and found to be about 120 μM. Hence, the number of heads per half sarcomere with a cross-sectional area of 1 cm^2 is $2 \times 120 \times 10^{-6} \times 10^{-3} \times 1.1 \times 10^{-4} \times 6.06 \times 10^{23} = 1.6 \times 10^{13}$. Hence, the energy release per head after covering a distance of ΔL cm is equal to

$$\frac{0.22 \times P_o \Delta L}{1.6 \times 10^{13}} = 2.06 \times 10^{-8} \text{ ergs}$$

Equating this to the enthalpy 1.6×10^{13} change of 6.7×10^{-3} erg per ATP molecule hydrolyzed we get $\Delta L = 325$ nm $= 0.325$ μm, which is 32.5 times larger than the value (of 10 nm) derived by Huxley. Just for the sake of illustration, if we stimulate a muscle that had been stretched to the point at which there is little overlap between the two sets of filaments (i.e., when the sarcomere lenghts are 3.65 μm), then following the hydrolysis of only three ATP molecules by each of the myosin heads, the (unloaded) sarcomeres will shorten to become $3.65 - 3 \times 2 \times 0.325 =$ 1.7 μm long, which is shorter than the sarcomere length at rest (2.2 μm), that is, there will then be *double* overlap.

B. The Two Observations

One was the finding (Slayter and Lowey, 1967) that the enzymatically active globular portion of the myosin molecules is composed of two elongated heads (about 18 nm long each). This length was *somehow reminiscent* of the value of about 10 nm calculated erroneously for the s.d. The second observation was that in muscle in rigor the c.b.s form "arrowheads" at an angle of 45° with the actin they contact that point toward the center of the sarcomere (Reedy et al., 1965). Similar "arrowheads" have been observed in actin-S-1 in solution in the absence of ATP.

C. The Speculation

Because it has been assumed that the hydrolysis products of ATP (ADP and Pi) must be released at the end of the *enzymic* cycle for a new ATP molecule to bind and start a new cycle, it has been taken *for granted* that the last step in the *mechanical* "cycle" in muscle must be the formation of a rigor complex between the two proteins, such as the one formed in solution. This is absolutely true when each of the myosin heads consumes the last ATP molecules available, at the end of the depletion of a limited amount of ATP. However, in living muscle, where a millimolar level of ATP is maintained, there exists the possibility that a fresh ATP molecule binds to the myosin head *before* this has the chance of forming a tight, rigor complex with actin. However, this point was not considered at that time. It was intriguing to *believe* that myosin forms similar arrowheads also in active muscle at the end of the enzymic cycle. There has never been any discussion of the plausibility of this belief. This led to the speculation that after activation the myosin heads, that had not been in contact with the actin filaments during relaxation, and claimed by Reedy et al. to be then perpendicular to them, split the bound ATP, bind to actin at 45° with their tips pointing toward the Z-band in the *same* half sarcomere, then undergo a conformational change that rotates them by 90° while *firmly* bound to actin, thus pointing at the end of the cycle at an angle of 45°

relative to the actin filament (the so-called "arrowheads"), this time toward the center of the sarcomere after having pulled the actin filament over a distance equal to the s.d. The latter must then be equal (for a right angle triangle with two 45° angles) to $(2L)^{1/2} = 1.41L \cong 1.4 \times 18 \cong 25$ nm, where L is the length of the myosin head, which was considered to be "similar" to the value of 10 nm calculated erroneously by Huxley. Unfortunately, even the larger value of 25 nm is still 13 times smaller than the corrected value of 325 nm derived previously.

The electron microscopic study (and obviously the study in solution) have been carried out in the absence of a load, and there was no reason to believe that the same tilt angle should be reached at the end of the enzymic cycle in an *isometrically* contracting muscle — the tension generated should be entitled to affect the nature of the process that created it, for example, the ATP turnover rate — as it really does (cf. Kushmerick and Davies, 1969) and, particularly, the force-generating step (just as hydrostatic pressure does, cf. Coats et al., 1985) and also the capability of the c.b.s to rotate in order to arrive at the tilt angle of 45° even if they wish to do so.

This is, essentially, the swinging crossbridge theory (Huxley, 1969). An estimate of a s.d. of a similar magnitude came from modeling the force-velocity and energetic properties of shortening muscle (Huxley, 1957). The value calculated was about 16 nm. However, a few years later Hill (1964) found that the shortening heat per unit length shortening, which was taken to be constant in the evaluation of the s.d. (on the basis of earlier measurements by Hill) actually varied significantly with load. Moreover, as mentioned previously, no shortening heat has been detected in various striated muscles.

Studies in which a shortening step was imposed on a single muscle fiber during an isometric tetanus showed that tension initially drops down to zero for steps larger than about 14 nm per half sarcomere (cf. Ford et al., 1977). This was interpreted to indicate that the size of the "working stroke", that is, the s.d., is about 12 nm. Let us denote this s.d. by ΔL_2 and the s.d. obtained by H. E. Huxley by ΔL_1. Because the latter was derived in the basis of heat measurements (assuming that these represent ATP turnover rates) it might be considered as a "thermal" s.d., whereas ΔL_2 is a "mechanical" s.d. Had H. E. Huxley obtained the correct value of 325 nm rather than the erroneous one of 10 nm, I wonder whether he would have suggested the swinging crossbridge theory and, moreover, whether anybody would have "jumped" to compare *his* value of 10 nm to (1) the value of 16 nm obtained by A. F. Huxley from modeling the force-velocity and energetic properties of shortening muscles (which were based on incorrect and archaic data, cf. Oplatka, 1994d, 1996a), (2) the value of 12 nm derived from mechanical transients, and (3) the length of about 18 nm of the long axis of the myosin head. The values 10, 16, 12, and 18 nm are, indeed, comparable, but, unfortuantely, this does not necessarily mean that all of them are measures of the *same* entity, especially as the true value for the s.d. nicely and simply defined by H. E. Huxley appears to be 32.5 times larger than 10 nm.

The value of the "mechanical" s.d. ΔL_2 was anticipated to be the same as ΔL_1, that is, 10 to 20 nm. However, this s.d. must involve many myosin heads that contribute different forces at the moment the rapid shortening is applied because, due to their asynchronous operation, they are at different stages of the mechanical and enzymic cycles. Its definition is thus quite different from that of the "thermal" s.d., and this should be even clearer when the actin filaments slide past a single myosin head or

molecule. Hence there is no reason why should the values of the two sliding distances be the same.

A basic feature of the SCBT is the idea that the myosin heads undergo rotation while *firmly bound* to the actin filaments. If we carefully analyze this postulate we find that it actually contains *two* independent axioms.

1. That the heads rotate. Unfortunately, no such rotation has so far been observed, despite numerous highly sophisticated experiments (Martin-Fernandez et al., 1994; Hirose and Wakabayashi, 1993). The excuse of some of the followers of the theory is that we shall probably detect a rotation when we attach the proper probe to the proper site. The question is how many more years should be devoted to this task in view of the fact that more than 25 years have already been spent (or, most probably, *wasted*) and of the possibility that the whole idea is wrong. A growing number of researchers apparently got despaired of the endless effort (more than 40 years of wandering in the desert since the sliding-swinging Dogma was announced, without, and probably never, reaching the Promised Land) to "force" the heads to rotate and recently it has become fashionable to turn to a new idol: the so-far practically neglected "passive co-factor", that is, actin but, *again*, looking for *conformational* changes (that are now expected to be more modest) and, *again*, involving *one* protein only. One group (Schutt and Lindberg, 1992) was so daring as to give their paper the iconoclastic and most determined title *Actin as the Generator of Tension during Muscle Contraction.* Unfortunately (or may be fortunately), it takes *two* to make a couple and, on *a priori* grounds, there is no *a priori* reason to prefer and, moreover, to give the role of the *only* active partner to *either* myosin or actin (it takes a mother, in addition to to a father, to give birth to a child). The very *use* of the word crossbridges *as* the force generators *automatically* implies that this part of myosin is *solely* responsible for tension generation. The modern, fashionable, and highly sophisticated term "motor" also *automatically* implies that kinesin, dynein, myosin, etc. (i.e., the ATPase component of biological engines) is the source of power, the actin filaments and the microtubules serving only as the railway or the highway on which the train or the car (that contain the motor, i.e., the engine) runs. After all, is it not the engine that contains the gasoline and burns it? It is the car that is moving while the road lies there most passively? We must coin terms in order to describe items and actions, but quite often we become servants rather than masters of our own words.
2. The second axiom is that the heads are *tightly* attached for propulsion to occur. In the following section I present experimental data that, to say the least, does not unambiguously imply that crossbridges are actually bound to the actin filaments at any time in *active* muscle.

III. ARE, INDEED, MYOSIN HEADS "CHEMICALLY" BOUND TO THE ACTIN FILAMENTS AT ANY STAGE OF THE MECHANOCHEMICAL CYCLES AND, IN PARTICULAR, DOES A RIGOR STATE EXIST IN ACTIVE MUSCLE?

It has been stated frankly by A. F. Huxley (1980) that "although it is likely

that the rigor state corresponds to one step in the events which occur during contraction, it has to be admitted that this is not certain and the changes in the X-ray diffraction pattern during contraction do not unambiguously imply that crossbridges are actually attached to the thin filaments". Thus,

1. An enormous effort has been invested during many decades, especially by H. E. Huxley (cf. Sengen et al., 1987), employing X-ray diffraction techniques, in an attempt to demonstrate that in active muscle the heads really *touch* the thin filaments. Most unfortunately, no evidence for attachment has been found. The center of mass of the c.b.s was observed to move closer to actin but less than in the case of muscle in rigor where the heads seem to contact actin, and they really do. In the words of Brenner and Yu (1993): (while) "in the relaxed state, the center of (the c.b.s) centered around the myosin (filament), in the active state .... it has moved out a little bit". Quoting Martin-Fernandez et al. (1994): "in presenting our (X-ray) results we have hitherto spoken of myosin heads diffracting with actin-based periodicity, or forming an actomyosin complex, rather than as being 'attached'. This is because our evidence does not necessarily prove that the heads *attach* to actin in a physical sense, at any stage of the contractile cycle." Wakabayashi et al. (1993) also claimed that "the lack of any appreciable intensity increase in the inner region of the first layer line during contraction indicates that there is very little sign of rigor-like specific binding of the myosin heads to the actin filaments in native muscles".

   In addition,

2. Hirose and Wakabayashi (1993) have claimed recently, on the basis of *cryo-electron microscopic* observations, that in an isometrically contracting muscle, the mass of the c.b.s is not as close to the thin filaments as in rigor.
3. It has been observed that c.b.s in isometrically contracting muscle appear to be angled nearer to 90° than rigor crossbridges, with no indication for the 45° angle of rigor muscles (Martin-Fernandez, 1994; Hirose and Wakabayashi, 1993).
4. Most recently, Brenner (1997) wondered whether, indeed, "the rigor-like c.b. represents the end of the power stroke and thus is the state significantly populated during isometric contraction?" To his surprise he found "no evidence for a detectable fraction of c.b.s. occupying a rigor-like stereospecific conformation during isometric state contraction" after recording 2D X-ray diffraction patterns. Moreover, a titration of ATPγS in the presence of calcium was carried out to study the features of different mixtures of nucleotide-free (rigor-like) and weakly found c.b.s. However, none of the diffraction patterns was comparable to the pattern under isometric contraction, thus making it unlikely that a significant fraction of rigor-like c.b.s. is present during active contraction.
5. There is a *basic* difference between active muscles and muscles in rigor and that is that the latter do *not* contain MgATP that is entitled to have more than one effect, in addition to being hydrolyzed, e.g., to dissociate A·M·ADP. Pi and/or A·M·ADP *before* A·M is produced [A and M representing, respectively, actin and myosin]. The ATPase cycle does not have to wait for its completion or ADP to be *dissociated* — the latter can be kicked

out rapidly by the abundant ATP with its very high affinity to myosin.

6. The claim that at any time part of the probes attached to the -SH1 group in S-1 are at the same angle in active as in rigor muscle should be taken cautiously because the ATPase activity has been reported to be appreciably affected by the probe (Fajer et al., 1990). Thus, poisoning of $-SH_2$ in S-1 led to the abolishment of mechanical activity, even though the enzymic activity was unaffected (Strivastava et al., 1981). Hence, the problem of whether the fluorescent or EPR probe indeed attaches parallel to the head's axis is not the only problem that should worry us — there is *always* another, much more important problem in this kind of measurements: that even the modification of a *single* functional group (-SH, $-NH_2$, etc.) might profoundly affect the enzymic and/or the mechanical cycle (in particular, the force-generating step), not to mention the possible formation of *permanent rigor complexes* between some of the heads and actin that might be responsible for the fraction of the probes observed by Fajer et al. to exhibit the rigor angle.
7. Sugi and his collaborators (Hatta et al., 1988; Tsuchiya et al., 1993) have continuously recorded the stiffness changes in glycerinated frog skeletal muscle by measuring the propagation velocity of ultrasonic waves both when a resting muscle was put (isometrically) into a rigor or into activating solution. Both the longitudinal and the transverse stiffness increased in the rigor state as anticipated as it is generally believed that stiffness is a measure of the number of attached c.b.s. In activated muscle, however, while longitudinal stiffness increased, transverse stiffness *decreased.* It was stated that in rigor the c.b.s attach permanently to the thin filaments so that it was not surprising to observe that both *stiffnesses* increased, indicating a nearly isotropic nature of rigor muscle. The behavior of active muscle was different and showed a highly anisotropic nature. As it is widely held that the configuration of the c.b.s immediately after their "power stroke" (i.e., the stage in the enzymic-mechanical cycle in which force is generated) is that of rigor, the highly anisotropic nature observed suggested to the authors that the proportion of the c.b.s with the rigor-like configuration, *if any*, must be very small during contraction. However, a crosslink is a crosslink whether we measure stiffness longitudinally or transversely and the same number of presumably attached, force producing, c.b.s must exist in the two cases. Even if the contributions of the c.b.s to transverse and to longitudinal stiffnesses are different, they must always be *positive,* that is, they must give rise to an *increase* in stiffness, small as it might be, but never a *decrease,* that is, *negative* stiffness. Because the whole idea that the stiffness of active muscle is due to the c.b.s while *attached* (and it does not matter whether strongly or weakly) to the thin filaments, it is legitimate at this point to wonder if the *decrease* in stiffness after activation could not be considered as an additional argument against the *speculation* that for force to be generated the myosin heads *must* be *tightly bound* to actin.
8. The 100% rigor c.b.s formation (i.e., a tilt angle of 45°) observed by Pollard et al. (1993), both in the presence and in the absence of ATP, might be due to the fact that they employed F-actin and S-1 in *solution* in which actin is free to

slide and create water flows (Tirosh et al., 1990; Burlacu and Borejdo, 1992, Oplatka, 1994b), which might cause the bending and tilting of the heads so as to form arrowheads with an angle nearer to 45° than to 90°. Thus, Broachard-Wyard (1993) has discussed the deformation of *branched* polymers in strong flows. The 90° tilt angle observed in isometric contractions simply indicates that this is the angle (and the only one) in which force is generated (without necessitating any rotation) and that even if *after* force is developed the heads rotate during an isotonic (particularly unloaded) shortening (as in Pollard et al.'s experiments), they should not rotate under isometric conditions because, assuming (as in the SCBT) that they are firmly bound to the thin filaments, this will cause shortening, that is not possible because the length of the muscle is fixed.

