Symposia Biologica Hungarica 17

SYMPOSIUM ON THE MUSCLE

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17

Akadémiai Kiadó, Budapest

SYMPOSIUM ON THE MUSCLE

(Symposia Biologica Hungarica 17)

Edited by

E. N. A. BÍRÓ and N. GARAMVÖLGYI

The European Muscle Club held its third meeting in Budapest in 1974. This book contains the papers of outstanding researchers and scientists discussing the most timely problems of muscle research and supplying numerous valuable data on the subject. Some contributions report on completed experiments while others deal only with the most significant aspects of a research work. The meeting of the European Muscle Club is of great importance as the quick motorization and mechanization seriously endanger the humanity and its environment.

The volume is recommended for those working in this special field of science.

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EXCITATION

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LIST OF PARTICIPANTS

AJTAI, K. Department of Biochemistry, Eötvös Loránd University, Budapest, Hungary

ALBRECHT, V. Research Center for Molecular Biology and Medicine, Central Institute for Alimentation, Academy of Sciences of the GDR, Potsdam—Rehbrücke, GDR

ALLERA, A. Institute for Cytology, Bonn, FRG

APOR, P. Research Unit, Hungarian College of Physical Education, Budapest, Hungary

BÁLINT, M. Department of Biochemistry, Eötvös Loránd University, Budapest, Hungary

BARYLKO, B. Nencki Institute of Experimental Biology, Polish Academy of Sciences, Warsaw, Poland

BELÁGYI, J. Biophysical Institute, Medical University of Pécs, Pécs, Hungary

BERGSTRÖM, K. Karolinska Hospital, Stockholm, Sweden

BÍRÓ, E. N. A. Department of Biochemistry, Eötvös Loránd University, Budapest, Hungary

BOTTAZZO, G. F. Department of Immunology, The Middlesex Hospital Medical School, London, England

BRUGGMANN, St. Institute of Pharmacology and Biochemistry, Zürich, Switzerland

CARAFOLI, E. Institute of General Pathology, Modena, Italy

COX, J. A. M. Department of Biochemistry, University of Geneva, Geneva, Switzerland

DALLA LIBERA, L. Institute of General Pathology, University of Padova, Padova, Italy

DAMBROWSKA, R. Nencki Institute of Experimental Biology, Polish Academy of Sciences, Warsaw, Poland

D'HAESE, J. Institute for Cytology, Bonn, FRG

DRABIKOWSKA, G. Nencki Institute of Experimental Biology, Polish Academy of Sciences, Warsaw, Poland

DRABIKOWSKI, W. Nencki Institute of Experimental Biology, Polish Academy of Sciences, Warsaw, Poland

DYDYNSKA, M. Nencki Institute of Experimental Biology, Polish Academy of Sciences, Warsaw, Poland

EBASHI, S. Institute of Pharmacology, University of Tokyo, Tokyo, Japan

ERMINI, M. Institute of Pharmacology and Biochemistry, Zürich, Switzerland

Ernst, E. Biophysical Institute, Medical University of Pécs, Pécs, Hungary
Fábián, F. Department of Biochemistry, Eötvös Loránd University, Budapest, Hungary
Fazekas, S. 2nd Department of Biochemistry, Semmelweis Medical University, Budapest, Hungary
Ferencz, K. Department of Biochemistry, Eötvös Loránd University, Budapest, Hungary
Focant, B. Department of General Biology, Institute Ed. Van Beneden, Liège, Belgium
Gabbiani, G. Institute of Pathology, Geneva, Switzerland
Garamvölgyi, N. Research Unit, Hungarian College of Physical Education, Budapest, Hungary
Gergely, J. Boston Biomedical Research Institute, Boston, Mass., USA
Guba, F. Department of Biochemistry, Medical University of Szeged, Szeged, Hungary
Hamoir, G. Department of General Biology, Institute Ed. Van Beneden, Liège, Belgium
Hardwicke, P. MRC Research Unit of Biophysics, Kings College, London, England
Hegyi, G. Department of Biochemistry, Eötvös Loránd University, Budapest, Hungary
Hinssen, H. Institute for Cytology, Bonn, FRG
Jenny, E. Institute of Pharmacology and Biochemistry, Zürich, Switzerland
Joassin, L. Department of Zoology, Institute Ed. Van Beneden, Liège, Belgium
Jólesz, F. Kálmán Kandó College of Electrical Engineering, Budapest, Hungary
Kakol, I. Nencki Institute of Experimental Biology, Polish Academy of Sciences, Warsaw, Poland
Kalamkarova, M. B. Institute of Biophysics, Academy of Sciences of the USSR, Pushchino, USSR
Katona, G. 2nd Department of Biochemistry, Semmelweis Medical University, Budapest, Hungary
Kohler, L. Department of Biochemistry, University of Geneva, Geneva, Switzerland
Kónya, L. Central Research Laboratory, Medical University of Debrecen, Debrecen, Hungary
Kövér, A. Central Research Laboratory, Medical University of Debrecen, Debrecen, Hungary
Lefvert, A. K. Karolinska Hospital, Stockholm, Sweden
Lerch, R. Medical Polyclinic University of Zürich, Zürich, Switzerland
Lowy, J. Biophysics Institute, Aarhus University, Aarhus, Denmark
Makariewicz, W. Institute of Biochemistry, University of Krakow, Krakow, Poland
Makinose, M. Department of Physiology, Max-Planck-Institute for Medical Research, Heidelberg, FRG
Margreth, A. Institute of General Pathology, University of Padova, Padova, Italy

Medugorac, I. Institute of Physiology, University of Tübingen, Tübingen, FRG

Müller, G. Institute of Pharmacology and Biochemistry, Zürich, Switzerland

Mulvany, M. J. Biophysics Institute, Aarhus University, Aarhus, Denmark

Nagy, B. F. Boston Biomedical Research Institute, Boston, Mass., USA

Nowak-Olszewska, E. Nencki Institute of Experimental Biology, Polish Academy of Sciences, Warsaw, Poland

Oplatka, A. The Weizmann Institute of Sciences, Rehovot, Israel

Pápai, M. 2nd Department of Biochemistry, Semmelweis Medical University, Budapest, Hungary

Paul, R. J. Physiology Department, Harvard Medical School, Boston, Mass., USA

Pechère, J. F. Department of Biochemistry of Macromol. CNRS, Montpellier, France

Perry, S. V. Department of Biochemistry, University of Birmingham, Birmingham, England

Pfister, M. Institute of Pharmacology, University of Zürich, Zürich, Switzerland

Pilarska, M. Nencki Institute of Experimental Biology, Polish Academy of Sciences, Warsaw, Poland

Pinset, I. Centre of Nuclear Studies de Saclay, Gif sur Yvette, France

Pringle, J. W. S. Department of Zoology, Agricultural Research Council Unit, Oxford, England

Riedhammer, H. 2nd Institute of Physiology, University of Heidelberg, Heidelberg, FRG

Riegler, B. Research Unit of the Hungarian College of Physical Education, Budapest, Hungary

Rüegg, C. 2nd Institute of Physiology, University of Heidelberg, Heidelberg, FRG

Salvatori, S. Institute of General Pathology, University of Padova, Padova, Italy

Salviati, S. Institute of General Pathology, University of Padova, Padova, Italy

Schaub, M. C. Institute of Pharmacology, University of Zürich, Zürich, Switzerland

Sréter, F. A. Boston Biomedical Research Institute, Boston, Mass., USA

Stankiewicz, A. Department of Biochemistry, University of Krakow, Krakow, Poland

Strzelecka-Golaewska, H. Nencki Institute of Experimental Biology, Polish Academy of Sciences, Warsaw, Poland

Svendsen, K. H. Biophysics Institute, Aarhus University, Aarhus, Denmark

Syrovy, I. Institute of Physiology, Czechoslovak Academy of Sciences, Prague, Czechoslovakia

Swynghedauw, B. Department of Biomedical Physiology, INSERM-U 127, Paris, France

Szabó-Kelemen, G. Department of Biochemistry, Eötvös Loránd University, Budapest, Hungary
Székessy-Hermann, V. 2nd Department of Biochemistry, Semmelweis Medical University, Budapest, Hungary
Szilágyi, L. Department of Biochemistry, Eötvös Loránd University, Budapest, Hungary
Szvetkó, D. Department of Physical Education, Memorial University, St. Johns, New Foundland, Canada
Takács, Ö. Department of Biochemistry, Medical University of Szeged, Szeged, Hungary
Trombitás, K. Central Research Laboratory, Medical University of Pécs, Pécs, Hungary
Váczy, K. Research Unit, Hungarian College of Physical Education, Budapest, Hungary
Varga, E. Department of Physiology, Medical University of Debrecen, Debrecen, Hungary
Wallimann, T. Institute for Cellular Biology, Zürich, Switzerland
Watterson, J. Institute of Pharmacology, University of Zürich, Zürich, Switzerland
Werber, H. The Weizmann Institute of Sciences, Rehovot, Israel
Will, H. Academy of Sciences, Central Institute for Heart and Circulatory Research Division of Cellular and Molecular Cardiology, Berlin-Buch, GDR
Wolf, I. Department of Biochemistry, Eötvös Loránd University, Budapest, Hungary
Zelck, U. Institute for Pharmacology and Toxicology, University of Rostock, Rostock, GDR
Zubrzycka, E. Nencki Institute of Experimental Biology, Polish Academy of Sciences, Warsaw, Poland

FOREWORD

This volume contains the papers presented at the Third Meeting of the European Muscle Club held in Budapest on 31 August and 1 September, 1974. Some authors described completed experiments while others outlined only the most important aspects of a research work. Finally the editors included the abstracts of a few contributions supplying valuable information on the subject as a whole. Thus, the diverging topics give an overall picture of the work of the meeting.

The Muscle Club was organized simultaneously with the 9th FEBS Meeting held in Budapest in 1974 when one of the symposia dealt with the "Proteins of Contractile Systems". Our aim is to study and discuss the most important aspects of the muscle functions in co-operation with the researchers working in this special field of science all over the world.

E. N. A. Bíró

OPENING ADDRESS

Ladies and Gentlemen,

It is my pleasant duty to welcome our guests on behalf of the Organizing Committee. After Belgium and Switzerland it is now Hungary's turn to be the venue for the yearly meeting of the European Muscle Club. The very fact, that this is already the third meeting on this subject, clearly shows how useful the idea of establishing the European Muscle Club was. A club of informal character, without any formal membership or organization which does not belong to any national or international organ seems to be the best means for extensive discussions and for creation of personal connections.

Though our club is in Europe, we are most happy to be able to greet outstanding scientists from overseas countries as well, as such Prof. Ebashi from Japan, Prof. Gergely from the United States and Prof. Oplatka from Israel. We sincerely regretted that our British colleagues were not present at the first two meetings and it is now a pleasure to welcome here Prof. Pringle, Prof. Perry and other British experts. I should like to express my hope that the meeting of the European Muscle Club will not be restricted to a continental character in the future.

Following our traditions in muscle research we have organized this meeting with the greatest pleasure, though we have to apologize for some possible organizational failures.

By way of introduction I should like to point out the special importance of muscle research in our days. In modern times, automatization of labour, increasing motorization and mechanization endanger the humanity—simultaneously with environmental contamination—leading to the impoverishment of movement. Muscle represents the basis of all human movements from the most simple ones to physical education and sport by which this danger can be overcome. It is not too difficult to predict that in a few years muscle research will be regarded as important as are today to protect the environment and to prevent the hazards of radiation.

With these thoughts I open the Third European Muscle Meeting.

N. Garamvölgyi

CALCIUM AND REGULATION

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EARLIER AND RECENT WORK ON PARVALBUMINS*

by

G. HAMOIR

LABORATORY OF GENERAL BIOLOGY, FACULTY OF SCIENCES,
UNIVERSITY OF LIÈGE, B-4000 LIÈGE, BELGIUM

The parvalbumins (PA), initially described 20 years ago (Henrotte, 1955) have received at first little attention. They have been originally considered as strange sarcoplasmic proteins of unknown function occurring only in the white muscles of fish and amphibia. But the recent discovery of their phylogenetic relationship to troponin C (TN-C) and to both alkali- and DTNB-light chains (LC) has drawn the attention towards them. I shall review here briefly the earlier work carried out on PA and examine the present development of the subject. Before that, I would like to mention some of my collaborators who have contributed largely to the work done in Liège: Henrotte who isolated and crystallized carp PA III in 1955; Konosu, Pechère, Focant, Bhushana Rao, Gerday and Gosselin-Rey.

As soon as a comparative study of the muscle proteins of the carp has started (Hamoir, 1951), it became clear that the fish sarcoplasmic extracts differ very much from the rabbit ones. This is already visible by ultracentrifugation (Fig. 1). Rabbit myogen separates into two peaks having sedimentation coefficients of about 5 and 7 Svedberg units. But in the case of the carp, three peaks are seen of about 1.5, 5 and 7 Svedberg units. The slow peak which was first observed by Deuticke (1934) in frog extracts, corresponds to the low molecular weight proteins of fish and that of the amphibians' white muscles. It was soon realized as a result of a comparative

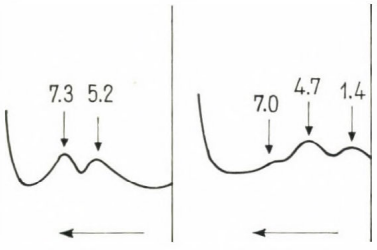

Fig. 1. Ultracentrifugal patterns of rabbit (left) and carp (right) sarcoplasmic extracts (white muscle) after ca. 1 h at 60,000 rpm. The sedimentation coefficients of the peaks are given in Svedberg units

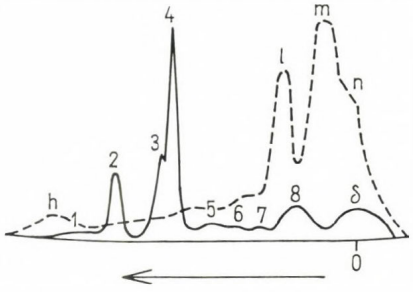

Fig. 2. Comparison of the ascending moving boundary electrophoretic patterns of rabbit (broken line) and carp (solid line) white muscle sarcoplasmic extracts at I 0.075 and pH 7.3 after ca. 5 h electrophoresis at 5.8 V/cm

*Invited paper

work on plaice (Jebsen and Hamoir, 1958) and cod (Connell, 1958) that these proteins generally occur in the muscle low ionic strength extracts of the lower vertebrates and correspond to 25 ($\pm$10) % of these extracts.

The composition of the slow ultracentrifugal peak of the carp extracts has been determined first by moving boundary electrophoresis. The differences between the rabbit and carp sarcoplasmic extracts are illustrated in Fig. 2. The broken line corresponds to the ascending pattern of the rabbit and the solid one to the ascending pattern of the carp. All proteins are negatively charged at neutral pH and migrate from the right to the left. In the rabbit extract, the only acidic electrophoretic component detected by this method, (h) corresponds to serum albumin, but the carp extract contains 5 components migrating in front of the diagram. The fourth is creatine kinase (EC 2.7.3.2) which has always a higher electrophoretic mobility in fish than in warm-blooded vertebrates. The slow ultracentrifugal peak is made up of the components 1, 2, 3 and 5, which has been unequivocally detected by filtration of the sarcoplasmic extracts on Sephadex G-75 (Pechère and Focant, 1965; Fig. 3). The proteins of low molecular weight separate completely from the other proteins. The retarded peak measured by UV absorption is much smaller than that measured by biuret, this discrepancy, due to the low absorption of these proteins at 254 nm, indicate their low tryptophan and tyrosine content.

The carp components 2, 3 and 5 were isolated by ammonium sulphate fractionation, crystallized and described between 1955 and 1965 (Konosu et al., 1965). It is the carp component 3 crystallized initially by Henrotte (1955) (Fig. 4) which has been used by Kretsinger to determine the tertiary structure of these proteins (Kretsinger and Nockolds, 1973).

The study of these low molecular weight proteins was extended from 1965 up to now to many species of fish and of other lower vertebrates using

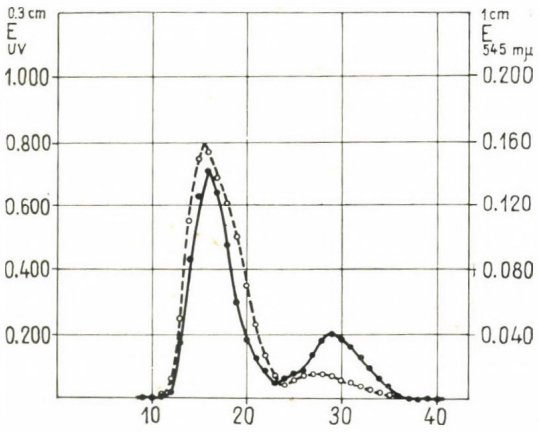

Fig. 3. Separation of the sarcoplasmic proteins of carp white muscle by filtration on a Sephadex G-75 column. Full symbols: biuret determinations; empty ones: ultraviolet absorption. The main fraction corresponds to the unretarded glycolytic enzymes

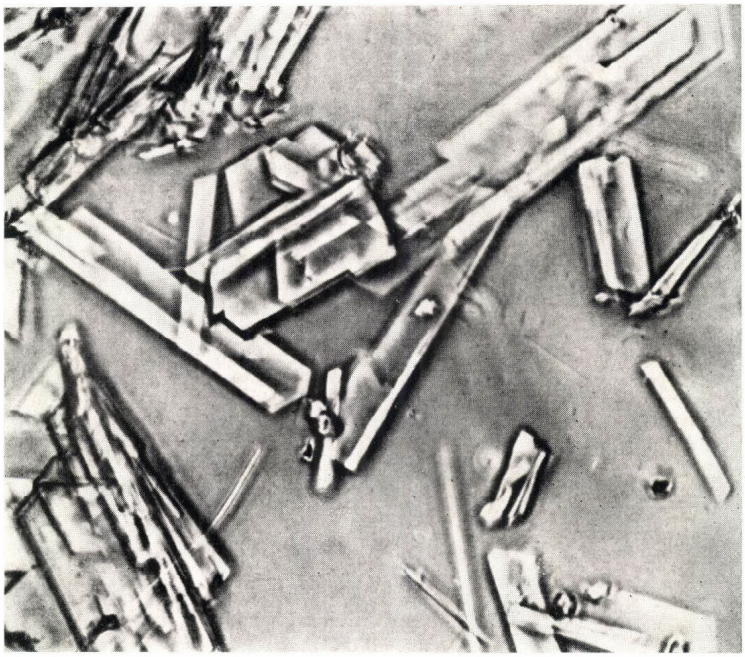

Fig. 4. Phase contrast picture of crystals of the first isolated parvalbumin (carp component 3) (Henrotte, 1955) ×450

different analytical and separation methods: acetone fractionation, filtration of Sephadex G-75, DEAE cellulose chromatography, starch gel and polyacrylamide gel electrophoresis. In 1968 Pechère proposed to designate these proteins of low molecular weight parvalbumins, in view of their small molecular weight and their great solubility. One year later we proposed also a new nomenclature based on the electrophoretic mobility in starch gels (Bhushana Rao et al., 1969). At that time we compared many species of lower vertebrates by starch gel electrophoresis in collaboration with R. K. Scopes. This work has made clear that PA migrate usually with the largely different mobilities shown by the carp components. This regularity is illustrated in Fig. 5 which represents starch gel electrophoretic patterns at pH 8.5 of muscle extracts of carp, of Tilapia macrochir, a fresh water fish living in African lakes and rivers, of codling and of pike. The roman capitals I, II, III and IV designate the different types of PA which can be encountered. Recently a slower component (V) has been described by Piront and Gerday (1973) and a still slower one (VI) exists in few species of fish (Hamoir and Gerday, unpublished).

While this comparative work was in progress, some attempts were made, of course, to determine the biological function of these proteins. Before examining this point, let us first consider some of their general properties (isoelectric points, the sedimentation coefficients, the diffusion coefficients of 9 PA, etc.) which are summarized in the Tables I and II. These proteins have isoelectric points situated between 4 and 5.5 and their molecular

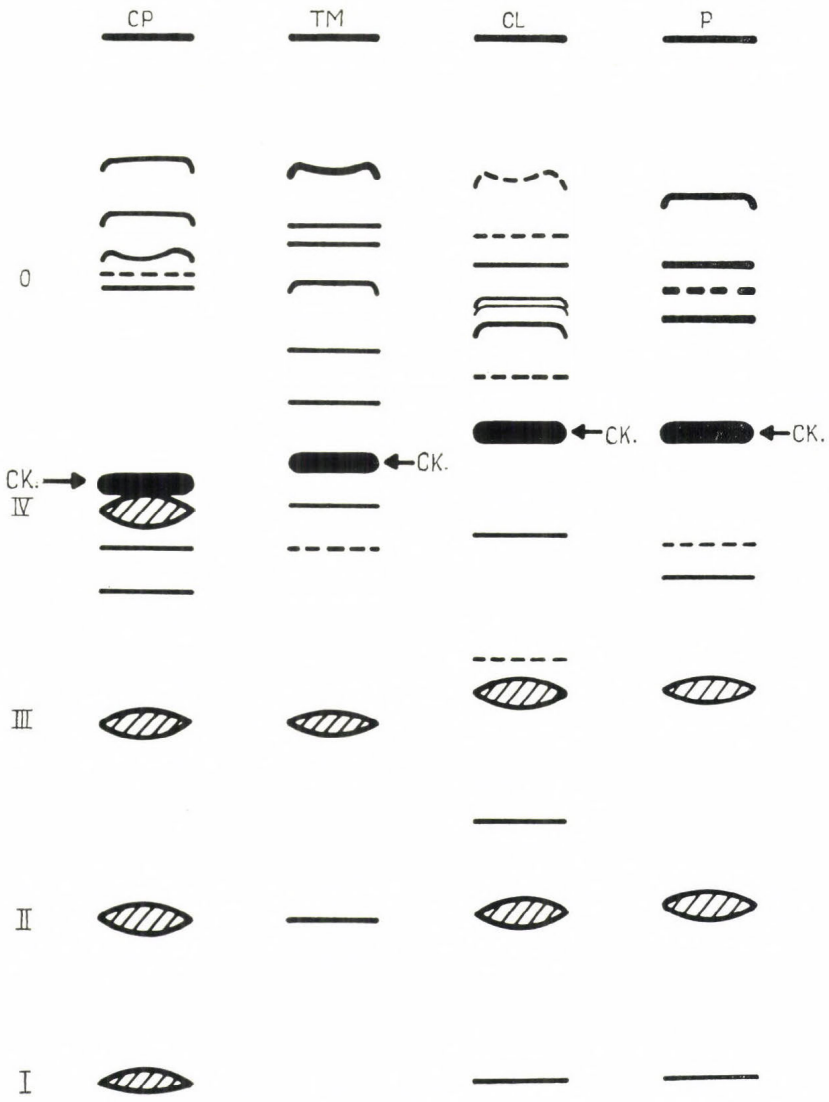

Fig. 5. Schematic representation of starch gel electrophoretic patterns at pH 8.5 of sarcoplasmic extracts of white muscles of carp (CP), Tilapia macrochir B (TM), codling (CL) and pike (P). Location of the insertion slot: 0. The bottom corresponds to the anode. The position of creatine kinase (CK) and of the PA occurring in these species is shown on the left

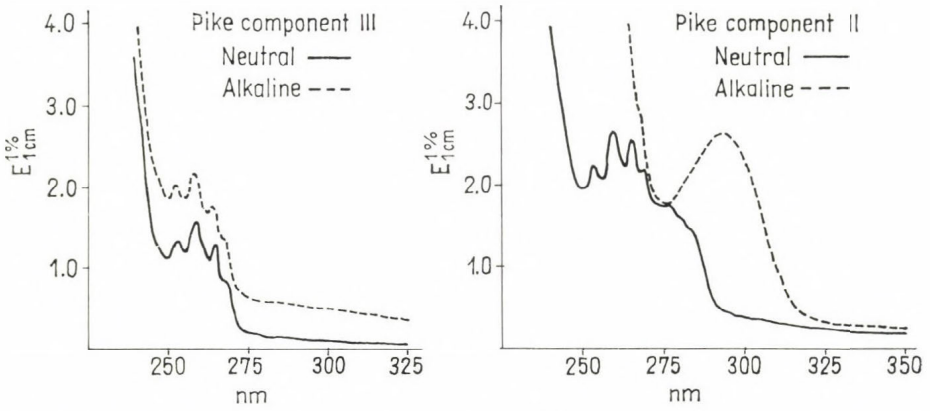

Fig. 6. Ultraviolet absorption spectra of the pike PA II and III in phosphate-NaCl buffer at pH 7.3 and in 0.1 N NaOH

weights range between 11,000–12,000. Some can be crystallized fairly easily. As Henrotte has observed with the carp component III, their ultraviolet absorption is quite unusual. They contain approximately 10 residues of phenylalanine per molecule, 0 or 1 residue or tyrosine and usually no tryptophan. The spectra of the two pike PA, shown in Fig. 6, correspond to component III devoid of tryptophan and tyrosine and to component II which contains one residue of tyrosine per molecule and gives therefore the usual shift and alkaline pH (Bhushana Rao and Gerday, 1973).

The amino acid compositions of 10 PA from 5 teleosts and 2 rays (Gerday and Teuwis, 1972) are compared in Table II. All PA are as a whole made up of a single polypeptide chain of 108 (± 1) amino acids. Several AA are absent or present as one or no more than 2 residues per molecule. This is the case of tyrosine and tryptophan, of the sulfur-containing amino acids, of proline, of histidine and of arginine. The amount of this last amino acid is particularly constant: all the PA examined so far contain a single residue of arginine per molecule except the frog one which contains 3 arginine residues (Pechère et al., 1971). In view of all these analogies, it was obvious that the PA represent a family of homologous proteins (Pechère, 1968; Gerday and Bhushana Rao, 1969) which appeared at first very different from other proteins described at that time.

But these properties did not indicate what function these proteins could play in the muscle cell. The first interpretation was that they were involved in some way in glycolysis. As Sephadex G-75 allows to fractionate the sarcoplasmic extracts into an unretarded fraction and a retarded one containing the PA, the glycolytic powers of both fractions were easily examined. Pechère and Focant showed in 1965 that glycogenolysis is carried out only by the unretarded fraction and is not influenced by the addition of PA. The comparison of the PA content of fish white and red muscle afforded also another argument against the contribution of these proteins to anaerobic glycogenolysis. In fish, the red muscle of the lateral line (Fig. 7)

Table I

Some properties of PA of fish white muscle

Component	Carp (1) (2)			Tilapia macr. B (3)		Cod (Gadus call.) (4)		Pike (5)	
	II	III	IV a + b	IIIa	IIIb	II	III	II	III
I.P.	3.95	4.25	4.37–4.47	4.1	4.4	4.4	4.1	4.2	5.0
$S_{20,w}$ (S)	1.7	1.6	1.7	1.6	1.6	1.7	1.7	1.55	1.55
$D_{20,w}{}^{x}\,10^{7}$	—	12.0	15.0	11.0	11.0	10.7	10.7	—	—
M.W. (UC)	—	9.300–12.500	11.100–11.300	13.100	13.100	14.700	14.800	—	—
(AA)	12.300	11.500	11.300	9.900	10.600	11.500	11.200	11.700	11.700
Crystallization	+	+	+	+			+	+	
UV. abs. Res. Phe/mol.	9.6	9.4	10.9	9.8	10.7	13.0	13.0	12.6	9.9
Res. Tyr/mol.	1.0	0	0	0	0	0	1.3	1.25	0
Res. Try/mol.	0	0	0	0	0	0	0.7	0	0

References (1) Konosu et al. (1965)

(2) Pechère et al. (1971)

(3) Piront et al. (1968)

(4) Bhushana Rao et al. (1969)

(5) Bhushana Rao and Gerday (1973)

Table II

Amino acid composition of some PA of fish and amphibian white muscle

Amino acid	II	Carp		Hake	Cod		Whiting	Pike		Ray thornback (clavata)	Ray spotted (montagui)	Frog/pI 4.50
		III*	IV a + b	II*	II	III	III*	II*	III*			
Lys	11	13	12 (14)	12	12	12	12	12	17	11	13	11
His	—	1	1	1	—	1	—	1	2	3	4	—
Arg	1	1	1	1	1	1	1	1	1	1	1	3
Asx	15	17	15	12	12	15	12	15	16	16	17	13
Thr	4	5	6	5	2	3	2	2	3	5	7	2
Ser	6	5	8	5	7	7	6	8	4	11	11	10
Glx	11	8	9 (8)	10	9	9	9	8	9	10	8	12
Pro	1	—	—	—	—	1	—	1	—	1	1	—
Gly	9	8	9 (8)	12	9	9	10	7	7	8	7	9
Ala	20	20	16	19	23	19	22	20	19	12	12	15
Cys	1	1	1	1	2	1	3	1	—	2	2	—
Val	4	5	6	4	5	5	6	5	6	4	4	7
Met	—	—	—	1	—	1	—	2	1	2	1	—
Ile	6	5	5	7	5	7	5	4	5	6	6	7
Leu	9	9	9	8	8	8	8	9	10	9	10	9
Tyr	1	—	—	—	1	—	1	1	—	1	1	1
Phe	9	10	10	10	10	10	10	10	9	8	8	9
Trp	—	—	—	1	—	1	—	—	—	—	—	—
Total	108	108	108	109	106	110	107	107	109	110	113	108

References in Pechère et al. (1974) except for pike PA II (Gerday, 1974) and for whiting PA III (Joassin, 1974). An asterisk indicates the completely sequenced proteins: carp PA III (Coffee and Bradshaw, 1973); hake PA II (Capony et al., 1973); pike PA III (Frankenne et al., 1973); pike PA II (Gerday, 1974); whiting PA III (Joassin, 1974).

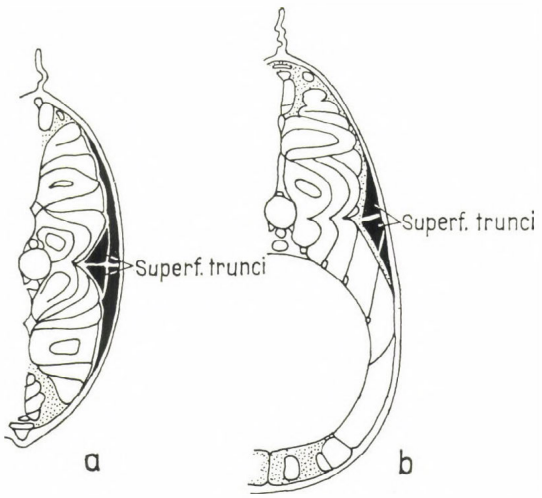

Fig. 7. Transverse sections through the tail (*a*) and the abdomen (*b*) of the carp showing the lateral red muscular region

contains only negligible amounts of PA. This conclusion is further reinforced by recent observations on fish heart muscle Starch-gel electrophoreses (Hamoir et al., 1972) and immunological investigations (Gosselin-Rey, 1974) have shown that this muscle contains only trace amounts of PA.

