Molecular and Physiological Mechanisms of Muscle Contraction

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To my wife Marie-Hélène, my son Philippe, my former students and coworkers
To my dear brother Yves who died in July 2014 and will never read this book
This monograph was written in Plouescat, Brittany, France, between 2001 and 2014

CONTENTS

PREFACE

Frog and rabbit are the principal animal species studied in this monograph. The passage from one species to another may make it difficult to follow the reasoning, but this passage is necessary because most of the experimental results concerning physiological properties (e.g. isometric tetanic tension and velocity of shortening) were obtained with intact frog fibres, whereas most of the enzymatic results concerning MgATPase activities were obtained with demembranated rabbit fibres.

This book comprises a long line of reasoning, with many interdependent and complex sections. I therefore recommend reading the titles of the most complicated chapters and sections carefully to facilitate understanding. Consulting the Index could also be useful.

In addition to reviewing and reanalysing the results of studies of my own and many other independent groups, I also report unpublished results for experiments with half-fibres (intact fibres split lengthwise) from white skeletal muscles of young adult frogs and with permeabilised fibre bundles from red skeletal muscles of young adult rats. From this many-faceted 'treatise', a hybrid model emerges, combining the swinging cross-bridge/lever-arm processes and lateral swelling mechanisms.

In these new experimental findings, the relative resting force, recorded at pH 7 and 10°C, in half-fibres from white skeletal muscles (*iliofibularis*) of young adult frogs (*Rana pipiens*), held around the slack length, increased very slightly when the bulk ionic strength was lowered from 180 mM to ~40 mM. Between ~40 mM and ~30 mM, the relative resting force increased very rapidly with further decreases in ionic strength, peaking at high levels, between ~30 mM and ~20 mM. Below ~20 mM, the relative resting force decreased sharply. The dependence of the relative resting force on ionic strength, the existence of a maximum and the rapid decrease at very low ionic strengths demonstrate that strong radial repulsive electrostatic forces are exerted between the myofilaments under resting conditions (see below concerning the conversion of radial forces into axial forces). These radial repulsive electrostatic forces are also effective in half-fibres (and all types of fibre, whether intact or demembranated), under isometric tetanic contraction conditions, and present qualitative characteristics similar to those at rest (only some quantitative features differ).

In another set of experiments, myosin heads were cleaved enzymatically (i.e. digested with α-chymotrypsin) from the rest of the thick myosin filaments, in permeabilised fibre bundles from red skeletal muscles (*tibialis anterior*) of young adult rats (Wistar), held around the slack length, in a buffer mimicking the physiological resting medium, at room temperature. Very small, sometimes tiny, but detectable, transitory contractures resulted from these enzymatic cleavages, demonstrating that thin actin and thick myosin filaments are

tethered by a small number of 'resting' (weakly bound) cross-bridges, exerting strong radial tethering forces (together with weak radial attractive/compressive forces) that counterbalance the radial repulsive electrostatic forces, under resting conditions.

Based on these two series of experimental observations, a hybrid model of muscle contraction is proposed, in which the radial tethering forces decrease drastically once contraction is triggered, leading to net radial expansive forces between the myofilaments. Under both resting and contracting conditions, the net radial repulsive (expansive) forces are turned into axial forces. Indeed, in the 1970s, based on theoretical and logical reasoning, mechanisms for this conversion were proposed that are valid at rest and during contraction, regardless of volume variations. In this hybrid model, under standard conditions (e.g. 10°C, slack fibre length, pH ~7, ionic strength ~180 mM), part of the isometric tetanic tension (~40%) results from lateral swelling mechanisms (the usual name for mechanisms involving radial expansive forces inducing axial contractile forces) and another part (~60%) results from swinging cross-bridge/lever-arm processes plus, possibly, the impulsive mechanism developed by Elliott and Worthington plus, possibly, the 'step-wise' mechanism developed by Pollack's group, and other models (e.g. ~30% of swinging cross-bridge/lever-arm models, ~10% of impulsive model, ~10% of Pollack's model, and ~10% of other models). In this work, I neglect the 'unconventional' models and use only swinging cross-bridge/lever-arm mechanisms, with a proportion of ~60%.

The experimental findings, summarised here, and the hybrid model developed and discussed in this book provide the pretext for a long critical and constructive review and an analysis concerning many well-known properties of contracting muscle fibres, as well as complex phenomena (including unexplained, forgotten, ignored, even 'mysterious' experimental and semi-empirical results). Most of the 'forgotten' observations were not accounted for by swinging cross-bridge/lever-arm models and were, therefore, rarely taken into account in the many discussions presented in publications concerning muscle contraction and its molecular basis. By contrast, I think that the hybrid model answers many of these awkward questions.

In 2000, Cyranoski published a commentary paper concerning the symposium held in Osaka (Japan) on in vitro motility and its possible link to muscle contraction. The author provided a severe, but lucid, analysis of the approach of Yanagida and his coworkers (see also Chapter 9 in this book for supplementary arguments). Cyranoski also cited Molloy, who claimed, during the symposium, that 'The tightly coupled lever-arm idea is simple, predictive and inherently testable because of its more restrictive nature' (as opposed to 'the loose-coupled thermal ratchet model' defended by Yanagida and his group). Thus, in 2000, the general view, expressed by Molloy, was apparently clearcut. In this monograph, I demonstrate that the situation is much more complex than previously thought by many specialists in muscle contraction and in vitro motility.

The starting point for writing this book was essentially the conclusion of Cyranoski (2000), who cited A.F. Huxley: 'I came here confused about actin and myosin. Now, I am still confused, but at a higher level'.

The need to reopen the question of the universality of swinging cross-bridge/lever-arm theories and to search for innovative ideas, based on new experiments, has been expressed by Bryant et al. (2007), who wrote: 'the basic actomyosin motor has been embellished, altered, and reused many times through the evolution of the myosin superfamily'. More recently, Grazi (2011) wrote: 'With time clever hypotheses may be accepted as "facts" without being supported by solid experimental evidence. In our opinion this happened with muscle contraction where pure suggestions still occupy the scene and delay the progress of the research'. In the last few years, many authors, including Bryant et al. (2007), have focused on myosin VI and discovered unexpected properties of this motor protein, studied in vitro by brilliant techniques. The titles of the articles by Spudich and Sivaramakrishnan (2010) and Sweeney and Houdusse (2010) were particularly 'explosive': 'Myosin VI: an innovative motor that challenged the swinging lever arm hypothesis' and 'Myosin VI rewrites the rules for myosin motors', respectively.

I have made use of the time available to me since my retirement to read as many papers as possible in the domains of muscle contraction and in vitro motility, with the aim of resolving the confusion. I provide in this work my own analysis

of the various questions posed in the muscle and motility areas and suggest a synthesis, including the hybrid model. Moreover, to the best of my knowledge, the most recent monographs describing the traditional mechanisms of muscle contraction only are those by Bagshaw (1993), Simmons (1992) and Squire (2011).* They have the same titles and resemble descriptive textbooks, and the many experiments published during the last 30 years or so have never been critically analysed. In any event, this long and complex book will be a useful working tool for specialists in muscle contraction, professors, doctors in medicine, and graduate students.

Some important keywords emerge from this monograph that may help the reader to understand this work: head–head dimers, thick myosin filaments, radial repulsive electrostatic forces, radial tethering forces and translation of radial forces into axial forces.

This monograph was completed between 2008 and 2011, and the bibliography concerns the period before 2008–2011 (the first reference dates back to 1911). There are 1000–1100 references. In the addendum, I propose a supplementary list of about 200–250 references, corresponding to the period between 2008 and 2012–2013, with a few references from 2014 and 2015.

Finally, this book may be seen as an analysis and a synthesis of many experimental, theoretical and semi-empirical studies published over the last century or so. Even the most firmly 'unconvinced' reader will enjoy reading the long reference list.

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* After the acceptance of this monograph, Rall published a book on muscle contraction (2014; see Addendum). These two works are clearly complementary, with little or no overlap.

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AUTHOR

Jean Emile Morel was born in western France in 1940. In 1958, after passing his baccalaureate, he embarked on the demanding preparation for the prestigious 'Grandes Ecoles' and was accepted at the Ecole Centrale de Paris. This course led to postgraduate training in radiochemistry and thermodynamics and a PhD in physical chemistry. At that point, he began working as a researcher in biophysics and made the key decision to apply his knowledge of the mathematical, physical and chemical sciences to the field of biology. After obtaining a second PhD, this time in biophysics, he became a researcher at the Commissariat à l'Energie Atomique at Saclay, where he began to focus on the complex problem of muscle contraction. He remained at Saclay from 1980 to 2004, becoming professor of bioengineering, biophysics and cell physiology at the Ecole Centrale de Paris and Université Pierre et Marie Curie (Paris) and joint director of the DEA course of the Molecular Biophysics Doctoral School of Université Pierre et Marie Curie (Paris). In 1993, he founded the Laboratory of Biology at the Ecole Centrale de Paris. Throughout this period, he applied his critical thinking and knowledge of mathematics and physics to the question of how muscles contract, calling into question the established dogmas, trying to resolve the persistent inconsistencies of the accepted models and developing his own alternative models. Since his retirement in 2004, Professor Morel has devoted much of his time to reviewing the existing data and trying to resolve the conundrums of this field. He presents here the results of his analysis: an extensive critical review of the literature, including his own publications, focusing on the more difficult issues often neglected by the advocates of the traditional swinging cross-bridge/lever-arm models and other approaches, together with a new hybrid model, based on these findings, which fits the data and resolves many of the problems raised or left unresolved by previous models.

CHAPTER ONE

Introduction

HISTORICAL ACCOUNT AND OVERVIEW

A. V. Hill (1911, 1913a,b, 1922, 1925, 1932, 1939, 1948, 1949a,b, 1951, 1953) and Hill and Hartree (1920) were probably the first to describe and to quantify the performance of contracting skeletal muscles. Fenn (1923, 1924), Fenn and Marsh (1935) and A.V. Hill (1938a, 1964a,b) studied the physiology of whole frog muscles, particularly under active shortening conditions. Huxley (1953) performed the first x-ray experiments on muscle. Hill and Howarth (1959) studied the biochemistry of stretched contracting muscles. Drury and Szent-Györgyi (1929) were probably the first to study the relationship between adenine compounds and physiological activity of mammalian heart. Engelhardt and Ljubimova (1939) discovered that myosin, the major protein in muscle, has ATPase activity. A. Szent-Györgyi (1947, 1949, 1951, 1953) published the first monographs on muscle biochemistry and physiology. Using the available experimental data, Carlson and Sieger (1960), Dubuisson (1954), Gasser and Hill (1924), Hill (1953), A.F. Huxley (1964) and Perry (1956) tried to develop self-consistent analyses of the mechanics of contracting muscles. Carlson and Sieger (1960), Gergely (1964) and Perry (1956) were probably the first to try to analyse the mechanochemistry of muscle contraction. However, these articles and books were mostly descriptive. The authors did not, and indeed could not, take into account any molecular events occurring during isometric or isotonic contraction, because of the lack of experimental data in this area. In the 1940s and 1950s, Katzir-Katchalsky and his group presented experiments and interpretations, based on polyelectrolyte dilatation and contraction, to explain muscle contraction (e.g. Katzir-Katchalsky 1949; Kuhn et al. 1950). The 'revolutionary' sliding filament model (Huxley and Hanson 1954; Huxley and Niedergerke 1954) was developed in the 1950s and 1960s and is widely, probably universally, accepted. Since the publication of these two classical papers in a single issue of *Nature*, some analyses of the sliding filament model itself were proposed (e.g. Dijkstra et al. 1973) and several theories have been put forward, in the 1960s and 1970s, to describe the mechanisms of force generation (isometric conditions), and sliding of the myofilaments past each other (isotonic conditions) (e.g. Ashley 1972; Caplan 1966; Davies 1963; Morales and Botts 1979; Oplatka 1972; Shear 1970; Spencer and Worthington 1960; Ullrick 1967; Worthington 1962, 1964; Yu et al. 1970).