9. A stretching force applied to muscles in rigor did not change the c.b.s orientation. This has been demonstrated in X-ray diffraction, paramagnetic resonance, polarization fluorescence, and electron microscopic studies (Trombitas et al., 1988) and is at variance with the accepted idea that the distribution among the various strongly attached states during rotation should depend only on the force in the system. This suggests that the heads do not rotate at all and therefore that the rigor tilt angle might have nothing to do with the cycle.
10. A rigor state should impede smooth sliding thus making shortening a most inefficient and complicated process. Because the heads act asynchronously, as the number of heads interacting with a given thin filament is quite large, as each sarcomere contains an enormous number of filaments, and as one myosin molecule might interact with two actin filament simultaneously (Borejdo and Oplatka, 1981), the existence at any moment (even for a very short time) of even a single head bound to actin in the rigor state should disturb movement, cause the formation of kinks in the filaments, and act as an internal load. In the following I present a theory according to which no rigor-bond formation occurs in active muscle (contracting isometrically as well as isotonically) so that there is no room for such interference.

An extraordinarily large pressure (several atmospheres) is needed to squeeze out the final few layers (a few molecular diameters) of liquid between two solid surfaces forced to approach each other. When a liquid is confined in a narrow gap (as near a cell membrane or in a porous medium), a new dynamic behavior emerges. The flow of liquids under extreme confinement cannot be understood simply by intuitive extrapolation of bulk properties (Granick, 1991). We may dare assume that the same is true for two macromolecules such as myosin and actin molecules approaching each other during their Brownian movements. Stated differently, they must overcome repulsive hydration (and also electrostatic) forces. For this purpose, they must possess extremely large kinetic energies that are most probably not acquired by any molecule during its random collisions with water molecules or when kinetic energies of the order of magnitude of a few kT's are provided by the extrusion of water jets. In other words, the kinetic energy of the released water is large enough for ensuring the unidirectional movement of the heads and therefore requiring the minimal time for approach, bringing them as close as possible to the thin filament (thus creating the maximal

possible tension in isometric contractions) but too small for enabling physical contact between the proteins (that is not necessary for any purpose and can only impede shortening).

At this point I feel that I should refer again to Albert Szent-Györgyi's intuition (1973)

> "water tends to build solid structures if it finds a solid support. If the water surrounding two particles fuse, they will *hold* the two structures together (i.e., the two structures do not have to physically contact each other in order to be held together. A.O.). We wondered about the strength of this bonding and measured the forces by which water can bind two glass plates together. We found them surprisingly high. They depended on the thickness of the water layer. Below a thickness of 2000 Å we found it very low, approaching zero [in a figure presented, the force is shown to decrease exponentially with distance so that at a distance of a few molecular layers of water, it should be enormous]. So that we can say that below a distnace of 1000 Å water can act as a solid, and can make part and parcel of the living machinery."

Thus, in the back of his mind, Albert Szent-Györgyi, must have had rejected the idea that some kind of rigor complex formation is obligatory for tension development in association with rotation of the myosin heads.

In summary, the rigor state is characteristic of a *dead* muscle, that is not supposed to move. Is there any reason why it *should* be an essential part of the *active*, living process except for the fact that is was observed in dead muscle? If a muscle is held at a constant length and allowed to undergo a transition to the rigor state (after *all* the ATP molecules had been hydrolyzed) the heads apparently have to be deformed in order to be able to form several sterically well-defined "chemical" links (i.e., hydrogen and "hydrophobic" bonds, salt linkages) with actin in the muscle thus leading to the development of the *rigor force*. The tilt angle will then be entitled to differ from that of force-generating crossbridges in active muscle that, as stated previously, is close to 90° in an isometric tetanus. For rigor tension to exist, a continuous three-dimensional protein network must be formed. No such network is needed for the generation of *active* tension and therefore the soluble myosin fragments HMM and S-1 are capable of inducing movement and *active* tension generation but, unlike myosin, are incapable of generating *rigor* tension. This is evident from the fact that when muscle segments from which the myosin component had been inactivated (so that no tension could develop when ATP was added) were irrigated by myosin, the addition of ATP generated tension that was maintained after ATP was depleted, that is, rigor tension developed. The same thing happened when HMM was employed. However, if S-1 (which unlike myosin or HMM cannot give rise to the formation of a continuous three-dimensional network, cf. Borejdo and Oplatka, 1981) was added, tension went down to zero when ATP was depleted (see Borejdo and Oplatka, 1976, Figure 8). It should be noticed that in these systems there are no "elastic elements" (as required by the theory of Huxley and Simmons, 1971) that connect the heads to the myosin filament shafts and that are stretched by the rotation of the heads thus generating the "contractile" force.

Thus, it seems as if the myosin heads and actin can interact from *a distance* without necessarily forming "chemical" bonds (i.e., salt linkages, hydrogen, and hydrophobic bonds), the "interaction" leading to the enhancement of the ATPase activity of the heads to the generation of a "contractile" force and to the movement of the actin filaments.

If, indeed, the heads are supposed to rotate, how could they pull the actin filaments if they are not firmly bound to them? The solution of the problem whether the

heads are attached should logically precede the solution of the secondary question whether they rotate: the first axiom (rotation) logically dictates the second one (firm binding). Somehow, the second axiom is taken for granted despite the observations against it mentioned previously.

How can force be generated without physical contact being made between the proteins? Do water molecules in the space separating the proteins act as a sort of glue that could form if their hydration shells merge so as to form a common layer, or maybe the hydration layers play a more active role? (Oplatka, 1994a, 1996a,b).

An indication for the "recognition" of the actin filaments by the myosin heads while quite far away from each other when a muscle is stimualted is suggested from the work of Yagi and Matsubara (1980), who examined the claim that the movement of the myosin heads out of their helical positions as the muscle contracts may not require the presence of actin in their vicinity. This was based on X-ray diffraction studies of active muscle that indicated that the decrease in the layer-line intensity on activation did not change significantly when the extent of overlap between thick and thin filaments was approximately halved by stretching the sarcomeres. This has been interpreted as indicating that an increase in sarcoplasmic calcium concentration may directly cause the movement. Yagi and Matsubara stretched muscles to such an extent that the thick and the thin filaments no longer overlapped and the muscles produced little or no tension. The 42.9-nm myosin layer line shown in X-ray diffraction patterns did not become weaker, suggesting that the myosin heads did not move from the resting positions in the absence of actin. Thus, the myosin heads somehow "perceive" the thin filaments at a distance following activation. Hence, the very binding of Ca^{2+} *simultaneously* to both the regulatory light chain of myosin and to troponin in the thin filaments must cause a dramatic increase in inter-filamentous attractive force (or a decrease in repulsive forces). Because the distance at the start of movement, particularly for muscle at rest length, is too large for any attractive forces to operate, we are left with the long-range repulsive hydration and electrostatic forces, the sum of which must therefore diminish following activation. As the hydration force is associated with the hydration shells of the proteins and as the structure, stability and water content should be sensitive to the degree to which they "penetrate" each other as they come closer to each other, we should consider also the possibility that changes in the hydration shells might play a decisive role in the "awakening" of the myosin and of the actin filaments that are "dormant" in the relaxed state. This may be considered as the *first* step in a *series* of events (not just one) that we call "regualtion" but, obviously, it might also be regarded as the first step of the steering and docking processes that lead the myosin heads toward their target on the actin filaments in the fastest and most efficient route and, as we shall see later on, it is also the beginning of the very development of contractile force in isometric contractions. Thus, "regulation" and "mechanochemical conversion" are inseparable in muscle contraction and are just two aspects of the same process.

IV. THE ROLE OF WATER IN MUSCLE CONTRACTION: A CRITICAL REVIEW OF INDICATIONS FOR THE ACTIVE PARTICIPATION OF WATER IN THE "CONTRACTILE" PROCESS

Long ago (cf. Oplatka et al., 1974; Borejdo and Oplatka, 1976; Tirosh and

Oplatka, 1982; Tirosh et al., 1990; Oplatka, 1996a), following the discovery that *free* HMM (and quite frequently also S-1) in conjunction with actin give rise to tension generation and shortening in muscle preparations, active streaming, superprecipitation, contraction of actin threads, and acceleration of thin filaments in solution, it became obvious that the mechanochemical activity observed could not be due to rotation of the heads that stretches "elastic elements", which generates a contractile force. A theory has been proposed (Oplatka et al., 1974; Tirosh et al., 1979) according to which energy released during ATP hydrolysis is acquired by all participating species. Conservation of mechanical momenta was assumed, which means that relatively small species (i.e., the hydrolysis products and possibly also protons) acquire larger velocities than the much heavier proteins. Because kinetic energy is proportional to velocity square, nearly all the energy is possessed as kinetic energy by the small particles. This energy is imparted to water molecules, creating mini-flows, which have a component parallel to the actin filaments. The latter are then passively dragged toward the center of the sarcomere in isotonic contractions or, in the case of isometric contractions, exert a contractile force on a transducer. Hence, water flows have been considered to be responsible for movement and for tension generation.

More recently, I have noticed that in the literature there are many indications for the involvement of the hydration shells and for *release* of energetic water from the hydration sheels of actin and/or myosin and that there seems to exist an intimate connection between changes in the hydration layers and the contractile process (Oplatka, 1989; 1991a,b; 1993; 1994a,b; 1996a). Therefore, I decided to pay special attention to this *originally* kinetically active water rather than to the possibility that free water of the medium might create flows due to the acquisition of kinetic energy from vectorially ejected ADP and Pi. The reason for this preference is that we know for sure that originally bound water is ejected, whereas the idea of kinetically active ADP and Pi was an assumption.

1. It has been demonstrated by Coats et al. (1985) and by Geeves (1989) that upon mixing S-1 and F-actin, both in the presence and in the absence of ADP, a complex is formed that undergoes isomerization. It has been indicated that *both* the equilibrium constant of the isomerization reaction and tension generation in muscle fibers are diminished by low temperature, high ionic strength, and hydrostatic pressure and by the presence of ethylene glycol, which strongly suggests that tension generation occurs during or following the isomerization. Geeves and his collaborators (McKillop et al., 1991) have tested the effect of pressure on other steps of the enzymic cycle involving acto-S-1 and ATP and came to the conclusion that these are unlikely to cause the decrease in isometric tension that is induced by pressure, which supports the argument that the tension drop is due to an effect on the specific isomerization reaction. Because the latter is *inhibited* by hydrostatic pressure, it must be accompanied by an *increase* in volume. This is quite appreciable and amounts to $\Delta V^\circ = 110$ cm^3/mol heads. An increase in volume is characteristic of many protein-protein interactions and is commonly ascribed largely to a change in the hydration shells of the interacting proteins. Thus, it has been claimed that the predominant contribution to ΔV° is due to changes in the solvent structure when flagellin polymerizes (Gerber and Noguchi, 1967). The partial specific

volume of water bound to proteins is 0.83 ml/g (Rupley et al., 1983), that is, one should expect $\Delta V > 0$ for the liberation of such water and $\Delta V/V \cong 0.1 - 0.2$, where V is the volume of the freed water: in the case of the polymerization of tobacco mosaic virus protein (TMVP), water was found to be released and its amount measured directly, in parallel to an increase in volume (Lauffer, 1975). $\Delta V = 0.00787$, while $V = 0.033$, both in cm^3/g protein at 25°C. The value of ΔV for TMVP has been shown to be mainly due to the increase in volume as a result of the transition of the water molecules from a bound into a free state; only 16% of ΔV were attributed to H^+ binding. Correcting for this we get $\Delta V/V = 0.200$. The polymerization of TMVP is entropy driven, thus allowing the process to be endothermic. Unfortunately, we do not know of any contribution of H^+ binding to the value of $\Delta V°$ for the isomerization reaction of acto-S-1. Taking the previously value of $\Delta V/V$ for TMVP and the value of $\Delta V°$ for acto-S-1, we obtain $V = 462$ cm^3/mol heads, which corresponds to the release of 31 water molecules per head (Oplatka, 1994a).

2. Optical changes have been observed in stimulated striated muscles (cf. Huxley, 1957). During a twitch, there is a decrease in the amount of light scattered by the muscle, which follows roughly the time course of the tension. The strength of the birefringence falls during an isometric twitch, the amount of the fall being a maximum if the muscle is near its rest length when the degree of overlap of actin and myosin and the isometric tension are maximal. Both effects have been ascribed to the transfer of water from the myofibrils into the sarcoplasm (i.e., the medium surrounding them), which leads to a decrease in the difference of refractive index between the sarcoplasm and the fibrils.
3. The finding that the spacing between the filaments in the myofibrils decreases during an isometric contraction, in skinned (Matsubara et al., 1985) as well as in intact muscle fibers (Cecchi et al., 1990), supports this interpretation. This suggests that an osmotic pressure difference is established during an isometric contraction between the sarcomeres and their surrounding medium. How is this osmotic pressure difference related to the tension that develops in parallel? In view of the observed changes in the value of the isometric tension (as well as of the velocity of shortening) of glycerinated muscles in a medium to which an osmotic pressure difference has been imposed by *us* by adding impermeant macromolecules to the medium (mentioned previously) it is to be anticipated that the transfer of water associated with the *very generation* of tension affects the value of the tension observed. In summary, there appears to exist a close reciprocal relationship between water movement and tension generation — the latter is linked with water transfer, while removal of water may affect tension development; in other words, the two processes seem to be inseparable. According to the Le Chatelier principle, if a change is imposed on one of the factors under which a system is at equilibrium (e.g., temperature, pressure) the system tends to adjust itself so as to annul, as far as possible, the effect of that change. If we apply this principle to the steady state prevailing in a tetanic isometric contraction then we should expect that if the generation of tension leads to the

extrusion of water then if we deliberately extrude water (e.g., by the addition of a polymer to the surrounding medium) the contractile force should decrease. This really happens but not over the *whole* range of polymer concentrations: as mentioned previously, according to several reports, the force first increases, up to a maximum, and then decreases as the polymer concentration is increased (cf. Ford et al., 1991). However, if we consider the other, opposite alternative, namely, that the extrusion of water is not a *result* of but rather the *cause* for the generation of force (which may sound mad) then the lowering of the thermodynamic activity of water by adding a polymer should cause an *increase* in tension, that is what one really observes when the polymer concentration is increased up to a certain point. What is the reason for the decrease in tension following the maximum? In order to be able to answer this question let us first answer another one: Where does the transferred water come from? Is it from the inter-filamentous space or maybe it is water that had been bound to the filaments before activation? The very transfer of water causes a decrease in the concentration of the inter-filamentous water (which contains also soluble components, the concentration of which should increase). This should cause the decrease of the thermodynamic activity and of the chemical potential of the water. Both actin and myosin should possess hydration shells of bound water, just like other proteins and water-soluble macromolecules. Because the bound water must be at equilibrium with the free water in the medium, its activity and chemical potential should also decrease. This could occur in association with a decrease in enthalpy (H) and/or in increase in entroy (S). Release of bound water from proteins is known to be accompanied by $\Delta H > O$ and $\Delta S > O$ (see, for example, the previously mentioned results concerning the polymerization of the tobacco mosaic virus protein) so that water must be liberated from the hydration shells of either or both actin and myosin. The increase in entropy that accompanies the release of water bound to macromolecules is due to the transition of water from an ordered state into that of free water. If the concentration of the polymer becomes too high, then the hydratin shells lose too much water and therefore are unable to liberate water for the generation of force so that tension should drease continuously at relatively high concentration of applied polymer, as is indeed the case.