An unexpected observation has led to another suggestion about the role played by these proteins. In the course of amino acid analyses carried out in 1966 by Crokaert and myself on the two PA of Tilapia macrochir, we noticed that the ash content of the lyophilized PA powder, obtained after exhaustive dialysis of the protein solution against water, was abnormally high; it reached 1 to 2%. The composition of the ash was completely different from the salt composition of the intracellular fluid: it contained traces of magnesium and very large amounts of calcium corresponding to 2 ions per molecule (Fleron, 1967–68). Pechère described similar observations in 1971 and has drawn the attention to the fact that the specific binding of Ca strengthens the resemblance occurring between the PA and the calcium sensitizing factor, troponin-C or TN-C (Pechère et al., 1971). The general occurrence of two calcium ions per molecule of PA is now well established.

Let us now compare some properties of PA and TN-C. The wide zoological distribution of the troponin–tropomyosin regulatory system is now well documented; it is found in all muscles except in molluscs (Lehman et al., 1972; Szent-Györgyi et al., 1973), That of the PA appeared until last year limited to fish, amphibia and some reptilia living in water; in fact these proteins seem to occur also in muscles of higher vertebrates (cfr. infra). Some characteristics of these two types of protein are shown in Table III. They have several properties in common; they are acidic, bind calcium strongly though with different affinities. They are highly antigenic: passive transfer skin test reactions have always been positive with a cod

Table III

Comparison of PA and TN-C

Properties	PA	TN-C
Acidity	Acidic	Acidic
Molecular weight	11,500	17,850 (1)
Ca^{++}-binding moles/mole	2	4
Ca^{++}-binding K_d	1 and 4×10^{-7} (2)	10^{-6} (3)
Antigenicity	+ at 2×10^{-10} mg/ml (4)	Strongly antigenic
N-terminus	Acetylated (5)	Acetylated (6)
Contents (in residues per mole) Trp	0– 1	0 (6)
Tyr	0– 1	1– 2 (6)
Phe	8–10	8–10 (6)
His	0– 4	0– 1 (6)
Arg	1	5– 9 (6)
Met	0– 2	6–10 (6)
Biological role	Not regulatory (6)	Regulatory
Content (per kilo of muscle)	0.7 mM (carp)	0.07 mM (rabbit)

References (1) Collins et al. (1973)
(2) Benzonana et al. (1972)
(3) Perry (1973)
(4) Aas (1967)
(5) Pechère et al. (1974)
(6) Demaille et al. (1974)

PA preparation at the protein concentration of $2\cdot10^{-10}$ mg/ml (Aas, 1967). Their compositions in aromatic amino acids are very similar. On the other hand, the molecular weights and the contents in some amino acids as arginine and methionine are very different. Furthermore, PA occur together with TN-C in fish muscle and are not able to replace TN-C in synthetic systems. The similarities are still remarkable; they share a common ancestry as we will see later.

Another relationship has also been recently suggested between the PA and the myosin LC. As the biological function of TN-C is fulfilled in molluscs by the EDTA-light chain, it could be questioned if the similarities found between TN-C and PA could be extended to the myosin LC. Recently the LC have been purified and some analogies became obvious. Both rabbit alkali- and DTNB-LC have UV spectra reminiscent of the PA as shown by Perrie et al. (1973; Fig. 8). Both alkali-LC have as the PA, their N-terminus blocked (Frank and Weeds, 1974). This homology has been examined more closely by Collins (1974) who has compared the sequences of rabbit TN-C and alkali-LC with those of 3 PA. He concluded from this comparison that the extent of similarity is too great to have occurred by chance. These proteins seem thus to originate from a common remote ancestor.

As soon as it became obvious that the PA can be related to some contractile proteins, the determination of the tertiary structure of the carp

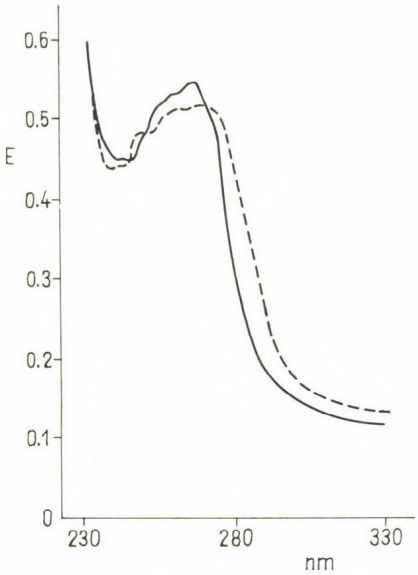

Fig. 8. UV spectra of the myosin alkali (solid line) and of the DTNB (broken line) light chains of rabbit skeletal muscle (after Perrie et al., 1973)

component III by method of Kretsinger received a great deal of attention. In fact Kretsinger started his X-ray study before being aware of the presence of two calcium in PA. Now the locations of these ions in the molecule are well established (Kretsinger and Nockolds, 1973; Hendrickson and Karle, 1973).

Figure 9 is a picture of the 1 cm-1 Å model built by Dr. Gerday in Oxford in Prof. Phillips' laboratory on the basis of Kretsinger's data. The dimensions of the protein are $30 \times 30 \times 36$ Å. The compact structure does not contain groves or pits typical of active sites of enzymes. It has a well-defined anhydrous hydrophobic core containing 8 of the 10 phenylalanin residues of the molecule; at the surface there are the hydrophilic groups and some hydrophobic ones which cannot be accomodated inside. The only polar internal groups are the carboxylic groups associated with the two calcium ions and an internal salt-bridge between the single Arg_{75} and Glu_{81}. The chain contains six helicoidal regions: A, B, C, D, E and F including 52 of the 108 residues. The first calcium is situated in the loop between C and D; the second one between E and F. Kretsinger (1972) has discovered repeats in the three-dimensional structure which are particularly obvious at the two calcium sites and has suggested that a gene triplication had occurred associating three fragments of about 33–36 residues: the fragment AB (without Ca^{++}), the fragment CD (1st Ca^{++}) and the fragment EF (2nd Ca^{++}). The internal salt-bridge between Arg_{75} and Glu_{81} occurs in a position relative to the AB loop similar to that of the Ca^{++} ions

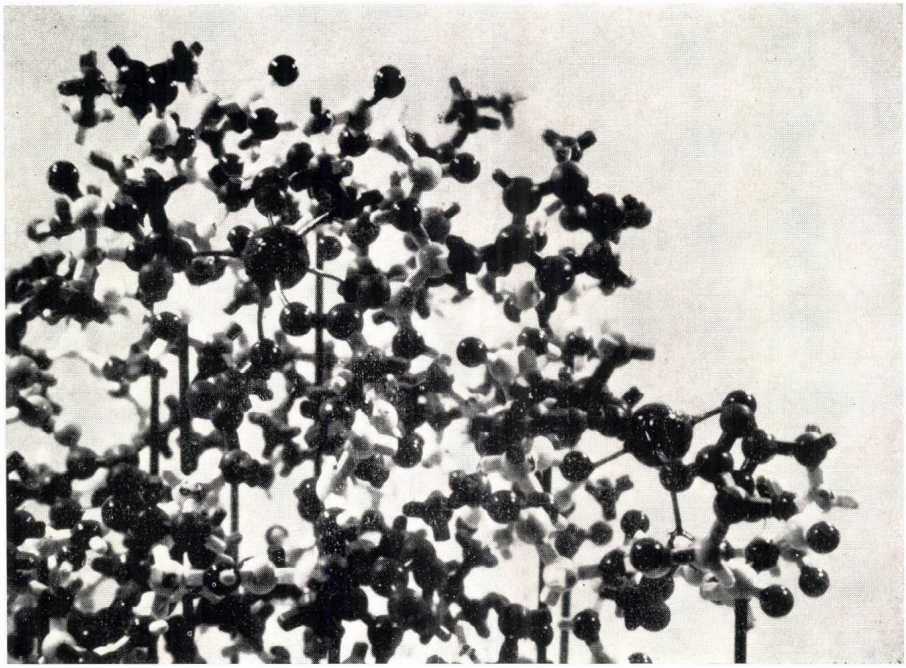

Fig. 9. Picture of a 1 cm-1 Å model of the carp PA III built on the basis of Kretsinger's data (Kretsinger and Nockolds, 1973) showing the fairly spherical shape of the molecule

in the two other fragments (Kretsinger, 1972). Selective modification of the single arginine residue in pike component III greatly alters the tertiary structure of the molecule as detected by immunochemical reactivity and leads to a reduced calcium-binding ability (Gosselin-Rey et al., 1973). The chain can thus be represented by the following formula indicating the location of the residues involved in calcium binding:

$$[A\text{–}B] - [C\text{–}Ca_{51-62}\text{–}D] - [E\text{–}Ca_{90-101}\text{–}F].$$

A mere inspection of the sequence reveals, however, no obvious homologies. This interpretation postulates that the three-dimensional structure of proteins evolves more slowly than the amino acid sequence.

If such a triplication of the PA exists, TN-C can arise from a tetraplication. This hypothesis has been put forward by Collins and coworkers (Collins et al., 1973). The homologies found are summarized in Fig. 10. The PA sequence has been divided into three fragments: AB, CD and EF. A delation of two residues has been assumed in AB. Vertical bars indicate helical regions and asterisks the residues of the CD and EF loops involved in calcium binding. As you can see, the CD calcium is co-ordinated by 6 well-defined oxygen atoms. The binding of the EF calcium is not so well established by the work of Kretsinger: 5 residues are involved, the 6th co-ordinate corresponds to a 2nd oxygen of Asp_{92} or to a water molecule.

PA			TN-C			
AB	CD	EF	I	II	III	IV
1 a		72 a	9 s		84 e	
2 f,y	34 g	73 d,g	10 y		85 d	
3 a,s	35 L	74 a	11 L	47 g	86 a	122 g
4 g	36 t,a,k	75 r	12 s	48 q	87 k	123 e
5 v,i	37 s,g	76 a	13 e	49 t	88 g	124 h
6 L	38 k	77 L	14 e	50 p	89 k	125 V
7 n,a	39 s,t	78 t	15 m	51 t	90 s	126 t
8 d	40 a,p	79 d	16 i	52 k	91 e	127 d
9 a	41 d,a	80 g,a	17 a	53 e	92 e	128 e
10 d	42 d	81 e	18 e	54 e	93 e	129 e
11 I	43 V,I	82 t	19 F	55 L	94 L	130 I
12 a,t	44 k	83 k,a	20 k	56 d	95 a	131 g
13 a	45 k	84 t,a	21 a	57 a	96 e	132 s
14 a	46 a	85 F	22 a	58 I	97 e	133 L
15 l	47 F	86 L	23 F	59 I	98 F	134 M
16 e,a	48 a	87 k	24 d	60 e	99 r	135 k
17 a	49 i	88 a	25 m	61 e	100 i	136 d
18 c	50 I	89 g	26 F	62 V	101 F	137 g
19 k,e	51 d*	90 d*	27 d	63 d	102 d	138 d
20 a	52 q	91 s	28 a	64 e	103 r	139 k
21 a,e	53 d*	92 d*	29 d	65 d	104 n	140 d
22 d,g	54 k	93 g	30 g	66 g	105 a	141 n
23 s	55 s*	94 d*	31 g	67 s	106 d	142 d
24 F	56 g,d	95 g	32 g	68 g	107 g	143 g
25 d,n,k	57 f*	96 k*	33 d	69 t	108 y	144 r
	58 I,V	97 I	34 I	70 I	109 I	145 I
	59 e*	98 g	35 s	71 d	110 d	146 d
26 h,e	60 e	99 v	36 v	72 f	111 a	147 f
27 k	61 d	100 d,e	37 k	73 e	112 e	148 d
28 a e	62 e*	101 e*	38 e	74 e	113 e	149 e
29 F	63 L	102 F	39 L	75 F	114 L	150 F
30 F	64 k	103 t,a	1 q 40 g	76 L	115 a	151 L
31 a,s,t	65 l	104 a	2 d 41 t	77 v	116 e	152 k
32 k	66 F	105 L,M	3 q 42 V	78 M	117 I	153 M
33 V	67 L	106 V	4 t 43 M	79 V	118 F	154 M
	68 q	107 k	5 a 44 r	80 r	119 r	155 e
	69 n	108 a,g	6 e 45 m	81 q	120 a	156 g
	70 F		7 a 46 L	82 M	121 s	157 V
	71 k,s		8 r	83 k		158 q

Fig. 10. Relationship between the primary structures of the carp and hake PA (residues from carp component III first, followed by carp components II, IV and hake III in the case of substitutions) and of rabbit skeletal TN-C. The alignment in PA is according to Kretsinger and Nockolds (1973). Vertical bars: helical regions of carp component III and predicted α-helices of TN-C. Asterisks: residues in PA involved in calcium binding. Horizontal lines and capitals: hydrophobic core residues in PA and corresponding residues in TN-C (after Collins et al., 1973)

TN-C has been, on the other hand, divided into four regions I, II, III and IV, each of which corresponds to a calcium binding site. The alignment chosen allows to get a good correlation with the 8 α-helical segments predicted from the amino acid composition as shown by the vertical bars, with the residues involved in Ca binding and with some hydrophobic core residues involved as shown by the horizontal lines. A structural similarity seems thus to be exist between the CD and EF regions and TN-C. The

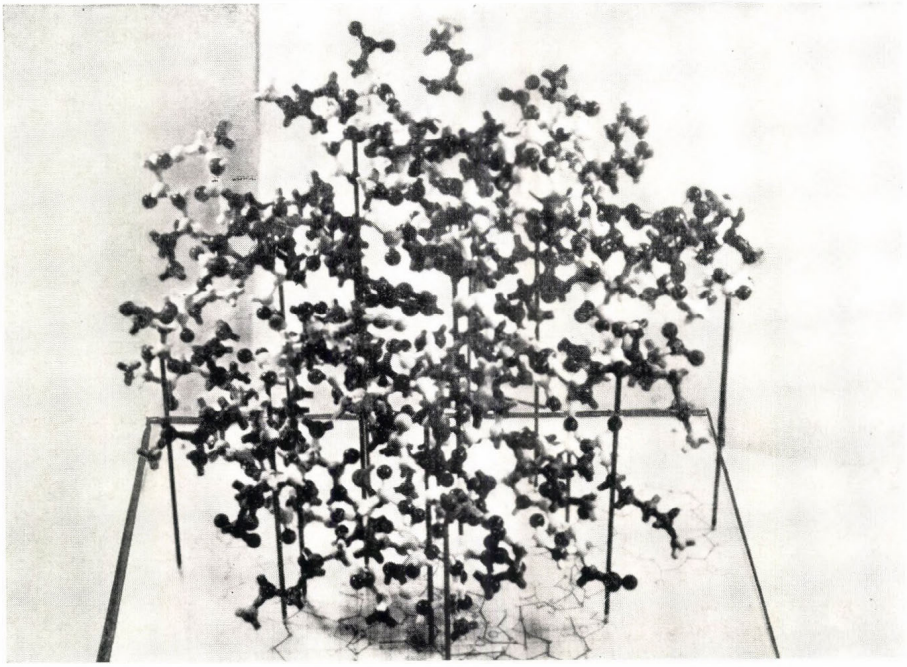

Fig. 11. Picture of a part of the model of the carp PA III of Fig. 9 showing the coordination of the two calcium ions and their different accessibility to the solvent

correlation with the AB region is less satisfactory. The location of the two calcium is shown more clearly in Fig. 11. The CD calcium is not exposed to solvent and the EF one looks less tightly bound: it can be replaced by a terbium ion (Moews et al., to be published).

Progress has also been made in the comparison of the primary structures of the PA. This field has been actively investigated by Coffee and Bradshaw in Saint Louis, by Capony and Pechère in Montpellier and by Gerday and his coworkers in Liège. The number of primary structures determined increases rapidly. A review of this comparative work appeared in Systematic Zoology (Pechère et al., 1974). The comparison of the sequences allows to draw some interesting conclusions. The N-terminus portion corresponding to the AB fragment varies much more than the two fragments CD and EF. The two calcium-binding acid regions (51–62 and 90–101) are invariable with regard to the residues involved in the co-ordination of the metal as well as the internal salt-bridge Arg_{75} Glu_{81}. The internal phenylalanine residues which contribute largely to the hydrophobic core, are also well preserved in all the sequences studied so far. On the other hand, the calculation of the distances between 7 PA has allowed to detect a new feature. An ancestral duplication has occurred; to lines of non-allelic genes have given rise to two genetic lines α and β. Similar calculations incorporating the two new primary structures of pike PA II (Gerday, 1974) and whiting PA III (Joassin, 1974) have allowed to build the phylogenetic tree repro-

duced in Fig. 12 which agrees with that of Pechère and collaborators (Gerday, unpublished). As you can see, this duplication which dates back to Million Years, is responsible for the occurrence of the two pike PA investigated in Liège. The carp components II, III and IV have appeared much more recently.

On the other hand, the observation of Aas and Jebsen (1967) that the hypersensitivity of some persons to fish is due to PA, has drawn the atten-

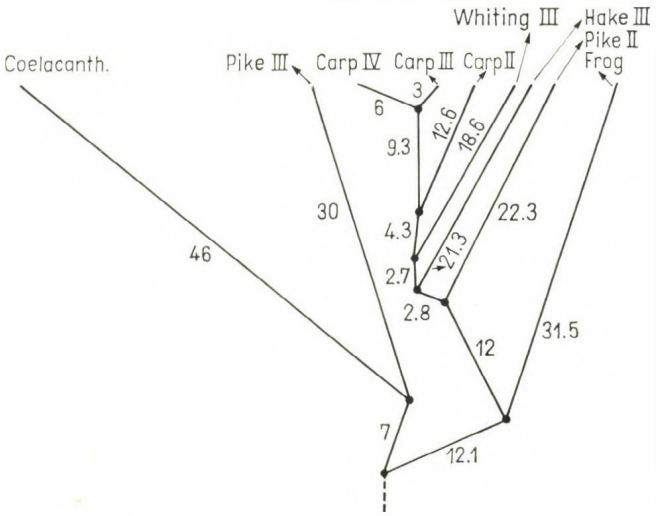

Fig. 12, Phylogenetic tree of PA drawn on the basis of the numbers of random evolutionary hits (REH). The length of the branches are proportional to the number of REH indicated (Gerday, unpublished)

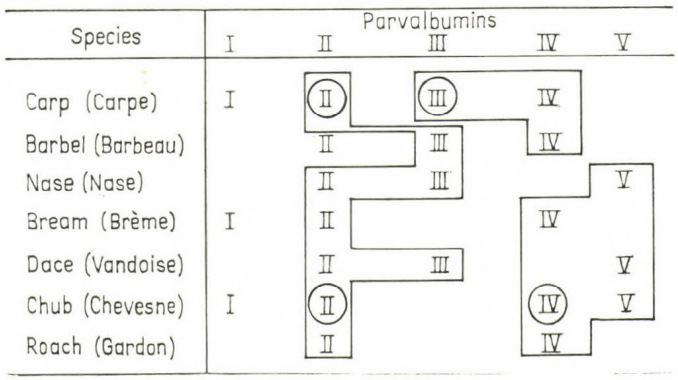

Fig. 13. Immunological relationships between the PA seven cyprinidae. Each frame encloses components displaying a similar immunochemical discrimination towards the antisera to the four encircled PA. The French names of the species are given between brackets (Pironi and Gosselin-Rey, 1974)

tion to the high antigenicity of these proteins. This opened a new approach to their study which has been investigated mainly in Liège. Dr. Gosselin-Rey has reviewed these immunological investigations at the Jablonna meeting (Gosselin-Rey, 1974). In general, cross-reaction among PAs is restricted to proteins originating from rather related fish orders and even lacks sometimes between PA of the same species. It is not only due to early divergence as in the case of pike PA III which displays no common antigenic determinant with pike PA II nor with any PA tested so far (the coelacanth one has not been examined), but it results also from the broad diversity of composition of the external shell. This high specificity has led to examine the immunological relationships occurring within a family of fish, the Cyprinidae (Piront and Gosselin-Rey, 1974). The results obtained are summarized in Fig. 13. The four encircled PA, two from the carp and two from the chub, have been isolated as pure as possible in order to obtain monospecific antisera. The components which cross-react are included in the same frame. The cross-reactions given by the carp component III and IV are in agreement with their fairly recent divergence determined by sequence comparison. The results found with the antisera against the carp and chub components II are more surprising in view of the earlier divergence of the carp component II. This component II appears thus to have retained properties which have been lost in the case of the less anodic components. It looks as being more ancestral than the others in the genetic line.

PA and TN-C have also been compared recently by this method. Heizman et al. (1974) have examined the cross-reactivity of PA and TN-C isolated from dogfish. Whereas native dogfish troponin or its TN-C subunit does not cross-react with antibodies against dogfish PA, the breakdown products of TN-C obtained by limited proteolysis show strong cross-reactivity. A new argument has thus been afforded from a quite different point of view, in favour of the relatedness between PA and TN-C.

Finally, I wish to mention two very interesting developments of which notice has just been given. Firstly, the zoological distribution of the PA is much larger than assumed so far. Minute amounts of PA are present in rabbit, (Pechère, 1974) chicken and human muscles (Lehky et al., 1974). The yield amounts to only 0.01 to 0.03% of the fresh weight while that from carp white muscle is of 0.7% (Konosu et al., 1965). Although TN-C and myosin LC have compositions related to PA and although some steps of the isolation procedure are carried out at pH 5.7, a pH at which catheptic degradation is possible, it is unlikely that this minor component arises from limited proteolysis occurring during the preparative procedure (Pechère, personal communication). The primary structures of the PA of higher vertebrates which are actively investigated will arouse great interest.

Another unexpected finding (Blum et al., 1974) could lead finally to the elucidation of the biological role of the PA. In the course of a study of the control of glycogenolysis in dogfish muscle, a protein kinase was found which does not activate phosphorylase kinase. The search for the function of this enzyme allowed to discover a phosphate-acceptor protein which unexpectedly has chemical and immunological properties identical to those of dogfish PA. One of the two methods of preparation of this protein involves heating at 80 °C for 1/2 h and precipitation with trichloracetic acid. This material is thus as stable as some PA. It differs, however, from dogfish PA

by its occurrence in three states of aggregation of about 350,000, 75,000 and 25,000 daltons. This last aggregated state is observed frequently with elasmobranch PA, the SH of which easily forms S–S bridges (Gerday and Teuwis, 1972) but higher ones have not been encountered so far. The high molecular weight components described by Fisher and coworkers appeal to correspond to various aggregation states of PA: they display the ultraviolet spectrum of dogfish PA, bind calcium in the same way and cross-react with antibodies to dogfish PA. Furthermore they dissociate completely in sodium dodecylsulfate into subunits of 11,000 and 13,000 daltons. These aggregates are readily phosphorylated by the cyclic AMP-independent protein kinase of dogfish muscle, one acid stable and alkali labile phosphate being introduced by subunit of 12,000. Dogfish PA on the contrary cannot be phosphorylated. These aggregates may thus occur in phosphorylated and unphosphorylated forms as the DTNB-LC, TN-I and TN-T, while TN-C could not so far be phosphorylated (Perry, 1974). It is difficult to assess the exact significance of this last paper until more work will be done along this line. It could give the clue to the biological function of these proteins which could be related in some way to the sarcoplasmic reticulum.

Although a large amount of work has been published on these proteins, their role in the muscle cell remained elusive and also their location is still uncertain. The most straightforward supposition is that these very soluble proteins are distributed homogeneously in the intracellular water including, in view of their small size, the space existing between the I and A filaments. But a disruption of the sarcoplasmic reticulum during the preparative procedure inducing the liberation of these constituents is possible and would agree with the occurrence of the high molecular weight aggregates described by Fisher and coworkers. A loose association with the A or I filaments is unlikely: as the PA are very unequally distributed between the white, red and cardiac muscles of the aquatic lower vertebrates, they do not seem to be located in the muscle machinery. On the other hand, the compact structure of the molecule stands against an enzymic function.

A recent work has made clear that some relatedness undoubtedly exists between LC and TN-C and the PA. The uneven distribution in different types of muscle is reminiscent of the differences shown by the LC. In the light of the present evidence, the PA appear more and more as a valuable candidate to a membership to the contractile machinery in spite of their high solubility and of their extractibility. They seem to constitute some kind of supplementary device for calcium regulation maintained in the skeletal white muscles of aquatic lower vertebrates.

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SOME RECENT RESULTS IN THE STUDY OF MUSCULAR PARVALBUMINS*

by

J. F. PECHÈRE

CENTRE DE RECHERCHES DE BIOCHIMIE MACROMOLÉCULAIRE, CNRS, MONTPELLIER, FRANCE

SUMMARY

The work on muscular parvalbumins, being now in progress in Montpellier is discussed in the present paper. This includes theoretical studies on parvalbumin phylogeny, the determination of the primary structure of the major component from Thornback Ray, the isolation of a parvalbumin as a minor component from rabbit muscle and the establishment of its amino acid sequence, and a study of the conformation of several parvalbumins through their luminescence properties.

THEORETICAL PHYLOGENETIC STUDIES

(in collaboration with M. Goodman, Dept. of Anatomy, Wayne State University)

The correctness of the topology presented earlier (Pechère et al., 1973) has been confirmed by several more elaborate, computerized methods based on the direct estimation of amino acid differences as well as on the reconstruction of ancestral nodal sequences (Moore et al., 1973). The existence of at least two different major genetic lines for the parvalbumins examined has thereby been corroborated.

SEQUENCE OF THE MAJOR PARVALBUMIN FROM THORNBACK RAY (*RAJA CLAVATA*)

(in collaboration with D. Thatcher)

The availability of an elasmobranch sequence is of particular interest for the preceding studies. The determination of the primary structure of the major parvalbumin from Thornback Ray has been undertaken. Presently, the following points appear to be well established. The protein having 110 amino acid residues is of α-type. The two Met are at the C-end of the molecule (105 and 108); two of the His appear as a doublet (26 and 27), as in the coelacanth 5.4 sequence, while the third one is at position 95; the single Tyr is at position 57, as in the coelacanth sequence; one of the two Cys (both Cys-SH, apparently) is often at position 18, the second

* Contribution No. 119. from the Centre de Recherches

one is at position 69; the single Pro is at position 7, a situation which corresponds to a region of the molecule just preceding the first helical stretch; a Lys-Lys doublet exists, at positions 37 and 38; position 60 is occupied by a Val and position 80 by a Lys. In addition, when the above sequence is compared to that of other parvalbumins, there appears to be a double gap in the region 21–24. A careful consideration of the stereochemical characteristics of the homologue of this region in the model of the carp 4.25 parvalbumin leads to the conclusion that the gap probably should be placed at positions 22 and 23.

Preliminary calculations indicate that a sequence as above would branch off from the coelacanth descent line in the α-domain after the Pike-Coelacanth nodal point. Such a situation is unexpected and needs further investigation.

ISOLATION OF A PARVALBUMIN FROM RABBIT MUSCLE

The possible existence of a parvalbumin in higher vertebrates has been the object of earlier observations (Pechère, 1968). An incident observation on rabbit myogen (Fig. 1) has been the starting point for a more careful investigation of this possibility. The major acidic component revealed by disc electrophoresis, first thought to correspond to a dimeric parvalbumin, was found to actually correspond to a different type of protein with normal UV spectrum. However, during these explorations a genuine monomeric parvalbumin was found in small amounts. It could be isolated by the usual

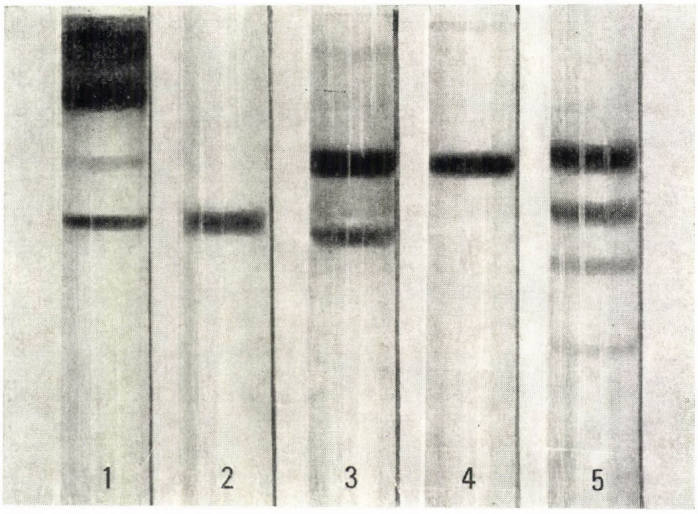

Fig. 1. Polyacrylamide gel electrophoresis (12% polyacrylamide, running pH ca 9.4) of rabbit myogen (1) and of the successive stages of the isolation of the parvalbumin (3, after G-75 Sephadex; 4, after DEAE-cellulose). The electropherogram of the purified major acidic component (2) and of the low molecular weight proteins from carp myogen (5) are presented for comparison

sequence of G-75 Sephadex and DEAE-cellulose chromatographies (Pechère et al., 1971). Some of its characteristics have been described by Pechère (1974). The protein isolated independently by Lehky and his co-workers (1974) has proved to be identical.

The physiological significance of this higher vertebrate parvalbumin, present in roughly 10 times smaller amounts than is found in lower vertebrates, remains to be clarified just as that of more abundant parvalbumins.

The determination of the amino acid sequence of rabbit parvalbumin (conducted in collaboration with J. P. Capony, H. Rochat, C. Pina and E. A. Fischer) is progressing rapidly.

LUMINESCENCE STUDIES

(in collaboration with E. A. Burstein, E. A. Permyakov and V. I. Emelyanenko, Institute of Biophysics, Academy of Sciences USSR, Puschino)

Muscular parvalbumins appear to be a material of choice for luminescence studies because of their frequent lack of Tyr and Trp residues. The examination of the phenylalanyl fluorescence of the carp 5.47 and hake 4.36 parvalbumins indicates high quantum yield values (up to 0.2) which are strongly dependent on the protein's structural state. In the case of the carp 3.95 component, the possibility also exists to independently study the single Tyr's fluorescence, and the Phe-Tyr energy transfer quantum yield which reaches 90%. A systematic exploitation of such parameters has led to the following conclusions. A denaturation of Ca-loaded parvalbumins takes place at pHs below 4 and above 10, and, at neutral pH, at temperatures above 60 °C; calcium removal by EGTA considerably narrows the pH stability zone and lowers the unfolding temperature to 20–40 °C. A conformational transition has also been found for all parvalbumins examined between pH 7 and 8 which could be of relevance to the possible physiological function of these proteins. Interestingly, such a change has not been observed in the case of troponin C (Lehrer and Laevis, 1974).

ACKNOWLEDGEMENTS

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A NEW CALCIUM-BINDING PROTEIN IN CRUSTACEAN TAIL MUSCLE

by

J. Cox, G. Benzonana, L. Kohler and E. A. Stein

DEPARTMENT OF BIOCHEMISTRY, UNIVERSITY OF GENEVA, GENEVA, SWITZERLAND

A couple of years ago, our laboratory became interested in the control of the muscular contraction in crustacea, specially fresh water crayfish. Recently we have shown (Benzonana et al., 1974) that the contraction in the crayfish tail muscle is regulated, as in mammalians, by a troponin–tropomyosin system and by a calcium pump. However, two differences were found between crayfish and rabbit. First, the molecular weights of tropomyosin and of troponin subunits are quite different in crayfish and rabbit muscle: 40,000 for crayfish tropomyosin compared to 34,000 for rabbit, and 54,000, 29,000 and 16,000 for crayfish TNT, TNI and TNC respectively, compared to 37,000, 24,000 and 20,000 for rabbit. Second, no particulate calcium sequestering protein could be isolated from crayfish sarcoplasmic reticulum, whereas in rabbit 3 classes of acidic calcium-binding proteins have been identified by the group of Mac Lennan (Ostwald and Mac Lennan, 1974).