These pioneering hypotheses did not receive wide approval, whereas there was a large consensus in favour of simple side-piece/cross-bridge models to account for force generation and active sliding in very different and innovative ways (A.F. Huxley 1957, 1965, 1969, 1971; Huxley and Simmons 1971, 1973). The cross-bridge theories were gradually modified and improved, from the conceptual, theoretical and semi-empirical points of view, by introducing, for instance, various experimental data, obtained over the years. These models are now described as swinging cross-bridge/lever-arm processes and many detailed analyses and theories have been developed, concerning these mechanisms (e.g. Baker and Thomas 2000; Barclay 1999; Brenner 1990; Brenner and Eisenberg 1987; Brokaw 1995; Cooke 1986, 1995,

1997, 2004; Cooke et al. 1994; Duke 1999, 2000; Eisenberg and Hill 1978, 1985; Eisenberg et al. 1980; Fisher et al. 1995a; Geeves 1991; Geeves and Holmes 1999, 2005; Goldman and Huxley 1994; Hill 1968a,b, 1970, 1974, 1975, 1977; Hill and White 1968a,b; Hill et al. 1975; Holmes 1997; Holmes and Geeves 2000; Huxley 1973b,c; A.F. Huxley 1988, 2000; Huxley and Kress 1985; Huxley and Tideswell 1996, 1997; Irving 1987; Julian et al. 1978a; Linari et al. 2009; Ma and Zahalak 1991; Martyn et al. 2002; Mijailovich et al. 1996; Pate and Cooke 1989; Piazzesi and Lombardi 1995; Piazzesi et al. 2002b; Rayment et al. 1993a; Schoenberg 1980a,b, 1985; Smith and Mijailovich 2008; Smith et al. 2008; Squire 1983). A comment should be made regarding the remarkable experimental work and its interpretation (in terms of the mechanisms of muscle contraction, in particular) presented by Rayment et al. (1993a,b). Indeed, the authors resolved the atomic structure of the myosin subfragment-1 (S1 or head), using crystals of extensively methylated S1. Unfortunately, Phan et al. (1994) demonstrated that 'methylation… causes a complete loss of in vitro motility of actin filaments over methylated HMM [heavy meromyosin, i.e. myosin subfragment containing the two heads, S1, plus the S2 part of myosin; see Figure 5.1 for definitions]… It is concluded that these relatively mild but numerous and important changes impair the function of methylated S1'. Thus, the promising work of Rayment et al. (1993a,b), which was seen as making a major contribution to our understanding of the myosin head, the head–actin interface and the molecular swinging cross-bridge/lever-arm mechanisms of muscle contraction, unfortunately seems to have been essentially a 'non-event' in terms of the mechanisms of muscle contraction.

Buonocore et al. (2004) proposed a hybrid model combining the swinging cross-bridge/lever-arm and biased Brownian motion concepts (the biased Brownian motion theories of muscle contraction were developed by the group of Yanagida; see, for instance, Kitamura et al. 2005; Yanagida et al. 2000a,b, 2007). Grazi and Di Bona (2006) proposed an unconventional model taking into account both swinging cross-bridge/lever-arm processes and the viscous-like frictional forces, already studied and discussed by Elliott and Worthington (2001). As pointed out by Elliott and Worthington (2001), the viscous forces are discounted in all traditional models but should be taken into account, particularly when the myofilaments slide past each other (isotonic conditions). The two very different hybrid models suggested by Buonocore et al. (2004) and Grazi and Di Bona (2006) differ considerably from that presented and discussed in this monograph. Indeed, the hybrid model presented and discussed here combines the swinging cross-bridge/lever-arm and lateral swelling theories, under isometric tetanic contraction conditions, but does not ignore the viscous forces under isotonic contraction conditions (see p. **281** in Section 8.11).

Many discussions and controversies regarding traditional approaches to muscle contraction have been published over the years. For example, Hoyle (1983) entitled one of the sections of his book 'Why do muscle scientists "lose" knowledge?' Considering in vitro motility as a model of muscle contraction and studying various factors influencing the movement of F-actin filaments propelled by HMM in vitro (see first column on this page for definition of HMM), Homsher et al. (1992) concluded that 'the results of motility assays must be cautiously interpreted'. In a meeting review, Alberts and Miake-Lye (1992) claimed that 'in no case is it understood how [chemo-mechanical] transduction happens' and that 'the problem with the rotating crossbridge hypothesis is that the major conformational change that it predicts for myosin heads during the power stroke has simply not been observed'. Taylor (1993) rephrased this problem more precisely: 'the fundamental problem with the rotating crossbridge model has been the failure to obtain convincing evidence for a large-scale change in the structure that could account for a movement of the crossbridge of 5 to 10 nm in the direction of motion'. However, Taylor (1993) remained optimistic and proposed that the problem would be resolved by taking into account both the crystallisation of myosin heads, with interpretation of the resulting experimental data (e.g. Rayment et al. 1993a,b), and studies of in vitro motility. Nonetheless, Huxley (1996) expressed doubts about swinging cross-bridge/lever-arm models, as they stood in the mid-1990s: 'The challenge to really understand the mechanism remains'. Analysing experimental data concerning stiffness of muscle fibres, Goldman and Huxley (1994) wrote: 'The studies raise many questions and prod us to reinterpret earlier experiments… We need explicit structural

models that explain the energetic discrepancies in both fibres and in vitro data'. A.F. Huxley (2000) was also dubious, stating that '… there is always a possibility—indeed, a probability—that our present concepts are seriously incomplete or even wrong'. Traditional cross-bridge models have been improved in recent years, but the main bases of swinging cross-bridge/lever-arm theories have not markedly changed and I think that the doubts of Alberts and Miake-Lye (1992), Goldman and Huxley (1994), Homsher et al. (1992), Huxley (1996), A.F. Huxley (2000) and Taylor (1993) remain topical. At this point, I believe that one of the major problems to be resolved concerns whether the main features of the usual models should still be considered 'unimpeachable'. The old and recent swinging cross-bridge/lever-arm models are mostly constructed from 'conventional cross-bridge models with one-to-one coupling between the mechanical and ATPase cycles' (Linari et al. 1998). These authors cited many works providing apparently convincing experimental evidence in favor of this traditional view of tight coupling. However, they pointed out that some previous assertions require revision, taking into account, for example, the marked compliance (opposite of stiffness) of the thin actin filaments. More generally, Linari et al. (1998) also raised the issue of the compliance of other structures present in a unit cell and even gave estimates for the compliance of cross-bridges, thick myosin filaments and thin actin filaments. Thus, since the end of the 1990s, swinging cross-bridge/lever-arm theories have become increasingly complex, because, for example, the various compliances must be taken into account and may interfere with interpretation of the experimental results, even potentially blurring the main feature of the swinging cross-bridge/lever-arm mechanisms. Huxley (2000) himself came to a similar conclusion. In this book, many other problems raised by the conventional approaches are analysed and discussed.

In a short abstract published in the *Scientific American*, Yanagida (2001) criticised the swinging cross-bridge/lever-arm theories and claimed that this kind of model 'is still popular because it posits that muscle contraction is, like the operation of ordinary motors, an easy-to-understand, deterministic process'. This is the 'eternal' viewpoint of Yanagida and his coworkers, as well as many other Japanese authors (e.g. Esaki et al. 2003, 2007; Kitamura et al. 1999, 2001, 2005; Oosawa 2000; Oosawa and Hayashi 1986; Shimokawa et al. 2003; Takezawa et al. 1998; Wakabayashi et al. 2001; Yanagida 2007; Yanagida et al. 2000a,b, 2007). This severe 'Japanese view' is not entirely new and the first cross-bridge models were nicknamed 'oar' theories, in the 1970s and 1980s, because of their 'anthropomorphic' aspect. Starting, in many instances, from the same general view as expressed by Yanagida (2001), but with very different concepts, many alternative models have been proposed since the beginning of the 1970s, most taking into account experimental data that were, and are still, generally forgotten (ignored?) (e.g. Brugman et al. 1984; Dragomir et al. 1976; Elliott 1974; Elliott and Worthington 1994, 1997, 2001, 2006; Elliott et al. 1970; Gray and Gonda 1977a,b; Harrington 1971, 1979; Iwazumi 1970, 1979, 1989; Iwazumi and Noble 1989; Jarosh 2000, 2008; Lampinen and Noponen 2005; Levy et al. 1979; McClare 1972a,b; Mitsui 1999; Mitsui and Chiba 1996; Morel 1975; Morel and Gingold 1979b; Morel and Pinset-Härström 1975a,b; Morel et al. 1976; Muñiz et al. 1996; Nielsen 2002; Noble and Pollack 1977, 1978; Oplatka 1972, 1989, 1994, 1997, 2005; Oplatka and Tirosh 1973; Oplatka et al. 1974, 1977; Pollack 1984, 1986, 1990, 1995, 1996; Pollack et al. 1988, 2005; Schutt and Lindberg 1992, 1993; Tirosh 1984; Tirosh and Oplatka 1982; Tsong et al. 1979; Ueno and Harrington 1981, 1986a,b; Wang and Oster 2002). As highlighted above, most of these models have been largely discounted, but, when taken into account, many criticisms have been raised against these unconventional theories and, particularly, against the lateral swelling models proposed to account for axial contraction. In most of the lateral swelling models, the radial repulsive electrostatic forces between the negatively charged myofilaments play a central role in generating axial contractile forces. In this context, it was suggested by April (1969), April et al. (1968, 1972) and Edman and Anderson (1968), using the 'external osmotic pressure technique' on intact fibres (with their sarcolemma), that increases in the internal ionic strength are associated with decreases in the tension-generating capacity of muscle. However, in these 'old' papers, which present experimental results that are a priori in favour of strong electrostatic forces (depending on ionic strength), the complex mechanisms proposed by the authors to explain the relationship

between external osmotic pressure, internal ionic strength, and axial contractile force in intact fibres appeared to be largely speculative.

The first lateral swelling models were presented by Elliott et al. (1970) and Ullrick (1967; this pioneering model did not involve radial repulsive electrostatic forces between myofilaments, but solely the elasticity of the Z discs and their possible role during contraction). These models were based on the isovolumic behaviour of intact muscle fibres under contraction conditions, leading to an automatic translation of lateral swelling into axial shortening. The constant volume relationship was first suggested by Huxley (1953) and demonstrated by stretching intact muscles or fibres from crayfish and frog, using traditional x-ray diffraction (e.g. April et al. 1971; Brandt et al. 1967; Elliott et al. 1963, 1965, 1967). However, April and Wong (1976) and Matsubara and Elliott (1972), still using traditional x-ray diffraction on mechanically skinned fibres from crayfish and frog, respectively, interpreted their experimental results as demonstrating that the constant volume relationship does not hold, when skinned fibres are stretched. From these two experimental studies, it was directly and certainly too hastily inferred by most specialists in muscle contraction that lateral swelling models cannot work, because radial repulsive electrostatic forces cannot be translated into axial contractile forces. From the experiments performed by April and Wong (1976) and Matsubara and Elliott (1972), the lateral swelling processes would therefore have canceled out. Nonetheless, Morel (1985a) and Morel and Merah (1997) demonstrated that x-ray diffraction experiments performed on demembranated fibres should be interpreted with great caution (the complex behavior of demembranated fibres is discussed in Sections 3.8, 4.4.2.1 and 8.7). However, based on x-ray diffraction from synchrotron radiation applied to intact frog fibres, Cecchi et al. (1990) claimed that, when the fibres pass from rest to isometric contraction, 'the myofilament lattice does not maintain a constant volume during changes in force' and that their 'observations demonstrate the existence of a previously undetected radial component of the force generated by a cycling cross-bridge. At sarcomere lengths of 2.05 to 2.2 micrometers, the radial force compresses the myofilament lattice'. This compressive force is weak, and, scrutinising the rather complex experimental study and discussion of these authors, I do not consider these conclusions to be clearcut (see also p. **157** in Section 4.4.2.5.4 for a brief analysis). More recently, Yagi et al. (2004) studied rat papillary muscles by x-ray diffraction from synchrotron radiation and laser light diffraction and concluded that 'the cell volume decreased by about 15% [corresponding to a decrease of ~7% in myofilament spacing] when the sarcomere length was shortened from 2.3 micro m to 1.8 micro m'. One problem with this result is that the thin actin filaments (~0.96–0.97 μm long; see p. **24** in Section 3.2) start to intermingle at sarcomere lengths between ~2 × (0.96–0.97) ~1.92–1.94 μm and ~1.80 μm, possibly inducing unexpected properties of the myofilament lattice. Nonetheless, the findings of these two independent groups could disprove the 'lateral swelling approach', but their conclusions are at odds with the observations presented in the next paragraph and the various experimental and semi-empirical results presented in this book, favouring the existence of strong lateral expansive forces in contracting muscles, regardless of changes in volume.