4. Is there any direct clue for the transfer of water from an ordered to a less-ordered state in activated muscle? Measurements of nuclear magnetic relaxation times for water protons in skeletal frog muscle suggested that a fraction of the intracellular water molecules have restricted rotational freedom and that this fraction decreases when the muscle contracts isometrically (Bratton et al., 1965). The NMR data were interpreted by a two-phase model that permitted for the amount released (20% of the total bound) to be, depending on an assumption made in the calculation, either 9×10^{-5}% or 3×10^{-2}% of the total water. Taking into consideration that about 25% of the water is present in the interspace between the fibers, 1.2×10^{-4} or 4×10^{-2}% of the interfilamentous water is liberated. Earlier microwave studies on cardiac muscle indicate that approximately the same quantity of bound water is liberated

(reversibly) during contraction (Bratton et al., 1965). Because muscle contains about 80% water, about 4×10^{-8} or 1.33×10^{-5} mol of water are liberated per cm^3 muscle. As the concentration of myosin heads, c, is about 2.4×10^{-7} mol/cm^3 (Squire, 1981), the number of water molecules released by a head should be either 0.15 or 55. The second value is much more probable than the first one. It is about twice the value of 31 derived previously (no. 1, for the isomerization of acto-S-1 from Coats et al.,'s work (1985).

The difference might at least partly be attributed to cooperatively between the two heads of the myosin molecules in living muscle. This could be related to the finding that S-1 is less effective than a head in the double-headed myosin fragment HMM in generating tension and movement in *in vivo* (Borejdo and Oplatka, 1976) and in *in vitro* (Tirosh et al., 1990) systems. Another reason is, in all probability, the fact that the value of 31 water molecules has been derived for acto-S-1 in the absence of any nucleotide, while the value of S-1 has been calculated for actomyosin dynamically interacting with ATP and with its hydrolysis products. Thus, it seems that the increase in volume accompanying the isomerization reaction is largely due to the release of bound water and that the NMR changes are mainly associated with the interaction between the myosin heads and actin.

5. A more recent dynamic ^{1}H-NMR investigation also led to the conclusion that part of the structured water becomes almost as free as pure water during contraction (Ogata, 1992).
6. The electrical resistance of the myoplasm in the resting state has been claimed to be very high compared with that in the activated state (Ogata, 1996). This is in accord with the observation that water is only slowly squeezed out from relaxed muscle fragments by applying pressure, compared with activated muscle fragments. These findings suggest that in relaxed muscles more water is retained by the proteins then in active muscles, that is, that after activation, water is liberated from hydration shells, most probably of the thick and the thin filaments before and/or during their force-producing interaction.
7. Laser temperature-jump and length-jump experiments (Davis and Harrington, 1993) recently suggested that tension generation occurs in a single endothermic (entropy driven) step in Huxley-Simmons phase 2 (Huxley and Simmons, 1971). Two different processes have been considered: the conversion of an organized protein structure of low entropy, having in mind the transition of the highly helical myosin subfragment-2 (S-2) into a random coil, and an order-disorder transition as the result of the exclusion of water from the interacting surfaces of the proteins, as in protein self-assembly (e.g., of myosin or actin). Because S-1 alone can, together with actin and ATP, generate tension and cause movement (Oplatka et al., 1974; Borejdo and Oplatka, 1976), it is clear that if the first possibility is true. It then cannot be taken as a proof that S-2 is the *only* generator of force (as was claimed by Harrington in 1971), even through S-2 appears to play a role in *amplifying* the *primary* force developed by S-1 or HMM (Oplatka, 1991a,b,c) by undergoing an helix-coil transition in muscle, and this could also contribute, together with the liberation of water from the hydration shells of the proteins, to the increase in entropy.

8. We have mentioned the experiments carried out by Hatta et al. (1988) and Tsuchiya et al. (1993) that showed that in isometrically contracting skeletal muscle the transverse stiffness decreases instead of increasing as anticipated by the SCBT, which takes it *for granted* that in active muscles the heads form tightly bound complexes with actin. This was deduced from the observation that the velocity of propagation of ultrasonic waves penetrating through muscle in the transverse direction *decreased* after stimulation (while the velocity of waves applied longitudinally increased). One of the mechanisms considered by these authors in order to explain this most surprising finding was that it is associated with a change in the structure of water molecules between the filaments, resulting in muscle "softening" in the transverse direction. They considered the possibility that water is squeezed out of the muscle, in accordance with the claim that the spacing between the filaments in skinned (Matsubara et al., 1985) and in intact (Cecchi et al., 1990) muscles decreases during isometric contraction. If indeed the water is first liberated from the hydration shells, as claimed previously, and then squeezed out, then the density should *decrease*, and this may then account for the decrease in transverse stiffness observed. This would be line with the fact that the compressibility of bound water is smaller then that of free water (cf. Gekko and Noguchi, 1979). In summary, the decrease in transverse stiffness after stimulation might be due to liberation of hydration water. The *increase* observed in longitudinal stiffness is another outcome of the *same* process, that is, the extrusion of water from the hydration layers of the thin filaments that should make them more rigid and less flexible, in full accordance with the finding of Burlacu and Borejdo (1992), who observed that the flexibility of F-actin in solution *decreases* in the presence of HMM + MgATP. I discuss stiffness measurements and their interpretation in more detail in Section VII.
9. As previously mentioned, Ford et al. (1991) and others have observed that after applying an osmotic stress on a glycerinated skeletal muscle (by continuously increasing the concentration of a polymer, in this case dextran, in the medium surrounding the muscle), the isometric tension first increased, up to 136% of control, and then decreased. If indeed water is liberated during the force-producing isomerization reaction, as we deduced from Coats et al.'s (1985) experiments, then the full reaction should be written as

'weak complex' <----> 'strong complex'
+ *water* (1)

The addition of dextran causes a decrease in the thermodynamic activity of water inside the lattice. The reaction should then be shifted to the right, that is, more 'strong' complexes should be formed and P_o should increase, as was indeed found. This observation may be taken as an additional indication for the release of water during the force-generating event. As speculated previously, the decline of P_o at higher concentrations of the polymer might be due, at least partially, to the depletion of so much water from the hydration shells of the proteins that there is no more room for a "phase transition" involving the liberation of water and the associated generation of force. From studies of the effects of pressure on the

equatorial X-ray diffraction from skeletal muscle, Knight et al. (1993) reported that the reduction in active tension under pressure seems *not* to be accompanied by c.b.s. "detachment" and concluded, in line with Equation 1, that pressure increases the proportion of "weakly bound" c.b.s.

Equation 1 and the shift to its r.h.s. after decreasing the chemical potential of water is reminiscent of other reactions in which water is liberated. Thus, the polymerization of G-actin, that is also accompanied by an increase in volume (as evidenced from the depolymerizing effect of applied hydrostatic pressure, cf. Swezey and Somero, 1985) must also involve the release of water, and therefore it is not surprising that the addition of polyethylene glycol to an actin solution increases the extent of polymerization (Tellam et al., 1983). The polymerizing effect of salt might also be partly due to the lowering of the activity of water, in addition to the diminuition of the electrostatic repulsion between the G-actin monomers.

10. A most important and illuminating report has been presented recently by Highsmith and collaborators (1996). Osmotic stress in a wide range, employing polyethylene glycol (PEG) of various molecular weights, was used to perturb the hydration of actin-myosin-ATP intermediates during steady-state hydrolysis. K_m for the interaction of S1·MgADP.Pi with actin decreased tenfold, suggesting that formation of actin. S1· MgADP·Pi involves net dehydration of the proteins. The dehydration volume increased as the size of the PEG was increased, as expected for a surface-excluded osmolyte. The experimentally determined volume was used to estimate the area of the "binding" interface and found to be consistent with the area determined from atomic structures of actin and myosin. The number of released water molecules per complex ranged between 330 and 2500. The authors indicated that these numbers are larger than the partial specific volume changes that have been determined from the effects of hydrostatic pressure on reaction rates and equilibria.

Most strikingly, PEG did not significantly affect the apparent K_m or the V_m for MgATP hydrolysis by S-1 *alone;* neither did osmotic stress affect various optical characteristics of S-1 in the presence of ATP, such as nucleotide-induced changes in the fluorescence intensities of S-1 tryptophans. We may therefore conclude that actin-activation of the ATPase activity of myosin, and with it in all probability also tension generation, contrary to the splitting of ATP by myosin alone, is associated with the release of appreciable amounts of bound water, presumably from the "combined" hydration shells. Because Ca^{2+} and EDTA-activations of the ATPPase activity of myosin are practically the same as that induced by actin, and because these activations do not give rise to tension generation and movement (just the contrary) it is suggested to check the possibility that Ca^{2+} and EDTA-activations are not accompanied by the extrusion of water, as can be evidenced from insensitivity of K_m to osmotic stress. Dehydration of F-actin by the application of osmotic stress was demonstrated by Grazi et al. (1993), who found that the diameter of actin filaments in solution was 9.0 nm at a stress of 1×10^5, and 6.8 nm at 9×10^6, dynes/cm^2 (at 1.81×10^5, the protein osmotic pressure in frog muscle, the diameter is 7.95 nm). They

also observed that tropomyosin-decorated actin filaments (as they exist in rabbit skeletal muscle) are also influenced by osmotic stress even though, at low pressures, they are significantly more resistant to compression than the undecorated filaments. Osmotic stress thus causes the extrusion of bound, as well as free water.

11. The activity of water can be decreased also by the addition of a water-miscible low-molecular-weight compound or organic solvent that, contrary to dextran, can penetrate into the lattice of a skinned or glycerinated muscle fiber. Endo et al. (1979) added sucrose to skinned *iliofibularis* muscles from African clawed toads. Their plot of P_o vs. sucrose concentration showed an initial increase followed by a rapid fall, just as when increasing concentrations of dextran were added (Ford et al., 1991). However, the authors have not paid any attention to the rise in tension as they were interested in tension inhibition that they ascribed to the high-viscosity media.
12. In continuation of the quotation at the beginning of this article, let me mention some more of Albert Szent-Gyorgyi's ideas that are nicely supported by the data presented previously on the involvement of water in muscular contraction. He claimed (1964) that "muscle contraction entails a profound change in physical state, I see only one possible way in which one can introduce a profound change in the physical state in a hydrophylic colloid and this is bringing two colloids together..... as we know from super-precipitation, actin and myosin, in the presence of ATP, form a hydrophobic precipitate. The (Huxleys') theory does not allow, or gives no room for, physical change". Water is usually thought of as a passive medium, necessary for the proper functioning of the "contractile" protein, but not forming part of the structure nor undergoing significant changes of state between rest and activity which, God forbid, may even be involved in the generation of mechanical forces. Albert Szent Györgyi had this vision. In his words (1973): "water is part and parcel of the machinery, and not merely its medium".

V. LIBERATION OF WATER IS THE DRIVING FORCE FOR TENSION GENERATION AND MOVEMENT

In the following I attempt to explain, in a preliminary, and therefore not too detailed manner, how the liberation of water from the hydration shells of actin and myosin can generate force in active muscle.

The value of the enthalpy change for the S-1-actin isomerization reaction can be calculated form the values presented by Coats et al. (1985) for the equilibrium constant at 2°C and 20°C in 0.1 *M* KCl, pH 7.0. Its value is $\Delta H^o = 21.9$ kcal/mol heads, which means that the reaction is entropy driven (Oplatka, 1993, 1994a,b, 1996a). It is interesting to note that the value of $\Delta H^o/\Delta V^o = 21.9/110 = 0.199$ kcal/cm^3 is quite close to 0.181 calculated from the data on the rod-forming, entropy driven polymerization of TMVP (Lauffer, 1975), which suggests that a similar "phase transition" occurs in the two cases involving the hydration shells.

Thus, during the isomerization reaction, water is liberated from the hydration shells of the proteins, probably from the region in which the protein surfaces get close to each other during activation. The "melting" of bound water is associated with an increase in entropy (just as in the case of melting

ice). The system can therefore "afford" an *increase* in enthalpy that can be utilized for the performance of work. This energy can be invested in order to bring the proteins closer together against the repulsive forces that operate between the "interacting" macromolecules, due to both the net negative charge of both proteins and the so-called "hydration force", which increases exponentially with decreasing distance between the hydration layers of neighboring macromolecules (cf. Leikin et al., 1994). Let us first deal with isometric contractions. It is probably easier for the myosin heads to move toward the "fixed" thin filaments than for the latter to approach the heads. I therefore limit myself to the kinetic energy acquired by the heads. This must eventually be fully exhausted. In view of what we have said previously about the improbability of rigor complexes formation in active muscle, exhaustion occurs *before* the two proteins get close enough to form a "chemical" complex. The repulsive force is then maximal and the heads, which cannot approach actin any further, are repelled down the repulsive force field until this vanishes (see Figure 1). The same forces that act on a head at any moment is of course perceived by the actin filament opposite it and this is conveyed to the transducer, contributing to the measured isometric tension. The rise and fall in force create a mechanical impulse (β), which is equal to the area covered by the force (φ) vs. time plot associated with the hydrolysis of one ATP molecule (see Figure 2). The average force per head $\bar{\varphi}$ is equal to the product of the impulse and the number of ATP molecules hydrolyzed by a head per second (ν) (Oplatka, 1972). Hence, rapid striated muscles, which exhibit relatively high ATPase activity compared with slow muscles (Bárány, 1967), must generate smaller impulses than slow muscles, that is, the rise and fall of the force occur more rapidly (Oplatka, 1972) (Figure 2a,b) as the values of P_0 (for similar concentrations of myosin) are practically the same for various striated muscles whose myosins may vary in their actin-activated ATPase activity by a factor of up to about 200 (as so, in parallel, their maximal velocities of shortening) [Bárány, 1967; Oplatka, 1972]). The value of P_0 is about the same for smooth as for striated muscles despite the fact that their myosin content is several times smaller. Therefore, the maximal value of φ for smooth muscles is much larger than for striated muscles (Figure 2c). The myosin heads may also be repelled from actin earlier than at the time the repulsive force is maximal if they bind ATP, which might affect the fields of force. This is obvious for the electrostatic forces because ATP is highly negatively charged, but it may apply also to the hydration forces that are ionic strength sensitive (cf. Rau et al., 1984). The myosin heads thus spend *all* their time without ever touching the actin filaments.

ATP in muscle seems to play two roles: (1) to avoid interaction between the myosin heads and the thin filaments in the relaxed state, (2) to be split when the muscle is activated so as to lead to the formation of myosin complex with ADP and Pi that can form a "weak complex" with actin in which the two proteins are separated by their close hydration shells. This "complex" can undergo an isomerization process that involves the release of bound water that creates tension (and movement). It is the energetics of this process that matters for the contractile process (cf. Oplatka, 1990, 1991a,b).