We then focused our attention on the soluble sarcoplasmic proteins of crustacea. A class of soluble calcium-binding proteins, called parvalbumins, was identified and isolated in lower vertebrates by Pechère (Pechère et al., 1973) and, recently, in turtle, chicken, rabbit and man by the group of E. H. Fischer (Lehky et al., 1974). In spite of a detailed structural knowledge, no physiological function could be ascribed as yet to the parvalbumins. If they are implicated in some basic function, they should also be present in the muscle of invertebrates, or otherwise this function should be accomplished by an analogous protein. This communication reports on the apparent absence of parvalbumin in crayfish and on the isolation from the sarcoplasm of crustacean tail muscle of a calcium-binding protein which seems to have little or no common properties with parvalbumin except a similar affinity for calcium and a low isolelectric point.

PURIFICATION OF THE CALCIUM-BINDING PROTEIN

The homogenized muscle was centrifuged at 2×10^5 g and the myogen was dialyzed against water, concentrated and fractionated by chromatography on Sephadex and DEAE cellulose. The profile obtained after chromatography on Sephadex G-100 at pH 7.0 is presented in Fig. 1. The high molecular weight fraction contains appreciable amounts of copper and zinc proteins. The low molecular weight fraction, which contains the calcium-binding protein, was fractionated on DEAE cellulose. Figure 2 shows that

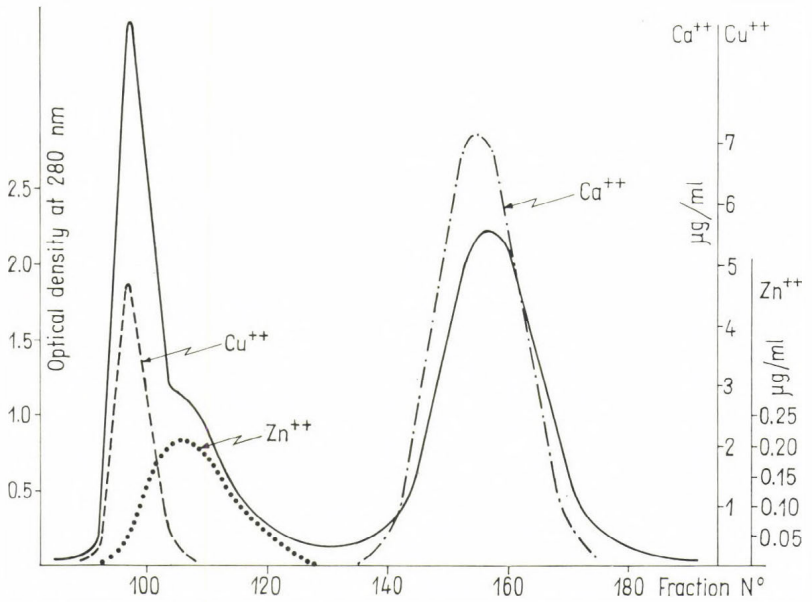

Fig. 1. Elution profile after a chromatography on Sephadex G-100 (4 × 75 cm column) of the albumin fraction of crayfish myogen. Elution was carried out at 6.5 ml per hour, volume of fractions 30 ml. Metals were determined by atomic absorption spectrophotometry

with a linear KCl gradient, two proteins are obtained, each as a single peak. The first protein is present in considerable amounts in the myogen; we have not been able yet to relate it to any known protein. The second peak, which coincides with the calcium-containing fractions, represents the calcium-binding protein. Six among the more typical fractions of the DEAE cellulose chromatography (Fig. 2) were analyzed by disc gel electrophoresis in the presence of sodium dodecyl sulphate and are shown in Fig. 3.

With the same procedure we were able to identify and isolate a pure calcium-binding protein from the myogen of lobster tail muscle.

CHARACTERIZATION

The pure calcium-binding proteins from crayfish and lobster have a classical UV spectrum with a maximum at 278 nm and a specific extinction coefficient of 10.6, based on dry weight. The calcium-binding protein thus differs from troponin C and from parvalbumins, both of which are characterized by a low absorption at 280 nm.

Table I summarizes the molecular weights of the calcium-binding protein and of peak 1 protein in crayfish as well as in lobster. The calcium-binding protein has a mol. wt of about 44,000 in its native state and dissociates in the presence of SDS and urea into two subunits with a mol. wt of about 21,000. In this respect also, the calcium-binding protein differs markedly from the parvalbumins, which consist of one polypeptide chain of about

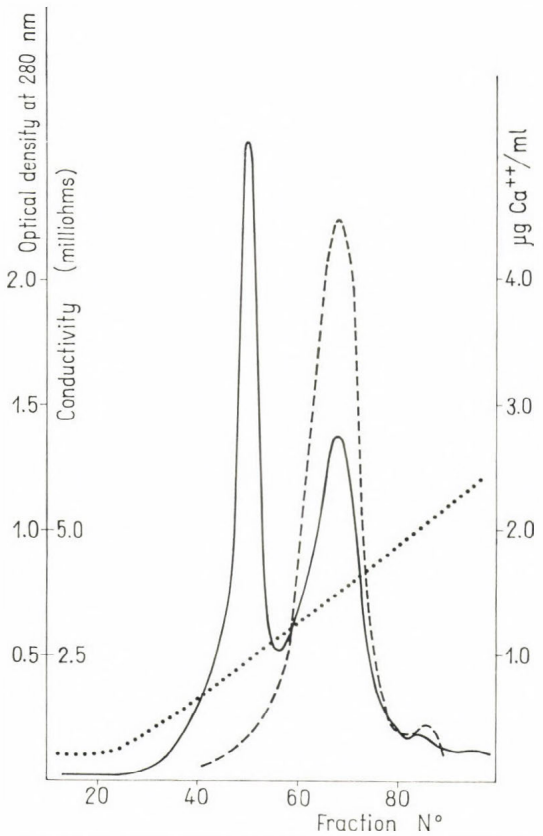

Fig. 2. Elution profile after a chromatography on DEAE cellulose (2 × 20 cm column) of the second peak fraction of Fig. 1. Elution was carried out at 25 ml per hour with a KCl gradient, volume of fractions 3.0 ml. Optical density at 280 nm (———); calcium concentration (- - - - - -); conductivity (. . . . . .)

Table I

Molecular weights of the calcium-binding protein and of peak 1 protein from crayfish and lobster

	Sephadex chromatography	SDS electrophoresis
Calcium-binding protein		
Crayfish tail	43,900	21,000
Lobster tail	46,500	21,500
Peak 1 protein		
Crayfish tail	37,000	47,000
Lobster tail	38,500	42,000

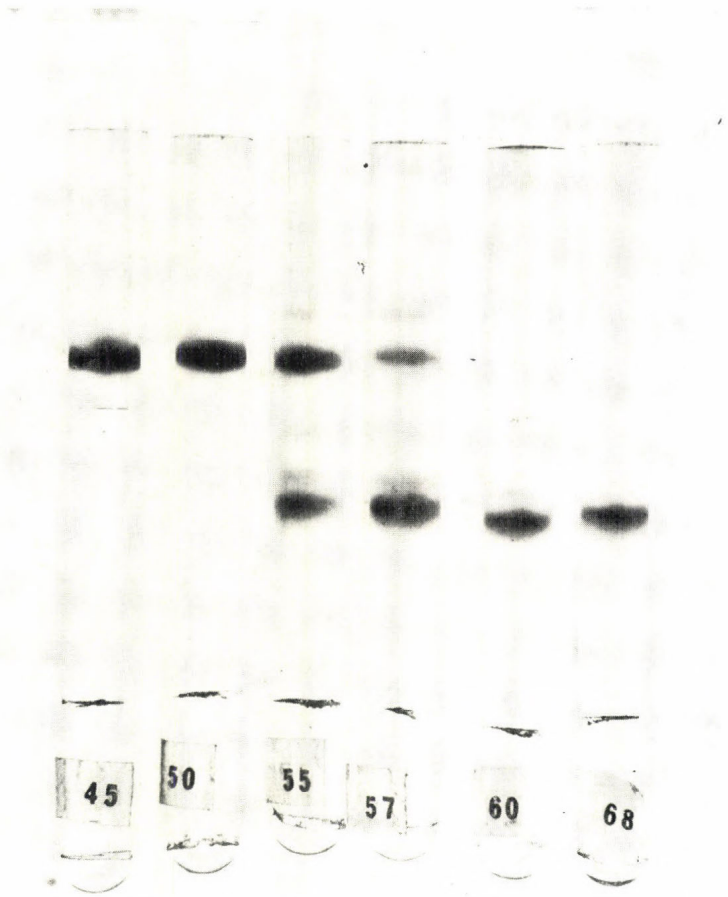

Fig. 3. Polyacrylamide (10%) disc gel electrophoresis in the presence of SDS of the eluate of DEAE cellulose chromatography. The numbers at the bottom of the gels correspond to the fraction numbers of Fig. 2

11,000 mol. wt. Peak 1 protein has a mol. wt of 42,000 (lobster) to 47,000 (crayfish) as determined by electrophoresis in SDS. The lower values found by Sephadex chromatography may indicate that the protein is highly asymmetric.

Metal content was measured by atomic absorption after extensive dialysis against a metal free buffer. The calcium-binding protein of both crayfish and lobster contains no other metal than calcium, which is present in a constant amount of 4 g atoms per mole of protein. Calcium can be completely and reversibly removed from the protein by dialysis against EGTA provided that Mg is present. The dissociation constant for calcium was determined by means of the Chelex method and by steady state dialysis,

and equals 3×10^{-7} M by both methods. With the same methods, we obtained a dissociation constant of 10^{-6} M for the B component of carp parvalbumin.

The amino acid composition of the calcium-binding protein of crayfish tail muscle was determined in the laboratory of Prof. E. H. Fischer in Seattle, and is presented in Table II. As expected from the UV spectrum, the protein has a normal Tyr and Trp content. Histidine, however, is absent. The high ratio of acidic amino acids, compared to basic, indicates that the calcium-binding protein has a low isoelectric point, as have parvalbumins. The last column of Table II represents the amino acid composition of peak 1 protein.

Table II

Amino acid composition of the calcium-binding protein and the peak 1 protein of crayfish tail muscle

	Calcium binding-protein		Peak 1 protein found
	found	assumed	
Gly	23.6	24	32.0
Ala	45.7	46	30.1
Val	25.7	26	22.1
Leu	25.9	26	36.7
Ile	21.6	22	18.6
Ser	14.2	14	19.4
Thr	10.6	11	20.6
Met	3.9	4	7.1
Cys	4.8	5	5.2
Pro	6.1	6	12.7
Phe	26.2	26	19.7
Tyr	19.0	19	12.1
Try	5.0	5	N.D.*
Asp	70.3	70	37.8
Glu	41.6	42	46.7
NH_3	54.7	55	30.8
His	0.0	0	9.2
Lys	28.4	28	34.2
Arg	13.7	14	20.0

Calculated molecular weight: 43,550

* Not determined

The concentration of calcium-binding protein in the myogen of crayfish tail muscle was determined by densitometric tracing following disc gel electrophoresis. A value of 10 g per litre of myogen was obtained. This corresponds to a concentration of bound Ca of 1 mM. Since the total calcium concentration of the myogen is 1.11 mM, it follows that there are little or no other calcium-binding proteins, such as parvalbumins, in crayfish myogen.

The calcium-binding protein has also been isolated from the claw muscle of crayfish. Interestingly enough, its concentration is 2 to 3 times lower in the claw than in the tail muscle. Similar differences have also been reported in the case of fish parvalbumins (Kretsinger and Nockolds, 1973), which are more abundant in white muscle than in red muscle. Work in progress shall answer the question whether the levels of calcium-binding protein in crustacea also show such a morphological difference.

CONCLUSION

For some time, it seemed that vertebrates could be divided in two classes according to their calcium-binding muscle proteins: the higher vertebrates possessing troponin but no parvalbumin and the lower vertebrates having parvalbumin but no troponin. However, recently troponin has been identified in several lower vertebrates (Demaille et al., 1974), and E. H. Fischer and collaborators found that parvalbumin was present in all higher vertebrates tested (Lehky et al., 1974). Thus parvalbumin and troponin are definitely not mutually exclusive. If we now concentrate on invertebrates, we may see that all organisms tested so far (except molluscs) possess troponin (Friedberg, 1974) whereas there is still much doubt about the presence of parvalbumin. As far as crayfish and lobster are concerned, we feel that parvalbumin is absent. Whether the calcium-binding protein described here for the first time substitutes for parvalbumin is a question that remains to be answered.

ACKNOWLEDGEMENT

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POSSIBLE MECHANISM BY WHICH CALCIUM AFFECTS THE MECHANICAL PROPERTIES OF RIGOR FROG MUSCLES

by

M. J. MULVANY

BIOPHYSICS INSTITUTE AARHUS UNIVERSITY, DK-8000 AARHUS C, DENMARK

INTRODUCTION

The structure of rigor and contracting muscles shows many similarities and for this reason it may be of interest for those investigating the molecular events associated with the contractile cycle. Recent results (Lowy and Mulvany, 1973) have shown that the resistance to stretch of a frog sartorius muscle, made rigorous by poisoning with iodoacetic acid (IAA-rigor), depends on the rate of stretch and on the fact whether this resistance can be reversibly reduced by withdrawing calcium.

These results, however, contradict to the characteristics of rigor glycerinated fibres. White (1970) has shown that their resistance to stretch is hardly affected by calcium and Heinl and co-workers (1974) have detected that this resistance does not depend on the rate of stretch. Thus it would have been desirable to repeat the IAA-rigor experiments using single fibres. However, the technical difficulties as well as the inherent qualities make difficult the putting single frog fibres into IAA-rigor (Brocklehurst, 1974). Until these difficulties are overcome this kind of experiments should be carried out with whole muscle.

METHODS

In the following experiments frog semi-tendinosus muscles were used and they were put into rigor by poisoning them with 0.4 mM IAA in solutions containing 1.8 mM calcium and repeatedly stimulating them ('Ca-rigor'). All experimental solutions were maintained at pH 7 at 2 $\pm$ 1 °C. Figure 1 shows the typical length-tension characteristics of a rigor muscle. Upon going into rigor the muscle developed a rigor tension. The muscle was then released, stretched and again released by 5%, 10% and 5% $\times L_0$ at a 'fast' rate. At the maximum stretch the tension approached the 'fast plastic limit'. The muscle was then stretched and released by 10% and 5% $\times L_0$ at a 'slow' rate. At the maximum stretch the tension approached the 'slow plastic limit', the value of which was about the half of the 'fast plastic limit'. The stretch rates used were defined so that further increase in the fast rate ($25 \times L_0$/s) or decrease in the slow rate ($25\% \times L_0$/h) produced little change in the characteristics.

Figure 1 shows the characteristics which would have been obtained if the slow cycle had been repeated. The characteristics would have remained almost unchanged but shifted along the axis. In this case, however, the calcium was withdrawn after the first slow cycle ('Ca-free-rigor') and the slow plastic limit was greatly reduced.

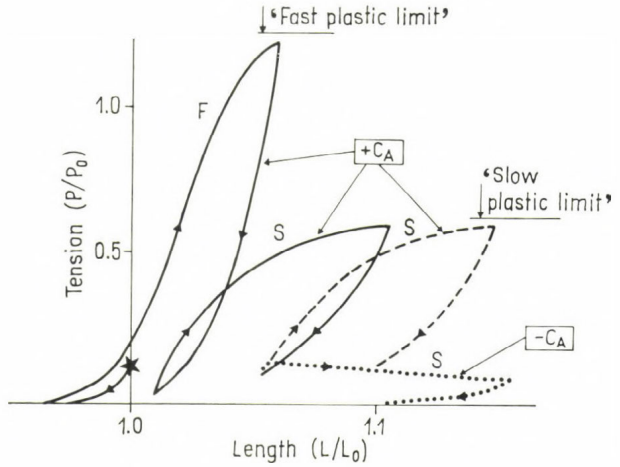

Fig. 1. Fast (F) and slow (S) characteristics of rigor muscles. (*) rigor tension; (———) This characteristic would have been obtained in case of repeated slow cycle; (- - - - -) Withdrawn Ca after the first slow cycle and reduced slow plastic limit; (. . . . .) The effect of Ca is reversible

Ca-RIGOR

X-ray diffraction studies (Huxley, 1968; Haselgrove, 1973) show that in rigor the cross-bridges move very close to the thin filaments, and it is generally assumed that the increased stiffness of rigor muscles is due to cross-linking between the contractile filaments. Thus resistance to stretch should, on the basis of the sliding filament theory, be proportional to the amount of overlap between the filaments. To check this point, the experiment shown in Fig. 2 was performed.

Using a laser muscles were set to sarcomere lengths of either 2.15 μ or 3 μ. They were put into rigor at these lengths and then released, stretched and released at the slow rate. The left-hand curves show the average net characteristics of the 2.5 μ muscles and the right-hand curves those of the 3 μ muscles. The bars show the standard deviation of the results. In each case the ratio of the fast to the slow plastic limit is the same, but the absolute values of the 3 μ muscles are only about the half of the 2.15 μ muscles. Thus both the fast and slow plastic limits appear to be proportional to overlap. This observation suggests that in fast and slow stretches of rigor muscles the strength of the cross-links determines the plastic limit, and that the combined resistance of the cross-links is greater during fast stretch than slow stretch.

Ca-FREE-RIGOR

Muscles were first put into Ca-rigor at sarcomere lengths of either 2.15 μ or 3 μ as described above. They were then placed in Ca-free solution containing EGTA and the calcium ionophore X537A for 3 h. Their subsequent

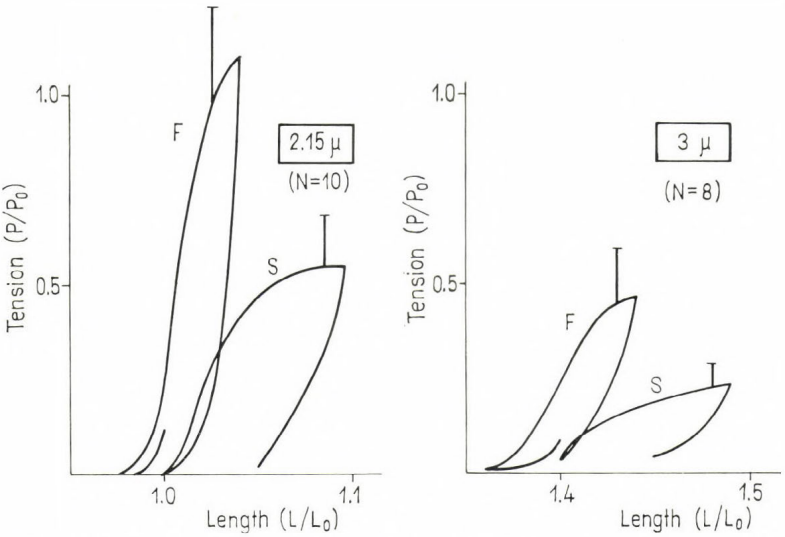

Fig. 2. Fast (F) and slow (S) net characteristics of Ca-rigor muscles

length-tension characteristics are shown in Fig. 3, where the left-hand and right-hand curves refer to the 2.15 μ and 3 μ muscles, respectively. They were then (as before) released, stretched and released at the fast rate and then stretched and released at the slow rate. These characteristics are shown by the full curves. For both the 2.15 μ and 3 μ muscles the fast plastic limit was slightly reduced compared with the Ca-rigor state, but the slow plastic limit was greatly reduced. Again comparison of the 2.15 μ and 3 μ muscles indicates that the Ca-free plastic limits are also approximately proportional to overlap.

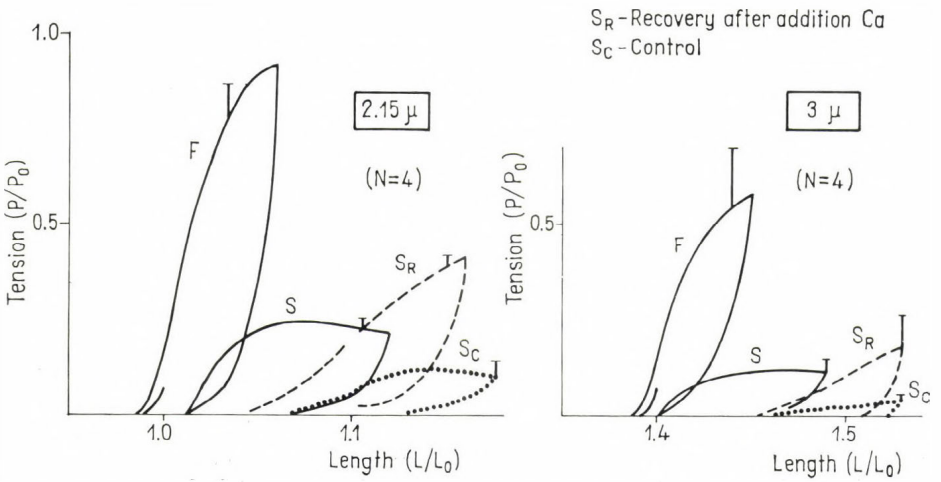

Fig. 3. Fast (F) and slow (S) net characteristics of Ca-free-rigor muscles

To demonstrate the reversibility of the calcium effect, half of the muscles has been replaced in Ca-containing solutions for 2 h, while the other half was kept in Ca-free solutions. The slow characteristics were then remeasured. The plastic limits of the muscles in the Ca-containing solutions (shown by the stippled curves) doubled, while those in the Ca-free solutions (shown by the dotted curves) decreased.

These results, therefore, confirm the previously published evidence that the Ca-effect is reversible. Furthermore they suggest that also in the Ca-free state the plastic limits are determined by the strength of the cross-links.

THE POSSIBILITY THAT RIGOR CHARACTERISTICS ARE DUE TO CHANGES IN CROSS-BRIDGE CONFIGURATION

The above results suggest that the 'fast' and 'slow' characteristics—both in the presence and absence of calcium—are determined by the properties of rigor cross-links between the contractile filaments. Thus the resistance of rigor cross-links is apparently greater during fast stretch than slow stretch, and while calcium withdrawal only slightly reduces the resistance to fast stretch, the resistance to slow stretch is greatly reduced. Such properties suggest that the cross-links could have 'visco-elastic' properties of the type proposed by Huxley and Simmons (1971). A simple adaption of this model is shown and briefly described in Fig. 4, in which the cross-link head (QX) has two possible configurations (configuration 1 or 2) and it takes up the configuration having the lower total potential energy.

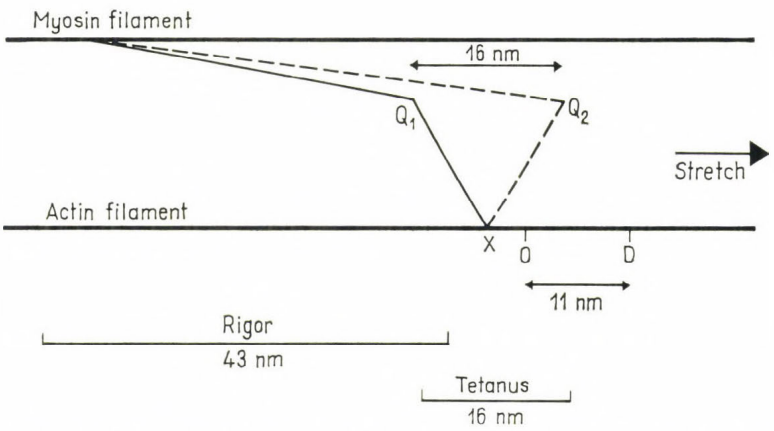

Fig. 4. Two configuration models of a cross-bridge and attachment distributions in rigor and tetanus

Properties: (*i*) head (QX) takes up configuration 1 (2) if attachment is to left (right) of O, (*ii*) Configurational changes are opposed by a viscous force. (*iii*) Link PQ is elastic but cannot exert compressive force. (*iv*) Link is unstrained if head is attached at 0 in configuration 1. (*v*) Attachments yield if stretched beyond D. (*vi*) Attachments are distributed as shown in the unstretched muscle

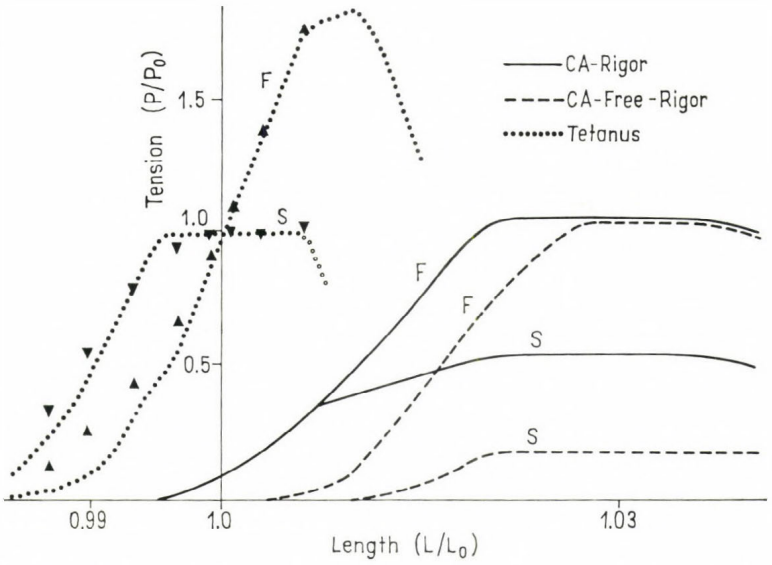

Fig. 5. Fast (F) and slow (S) characteristics of two-configuration model

Structural studies suggest that in rigor there is an even distribution of attachments along the actin filaments. Under certain conditions this implies that with reference to the point 'P' for each cross-link, the attachments are distributed as shown over a distance of 43 nm, the myosin repeat distance. The proposed model will then have the characteristics shown by the full lines in Fig. 5. The tension in 'slow' stretches is lower than that in 'fast' ones because attachments stretched through '0' at the fast rate will subsequently revert to configuration 1 at a rate determined by the 'viscous' force opposing this change. Making allowance for tendon compliance the model characteristics agree well with the experimental results described above.

On the basis of this model, calcium withdrawal might possibly be affecting the cross-link head equilibrium, for if this equilibrium is changed so that heads would only take up configuration 2 if they could do so without straining the link PQ, the characteristics shown by the stippled curves in Fig. 5 are obtained.

The dotted lines in Fig. 5 show that using a shorter attachment distribution length (corresponding to the stroke distance as shown in Fig. 4 as well as a much lower 'viscous' force opposing configurational changes) the model can also simulate the 'quick stretch/release' and 'initial recovery' characteristics of tetanised fibres as determined by Huxley and Simmons (1971). Their experimental results are shown by the triangles.

CONCLUSION

The Ca-rigor and Ca-free-rigor characteristics can be explained by supposing the cross-link head has two possible configurations (as proposed by Huxley and Simmons, 1971) with calcium withdrawal, affecting the head equilibrium. On the basis of this hypothesis structural studies of the Ca-rigor and Ca-free-rigor states may reveal different cross-link head configurations and so they support the model of Huxley and Simmons for the action of cross-bridges in living muscle.

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STUDIES ON THE BIOCHEMISTRY OF RIGOR MORTIS

(ABSTRACT)

by

V. Albrecht, W. Schulze,* B. Gassmann and S. M. Rapoport**

CENTRAL INSTITUTE OF NUTRITION, ACADEMY OF SCIENCES OF GDR, POTSDAM-REHBRUECKE, GDR

The discussion in the literature on the biochemistry of rigor mortis is focussed on three possibilities:

1. The post-mortem pH decrease elicits the onset and determines the time course of rigor mortis.
2. The breakdown of ATP elicits the onset and determines the time course of rigor mortis. The time course of rigor depends on the ATP content and the capacity of the ATP content and the capacity of the ATP-generating systems.
3. The post-mortem ATP decrease causes the release of calcium ions from sarcoplasmic reticulum. The time course of rigor mortis depends on the ATP content and on the capacity of the ATP-generating systems.

It is generally accepted, that rigor mortis follows Huxley's sliding filament model which describes physiological muscle contraction.

We have observed that variations of glycolytic flux rate due to the pretreatment of the rabbits with sodium chloride, epinephrine 4 h ante-mortem and epinephrine plus 1-fluoro-2,4-dinitrobenzene do not influence the onset and time course of rigor mortis. Statistical analysis has shown that no correlations exist between the glycolytic rate or the post-mortem behaviour of glycolytic metabolites and the onset or time course of rigor mortis.

Other results show that the variations of post-mortem ATP levels and the velocity of post-mortem ATP breakdown do not influence rigor characteristics such as extensibility and shortening. The variations of ATP levels of velocity of ATP breakdown were achieved by treating the rabbits *in vivo* with sodium chloride, 500 mg/kg body weight 1-fluoro-2,4-dinitrobenzene, 800 mg/kg body weight 1-fluoro-2,4-dinitrobenzene or sodium hexametaphosphate.

No correlations were found by comparing the correlation coefficients and regression coefficients of the ATP breakdown velocities and the rigor characteristics, respectively. Neither did we observe correlations between the ATP metabolites and the rigor mortis.

Oxalacetic acid was used to visualize the Ca^{++} ions within the muscle cells of the psoas major, under the electron microscope. One hour post

* Central Institute of Heart and Circulatory Research, Academy of Sciences of GDR, Berlin-Buch

** Institute of Physiological and Biological Chemistry, Humboldt-University, Berlin, GDR

mortem the calcium oxalate precipitate is located exclusively within the lateral cistern of sarcoplasmic reticulum close to the T-system. The T-system never contains any oxalate crystals. The location of calcium is identical with that of the calcium-accumulating ATPase activity in the living muscle demonstrated by the lead precipitation method. Four h post mortem calcium ions flow out. Some of the cisterns become empty, others just injured. Big crystals are formed, caused by a high local concentration of calcium ions near the injured membranes. Mitochondria are swollen, crystals of calcium oxalate lie near the membranes.

Though rigor mortis is accelerated by the application of sodium iodoacetate, which does not penetrate into the muscle cell of living animals; the post-mortem glycolytic rate and the breakdown of ATP are not influenced. The acceleration of rigor can be abolished by the application of muscle relaxants. A nervous induction of acceleration should be considered. The post-mortem calcium release and the characteristics of rigor run parallel in normal and accelerated rigor. The onset and time course of rigor mortis are determined by the post-mortem release of calcium ions from sarcoplasmic reticulum.

The shortening of sarcomeres is due to the shortening of all three bands. Actin and myosin bands shorten to a different extent, the actomyosin bands do not enlarge, they shorten, too. The maximum of ATPase activity corresponds with the onset of rigor mortis. Its activity is only 15 % of the myosin ATPase activity and is equal to the calcium-accumulating activity of the SR-system.

Within the estimated temperature range the rigor mortis does not follow Huxley's sliding filament model.

First results show that the actomyosin binding of the Ca^{++}-induced rigor is different from the ATP- and Ca^{++}-free rigor. The Ca^{++}-induced rigor is influenced by anionic detergents, the ATP-free rigor by cationic detergents.