The theoretical and logical arguments presented by Dragomir et al. (1976), Elliott (1974) and Morel et al. (1976) demonstrated that isovolumic behavior is not a prerequisite for the conversion of lateral swelling into axial shortening during contraction. Nevertheless, lateral swelling theories were 'definitively discounted/ignored', as also highlighted in the preceding paragraph. However, some forgotten (ignored?) experimental findings may generate new interest in lateral swelling models. For example, using intact frog fibres in normal Ringer solution (osmolarity estimated at 245 mOsM by Millman 1986, 1998), I calculated from Figure 8 in the review by Millman (1998) small increases in myofilament lattice spacing, amounting to ~3%–4%, when the fibres passed from resting to isometric contraction conditions (using traditional x-ray diffraction techniques). In his review, Millman (1998) commented on his own findings and those of other authors, obtained with the same biological material, also showing a small detectable increase in filament spacing during tension rise in a tetanus, using x-ray diffraction from synchrotron radiation (e.g. Bagni et al. 1994b; Griffiths et al. 1993). Millman (1998) proposed various explanations but did not take into account a possible significant active role of radial repulsive (expansive) forces, which are considered

to play only a passive role in the stability of the myofilament lattice. Using a stereomicroscope coupled with a mechanical apparatus on single intact frog fibres, Neering et al. (1991) showed that, on shifting from rest to isometric tetanic contraction, there was a non-uniform increase in cross-sectional area of between ~1% and ~40% (increase in fibre diameter of between ~0.5% and ~18%), with respect to the same fibre at rest, and an average increase in diameter, along the length of the fibre, of ~10% (estimated from Figures 2 and 3 in the paper by Neering et al. 1991, but not given by the authors). The authors tried to account rationally for their experimental observations, including the non-uniform increase in cross-sectional area in particular. However, they did not take into account a possible contribution of repulsive forces between the thick myosin and thin actin filaments, but I think that they unwittingly gave a good experimental argument for lateral swelling processes. Millman (1998) suggested that '… there is generally not a direct proportionality between fibre diameter and filament lattice under all conditions'. The ~10% difference in the width of frog intact fibre and the ~3%–4% difference in the lattice spacing of frog intact fibre when shifting from resting to isometric tetanic contraction conditions are consistent with this citation from Millman (1998). Further arguments are presented in the next paragraph.

Comparing the two series of experimental results obtained for intact frog fibres passing from rest to isometric tetanic contraction, presented in the preceding paragraph (Millman 1998; Neering et al. 1991), the increase in width when isometric tetanic contraction occurs would be approximately proportional to the cubic power of the lattice spacing [$(1.03–1.04)^3 \sim 1.09–1.12 \sim 1.10$]. Otherwise, Millman (1998) did not dismiss the possibility that the proportionality relationship may hold under particular conditions. In this context, Kawai et al. (1993) used a Na skinning solution at 0°C (chemical skinning) that 'dramatically improved the performance of muscle preparations' and, following the osmotic compression of their chemically skinned fibres from rabbit psoas muscle, they found that the decrease in fibre width was approximately proportional to the decrease in lattice spacing, during relaxation or after rigor induction. Thus, in this special case, the relative decrease in fibre diameter is similar to that in lattice spacing. Nonetheless, the behaviour of demembranated fibres from rabbit psoas muscle should be interpreted with caution (see Sections 3.8, 4.4.2.1 and 8.7). Very few experiments have been performed on frog demembranated fibres, and we may suggest that frog and rabbit demembranated fibres behave differently.

Thus, the situation is confusing and further experimental, semi-empirical and theoretical results should be presented to support 'lateral swelling hypotheses'. In particular, a demonstration of the existence of strong radial repulsive electrostatic forces between myofilaments and their translation into axial forces during contraction is required. This monograph, and the experimental part especially, provides such a demonstration. Discussions and critical analyses of various problems should also provide further support for both the presence of these forces and their active role in muscle contraction.

As early as 1975–1979, it was suggested that the lateral swelling of fibres during isometric contraction might result from a drastic decrease in radial attractive forces once contraction is triggered (Morel 1975; Morel and Gingold 1979b; Morel and Pinset-Härström 1975b; Morel et al. 1976). This may automatically lead to a net increase in radial repulsive forces, converted into axial contractile forces, independently of possible volume variations (see pp. **4–5** and **102–103** in Section 3.10). New experimental results, presented in this book, show that radial repulsive electrostatic forces are effective, both at rest and under contraction conditions. It is also demonstrated that there are strong radial tethering forces between the thick myosin and thin actin filaments at rest. Consequently, the main features of the 1975–1979 model are maintained in this work. At rest, it is demonstrated that there is, indeed, a balance between radial repulsive electrostatic forces, radial tethering forces and weak radial attractive/compressive forces, whereas, during isometric contraction, the radial tethering forces decrease considerably. This results in the automatic appearance of net radial repulsive (expansive) forces between myofilaments, which are translated into axial contractile forces. The 1975–1979 model is extensively revisited and considerably improved in this monograph, in the light of new experimental data and many published results cited in the preceding paragraphs and obtained by my own and other independent groups. Moreover, in addition to these experimental data, many simple

theoretical, phenomenological and semi-empirical approaches demonstrate that self-consistent conclusions can be drawn. The new experimental data show that muscle contraction is partly attributed to the lateral swelling mechanisms, with swinging cross-bridge/lever-arm mechanisms also playing a major role. A complete hybrid model of muscle contraction is proposed, with strong predictive and explanatory power. The notion of hybrid model was first put forward more than 15 years ago (see legend to Figure 5 in the paper by Morel et al. 1998a). The experiments and most of the discussions concern isometrically held intact fibres, half-fibres, mechanically or chemically skinned or permeabilised fibres, isolated myofibrils and intact unit cells (comparative studies of these various biological materials are highly informative; see Sections 3.7, 3.8, 4.4.2.1 and 8.7). Although the experimental data and discussions concern mostly isometric conditions, some major problems raised by the active shortening (isotonic contraction) are also discussed (see Sections 3.4.3.1.1, 3.4.3.1.2, 3.4.4 and 8.11).

The hybrid model addresses many previously forgotten (ignored?) issues relevant to in vivo/in situ conditions but irrelevant to in vitro conditions (see Chapter 9 for a circumstantial discussion). Indeed, the conditions prevailing in vivo/in situ and in vitro are extremely different. For example, in vertebrate skeletal muscle fibres, the myosin molecules are inserted into thick myosin filaments, arranged in a double hexagonal array (the thin actin filaments constitute the second series of myofilaments), whereas myosin or its isolated heads or synthetic myosin filaments are free in vitro. As recalled in Chapter 9, isolated myosin heads can generate movement in vitro. In this context, why does myosin have two heads and what are their roles in vivo/in situ? This is an old question, first posed by A.F. Huxley (1974): 'what is the significance of the fact that each myosin molecule has two heads?' This problem has been studied, from a structural viewpoint, by Morel and Garrigos (1982b) and Offer and Elliott (1978), using indirect reasoning, based, however, on experimental data. For example, Morel et al. (1999) provided experimental evidence to support the hypothesis of Morel and Garrigos (1982b). Briefly, the two independent groups of Morel and Offer gave structural reasons for the existence of two heads. In my group, we demonstrated that the two heads are intimately involved in the structure of the thick myosin filaments, whereas Offer and Elliott (1978) suggested that the two heads can bind to two different thin actin filaments. The major question, addressed by Huxley (1974) and others, principally concerns the mechanical roles of the two heads. For instance, Reedy (2000) clearly summarised this eternal view: 'what does the second myosin head do in skeletal myosin molecule? This is particularly relevant because myosin in contracting muscle appears to use only one head in a crossbridge'. Huxley and Kress (1985) and Huxley and Tideswell (1997) proposed models for the possible mechanical roles of each head in a given myosin molecule in vivo/in situ. In Section 5.1.1, particularly in Figure 5.1, and on pp. **170–172** and **174–176**, the roles of the two heads are identified from the hypotheses of Morel and Garrigos (1982b) and Offer and Elliott (1978) and the experimental demonstration of Morel et al. (1999). These roles are both structural and functional and, more generally, the two heads play a key role in the hybrid model proposed and analysed in this monograph.

From comparative experimental results, Tyska et al. (1999) deduced that myosin with two heads produces greater force and motion than a single head (S1) in in vitro assays. Based mostly on in vitro experiments, Albet-Torres et al. (2009), Duke (2000), Esaki et al. (2007), Jung et al. (2008) and Li and Ikebe (2003) demonstrated that myosin heads act cooperatively in the motion of myosin along a thin actin filament, with cooperation also occurring between both heads of a single myosin molecule. Cooperativity is demonstrated, in this book, to occur in muscle fibres too, under isometric and isotonic conditions, notably in the hybrid model. However, this cooperativity is not strictly identical to that observed in vitro.

Hill (1978) published a theoretical study on the binding of S1 (isolated myosin head) and HMM (see definition on p. **2**) to F-actin. From this letter to *Nature*, it can be inferred that these two subfragments do not behave similarly and that the presence of the two heads in HMM, assumed to bind to F-actin via the intermediary of either only one head or both heads, results in different binding characteristics. An experimental and semi-empirical study supports many conclusions drawn by Hill (1978) and demonstrates cooperativity between the two heads of HMM in binding to the thin actin filaments (Conibear and Geeves 1998).

Murai et al. (1995), building on the unconventional work of Tonomura and his group, posed questions similar to those posed by Huxley (1974) and Reedy (2000), and stated that: 'it remains unknown why myosin has two heads'. From their experimental studies, concerning MgATPase activity (particularly the Pi burst), performed on myosin heads (S1) in vitro, Murai et al. (1995) suggested 'the existence of two kinds of head in the myosin molecule'. These two families of heads differ enzymatically. This type of enzymatic difference was reviewed by Inoue et al. (1979) but has been largely discarded by many specialists, mostly because of the severe controversy between Taylor and Tonomura, in the 1970s. In the review published by Taylor (1979), the two heads were 'definitively' considered to be identical, from an enzymatic viewpoint. Ten years after the assertion of Taylor (1979), Tesi et al. (1989) demonstrated the existence, in vitro, of two enzymatically different heads, M and M′. According to these authors, who studied essentially the initial transitory phenomena, there are two different sites for MgATP on the two different myosin heads: M, where MgATP is bound and hydrolysed, and M′, where MgATP is trapped transitorily without hydrolysis. According to Iorga et al. (2004), '… it could be that both M and M′ bind actin and that ATP dissociates both actoM and actoM′: actoM with hydrolysis of the MgATP, actoM′ without hydrolysis'. As far as I know, the conclusions of Iorga et al. (2004) and Tesi et al. (1989) have not yet been discounted and can be seen as definitive. However, the 'mechanical' consequences of the enzymatic differences were not clearly evoked. Nonetheless, Murai et al. (1995) suggested that 'the two heads of myosin may play different roles in the sliding movement of myosin heads on the thin filament during muscle contraction'.