It has been suggested recently (Brune and Kim, 1994) that protein-ligand reaction rates, which are often limited by the rate of diffusional encounter, can be much greater if the components are given the correct orientation before reaction. They have considered "hydrodynamic forces produced when the water molecules between protein and

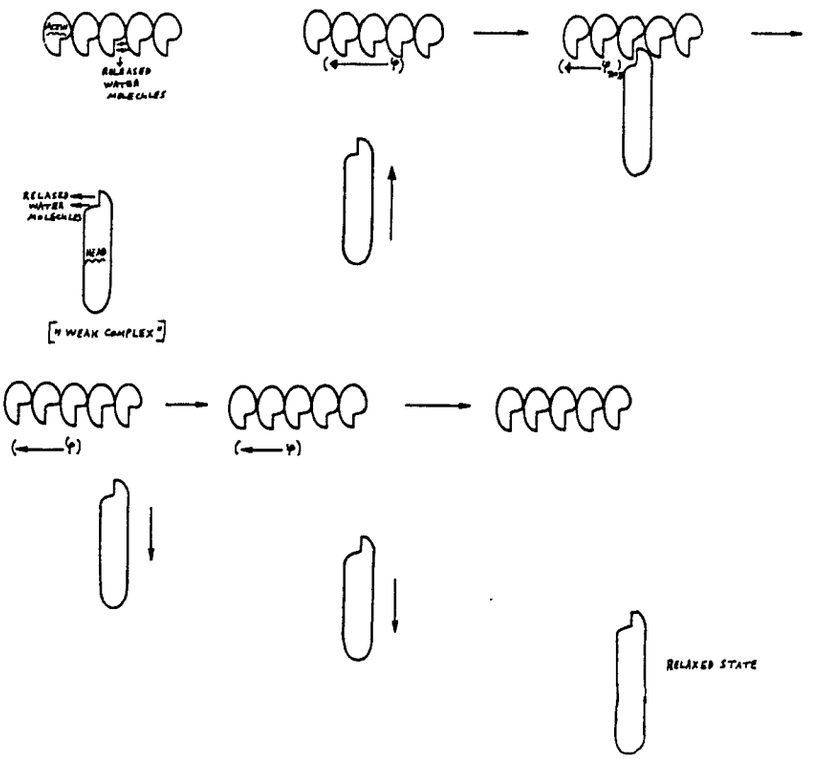

FIGURE 1. The approach and retreat of a myosin head from a (fixed) actin filament in an isometrically contracting muscle. The initial and the final distances have been largely exaggerated. In the first step (top left) the hydration layers of the two proteins (which presumably relatively weakly touch each other) release water (indicated by arrows). This led to the movement of the head toward the actin filament, that is, up the repulsive field. At a certain point (top right), the interacting surfaces get close to each other but they have no opportunity to get closer (when attractive short-range force operate so as to allow chemical, that is, rigor bond formation) because the "liberated" enthalpy has been fully used for the up-hill approach. The heads are then repelled by the maximal repulsive force reached and retreat down the repulsive forces field. The actin perceives an increasing force (from the moment the head starts its movement). This force first increases and then decreases in a cyclic manner, each cycle being associated with the hydrolysis of one ATP molecule and is equal at any moment to that acting on the head. The force acting on actin at any moment is opposed by an equal force developed in the transducer, a force that also varies cyclically. If the number of heads interacting with actin is relatively small, P_o will fluctuate (cf. Jackson and Oplatka, 1974).

ligand must be pushed out of the way to allow their encounter". Cleft enzymes have been used as a model system, as they could be expected to show strong hydrodynamic effects. "One particular type of hydrodynamic interaction stands out: a steering torque which occurs when the enzyme and substrate move toward each other in solution..... A simple model was used to demonstrate that the hydrodynamic torque can be two orders of magnitude greater than the electrostatic torque. The steering force is a long-range force..... Since many protein-ligand pairs exhibit complementary shapes (the so-called "lock-and-key paradigm"), if random thermal fluctuations push a protein-ligand together, the pair will be more favorably oriented for interaction by hydrodynamic interactions between the complementary shapes". Because both G-actin and

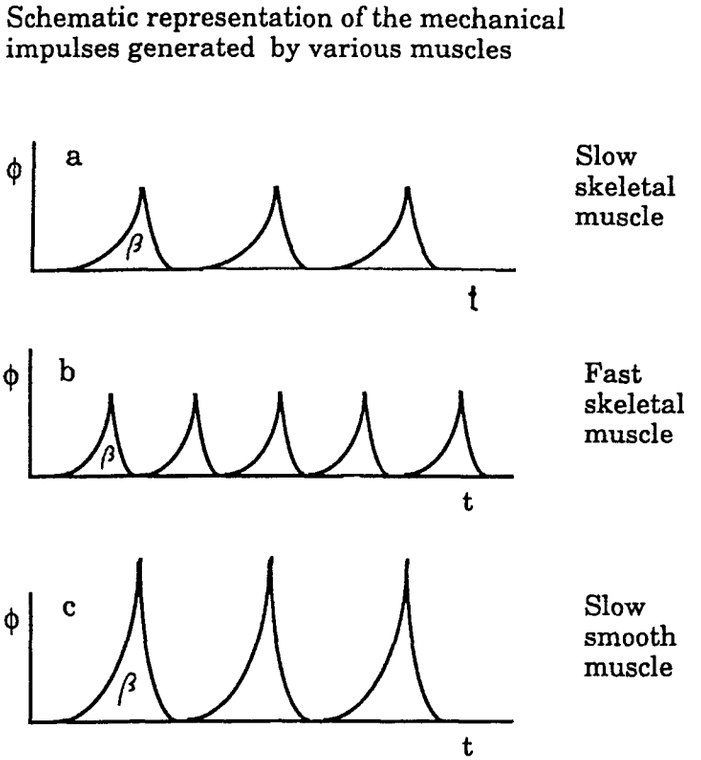

FIGURE 2. Schematic representation of the mechanical impulses generated by different muscles in an isometric tetanus. φ is the value of the repulsive force between a thin (i.e., actin) filament and an approaching or receding myosin head at time t. The area enclosed by each of the ascending-descending curves, that is, $\int \varphi \, dt$ is, by definition, the mechanical impulse (β). The number of such curves per unit time is equal to the number of ATP molecules hydrolyzed per head per unit time (ν). The *average* force detected by a transducer is $\bar{\varphi} = \beta\nu$ (Oplatka, 1972). For slow (Figure 2a) and fast (Figure 2b) skeletal muscles, the value of $\bar{\varphi}$ is nearly constant (as P_o is about the same for similar myosin contents, cf. Bárány, 1967). Figure 2c: apparently (see text), the maximal value of φ for smooth muscles is much larger than for striated muscles.

S-1 contain several "domains" that can give rise to "clefts", and as the approach of the myosin heads to the actin filaments in active muscle is not random and directional, hydrodynamic steering forces must be of special importance. Moreover, in this particular case, the driving force for the movement of the heads is the outcome of the liberation of water molecules, probably endowed with kinetic energy in excess of that of the already free water molecules in the inter-filamentous lattice. The distribution of the electrical charges and hydrophobic "pockets" at the surfaces of the proteins also affect, at least initially, the direction of flow of the liberated water molecules. These water flows determine the orientation of the moving heads, contribute to their velocity and kinetic energy, and hence can influence the distance of minimal approach to the actin filaments and therefore the magnitudes of the maximal and, the average force generated and of P_o. All these factors are probably responsible for Bagni et al.'s (1990) finding that changes in length, which affect the spacing between the filaments, can cause

a shift of the maximal force in an isometric tetanus to a smaller or a larger length, in hypertonic and hypotonic media, respectively. The fields of the repulsive long-range forces against which the proteins move can also be affected by the osmotic pressure, and this should also determine the path of movement. Muscular contraction is an extremely fast process, involving an enormous number of myosin heads. These simply cannot afford the luxury of exhibiting a random Brownian motion — they must be "delivered" promptly toward their target and hit a specific small region on or in the close vicinity of actin without any "trials and errors". This is particularly clear and obvious for shortening muscles where the actin filaments are continuously on the move. "Something" *outside* the protein molecules must *guide* them precisely and as quickly as possible to their target, producing the maximal force and the "best" movement. This complex role is played by the combination of the vectorially ejected water molecules (which were until then part and parcel of the protein molecules) and the hydration and electrostatic repulsive forces (which are determined by the surfaces of the proteins). Hence, the proteins are, at the same time, both the governors and the slaves of their fate. Similarly, two magnets put in close proximity move in the path determined by the magnetic fields that they themselves have created and that change continuously during motion.

The molecular mechanism of muscular contraction should in principle comprise *three* processes. (1) The signal that "arouses" the myosin heads and makes them "decide" to move toward the thin filaments when the muscle is stimulated. This must be associated with the binding of Ca^{2+} to both the myosin regulatory chain and to troponin. (2) The mechanism that *leads* the heads to their proper and exact site of interaction with actin, that is obviously more complicated in a shortening muscle. This question has never been asked or addressed to despite its fundamental and crucial importance. (3) The process by which force is generated. One should consider *two* different possibilities: (1) that the force develops while the heads are moving, and (2) that it is produced *following* attachment to actin. Only the second alternative has been considered by the SCBT and its modifications. Practically all efforts have been made in an attempt to solve the third problem, very few for the first and *none* for the second. A. F. Huxley (1980) has once referred, most vaguely, to the problem of steering by saying that "if one of the myosin-actin interactions which governs shortening speed were limited by the speed of the *restricted diffusion* movements that a myosin head presumably undergoes when it is not attached to a thin filament...." The major difference between the mechanism I propose and that adopted by the SCBT and by practically all other theories of muscle contraction is that isometric force is generated *while* the heads *approach* the thin filaments, whereas others take it for granted that force starts developing only *after* the head arrived at the actin filament and bound to it tightly; moreover, the "restricted diffusion movements" of the myosin heads are shown to be governed by the vectorial ejection of water and by the hydration and electrostatic fields of force, that is, it has nothing to do with "diffusion". It is part (actually the most important part) and parcel of the force-developing process.

So far, we have concentrated on isometric contraction. Activated unloaded muscles shorten at a constant velocity shortly after the initiation of shortening. This means that the net longitunal force acting on the muscle is zero, that is, no (net) force drives the movement. Liberation of water from the hydration shells of both proteins must occur in this case and this is very clear when an isometrically contracting muscle is suddenly

released. At this point it makes sense to believe that the water liberated from the hydration shells of the thin filaments is "ejected" in a direction parallel to the filaments, thus dragging them toward the center of the sarcomere. With respect to the myosin heads, they start moving toward the nearest thin filament but cannot approach it as much as in an isometric contraction where the maximal repulsive force can be reached. Thus, they make a partial contribution to the force "pushing" the thin filamets. The sum of this force and the force associated with the expulsion of water from the hydration layers of the thin filamets must be "neutralized" by an opposing force of equal value. This force is the sum of the load per filament and a sort of "viscous" force (Oplatka, 1972). Because the velocity of shsortening must decrease with increasing load, it is probable that in shortening loaded muscles, the contribution of the "ascend", followed by the "descend", of the myosin heads to the force balancing the load increases with increasing value of the load.

What makes the actin filaments slide past myosin in *unloaded* muscles when apparently no force is allowed to develop, that is, when the myosin heads do not seem to climb up the field of repulsive forces? The answer is probably that when muscle is stimulated the myosin heads are at a point at which the repulsive force starts operating, after the field of force had changed dramatically following the binding of Ca^{2+} ions to troponin, and the hydration layers of actin and myosin begin the release of water molecules. The "liberated" enthalpy is then endowed to these water molecules in the form of kinetic energy in excess of that of the surrounding water molecules. Due to topological and field factors associated with the surface of the proteins at the sites of ejection and to the existence of the fields of repulsive and attractive forces, these water molecules might create vectroial miniflows. Moreover, these flows might affect the orientation as well as the velocity and the direction of movement of the heads in isotonic as they do in isometric contractions. The actin filaments, which are supposed to liberate water from their hydration layers just like the myosin heads, will then be "carried" by the net flow that apparently has a component (or is fully) parallel to them (see Figure 1). As previously mentioned, this idea, in a vague form, was presented for the first time more than 20 years ago (Oplatka et al., 1974), when it was propsoed that the enthalpy change of ATP hydrolysis (that is *negative*) is *directly* converted into kinetic energy of all the participant of the process, that is, myosin, actin and particularly the hydrolysis products of the ATP hydrolysis reaction. The excessive kinetic energies were then supposed to be eventually transferred to the surrounding water molecules that would then flow in a well-defined direction, presumably along the actin filaments, causing their *passive* movement. This does not require myosin in a filamentous form. Indeed, as mentioned previously, actin filaments were found to move faster in solution when HMM and MgATP were added (Tirosh et al., 1990; Burlacu and Borejdo, 1992; Oplatka, 1994b). Long ago, *active* streaming against gravity of solutions containing acto-HMM, native tropomyosin, and MgATP in glass microcapillaries was observed (Tirosh and Oplatka, 1982).

Rome (1968) suggested that the formation and stability of parallel arrays of thick and thin filaments in striated muscles are the outcome of a balance between a repulsive electrostatic force and an attractive van der Waals force. Therefore, she was surprised noticing that after increasing the ionic strength, the interfilamentous spacing in relaxed glycerinated muscles *increased*, suggesting an increase in the repulsive force rather than the anticipated decrease. Because

repulsion between DNA molecules also increases on elevating the ionic strength and as this has been ascribed to an increase on the repulsive hydration forces (Rau et al., 1984) (which more than compensate for the decrease in the electrostatic repulsion), we may conclude that a repulsive hydration force operates also between the filaments in striated muscles, in addition to the electrostatic force, and that this also increases with increasing ionic strength, thus masking the dimunition of in the electrostatic force.

As indicated previously, Bagni et al. (1990) found that the force-length relationship of isometrically contracted muscle changed when the osmotic pressure of the medium was either lowered or increased relative to the "physiological" one, so that the maximum force was not observed at the point of maximal overlap but rather at a longer or at a shorter length, respectively. It should be stressed that the proportionality between the value of the isometric force and the degree of overlap of the two sets of filaments (for stretched muscles only) constitutes the major pillar of the SCBT. Unfortunately, many muscles do not seem to obey this Gordon et al.'s (1966) rule even at physiological osmotic stress. (For an extensive and thought-provoking survey see Pollack, 1983.) It is tempting to try and analyze these findings in terms of changes in the hydration shells of actin and myosin. For this purpose, let us examine ^{1}H-NMR studies of water in skeletal muscle, presented briefly by Yamada and Sugi (1989).

These authors confirmed and extended earlier work by Belton et al. (1972), who studied the transverse relaxation process of the intracellular water of frog skeletal muscle using ^{1}H-NMR spectroscopy and reported that relaxation represents three different processes corresponding to the protein-bound, inter-organelle, and free states of water. (Evidence for the existence of a minimum of two phases of ordered water in skeletal muscle was presented earlier by Hazelwood and Nichols [1969] on the basis of NMR signals by deuterium exchange and by vacuum drying.) When muscle bundles, immersed in normal Ringer solution, were stretched (from rest length) so that almost no overlap existed, the time constant of relaxation incresed by about 30%, indicating that water became more structured. The time constant increased also (by 150%) when the muscles were immersed in a hypotonic Ringer solution that caused an increase in the water content of the muscles. The reverse occurred in hypertonic solutions. It was concluded that the structure of the inter-organelle water changes depending on the physiological state, possibly due to structural changes of the actomyosin *lattice*. Thus, abolition of overlapping gives an effect on water that is similar in direction to that obtained by increasing the water content, even though spacing changed in *different* directions. This makes the suggestion that the changes in water structure are associated with the accompanying change in the *myofibril lattice* somewhat doubtful.

However, let us recall that in most of the range employed in osmotic stresses (and in many studies, in the whole range), the isometric force decreases after increasing the osmotic pressure, either by adding a nonpenetrating polymer or by increasing the ionic strength (cf. Ford et al., mentioned previously). I have tried to account for this by considering the possibility that after increasing the osmotic stress, more water is removed from the hydration shells thus damaging their capability to liberate kinetically active water molecules that are essential for the generation of "contractile" force. The reverse, that is, the increase in P_o after diminishing the ionic strength, suggests that then more and more c.b.s. are recruited. This explains the increase observed in the time constant of relaxation on decreasing the osmotic stress (and of its reversal after

increasing the tonicity). The fact that the value of this constant increases *also* after stretching the muscles therefore suggests that a decrease in spacing *also* leads to an elevation in the amount of bound water. *This* might be linked to the decrease in spacing per se in association with the increase in the repulsion exerted by *both* the hydration and the electrostatic forces. The observation that, in hypotonic media, the point of maximal tension is shifted to a length larger than rest length (i.e., to a length at which the degree of overlap is less than maximal) also suggests that the number of active c.b.s. is not the only factor that determines (at a given osmolarity) the value of P_o, and that the effect of the other operating factor(s) is better perceived at low osmolarity. It is not yet clear why is this (or maybe these) factor(s) much more pronounced for some types of stretched muscles and not for others under normal physiological conditions.