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EFFECT OF CALCIUM IONS AND ATP ON THE SERIES-ELASTICITY OF GLYCEROL-EXTRACTED MUSCLE FIBRES

by

J. C. Rüegg, H. J. Kuhn, J. W. Herzig and H. Dickhaus

2nd DEPARTMENT OF PHYSIOLOGY, HEIDELBERG, FRG

It is well known that in isometric contraction the series elastic elements are stretched as a result of the shortening of contractile elements. The tension developed in an elastic element can then be partially or fully discharged by a quick release. The series elastic modulus or stiffness can be measured by determining the tension drop for a given reduction in length. Such length changes, however, must be accomplished in less than one millisecond to avoid truncation effects in the initial tension drop (Huxley and Simmons, 1971).

Huxley and Simmons (1973) concluded from sarcomere length dependence of the series elastic modulus that it is located largely within the cross-bridges and that it depends on the number of cross-bridges attached to the actin filaments at any moment. Stiffness measurements in glycerol-extracted fibres were used to elucidate the influence of calcium ions and ATP on the elastic properties of cross-bridges attached to the actin filament.

Bundles of glycerol-extracted rabbit psoas fibres were stretched and released by up to 1% L_0 within 1.1 msec by means of a Ling-dynamics 101 vibrator. The vibrator was controlled by a displacement and velocity dependent servo-amplifier and tension was recorded using a RCA-5437 transducer with a resonance frequency of about 2500 Hz. The bundle was relaxed in a calcium-free (5 mM EGTA, $[Ca^{++}] < 10^{-8}$ M) ATP-salt solution. Then, various levels of contraction were produced by raising the calcium ion concentration of the ATP-salt solution by means of Ca-EGTA buffers.

Table I shows the effect of various ATP and calcium ion concentrations on isometric tension, the tension fall immediately following a quick release (of 0.9% L_0, compare inset Fig. 1) and stiffness at low calcium ion concentration (line I), high calcium ion concentration (line II) and in rigor following a contraction (line III). Note that at high calcium ion concentration the isometric tension and the extent of the tension fall for a given release are twice to three times higher than at low calcium ion concentration.

Changes in the length step resulted in proportional changes in tension. Within the range of $\pm 1\%$ L_0, the stiffness was found to be independent of the test-length step. The Hookean nature of the series elasticity is clearly seen in Fig. 1. The immediate tension (T_1) observed within 1 msec after a length step is plotted as a function of the size of stretch in Fig. 1. Note that the slope of the T_1-curve is constant over a wide range of tension or length. By extrapolation of the straight line it is found that about a 2% length change is required to abolish isometric tension. This is true for the

Table I

Series elastic stiffness of glycerol-extracted rabbit psoas fibres

Solution*		Isometric tension	Tension fall**	Stiffness
MgATP (mM)	Ca^{++} (μM)	(kg/cm²)	(kg/cm²)	(kg/cm²)
I 15	0.13	0.64	0.29	32
II 15	5.9	1.74	0.71	79
III —	5.9	1.67	1.34	149

* in addition to 20 mM histidine, 10 mM sodiumazide, 5 mM EGTA, pH 6.7, 7°C
** immediate tension fall after a quick release of 0.9% L_0

various calcium concentrations used. This measurement is about half of the value estimated by Podolsky and Teichholz (1970) and Wise et al. (1973) for skinned fibres, using force steps (completed within 10 msec) if extrapolated to lie in the same range of final length changes; while our value is about twice of that reported by Huxley and Simmons (1971) of the experiments on live muscle.

From the demonstrated proportionality between stiffness and tension Huxley and Simmons (1971) suggested that the length shift per half sarcomere required to abolish isometric tension may correspond to the size of the cross-bridge stroke.

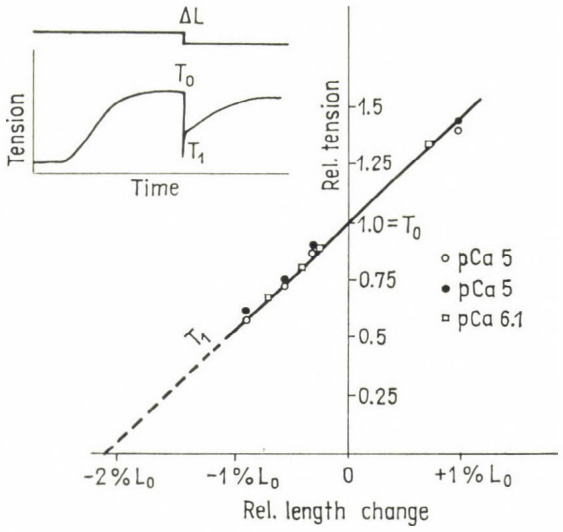

Fig. 1. Length-tension diagram of glycerol-extracted rabbit psoas fibre bundles in ATP-salt solutions at variable concentrations of the free calcium ions

Inset: Development of isometric tension and tension change observed after a quick release in length.
Diagram: Tension T_1 immediately following the length step as a fraction of the isometric tension before the length step plotted against the relative size of the length step (% L_0). (The absolute tensions (T_0) were 1.32 kg/cm² at Ca^{++} = 10 μM (○) and (●) and 0.39 kg/cm² at Ca^{++} = 0.8 μM (□).
Conditions: ATP, 15 mM; $MgCl_2$, 15 mM; EGTA, 5 mM; imidazole, 20 mM; sodiumazide, 10 mM; pH 6.7; 7 °C. Ca^{++} = 10 μM (○) and (●, control) or Ca^{++} = 0.8 μM (□).

If this is so our results indicate that—unlike tension—the extent of movement in a cross-bridge cycle is not affected by the calcium ion concentration (in the range of 10^{-7}–10^{-5} M).

In Fig. 1 the T_1-tensions are given as relative values with respect to isometric tensions before the quick release. Note that all the relative T_1-tensions lie on the same straight line irrespective of the calcium ion concentration. This result is in agreement with the view (Podolsky and Teichholz, 1970; Wise et al., 1973) that an increase in calcium ion concentration generates tension by increasing the number of crossbridges attached to the actin filament at any moment.

The stiffness of the fibres can also be increased by washing out the ATP. The glycerinated fibres were first brought to a state of isometric contraction, then the ATP was washed out in order to produce rigor with little loss of tension. Comparing line II and III of the Table I we can see that in ATP-induced contraction the immediate tension fall and stiffness are only about half as large as in rigor. This is true only provided that the tension before the release is about the same in contraction and rigor. It would be, however, too early to conclude from these experiments that in contraction about half as many bridges are attached as in rigor.

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Symp. Biol. Hung. 17, p. 57 (1974)

CORRELATED BIOCHEMICAL AND ULTRAHISTOCHEMICAL STUDIES ON Ca LOCALISATION AND Ca MOVEMENT IN SMOOTH MUSCLE

(ABSTRACT)

by

U. ZELCK, L. JONAS, U. KARNSTEDT and L. M. POPESCU

INSTITUTE OF PHARMACOLOGY AND TOXICOLOGY, UNIVERSITY OF ROSTOCK, ROSTOCK, GDR

Ca contents in nuclear, mitochondrial and microsomal fractions of pig coronary, partly of guinea pig ileum and guinea pig taenia coli were estimated by atomic absorption spectrophotometry and compared with the ^{45}Ca uptake and the Ca localisation in the smooth muscle cell. Ca localisation was determined by electron microscopy after oxalate precipitation and by electron-microscopic autoradiography using $^{45}CaCl$.

Results obtained clearly show that Ca oxalate precipitates in the cell membrane, in the sarcoplasmic reticulum, in the microvesicles of the mitochondrial membrane (including cristae) and in the nucleus membrane. There are remarkable correlations between the ^{45}Ca uptake of the subcellular fractions, their Ca contents and the Ca distribution in the smooth muscle cell.

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CLASSIFICATION OF HUMAN SMOOTH MUSCLE ANTIBODIES (SMA) BY IMMUNOFLUORESCENCE

by

G. F. Bottazzo, A. Florin-Christensen and D. Doniach

DEPARTMENT OF IMMUNOLOGY, THE MIDDLESEX HOSPITAL MEDICAL SCHOOL, LONDON, ENGLAND

Smooth muscle antibodies detected by immunofluorescence (IFL) have been studied for some years and the highest titres are found in patients with chronic active hepatitis (CAH) (Johnson et al., 1965; Whittingham et al., 1966). Low titres of these antibodies are seen in 12% of normal subjects and as a temporary phenomenon in about 80% of infectious hepatitis and mononucleosis cases, irrespective of age and sex or of the infectious virus (Holborow, 1972).

All positive sera react by definition with the smooth muscle layers of vessel walls in all organs, with the muscularis mucosae and interglandular smooth muscle fibres of gastrointestinal tract. In addition, some positive sera react with renal glomeruli (Whittingham et al., 1966) and with fibrils related to the plasma membrane in many epithelial structures, suggesting that several related tissue antigens may be involved. Smooth muscle antibodies have also been described in monoclonal proteins (Wager et al. 1971; Roux et al., 1974) and this provides an opportunity to analyse the IFL patterns obtained in relation to a single antigen.

Using sera reacting to more than 1 : 80, mainly from patients with chronic liver diseases, we have been able to distinguish three patterns related to the distribution of IFL in different tissues. The most frequent pattern (SMA 1) includes in addition to actual smooth muscle fibres, diffuse glomerular staining (Whittingham et al., 1966), small fibrils situated near the tubular basement membrane and near the brush border, polygonal pericellular IFL in liver and/or thyroid cells. A smaller group of positive sera (SMA 2) produce a lobulated mesangial glomerular IFL and does not react with any of the plasma-membrane associated fibrils in epithelial cells. In the third pattern (SMA 3) staining is limited to actual smooth muscle fibres. We have also studied five monoclonal SMA sera. Three were myeloma proteins (2 kindly donated by Dr. O. Wager) and two were from non-myelomatous benign gammapathies. Two monoclonal sera showed the SMA 1 distribution, one myeloma protein resembled SMA 2 and two sera were SMA 3. The paraproteins were of IgG class in two instances, IgA in two cases and one was a Waldenström's macroglobulin. In the myeloma proteins, SMA titres reached up to 1 million.

The SMA-related antigens have not been fully identified. Smooth muscle proteins injected into rabbits (Tranchev et al., 1974) yielded antibodies giving IFL patterns which resembled those shown by human sera and human SMA were absorbed with platelet extracts rich in actin (Gabbiani

et al., 1973). In our own experiments we were unable to absorb out any of the three SMA patterns with human actomyosin but some absorption of SMA 1 occurred with actin.

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SARCOPLASMIC RETICULUM AND TRIADS IN HUMAN STRIATED MUSCLE

by

N. GARAMVÖLGYI

RESEARCH UNIT, HUNGARIAN COLLEGE OF PHYSICAL EDUCATION, BUDAPEST, HUNGARY

There are very few data available on the ultrastructure of normal human muscle, because it is not very easy to obtain samples from healthy men, except surgical cases. When I have worked in the Postgraduate Medical School, Budapest, I had the opportunity to obtain carefully prepared muscles fixed at constant length immediately after their excision. These samples were taken from the oblique abdominal muscle, when in case of e.g. appendicitis is not affected by any sickness and may be considered normal. The careful preparation of the muscle specimen made electron micrographs of

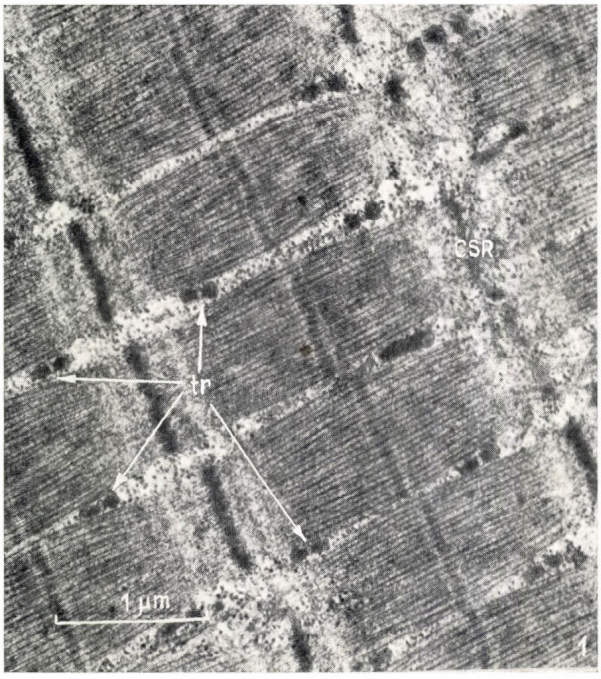

Fig. 1. Longitudinal section of a fast fibre of human oblique abdominal muscle. The triads allocated close to the I-A junctions (tr.). On the level of the Z-line a circular component of the sarcoplasmic reticulum (CSR) surrounds the myofibrils

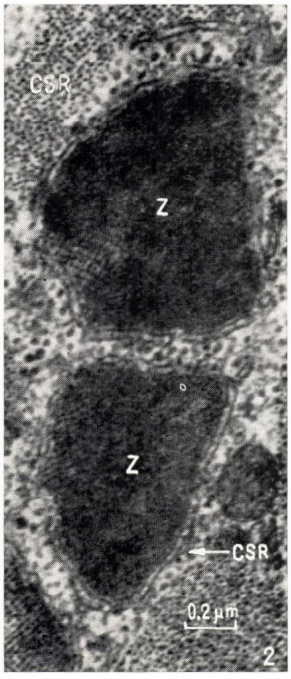

Fig. 2. Cross-section cut on the level of the Z-line. Note the circular sarcoplasmic reticulum (CSR) around the myofibrils

high quality possible, similar to those of experimental animals. As a consequence, in an earlier paper I could write about the slow and fast fibre types of human muscle (Garamvölgyi, 1972). In this paper I shall deal merely with the structure of the sarcoplasmic reticulum and T-system. (This is of great importance, because serious mistakes may occur. Clinicians usually speak of an 'expansion of the sarcoplasmic reticulum' even at sites where there is no SR.) Our knowledge in this respect is insufficient, as shown by the fact that in her excellent review on the sarcoplasmic reticulum Clara Franzini-Armstrong (1972) does not even mention human striated muscle.

Near the resting length the triads are located close to the A-I junction (Fig. 1), more or less regularly at mid-distance between the Z- and M-line. When the muscle is stretched and the I-bands become long, the triads are drawn to the level of the I-bands.

The triads as seen in longitudinal sections appear to consist of a profile of a T-tubule, very closely connected to two terminal cisternae of high electron density. There is no visible space between the T-tubule and the terminal cysternae.

On the right side of Fig. 1 the plane of the section runs above (or below) the surface of the myofibril and, as a consequence, a system of tubules and vesicles surrounding the myofibril at the level of the Z-lines becomes visible. This circular sarcoplasmic reticulum (CSR) is not to be confounded with

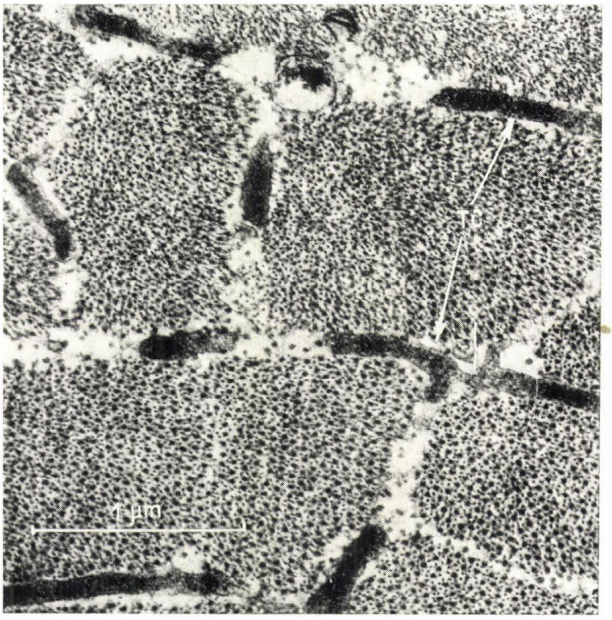

Fig. 3. Cross-section cut on the level of the triads. The dense terminal cysternae (TC) are visible between the myofibrils, they do not surround them, however, completely

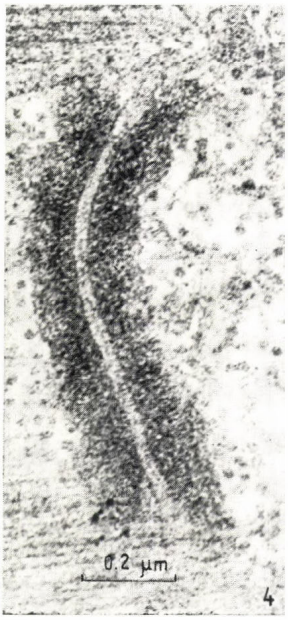

Fig. 4. Tangent section of a single triad. The terminal cysternae are filled out by amorphous dense material. Note the regularly arranged dense particles close to the T-tubule

the T-system. The same circular system of the sarcoplasmic reticulum can be seen also in cross-sections cut exactly at the level of the Z-lines (Fig. 2).

This muscle is very poor in longitudinal sarcoplasmic reticulum, much poorer than frog, reptilian or avian muscles known from the literature. In cross-sections longitudinal tubuli occur only sporadically between the myofibrils. There is no 'sausage-skin'-like arrangement of the sarcoplasmic reticulum, nor is there any special structural arrangement at the level of the M-line. It is therefore highly unprobable that the sarcoplasmic reticulum could considerably contribute to the resting elasticity of this muscle. Cross-sections cut exactly at the level of the triads show the latter to surround the myofibrils incompletely (Fig. 3). This may explain the inconsistent presence of triads in longitudinal sections, particularly in slow fibres.

In fortunate cases triads can be seen as tangent sections (Fig. 4) (*see also* Garamvölgyi, 1972). The terminal cisternae seem to be filled by amorphous, granular dense material. On the edge towards the T-tubule there is a regular arrangement of globular bodies. These structures have been described by Ashhurst (1969) in pigeon breast muscle as bridges connecting the membrane of the terminal cisterna to the wall of the T-tubule. Our figures indicate that the connection between terminal cisternae and T-tubules is more intimate and the mentioned regular structures are arranged alongside the internal wall of the terminal cisternae. At any case, they probably have something to do with impulse transmission and calcium-release.

I wanted to point out the special features of well-preserved normal human muscle, which is highly important from practical point of view, without wanting to state anything new in principle.

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MECHANICS AND ENERGETICS

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THE RESTING ELASTICITY OF INSECT FLIGHT MUSCLE*

by

J. W. S. PRINGLE

AGRICULTURAL RESEARCH COUNCIL UNIT OF MUSCLE MECHANISMS
AND INSECT PHYSIOLOGY, DEPARTMENT OF ZOOLOGY, OXFORD, ENGLAND

SUMMARY

1. The mechanical properties of relaxed insect fibrillar flight muscle are shown to depend critically upon the rate of stretching and the extent of stress relaxation. There is no true 'equilibrium' tension.
2. The properties of the relaxed muscle are best explained by the presence of a hydrated gel of paramyosin which effectively glues the end of the myosin filaments to the Z-line. Filaments become visible in this material only after stretching.

INTRODUCTION

One of the incompletely solved problems about insect flight muscle is the location and nature of the structure responsible for the high elasticity of the relaxed fibres. The problem is simply stated. In the classical picture of the fine structure of a striated muscle, neither the myosin filaments nor the actin filaments are continuous throughout the myofibril and in the relaxed condition the myosin cross-bridges are detached from the actin

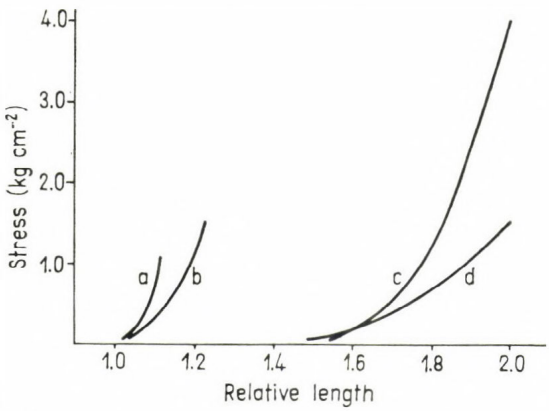


Fig. 1. Tension-length relationship in relaxed muscles, referred to the length at which the fibres are just slack; *a* insect fibrillar flight muscle (beetle) (after Machin and Pringle, 1959); *b* insect non-fibrillar flight muscle (locust) (after Weis-Fogh, 1956); *c* crustacean muscle (crayfish) (after Zachar and Zacharova, 1966); *d* vertebrate skeletal muscle (frog semitendinosus) (after Ramsay and Street, 1940)

*Invited paper

filaments and are performing their cycle of attachment and detachment either not at all or at a slow rate and in small numbers. Consistent with this picture, fibres from frog leg or rabbit psoas muscles, either intact and relaxed or glycerol-extracted and placed in relaxing solutions, can be extended to the point of no overlap with little development of tension if the stretch is performed slowly. Rapid stretches give rise to a transient tension which decays quickly to zero.

By contrast, in many invertebrate muscles and particularly in the two types of insect flight muscle, significant and apparently maintained tension is developed by small, slow stretching of relaxed fibres (Fig. 1). This is not merely a quantitative difference. The rise of tension above 1.6 l_0 in frog muscle is due to the orientation of a helical network of collagen fibrils in the sarcolemma (Schmalbruch, 1974) but Buchthal and Weis-Fogh (1956) showed that in insect flight muscles the sarcolemmal sheath is virtually absent. Furthermore, a tension-length curve similar to line *a* of Fig. 1 is obtained from single glycerol-extracted fibres from water-bug *Lethocerus*, which, like beetles, has fibrillar flight muscles (White, 1967). The resistance to stretch in insect flight muscles is thus a property of the myofibrils. The problem is to determine the reason why these muscles differ in this mechanical property from the muscles of the frog and rabbit.

This paper reviews previous studies on the problem and gives a preliminary report of work in progress in Oxford which suggests that the I-band of insect fibrillar flight muscle contains a gel of paramyosin which makes a significant contribution to the mechanical properties of relaxed fibres.

MECHANICAL PROPERTIES

It is first necessary to appreciate the nature of the mechanical property commonly described as 'resting elasticity'. Figure 2*a* shows the tension developed when glycerol-extracted fibres of water-bug flight muscle are given a quick stretch of 2%; Figure 2*b* shows similar results plotted on log-log scales for various amplitudes of stretch. Because of stress relaxation, there is no truly maintained tension and over a time period of three log units the tension decay curve is of the form;

$$T = A \cdot t^{-k}$$

where A is a constant depending on the amplitude of stretch. In these experiments the exponent $k = 0.08$, if t is in seconds.

This means that graphs of the relationship between tension and extension depend critically on the time after the stretch at which tension is measured. Figure 3 is a published example of such a graph, the values of tension being those reached after 4 min of stress relaxation. There is no true 'equilibrium tension' (Garamvölgyi and Belágyi, 1968*a*), although the decay of tension after several minutes is so slow that special precautions are needed to detect it.

Tension-length curves, plotted for any degree of stress relaxation, also depend on the direction of length change. Figure 4 shows such curves,

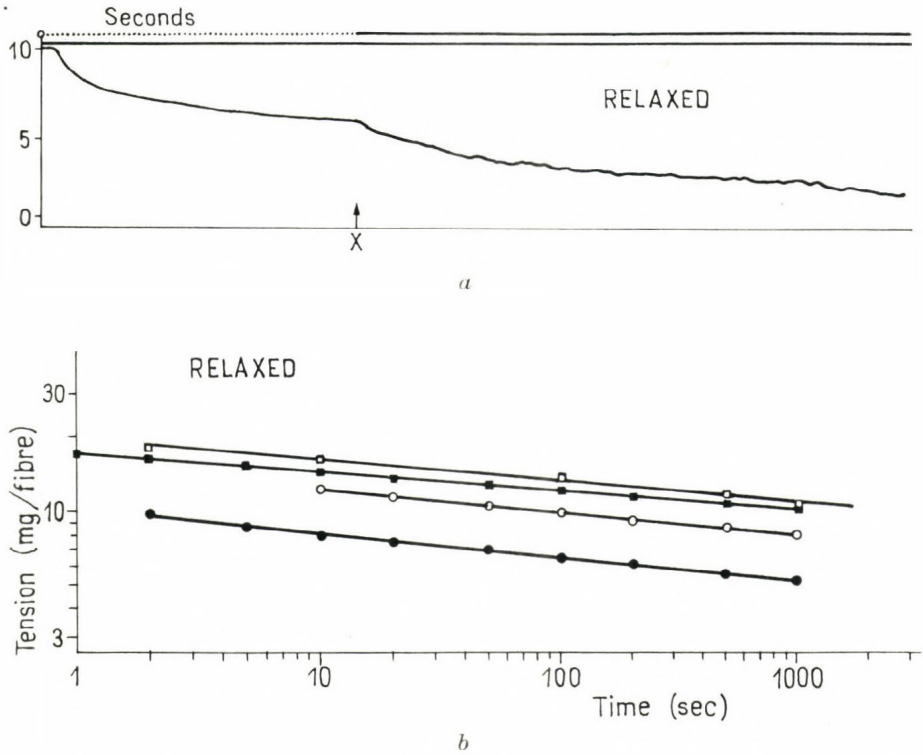


Fig. 2. Stress relaxation of relaxed glycerol-extracted *Lethocerus* flight muscle fibres. *a* After a quick stretch of 2%; the time scale slows by a factor of 10 at point X; *b* similar results, for quick-stretches of 3%, 5%, 7% and 9%, plotted on log-log axes (after White, 1967)

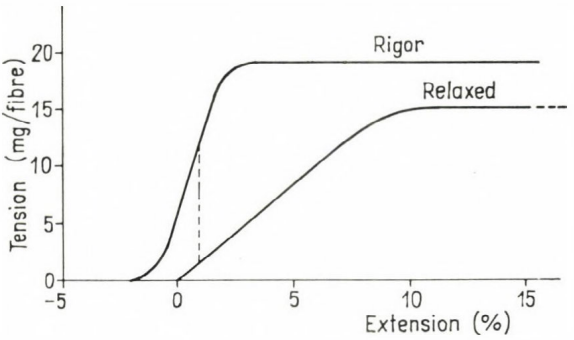


Fig. 3. 'Equilibrium' tension-extension curves for glycerol-extracted *Lethocerus* flight muscle fibres in rigor and relaxing solutions. Stress relaxation was allowed to proceed for 4 min (Pringle, 1967)

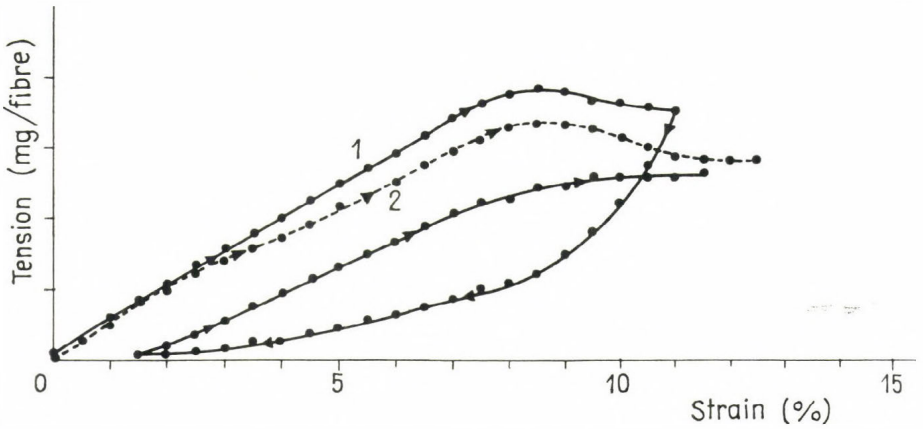


Fig. 4. Tension-strain curves for glycerol-extracted *Lethocerus* flight muscle fibre in relaxing solution. In curve 1 the length of the fibre was increased by 11% in 0.5% steps every four minute then decreased and increased again; curve 2 is another example of a tension-strain curve for successive extensions (White, 1967)

from an experiment in which 0.5% stretches were applied every 4 min up to 11% strain, followed by similar step decreases in length. There is pronounced tension hysteresis and if a second series of stretch is then given, the curve is displaced along the extension axis. Only after a long wait at rest length can the original curve be obtained again.

An alternative way of studying resting elasticity is to apply very small sinusoidal changes of length and measure the resulting sinusoidal changes of tension. Such results are best displayed as a Nyquist plot, on which the X and Y coordinates represent the components of tension in phase and in quadrature with the length change for each frequency of imposed oscillation. In the similar 'vector modulus' (Pringle, 1967), the tension values are divided by the cross-sectional area of the fibres to give absolute values of the elastic and 'viscous' moduli of the preparation. On such plots, 'stiffness' is the length of the origin vector of each frequency point, but the X coordinate can be used as a good approximation to this if the viscous modulus is small.

Figure 5 shows a Nyquist plot of glycerol-extracted fibres in relaxing solution. At 1 Hz, relaxed fibres show an almost pure elasticity; at higher and lower frequencies, there is a small viscous component. The distance of the points from the origin gets less as the frequency of oscillation is reduced; this corresponds to the stress relaxation seen in quick stretch experiments.

The experiments of White (1967) described so far were done using glycerol-extracted fibres from water-bug flight muscle. Garamvölgyi and Belágyi (1968*a*, *b*) performed similar experiments using intact relaxed honey-bee flight muscle and obtained a tension-length curve differing in one important respect from that of Fig. 3. Figure 7 from their paper shows a steady increase in 'equilibrium' tension up to an extension of 2.5 mm which is

a 100% increase in fibre length; the curve for relaxed fibres in Fig. 3 reaches a plateau at about 9% extension. Before concluding that this is real difference between the two species of insect, it is necessary to examine closely the conditions under which the experiments were performed.

In their experiments, Garamvölgyi and Belágyi employed successive stretches of 2% fibre length, applied in about 0.5 sec, and then allowed stress relaxation to proceed for 30 sec before measuring 'equilibrium tension'. The bundle of fibres from one dorso-ventral flight muscle was left *in situ* in the thorax, all other connection between the top and bottom of the thorax being severed. Connection to the apparatus was made with 0.1 mm flexible copper wires bound round the wings and the base of meso-thoracic legs, respectively. The stiffness of the force transducer is not given. In White's experiments, bundles of about 10 fibres were glued to an apparatus which was two orders of magnitude more stiff than the preparation. Successive quick stretches of 0.5% were employed and stress relaxation allowed to proceed for 4 min before measurement of tension.

It seemed possible that these differences in technique, rather than the difference in source of flight muscle, might be responsible for the difference between the results. Experiments have therefore been performed in Oxford using glycerol-extracted fibres from the large tropical carpenter bee, *Xylocopa* (*Koptorthosoma*) *flavo-rufa*, de Geer, which is very similar to the honey-bee, *Apis mellifera*, but from which fibres of reasonable length can be prepared for attachment to measuring apparatus. We have confirmed that, however resting tension is measured, there is a discontinuity in the tension-length relationship as extension proceeds, but the value of strain at which this occurs depends markedly on the conditions of the experiment.