Regardless of the possible 'enzymatic differences' in vitro, the problem of the mechanical roles of the two heads remains unresolved, despite the possible mechanisms proposed by Huxley and Kress (1985), Huxley and Tideswell (1997) and Esaki et al. (2007). At this point, it should be recalled that Oplatka and his coworkers, in the 1970s and 1980s, demonstrated that movements can be detected upon interaction of the enzymatically active part of myosin (head, S1) with F-actin filaments in vitro. Unconventional models of muscle contraction have been deduced from these observations (e.g. Hochberg et al. 1977; Oplatka 1989, 1994; Oplatka and Tirosh 1973; Oplatka et al. 1974; Tirosh 1984; Tirosh and Oplatka 1982). Moreover, Cooke and Franks (1978) found that, in vitro, threads of single-headed myosin and thin actin filaments (F-actin) can generate tension, and Harada et al. (1987) demonstrated that single-headed myosin can slide along F-actin. Thus, as isolated soluble myosin heads and single-headed myosin can generate movement in vitro, another major question remains: what is the role of the thick myosin filaments in vivo/in situ? In this context, it should be recalled that, in the 1980s, much experimental work was carried out on in vitro motility generated by the interaction of S1 and single-headed myosin with F-actin, without any reference to the pioneering work of Oplatka and his group (see also the similar opinion of Oplatka himself, 2005). All the authors of these studies claimed that in vitro motility and muscle contraction obey the same biological and physical laws. As an illustrative example, Bagshaw (1987) stated that 'S2 [see Figure 5.1 concerning this subfragment] and the second head of myosin are probably useless in muscle contraction'. To be deterministic, Morel and Bachouchi (1988a) noted that 'if this were the case, they [S2 plus the second head] would have disappeared during the course of evolution'. Small (1988) was also dubious about the assertion of Bagshaw (1987) and claimed that 'those who were impressed by the elegantly simple demonstration that single myosin heads bound to a substrate can alone translocate actin filaments may doubt that the rest of the molecule is necessary for motility'. In Chapter 9, a comparison of in vitro motility and muscle contraction is presented and it is concluded that these two processes do not obey the same biological and physical laws.

Other problems, of a physiological nature, are also very important. How can we account for unexplained heat in frog muscles? Where does the negative delayed heat observed in the case of short tetani and twitches in frog muscles come from? What is the origin of temperature-induced contracture in muscle fibres and whole muscles within the body? I have tried to answer these and other problems, by providing as complete a monograph as possible, dealing with many old and recent experimental results that remain unexplained, even somewhat 'mysterious' (see Chapter 8).

Elliott (2007) claimed that 'there are still unknowns in the current view of the contractile event. Modern [and older] work has recently been reviewed by Geeves and Holmes (2005), who present what might be called the majority viewpoint'. As recalled at the beginning of this introduction, this majority view is not a 'universal viewpoint' and I agree with Elliott (2007), who also wrote that 'The discussion continues'. The opinion of Elliott merits careful consideration. One of the many reasons for publishing this book is to provide an alternative to recent 'traditional' reviews, including that presented by Geeves and Holmes (2005), which mostly considers limited aspects of the swinging cross-bridge/lever-arm theories from a purely molecular viewpoint, ignoring many other aspects, such as those recalled in the preceding paragraph, together with many available physiological data that have not yet been taken into account by most authors. Another major reason for publishing this monograph is to propose a new approach, undoubtedly with its own flaws and lacunae, but nonetheless contributing to a better understanding of muscle contraction. In this context, an important experimental study was published by Martin-Fernandez et al. (1994), potentially invalidating the traditional approach, but was ignored by most investigators. Indeed, the authors studied both isometric and isotonic contractions of frog whole muscles, at 8°C, with x-ray diffraction techniques from synchrotron radiation, and stated: 'we conclude either that the required information is not available in our patterns or that an alternative hypothesis for contraction has to be developed'. This comment is, at least partly, consistent with this monograph, which presents such an alternative model. However, using a similar physical technique, but working on single frog fibres, at 4°C, Piazzesi et al. (1999) challenged the conclusions of Martin-Fernandez et al. (1994). Indeed, Piazzesi et al. (1999) concluded from their experimental studies that 'the myosin head conformation changes synchronously with force development, at least within the 5 ms time resolution of these measurements'. This inference clearly supports the traditional view, according to which there is a 'one-to-one coupling between the mechanical and the ATPase cycles' (Linari et al. 1998). However, the experiments of Martin-Fernandez et al. (1994) and those of Linari et al. (1998) and Piazzesi et al. (1999) were performed neither on the same biological material (whole frog muscles vs. single intact frog fibres, respectively) nor at the same temperature (8°C vs. 4°C). Differences in temperature may account for, at least, some of the discrepancies between the results obtained by the two independent groups. In fact, it is demonstrated throughout this book that temperature is an extremely important parameter, for both MgATPase activity and isometric tetanic tension (see Sections 3.4.3.2 and 8.8, respectively). Moreover, Piazzesi et al. (1999) strongly suggested that it would be risky to compare whole muscles and intact fibres. This opinion is consistent with my own experimental and semi-empirical findings, according to which there are major differences between intact unit cells lying in the centre of intact fibres and whole intact fibres, particularly in terms of the isometric tetanic tensions developed by these two types of biological materials (see Section 3.7). A comparison of the conclusions of Martin-Fernandez et al. (1994) with those of Linari et al. (1998) and Piazzesi et al. (1999) again demonstrates that the situation is confusing.

My aim, in this monograph, is not to discuss salient recent and detailed experimental data interpreted on the basis of the traditional approach to muscle contraction (swinging cross-bridge/lever-arm models) or to choose between the conclusions drawn by independent 'conventional' groups, although I give my own opinion in this area. Instead, I focus on the development of a hybrid model, on the basis of new experimental observations presented here and new phenomenological/semi-empirical lines of reasoning. This hybrid model has a good predictive and explanatory power (see Chapter 8). Moreover, as an illustrative example, introducing the hybrid model into the rather divergent interpretations presented by Martin-Fernandez et al. (1994) and Piazzesi et al. (1999), for example (see the preceding paragraph), might make it possible to account entirely for some recent experimental data that do not fit the 'mould' of swinging cross-bridge/lever-arm theories.

Many generalist books, monographs, reviews, minireviews, comments, criticisms, news and views, perspectives and reflections concerning muscle (from biochemical, biophysical, enzymological, physiological and structural points of view) and the possible mechanisms of muscle contraction, in vitro motility and cell motility have been published since the 1970s (e.g. Alberts

et al. 2007; Bagshaw 1987, 1993; Bárány 1996; Barclay et al. 2010; Barman and Travers 1985; Block 1996; Borejdo et al. 2006; Bottinelli and Reggiani 2000; Bray 2000; Brenner 1987, 1990; Brenner and Eisenberg 1987; Cooke 1986, 1990, 1995, 1997, 2004; Craig and Woodhead 2006; Curtin and Woledge 1978; Dijkstra et al. 1973; Duke 1999, 2000; Eisenberg and Greene 1980; Elliott 2007; Ferenczi et al. 2005; Geeves 1991; Geeves and Holmes 1999, 2005; Geeves et al. 2005; Gergely and Seidel 1983; Goldman and Huxley 1994; Goody 2003; Gregorio et al. 1999; Harrington and Rodgers 1986; Herzog et al. 2008; Hibberd and Trentham 1986; Hill 1968a,b, 1970c; Holmes 1996, 1997; Holmes and Geeves 2000; Houdusse and Sweeney 2001; Howard 1997, 2001; Hoyle 1969, 1970, 1983; A.F. Huxley 1974, 1980a, 1988, 1998, 2000; Huxley 1971, 1973b,c, 1975, 1980b, 1990, 1996, 2004; Irving 1985, 1991, 1995; Irving and Goldman 1999; Julian et al. 1978a; Kawai 2003; Koubassova and Tsaturyan 2011; Kuhn 1981; Lehninger 2008; Maciver 1996; Mehta 2001; Mehta and Spudich 1998; Mehta et al. 1999; Molloy 2005; Morel and D'hahan 2000; Morel and Merah 1992; Morel and Pinset-Härström 1975a,b; Offer 1974; Offer and Ranatunga 2010; Oplatka 1994, 1997, 2005; Pollack 1983, 1984, 1986, 1988, 1990, 1995, 1996; Pollack et al. 1988, 2005; Pollard 2000; Reedy 2000; Reggiani et al. 2000; Sandow 1970; Sellers 2004; Simmons 1983, 1991, 1992a,b, 1996; Simmons and Jewell 1974; Simmons et al. 1993; Sleep and Smith 1981; Small 1988; Smith et al. 2005, 2008; Spudich 1994, 2001, 2011a,b; Spudich and Sivaramakrishnan 2010; Spudich et al. 1995; Squire 1983, 1989, 1994, 1997, 2011; Sweeney and Houdusse 2010a; Taylor 1972, 1979, 1989, 1993; Thomas et al. 1995; Titus 1993; Trentham 1977; Trentham et al. 1976; Vale and Milligan 2000; Vol'kenstein 1970; Woledge 1971, 1988; Woledge et al. 1985, 2009; Wray et al. 1988). Most authors favour the swinging cross-bridge/lever-arm processes, both in vivo/in situ and in vitro, but there are several bones of contention. For instance, Pollack (1988) presented severe criticisms against the swinging cross-bridge/lever-arm theories. Pollack (1990) has also published a monograph in which unconventional ideas are developed, concerning possible molecular mechanisms of muscle contraction and in vitro motility (particularly the stepwise process for muscle contraction). Jontes (1995) has proposed a 'calmer' analysis of many mechanisms accounting for muscle contraction.

In this book, I demonstrate that, taking into account experimental data from my group (including new experimental results presented and discussed here) and from many other independent groups, alternative approaches can be proposed that are different from the mechanisms suggested by Elliott and Worthington, by Pollack and his collaborators, and, more generally, by many independent specialists in mechanisms of muscle contraction. Regardless of the choice of the best model, I also take the opportunity to analyse and dismiss a number of pointless discussions and so-called well-established concepts. The experimental work of Bagni et al. (1990a), concerning the relationship between myofilament spacing and force generation in intact frog fibres, using normal and hypertonic or hypotonic Ringer solutions, at 10°C–12°C, put a premium on this view. Indeed, these authors claimed that '… the separation distance [between the myofilaments] influences the force generating mechanism… Even if this effect is not sufficient to challenge the idea of cross-bridges acting as independent force generators, it should be considered in models of the force generation mechanism'. The old notion of cycling cross-bridges acting independently was called into question, even dismissed, by Bachouchi and Morel (1989a), Morel (1984a) and Morel and Merah (1995), who demonstrated that the kinetic characteristics of the cycling cross-bridges (constants of attachment, f, and detachment, g), necessarily depend on sarcomere length, that is, also on myofilament spacing. From the 'mitigated opinion' of Bagni et al. (1990a) and the assertions of Bachouchi and Morel (1989a), Morel (1984a) and Morel and Merah (1995), it appears clear that swinging cross-bridge/lever-arm theories should be revisited. This is done throughout this book, because the 'well-established' notion of cross-bridges acting independently is no longer valid, and this view is strongly, probably definitively, supported in this work.