Despite the lack of evidence for the existence of a rigor state during the enzymic cycle, and as it is indeed difficult to think of tension being developed without even very short-lived tight bonds between the myosin heads and actin, it would only be fair to consider the possibility that such contact really exists even though it is most difficult to detect. This, however, will not bring to life the "elastic elements" developing the contractile force (due to the doubtful rotation of the heads) because of the most *unpleasant* finding that *free* HMM can induce tension generation and movement, particularly the acceleration of the movement of *free,* isolated actin filaments in solution (with no possible involvement of titin) (Tirosh et al., 1990; Burlacu and Borejdo, 1992, Oplatka, 1994 b). The S-1 and HMM employed in the *in vitro* motility assays are *not* free; they are nonspecfically bound to an artificial substrate and therefore can "afford" to give rise to movement and force by wigwagging a tail containing the light chains (cf. Spudich et al., 1995). Unfortunately, *all* the tails, in addition to the actin and ATP binding sites of some of the heads, *must* be *buried* in the nitrocellulose substrate and hold strong to it (which suggests that the substrate did *something* to the tail, including limiting its freedom to wiggle) and thus, making the values obtained for the velocity of movement of the actin filaments and for the sliding distance most doubtful. This must have been the *main* "scientific" reason for the 24 years of total neglect of the observation made with *free* active myosin fragments. To be fair, there was one exceptional case — an honorable and interesting statement made recently by A. F. Huxley (1993): "I am glad that professor Sugi reminded (?) us of the experiment of putting S-1 *back* (?) into ghost preparation and getting motion [referring to Borejdo and Oplatka, 1976], but I think most of us are more *conscious* (?) of the *in vitro* filament assays", that is, the assays that "re-discovered" the mechanochemical capability of active myosin fragments, with all that this rediscovery implies (see all the papers on this subject by Drs. Spudich and Yanagida who have *always* pretended to be the first, *totally* ignoring our much earliler work. See accusation of Dr. Yanagida on this matter by Dr. Sugi and Yanagida's false but smart evasion [Sugi, 1993]).

If indeed the myosin heads really "touch" the actin filaments then.

1. In *isometrically* contracting muscles the tilting angle, while in the rigor state at the end of a cycle cannot be 45° (the arrowheads pointing toward the center of the sarcomere) as in acto-HMM in solution in the absence of ATP or in unfixed rigor muscle because this would mean that the actin filaments slided. Hence, this rigor state is not identical with the "real" rigor state.

2. Frado and Craig (1992) reported that, in the absence of ATP, HMM molecules generally showed a regular, angled appearance, with both heads attached to the actin filament. In the presence of ATP, attached molecules showed a less-ordered structure, often with only one head attached. They concluded that a configuration *other* than the rigor structure occurs during the actomyosin crossbridge cycle. It is interesting that they ascribe the apparent binding to *weakly* attached head-ATP and head-ADP-Pi, rather than to bare heads, that is, heads that are *strongly* bound, forming a *real* rigor complex. Indeed, Frado and Craig state quite frankly that the finding that actin is decorated with HMM in the presence of ATP is surprising, considering the weak affinity of these proteins for each other under these conditions. They add that "in interpreting the results we should, therefore, consider possible, non-physiological reasons for the observations", which may cast doubts on the very attachment of the myosin heads to actin in their electron microscopic study of negatively stained preparations.
3. Any kind of rigor complex formation (between actin and myosin or myosin-ADP) should be expected to be accompanied by the liberation of water because it involves hydrophobic bonds formation and salt linkages, both of which are generally accompanied by the release of bound water. In the special case of actomyosin, much research has suggested that water is liberated when a rigor complex is formed due to the presence of well-defined, and now known (cf. Rayment et al., 1993), "patches" of both hydrophobic and ionic groups in both actin and S-1, which are believed to interact specifically with each other (cf. Zhao and Kawai, 1995; Highsmith, 1977, Diaz Banos et al., 1996); *also,* Tonomura and Takashita, 1972, for earlier experiments). I have previously discussed the work by Coats et al. (1985) on the isomerization of acto-S-1 from which I derived the number of water molecule (per head) liberated during the isomerization reaction that also occurs in the presence of ADP (Geeves, 1989) and is supposed to take place during or before the release of Pi, that is, before the formation of a rigor complex. If a rigor state really follows them, then *more* water must be liberated due to the bonds (mentioned previously) formed. This liberation, just like that occurring during the isomerizaton reaction, could be characterized by vectorial flows of water molecules, the direction of which being dictated by the chemical and electrostatic topology at the site(s) of ejection; furthermore, the water molecules must in this case also possess extra energy due to the increase in entropy (associated with the "melting" of bound water) enabling ΔH to be positive. The flows act on the actin filaments that are either passively dragged (in isotonic contractions) or stretched (in both isometric and loaded isotonic contractions thus contributing to the tension). The same process operates both in living muscle and in systems in which the myosin species is free HMM or S-1, or *free* one-headed myosin (myosin I).

In summary, liberation of water might take place in two steps: during the first one actin and the myosin heads are separated and the ejection of water during the isomerization reaction serves two purposes: (1) the steering and docking of the heads so as to ensure their quick and least wasteful arrival

at the proper point(s) of contact with actin; (2) the development of contractile force. The second step involves rigor bond(s) formation that also leads to extrusion of vectorially-flowing water, which also gives rise to force and movement.

The relevance is that if rigor complexes are or are not formed, tension generation and movement are due to the vectorial ejection of energetic water molecules from the hydration shells of the proteins.

VI. ANALYSIS OF THE MEANING OF THE VARIOUS SLIDING DISTANCES AND OF THE FACTORS AFFECTING THEIR VALUES

During the last 12 years, many members of the scientific community have devoted (too) much time in the desperate attempt to experimentally obtain values as close as possible to H. E. Huxley's sacred number of 10 nm for the sliding distance. Experiments have been carried out involving the measurement of the distance moved by an actin filament either in an *in vitro* assay (in which myosin, or a myosin active fragment, was immobilized on a surface) or in a myofibril and of the number of ATP molecules hydrolyzed per head at the same time. Surprisingly enough, the value of this "chemical" s.d., $\Delta L_3 = V_m/\upsilon$, varied from one laboratory to another: while Spudich and his colleagues claimed it was 5 to 8 nm, which, according to them, is "well within the geometric constraint of conformational change imposed by the size of the myosin head", that is, less than twice the chord length of the myosin head, $2 \times 18 = 36$ nm, Yanagida and others found much higher values — up to about 200 nm (cf. Burton, 1992; Oplatka, 1990, 1991a,b, 1994c). *Both* 5 to 8 and 200 nm deviate appreciably from twice the cord length of the myosin head. The only satisfying finding is the value of 8 nm, which is strikingly close to the erroneous value of 10 nm derived by H. E. Huxley.

Strangely enough, Yanagida, the most pathetic advocate of the idea that $\Delta L > 10$ nm, has not paid any attention to the fact that his first value for ΔL, determined for myofibrils (Yanagida et al., 1985), should differ, *by definition,* from that suggested by H. E. Huxley (defined as the distance covered by an actin filament when *each* of the myosin heads interacting with it splits one ATP molecule). On the other hand, Yanagida derived his s.d. as the unloaded velocity of a thin filament divided by the number $\times$ of ATP molecules hydrolyzed per second by *all* the myosin heads interacting with that filament. The latter is equal to the product of the average number of heads $\times$ times the turnover rate (number of ATP moleucles hydrolyzed per head per second). This means that his s.d. should be $\times$ times *smaller* than that employed by Huxley.

Some authors have "solved" the problem of the discrepancy between the large experimental values of ΔL_3 and the length of the myosin head by concluding that a head must undergo many *cycles* of attachment-detachment, that is, many rotations, before consuming a *whole* molecule of ATP. This, of course, leaves us with another question and that is what determines the *number* of such repetitive cycles, which appears to vary in a wide range. Moreover, how does this novel idea of multiple rotations reconcile with the principle, advocated for many years by practically all scientists, that there must be a *one-to-one* relationship between each step of the mechanical cycle and a corresponding stage in the enzymatic cycle, both involving a given head and only one (or maybe two) *specific* actin subunit(s) (cf. Irving, 1987; Botts et al., 1984).

Another problem that arose in this connection was, Is there, or is there no, *full*

coupling between ATP hydrolysis and the step distance? No doubt that movement is coupled to the utilization of ATP. However, does this necessitate a one-to-one relationship between the hydrolysis of one ATP molecule and a *particular* distance covered in an *isotonic* contraction that *must* be independent of state parameters, such as the magnitude of the load, temperature, ionic strength, presence of an organic solvent, osmotic stress, hydrostatic pressure, etc.? No doubt that the movement of a car is coupled to the burning of fuel, but would anyone claim the existence of "full coupling" only if the distance covered by a car per gallon of gasoline is exactly 1000 times the length of the car? We all know that the efficiency of the fuel depends on the speed; furthermore, we can uncouple the mechanochemical process if we put the gear box in neutral. With respect to the effect of load on ΔL_3: there is no movement, that is, $\Delta L_3 = 0$ if the muscle is held at a constant length despite the continuous splitting of ATP. In summary, $\Delta L_3 > 0$ for $P < P_o$ and is equal to zero when $P = P_o$. If indeed the value of ΔL_3 is determined only by the length of the myosin head, then we should expect it to be the same for *any* value of P except P_o. Obviously, the larger P is, the more "difficult" it will be for the heads to fully rotate from their initial angle to the final one of 45°; however, this should be considered as a matter of "convenience" but not of geometry. Interestingly enough, nobody has paid attention to an observation that is exactly as old as H. E. Huxley's rotating crossbridges theory. In the same year, Kushmerick and Davies (1969) measured the ATP turnover rate $\dot{n}$ as function of V for the frog sartorius muslce. Had ΔL_3 been constant then the plot of $\dot{n}$ vs. the velocity (which increases with decreasing load) should have been linear, that is, $\dot{n}$ being *proportional* to V, with a *discontinuity* at $V = 0$ when $\dot{n} = 0$, and the slope representing $1/\Delta L_3$. Inspection of the curve reveals that the value of ΔL_3 changes continuously with V (or P), reaching a maximum at $P = 0$. Indeed, Higuchi and Goldman (1991) found that the value of ΔL_3 decreases with increasing load.

There is no reason why at least some of the other parameters mentioned previously should not (in addition to the load) also affect the value of ΔL_3. Indeed, it was found (Maruyama et al., 1989) that the addition of 20% ethylene glycol caused a decrease of ΔL_3 by a factor of 12. An increase in temperature lowers ΔL_3, whereas after increasing the ionic strength, ΔL_3 increases. In summary, ΔL_3 cannot be a constant (Oplatka, 1990; 1991a,b, 1994c). Tension generation requires the breakdown of ATP, but we are familiar with muscle in rigor in which tension is maintained without the splitting of ATP so that in this case there is no room for discussion of the degree of coupling between a geometrical-mechanical parameter and ATP hydrolysis. Coupling is full in electrochemical cells where the chemical change is proportional to the amount of electrical charge passed. If we apply a counter voltage that exactly balances the electromotive force produced by the cell, there will be neither electrical current nor chemical change. This is because each ion has fixed mass and electrical change.

The value of ΔL_3 is entitled to vary, just like the average force generated per myosin head, not only after changing the environmental conditions, but also when it is measured for different actomyosin systems such as whole muscles, myofibrils, *in vitro* motility set-ups employing S-1, HMM, and molecular and filamentous myosins (on different substrates).

According to the picture presented previously the value of the "mechanical" s.d. (ΔL_2) obtained from length transients applied to isometrically contracting muscles during tetanus is the maximal distance cov-

ered by a head when it approaches actin against the repulsive force(s), that is, from the point at which water starts being liberated (the "weakly attached complex") to the point at which the driving ΔH vanishes and the force assumes its maximal value. From these experiments, the value appears to be about 12 nm (cf. Burton, 1992), which is indeed "well within the geometric constraint of conformational change imposed by the size of the myosin head", that is, less than twice the cord length of the myosin head, but, unfortunately for the followers of the SCBT, its origin and meaning have nothing to do with a geometrical parameter of the head. This value is close to the value of the maximal distance between two neighboring DNA molecules at which the repulsive hydration force is perceived by the macromolecules (Rau et al., 1984).

What determines the value of the "chemical" s.d. (ΔL_3), which has been estimated mainly from motility assays? In view of the enormous variation of the values obtained by various authors (in the range of 5 to 200 nm, differing by a factor of up to 40 or more), there is room for suspicion that something might be essentially wrong with the technique. Variation may originate from a wealth of artifacts (cf. Oplatka, 1990, 1991a,b, 1994c) associated with the nonspecific, ill-defined way the myosin molecules (even in the form of filaments), not to say S-1, bind to the nitrocellose (and in all probability differently to siliconized glass or to polylysine, etc.). The site that is attached to the surface (this could include the ATP- and/or the actin-binding site) is entitled to vary, it may assume different orientations or be located at points that are outside the track of the locomoted actin filaments. Methylcellulose is usually added in order to avoid the detachment of the actin filaments from the surface. The very effectivity of this substance must be due to an increase in the affinity of actin to the myosin heads, that is, the interaction does not take place between actin and myosin as such anymore, and the "complex" formed is rather actin-nitrocellulose-myosin, which in all probability does not exist in living muscle.

The staining of the actin filaments by a fluorescent derivative of phalloidin is not without effect on the enzymic properties and the mechanochemical capability of the filaments, as evidenced from seveal works carried out both *in vivo* and *in vitro* (Oplatka, 1994c). It is entitled to influence the essential force-producing isomerization reaction — just like the presence of ethylene glycol or a change in ionic strength in a solution containing acto-S-1 (cf. Coats et al., 1985). Therefore, it does not make too much sense to argue about the "true" value of ΔL_3.

Its determination by Yanagida et al. (1985) by measuring simultaneously the maximal velocity of shortening and the ATPase activity of *myofibrils* (about 60 nm) is much more reliable than those derived from the *in vitro* motility assays both because of the absence of the many artifacts mentioned previously and elsewhere (Oplatka 1990, 1994c) and because the number of active and properly oriented myosin heads was precisely known as well as the *true* and *exact* value of the number of ATP molecules hydrolyzed in association with the movement of the thin filaments. Unfortunatly, the value of 60 to 68 nm obtained by Yanagida is *not* at all that of ΔL_3 (which was evaluated later on for muscle fibers by Higuchi and Yanagida, 1991) but rather of another ΔL_1, ΔL_4: these values were found after dividing ΔL_3 by the number of myosin heads interacting with a given thin filament. From Yanagida's data

$$\Delta L_3 = \frac{V_m}{\upsilon} = \frac{5.3 \times 10^3}{1.0/2} = 10{,}600 \text{ nm (at } 5°C)$$

This enormous number should have made Yanagida even happier as it is 1060

times (and not just 6 to 8) larger than H. E. Huxley's value of 10 nm. As shown previously, after correcting Huxley's calculation of ΔL_1 (that is identical by definition with ΔL_3, the only difference being that it was derived on the basis of heat rather then chemical data) we get 325 nm. The discrepancy between the values of ΔL_3 and ΔL_1 (both determined for a muscle preparation and not from *in vitro* motility assays) is thus by a factor of 32.6. This might partly be ascribed to the fact that the shortening velocity of rabbit psoas myofibrils studied by Yanagida et al. is more than five times higher than the maximal velocity of the corresponding muscle fibers (Arata et al., 1977). Other possible reasons might include the employment by Yanagida of proteolytic enzymes for the complete removal of the Z-lines that could in principle profoundly affect various characteristics of contraction. It is most surprising but also interesting to note that Yanagida, after having published many papers in which it was claimed that ΔL_3 is *always* larger than the length of the myosin head, arguing with Spudich and collaborators, who have been loyal to the SCBT and *always* succeeded in getting "proper" values for ΔL_3 (even though variable and even though I wonder if the value of 5 nm is still proper), suddenly surrendered and finally came out with ΔL_3 of 17 nm (Ishijima et al., 1994). In order to save face, he says that "many power strokes (?) might be continuously produced during one ATPase cycle at low load, in agreement (?) with our previous results", that is, the value of 60 nm *he* originally obtained employing myofibrils (Yanagida et al., 1985) (cf. Burton, 1992, for a review of the works in which ΔL's were evaluated).