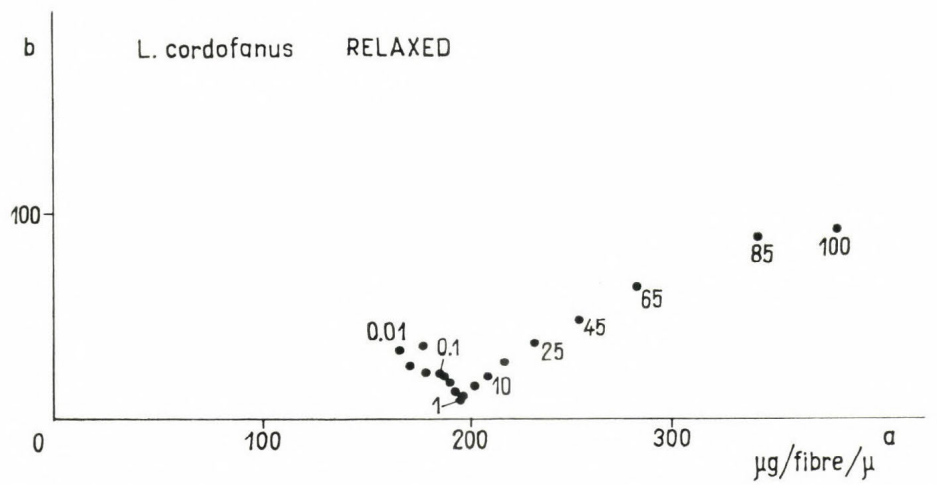


Fig. 5. Nyquist plot of mean results from measurements of tension change during small-amplitude length oscillation superimposed on 1% stretch of relaxed *Lethocerus* flight muscle fibres. The figures against the plotted points give the frequency of oscillation. See text for explanation of the meaning of this plot (White, 1967)

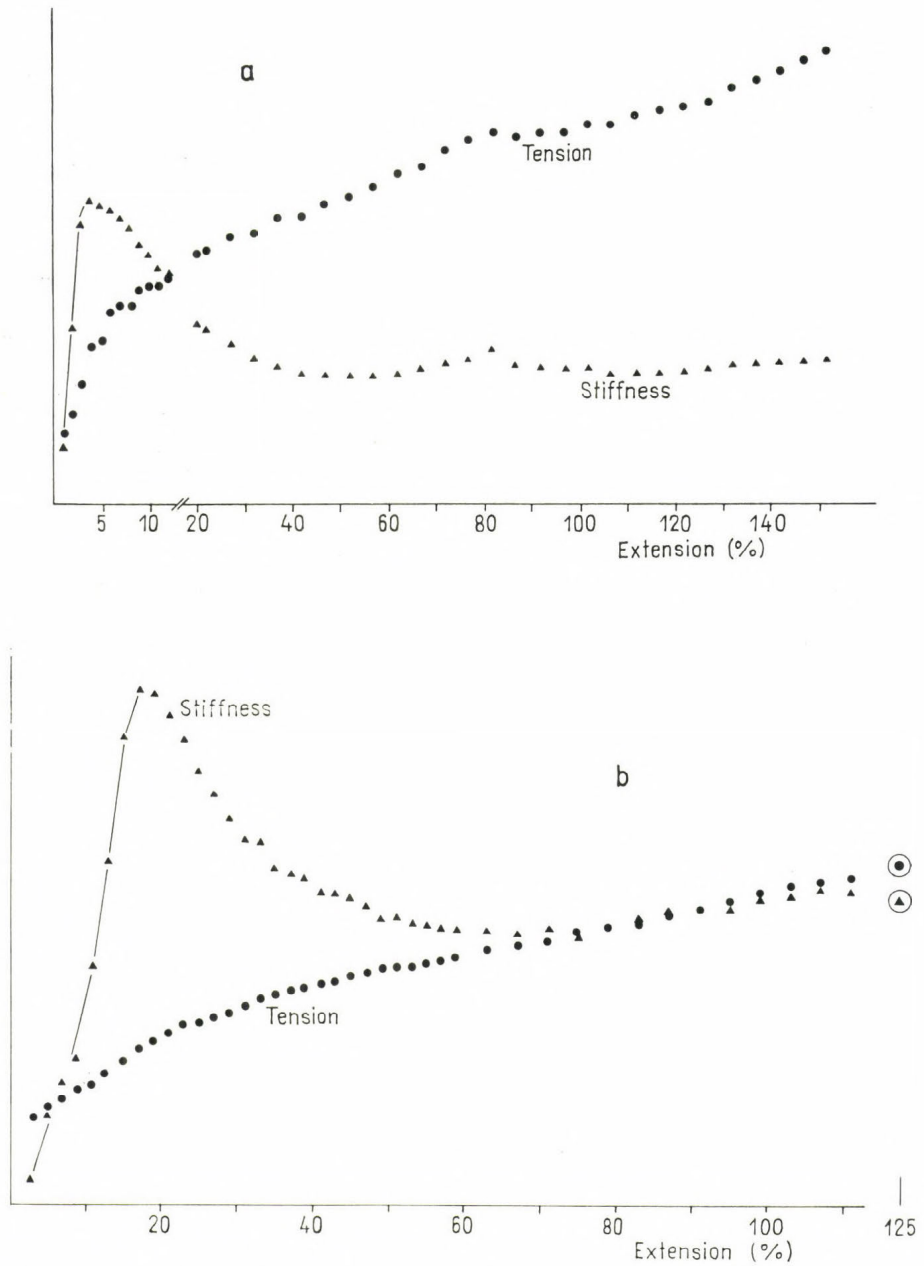


Fig. 6. Tension–extension and stiffness–extension curves for relaxed *Lethocerus* flight muscle fibres; 'stiffness' is the sinusoidal tension developed during small-amplitude sinusoidal length modulations; vertical scale is arbitrary. *a* Extension by 1% steps in 1 sec, followed by 24 sec stress relaxation (note break in extension scale); *b* extension by 2% steps in 21 sec, followed by 40.5 sec stress relaxation (experiments by Dr R. H. Abbott)

We have also measured fibre stiffness using small-amplitude sinusoidal length oscillations at various extensions and find that this reaches a peak and then declines; the value of strain at which stiffness is maximal varies with experimental conditions in the same way as does the discontinuity in the tension-length curve.

Figure 6 shows the results of two different experiments performed in the manner described in the legends. When successive stretches of 1% in 1 sec are used and stress relaxation is allowed to proceed for 24 sec, the discontinuity in the tension-length curve is at about 6% strain and the peak of stiffness is at about 4% strain. When successive stretches of 2% are given in 21 sec and stress relaxation allowed to proceed for 40 sec the tension-length discontinuity is at about 20% strain and the stiffness peak at about 17% strain. After the discontinuity, tension continues to rise at a slower rate and there is no sign of a change in the slope of the curve at 100% extension.

We conclude that the tension discontinuity at 100% extension found by Garamvölgyi and Belágyi (1968*a*) is either due to the presence of considerable series elasticity under the conditions of their experiments or is a different phenomenon from the discontinuity we have observed. The variation in the exact point at which our discontinuity occurs reflects a real difference in mechanical properties of the preparation at different rates of stretching.

The peak of stiffness found in our experiments may correspond to a phenomenon observed by Garamvölgyi and Belágyi. They note that the magnitude of the tension transient following quick stretches increases over the range of extensions up to about 10% extension and then either remains constant or decreases. Without measurements also of the time constants of the tension transients, the two results cannot be compared exactly, but are probably a measure of the same mechanical property of the preparations.

POSSIBLE EXPLANATIONS

There are five possible types of explanation for the resting tension in insect flight muscles:

1. Fixed or very slowly turning-over cross-bridges;
2. S-filaments connecting actin filaments in the middle of the sarcomere;
3. C-filaments or material connecting myosin filaments to the Z-line;
4. T-filaments in parallel with actin and myosin filaments;
5. A longitudinal force due to lateral filament interactions.

These will be discussed in turn, in order of the ease with which they can be eliminated.

Alternatives 2 and 4

The term S-filament was used by Hanson and Huxley (1955) to describe a highly elastic structure which, they considered, must exist between actin filaments in the centre of the sarcomere in order to account for the fact that fibrils stretched to the point of no overlap return to their original

length when released. No filamentous structure in this position has ever been found by electronmicroscopy in any striated muscle. The term T-filament was used by Hoyle (1967) to describe very thin filaments extending from Z-line to Z-line, which could provide an alternative explanation of the same observation; electron microscope evidence for filaments 25–40 Å in diameter and distinct from actin filaments (diameter 75 Å in their preparations) was presented by McNeill and Hoyle (1967).

These supposed structures share the feature that they are independent of the myosin filaments. Myosin can be dissolved out from glycerol-extracted flight muscle fibres by treatment with 0.6 M KCl; unlike vertebrate muscle, the protein is first removed from the region of the myosin filaments in the centre of the sarcomere. After 30 min in 0.6 KCl, the resting elasticity is lost and the fibres become very weak. This argues against S- or T-filaments as the element responsible, unless they are also dissolved by treatment with 0.6 KCl.

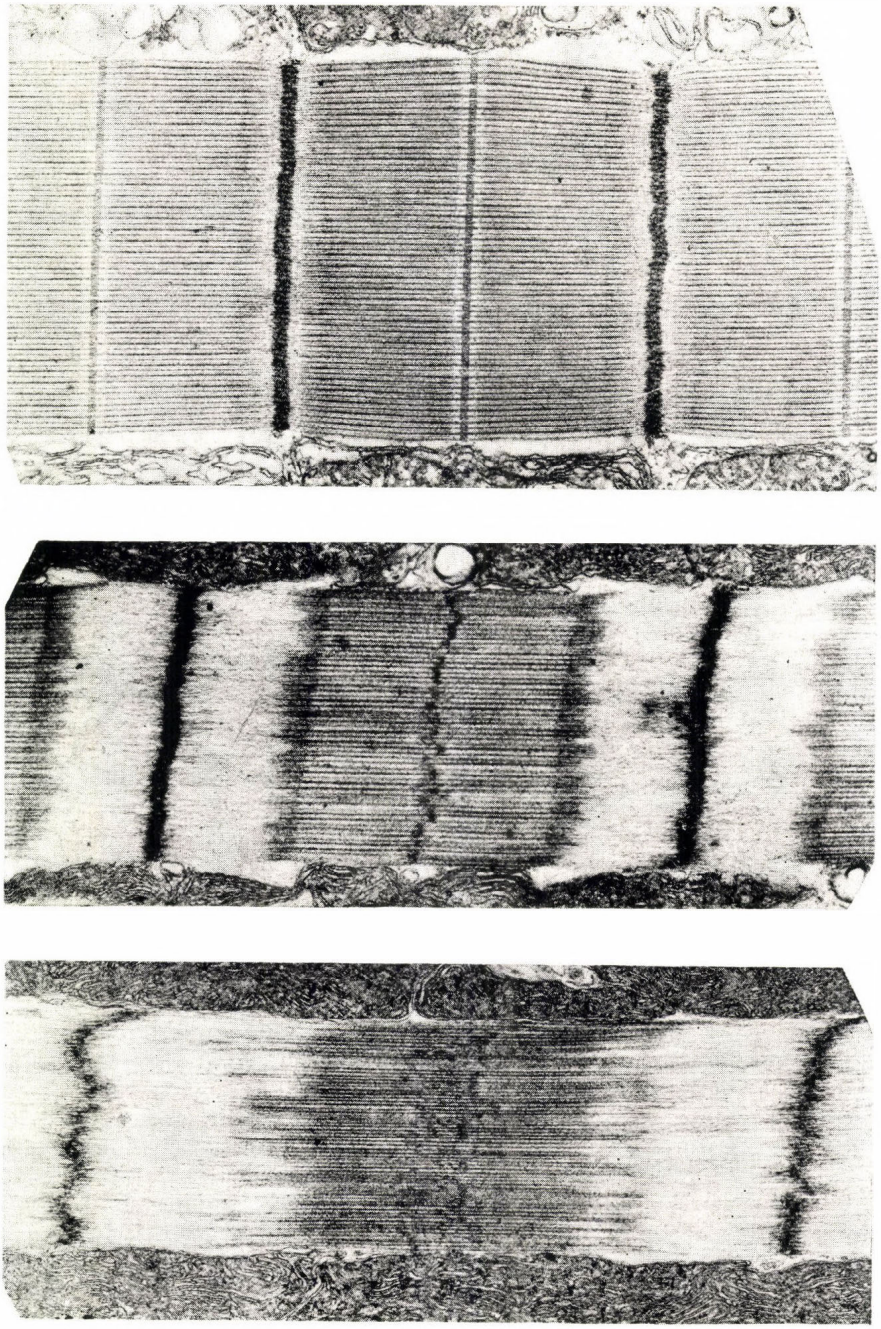
The best evidence against these explanations as the main cause of resting elasticity comes from the appearance of fibres stretched by about 50%. Figure 7*b* shows such a sarcomere from *Xylocopa* flight muscle. The boundary of the H-zone is sharp, showing that the actin filaments remain in good transverse register, whereas the boundaries of the A-band and M-line are irregular. Some myosin filaments have moved in one direction and some in the other, as would be expected if a mechanical element connected them to the Z-line. White (1967) has a similar micrograph from *Lethocerus* flight muscle, showing the effect to an even greater extent. It would not be expected from the presence of S or T filaments.

S-filaments probably do not exist. T-filaments may indeed be present, since a backbone material remains after extraction of both myosin and actin with 1 M KI (Hanson, 1956). Although this backbone material preserves the integrity of the myofibril after such extraction, it has no measureable mechanical strength and is unlikely to be responsible for the resting elasticity of intact fibres.

Alternative 1

The appearance shown in Figure 7*b* could possibly be explained by the presence of irregular cross-bridge interactions. The best evidence against the hypothesis that cross-bridges are responsible for the resting elasticity is provided by the fact that tension in relaxed fibres is not proportional to the degree of overlap of the filaments. As described above, the tension discontinuity and the stiffness maximum occur at different extension depending on the rate of stretch and the extent of stress relaxation but the range in our experiments was from 6 to 20% using *Xylocopa* muscle. In insect flight muscles, the point of zero overlap is at nearly 100% extension.

Fig. 7. Longitudinal sections of *Xylocopa* flight muscle, fixed in relaxing solution. *a* Unstretched; *b* stretched by about 50%; *c* stretched by about 100% (electron micrographs by Barbara M. Luke)


a
b
c
1 µm

Alternative 5

This alternative is at present largely theoretical and it is included mainly because Garamvölgyi (1972) and Garamvölgyi et al. (1973) have recently published calculations suggesting that a significant longitudinal force might be developed in insect flight muscles which do not preserve a constant volume in the overlap region on extension. No attempt has yet been made to show that such a mechanism could explain in detail the properties of relaxed fibres as revealed by the mechanical studies.

Evidence for alternative 3

The chief difficulty in accepting this explanation for the resting elasticity has been the failure to demonstrate by electronmicroscopy the existence of a connexion between the myosin filaments and the Z-line. Such a connexion was claimed by Auber and Couteaux (1963) in blow-fly flight muscle in the form of material, of lower density than myosin filaments or Z-line, visible in the correct position in transverse sections. Ashhurst (1967) was, however, unable to find such material in water-bug muscle and it has not been demonstrated in any longitudinal sections of intact fibres.

Clear evidence for the presence of such connecting material can, however, be obtained under a special condition. When fibres in rigor are stretched, the actin filaments become detached from the Z-line, leaving a zone of very low density on one or both sides of the Z-line. This appearance was first described by Hanson (1956), who remarked that the 'clear zone' showed considerable birefringence in spite of its very low optical density. Electron micrographs of this region (transverse, White, 1967; longitudinal, Reedy, 1972; *see also* discussion in White and Thorson, 1973) show filamentous material which is continuous with the myosin filaments. White and Thorson (1973) stress the significance of the fact that clear zones often occur on both sides of a Z-line (this could not occur in the absence of continuity, because otherwise whichever side broke first would be unable to exert any force to pull the Z-line away from the adjacent array of A and I filaments), and that clear zones are seldom seen with widths greater than about 1 μm. The maintenance of tension in rigor fibres stretched beyond the plastic limit implies that the clear zone material has considerable mechanical strength.

Evidence of a different nature, indicating also the nature of the connecting material, has been obtained by Bullard (unpublished). Insect flight muscles contain significant amounts of the protein paramyosin (6.3% in water-bug, 9.5% in a beetle; Bullard et al., 1973). Preliminary results using antibody staining of glycerol-extracted fibres show that this protein is located on each side of the Z-line in a narrow band extending over the ends of the myosin filaments, where a zone of higher density is regularly seen in longitudinal electron micrographs. If further work confirms that antiparamyosin stains in the I-band of stretched fibrils and the clear zones of fibres stretched in rigor, the chemical nature of the connecting material will, at last, have been resolved.

A HYPOTHESIS FOR THE MECHANISM OF RESTING ELASTICITY

White (1967) pointed out that the mechanical properties of relaxed insect flight muscle are not well explained by a model containing elastic structures representing myosin, actin and connecting filaments with viscous interaction. This type of analysis is often used in studies of muscle; it leads to a formulation in terms of Maxwell or Voigt elements (e.g. Fig. 13 of Pringle, 1967) and to an interpretation of the tension decay curves as the sum of a number of exponentials (Abbott and Lowy, 1957). In fact, the straight line plot of stress relaxation on logarithmic axes (see Fig. 2*b*) is the type of response expected of a lightly cross-linked, high molecular weight polymer. Paramyosin, with a molecular length of 1,280 Å and an α-helical content of 87% satisfies the requirements for such a material if it is present in unstretched fibres in the form of a lightly interacting and strongly hydrated gel, which effectively glues the ends of the myosin filaments to the Z-line. Ordered filaments would not be demonstrable in such a material. Extension up to the tension discontinuity would produce in such a material an increasing orientation of the paramyosin molecules and a correspondingly increasing strain upon them; tension and stiffness should increase. At greater extensions, slippage would begin to occur between the oriented macromolecules and the material should show plastic behaviour, with pronounced tension hysteresis. With improved orientation, it is to be expected that lateral molecular association would increase, giving rise to filamentous structures which would ultimately become visible in fixed and stained preparations. As we have seen, filaments are only demonstrable in the connecting material when it is stretched.

There is considerable similarity between the properties of relaxed insect flight muscle and of mammalian skin, which contains a poorly oriented network of collagen fibrils. Skin shows the same combination of short-range elasticity and long-range plasticity as is found in the muscle (Tregear, 1968). In skin, it has been shown that high extension causes extrusion of fluid and changes the staining properties of the collagen (Craik, 1966). A similar effect may account for the difficulty of demonstrating 'C-filaments' in insect flight muscle. It is therefore preferable to abandon this term and refer to connecting material between myosin filaments and Z-line as the explanation of resting elasticity.

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MECHANOCHEMICAL COUPLING IN SYSTEMS IN WHICH THE MYOSIN HEADS DO NOT FORM PART OF AN AGGREGATED STRUCTURE AND MAY OR MAY NOT FORM PAIRS

by

A. Oplatka, R. Tirosh, N. Liron and J. Borejdo

DEPARTMENTS OF POLYMER RESEARCH AND APPLIED MATHEMATICS, THE WEIZMANN INSTITUTE OF SCIENCE, REHOVOT, ISRAEL

The structure of myosin is rather unique. Its molecule contains two globules ('heads') which are connected to an elongated rod (LMM). The heads are capable of splitting ATP and of combining with actin, being cut from the rest of the molecule, either as single heads (HMM S-1) or as a pair (HMM). These two entities are soluble under physiological conditions, contrary to the LMM rods which aggregrate parallel to each other to form long filaments in striated muscle. The interaction of the protruding heads with the surrounding actin filaments in active muscle, associated with the splitting of ATP and leading to the generation of force, apparently does not strongly affect the association of the rigid LMM rods so that the length of the myosin filament does not change during contraction. Now, there is no reason to suppose that the mechanochemical transformation occurs far away from the region of interaction of the myosin heads with actin. The sliding of a myosin filament core past the actin filaments then it appears to be a *passive* movement, something like a flag attached to a moving car. One cannot, however, consider the movement of the actin filaments as a passive process since the mechanochemical transduction involves actin as well as the myosin heads, irrespective of whether the 'flag' is present or not. In other words, movement of the actin filaments and of the myosin heads, relative to each other and to the surrounding aqueous medium, is expected to occur in systems containing actin, HMM and MgATP. The fact that all attempts to resolve HMM S-1 into two preparations, differing in their behaviour towards ATP and/or actin have up till now failed, (Lamed and Oplatka, 1974; Oplatka et al., 1976) suggests that single isolated heads may also exhibit mechanochemical transduction.

Since actin filaments are polarized, as visualized by 'arrow-head' formation with HMM (Huxley, 1963), the mechanical effect produced by each of the globular parts of all the myosin molecules, interacting with a given actin filament probably has a component which is parallel to the filament and points in the same direction. As the movement of the protein species relative to water obviously means also the movement of water past the protein, the polarity of the actin filament should result in a net transport of water molecules parallel to the filament. Unfortunately, one cannot fix a single actin filament at a constant length and measure the developed tension or follow any movement of the vicinal water molecules as a result of interaction with HMM. The experimental verification of the above conclusions required the *amplification* of the effect. This could, in principle, be achieved by parallel arrays of actin filaments, being at our disposal all (or

most) pointing in the same direction. As we were not sure whether this is the situation in actin paracrystals, the following possibilities have been examined: (*i*) Would the tension develop by the action of molecularly dispersed active myosin derivatives plus MgATP on a muscle in which myosin has previously been 'neutralized'; (*ii*) Would myofibrils and actomyosin threads shorten under similar conditions; (*iii*) Would water be extruded from the above mentioned systems, and (*iv*) whether solutions containing actomyosin, particularly from non-muscle cells, would exhibit streaming in the presence of MgATP when confined in fine glass capillaries which are 'geometrically analogous' to e.g. the plasmodia of the myxomycete *Physarum polycephalum* whose cytoplasmic streaming is exceedingly vigorous. Another system which strongly suggested water streaming was superprecipitating actomyosin, the most characteristic feature of which is water extrusion. Such experiments were supposed to demonstrate the movement of water and/or 'contraction'.

Myofibrils and muscle fibres of striated muscle (rabbit psoas) and fibres of smooth muscle (taenia coli) have been studied. In order to enable the penetration of the myosin species into the filamentous space, glycerinates or detergent-treated muscle fibres and myofibrils were used. Neutralization of the filamentous myosin in these systems as well as that of the myosin in actomyosin threads, was achieved either by its extraction (in the case of myofibrils, to form 'ghost' fibrils) or by poisoning it with various chemical reagents (in all three systems) or, in the case of striated muscle fibres, by stretching them as far as there is no overlap between the two sets of filaments. The reagents used were salyrgan, p-chloromercury benzoate, p-phenylene dimaleimide and trinitrobenzene sulfonate (TBS). The inactivated contractile systems were irrigated with either of the molecularly dispersed myosin derivatives HMM, HMM S-1, myosin which was rendered water-soluble by growing poly-DL-alanine chains on its LMM tail or myosin (or HMM) in which actin-activation of the ATPase activity of one of its heads was abolished (by trinitrophenylation with TBS). After irrigation, MgATP was added and changes in isometric tensions were recorded (muscle fibres) or contraction was followed either under a phase contrast microscope or turbidimetrically (myofibrils) or by photographing at various times (actomyosin threads). In order to eliminate the possibility that any effects observed were due to traces of intact myosin in the irrigating solution, the protein preparations were further purified by Sepharose-ATP affinity chromatography columns and checked for myosin. The concentration of any myosin contamination present was smaller than the minimal concentration required for contraction. Control experiments were performed in which the soluble myosin species added were previously inactivated either thermally or by treatment with one of the above-mentioned chemical reagents. Binding of protein to myofibrils or fibres was checked either by measuring the amount adsorbed or by observing changes in optical density of the I-bands (in myofibrils).

Glycerinated rabbit skeletal muscle fibres in which active tension has been abolished (either by stretching or by chemically modifying the myosin) developed tension after irrigation with HMM. For salyrgan-poisoned muscle, the tension was proportional to the HMM concentration and amounted to about 10% of the maximal isometric tension at rest length of the native

fibres when the concentration was 20 mg/ml. The isometric tension of the skeletal muscle fibres at rest length decreased in each successive contraction. However, after the third contraction, the tension generated after irrigation with 2.2 mg/ml was greater than the decrease. Similar results were obtained with HMM S-1. However, for the same concentration of 'heads', HMM S-1 produced a 2–4 times smaller tension. Both HMM and HMM S-1 were strongly adsorbed by the fibres. Irrigation with proteins which have been treated with reagents that abolish actin-activated ATPase failed to induce tension development (Oplatka et al., 1974*a–c*; Gadasi et al., 1974).

The active isometric tension of glycerinated taenia coli muscles of guinea pig increased after irrigation with HMM. Tension which has been nearly completely abolished by chemical modification of the smooth muscle myosin with salyrgan could be partly regenerated after irrigation with HMM. Poisoned HMM was ineffective. However, irrigation with HMM S-1 resulted in depression of the mechanical response of the muscle. Both proteins were found to bind to, and to be capable of, displacing protein from the muscle at low ionic strength. Bands corresponding to actin and tropomyosin could be identified by SDS-polyacrylamide gel electrophoresis. The observed decrease in tension following irrigation with HMM S-1 may be due to the removal of actin; at the same time, any loss of tension due to the displacement of actin by HMM would be more than compensated by a higher mechanomechanical efficiency of the double-headed HMM (as in the case of skeletal muscle).

A stronger effect of HMM is revealed also by the fact that the extent of shortening of p-phenylene dimaleimide-poisoned actomyosin irrigated with HMM is the double of that observed with HMM S-1 (Oplatka et al. 1975) and that there is a better chance for observing contraction of HMM-S-1-irrigated ghost myofibrils if the MgATP solution contains additional protein, (a procedure which may not be necessary in the case of HMM under similar conditions). Such a difference in efficiency might be expected on the basis of statistical considerations: the probability of attachment of an HMM head, which may increase once its twin head, is bound while the probability of detachment of the double-headed HMM may be smaller than that of the single-headed HMM S-1, leading to an increase in effective concentration of active sites in the vicinity of actin filaments. Preliminary experiments indicate that actin is released also from skeletal muscle by the myosin fragments at low ionic strength but that the proportion released is substantially smaller than in the case of smooth muscle.

As already mentioned above, both HMM and HMM S-1 were capable of inducing the contraction of ghost or poisoned myofibrils. Again, the inactivated proteins were ineffective even if they could bind in the absence of MgATP.

Active streaming of actomyosin solutions associated with the splitting of ATP was demonstrated in microcapillaries. The oscillatory movement of suspended particles which could be observed microscopically was very reminiscent of cytoplasmic streaming in *Physarum* and was obtained with actomyosin from *Physarum*, blood platelets, chicken gizzard smooth muscle and rabbit skeletal muscle (Oplatka and Tirosh, 1973; Tirosh et al., 1973; Cohen et al., 1974). Acto-HMM solutions behaved similarly. Cytoplasmic streaming is usually considered as the outcome of a 'contractile' process

involving a network of actin and myosin filaments. It is difficult to ascribe the active streaming exhibited by acto-HMM solution to a network formation. It is also not easy, on the basis of the conventional theories, to explain the HMM-induced contraction of ghost or poisoned myofibrils and muscle fibres. In the following we present in brief a hydrodynamic model which may account for our observations.

The splitting of ATP is considered to be a vectorial reaction involving the relative movement of the components (myosin heads, actin, hydrolysis products) in well-defined local spatial geometry and sequence in time. The total excess kinetic energy possessed by the components is taken as equal to the enthalpy change. Assuming conservation of energy and of mechanical moments, most of the energy will be acquired by the lightest components. The energy must be dissipated by collisions with the surrounding water molecules in a well-defined direction relative to the reaction locus, the resulting water movement probably having a component parallel to the actin filament. Since the reactive sites of actin are arranged in a polarized monomer, a *net* movement of water may occur. The streaming observed in microcapillaries strongly suggests the existence of super structures in which actin filaments are aligned parallel to each other, pointing in the same direction. The oscillations observed might then reflect changes in direction of these arrays. There is no reason to believe that the picture is basically different when ATP is split by *actomyosin* confined in a microcapillary or, for that matter, by HMM-irrigated myofibrils or by living intact muscle. In other words, we may expect living muscle contraction to be the outcome of an active streaming process. It is well known that cytoplasmic streaming may be slowed down or even stopped by applying a counterhydrostatic pressure. We suggest that in an analogous manner, an external load applied to a contracting muscle is the origin of a hydrostatic pressure difference against which the mechanochemical engines operate: while the external load creates a pressure gradient which tends to stretch the muscle, the engine in each half sarcomere pumps water towards the center of the sarcomere. The lightest components of the ATP splitting reaction, the movement of which is responsible for the streaming of water must thus move against the pressure gradient. In analogy to the Archimedian buoyancy force which originates from a pressure gradient due to gravity, the force opposing the movement of a particle (myosin head, ADP, inorganic phosphate and water) will be equal to the product of its volume (v_i) and the pressure gradient (ΔP). The movement of the energetic particles will thus be a dissipative process and the heat produced by a contracting muscle will originate from this process as well as from the viscous resistance of the medium to the sliding filaments. In an isometric contraction, the enthalpy change per ATP (Δh), will be equal to the total sum of the products of the opposing force (f_i) times the corresponding paths (Δl_i):

$$\Delta \bar{h} = \sum_i f_i \Delta l_i = \sum_i v_i \Delta P \cdot \Delta l_i = \frac{\Delta P}{L} \sum_i v_i \Delta l_i = \frac{P_0}{L} \sum_i v_i \Delta l_i$$

where ΔP and P_0 are the hydrostatic pressure difference and the isometric tension, respectively, while L is the length of the overlap region in half a sarcomere. There is no reason for $\Delta \bar{h}$ or for $\sum_i v_i \Delta l_i$ to depend on the rate

of ATP splitting per myosin molecule. We may thus conclude that P_0 is proportional to the degree of overlap and independent of the specific turnover rate. In other words: P_0 should not vary much from one muscle to another or, for a given muscle with temperature. This is in agreement with the experimental facts. In an unloaded isotonic contraction, the whole enthalpy is dissipated through the viscous drag of the filaments. Assuming the viscous force to be proportional to the maximal velocity of contraction V_m (Oplatka, 1972), the heat output per half thick filament will be proportional to V_m^2. Contrary to P_0, the value of V_m is thus expected to vary with the rate of enthalpy change and therefore with the turnover rate of the corresponding actomyosin (V_{am}, which is in line with experimental observations; moreover, the relationship $V_{am} \propto V_m^2$ appears to hold quite nicely for the change with temperature of V_m and V_{am} (for frog sartorius and for tortoise iliofibularis muscles) (Bárány, 1967, Table IV).

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EFFECT OF Ca-IONS ON CONTRACTION SPEED AND FORCE GENERATION IN GLYCERINATED HEART MUSCLE

by

J. W. Herzig and U. B. Herzig

2nd DEPARTMENT OF PHYSIOLOGY, HEIDELBERG, FRG

INTRODUCTION

The phenomenon of stretch activation present in insect flight muscle is a physiologically significant process. This stretch activation can be observed even in glycerinated muscle fibres, which are deprived of the muscle membrane and a functional SR system (Jewell and Rüegg, 1966). Thus stretch activation is independent of the membrane potential and Ca^{++}-concentration, provided the intracellular Ca^{++}-concentration is higher than 10^{-7} M.

As shown by Steiger (1971), cardiac contractile structures, isolated by extraction of heart muscle with ether and glycerol, also show stretch activation. As the same effect has been previously described for skeletal muscle, the capacity for stretch activation appears to be a general property of striated and cardiac muscles.

According to the sliding filament model, force generation is a function of the relative number of links formed between actin and the myosin cross-bridges. Thus stretch activation is interpreted as a synchronization of activated cross-bridges. This hypothesis is supported by the observation that under certain conditions, stretch activated contractile structures can be forced to perform damped isometric oscillations (Schädler et al., 1971).