When reading the many papers cited in the reference list, I noted that the experimental results and their interpretation are extremely muddled and frequently self-contradictory. I have devoted a great deal of work, over the last 12–13 years, to trying to unravel this problem and hope that I have succeeded in this monograph. This notion of complexity was clearly put forward by Pollack

(1988), who wrote, in his critical analysis of the traditional cross-bridge theory: 'the complexity would be quite staggering. Easiest is to ignore what was found last year'. In this 'unclear' context, MgATPase activity is a case of special interest (A.F. Huxley 1998, 2000; Huxley 1971, 1980, 1990, 1996, 2004). The numerical values differ considerably between and within papers, even under 'simple' isometric tetanic conditions. This is, in my opinion, a major stumbling block when trying to identify an appropriate model of muscle contraction. Indeed, on p. **52** in Section 3.4.3.2, the 'isometric' MgATPase activity of isometrically contracting permeabilised rabbit fibres is found to be ~0.58–0.70 s^{-1} at the reference temperature of 10°C used in this monograph. However, He et al. (1997), also working on permeabilised rabbit fibres, at 12°C, obtained ~18.8 ± 1.5 s^{-1}, ~17.1 ± 1.0 s^{-1} and ~15.5 ± 0.8 s^{-1} (mean ± SE; $n = 14$) for the first, second and third MgATP turnovers, respectively (see their Table 1). In Section 2.1, it is recalled that $SD = n^{1/2}SE$ is the most suitable statistical test. Thus, at 12°C, the maximal MgATPase activity (first turnover) is ~$18.8 \pm 14^{1/2} \times 1.5$ s^{-1} ~ 24.4 s^{-1}, that is, ~19.7 s^{-1} at 10°C, taking Q_{10} ~ 2.9 obtained by He et al. (1997). The ratio of these two series of results is therefore ~19.7 s^{-1}/(0.58–0.70) s^{-1} ~ 30–35. He et al. (1997) also highlighted this problem of a spectrum of MgATPase activities under isometric conditions. For example, they found that, in a single permeabilised fibre from rabbit psoas muscle, under isometric tetanic conditions, at 20°C, MgATPase activity was initially ~40.6 s^{-1} but only ~3 s^{-1} after 600 ms of contraction (ratio of ~13–14). These large uncertainties on the value of MgATPase activity, for a single fibre or a given type of fibre, at a given temperature, lead to difficulties in clearly defining the notion of 'isometric' MgATPase activity under steady-state conditions. As demonstrated in Section 3.4.3.2 and, more generally, in Section 3.4, determining the most appropriate rate of MgATP breakdown in any biological material, at any time, at a given temperature, during isometric tetanic or isotonic contraction, is a complex problem that, I hope, is correctly unraveled in Sections 3.4.3.2 and 3.4.4 and, more generally, throughout this book.

CHAPTER TWO

Materials and Methods

2.1 PRELIMINARY REMARKS ON THE SIMPLE STATISTICAL TESTS EMPLOYED

I have observed a lack of rigor in the use of SE (standard error) and SD (standard deviation) between and within papers. Thus, it is useful to recall briefly the simplest definitions of these two statistical tests (see handbooks on statistics; the precise definitions are rather complex, but the simplified formulae presented here correspond to traditional usage). The first definition is $SD = \left[\sum (x_i - x_{mean})^2 / n\right]^{1/2}$, where n is the number of values measured, x_i is each value measured ($i = 1, 2, \ldots, n$) and $x_{mean} = \left[\sum x_i\right]/n$, the mean of the n values. The second definition is $SE = SD/n^{1/2}$. In this book, as in most of the papers that I have read, I frequently use SE, except when the authors cited give only the SD and do not give the number n of measurements and in cases in which the use of SD appears to be more appropriate than the use of SE. Indeed, a 'forgotten' advantage of SD is that it is little dependent on n, whereas SE decreases with increasing n and is even close to zero when n is very high, making it hard to obtain a clear notion of the scattering of the experimental points. In this context, I think that SE is potentially misleading, and certainly a much more restrictive statistical tool than SD. Throughout this book, I use these two statistical tests. In some instances, I average SE and SD and use the unconventional test (SE + SD)/2. Finally, it should be stressed that the number n of measurements is an established denomination and should not be mistaken for the proportion n of attached cross-bridges under contraction conditions, which is also an established denomination.

2.2 HALF-FIBRES (SPLIT FIBRES) FROM YOUNG ADULT FROGS (*RANA PIPIENS*)

Half-fibres (split fibres) were prepared from the *iliofibularis* muscle of young adult *Rana pipiens* frogs (length ~ 10–11 cm), using an unconventional and powerful technique first described by Endo et al. (1970) and later improved by Horiuti (1986), Villaz et al. (1987) and Vivaudou et al. (1991), with further improvement introduced here. All dissections were performed in a cold room equipped with a dehumidifier (temperature, 4.0°C ± 0.5°C; relative humidity, 25% ± 5%). All muscles were dissected under a stereomicroscope, in Ringer solution containing 116 mM NaCl, 1.65 mM KCl, 1.80 mM $CaCl_2$, 2.15 mM Na_2HPO_4 and 0.85 mM KH_2PO_4 (pH ~ 7.2 at 4°C, but very little dependent on temperature) (see Horiuti 1986; Villaz et al. 1987). A single intact fibre was then isolated, under the stereomicroscope, in a relaxing buffer similar to that used by Villaz et al. (1987), consisting of 108.5 mM KMS, 5.4 mM Na_2ATP, 10.5 mM $Mg(MS)_2$, 10 mM EGTA and 10 mM PIPES, brought to pH ~ 7.1 at 4°C with KOH, so that the pH was ~7.0 at the working temperature (10°C). Methanesulphonate (MS^-) was used for studies of half-fibres (see the next paragraph, pp. **13–14** and many sections concerned with new experimental data presented in this monograph), because of the advantages of this biological material. The dissection process took ~15–20 min. The intact fibre was carefully split lengthwise under the

stereomicroscope (see Vivaudou et al. 1991, particularly their Figure 1 and corresponding comments, for precise details on the technique used to split the fibre). This splitting was carried out in the relaxing buffer, described above, and took ~15 min. Binding to the force transducer (AM 801E, Ackers, Horton, Norway) in the trough and measurements of lengths and apparent diameters of the half-fibres (see pp. **12–13**) took ~10 min. Forces were therefore recorded after ~15 min + 10 min ~ 25 min. Force measurements took ~25 min. Thus, the total duration of handling of the half-fibres was ~50 min.

The half-fibres contained half the sarcolemma, but the internal compartment was exposed to the external medium. The sarcoplasmic reticulum was functionally intact, as demonstrated by Villaz et al. (1987, 1989) and confirmed in Section 4.2.2 (see Figure 4.1 and the arrows C corresponding to injection of 30 mM caffeine, and corresponding comments). Using electron microscopy, Asayama et al. (1983) showed that, in mechanically skinned fibres (traditional techniques), the sarcoplasmic reticulum was 'markedly swollen and possibly fragmented', except when the buffers contained 50 mM sucrose. However, the authors used relaxing buffers containing propionate as the major anion. Andrews et al. (1991) demonstrated experimentally that this anion is highly deleterious, as is also Cl^- (in the experimental part of this monograph, care is taken not to use these two anions). Thus, the experimental results obtained by Asayama et al. (1983) probably cannot be extrapolated to half-fibres studied in the presence of MS^- as the major anion. In any event, neither I nor Villaz et al. (1987, 1989) were able to verify by electron microscopy whether, in half-fibres and in the presence of MS^- as the major anion, the sarcoplasmic reticulum was adulterated. However, this kind of behaviour is highly unlikely for half-fibres in the presence of MS^- (see above and Section 3.3, in which it is demonstrated that half-fibres develop the same isometric tetanic tension as intact fibres). This conclusion is clear-cut, particularly as Andrews et al. (1991) demonstrated that MS^- is the least deleterious anion and has many other advantages. Thus, in half-fibres in the presence of MS^-, the sarcoplasmic reticulum is structurally and functionally intact (see the experimental part of this book, concerning split fibres; see also pp. **221–222** in Section 7.2 for some quantitative data, regarding the absence of any detectable deleterious effects of MS^-, by contrast to Cl^-, for example).

The intact sarcoplasmic reticulum around each myofibril in the half-fibres, prepared and used in the presence of MS^- as the major anion, is important for the maintenance of resting conditions at any ionic strength (see pp. **113–115** in Section 4.2.1). Half the sarcolemma and MS^- contribute to the 'similarity' of half-fibres and intact fibres (see pp. **13–14**). It should be pointed out that split fibres are rarely used nowadays by specialists in muscle and muscle contraction, despite their inherent potencies (see the preceding paragraph). The half-fibres were used by Villaz and his group, mostly for studying the sarcoplasmic reticulum. It would have been interesting to measure a possible increase (or decrease?) in lattice spacing (e.g. d_{10}) of the half-fibres when shifting from rest to contraction. As highlighted by Millman (1998), measuring fibre diameter would lead to conclusions concerning the behaviour of the myofilament lattice that could be justified to various extents from the quantitative viewpoint (see also pp. **4–5** in the Introduction). No x-ray apparatus was available in my laboratory and I used a stereomicroscope for observations of possible marked lateral swelling (or shrinkage?).

Each half-fibre was prepared immediately before use. Preliminary experiments were performed before isometric forces were recorded. Only 14 'suitable' half-fibres, from a total of 30, were used for this purpose. The remaining 30 − 14 = 16 half-fibres were rejected because of 'unsuitable' dissection (a high proportion of unsuitable half-fibres as a result of the many problems raised by the difficult preparation). Four of the 14 suitable half-fibres were used for calcium measurements with fura 2 (see p. **15** and p. **113** in Section 4.2.1) and only three half-fibres were used for measurements of isometric forces (mostly resting forces; see Section 4.2.2, Figure 4.2 and corresponding comments). The useable lengths of the remaining 14 − 4 (four half-fibres were used for calcium measurements with fura 2; see above) = 10 half-fibres were measured under a binocular microscope (after binding to the force recorder), and their apparent diameters were determined under the stereomicroscope. Both series of measurements were performed in a relaxing solution similar to that used for lengthwise splitting (see p. **11**), after placing the half-fibre in the trough (the forces were recorded for only three

half-fibres, arbitrarily chosen from the 10 selected; see above). The useable length of the 10 half-fibres was ~3.0 ± (0.2 or 0.6) mm (mean ± SE or SD; $n = 10$) and their 'apparent' diameter was ~85 ± (10 or 32) μm (mean ± SE or SD; $n = 10$). It can be deduced from the experiments of Edman (1979), performed at a mean sarcomere length of ~2.25 μm, that the diameter of intact fibres from young adult frogs *Rana temporaria* was ~130 ± (8 or 25) μm (mean ± SE or SD; $n = 10$), corresponding to ~123 ± (8 or 15) μm at the sarcomere length of ~2.5 ± 0.2 μm used here (see p. **14**) (isovolumic behaviour of intact fibres; e.g. April et al. 1971; Brandt et al. 1967; Elliott et al. 1963, 1965, 1967). Half this diameter is therefore ~62 ± (4 or 8) μm (mean ± SE or SD), lower than that obtained here for half-fibres from young adult frogs *Rana pipiens* (~85 ± (10 or 32) μm; see above), but of the same order of magnitude and statistically consistent, if SD is used (see Section 2.1 for precise details concerning the advantage of using SD). Thus, the 10 half-fibres selected had cross-sectional areas of ~5.67 ± 1.34×10^{-5} cm^2 (mean ± SE for the diameter). The technique of Blinks (1965), widely used to measure cross-sectional area, was not employed here, because this technique would have led to values difficult to interpret in the case of half-fibres. The volume of the 10 half-fibres was therefore ~$1.70 \pm 0.38 \times 10^{-5}$ cm^3 (only the SEs for the lengths and diameters are taken into account in this estimate). The apparent diameters, cross-sectional areas and volumes correspond to resting half-fibres in relaxing buffers with bulk ionic strengths exceeding ~40 mM (the compositions of the various relaxing buffers are given on p. **118** in Section 4.2.2). Indeed, for bulk ionic strengths above ~40 mM, no variations in the apparent diameters of the three half-fibres used to record resting forces could be detected, whereas below ~40 mM, the resting half-fibres swelled considerably (see pp. **165–167** in Section 4.4.2.8 for a discussion of the phenomena occurring at low ionic strengths).