The value of ΔL_3 is probably governed by the value of ΔH^o that provides the kinetic energy to the flowing water molecules (and we shall see later on that ΔH^o might be different for smooth and striated muscles), by the ATP turnover rate (which differs, and should differ, from that of isometrically contracting muscles, cf. Kushmerick and Davies, 1969), and by the "resistance" to the movement of the actin filaments (Oplatka, 1996a). Hence, again, there is no reason for it to be constant, just like the value of $\bar{\varphi}$, the average maximal force per head, which also is a function of temperature, ionic strength, etc. The same factors obviously affect the values of the ATP turnover rate, the maximal velocity of shortening, the isometric force, and therefore the force-velocity relationship.

An interesting and most original outlook and interpreation of ΔL_3 has been presented recently by Spudich et al. (Uyeda et al., 1994). They state that "previously, a comparison of the enzymatic and motile activities of muscle (both skeletal and smooth) myosins from several animal species demonstrated a good correlation (actually proportionality, A.O) between ATPase activity and contraction speed (Bárány, 1967). In terms of kinetics, however, it is not clear why these two parameters should be tightly coupled". The last sentence is quite surprising in view of the fact that Spudich has done his best in recent years to convince everybody, employing his *in vitro* motility assay, that the value of ΔL_3 is compatible with the SCBT, that is, is close to the length of the myosin head (that is practically the same for all skeletal muscle myosins). With ΔL_3 as a constant, the maximal velocity of shortening (or movement of actin filaments), V_m, should be given, according to the very definition of ΔL_3, by $V = \Delta L_3.\nu$, where ν is the number of ATP molecules hydrolyzed per head per second, that is, V should be proportional to ν as found by Bárány. In other words, the SCBT claims that the velocity and the tunover rate should be *tightly* coupled. Spudich had fought Yanagida, who announced that because his ΔL_3's have always been much larger, that

kind of coupling does not exist. Conversely, (1) Yanagida *now* admits that $\Delta L_3 = 17$ nm (that is practically equal to the length of the myosin head, 18 nm) (Ishijima et al., 1994) and (2) "it is not clear" to Spudich why "these two parameters (i.e., velocity and ATPase) should be coupled". Spudich continues by saying, "It is believed that the force producing portion of the cycle follows or coincides with P_i release. The rate-limiting step of this cycle is proposed to go somewhere in the weakly bound state. Conversely, the contraction speed should be related to the force-generating strongly bound state and thus be relatively unaffected by the weakly bound state. Therefore, the observed correlation between the ATPase and motile activities of the skeletal muscle myosins is probably a consequence of functional optimization (?) of these proteins for particular purposes (?) rather than being casual or *obligatory*. It is reasonable that different structural features of the motor limit each of these activities and that mutagenesis should alter one activity without affecting the other" (Uyeda et al., 1994). Indeed, they constructed myosin mutants in which the actin-activated ATPase activity and the sliding velocity were disproportionately altered, which exhibit different values for ΔL_3.

Interestingly enough, despite his devotion to the SCBT, Spudich is not aware of the fact that in the paragraph quoted previously he deviates seriously from the picture advocated by the SCBT; according to the latter, the heads first rotate. This will stretch the mysterious "elastic elements" that generate force that pushes forward the attached thin filament by a distance that is dictated by the length of the head. The *value* of the force should not matter in unloaded isotonic contractions; according to the SCBT, the force generated in the "elastic elements" makes the thin filaments slide a distance of ΔL_3, that is determined by the geometry of the myosin head and then vanishes. Imagine a muscle in which the force assumes a value 1.6 times that developed in a rabbit skeletal muscle. Will the value of ΔL_3 be then, say 1.4×18 nm, 1.4 times the length of the head?

However, inspection of the ratios of the sliding velocity and the ATPase activity of the *parent* myosins (skeletal, cardiac, and smooth) reveals that for *these also* the ratio was not *constant* and varied from 0.2 to 0.8. (It was different for each of the four myosins.) Different by a factor of 4, while in the table presented by Bárány the ratio (for the same temperature, 35 to 36°C) varied between 0.51 and 0.80 units (for six myosins) and was not much different for 10 more myosins, including one from smooth muscle, which were examined at other temperatures. The actin-activated ATPase activities and the maximal velocities of shortening of the muscles from which the myosins had been extracted varied by a factor of 200. The value of ΔL_3 is sensitive to temperature and increases after lowering the temperature, that is one of the indications for its variability and its dependence on the environmental conditions (Oplatka, 1990, 1991a,b, 1994c).

The variability of the values of ΔL_3 derived by Spudich et al. for both the parent and the chimaeric myosin might be *another* indication for the fact that the *in vitro* motility assays are artifacts laden (Oplatka, 1990, 1994c). It is proposed that Spudich and Yanagida employ their *in vitro* motility assay for the elucidation of the value of ΔL_3 for the 16 muscles studied by Bárány (1967), but for God's sake use the *same* substrate (e.g., nitrocellulose), add a constant amount of either HMM or S-1 or molecularly dispersed myosin, spread it *evenly* on the substrate, and always work at the *same* temperature, ionic strength, etc. The fact that the value of ΔL_3, which came out from different laboratories at different times, has varied in such a wide range is not accidental

and reflects on the "reliability" of the assay. Spudich should therefore be more cautious when he interprets the variability he observed for the value of ΔL_3 for different *chimaeric* myosins he prepared.

In another communication, Spudich et al. (1995) tell the story of a mutant myosin that had a higher than normal ATPase rate, but it moved actin *in vitro* at only 1/10 the velocity of wild-type myosin. They concluded that the chemomechanical coupling has been affected severely. We must therefore conclude that while certain myosins obey the SCBT and exhibit maximal velocities of movement that are proportional to the ATP turnover rate, the proportionality constant being about equal to the length of the myosin head (which indicates that tension generation is due to the rotation of the myosin heads while tightly bound to actin), whereas other myosins are "uncoupled" (that is an elegant word for not behaving "properly") and their heads have either to attach and detach an unspecificed number of times for each ATP molecule hydrolyzed (as had been suggested by Yanagida), or when (as in the case of Spudich's mutant) the velocity is 1/10 the velocity of wild-type myosin, while its ATPase activity is higher, the value of ΔL_3 must be much smaller (e.g., 5 nm or less) than the length of the myosin head, which should create a problem of a different (opposite) type to the SCBT.

In a paper entitled *How Molecular Motors Work,* Spudich (1994) pretends he has solved the puzzle, without forgetting for a moment his long-term loyalty to the SCBT or having the slightest doubts about the reliability of the *in vitro* motility assays when it comes to *quantitative* measurements (of velocity, force, and sliding distance). He says, "Although the V_{max} values for the actin-activated ATPase activities of the various chimaeras differ by as much as a factor of nearly six, their velocities in the *in vitro* motility assay are the same within a factor of two. How can this be? *The answer to this question may be at the heart of how molecular motors (*i.e., the myosin heads, the actin molecules not being entitled to be an integral part of the motor but rather considered as passive rails) work". The answer is when the number of myosin heads interacting with an actin filament exceeds a certain small number, the velocity of movement is *not* given by d/ts, where d ($=\Delta L_3$) is the sliding distance and t_s is the time in which the strongly bound state continues. However, *by definition,* the maximal velocity of movement (or shortening) is d/t_c, where t_c is the total ATPase cycle time ($1/t_c$ is the turnover rate, the number ν of ATP molecules hydrolyzed per head per second). Instead of deriving the value of the unknown d from the values of the velocity and of the turnover rate, Spudich calculates the value of t_s as the ratio of d, which he considers as an universal constant with the value of 10 nm (i.e., about half the length of the myosin head) and the velocity. By the way, Spudich et al. claim that if the number of myosin heads interacting with an actin filament is relatively small, than the smaller the number the smaller should the velocity be so that, in the case of a single head, the velocity should be 25 times smaller than the maximal. This does not seem to be in accordance with one of the greatest predictions of the SCBT, that is, that the maximal velocity of shortening should be independent of overlap, that is, of the number of c.b.s facing a given thin filament in muscle (and the velocity is indeed constant, Huxley, 1980).

VII. MUSCULAR STIFFNESS

It seems obvious that the longitudinal stiffness of a muscle in any state requires some cytoskeletal continuity, that is, the presence of the protein network such as that

undoubtedly exists in rigor muscle. Until now it has been taken for granted that a similar network is created after activation, comprised of thick and thin filaments crosslinked to each other at many points by the c.b.s. This kind of crosslinking should cause also an *increase* in the lateral stiffness, just as is the case for a crosslinked rubber strip, the stiffness of which increases after increasing the degree of crosslinking. However, surprisingly enough, as already mentioned (Section III), Hatta et al. (1988) and Tsuchiya et al. (1993) reported that *both* longitudinal and transverse stiffnesses *increased* when a glycerinated muscle was transferred from a relaxing into a *rigor* solution, whereas when the muscle was activated the transverse stiffness *decreased,* while the longitudinal increased. This observation, to say the least, is not in line with the idea that crosslinking occurrred. In Section IV, an explanation has been presented for the decrease in transverse stiffness, which does *not* involve c.b. attachment. We are therefore entitled to consider other setups that may enable continuity, for example, that provided by the long titin molecules that extend between the two Z-lines of the same sarcomere. It has been claimed that titin is responsible for most of the stiffness of relaxed muscles. The increase in stiffness after activation might therefore be ascribed, at least partly, to an increase in the stiffness of titin, presumably by its increased affinity to the thin filaments, induced by Ca^{2+}, that was reported by Kellermayer and Granzier (1996). It is not impossible that after activation, the hydration shells of titin and/or nebulin are also affected thus contributing to the changes in both P_o and stiffness. The bound thin filaments themselves contribute to the increase in stiffness also because of the loss of water due to both the binding of Ca^{2+} to troponin and their interaction with the myosin heads. Thus, as mentioned previously, it was found by Burlacu and Borejdo (1992) that the flexibility of F-actin in solution *decreases* in the presence of HMM and MgATP. This is probably the outcome of the liberation of water bound to actin. Because, in an isometrically contracting muscle the same force should act on a thin filament in the I-band as in the region of overlap with myosin, it makes sense to believe that the stiffness induced in that part of the thin filament that interacts with the myosin heads should be relayed to the I-band part, presumably by forcing it also to extrude water. A threefold decrease in torsional rigidity of F-actin has been reported recently (Prochniewicz et al., 1996) after binding a single gelsolin molecule at the barbed end of the filament. In other words, a single small protein molecule can affect the structure and dynamics of a whole filament.

As we have seen previously, osmotic stress can affect the value of P_o, that is, either increase it initially (due to a shift of Equation 1 to the right) or, at higher stresses, cause a continuous decrease due to the "drying" of the hydration layers of the proteins (Ford et al., 1991). Similarly, it has been reported (Roos and Brady, 1990) that the stiffness of relaxed skinned cardiac myocytes increased in the presence of nonpenetrating polymers in a concentration-osmotic pressure-dependent relationship. The authors addressed the possible contribution of the megadalton proteins titin and nebulin that are associated with the thick (titin) and thin (titin and nebulin) filaments.

If this is true, then the increase in stiffness after activation is *not* necessarily due to the crosslinking of the thick to the thin filaments by the tightly bound c.b.s, as is the case in rigor muscle; moreover, the contribution of the thin (and very possibly also the thick) filaments to the muscle compliance must be *different* for active and rigor muscles because the degrees of hydration are most probably *not* the same; as indicated previously, water seems to be liber-

ated from the thin filaments both due to the binding of Ca^{2+} to troponin (that is probably relayed to the hydration shells of both tropomysin and actin) and to the force-producing isomerization reaction. In muscle in rigor (particularly in the absence of Ca^{2+}) neither of these two water-extruding process occurs (actually no process is occurring), and there is no reason to believe that the amount (and origin) of the water that had been ejected (once in the past) after forming the rigor complexes is the same as in the dynamic active muscle in which, at any moment, there exists a whole spectrum of states, both with respect to the extent of dehydration and to the distance from the thin filaments of the asynchronously operating heads. Hence, there is no sense in Higuchi et al.'s (1995) pretension to estimate the mechanical compliance (reciprocal of stiffness) of the thin filaments in active muscle from the compliance of skinned muscle fibers in *rigor*. They concluded that, considering that the sarcomeres are ~1.25 more compliant in active isometric contractions than in rigor, the thin filaments contribute ~44% to sarcomere compliance during isometric contraction. This value is considerably larger than other reported values (less than 20%) referred to in Higuchi et al.'s paper, in which measurements were performed with *stimulated* frog muscle fibers. We may therefore wonder if the difference could not derive from the employment of different muscles (skeletal rabbit and frog), that is hard to believe in view of the high degree of conservation of actin or, more probably, is associated with the fact that muscles at different *states* have been investigated in line with the arguments presented previously. Therefore, we should not, be at all surprised if the compliances of the thin filaments in active and in rigor muscles differ markedly, considering the differences in the nature, amount, and dynamics of the various interacting sepceis. The Ca^{2+} sensitivity of reconstituted thin filaments in the presence of an enzymatrically active myosin fragment is affected profoundly by the existence of a few rigor complexes in the presence of relatively low concentrations of ATP (Bremel and Weber, 1972). Following the "discovery" that the c.b.s; are not the *only* compliant elements in active muscle (cf. Huxley et al., 1994), stiffness stopped being a measure of the number of active c.b.s: it has earlier been taken *for granted* by advocates of the SCBT, contrary to experimental data (Oosawa et al., 1973), that the filaments are always rigid, irrespective of the state of muscle or of environmetnal conditions.

We may conclude that the factors affecting stiffness and the isometric force are not the same. Therefore, it is no surprise that

1. Stiffness is not proportional to P_o
2. That there is a time-lag between the developments of stiffness and of the isometric tension when a muscle is stimulated (cf. Bagni et al., 1988)
3. That P_o is significantly reduced by increases in tonicity, while the stiffness is hardly affected (Mansson, 1993)
4. That, at short sarcomere lengths, stiffness (as well as ATPase) remain elevated and close to their respective values at rest length even when the isometric force decreased to near zero levels (Stephenson et al., 1989). There are more cases from which it is clear that stiffness and isometric tension are not measures of the *same* parameters, that is, the number of active c.b.s. Each is affected differently by changes in the parameters of state and, above all, none of them, not even P_o, is (or should be) *proportional* to the number of active c.b.s (see detailed analysis of the concept of "independent force generators", its history and its very necessity in Oplatka, 1996a).