In our present work, the influence of Ca-ions on stretch activation in heart muscle was investigated with special regard to the number of cross-bridges (Podolsky and Teichholz, 1970; Wise et al., 1973) and the kinetics of cross-bridge movement. This problem has been briefly discussed also by Herzig (1973).

METHODS

Trabecular preparations approximately 4 mm in length and 0.3 mm in diameter were dissected from various vertebrate hearts. In order to isolate the contractile elements, the membraneous systems and the sarcoplasmic reticulum were destroyed. Three different methods of extraction were used:

1. Extraction with ether and glycerol

The preparations have been shaken for 2 min in ether and then extracted for 2×24 h at 0 °C. The extracted solution contained 50% water and 50% glycerol, buffered with histidine to a pH of 6.7. To suppress bacterial growth, the solution contained 10 mM NaN_3.

2. Chemical skinning

The fresh preparations have been suspended for 45 min in a solution containing 140 mM KCl, 3 mM EDTA, 5 mM ATP and 10 mM Tris-buffer, pH 6.7.

The functionally isolated contractile structures were then glued between a Ling dynamics 101 vibrator and a RCA 5734 force transducer and placed in contraction solutions with well-defined Ca^{++}-concentrations, using EGTA (Winegrad, 1971).

3. Test solutions

The relaxation solution contained 15 mM ATP; 17.5 mM $MgCl_2$; 20 mM Imidazol; 5 mM EGTA; 10 mM NaN_3, with a free Ca^{++}-concentration less than 10^{-8} M at pH 6.7.

The contraction solution was identical to the relaxation solution with the exception of 5 mM $CaCl_2$ resulting in a free Ca^{++}-concentration of 10^{-5} M at pH 6.7. The Ca^{++}-concentration of the contraction solution was varied by mixing relaxation and contraction solutions showing distinct ratios. The Mg^{++}-concentration varied between 15 mM and 30 mM.

The preparations showed stretch activation in response to a quick stretch in the range of 0.5–2% of the initial length, when suspended in contraction solutions containing more than 10^{-7} M Ca-ions. The kinetics of the delayed tension development were evaluated in terms of the time constant, τ. This is defined by the period of time extrapolated from the exponential tension rise. It reaches a value 1/e less than the maximum.

RESULTS

In the following experiments the dependence of the delayed tension time constant on the Ca^{++}-concentration was studied. Ether-glycerol-extracted and chemically skinned trabecular preparations were rapidly stretched within 5 msec in contraction solutions containing 10^{-7}–10^{-5} M Ca-ions. Preparations from chicken, rabbit, cow and pig were tested at 37 °C. Isometric incubation of the preparations in the Ca^{++}-containing Mg-ATP solutions produced a tension, the level of which depended on the Ca^{++}-concentration. Tension increased from 0 to about 0.35 kg/cm², the maximum was reached at about 8×10^{-6} M Ca (Fig. 1). The amplitude of the quick stretch varied between 0.5 and 2.0% initial length in order to produce stretch activation.

The preparations showed first a rapid tension rise, in phase of the quick stretch, followed by a quick visoelastic tension drop. After this tension peak a delayed exponential tension developed and the tension declined slightly to a constant value.

The extent of delayed tension rose sigmoidally with increasing Ca^{++}-concentration and reached a maximum value of about 0.06 kg/cm² (rabbit trabecular muscle, 1% quick stretch) near 8×10^{-6} M Ca (see Fig. 1). When the Ca^{++}-concentration was gradually diminished from 10^{-5} M to 10^{-6} M, the Ca^{++}-induced tension level, the stiffness and the extent of delayed tension decreased, whereas the kinetics of the delayed tension development

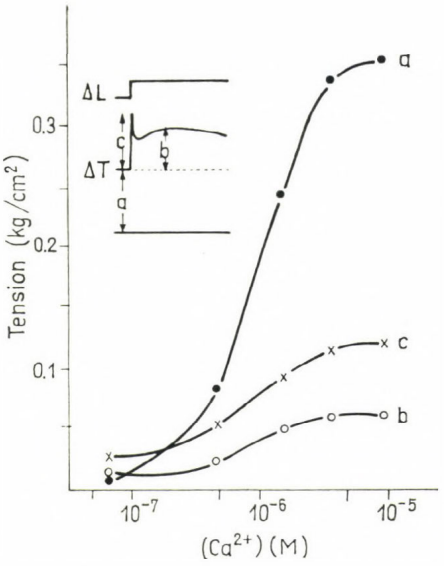

Fig. 1. Ca^{++}-dependence of *a* Ca^{++}-induced tension; *b* extent of delayed tension, and *c* stiffness. Glycerinated rabbit trabecular muscle, 15 mM Mg-ATP, pH 6.7, 37 °C, 0.5% quick stretch

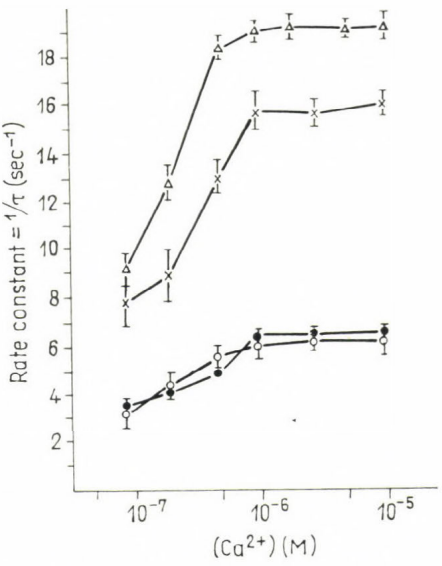

Fig. 2. The effect of Ca^{++}-concentration on the kinetics of the delayed tension development following quick stretch. Glycerinated trabecular preparations from chicken (△), rabbit (×), cow (○) and pig (●). 15 mM Mg-ATP, pH 6.7, 37 °C

measured by the time constant τ, did not change. On the basis of this observation the kinetics of the delayed tension rise in heart muscle have previously been considered to be Ca^{++}-independent (Steiger, 1971).

If the Ca^{++}-concentration was diminished to 10^{-7} M, Ca^{++}-tension, stiffness and extent of delayed tension development were not independent of the Ca^{++}-concentration. The time constant rose with the decreasing Ca^{++}-concentration. The rate constant (the reciprocal value of the time constant) doubled when the Ca^{++}-concentration was increased by a factor 10 (Fig. 2). At Ca^{++}-concentrations lower than 10^{-7} M it is impossible to measure the time constant as the contractile system relaxes and stretch activation can be no longer observed. Increasing Mg^{++}-concentration from 15 mM to 30 mM had no effect on this behaviour. The temperature coefficient of the time constant was found to be about 2.5. Experiments performed on ether-glycerol-extracted and chemically skinned preparations gave the same results.

DISCUSSION

The experiments show that Ca-ions have two effects on the contractile elements of the myocardial cell. The increase of Ca^{++}-concentration up to 10^{-5} M leads to an increment in isometric tension and the extent of delayed tension, following quick stretch. On the other hand, the kinetics of the delayed tension development are Ca^{++}-independent in the range of Ca^{++}-

concentrations higher than 10^{-6} M, but strongly Ca^{++}-dependent between 10^{-6} M and 10^{-7} M. This indicates that Ca-ions may influence myocardial contraction in two different ways. According to a theory proposed by Thorson and White in 1969 for insect flight muscle, the rate constant of the delayed tension development is closely related to the rate of cross-bridge cycling. The rate constant was postulated to correspond to the (rate limiting) detachment step of the cross-bridge cycle. Hence, Ca-ions in some way influence the kinetics of the cross-bridge cycle in heart muscle. There are two explanations which we find reasonable.

Possibly, the cross-bridge kinetics are influenced by Ca-ions because of a combined effect as discussed by Bremel and Weber in 1972. On the other hand, one could assume that Ca-ions directly affect the rate limiting step of the ATP splitting in heart muscle.

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IRREGULAR CHANGES OF THE STRIATED PATTERN IN FLIGHT MUSCLE OF THE INSECT

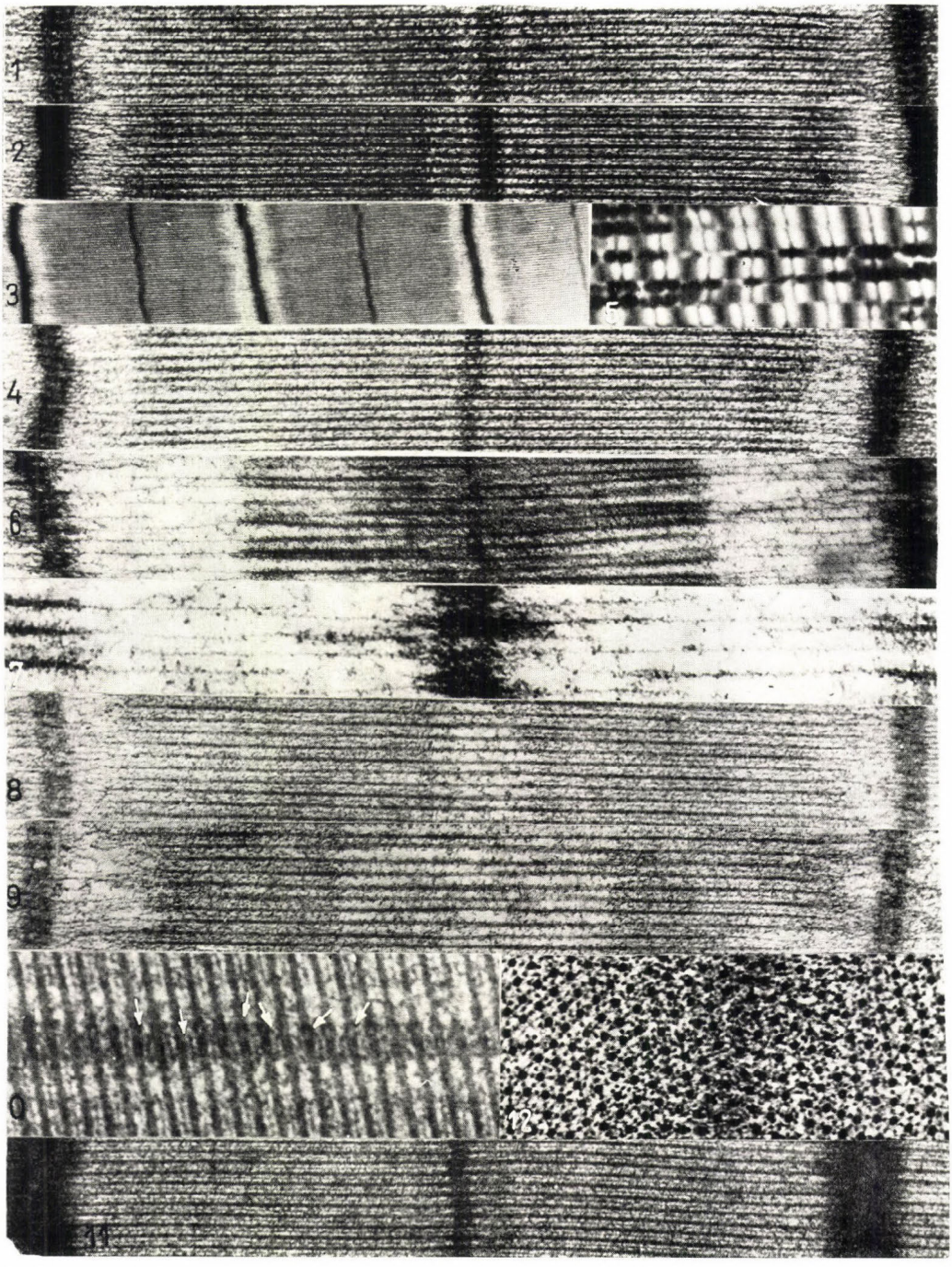
by

K. Trombitás and A. Tigyi-Sebes

CENTRAL LABORATORY, MEDICAL UNIVERSITY, PÉCS, HUNGARY

We performed our electron-microscopic experiments on fresh flight muscle of the honey-bee. The sarcomeres of this muscle contain very narrow I-bands and H-zones at the resting length, where the sarcomere length is 2.7 μ (Fig. 1). The Z-line and M-line, the thick and thin filaments in the M-line are distinctly visible. In stretched fibrils—sarcomere length is 3.1 μ—the length of H-zones corresponds to that of the I-bands (Fig. 2). It seems that the H-zone beside the thick filaments contains yet an additional material. Beside the normal striated pattern we often observed fibrils where the cross-striated pattern has changed irregularly (Fig. 3). In the fibrils on this figure the sarcomere length is about 3.2 μ. These fibrils have I-bands but the H-zones are missing. The thin filaments are uniformly located between the thick filaments as far as the M-line (Fig. 4). Comparing the 2nd and 4th figures, one can see that the A-band has equal lengths, therefore the shrinkage of the A-band does not cause this phenomenon. Since in the structure of the I-bands no change can be seen, it is probable that the thin filaments are stretched in the I-band. Similar finding was described by Garamvölgyi (1971) who observed the stretching of the actin filaments in extremely stretched human muscle. In fibrils of 4 μ or longer sarcomere length we often observed that the thin filaments had broken along the Z-line. In these cases the structure of the fibrils shows a change when studied with the light microscope in thick sections (Fig. 5).

Here wide dense zones can be seen in the place of M-lines. These zones could also be observed electron-microscopically. In addition, the I-bands become transparent (Fig. 6). It is probable that after the thin filaments had broken at the Z-line they contracted towards the M-line and only the connecting filaments remained in the I-band (Fig. 7). We do not know the reasons of the irregular changes of the striated pattern. It is supposed that these fibrils were stimulated by preparation before the stretch and the tetanic contraction blocked the sliding movement. We extracted the M-line from the fresh stretched muscle with a solution of low ionic strength which contained 5 mM Tris buffer (pH 7.8), 1 mM dithiothreitol (Morimoto and Harrington, 1973; Samosudova, 1966; Stromer et al., 1967) (Fig. 8). After extraction the additional material of the H-zone disappeared from the thick filaments together with the material of the M-line and the thin filaments contracted towards the Z-lines. Large H-zones developed without any change taking place in the sarcomere length (Fig. 9). Comparing the normal sarcomere (see Fig. 2) and the extracted one (see Fig. 9) it can be observed that the additional material being in the H-zone is missing from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the extracted muscle. This material has in some places a structure similar to filaments and it is probable that this structure corresponds to the super-thin filaments, observed by Hoyle et al. (1968). It is supposed that the extraction of the M-line made the contraction of the thin filaments towards the Z-line possible which led to an increase in the H-zones. If this is true then it means perhaps that the thin filaments are linked to the M-line. On the effect of the stretch this connection does not break off but the thin filaments suffer an elongation in the H-zone. Earlier we saw an example (see Figs 3 and 4) when no H-zone had developed with the increase in sarcomere length. In that case the thin filaments were stretched in the I-band.

A suspicion arises that the S-filaments, suggested earlier by Hanson and Huxley (1955) exist, though they do not bind two thin filaments only the M-line and the thin filaments. The M-filaments can be part of this structure and it seems that the M-filaments bind the S ones to the M-line (Fig. 10, arrows). This suggestion is supported, on the one hand, by the fact that it is very difficult to produce double overlapping zones in fresh muscle of the insect. On the other hand, we succeeded in producing supercontraction by cooling off insects and we could observe double overlapping zones, where sarcomere length was 2.3 μ (Fig. 11). In cross-section we can see the increase in the number of thin filaments on both sides of the M-line (Fig. 12), which proves that thin filaments broke through the M-line. This phenomenon is inconsistent with a rigid connection between the M-line and the thin filaments. If the connection exists, it must have a functional character. The conclusion drawn from these figures are only bold speculations and they remain yet to be proved. We only wanted to call attention to the fact that in this muscle the M-line can play an important role in the mechanism of contraction.

Fig. 1. The sarcomere of the flight muscle of honey-bee resting length. ×61,000
Fig. 2. Stretched sarcomere. Besides the thick filaments the H-zones contain even an additional material. ×54,000
Fig. 3. In this stretched fibril the striated pattern changed irregularly, the H-zones are missing. ×14,000
Fig. 4. Higher magnified electron micrograph of the previous fibril, showing the arrangement of the thin and thick filaments. ×52,000
Fig. 5. Highly stretched fibrils, having wide dense-zones, the places of the M-lines. ×1,500
Fig. 6. Electron micrograph from the area of the previous figures. The I-bands became transparent. ×40,000
Fig. 7. Highly magnified electron micrograph of the same area, showing the connecting filaments in the I-band. ×90,000
Fig. 8. Extraction of the M-line with low ionic strength solvent. The other structures remained intact. ×60,000
Fig. 9. After M-line extraction large H-zone was developed. ×60,000
Fig. 10. Fine structure of the M-line as seen in longitudinal section. ×130,000
Fig. 11. Contracted sarcomere. There is a double overlap zone in the center of the A-band. ×70,000
Fig. 12. Cross-sections of the overlap zone of the A-band of the contracted sarcomere. ×90,000

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COMPARISON OF INITIAL AND RECOVERY METABOLIC PROCESSES IN ISOMETRIC TETANI OF FROG SARTORIUS MUSCLE

by

R. J. Paul and M. J. Kushmerick

PHYSIOLOGY DEPARTMENT, HARVARD MEDICAL SCHOOL, BOSTON, MA 02115 USA

INTRODUCTION

During muscular contraction, there is a rapid hydrolysis of ATP (coupled by the Lohmann reaction to phosphorylcreatine, PCr, breakdown) with various 'recovery' processes occurring thereafter. Though both the initial and recovery processes have been studied in detail, they have not been made jointly on the intact muscle. In fact, our knowledge of the recovery processes stems largely from studies of mitochondria or other subcellular systems, while measurements of recovery O_2 consumption in whole muscle have rarely been made under conditions where comparison to initial chemical breakdown can be made. Using rapid-freezing techniques for measurement of the initial chemical reactions, and a sensitive polarographic system for measuring O_2 consumption, we have measured the metabolic events incurred in a single isometric tetanus in frog sartorius muscle at 0 °C. In some cases, the initial chemical reactions and recovery O_2 consumption were measured on the same muscle. These conditions, chosen to facilitate comparison to previous thermal, mechanical and chemical data, in particular, offer unique advantages to the study of the control of metabolism, as the initial reactions are almost completely separated from the recovery metabolism at this temperature. The comparison between initial and recovery metabolic events in whole muscle can provide unique information on *in situ* mitochondrial performance. In addition, as the classical interpretation of heat production measurements in terms of the known chemical reactions are being questioned (Curtin et al., 1974), our techniques allow a total biochemical evaluation of muscle energetics.

METHODS

Rana pipiens, stored at 10 °C were decapitated and the sartorii dissected free, preserving the *in vivo* attachments to the pelvic bone. Our physiological saline solution contained: 115 mM NaCl, 2.5 mM KCl, 1.8 mM $CaCl_2$, 1.0 mM $MgSO_4$ and 3 mM Na_2HPO_4/NaH_2PO_4, pH 7.1. O_2 tensions between 75 and 150% of that of air were used.

O_2 consumption was measured using a Clark-type electrode in a glass and stainless steel chamber of 4 ml volume. The design of this system is crucial, as very high sensitivity is necessitated by the low metabolism at 0 °C; details of this system are described elsewhere (Kushmerick and Paul, 1976*a*).

For chemical analysis, a pair of sartorii were frozen on a hammar apparatus of similar design to that described by Kretzschmar and Wilkie (1969). ATP, phosphorylcreatine (PCr), creatine (Cr), inorganic phosphate (P_i) and lactate were extracted from the thin frozen muscles by simple diffusion into a mixture of equal volumes of methanol and 10 mM EDTA, pH 8, at −35 °C for 4 days. Standard chemical assays were used (Kushmerick et al., 1969; Hohorst, 1963). The change in chemical content is the difference between the stimulated and control members of a sartorii pair.

RESULTS

Initial reactions. In this work single isometric tetani were studied. The extent of the initial PCr breakdown was directly measured and independently, in terms of the increase in Cr and P_i. To the first order, the stoichio-

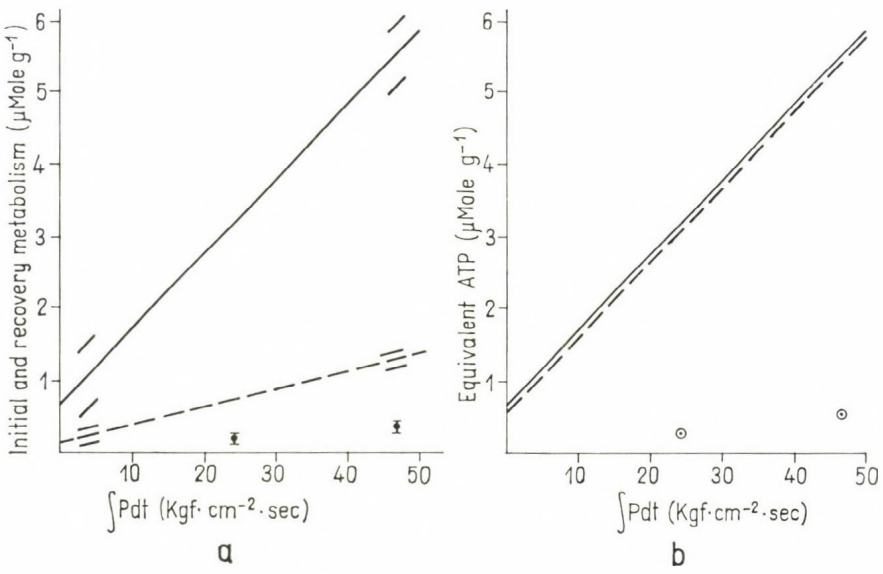

Fig. 1. The relation between the initial chemical reactions, aerobic recovery metabolism and the tension time integral. *a* the solid line represents the initial chemical reactions, $\Delta \sim P$ (see text) of 36 muscle pairs for durations of 5, 10 and 20s; the broken line represents the recovery O_2 consumption of 15 muscles for the same durations. Least square linear regression techniques were used; the short parallel lines near the origin and end of the lines indicate the standard error of estimate so obtained. The regression parameters were:

$$\Delta \sim P = 0.685\ (\pm 0.229) + 0.104\ \ (\pm 0.0075) \cdot \int Pdt$$

$$\Delta O_2 = 0.138\ (\pm 0.058) + 0.0263\ (\pm 0.0019) \cdot \int Pdt$$

The open circles represent the lactate production following 10s (n = 3) and 20s (n = 4) tetani; the bars, the standard error of the mean. *b* the same legend as *a* with the extent of the chemical reactions expressed in terms of the equivalent synthesis or breakdown of ATP (see text). The ordinate units are μmole per gram blotted weight; the abscissa units are $kgf \times cm^{-2} \times s$, the approximation $A = M/L_0$ used for the muscle cross-section area

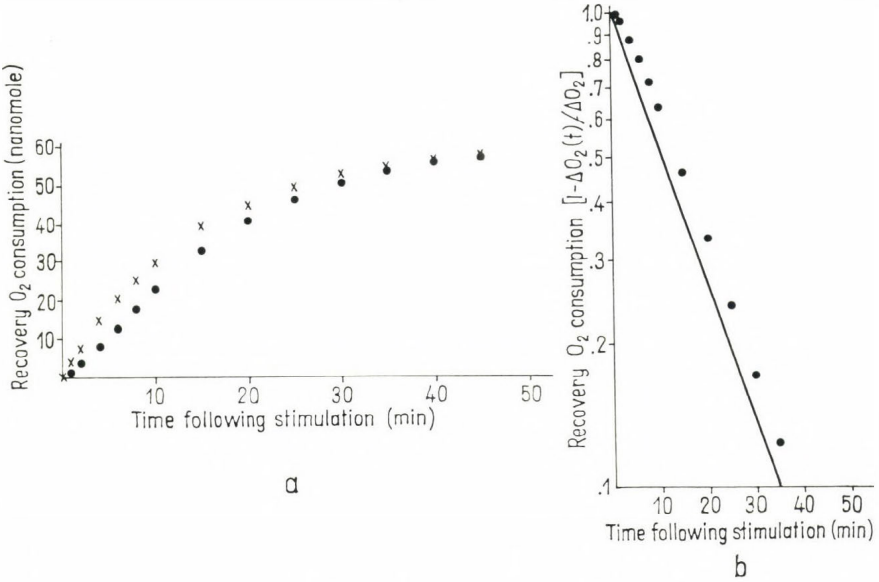

Fig. 2. Time course of the suprabasal O_2 consumption following a 10s isometric tetanus for a typical sartorius. *a* Filled circles represent the measured time course; crosses represent an exponential, whose time constant (15 m) was taken from the line fitted to the linear region ($t > 8m$) in the logarithmic plot shown in *b*. The lag between the measured ΔO_2 and a true exponential can be explained in terms of non-steady state diffusion (see text)

metry of the Lohmann reaction was found to be valid and thus these three measurements per sartorii pair were averaged to obtain the most precise estimate of the high-energy phosphate breakdown ($\Delta \sim P$). A linear regression of $\Delta \sim P$ vs. the tension-time integral ($\int Pdt$) for tetanic durations of 5, 10 and 20s is shown in Fig. 1*a*. For long tetani ($> 20s$), statistically significant deviations from the Lohmann reaction were observed ($\Delta P_i < \Delta PCr = \Delta Cr$). Though of potential significance (Kushmerick and Paul, 1976*b*), the effect of this deviation is negligible for the purposes of this report ($< 10\%$ on the $\Delta \sim P$ vs. $\int Pdt$ regression given in Fig. 1*a*).

Recovery aerobic metabolism. Within 1 to 3 min after the tetani, the O_2 utilization increased above baseline (7 nanomole/min $\times$ g) and 40 to 80 min was required before the baseline was reattained. The suprabasal O_2 consumption (ΔO_2) as a function of time is given in Fig. 2*a*. An exponential fitted to the latter points (Fig. 2*b*) represents the tissue O_2 consumption, the early lag below a true exponential being adequately explained in terms of non-steady state diffusion (Kushmerick and Paul, 1976*a*). A small but finite amount of aerobic glycolysis was also measured, which, within the limits of chemical resolution, occurs entirely after the tetani (Paul and Kushmerick, 1974*a*). Lactate production increased markedly in contractions $> 20s$, coinciding with the predicted anoxia in parts of the muscle. The relation of ΔO_2 and lactate production to $\int Pdt$ for 5, 10 and 20s tetani is given in Fig. 1*a*.

DISCUSSION

A striking characteristic of both the initial chemical reactions and the aerobic recovery metabolism is the linear dependence on the tension-time integral, in contrast to the curvilinearity seen when time is used as the independent variable. This linearity has been extended to tetanic durations of 60s for the case of $\Delta \sim P$ vs. $\int$ Pdt (Kushmerick and Paul, 1976*b*), quite remarkable in view of the more than 50% loss in tension and similar reduction in the PCr content.

A somewhat surprising finding was that the extent of aerobic glycolysis was also linearly related to $\int$ Pdt and hence in constant proportion to the extent of the oxidative recovery metabolism; this was valid as long as no part of the muscle lacked O_2, anoxia occurring when tetanic duration exceeded 20 to 40s depending on the size of the muscle. The contribution of aerobic glycolysis to the total aerobic recovery metabolism, however, was small ($< 10\%$) as can be seen from Fig. 1*b*. In this figure the ATP synthesis or breakdown has been plotted against $\int$ Pdt, assuming 1 mole of ATP per PCr, 1.5 mole per mole lactate and a P : O ratio of 2.

The finding of a P : O of 2 is somewhat surprising in view of the standard biochemical ratio of 3 found for isolated mitochondria. However, as can be seen from the figure, both $\Delta \sim P$ and the ΔO_2 can be superimposed using this ratio ($P : O = 2$, $\Delta \sim P : \Delta O_2 = 4$). One possibility is that a breakdown of high-energy phosphate, proportional to the $\int$ Pdt occurs after contraction and is hence not measured by our rapid-freezing protocol. This, however, is contrary to current evidence (Mommaerts and Wallner, 1967; Kushmerick and Paul, 1976*b*). It remains that the *in situ* P : O ratio of mitochondria in intact muscle is closer to 2 than to the normally assumed value of 3.

Within the last few years, discrepancies between muscle heat production and the predicted (assuming a constant enthalpy of reaction) initial chemical reactions have been reported. One possible interpretation of these results is that there exists a 'missing' reaction, i.e., one not involving ATP hydrolysis linked to PCr. The superposition of $\Delta \sim P$ on ΔO_2 argues against this interpretation, as the discrepancy between heat and ΔPCr is reported to occur only for short contraction durations (Gilbert et al., 1971) or only for long durations (Canfield et al., 1973).

The intercepts seen in the regressions presented in Fig. 2 are statistically significant. This suggests for tetani of short duration (< 5s) either the presence of a non-actomyosin ATP requiring process, or a non-linear actomyosin ATPase. The dependence of this intercept on the interval between repeated contractions (Paul and Kushmerick, 1974*b*) argues for the former. Thus, the interpretation of the ATP breakdown determined in whole muscle studies in terms of actomyosin interaction must be limited by these results to only the steady state region. It is interesting to note that the stationarity in the chemical events occurs well after mechanical stationarity has been achieved.

The results of our total biochemical approach to muscle energetics suggest a more complex picture than generally assumed, but nonetheless, an extremely well coordinated system for control of metabolic events in response to the chemical energy demands of contraction.

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EXCITATION

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INVESTIGATION OF METABOLIC DEPENDENCY OF MEMBRANE POTENTIAL OSCILLATION EVOKED BY VERATRINE ON SKELETAL MUSCLE

by

E. VARGA, M. DANKÓ, J. DOMONKOS and I. GESZTELYI

DEPARTMENT OF PHYSIOLOGY, UNIVERSITY MEDICAL SCHOOL, DEBRECEN, DEPARTMENT OF NEUROLOGY AND INSTITUTE OF BRAIN RESEARCH, SZEGED, HUNGARY

Two years ago we published our experiments showing that following veratrine treatment the sartorius muscle exhibits expressed rhythmic membrane potential oscillations for hours (Varga et al., 1972). In our experiments we recorded the veratrine evoked oscillations of membrane potential generally by extracellular conduction from all fibres of the aneural part of the sartorius muscle according to the method of Gesztelyi (Gesztelyi, 1973). The rhythmic membrane potential oscillation caused by veratrine could also be observed, however, by intracellular conduction from one superficial fibre of the sartorius muscle (Varga et al., 1975).

In our earlier experiments we established the temperature dependency of the phenomenon. A critical value of temperature about 13–14 °C was found below which practically no oscillation can be observed and above which the further increase of the temperature does not change it anymore (Varga et al., 1975).

We have observed further that the developed oscillation of membrane potential can be inhibited by 1 mM monoiodic acetic acid, and 10 mM Na-fluoride, respectively. The inhibiting effect of Na-fluoride could be reversed by a treatment of fluoride-free Ringer containing Mg (Varga et al., 1975).

In our above experiments we investigated first of all how oscillation of the membrane potential evoked by veratrine is influenced by oxygenation of the incubating Ringer's solution. In the studies the muscle specimen incubated in oxygen-free medium was used as control, while the incubating solutions of other samples were oxygenated for different time during the experiment (Fig. 1).

Upon the effect of oxygenation the frequency of oscillation increased from 0.4 cycle/min to 0.6 cycle/min. The amplitude began to decrease later and oscillation ceased after about a half an hour.