Unlike mechanically or chemically skinned or permeabilised fibres in the presence of the anions generally used (e.g. Cl^-, acetate, propionate and sulphate), half-fibres in the presence of MS^- as the major anion (see below, in this section, for precise details) were used over very long periods (~50 min; see p. **12**), with no detectable impairment. Villaz et al. (1989) observed the same kind of behaviour on the same type of biological material, except that they employed *Rana esculenta*, rather than *Rana pipiens*. They assessed the calcium release induced by caffeine, by measuring the various characteristics of the caffeine-induced transitory contraction, and noted that 'one single [split] fibre could be challenged many times (up to 61) with each assay lasting about 6 min'. Thus, a full set of experiments could last up to ~6 × 60 ~ 360 min (~6 h) in the experiments of Villaz et al. (1989). In the study described here, the duration of the experiments on each half-fibre could not exceed ~1 h. Part of the difference in the maximal duration of experiments (~1 h vs. ~6 h) may be attributed to the use of two different species of frog (see pp. **49–50** in Section 3.4.3.2 for a discussion of some problems relating to different experimental results obtained with different species of frog). The difference in the two durations is also probably partly attributed to the difference in the two types of experiment: caffeine-induced contraction (Villaz et al. 1989), also called 'contracture' by Horiuti (1986), versus resting tension studied in the experimental part of this monograph (see, Sections 4.2.2 and 4.4.2.8).

The unusually long period over which half-fibres can be studied probably results from both the presence of half the sarcolemma and the use of MS^- as the major anion. In this context, many uncontrolled phenomena occur in traditionally mechanically skinned frog or rabbit fibres studied in the usual buffers: disruption/damage/impairment, in the regular arrangement of the myofilament lattice, including disorder in the peripheral myofibrils and at the periphery of each myofibril (e.g. Ford and Surdyk 1978) (furthermore, x-ray diffraction data for such impaired lattices are misinterpreted; see Morel 1985a; Morel and Merah 1997). The same kind of lattice disorder/irregularities occur in chemically skinned frog fibres (e.g. Magid and Reedy 1980). Moreover, in chemically skinned rabbit fibres, the dissolution of myosin, actin and other proteins and their release into the bathing medium were also observed and quantified (see p. **221** in Section 7.2). This protein dissolution phenomenon was not studied in mechanically skinned frog fibres, but it probably also occurs in this biological material. Owing to the many advantages of using half-fibres in the presence of MS^-, as highlighted at many places in this monograph, only three half-fibres were used for studies of resting forces and, occasionally, active forces (see Section 4.2.2, including Figures 4.1 and 4.2). As an illustrative example,

a single half-fibre, used for ~25 min (see p. **12**), displayed no significant loss of resting force (no more than ~4%–6% over ~25 min). Many experimental points could therefore be obtained with a single half-fibre. For durations of up to ~25 min, no impairment of the half-fibres was detected under the stereomicroscope and resting tensions remained unaltered, within the limits of experimental error. However, beyond ~30–40 min, the three half-fibres were gradually destroyed, as shown by examination under the stereomicroscope, and there was a gradual decrease in cross-sectional areas, also detected under the stereomicroscope.

MS^- was used as the major anion, rather than Cl^-, which is used in experimental studies performed by many independent groups. The first reason for using MS^- was to avoid the Cl^--induced release of Ca^{2+} from the sarcoplasmic reticulum (e.g. Allard and Rougier 1994; Endo et al. 1970; Ford and Podolsky 1970; Sukharova et al. 1994), resulting in limited, uncontrolled contractions. This Cl^--induced release of Ca^{2+} was observed but not clearly quantified by Endo et al. (1970) and Ford and Podolsky (1970) and was later reinvestigated with greater precision by Asayama et al. (1983). The use of MS^- is therefore essential when resting forces are recorded, as in most of the experiments presented in this book. The second reason for using MS^- is to replace Cl^- with a benign anion (chemically, biochemically and physiologically almost neutral). Indeed, Cl^- and many of the other anions often used (e.g. acetate, propionate or sulphate) are not benign and changes in their concentration affect several parameters, at least in chemically skinned and permeabilised fibres. In this context, in a remarkable comparative experimental study, Andrews et al. (1991) demonstrated, on chemically skinned fibres from rabbit psoas muscle, that MS^- was the least deleterious anion in studies of various properties of skinned fibres bathed in buffers containing various major anions. The authors found that isometric tetanic tensions were maximal in the presence of MS^- and much higher than with any other anion studied (e.g. Cl^- or propionate). Moreover, in Section 3.3, it is shown that the three half-fibres studied here, in the presence of MS^-, developed isometric tetanic tensions similar to those recorded in intact fibres, which is not the case for other skinned fibres in the presence of other anions (see Section 3.8.1). This demonstrates that the resting and contracting half-fibres in the presence of MS^- were not swollen, except at low and very low bulk ionic strengths (see pp. **165–167** in Section 4.4.2.8 for a discussion). This implies indirectly that, with the 'half-fibres plus MS^- material', the sarcoplasmic reticulum was not 'enormously swollen', by contrast to mechanically skinned fibres (Asayama et al. 1983). Such a swelling of the sarcoplasmic reticulum provides a non-negligible contribution to the overall lateral swelling upon total or partial demembranation (see Section 8.7) and to a decrease in isometric tetanic tension, for purely geometric reasons. Thus, half-fibres in the presence of MS^- can be used as models of intact fibres. Many other advantages of this biological material are described at many places in this book.

Six different fresh stock solutions were prepared and stored for very short periods, at ~4°C, in the cold room (see p. **11**), as described by Horiuti (1986): 1 M KMS, 0.1 M $Mg(MS)_2$, 0.1 M $Ca(MS)_2$, 0.1 M EGTA (brought to pH ~ 7 with KOH), 0.2 M PIPES (brought to pH ~ 7 with KOH) and 0.05 M Na_2ATP. The various chemical compounds were purchased from Sigma France. The fresh stock solutions were mixed, immediately before use, in appropriate quantities to produce the various relaxing and contracting solutions. The final pH was adjusted with KOH, at ~4°C, in the cold room, to give pH 7.0 at the 'experimental' temperature (10°C). Solution compositions were calculated from the stability constants given by Horiuti (1986), with a simple routine.

The half-fibres were attached to the force transducer in a small trough (0.4 ml). The solution in the trough could be changed within ~300 ms, by injecting the new medium from a syringe and removing the overflow by aspiration. The whole apparatus, trough and solution reservoirs were kept at 10.0°C ± 0.2°C (homemade apparatus). The half-fibres were slightly stretched, as in most independent groups, and their length was adjusted to give sarcomere lengths of 2.5 ± 0.2 μm, determined by diffraction (He–Ne laser beam).

The calcium contamination of freshly distilled water is of major importance when resting forces are recorded. This contamination was estimated by atomic spectroscopy absorbance and was found to be ~5 μM (mean value). Freshly distilled water containing more than ~7–8 μM calcium was not used. Calcium contamination of the various stock solutions was estimated by the same method. As pointed out by Horiuti (1986),

much of the contaminating calcium stems from ATP and this contamination adds to the ~5 μM from freshly distilled water. Indeed, studies of the various stock solutions, other than that containing 0.1 M $Ca(MS)_2$, of course, showed that the stock solution containing 0.05 M (5×10^{-2} M) Na_2ATP contained concentrations of ~50 μM Ca^{2+} (5×10^{-5} M; mean value). Stock solutions of Na_2ATP containing more than ~70 μM Ca^{2+} were discarded. The value of ~50 μM for Ca^{2+} contamination gives a Ca/ATP ratio of $\sim 5 \times 10^{-5}$ M/5×10^{-2} M ~ 10^{-3}, lower than the maximal value of $\sim 5 \times 10^{-3}$ reported by Horiuti (1986), due simply to improvements in the techniques of ATP purification by the Sigma Company. Two concentrations of ATP were used in the experiments presented in Section 4.2.2: 0.4 mM and 2 mM (see, in Section 4.2.2, p. **118**). Using a Ca/ATP ratio of $\sim 10^{-3}$ (given above), the estimates of the contaminating calcium concentration stemming from ATP were therefore $\sim 0.4 \text{ mM} \times 10^{-3} \sim 0.4$ μM at 0.4 mM ATP and $\sim 2 \text{ mM} \times 10^{-3} \sim 2$ μM at 2 mM ATP, respectively.

The other stock solutions contained only trace amounts of Ca^{2+}, frequently undetectable, corresponding to Ca/Y ratios not exceeding $\sim 10^{-6}$, where Y is the concentration of any chemical compound mentioned on p. **14**. All stock solutions with a Ca/Y ratio greater than $\sim 1.5 \times 10^{-6}$ were discarded. The stock solution with Y = 1 M KMS contained a maximum of ~1 μM Ca^{2+} and the maximal bulk ionic strength used here (180 mM), corresponding to ~160 mM KMS, resulted in a mean concentration of contaminating Ca^{2+} of only $\sim 0.160 \text{ M} \times 10^{-6} \sim 0.16$ μM. Adding the contaminations of calcium stemming from Na_2ATP (see end of the preceding paragraph) and distilled water (see beginning of the preceding paragraph), we obtain the maximal concentrations of Ca^{2+} of ~5 μM + 0.4 μM + 0.16 μM ~ 5.56 μM and ~5 μM + 2 μM + 0.16 μM ~ 7.16 μM, respectively (for the two concentrations of ATP used; see end of the preceding paragraph). At low concentrations of KMS, the concentration of contaminating Ca^{2+} is very low and can be neglected, giving maximal concentrations of Ca^{2+} of ~5 μM + 0.4 μM ~ 5.40 μM or ~5 μM + 2 μM ~ 7.00 μM. Thus, regardless of the composition of the buffers, the two reference concentrations of contaminating Ca^{2+} can be taken as ~ (5.40 + 5.56)/2 ~ 5.48 μM ~ 5.5 μM and ~ (7.00 + 7.16)/2 ~ 7.08 μM ~ 7.1 μM. EGTA was present at a concentration of 0.5 mM or 1.0 mM in the various bathing buffers used here and surrounding the half-fibres (see throughout Section 4.2). Thus, the Ca^{2+} concentrations within resting half-fibres were necessarily much lower than ~5.5 μM (at 0.4 mM ATP) or ~7.1 μM (at 2.0 mM ATP) (see Section 4.2.1, for calculations, estimates and conclusions concerning the concentrations of Ca^{2+} in the various buffers).

As repeatedly pointed out above, in this section, the sarcoplasmic reticulum is functional in the three half-fibres used in the experimental part of this monograph (see Figure 4.1 and corresponding comments) and it certainly pumps, at least partly, the remaining trace amounts of Ca^{2+}. In this context, it is interesting to estimate the capacity of the sarcoplasmic reticulum to accumulate Ca^{2+}. For skeletal muscle fibres from young adult rabbits, the sarcoplasmic reticulum can accumulate ~10 mM Ca^{2+} in vivo or in vesicles studied in vitro (Philippe Champeil, personal communication). This value is assumed to be valid for young adult frogs too. Regardless of the calculations presented in Section 4.2.1, experimental estimates of the concentrations of Ca^{2+} in the sarcoplasmic medium of resting half-fibres were made, with fura 2 (purchased from Sigma France) used at a concentration of 2 μM. This chemical marker and technique were first described by Grynkiewicz et al. (1995) and have been successfully used by my group (D'hahan et al. 1997). The experiments were performed in a Hitachi F-2000 spectrofluorometer, regulated at 15.0°C ± 0.1°C (the lowest temperature that can be used without problems of water condensation at room temperature). The buffer (with a volume of 0.5 ml, corresponding to the minimal useable value in the spectrofluorometer) was introduced into the fluorescence cuvette and gently stirred. In parallel, one half-fibre, with an apparent diameter of ~85 ± 10 μm, was prepared and the two ends were cut off to obtain a length of ~3 mm (see p. **12**). This half-fibre was prepared immediately before use and rapidly introduced into the cuvette. Four half-fibres were prepared and studied at four different ionic strengths (see p. **113** in Section 4.2.1). A problem when measuring free Ca^{2+}, even with fura 2, is that it is impossible to detect Ca^{2+} at concentrations lower than ~10 nM (pCa ~ 8). At this pCa, partial activation can occur and the major point relating to the possible presence of trace amounts of calcium under any of the conditions studied here (particularly at low and

very low ionic strengths) and under supposedly resting conditions is discussed in Section 4.2.1, to allow definitive conclusions to be drawn.