VIII. REGULATION MIGHT ALSO BE ASSOCIATED WITH RESTRUCTURING OF THE HYDRATION LAYERS OF THE "REGULATORY", IN CONJUNCTION WITH THE "MECHANOCHEMICAL", PROTEINS

Changes in hydration shells might account also for the regulatory role of Ca^{2+} in muscle; the binding of this ion to troponin probably affects the hydration shells of troponin and possibly also of tropomyosin and therefore their interaction with actin, thus making the hydration shell of the thin filaments in muscle similar to that of troponin-tropomyosin-free F-actin, which enables them to undergo the force-generating isomerization reaction together with the myosin heads (and also enhance the ATPase activity). The binding of Ca^{2+} ions to troponin must be accompanied by the release of part of the strongly bound water of the Ca^{2+} ions and of troponin. Thus, the reaction is actually

$$nCa^{2+} + \text{troponin} \leftrightarrow nCa^{2+}\text{-troponin} + \textit{water} \quad (2)$$

Application of osmotic stress should then shift the equilibrium to the right by removing water, and this means increase in Ca^{2+} sensitivity that has indeed been observed by Stephenson and Wendt (1984). However, there is no reason why should the Ca^{2+} requirements for the stimulation of the ATPase activity and for mechanochemical coupling be the *same*. Therefore, it is not be surprising that whereas translocation of fluorescent reconstituted thin filaments in the presence of myosin and MgATP could be observed at $pCa^{2+} > 6$, no movement could be detected when the pCa^{2+} was lowered, despite the fact that the actomyosin still exhibited a significant ATPase activity (Honda et al., 1986; Oplatka, 1989). Similar arguments can be applied to the mechanism of myosin-linked regulation; binding of Ca^{2+} to the myosin regulatory light chain (both in scallop and in rabbit myosin) should lead to the restructuring of the hydration shells of both Ca^{2+} and the light chain and involve the liberation of water, thus "permitting" the existence of the force-producing isomerization reaction that is apparently inhibited in the absence of Ca^{2+}, as is the case of actin-linked regulation (as claimed by Geeves and Halsall, 1987).

A decrease in the Ca^{2+} sensitivity of troponin- and tropomyosin-containing actomyosin (from rabbit skeletal muscles) was observed after removing the "DTNB" light chain of myosin with DTNB (5,5′-dithio-*bis*–2-nitrobenzoic acid), which binds Ca^{2+} but does not induce Ca^{2+} sensitivity in regulatory protein-free actomyosin. This suggested that, in skeletal muscle, regulation (that is generally considered to be linked *only* to actin) is a process involving the DTNB light chain also, probably *in cooperation* with troponin and tropomyosin (Werber and Oplatka, 1974). This cooperativity is probably mediated by the combined hydration shells of the myosin (heavy as well as light) chains and the actin complex rather than by a physical contact (i.e., by rigor complex formation) between the regulatory light chains of myosin and the regulatory proteins associated with actin. The removal of the light chain by DTNB probably causes a change in the hydration layer of myosin and, therefore, may affect the regualtive capability of troponin-tropomyosin. Needless to say, the structures of a hydration shell are determined mainly by that of the protein surface (and vice versa), just like the relationship between glove and fingers.

H. E. Huxley's steric blocking mechanism of muscle contraction, just like the

swinging crossbridge theory, takes it *for granted* that the myosin heads *must* firmly bind to the thin filaments for tension to develop. The attachment of Ca^{2+} ions to troponin is assumed to cause a shift of the troponiosin molecules so as to "uncover" the masked (during relaxation) sites on actin to which the heads have to bind. However, as I have tried to demonstrate, there is no such binding during tension generation or movement so that, unfortunately, *both* the steric blocking mechanism and the SCBT should face the same fate, that is, *both simultaneously* must become obsolete (Oplatka, 1996c, 1997). Moreover, as hinted previously, both tension generation and regulation appear to be based on structural changes in the hydration shells of all the proteins involved, that is, both the "mechanochemical" and the "regulatory" proteins. Because in muscle, actin, tropomyosin, and troponin are intimately bound together (as are the regulatory light chain, the "alkali" light chains, and the heavy chains of myosin), and as the hydration shells of *all* these proteins "recognize" each other, the very distinction between regulation and mechanochemical transduction becomes meaningless. It is as real as the artificial and harmful old separation between the "maintenance heat" (i.e., the heat evolved by isometrically contracting muscles) and the "shortening heat". In other words, in *muscle*, tension generation and regualtion are inseparable, the hydration shells of the "contractile" and the "regulatory" proteins " communicating" with each other and undergoing intimately linked structural changes (involving the ejection of vectorially moving and mechanically active water molecules) associated with the binding of Ca^{2+} and with the force-generating isomerization reaction.

Vectorial injection of water molecules after binding of Ca^{2+} ions to both troponin and the regulatory light chain of myosin may account also for the slight decrease in tension (originally resting) that precedes the development of active tension in isometrically stimulated muscles (named "latency relaxation", cf. Sandow, 1966). The force associated with this water ejection must then have a component that is parallel to the muscle fiber axis, pointing toward the Z-bands. The other component, that is perpendicular to the arrays of thin and thick filaments, must be cancelled due to its pointing in different directions along, at least, the thin filament, resulting from the helical structure the latter. It was reported (Eberstin and Rosenfalck, 1963) that during a twitch there is a decrease in the amount of light scattered by muscles, which follows roughly the time course of the tension. This has been ascribed to the transfer of water from the myofibrils into the sarcoplasm. The same explanation was given to the parallel decrease in birefringence. The facts that the latter starts *decreasing* before tension starts to develop and that it reaches its minimum before tension becomes maximal may be accounted for if we assume that water ejection by the Ca^{2+} "*excitation*" of the proteins preceeds water release associated with the "contractile" force generation by the *interacting* proteins. In line with this, we may predict that in a twitch water starts to be reabsorbed prior to the beginning of the decline of P_o. This can be followed with optical techniques.

In the following, I discuss several cases in which muscles do not generate force or shorten despite the fact that they are fully enzymatically active in light of the idea that the liberation of water is responsible for force generation and movement.

IX. THE SIGNIFICATION OF UNCOUPLING BETWEEN MECHANICAL PERFORMANCE AND ATPase ACTIVITY

Long ago (Oplatka et al., 1983) it has been proposed that the study of cases in

which muscles and other model actomyosin systems are incapable of generating tension, shortening, or exhibiting superprecipitation while being enzymatically active (i.e., *uncoupled* systems) should in principle help us in understanding what is required for *coupling* to exist, that is, the molecular mechanism of the mechanochemical *transduction* process, which is the main goal of muscle research. Many cases have been reported in which chemical modification of either actin or myosin led to the abolishment of mechanochemical transduction, even though the actin-activated ATPase activity of myosin (or of enzymatically active myosin fragments) had not been impaired. Thus, after treating F-actin with glutaraldehyde, crosslinking of the actin subunits to each other occurred (Gadasi et al., 1974). Activation of the ATPase activity of myosin by such actin was not impaired (it often *increased*) but superprecipitation, which has been utilized extensively for many years as an *in vitro* mechanochemical assay (which does not involve the many artifacts inherent in the nowadays fashionable so-called "*in vitro* motility assays", cf. Oplatka, 1990, 1994c), could not be detected. Furthermore, myosin at high ionic strength (that is molecularly dispersed) attaches to ghost myofibrils (i.e., myofibrils from which myosin had been extracted), as evidenced from the darkening under the microscope of the remaining actin-containing I-bands after rendering the latter insoluble in KI by glutaraldehyde, but no contraction would occur following the addition of MgATP at low ionic strength (Oplatka et al., 1983). Intermolecular crosslinking of the G-actin subunits by itself was probably not responsible for the *uncoupling* of ATPase activity and mechanical performance as F-actin intermolecularly crosslinked by *p*-phenylene dimaleimide is competent both enzymatically and with respect to superprecipitation (Knight and Offer, 1978). Furthermore, when ghost myofibrils were treated with phalloidin, that is known to specifically and noncovalently crosslink actin subunits in F-actin, the I-bands do not disappear following the addition of KI, myosin would bind and contraction would occur (Oplatka et al., 1983). Furthermore, the enhancement of translational motion of reconstituted thin filaments in solution by HMM and MgATP was found to be abolished when actin had been treated previously with glutaraldehyde (Tirosh et al., 1990). Apparently, glutaraldehyde makes actin hydrophobic by covalently binding to a multitude of hydrophilic groups on its surface, this drastically modifying its hydration shell. The fact that the enzymic activity was preserved suggests that

1. Activation of the ATPase activity by actin is necessary but not sufficient for mechanical performances
2. The hydration shell of actin seems to play a role in mechanochemical transduction, in support of the discussion presented previously
3. If the isomerization reaction (that is accompanied by the liberation of water molecules from the hydration shells of the proteins and appears to be responsible for tension generation) cannot occur in actomyosin in which the hydration shell of one of the components (in this case actin) is not anymore that particular shell that exists in active muscle, then mechanochemical transduction should be abolished if indeed liberation of water molecules from the hydration shells is necessary for it to happen

Obviously, as the hydrolysis products of ATP (at least ADP) are released from the myosin-crosslinked actin system without the existence of the isomerization reaction, the enzymic cycle *must* follow a different path.

This idea supports by the claim made by Geeves and Halsall (1987) that troponin-tropomyosin inhibits actomyosin ATPase by inhibiting the isomerization reaction in the absence of Ca^{2+}. Poo and Hartshorne (1976) confirmed the finding that glutaraldehyde-crosslinked F-actin retains the ability to activate the ATPase activity of S-1 but the resultant ATPase was *not* controlled by the regulatory proteins. Fluorescent energy transfer measurements implied that the crosslinked actin was "frozen" in the active state. Hence, the interaction between the hydration shells of tropomyosin-troponin and that of actin apparently changes following the modification of the actin shell and this can affect Ca^{2+} sensitivity. Uncoupling by glutaraldehyde treatment of F-actin has been "re-discovered" recently by Prochniewicz and Yanagida (1990) employing the more fashionable *in vitro* motility assay. The same group (Prochniewicz et al., 1993) also observed inhibition of sliding of copolymers of unmodified with internally crosslinked actin monomers despite the retainment of the V_{max} of the actin-activated ATPase activity. Kwon et al. (1994) have treated only lysine-326 and lysine-32 in G-actin by a photolabile crosslinker and found that photolysis enhanced actin-activated ATPase activity of scallop myosin 3 to 4 fold, but the filaments exhibited "poor" movement on HMM (only 20% of the filaments moved, at a velocity that was only 50% of the "normal"). This was quite surprising to the authors who stated, "the sliding force in muscle contraction is presumably produced by the conformational change of myosin (which was presumably intact in their experiments) during the cycle of ATP hydrolysis *while* it *attaches* to actin filaments that transmit the sliding force *produced by myosin*"

Modification of myosin also can lead to uncoupling. Thus, the blocking of the -SH2 group in the myosin heads by *N*-ethyl maleimide (Strivastava et al., 1981) abolished the contraction of actomyosin threads, while the actin-stimulated ATPase activity was not affected. The fact that modification of just *two* lysines in F-actin (Kwon et al., 1994) or of only *one* functional group in myosin was sufficient for the inhibition of mechanochemical transduction suggests that even an apparently minute change in the hydration shell of one of the proteins can be harmful, probably because it is propagated in a much larger area of the hydration shell, thus presumably abolishing the force-generating isomerization reaction while forcing the ATPase cycle to follow a different, nonforce producing, route (that can certainly be easily tested). Removal of the regulatory light chain from clam foot myosin was found to lead not only to the loss of Ca^{2+} sensitivity but also to inhibition of superprecipitation with actin (Ashiba et al., 1980). Another chemical modification of S-1, the result of that is nowadays considered to have caused a "revolution" in muscle research, has been presented recently by Rayment and collaborators (cf. Rayment et al., 1993); at last, it was possible to obtain S-1 crystals suitable for a high-resolution X-ray structural analysis, a goal that has strongly been believed to practically solve the problem of the molecular mechanism of muscle contraction (as if myosin *alone* is involved). This was achieved by treatment with formaldehyde (which should modify the hydrophilic groups similarly to glutanaldehyde and could lead to similar effects on the hydration layer of S-1 molecules). Thus, essentially all the lysine (97%) residues have been methylated, which means that practically all the hydrophilic -NH2 groups have been rendered hydrophobic. According to the authors, this was the *key* step to obtain S-1 crystals that are adequate to determine a high-resolution structure. After comparing this structure with that of actin, the authors have speculated on the geometry and chemistry of the interac-

tion (i.e., *in rigor* complex formation) between the two proteins. Unfortunately, in a later publication (White and Rayment, 1993) it was admitted that the maximum steady-state rate of ATP hydrolysis by the methylated S-1 at saturating actin is less than *one tenth* that observed for the unmodified protein.

Even sadder are the facts (Phan et al., 1994) that the methylation significantly decreases the affinity for actin in rigor, impairs the "coupling" between the actin- and nucleotide-binding sites, as well as the "communication" between other sites on S-1 (including that between the nucleotide-binding site and -SH1, which have been followed so extensively with the hope that it will reveal the secret of the mechanism of muscle contraction [cf. Botts et al., 1984]) and, above all, causes a *complete* loss of *in vitro* motility of actin filaments over methylated HMM. It was concluded by Phan et al. that, "these relatively mild (?) but numerous and important changes impair the function of S-1" (and it is the *function* that we all are trying to elucidate). It is a basic principle of scientific research that a measurement should not (or only minimally) affect the properties of the system investigated. The beauty of the S-1 crystals (and the possibility to use them in X-ray studies) cannot be considered to be more important than the scientific truth (that is undoubtedly beautiful, too). Apparently, the exhaustive methylation does not allow the most probably profoundly modified hydration layer of the myosin heads to interact properly with that of actin so that the isomerization reaction, including its accompanying liberation of water molecules and the rise in enthalpy, cannot occur and/or that the hydration forces are now much smaller and/or that less water molecules are ejected and/or that ejection is now less vectorial or less energetic or in the wrong direction. These changes are reminiscent of the changes induced by binding a fluorescent or EPR probe to -SH1, that is, *single* group modification that has been used so widely in the attempt to follow changes in the tilt angle of myosin heads during contraction. I have already mentioned Fajer et al.'s (1990) report that after binding of an EPR probe to -SH1, a fraction of the heads exhibited the rigor tilt angle of 45°. This did not withhold them from believing that this means that the heads undergo *rotation* (because they must have assumed a different angle before activation), despite the fact that the ATPase activity of their muscles was about half that of unmodified muscles. Apparently, this seemingly slight chemical modification was capable of rendering many heads incapable of *actively* interacting with actin and behave as if ATP was absent, which made them form permanent rigor complexes, leading to a lower ATPase activity and to a (permanenent) tilt angle of 45°.

In conclusion, generally speaking, uncoupling following chemical modification of either actin or myosin heads is probably associated with the abolishment of the isomerization reaction or of its capability to liberate water, or in the energy and directionality of the liberated water or with a drastic change in the hydration and electrostatic repulsive force fields. ADP and Pi are then released, but presumably by a *different* route. The fact that in some of the cases discussed previously the actin-activated ATPase activity is higher, that is, not equal to that of the unmodified proteins, might lend support to this idea (as there is no reason why the different enzymic cycles should proceed at the same rate). It is therefore suggested to study possible changes in the ATPase cycle of the previously-mentioned (and other) uncoupled systems in solution and, particularly, to check for the very existence of the isomerization reaction, including the evaluation of the temperature dependence of its equilibrium constant from which the value of ΔH° can be

calculated. Also, the effect of hydrostatic pressure, which, as mentioned previously, provides us with the value of $\Delta V°$, which is a measure of the amount of liberated water, should be elucidated.