Further we investigated the effect of two compounds having characteristic metabolic site of action (Fig. 2).

This characteristic record shows the effect of K-cyanide which inhibits terminal oxydation. In these experiments oscillation of the membrane potential was evoked on both members of the muscle pair incubated in oxygenated medium. Later one of them was treated with 1 mM K-cyanide, as a result of which the oscillation entirely ceased in about 15 min (Fig. 3).

The effect of 0.1 mM dinitrophenol is shown in an oxygenated medium. In this concentration which uncouples oxidative phosphorylation dinitrophenol inhibits the oscillation of the membrane potential, too.

Finally the pH-dependency of the phenomenon was studied by compar-

ing the frequency of veratrine evoked membrane potential oscillation on muscles incubated in solutions adjusted to pH 6, 6.5, and 7, respectively (Fig. 4).

In the experiment shown in Fig. 4 the muscle specimens were incubated in Ringer's solution adjusted to pH 6 and pH 7, respectively, for one hour before the treatment with veratrine. It can be seen that rhythmic potential

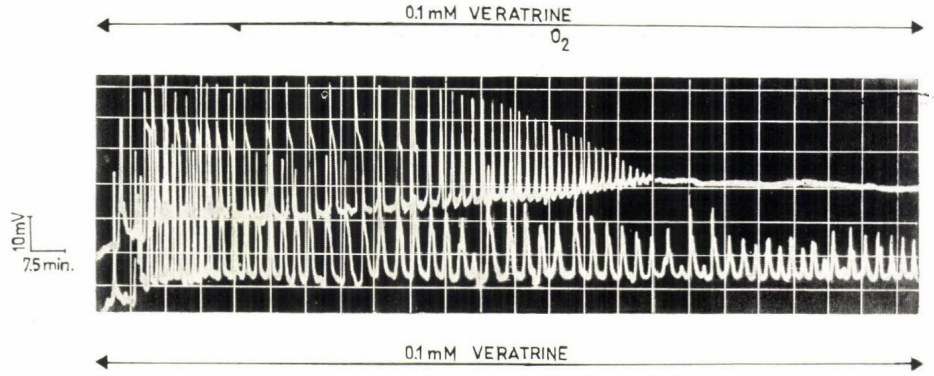

Fig. 1. Effect of oxygenation on veratrine-induced membrane potential oscillation. The specimens of the sartorius muscle were placed in separate chambers and were treated with 0.1 mM veratrine. One member of the muscle pair was oxygenated from the 28th min of the treatment (upper curve)

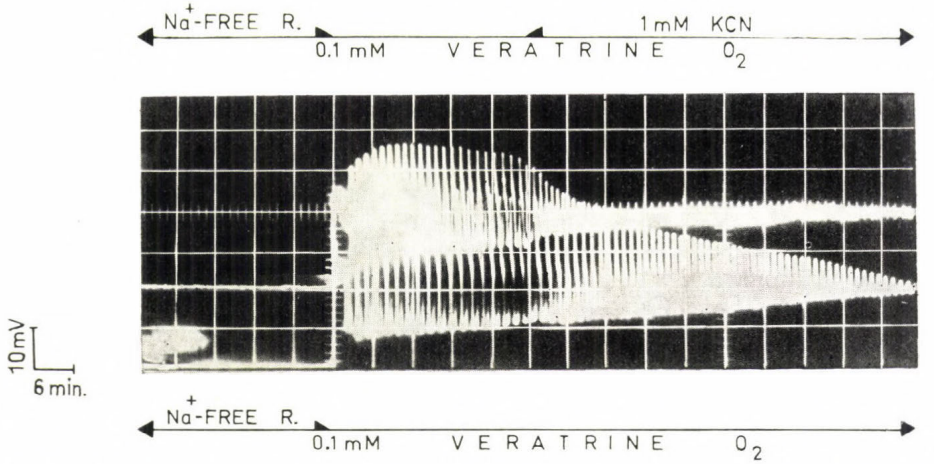

Fig. 2. Effect of K-cyanide on the veratrine-induced membrane potential oscillation. Both members of the muscle pair were incubated in veratrine-containing, Na-free Ringer's solution in the first half hour of the experiment, then the two incubating solutions were changed to a Na-containing medium simultaneously. This process has the advantage that the oscillation starts on both members of muscle pair at the same time, thus the results are better comparable. The upper recording demonstrates the decrease of membrane potential oscillation due to the effect of K-cyanide, while the lower one shows the oscillation of membrane potential of a control muscle occurring on the effect of veratrine alone

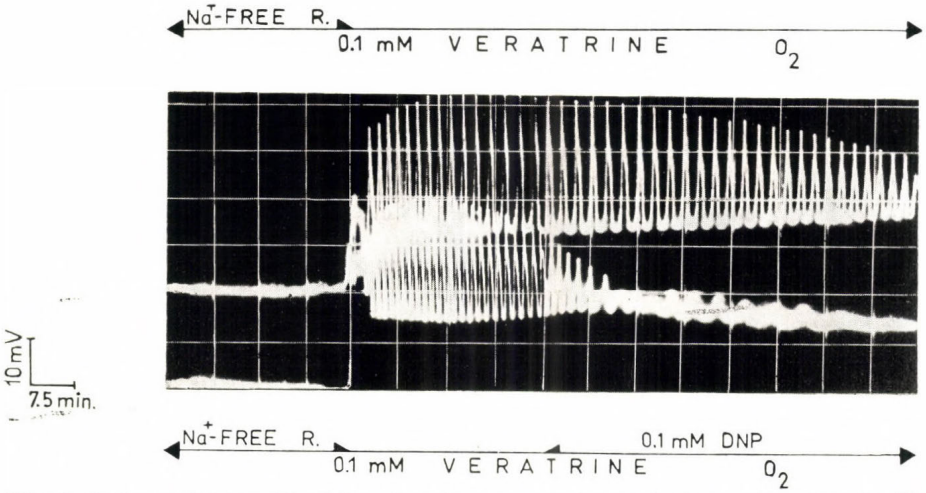

Fig. 3. Effect of 0.1 mM dinitrophenol on veratrine-evoked membrane potential oscillation. The method is the same as described in Fig. 2

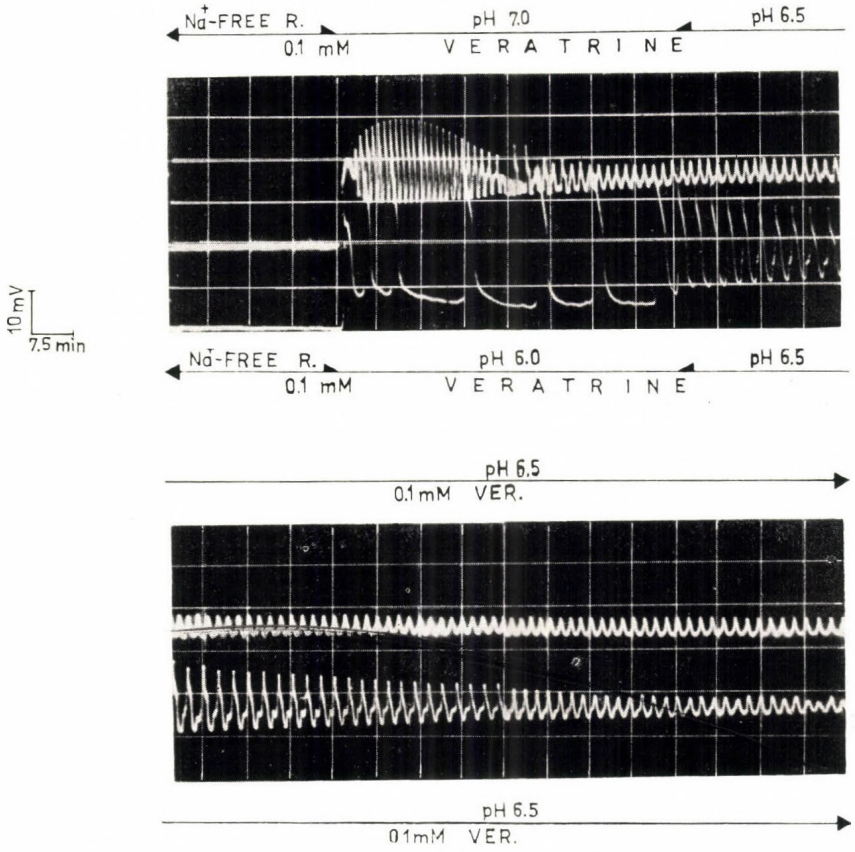

Fig. 4. Effect of H^+-ion concentration on the veratrine-induced membrane potential oscillation

oscillation could be evoked by veratrine on both members of the muscle pair, but while at pH 7 the characteristic curve with usual frequency was obtained, the frequency of the oscillation was considerably smaller, only about 0.1 cycle/min, of the muscle incubated at pH 6. After 90 min both muscles were placed into Ringer's solution adjusted to pH 6.5. It can be seen that the oscillation activity of the muscle incubated at pH 6 previously increased to a higher frequency, while that of the other muscle remained unchanged. In the 4th hour of the experiment oscillation frequency of both muscles incubated now at the same pH was identical.

For the evaluation of our result it is necessary to mention our earlier observation showing marked veratrine evoked membrane potential oscillation in muscles incubated in Na-free and Li as well as 0.1 mM ouabaine containing solutions for 60 min before treatment with veratrine, though the Na-pump is not acting under such circumstances (Varga et al., 1975).

We suggest that the rhythmic potential oscillation of the membrane, being in a metastable stage due to the effect of veratrine, is in connection with the changes occurring in the balance of chemical energy formation and utilization.

Considering the question which metabolic processes could be made responsible for the pace-maker activity it would be too early to take a stand. We do not preclude the possibility, however, that the glycolytic oscillation, with phosphofructokinase being in its centre, may be the primary pacemaker.

The significance of our results lies in the fact that according to our hypothesis this membrane potential oscillation evoked artificially may be an appropriate model for studying the relationship between the rhythmic metabolic processes taking place inside the cell and the rhythmic oscillations of membrane potential, e.g. the mechanism of myogen rhythm characteristic of the smooth muscle.

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STUDIES ON THE MECHANISM OF ELECTRO-MECHANICAL COUPLING IN FROG SKELETAL MUSCLE

by

L. KÓNYA and A. KÖVÉR

CENTRAL RESEARCH LABORATORY AND DEPARTMENT OF PHYSIOLOGY, UNIVERSITY MEDICAL SCHOOL OF DEBRECEN, DEBRECEN, HUNGARY

According to general use and accepted definitions, electro-mechanical (E-M) coupling comprises processes between the depolarization of the sarcolemma and the contraction of the muscle fibre (Sandow, 1965, 1970). At different stages of coupling, calcium transients are cardinal and exert triggering and control effects on the contractions. Data on the localization of calcium in the resting muscle, its binding properties and the dynamics of its transients in muscle contraction may contribute to the understanding of the mechanism of the coupling, and also to features which are probably common to biological membranes. The different experiments described below had as their aim the elucidation of these problems.

Fiber bundle preparations of frog m. semitendinosus were used in all experiments. They were attached to glass rods in the radioisotope experiments and were transferred through a series of test tubes containing ^{45}Ca ('uptake'), or inactive Ringer's solution ('wash-out' experiments). In order to get information about ^{45}Ca fractions exchanging both quickly and slowly in the muscle, wash-out of the activity was started immediately after an incubation period of 2 h for the muscles in ^{45}Ca containing Ringer. The wash-out process was continued for 4 h, varying the washing times from 10 sec to 10 min according to a program. From activity measurements data desaturation curves were constructed then analysed graphically from which the parameters (size and time constants) of the calcium compartments were determined (Fig. 1).

These experiments were performed both in normal Ringer and Ringer containing 1 mM La^{3+}. The arithmetic means of the parameters are depicted by column diagrams in Fig. 1. Calcium compartments are designated according to their supposed localization. In the figure a remarkable reduction in the size from about 45 to 10 rel. % of the fast exchanging muscle calcium compartments can be seen (3rd + 4th compartments).

To record contractions, muscles were mounted in Sartorius vessels, in which the exchange of solutions was effected by flushing with a syringe from below. Care was taken to avoid artifacts on the smoked drum used for registration. Potassium contractures were elicited on muscles equilibrated in Ringer's solution or Ringer containing 1 mM La^{3+} by isotonic KCl. 1 mM La^{3+} had reversible blocking effect on the potassium contractures (Fig. 2). If, however, instead of 1 only 0.1–0.3 mM lanthanum was used before the application of isotonic KCl, potassium contractures showed substantially different features (Fig. 3). The amplitude of the contracture, the speed of development and of the spontaneous relaxation increased and,

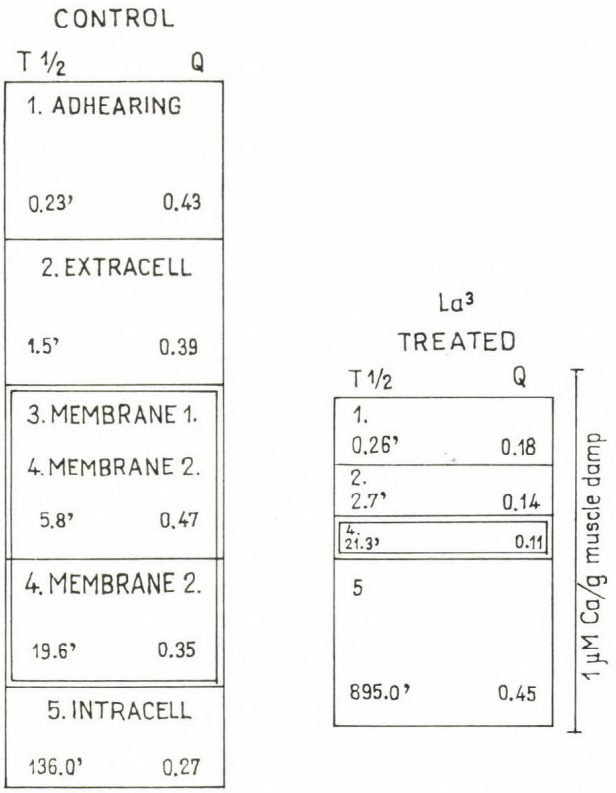

Fig. 1. The compartmentalization of 45-Ca in control and 1 mM La^{3+} treated muscles after 2 h incubation in the active solution. The parameters are the arithmetic means of 39 control and 9 La^{3+} treated muscles ($T_{1/2}$ = half time in minutes; Q = size in μM Ca/g wet muscle)

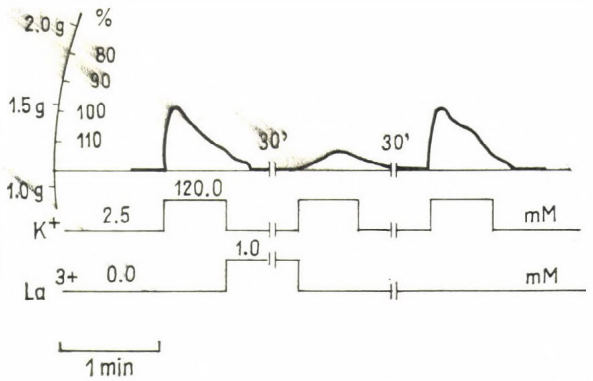

Fig. 2. Quasi-isotonic contractures of muscles elicited by isotonic potassium chloride solution after equilibration in normal Ringer or in Ringer containing 1 mM La^{3+}. Alterations in the tension and muscle length corresponding to the contracture are represented on the ordinate in grams and compared to the resting length of the muscle (= 100%)

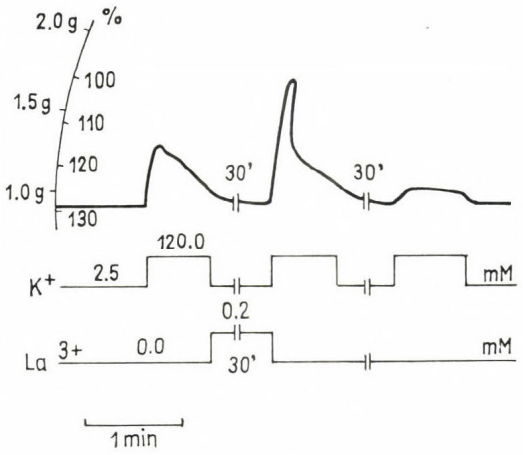

Fig. 3. Demonstration of the biphasic effect of 0.2 mM La^{3+} preincubation on potassium contractures

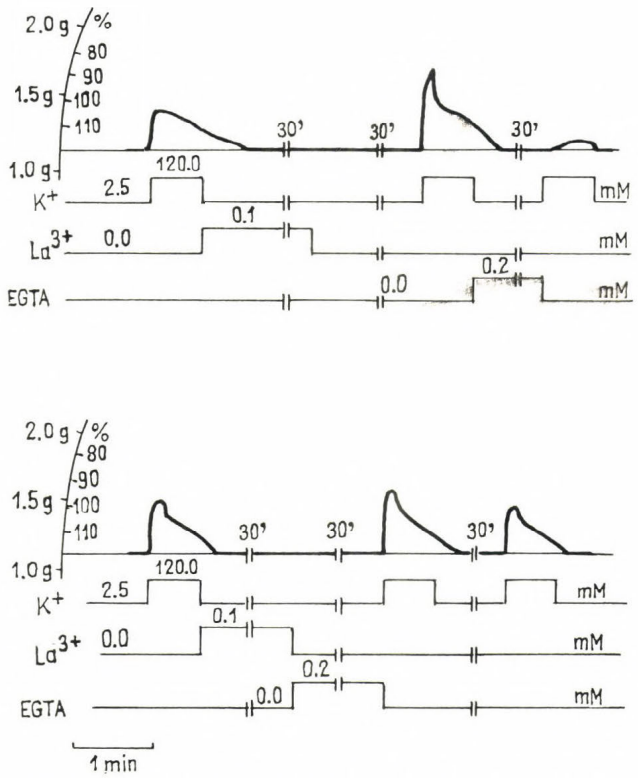

Fig. 4. The influence of EGTA on the biphasic La^{3+} effect on the muscle potassium contractures

as a consequence, the duration of the contracture decreased. Later on however, potassium contracture became irreversibly blocked (Fig. 4) even if the muscles were washed with Ringer containing the appropriate amount (0.2 mM) of the complexing compound EGTA. Before eliciting potassium, contracture but after incubation of the muscles with 0.1 mM La^{3+}, EGTA was effective in suspending this biphasic effect of lanthanum (facilitating, then blocking).

The La^{3+} effect relating both to the ^{45}Ca compartmentalization and potassium contractures can be explained in connection with the theory of Bianchi (1969, 1973). He supposed the existence of two, a superficial (stabilizing = S), and a deeper (releasing = R) calcium fractions bound to the surface membranes of the muscles (sarcolemma and T-tubule membrane). They can have different function in the mechanism of E-M coupling. 1 mM La^{3+} can replace S-calcium and by this blocking the development of potassium contractures reversibly.

Physicochemical parameters of Ca^{2+} and La^{3+} (the ionic radius differs in water by less than 10 rel. %, while the surface charge density of La ions is much more than that of calcium ions) suggests that, binding of lanthanum to calcium binding sites is favoured. Electron-microscopic data (Langer and Frank, 1972; Waugh et al., 1973) support the above findings and suppositions. The reversibility of the blocking effect of La^{3+} is consistent with the findings that lanthanum does not cross cellular membranes, and can be used as a marker for the extracellular space (Karnovsky and Revel, 1966).

In the case of smaller concentrations of lanthanum it can be supposed that, during potassium depolarization, La^{3+} can get to the R-binding sites and the replacement of calcium there may be responsible for its biphasic effect. In this way both, the irreversible blocking effect of La^{3+} and the ineffectiveness of the EGTA on its suspension can also be explained.

Bianchi and Bolton (1967) also stated that caffeine exerts its effect on the surface membranes (besides effects at other places) of the muscles by releasing calcium.

In our experiments the effect of 10 mM caffeine, if applied 3–10 min after the spontaneous relaxation and repolarization of a muscle in normal Ringer, seems to support this mechanism (Fig. 5). After washing in normal Ringer the effect of caffeine was always a biphasic contracture the first peak being reached within 20 sec the second by the 10th min from caffeine application. The amplitude of the first contracture was comparable to that induced by potassium, while the second proved to be supermaximal and irreversible, the muscles contracting to less than 50% of their resting length. In isotonic potassium chloride (Fig. 6), i.e. during long depolarization, the development of the first phase of caffeine contracture became more and more retarded until it disappeared after 30 min. The second phase developed full amplitude in this case, although over a longer time. In isotonic potassium sulphate, however, both phases of the caffeine contracture remained unchanged even after 1 h of depolarization (Axelsson and Thesleff, 1958). This suggests, that the reactivity of the muscle to caffeine is not much changed by the depolarization itself, but more probably it is influenced by the swelling caused by the isotonic potassium chloride.

The preliminary results of these experiments have already been reviewed

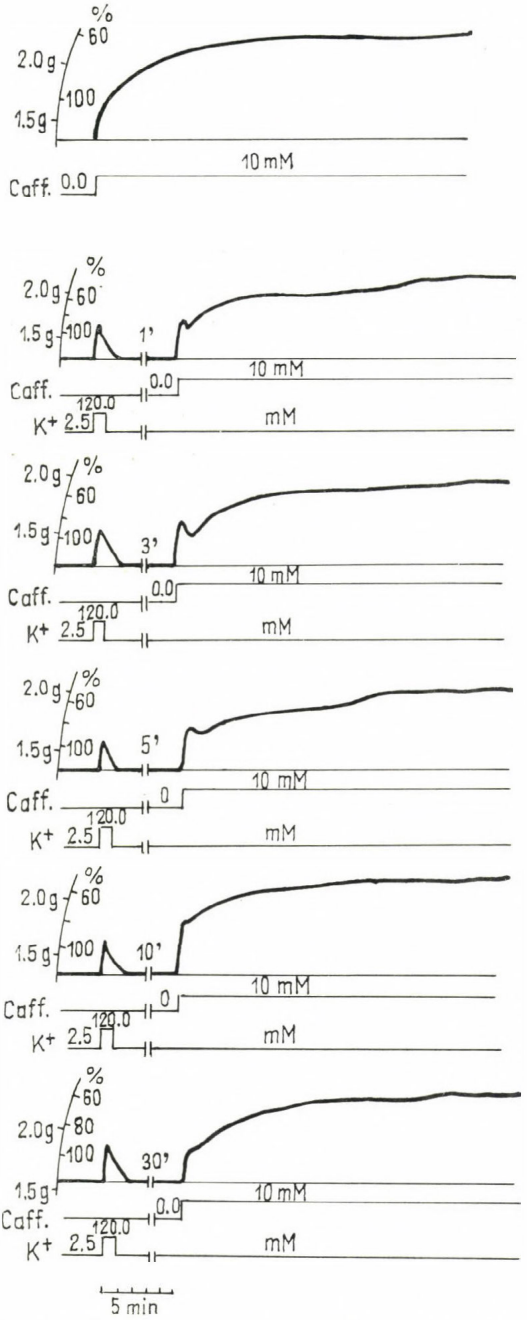

Fig. 5. Alteration in the character of caffeine contractures depending on the time interval elapsing between the initiation of the washing of muscles with normal Ringer after a potassium contracture and the application of caffeine

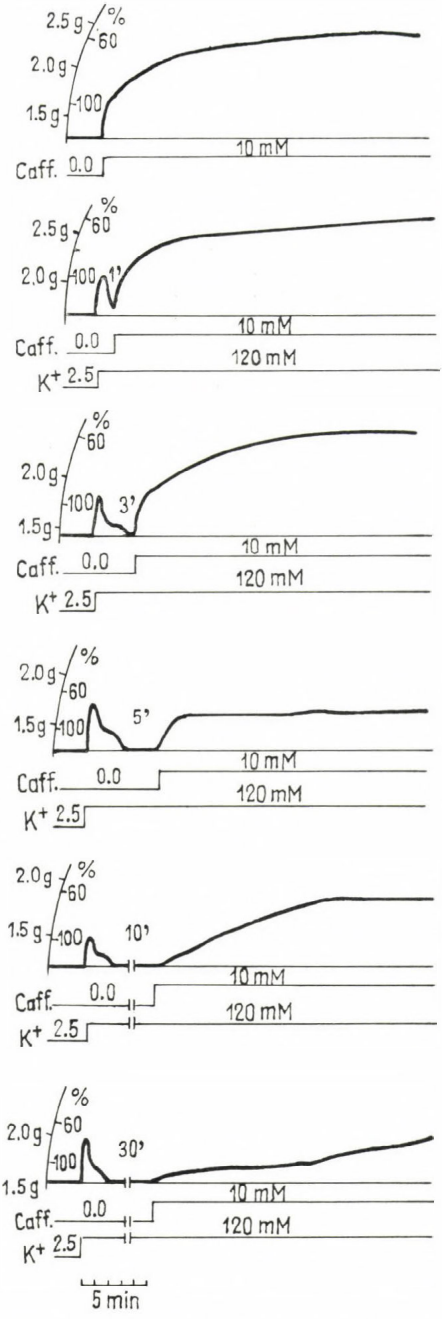

Fig. 6. Changes in the character of caffeine contractures depending on the time of the depolarization of the muscles in isotonic potassium chloride

(Kónya and Kövér, 1972; Kónya et al., 1973) and a detailed description will soon be published. They draw attention to the significance of the junctional connection between T-tubule membranes and terminal cysternae of the SR in the mechanism of function of E-M coupling.

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COMPARATIVE STUDIES ON SOME MEMBRANE-BOUND ENZYMES IN NORMAL AND DYSTROPHIC MUSCLE

by

G. KATONA and VILMA SZÉKESSY-HERMANN

2nd INSTITUTE OF BIOCHEMISTRY, SEMMELWEIS UNIVERSITY OF MEDICINE, BUDAPEST, HUNGARY

In our earlier investigations concerning the role of vitamin E in the regulation of muscle metabolism we observed a marked increase of cholinesterase and a less considerable enhancement of AMP-deaminase activity in muscular dystrophy caused by vitamin E deficiency (Vodnyánszky et al., 1962, 1966). There are several possibilities regarding the molecular mechanism underlying the observed changes in enzyme activities. Since evidences have been obtained about the increase of the turnover of protein and nucleic acid synthesis in vitamin E deficiency (Dinning et al., 1955, 1956*a*, *b*; Antoni et al., 1957), efforts were made to find connection between these processes and the key-effect of vitamin E. Such investigations resulted in a theory stating the role of vitamin E in the regulation of the repressed–derepressed state of certain enzyme genes (Olson and Carpenter, 1967).

On the other hand, it has been demonstrated that vitamin E deficiency is characterized by a desintegration of muscle ultrastructure, the degree of which depends on the species studied (Dhalla et al., 1971; Yu-Yan-Yeh and Johnson, 1973; Diplock et al., 1971). Following the reasoning of Coleman that 'the enzymes may be viewed as sensitive indicators of membrane ultrastructure", it can be supposed that also in vitamin E deficiency these structural changes might be responsible for the increased activity of membrane-bound enzymes. In our present work comparative investigations have been carried out on the effect of detergent treatment, which seems to be a successful method for the assay of membrane-associated enzymes (Miller, 1970; Norstrand and Glattz, 1973; Srinivasan et al., 1972; Millar et al., 1973; Wright and Plummer, 1973). From the variety of detergents applied we have chosen the nonionic reagent TRITON-X-100, widely used for the estimation of cholinesterase.

In preliminary experiments, however, a pronounced effect of the ionic strength on the extracted enzyme activity has been found. Therefore, TRITON-X-100 treatment was carried out at various KCl concentrations. The following methods were used: The vitamin E deficient rabbits were kept on Goettsch–Pappenheimer diet until the manifestations of dystrophy became evident (after about six weeks). The mixed, minced muscle was homogenized in a Waring-blendor, in the absence and in the presence of 0.5 per cent (volume/volume) TRITON-X-100. The KCl concentrations used were 0.15, 0.5 and 1.0 mol/liter. The homogenates were maintained at 4 °C for 30 min. The insoluble material was removed by centrifugation. The cholinesterase activity was measured by the method of Ellmann and co-workers (1961), using acetylthiocholine iodide as substrate. The AMP-

deaminase activity was determined on the basis of the amount of ammonia produced, measured by the Nessler–Winkler method. Protein concentration was assayed by the method of Folin–Lowry. Enzyme activities were expressed in μmol (or nanomol) of substrate converted in one minute and were related to the wet weight of muscle or to mg protein.