2.3 PERMEABILISED FIBRE BUNDLES FROM YOUNG ADULT RATS (WISTAR)

Bundles of ~4–6 fibres were prepared from small red muscles from the legs (*tibialis anterior*) of young adult rats (Wistar, weight ~ 400 g). Rat muscles were used because the experiments described in Section 4.3.1 (digestion with α-chymotrypsin) proved impossible with bundles of fibres from young adult frogs *Rana pipiens* (species used for studies of half-fibres; see Section 2.2). Bundles of fibres were used because they develop more force than single fibres. This is necessary to record very small transitory forces (see Section 4.3.1). All the dissection experiments were performed in the dehumidified cold room (~4°C; see p. **11** in Section 2.2). Muscles were dissected in Ringer solution (see composition on p. **11** in Section 2.2) and many bundles were isolated. The isolated bundles of fibres were incubated at room temperature (20°C–26°C) in a relaxing medium (see composition in the next paragraph) containing 1% Triton X-100, for ~1 h, and used immediately after this process. Using this soft permeabilisation technique, the sarcolemma of each fibre was permeabilised and the sarcoplasmic reticulum was almost certainly completely removed (e.g. Aldoroty and April 1984; Aldoroty et al. 1987), but the mitochondria remained intact (Pierre Vignais, personal communication). As observed under a binocular microscope, the useable length of the 24 bundles used (see legend to Figure 4.3, concerning the number of 24) was ~3.0 ± (0.1 or 0.5) mm (mean ± SE or SD; $n = 24$). The diameter of these 24 bundles, measured under a stereomicroscope, was ~185 ± (7 or 34) μm (mean ± SE or SD; $n = 24$), corresponding to a mean cross-sectional area of ~2.7 ± (0.2 or 1.1) × 10^{-4} cm^2 (mean ± SE or SD; $n = 24$). This value is valid for fibre bundles previously incubated as described above and therefore include the unavoidable initial lateral swelling occurring during the permeabilisation process (see Section 8.7) and other complex phenomena also occurring during this process (see pp. **97–98** in Sections 3.8.1 and 3.8.2). The volume of these bundles was therefore ~8.1 ± 4.6 × 10^{-5} cm^3, if the SDs on the length and the diameter of the bundles are taken into account (see Section 2.1 for precise details on the advantage of using SD rather than SE).

The freshly prepared relaxing medium consisted, in all experiments, of 3 mM $Mg(MS)_2$, 3 mM Na_2ATP, 10 mM EGTA, 0.3 mM DTT, 12 mM creatine phosphate and 30 mM imidazole, pH 7.0 (at room temperature), ionic strength ~100 mM (all the chemical compounds were purchased from Sigma France). Atomic absorbance spectroscopy experiments were performed on the relaxing medium used here and showed no detectable traces of Ca^{2+} to be present, consistent with the following estimate. On p. **14** in Section 2.2, it is shown that the mean concentration of contaminating calcium from freshly distilled water was ~5 μM. On p. **15** in Section 2.2, it is also shown that Ca/ATP ~ 10^{-3} and, in the absence of 10 mM EGTA, the concentration of contaminating Ca^{2+}, mostly from 3 mM Na_2ATP, would have been ~3 mM × 10^{-3} ~ 3 μM, giving a total concentration of contaminating calcium of ~5 μM + 3 μM ~8 μM. Thus, in the presence of 10 mM EGTA, the concentration of contaminating calcium could not have exceeded ~$10^{-9.5}$ M (pCa ~ 9.5; the equilibrium constant is taken as $10^{6.4}$ M^{-1}; see p. **124** in Section 4.3.2), confirming that the solutions used were actually relaxing buffers (pCa ~ 9.5; Gordon et al. 1973 and Reuben et al. 1971 considered values of pCa ~ 8.7 and pCa > 9, respectively, to correspond to full resting conditions, in buffers mimicking the physiological sarcoplasmic medium, which is approximately the case here). Bulk ionic strength was deliberately fixed at ~100 mM, rather than the reference ionic strength of 180 mM in half-fibres (see, for instance, in Section 4.2.2, pp. **117** and **118** and Figure 4.2), to ensure that the proportion of weakly bound cross-bridges was higher than at 180 mM, because this proportion increases with decreasing ionic strength (see pp. **165–167** in Section 4.4.2.8 for a circumstantial discussion).

As for the three half-fibres selected in Section 2.2 (see p. **14**), the sarcomeres were ~2.5 ± 0.2 μm long, as shown by diffraction (He–Ne laser beam). It was necessary to use techniques different from those employed for half-fibres (see p. **14** in Section 2.2). The apparatus was different and all experiments were performed manually. The cuvette had a volume of 2.5 ml (homemade apparatus). Moreover, the experiments were performed at room temperature, without regulation, to try to increase the resting tensions (temperature of ~20°C–26°C vs. 10°C for the half-fibres; see p. **14**

in Section 2.2). The buffer in the cuvette was gently stirred to accelerate diffusion of α-chymotrypsin (see the beginning of the first paragraph) into the fibre bundles. By contrast to the situation for the half-fibres (described in Section 2.2 and studied, for example, in Section 3.3), an initial slow swelling of the permeabilised fibre bundles and other uncontrolled phenomena were expected during the ~1 h of incubation (see the first paragraph). These phenomena were not quantified and only the cross-sectional areas corresponding to the end of incubation were taken into account. The transitory increase in force recorded during the in situ digestion of myosin heads with α-chymotrypsin (see Section 4.3.1, especially Figure 4.3 and its legend) should therefore be compared with the forces developed by the 'swollen' permeabilised bundles of fibres immediately before digestion (see pp. **127–128** in Section 4.3.2). The force transducer was the same as that used for frog half-fibres (see p. **11** in Section 2.2).

2.4 PREPARATION OF SYNTHETIC THICK MYOSIN FILAMENTS (MYOSIN FROM THE BACK AND LEGS OF YOUNG ADULT NEW ZEALAND WHITE RABBITS). LIGHT SCATTERING EXPERIMENTS

Myosin from skeletal muscles (back and legs) of young adult New Zealand white rabbits (4 months old, ~2.5–3.0 kg) was prepared as described by Grussaute et al. (1995 and references therein). The reasons for choosing young adult rabbits were explained in the paper by Morel et al. (1999), who demonstrated that natural thick myosin filaments from old rabbits (weighing ~5 kg or more) have 'anomalous' properties (two-stranded filaments and rather low specific MgATPase activity per myosin head, with respect to the three-stranded filaments and rather high specific MgATPase activity per myosin head for young adult rabbits). In this context, to obtain the best experimental results, Schiereck (1982) and Linari et al. (2007) also used young rabbits, weighing ~2.0–2.5 kg and ~2.7–4.2 kg, respectively. Synthetic thick myosin filaments were prepared by the slow dilution technique (Morel et al. 1999; Pinset-Härström 1985; Pinset-Härström and Truffy 1979), at ~0°C (on ice in the dehumidified cold room, regulated at ~4°C; see p. **11** in Section 2.2), in the presence of 1.5 mM MgATP. It has been shown by my group that, in the presence of 0.5 mM MgATP and at ~0°C, the synthetic thick myosin filaments are two-stranded with half the heads inserted into the core of these filaments (F-filaments; Morel et al. 1999). A similar behaviour of synthetic thick myosin filaments was described by Pinset-Härström (1985), who found that these filaments can fray into two subfilaments only. In the presence of 4 mM MgATP and at ~0°C, we obtained synthetic 'antifilaments' (AF-filaments; Morel et al. 1999), in which most of the heads were inserted into the filament core. The intermediate concentration of 1.5 mM MgATP used here probably corresponds to a mixture of ~60%–70% (e.g. ~65%) F-filaments and ~30%–40% (e.g. ~35%) AF-filaments, although no electron microscopy observations were made (no apparatus available). The use of 1.5 mM MgATP, instead of 0.5 mM, was based on the need to avoid too large a decrease in the concentration of MgATP because of a possible rapid cleavage on the 'external' heads, between the end of synthetic thick myosin filament preparation and the end of the light scattering experiments, particularly at temperatures higher than ~35°C (see below, in this section). The definition of the external heads is given on pp. **170–171** in Section 5.1.1; see also Figure 5.1. Finally, the presence of a limited proportion of antifilaments does not modify the interpretation of the experimental results obtained here.

Essential preliminary experiments were performed to locate the phenomena occurring when the temperature increased to ~40°C–41°C (see Section 5.2 for precise details) and to check whether temperature-dependent filament–filament interactions could interfere with other phenomena. Indeed, experimental studies have shown that filament–filament interactions occur in vitro between myosin minifilaments, via head–head junctions (Podlubnaya et al. 1987). In vivo/in situ, for highly stretched fibres in the absence of an overlap between the thick myosin and thin actin filaments, neighbouring thick myosin filaments are naturally cross-linked (Baatsen et al. 1988; Magid et al. 1984; Suzuki and Pollack 1986). As previously suggested by Suzuki and Pollack (1986), Pollack (1990) proposed that the thick-to-thick cross-links described by his group and by Magid et al. (1984) were 'almost certainly built of myosin S-1 [head]. Possibly, each link is a dimer consisting of S-1 heads projecting toward one another from adjacent thick filaments'. In

my group, such filament–filament interactions of external heads were observed in vitro, at 20°C (the only temperature used by Morel et al. 1999). These interactions occur between pure F-filaments and also between pure AF-filaments (Morel et al. 1999, in these filaments, there are some external heads which can interact) but with different features. Both types of filaments were present in this study (see the preceding paragraph), and crossed interactions (of F- and AF-filaments) are almost certain to occur. Moreover, experimental studies, performed on isolated heads (S1), obtained by digesting myosin synthetic filaments with α-chymotrypsin (see Margossian and Lowey 1982 for the principle of the technique) have shown that these heads can dimerise in the presence of MgATP (e.g. Bachouchi et al. 1985; Grussaute et al. 1995; Morel and Garrigos 1982a; Morel et al. 1998a; in this last paper, see the 'supporting information available', for a long list of precautions that must be taken to prepare native S1 able to dimerise). In my group, we also found that when the temperature was gradually increased from ~18°C to ~25°C, the S1 dimers entirely dissociated at ~21°C–22°C, in the presence of 0.15 mM MgATP (Morel and Guillo 2001). Thus, the filament–filament interactions, via the external heads, observed at 20°C (see above) would disappear with increasing temperature, with inevitable consequences for the intensity of light scattered at various temperatures.

In the light of the comments and discussions presented in the preceding paragraph, it was necessary to perform preliminary experiments. Four filament suspensions, prepared as recalled on p. **17**, were studied in their dilution buffers. A volume of 2.5 ml of each suspension was introduced into a fluorescence cuvette (see below), previously regulated at 30.0°C ± 0.1°C. Immediately after injection into the cuvette, the temperature of the suspension was ~6°C–8°C. The cuvette contained 2 mg ml^{-1} myosin (MW ~ 470 kDa, as determined by Morel and Garrigos (1982a), giving a concentration of ~4.26 μM). After injection of filament suspensions into the cuvette, the temperature in the cuvette was increased very rapidly, at a rate of ~+18°C min^{-1} (in these experiments, the phenomena induced by increasing temperature were studied; the case of decreasing temperature is presented on pp. **20–21**). This rate was chosen to prevent denaturation, which occurred at ~40°C (see Figure 5.2). Lower rates (e.g. ~+10°C min^{-1}) resulted in the systematic adulteration of filaments between ~30°C and ~35°C. For purely technical reasons, it was impossible to reach rates higher than ~+18°C min^{-1}. The experimental technique used to increase the temperature in the cuvette so rapidly was merely to adjust very quickly (in less than ~5–10 s) the temperature setting of the thermostat to ~95°C–97°C and to record simultaneously the temperature in the cuvette and the intensity of the scattered light, on a homemade recorder specially equipped to report directly variations in the intensity of the scattered light with temperature in the fluorescence cuvette. The suspension was gently stirred to prevent rapid sedimentation of the synthetic myosin filaments and was studied in a Hitachi F-2000 spectrofluorometer. The intensity of the light (wavelength, 500 nm; arbitrary units) scattered at 90° was studied as a function of temperature. The total time elapsed from the end of the formation of the synthetic myosin filaments (at ~0°C, in the cold room; see p. **17**) to attainment of the maximal temperature in the cuvette (~40°C–41°C) was ~2.5 min (~150 s) in all the scattering experiments.