It should also be interesting to repeat Coats et al.'s (1985) experiments with acto-HMM, predicting that the value of $\Delta V°$ per head will be larger than that calculated from the acto-S-1 experiments; as mentioned previously, the number of water molecules liberated per head by active muscle from Bratton et al.'s (1965) NMR studies seem to be about twice as large as that calculated from Coats et al.'s experiments in which acto-S-1 in solution in the absence of nucleotide(s) was investigated. It should not be surprising if the value of $\Delta H°$ per mole HMM to be derived from acto-HMM studies will turn out to be larger than twice the value found per mole of S-1 from the acto-S-1 experiments. This could also account for the finding that after irrigating by HMM skinned muscle strips in which myosin had been poisoned, the tension generated on adding MgATP is larger than that obtained with S-1 at the same concentration of heads (Borejdo and Oplatka, 1976).

There are also cases in which tension generation and the enzymic activity become uncoupled without chemically modifying any of the proteins. Thus, Krasner and Maugham (1984) have studied the relationship between isometric tension and ATPase activity in osmotically compressed skinned muscle fibers of the rabbit (by adding increasing concentrations of dextran to the bathing media). An increase in tension was initially observed, followed by a sharp decrease, as in Ford et al.'s (1991) work mentioned previously. At the same time, the average ATPase of the fibers decreased monotonically. With 0.22 g/ml, polymer tension was abolished, while ATPase was still 20% of the value in dextran-free solution. The fall in tension might be due to a decrease in the value of the equilibrium constant of the isomerization reaction. It is to be expected that in the partly dehydrated shells only very strongly bound water is left; moreover, there is much less water left to be liberated, that is, the isomerization reaction cannot take place anymore, or only to a small extent. The hydration forces between hydrated lipid layers have been found to depend on the water activity (Cevc and Marsh, 1987). It is therefore reasonable to assume that osmotic stress, by decreasing the water activity, affects the repulsive hydration forces also between F-actin and the myosin heads, thus leading to a decrease in tension. It has been found that the addition of polyethylene glycol to a biological membrane caused aggregation of Na^+, K^+–ATPase molecules (Esmann et al., 1994). This has been ascribed to dehydration caused by the reduction in water activity. Thus, it is possible that, in the presence of relatively high concentrations of dextran, dehydrated myosin heads and actin might form a sort of rigor complexes that are incapable of producing force, thus leading to decline of P_o.

I have mentioned previously (Section VII) another case in which uncoupling occurs without any chemical modification of either of the proteins, in this case by changing the Ca^{2+} ions concentration. While translocation of fluorescently labeled reconstituted thin filaments in the presence of myosin and MgATP could be observed at $pCa^{2+} > 6$, no movement could be detected when the pCa^{2+} was lowered, despite the fact that the actomyosin still exhibited a significant ATPase activity (Honda et al., 1986; Oplatka, 1989). Thus, an "excess" of Ca^{2+} ions abolishes mechanochemical transductions, probably by binding to relatively low-affinitly binding sites in one or more of the proteins involved (troponin, actin, myosin, and tropomyosin). It makes sense that excessive dehydration due to excessive Ca^{2+} binding prevents the force-generating

isomerization reaction. In summary, uncoupling may result from chemical modification, osmotic stress, or excessive cation binding (or EDTA). All these are probably associated with alterations in the hydration shells of the proteins, particularly involving dehydration.

Thus, it seems that the requirements for mechanochemical transduction are more strict and limited to narrower regions of various paramenters than those needed for the activation of the enzymic activity of myosin (which, it must be stressed, can occur even in the absence of actin). I am convinced that this point is extrememly important and much thought should be devoted to it. Just as an illustration for the claim made previously that in uncoupled systems the ATPase cycle probably follows a different route that is devoid of the isomerization reaction: it is obvious that ATP hydrolysis during Ca^{2+} activation of the ATPase activity of myosin must assume a different path than in muscle. This may be considered as another illustration for the mechanochemical *impotence* of the myosin "motor" when actin is absent.

As indicated previously, Burlacu and Borejdo (1992), who observed (like Tirosh et al., 1990) that the addition of ATP to acto-HMM in solution accelerated the motion of the actin filaments, also noticed that ATP caused a *decrease* in the flexibility of the filaments (contrary to Oosawa et al., 1973). It makes sense that the decrease in flexibility, that is, the increase in rigidity, of mechanochemically active F-actin (with or without troponin-tropomyosin) is associated with the loss of water from its hydration shell, due to the binding of Ca^{2+} to troponin (see Equation 2) and/or to the isomerization reaction (Equation 1). On the other hand, these authors reported that the addition of HMM to F-actin in the absence of ATP did not cause any change in the flexibility of the filaments. As mentioned previously, rigor complex formation has been claimed by many authors to involve the formaiton of hydroptobic bonds and of salt linkages, both of which are supposed to lead to the release of water (cf. Zhao and Kawai, 1995; Highsmith, 1977, 1996; Diaz Banos et al., 1996). Thus, it seems that "active" actomyosin "complexes" differ from rigor complexes in *at least* two respects: (1) in the first actin is less flexible than free actin, while in the latter there is no change in flexibility, and (2) water appears to be liberated from actin (and probably also from the myosin heads) as evidenced from the fact that in the active state they differ in shape from free S-1 (cf. Frado and Craig, 1992; Hirose and Wakabayashi, 1993), while water is released only from the hydration shells of the heads after forming a rigor complex. Therefore, it would be most interesting to measure the flexibility of actin filaments, with and without HMM or S-1, both in the presence and in the absence of MgATP, in systems in which mechanochemical coupling had been partly or fully abolished (while the ATPase activity is retained) by chemical modification or by other means, such as osmotic stress, and compare the flexibility to that in coupled systems.

It is also suggested to prepare muscles in which one of the two myosin heads had been cut or poisoned and study the influence of temperature, hydrostatic pressure, and ionic strength on tension and on the equilibrium constant of the isomerization reaction of the corresponding actin-HMM preparations in solution. Similarly, the effect of these variables on the isomerization reaction in systems in which troponin (or one of its components) had been removed and/or in which troponin and/or tropmyosin had been chemically modified. In all probability, the hydration layers of *all* the participants of the process of mechanochemical energy transduction in muscle, that is, actin, tropomyosin, troponin, and myosin

(both heavy and light chains), have been tailormade for this purpose, just like, and together with, the proteins themselves.

X. IMPLICATIONS TO OTHER BIOLOGICAL ENGINES AND TO ION AND WATER TRANSPORTERS

A. Smooth Muscles and Actomyosin Engines in Non-Muscle Cells

Smooth muscles exhibit a bell-shaped force-length relationship, just like striated muscles (cf. Gordon and Siegman, 1971). It would be awkward to explain this relationship for smooth muscle on the basis of "the structural basis of muscular contraction", when the structures formed by actin and myosin are so different for the two types of muscle. However, if we accept the idea that the words "structural basis" should apply to the structural changes undergone by the hydration layers rather than by the proteins to which they are bound (sometimes called "phase transitions"), then a solution to the problem might be found: the relationship, for *both* smooth *and* striated muscles, may be associated with a change with length of the hydration and electrostatic forces and not only with a variation in the *number* of "independent force generators". Let us just recall again the challenge made by the results obtained by Bagni et al. (1990) to the force-length relationship of Gordon et al. (1966), who claimed that under "physiological" conditions the maximal isometric force in a tetanically contracting muscle is attained at the maximal degree of overlap between the two sets of filaments, that is, when all the c.b.s are entitled to generate force by interaction with actin, and the force then decreases linearly with length as the muscle is stretched. Bagni et al. found that the force-length relationship changed when the osmotic pressure of the medium was either increased or decreased: the maximal force was not observed any longer at the length corresponding to maximal overlap but rather at a shorter or at a longer length (respectively), that is, the maximal force was exhibited when the number of "force generators" differed from the maximal. Like Bagni et al., I associate this with the change in spacing that accompanies length changes that, for the same length, must be different for different osmolarities. However, trying to answer this obvious question *how* could spacing affect tension, I draw attention to the change in the thermodynamic activity of water that would cause a shift, either to the right or to the left, in Equation 1, the extent of which, as well as the shape of the fields of repulsive forces, might depend on the spacing and therefore on the length in such a manner as to give a bell-shapped force-length relationship with smooth muscles also and to possibly shift this relationship (that is similar but not identical with that for striated muscle) to the left or to the right as in the case of striated muscles after changing the osmolarity. This can be checked. It should not be surprising if similar shifts will be observed *also* after changing the temperature, ionic strength, hydrostatic pressure, and by adding an organic solvent.

The mystery of the isometric tension exhibited by smooth muscles being larger than that of striated muscles despite the fact that much lower concentrations of myosin are present (cf. Murphy et al., 1974) might be deciphered if we assume that the values of $\Delta V°$, $\Delta H°$, and $\Delta S°$ (and therefore the number of water molecules liberated per head) of the isomerization reaction (if this really exists) are much larger in smooth muscles, thus possibly enabling a higher value for $\Delta H°$, which will allow the heads to

get closer to the actin filaments, that is, to reach a higher maximal force than in striated muscles and therefore to have larger impulses, β, which would lead, for the same ATP turnover rate, to P_o larger than that exhibited by striated muscles (Figure 2a,c).

As is well known, the non-muscle cells myosin II, double-headed myosin that contains a tail (like muscle myosin), is more similar to smooth than to striated muscle myosin (cf. Korn and Hammer, 1988). There is clear evidence that it actively participates together with actin in various motility phenomena such as cytoplasmic streaming and amoeboid locomotion. Similarly, the single-headed, tailless myosin-I is mechanochemically active (cf. Pollard et al., 1991). The *real* (i.e., not from *in vitro* motility assays) value of the force that myosin I and myosin II, together with actin, generate is not known, but it makes sense to speculate that because of the similarity to smooth muscle myosin and as their concentrations are far below even that of smooth muscles (and still they must fulfill many different roles and therefore be organized or fitted into different structures in different parts of the cell at different times), the force they can generate exceeds that of their counterparts in smooth, not to say in striated, muscles.

B. Microtable Based Engines

Because the laws governing the behavior of hydration and electrostatic forces are similar for different macromolecules, for example, collagen, DNA (Leikin et al., 1994); Rau and Parsegian, 1992), it makes sense to believe that there is a similarity between the basic principles underlying the operation of actomyosin engines and the so-called "microtubule-based motors", which actually means engines comprising not only the "motors" kinesin or dynein, etc. (which are ATPases like myosin), but also microtubules, the *linear* polymer analogous to the F-actin. The microtubules (m.t.), just like F-actin, activate the ATPase of their partners and are translated past them in the presence of ATP. It is therefore not surprising that the force generated by a dynein molecule interacting with a m.t. is strikingly similar to that developed per myosin head in striated muscles (which does not differ much from one striated muscle to another) (Kamimura and Takayashi, 1981; Oplatka, 1972). Similar values have been measured recently for kinesin (Svoboda and Block, 1994). This fact has not raised the trivial and fundamental question how could these systems, which are so different both chemically and structurally, share practically the same capability to develop force. It is generally accepted (by practrically everyone except A. F. Huxley) that the SCBT must apply, maybe with some modifications, also to the m.t.-based "motors". Because, according to the SCBT, the force is developed in "elastic elements", which are stretched when the myosin heads rotate or tilt, we should, for consistency reasons, conclude that the two different families of engines contain similar elastic elements, that is, the element nobody knows of its nature or exact location except that it connects the heads to the core of the myosin filaments or, in the case of so-called *in vitro* motility assays, to the nitrocellulose or silicone coating of a glass surface in which myosin or kinesin heads are embedded.

Therefore, it is suggested to check the possibility that the interaction of dynein, kinesin, etc. with m.t.s in solution also involves an isomerization step similar to that found by Coats et al. (1985) for acto-S-1, and that the equilibrium constant for this reaction varies in the same direction as the value of the force generated when the m.t.s are not allowed to move, after raising the

temperature, the ionic strength or applying hydrostatic pressure or adding a water-miscible organic solvent, as is the case with acto-S-1. From the variation with temperature and pressure of the equilibrium constant, the values of $\Delta V°$, $\Delta S°$, and $\Delta H°$ can be calculated and an estimate made of the number of liberated water molecules, when compared with the corresponding values for striated (and smooth) muscles acto-S-1 or HMM. The similarity of the values of the force generated "by" myosin, dynein and kinesin already suggests that the values of $\Delta H°$, $\Delta S°$, and $\Delta V°$ should not be much different — if indeed an isomerization reaction of a similar type exists also during the enzymic cycle of the "m.t.-based motors".

XI. CONCLUSIONS

Muscle has been considered to be an enthalpy-driven device because ATP hydrolysis, the ultimate energy source, is accompanied by a *drop* in enthalpy. In contrast, the bacterial flagella motor, that is a rotary device, can be entropy driven (by a trans-membrane pH difference) as well as enthalpy driven (by an electrical potential difference) (Macnab, 1983). According to the picture depicted previously, the generation of force and movement in muscle is associated with a *rise* in enthalpy, which is enabled by an increase in entropy $\Delta S > O$. The process is thus *directly* driven by a change in enthalpy (positive rather than negative), but *indirectly* is an entropy-driven process like many other important biological processes, such as protoplasmic streaming, division of fertilized eggs, formation of the spindle apparatus during cell division, etc., underlying each of that is a chemical reaction including the interaction of myosin with actin filaments or of dynein (kinesin etc.) with microtubules and the polymerization of actin or tubulin. All these reactions are endothermic, entropy driven, and all are apparently associated with the release of water from the hydration shells of the proteins. It was Albert Szent-Györgyi who, many years ago, was the first to claim that "what is driving muscle is the increase in entropy. Eventually the splitting of ATP has to foot the energy bill" (1973). Lauffer (1975) concluded that water is more than a mere vehicle for biochemical reactions (just as a solvent and as the provider of one molecule for the hydrolysis of each ATP molecule). It is a *crucial* contributor to biological structures and processes. It is capable of carrying its role by undergoing changes in structure that, to say the least, are not less important than the much-talked about conformational changes occurring in proteins. Needless to say, the two types of change are in all probability *inseparable* because they must be *coupled,* and it does not make any sense to argue that is the chicken and that is the egg. In other words, the two occur together as two aspects of the *same* happening. *Major* conformational changes are not a must, neither does the amount of water released have to be very large. Thus, the rotation of the myosin heads required by the SCBT seems to be quite coarse in comparison with the subtle changes in the hydration shells that involve only about 30 to 50 water molecules per myosin head facing one or two actin subunits. Changes in hydration shells are probably also the origin of force generation when kinesin heads approach a microtubule, when G-actin or tubulin polymerize (and possibly depolymerize), when ions are transported selectively and in the proper direction across biological membranes, and possibly also when *any* two (or more) protein molecules (or a protein molecule and a ligand, including enzyme and substrate) "get together" and, generally speaking, when two similar or different macromolecules (e.g., DNA and

protein molecules) are going to form a complex. The molecules first repel each other (with net repulsive force) due to the operation of long range (hydration and electrostatic) forces, that is, they have to climb up an energy barrier, which requires energy that can be supplied by their Brownian motion but also by a water-liberating process linked to the interaction of the hydration shells. A special case is the release of water molecules induced by an osmotic stress. If the supplied energy is large enough to overcome the energy barrier, the macromolecules may get so close to each other that chemical bonds are allowed to form, thus stabilizing the complex. In the case of active muscle, this process is repeated cyclically without the formation of rigor bonds because ΔH is (*on purpose*) not large enough and/or due to the dissociating action of ATP. The myosin heads can form tight "chemical" bonds also with actin – but this happens only *once* in a lifetime: when a creature dies and its muscles (and in all probability also other actomyosin and kinesin-tubulin systems) go into rigor, following the depletion of ATP that is not produced anymore.

For a comprehensive critical review of the SCBT and the way it led to the present theory see Oplatka, 1996a. For preliminary discussions of the role of structural changes in bound water in the mechanism of muscular contraction see Oplatka, 1989, 1990, 1991a,b, 1993, 1994a,b, 1996b,c, 1997.

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