The intensity of the scattered light was fairly constant, at first, when the temperature gradually increased from ~6°C–8°C to ~27°C. It decreased significantly thereafter, between ~27°C and ~29°C, and was constant beyond ~29°C. The relative amplitude of the decrease, between ~27°C and ~29°C, was ~30%. From Equations 5.1 and 5.2 and the corresponding comments, there appears to be a rather complicated relationship between the molecular weight of a particle and the intensity of the light scattered, even for small particles. It is also recalled that Equations 5.1 and 5.2 are valid solely for small particles, but that they can be used, as a first approximation, for large and very large asymmetrical particles, as here. Equation 5.1 (which is valid over a wider range of temperature than Equation 5.2) shows that the intensity of the light scattered should decrease with decreasing 'apparent' molecular weight of the scattering particle (simple calculations; not shown). Morel et al. (1999) strongly suggested that, at ~20°C and for MgATP concentrations of the same order of magnitude as that used here (see p. **17**), heaps of ~4–6 filaments interact by forming head–head dimers between few external heads from different filaments. Thus, the ~30% decrease in the intensity of the scattered light is certainly related to a decrease in the apparent molecular weight of the scattering objects, resulting from dissociation of the external head–head dimers and, therefore,

disappearance of the heaps of filaments, leading to the presence of only isolated filaments above ~29°C. Otherwise, the experimental findings of Morel and Guillo (2001) demonstrated that free isolated myosin head (S1) dimers, in solution, dissociate at ~21°C–22°C (see also the second column on this page). Here, the extruding myosin heads belong to complex structures: the myosin molecules are arranged in synthetic thick myosin filaments, almost certainly providing an explanation for the temperature transition being shifted to higher values than those reported by Morel and Guillo (2001) for S1 in solution (~27°C–29°C vs. ~21°C–22°C, respectively). In any event, beyond ~29°C, the intensity of the scattered light stabilised at a new level, corresponding to 'non-interacting' filaments only. Thus, beyond ~29°C, there are no problems of filament–filament interactions, and the phenomena described in Section 5.2 can therefore be interpreted in a straightforward way.

In the experiments described in the preceding paragraph, too large a decrease in MgATP concentration from the end of the preparation of synthetic thick myosin filament suspensions to the end of the light scattering experiments may occur, owing to hydrolysis by the myosin heads. This is a major problem, because a large decrease in MgATP concentration may result in partial or total dissociation of the internal head–head dimers buried within the synthetic thick myosin filament core (see Section 5.1.1 for a circumstantial discussion of the internal heads and related problems). Indeed, Morel and Guillo (2001) and Morel et al. (1998a) showed that, in the absence of MgATP, only the head monomer is present in vitro. Such a depletion of MgATP would therefore lead to a misinterpretation of the experimental results obtained in Section 5.2. Morel et al. (1999) presented an experimental study of the synthetic thick myosin filaments from young adult rabbit skeletal muscles. The various experimental studies included measurement of the initial rates v_0 of MgATP splitting, after the transitory periods (H^+ and Pi bursts), under steady-state conditions, at 20°C, for MgATP concentrations between ~20 μM and ~1000 μM. Regardless of the interpretation proposed by Morel et al. (1999), at 1500 μM MgATP (i.e. the 1.5 mM used here), it can be deduced from their Figure 2 (extrapolation) and the corresponding equations that, for both F- and AF-filaments, $v_0 \sim 0.20$ μM MgATP s^{-1}. Moreover, this Figure 2 also demonstrates that decreasing MgATP concentration results in an increase in v_0, followed by a flat maximum, and then a decrease for low MgATP concentrations. For the F-filaments, the value of v_0 corresponding to the flat maximum is ~1.1 μM MgATP s^{-1} (at MgATP ~ 400 μM) and, for the AF-filaments, the value of v_0 corresponding to the flat maximum is ~0.7 μM MgATP s^{-1} (at MgATP ~ 300 μM). On p. **21**, it is suggested that there are ~65% F-filaments and ~35% AF-filaments in the filament suspensions. Thus, a rough estimate of the value of v_0 corresponding to the flat maximum for the suspension of synthetic thick myosin filaments used here would be ~ $(1.1 \times 0.65 + 0.7 \times 0.35)$ μM MgATP $s^{-1} \sim 0.96$ μM MgATP s^{-1}. The concentration of MgATP necessarily decreases during scattering experiments and a maximal estimate for v_0 in the course of these experiments would be $v_{0,max} \sim (0.96 + 0.20)/2$ μM MgATP $s^{-1} \sim 0.58$ μM MgATP s^{-1}, and the 'true uniform' value to be used should be $v_0^* < 0.58$ μM MgATP s^{-1} (at 20°C). In this estimate, the concentration of MgATP at the end of each scattering experiment is assumed to be much greater than the ~300–400 μM (see above), ascertained experimentally below, in this section.

We now need to estimate the Q_{10} for v_0. In Figure 2 of the paper by Morel and Guillo (2001), the rate of MgATP splitting by S1 is characterised by kat, another kinetic parameter, probably with a dependence on temperature similar to that of v_0 (see also the next paragraph). In buffers mimicking physiological conditions (pH 7; ionic strength ~100 mM), it can be deduced from this Figure 2 (Morel and Guillo 2001) that, between ~18°C and ~21°C–22°C, $Q_{10} \sim 2.7$ for kat values corresponding to both the monomer and the dimer. Between ~21°C–22°C and ~25°C, $Q_{10} \sim 4.2$, for the monomer only, because, in these conditions, the dimer is entirely dissociated, as recalled in the first column on this page (see Morel and Guillo 2001, for precise details; the estimates of Q_{10} were not presented by the authors and are especially calculated here). A careful study of the rate of MgATP breakdown in permeabilised fibres from rabbit psoas muscle, under relaxing conditions, gives $Q_{10} \sim 2.5$ within the range ~7°C–25°C, and $Q_{10} \sim 9.7$ within the range ~25°C–35°C (Hilber et al. 2001). The origin of this behaviour (very high value of Q_{10} at high temperatures) in resting skinned fibres is, in principle, not known, although Hilber et al. (2001) suggested a mechanism based on the fact that 'some active force may have been generated in

relaxing solution at temperatures above 25°C'. In any event, I strongly suggest that there is a correlation between the results obtained in situ by Hilber et al. (2001) and in vitro by Morel and Guillo (2001), as discussed in the next paragraph, where the value of $Q_{10} \sim 9.7$ is analysed and criticised.

As highlighted by Ma and Taylor (1994), there are no major differences between the kinetic characteristics of MgATPase activity in acto-S1 systems in vitro and myofibrils (see the Appendix 2.I for some comments and references on myosin and PGK enzymology), and we can see, in the preceding paragraph, that this inference is also valid when comparing free S1 and permeabilised fibres, supporting the assumption that v_0 and kat depend similarly on temperature (see also the preceding paragraph). Indeed, the experimental values of Q_{10} are similar for the two biological materials, as are the two parameters used in this and the preceding paragraphs (v_0 and kat) and we can choose, as a first approximation, $Q_{10} \sim (2.7 + 2.5)/2 \sim 2.6$ within the ~7°C–20°C range, extended to the ~0°C–20°C range, $\sim (4.2 + 2.5)/2 \sim 3.3$ between ~20°C and ~25°C and $\sim (4.2 + 2.5 + 9.7)/3 \sim 5.5$ between ~25°C and ~35°C (extended to the ~25°C–39°C range; 39°C is the body temperature of the rabbit) and also to the range ~25°C–40°C (~40°C is the maximal temperature reached in the present experiments; between ~40°C and ~42°C, the filaments are adulterated; see Figure 5.2) (the four values of Q_{10} ~2.5, 2.7, ~4.2 and ~9.7 are given in the preceding paragraph). Thus, from the true uniform value $v_0^* < 0.58$ μM MgATP s^{-1} at ~20°C (see p. **19**), we deduce that $v_0^* < 0.09$ μM MgATP s^{-1} at ~0°C, $v_0^* < 0.22$ μM MgATP s^{-1} at ~10°C, $v_0^* < 0.36$ μM MgATP s^{-1} at ~15°C, $v_0^* < 1.05$ μM s^{-1} at 25°C and $v_0^* < 17.54$ μM MgATP s^{-1} at ~40°C.

As pointed out on p. **18**, the total time taken to pass from ~0°C to ~40°C is ~2.5 min (~150 s). A rough estimate of v_0^*, between ~0°C and ~40°C, would be $< (0.09 + 0.22 + 0.36 + 0.58 + 1.05 + 17.54)/6 \sim 3.31$ μM MgATP s^{-1}, regardless of temperature. Thus, when passing from ~0°C to ~40°C, the utilisation of MgATP by the filament suspensions is almost certainly <3.31 μM $s^{-1} \times 150$ s ~ 496 μM (~0.5 mM). The concentration of MgATP therefore decreased, at most, from 1.5 mM to ~1.0 mM. At this stage, it should be stressed that the value of $Q_{10} \sim 9.7$ above 25°C, reported by Hilber et al. (2001) (see the preceding paragraph), is almost certainly unsuitable, because of inevitable adulteration/fragility of the permeabilised fibres at temperatures within the 25°C–35°C range (see the preceding paragraph concerning this temperature range). The problem of adulteration/fragility is strongly suggested on p. **52** in Section 3.4.3.2, p. **200** in Section 6.3.2, p. **202** in Section 6.3.3, p. **208** in Section 6.3.5 and p. **238** in Section 8.5. For the sake of simplicity, I propose to extend the values of Q_{10} obtained between 20°C and 25°C to the range 25°C–40°C, ignoring adulteration. Thus, the value of Q_{10} becomes $\sim (3.3 + 4.2)/2 \sim 3.8$ between 25°C and 40°C (rather than ~5.5 as used in the preceding paragraph; the value of ~3.3 is valid between ~20°C and ~25°C and that of ~4.2 is valid between ~21°C–22°C and ~25°C; see the first column on this page concerning the values of ~3.3 and ~4.2). Thus, the value of 17.54 μM MgATP s^{-1} at 40°C (see the end of the preceding paragraph) should be replaced by 8.37 μM MgATP s^{-1} and a more suitable estimate of v_0^*, between ~0°C and 40°C, would be $< (0.09 + 0.22 + 0.36 + 0.58 + 1.05 + 8.37)/6 \sim 1.78$ μM MgATP s^{-1}, corresponding to ~ 1.78 μM $s^{-1} \times 150$ s ~ 267 μM MgATP (~0.3 mM). There is therefore a limited maximal decrease in MgATP concentration, which would pass from ~1.5 mM to ~1.2 mM, with no effect on the conclusions drawn below and in Section 5.2. Indeed, it is recalled on p. **17** that, at 0.5 mM MgATP, F-filaments (in which the head–head dimers are buried within the core) were obtained by Morel et al. (1999) and these F-filaments remain stable for at least ~10 min at room temperature (unpublished observations). The lowest estimate for MgATP concentration of ~1.2 mM, and even ~1.0 mM (see above), is well above 0.5 mM, and there are, in the experiments performed here, neither problems of dissociation of the internal head–head dimers nor problems of stability of the synthetic filaments, owing to MgATP depletion.

From the experiments and calculations presented above, in this section, it appears that (i) the limited depletion of MgATP does not affect the structure and stability of the synthetic thick myosin filaments and (ii) filament–filament interactions do not occur beyond ~29°C. Thus, above ~37°C, nonambiguous and extremely interesting phenomena were observed, which are described and discussed in Section 5.2 (see particularly Figure 5.2, its legend and Appendix 5.II).

When the temperature was decreased below ~39°C, other filament suspensions were used,