Figure 1 shows cholinesterase activities of normal and dystrophic muscle homogenates obtained at different KCl concentrations in the absence of TRITON-X-100. Each column represents (here and also in the other figures) the average level of the activities of seven to nine animals, in this case related to the wet weight of muscle. In order to make the data more comparable, the activities are expressed also in per cents of the lowest value, that is, as per cents of the activity measured using 0.15 M KCl concentration. The figure shows an enhancement of the total activity with increasing ionic strength of the extracting medium, in the case of both normal

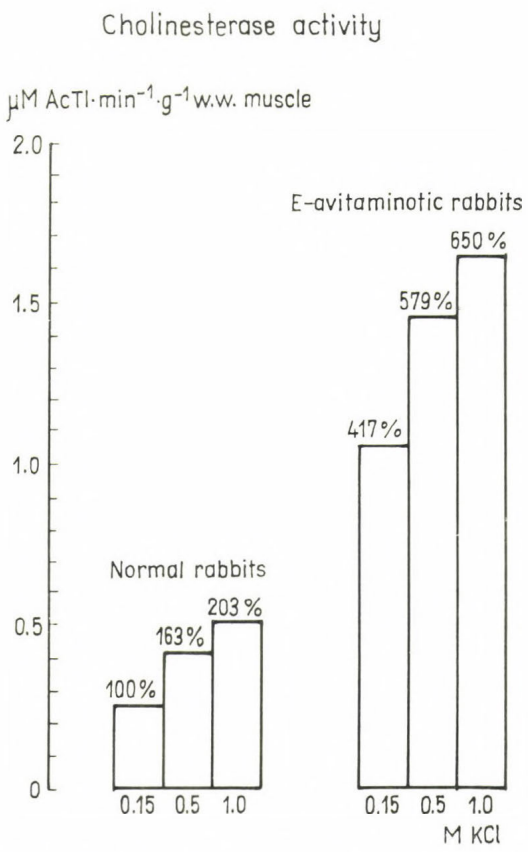

Fig. 1. The reaction medium contained: 1.5 micromole acetylthiocholine iodide, 10^{-} milimole KCl, 0.28 milimole phosphate buffer pH 8.0, 10 mg muscle, 1.0 micromole 5 : 5-dithiobis-2-nitrobenzoic acid (DTNB), in a final volume of 3.12 ml. The reaction occurred at room temperature

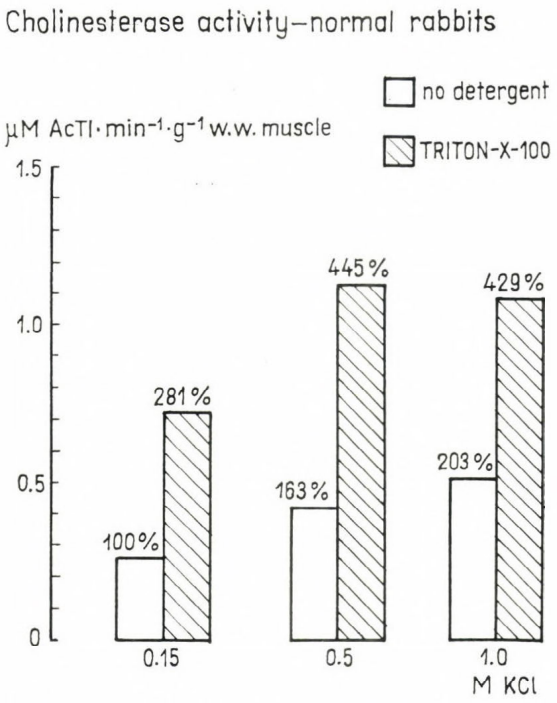

Fig. 2. Constitution of the reaction medium: see in the legend of Fig. 1

and vitamin E deficient animals. However, in the case of dystrophic muscle the increase is less pronounced, consequently the ratio of the activities of these two types of muscles is smaller. This difference between the normal and dystrophic muscles concerning the effect of the ionic strength is even more pronounced in the presence of TRITON-X-100. Figure 2 shows the activities of normal muscle homogenates, in the absence and in the presence of the detergent. It can be seen, that TRITON-X-100 treatment causes a considerable increase of the cholinesterase activity at all KCl concentrations used. The relative increase in the case of 0.15 and 0.5 M KCl concentrations is about the same and only in the case of 1.0 M KCl is it smaller. Figure 3 indicates the activities of homogenates prepared from dystrophic muscle. It can be seen, that contrary to the normal muscle, the activities obtained at various KCl concentrations in the presence of TRITON-X-100 are very similar. On the basis of the protein concentration, we have calculated the specific activities of the homogenates. In Fig. 4 the average specific activities of normal muscle homogenates are indicated, expressed in nanomol of substrate per minute per mg protein. It is demonstrated that, in contrast to the total activity, the specific activity increases markedly on addition of the detergent only at low ionic strength. The same is valid in connection with the specific activities of vitamin E deficient muscle homogenates (Fig. 5), reflecting the fact that

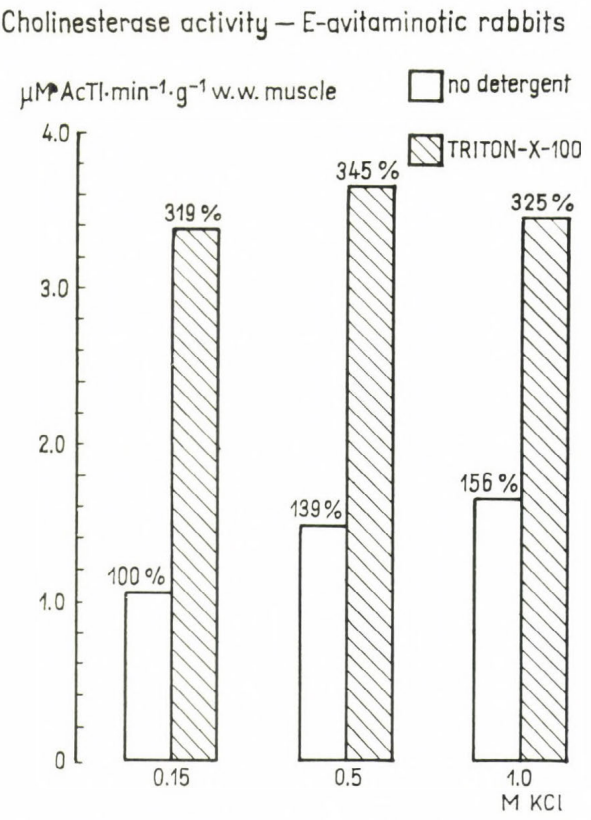

Fig. 3. Constitution of the reaction medium: see in the legend of Fig. 1

the protein concentrations of the homogenates obtained from normal and dystrophic animals do not differ significantly from each other.

As to the AMP-deaminase activity, we have obtained results entirely different from those with the cholinesterase activity. In Fig. 6 the activities of normal homogenates related to the wet weight of muscle are shown. It can be seen that parallelly with the increasing KCl concentration from 0.15 to 0.5 M the AMP-deaminase activities increase to a much higher extent than the cholinesterase activities do. The presence of TRITON-X-100 affects these enhanced activities only slightly. We can see a considerable influence of this detergent on the AMP-deaminase activity only at low ionic strength. The results obtained with dystrophic muscle are very similar (Fig. 7); only in the absence of TRITON-X-100 at low KCl concentration is the average activity higher than in the case of normal animals. The specific activities of AMP-deaminase exhibit the same relation to the total activities, as in the case of cholinesterase.

The different behaviour of the two types of muscles toward the changes of the extracting solution suggests a less stable binding of the studied

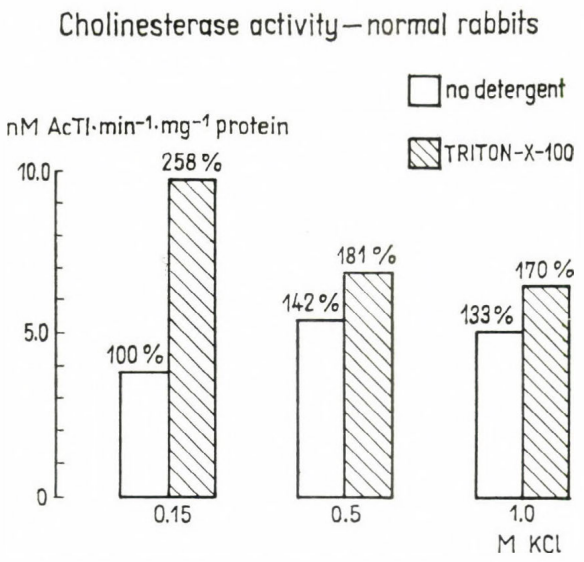

Fig. 4. Constitution of the reaction medium: see in the legend of Fig. 1

membrane-associated enzymes in the striated muscle of vitamin E deficient rabbits than found in normal muscle. However, there is a remarkable difference between the AMP-deaminase and cholinesterase activity in this respect. Concerning the change of enzyme activity caused by vitamin E deficiency we can make the following comparison between the two enzymes.

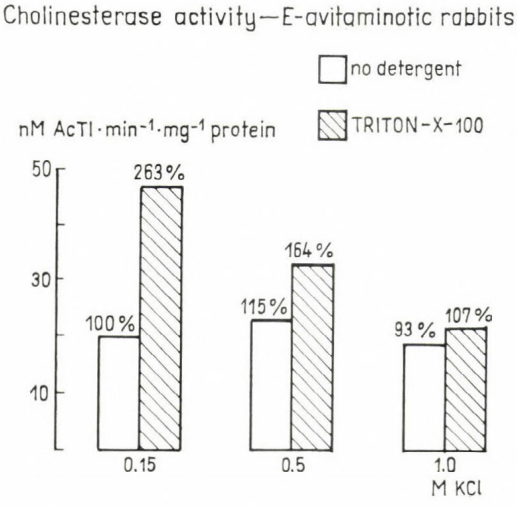

Fig. 5. Constitution of the reaction medium: see in the legend of Fig. 1

The AMP-deaminase activity at 0.15 molar KCl concentration shows a value about fifty per cent higher in the dystrophic than in the normal muscle homogenates. In the presence of TRITON-X-100 at low ionic strength, or at higher 0.5 and 1.0 M KCl concentrations both in the absence and presence of the detergent, the activity difference between the two types of muscles disappears.

The cholinesterase activity at low ionic strength in homogenates of dystrophic muscle is about four times higher than in normal homogenates. In the presence of TRITON-X-100 the activity increases to a level three times higher than that in the absence of the detergent, in the case of both normal and vitamin E deficient rabbits. With increased ionic strength the difference between both types of muscles diminished. However, even at 1.0 M KCl concentration and in the presence of TRITON-X-100, the activity of dystrophic muscle homogenates is roughly three times higher than that of normal homogenates.

On the basis of these results it has been concluded that in the case of AMP-deaminase the difference of activities observed between the normal

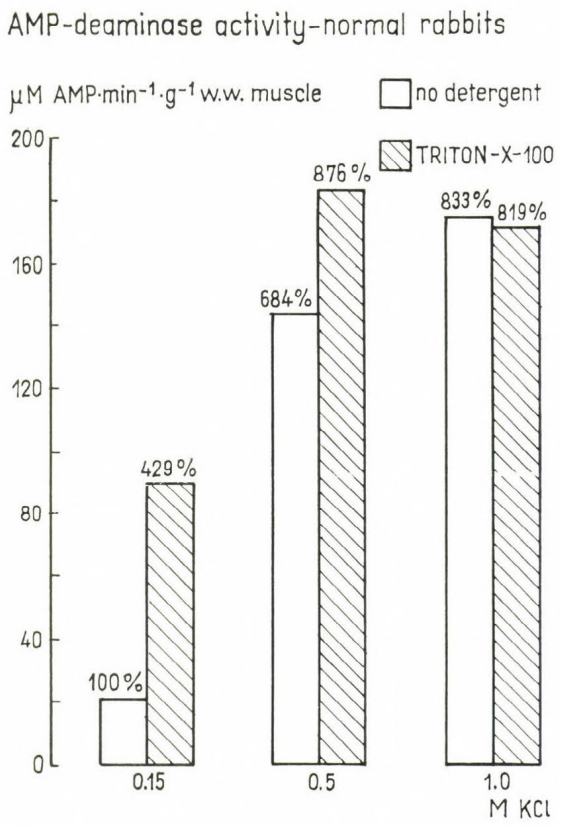

Fig. 6. The reaction medium contained 8 micromole AMP, 0.66 milimole succinate buffer pH 6.1, 1.0 milimole KCl, 5–10 mg muscle, in a final volume of 5.0 ml. The reaction occurred at 37°C

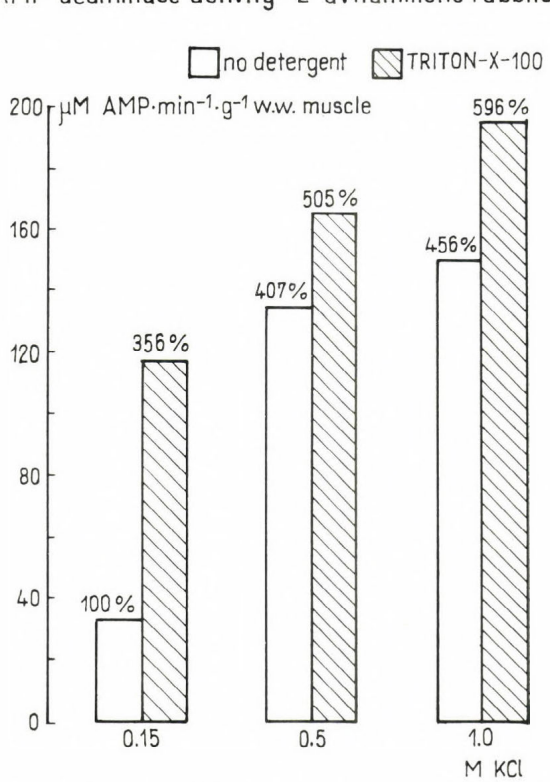

Fig. 7. Constitution of the reaction medium: see in the legend of Fig. 6

and dystrophic muscle homogenates may be ascribed entirely to a less stable association of this enzyme to the membrane. Any change of the total potential activity seems to be most improbable. On the other hand, the large increase of cholinesterase activity caused by vitamin E deficiency remains considerable in spite of some reduction in extracts obtained at high ionic strength and in the presence of the detergent. Consequently, to explain this increase another mechanism must be taken into account in addition to the change of membrane stability.

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EFFECT OF RAPID DIGITALISATION ON EARLY ISCHAEMIC HEART FAILURE IN DOGS

(ABSTRACT)

by

R. Lerch, W. Rafflenbeul, A. Kleber, W. Meier, E. Jenny and W. Rutishauer

MEDICAL POLYCLINIC, UNIVERSITY OF ZÜRICH, ZÜRICH, SWITZERLAND

Left ventricular (LV) performance, myocardial ATP, creatinephosphate (CrP) and lactate (La) content and La production were determined in early ischaemic heart failure before and after administration of acetyl strophanthidin (AcS). In open chests of 6 dogs the left coronary artery was cannulated and perfused from a femoral artery by means of a roller pump. From LV tipmanometer pressure LV dP/dt and the isovolumic velocity of contractile elements ($V_{CE} = dP/dt$) (P. 28) were obtained instantaneously. LV end-diastolic volume (EDV) was determined by cineangiocardiography. In myocardial tissue samples ATP, CrP and La contents, in arterial and coronary venous blood samples oxygen and La content were determined. LV failure was produced by stepwise reduction of coronary flow until LV end-diastolic pressure (LVEDP) exceeded 12 mmHg.

An average reduction from 84 to 46 ml/min $\times$ 100 g was followed by an increase of LVEDP from 5 to 19 mmHg and a decrease of LV peak systolic pressure from 122 to 98 mmHg ($P < 005$), LV dP/dt from 2,812 to 1,553 mmHg/sec ($P < 001$) and peak measured V_{CE} (Vpm) from 2.46 to 1.19 sec^{-1} ($P < 001$). Heart rate did not change significantly. EDV increased from 20 to 36 ml/10 kg ($P < 005$). Whereas oxygen consumption decreased from 8.4 to 6.4 ml/min $\times$ 100 g ($P < 005$) La production rose from -50 to $+41$ μmoles/min $\times$ 100 g ($P < 001$). Myocardial ATP, CrP and La contents showed no significant changes. AcS 20 γ/kg lead to a decrease of LVEDP to 11 mmHg ($P < 005$) and EDV to 29 ml/10 kg ($P < 002$) and an increase of Vpm to 1.49 sec^{-1} ($P < 001$), while none of the other above-mentioned parameters showed a significant change.

In summary, early ischaemic heart failure is not accompanied by changes in myocardial ATP, CrP and La contents and rapid digitalisation leads to diminution of LVEDP and EDV without affecting myocardial oxygen consumption, high energy phosphate or La content.

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THEORETICAL ANALYSIS OF MUSCLE FATIGUE AND THE MUSCLE AS MACROSYSTEM

by

F. A. Jólesz, I. Illés, M. Rapcsák and R. Frenkl

KÁLMÁN KANDÓ COLLEGE OF ELECTRICAL ENGINEERING,
HUNGARIAN COLLEGE OF PHYSICAL EDUCATION, BUDAPEST, HUNGARY

The earlier physiological studies attempted to determine, how the chemical energy contained in muscle is converted to work and what is the biochemical basis of muscle fatigue.

The analysis of fatigue curves (Fig. 1) still relies on the biochemical aspect. Furthermore the mathematical analysis employed is very simple, based on the time of fatigue, the trend of the curves, etc.

Previous models and stimulations of striated muscle have been based on single functional units. Typically, these single units are descriptively related to gross muscle features. We describe a method of analysis of fatigue which is based on the assumption, that living systems like muscle are macroobjects according to the law of physics and need analysis in terms of it.

Thus the striated muscle may be represented by multiple distributed elements, i.e. by microobjects. Physiologists often study the muscle

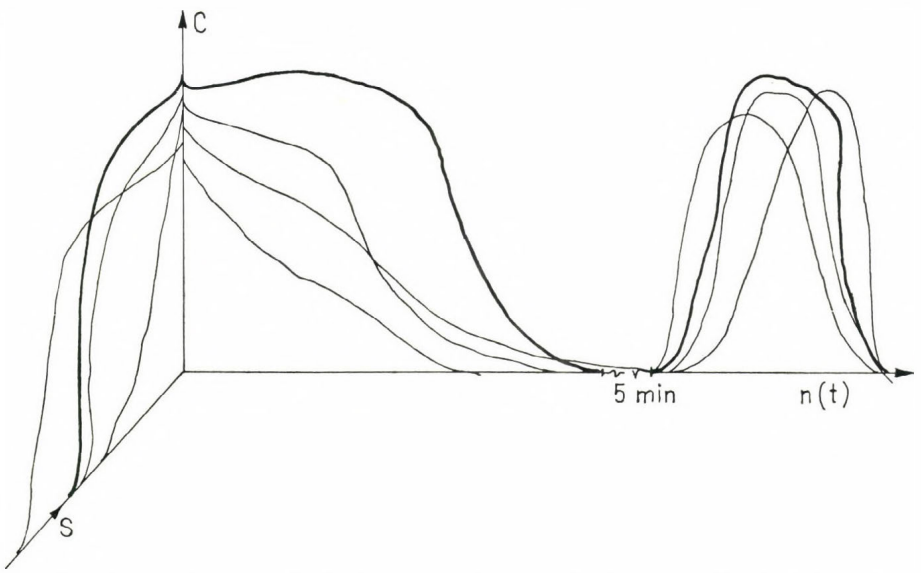

Fig. 1. Quantal summation, fatigue and regeneration curves. S = stimuli; C = amplitudes of contractions; n(t) = stimulus number

structure and function by analyzing only these microobjects assumed to have uniform characteristic. This is an idealization, however.

To overcome these shortcomings we introduce a model which emphasizes not the individual elements but rather the properties of populations composed of multiple distributed elements. Physiological evidences for the existence of muscle populations are the quantal summation, fatigue and regeneration curves (see Fig. 1). These findings indicate that within a muscle there exist many elements with different responses to identical stimuli.

The complex muscle seems to be a macrosystem or macroobject which in part of its behaviour is purely mechanical not thermodynamical or statistical. These ideas are closely connected with the stability of dynamical systems such as muscle (Ashby, 1956).

We have to recall that dynamic system is in stable equilibrium if under a small perturbation the forces operate successfully within the system so as to restore the equilibrium state. Is it true considering the process of fatigue? Is the muscle a stable system?

An ultrastable system is one possessing many states of stable equilibrium. Any perturbation in one state produces a disturbance within the system which operates until a new state of equilibrium is reached.

The microobjects within the muscle macroobject ($m_1, m_2, \ldots, M$ in Fig. 2) are ultrastable systems. The complex muscle or the multistable system (Ashby, 1954) is a collection of loosely coupled ultrastable subsystems, not all of which are active at any given instant. Further it has the important property of being able to change the configuration of active subsystems. This multistable system is adaptive and displays, as a consequence, the properties of fatigue. From the analysis given for the multistability of complex dynamical systems as muscle, several important properties of such systems are deduced:

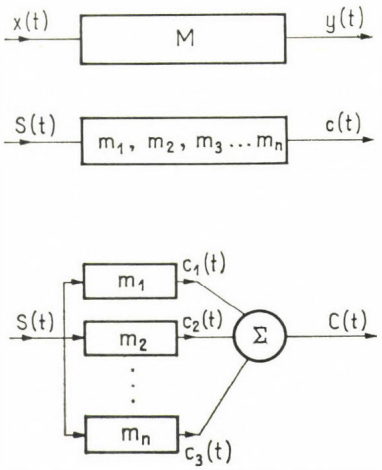

Fig. 2. The block diagram of muscle. M = the complex muscle system; m = elements of complex muscle; S(t) = stimulus; c(t) = amplitudes of contraction

1. Not all parts are active at any time.

2. The system is heterogenous. All the subsystems are not similar in structure and function and there are some randomness of functional interconnexions.

3. There is a large number of interconnected nonlinear elements, the components of the muscle tissue.

4. Considering the quantal summation curve, this is the case of nonlinear system, and corresponds to the fact that different initial conditions may lead to distinct modes of response. The muscle is an ensemble, a collection comprising a large number of approximately identical copies of the subsystem.

5. Each member of the ensemble reacts with a different amplitude and the response of the ensemble is taken to be the summation of all individual responses of the members. In this way the quantal summation curve has a spectral characteristic and the assembly has an integrative property. It is important to note that this integrative property depends on the non-linearity of the individual elements of the assembly.

Our point of view was expressed in the hypothesis of probability-statistical principle of the organization of muscle elements in the complex functional system of the muscle. According to this hypothesis the ensembles of muscle elements form the probabilistic distribution of activity in space and time. A large number of randomly acting elements determine statistically the resulting global pattern of the regular reaction of the system. One can draw the conclusion that the transition from the schematic organization of muscle elements to the probability-statistical organization is made by the structural organization of the coupled approximately homogenous elements into a dynamic mozaic of elements with heterogenous functional states.

We define the variables characterizing the dynamics of a muscle system or population. We shall first obtain expressions for the proportion of subunits receiving at least threshold excitation (Fig. 3). We call this subpopulation response function (Wilson and Cowan, 1972) because it gives the expected proportion of subunits if a subpopulation which would respond to a given level of excitation ($\bar{S}$). There is a distribution of individual thresholds ($D(\Theta)$) within a subpopulation characterized by the distribution function. But let there be a distribution of the amplitudes of contraction (L/c) so the subpopulation response function takes the form as shown

$$P(\bar{S}) = \int_0^{\bar{S}(t)} D(\Theta)\, d\Theta$$

$$P'(\bar{S}) = \int_0^{\infty} L(c)\, dc$$

Fig. 3. Subpopulation response functions

in Fig. 3. There are two important distributions as defined in either equations, the distribution of individual thresholds and the distribution of forces.

The quantal summation curve represents these distribution functions in the initial state. After using supermaximal impulse series the condition of the muscle is different from that of a normal resting state. The threshold and the contractibility are known to undergo a pronounced change and the fatigue curve represents the time course of the gradual change with increasing stimulus number. A transient threshold and contractibility distribution variation are generated following successive stimuli. In the initial state as shown on the quantal summation curve, the distributions

$$\left.\begin{aligned} N(t) &= N_0 \cdot e^{-\frac{(t-t_0)^{\beta}}{\alpha}} \\ \lambda(t) &= \frac{\beta}{\alpha}(t-t_0)^{\beta-1} \\ \frac{d \ln N(t)}{dt} &= \lambda(t) \text{ 'fatigue' coefficient} \end{aligned}\right\} \text{ if } t > t_0$$

$$\log\left[\log \frac{N(t)}{N_0}\right] = \log\left(-\frac{1}{\alpha}\right) + \beta \log (t - t_0)$$

$$\log Y' = \alpha + \beta T \quad \text{if} \quad t_0 = 0$$

$$\boxed{Y = \alpha + \beta T}$$

Fig. 4. The Weibull distribution function

are approximately normal, but the fatigue curve represents another distribution, of the type shown in Fig. 4. This is the Weibull distribution (Jólesz and Frenkl, 1971).

The muscle has the important property of being able to change the configuration or distribution of active elements so as to remove disturbances caused by any stimuli. It is a multistable system, it is adaptive and displays, as a consequence, the properties of fatigue.

The model is based on the main property of the living systems; their changeability and adaptability. We suppose on the ground of physiological facts, that these properties are caused by the probability of distribution of activity in ensembles of ultrastable muscle elements and by the statistical results of their dynamic function.

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MYOFIBRILLAR PROTEINS

Symp. Biol. Hung. 17, p. 127 (1974)

ACTIN IN NONMUSCULAR CELLS

(ABSTRACT)

by

G. GABBIANI

DEPARTMENT OF PATHOLOGY, UNIVERSITY OF GENEVA, GENEVA, SWITZERLAND

Few years ago, Johnson et al. (1965) described in patients with chronic aggressive hepatitis an autoantibody which fixed selectively on smooth muscle (smooth muscle autoantibody). This antibody was successively found to be present in patients with acute hepatitis, infectious mononucleosis and several malignant diseases.

Further studies indicated that the antigen of smooth muscle autoantibody was a contractile protein and actin in particular (antiactin autoantibody or AA). This was demonstrated (Gabbiani et al., 1973) by abolishing the fixation on smooth muscle of AAA after incubation of this antibody with thrombosthenin-A (the actin moiety of thrombosthenin). When thrombosthenin-A is incubated with AAA, a precipitate is formed which fixes anti-human IgG but not anti-rat IgG.

By means of immunofluorescence it has been shown that AAA fixes on the I bands of striated muscle and that this fixation is abolished when AAA, containing serum is incubated with thrombosthenin-A. Hence it appears that actins present in striated muscle, smooth muscle and platelets are antigenically similar.

Using immunofluorescent techniques, actin can be shown in various nonmuscular cells such as lymphocytes, epidermal cells—growing over an open wound—hepatic cells, β-cells of Langerhans islets and adrenocortical cells.

The presence of actin is confirmed by the electron-microscopic observation of intracytoplasmic microfilaments, 40–70 Å in diameter. In many instances the amount of proteins present in the cytoplasm varies with the functional activity of the cell.

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A PROTEIN FACTOR INHIBITING THE POLYMERIZATION OF ACTIN

by

MÁRIA B. PÁPAI

2nd INSTITUTE OF BIOCHEMISTRY, SEMMELWEIS UNIVERSITY OF MEDICINE, BUDAPEST, HUNGARY

In 1970, Ebashi and Maruyama proposed the concept of 'regulatory proteins'. These proteins, the troponin complex, α-actinin, β-actinin and M protein, play a role in the muscle function either by controlling the myosin–actin interaction or by arranging the steric correlation of myofilaments to form the ordered structure of the sarcomere. I would like to discuss some earlier and recent works on an inhibitor protein of actin polymerization, previously found in crude actin extracted from dystrophic rabbit skeletal muscle.

In 1961 Prof. Székessy-Hermann called attention to the fact that in case of muscle dystrophy induced by vitamin E deficiency one might extract a protein accompanying actin that inhibited the G–F transformation of actin. It is very likely that the presence of this protein factor in the aceton-dried muscle powder of vitamin E deficient rabbits could provide one of the explanations for the well-known reduced polymerizing ability of actin isolated from dystrophic skeletal muscle. This assumption is supported by the following observation. If actin isolated from the striated muscle of rabbits with vitamin E deficiency by Straub's extraction procedure and is further purified by depolymerization and repolymerization according to Mommaerts, its properties become similar to that of normal actin, as far as polymerizing ability and interaction with myosin are concerned. Furthermore, in the supernatant obtained by the ultracentrifugal purification the inhibitor protein of actin polymerization is present.

As early studies from our laboratory demonstrated considerable differences of myosin obtained from vitamin E deficient and normal rabbit muscle, it seemed interesting to study whether there might be an inhibitor protein of actin polymerization as an impurity in the "dystrophic" myosin preparations. For this reason a sample of myosin from rabbits with vitamin E deficiency was dialysed against 0.1 M KCl solution at pH 7.4, and the effect of protein which did not precipitate was examined on the polymerization of G actin. As shown in Fig. 1, the presence of the dialysed 'dystrophic' myosin supernatant causes a considerable loss in the ability of G actin to polymerize.

The question arises whether the inhibitor is a characteristic feature of the dystrophic muscle or it may occur in the skeletal muscle of normal rabbits, too. If yes, does the structural damage of striated muscle in vitamin E deficiency cause any difference in its extractibility?

Experiments have shown that it is possible to extract an inhibitor of actin polymerization from the striated muscle of healthy rabbits, too.

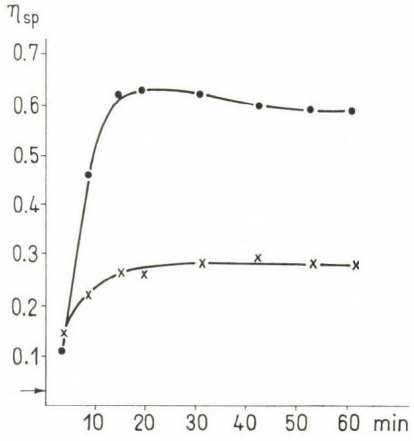

Fig. 1. Actin polymerization in the presence of a protein fraction obtained from 'dystrophic' myosin by dialysis. Dialysis was performed against 0.1 KCl in 0.01 M Tris-HCl buffer, pH 7.4. The supernatant remaining after precipitation was applied. Conditions: 0.1 M KCl, 1 mM $MgSO_4$, 50 mM Tris-HCl, pH 7.4, final volume 6 ml, temperature 24 °C. Polymerization of 4.0 mg G actin in the absence (●) and in the presence (×) of 2.4 mg non-precipitated protein. Polymerization was started at time 0 by the addition of G actin to the reaction mixture. Arrow indicates specific viscosity of G actin in water

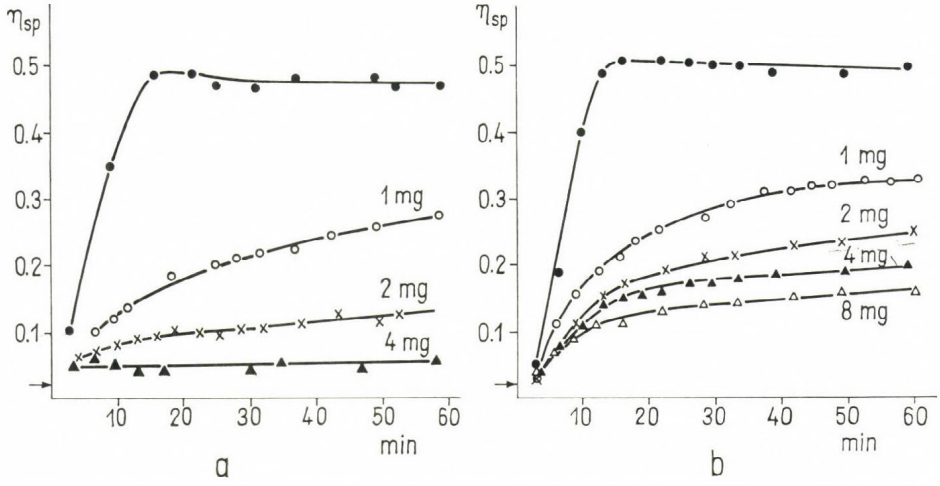

Fig. 2. The effect of various amounts of inhibitor protein factor from dystrophic and normal rabbits on the polymerization of G actin. Conditions: 0.1 M KCl, 1 mM $MgSO_4$, 50 mM Tris-HCl, pH 7.4, final volume 6 ml, temperature 24 °C. At time 0, 4.0 mg of G actin was added to the reaction mixture. Arrow indicates specific viscosity of G actin in water. *a* Inhibitor protein factor isolated from the muscle of healthy rabbits. The amounts of inhibitor protein as indicated. *b* Inhibitor protein factor isolated from the muscle of rabbits with vitamin E deficiency. The amounts of inhibitor protein as indicated

It can be isolated from muscle homogenate, from glycerol-extracted muscle fibres and from myofibrils, as well.

I should like to summarize the main steps of the isolation procedure from muscle homogenate. All operations are carried out at 0 5 °C. Freshly minced muscle is washed with 0.04 M KCl, dissolved in neutral phosphate buffer. After centrifugation the muscle residue is extracted four times with Hasselbach Schneider solution in order to remove myosin. Following the last centrifugation the residue is homogenized in a Waring-Blendor and washed first with 1 mM $KHCO_3$ and then with 0.1 M KCl solution. To the last pellet 3 volumes of KI is added at a final concentration of 0.9 M in order to extract the inhibitor protein. The suspension is centrifuged and the supernatant is dialysed. After dialysis against 0.1 M KCl containing 0.01 M Tris-HCl, pH 7.4, the precipitate is centrifuged. The supernatant is centrifuged again in a preparative ultracentrifuge at $55{,}000 \times g$ for 2 h. The resulting supernatant contains the inhibitor protein of actin polymerization in an unpurified form. This solution containing approximately 5 mg protein per ml was used in the experiments presented here.

Figure 2 shows the effect of the protein factor on the polymerization of G actin. The polymerization was followed by viscometry. Inhibitor was prepared from the striated muscle of both normal and vitamin E deficient rabbits by the same procedure. Comparing the concentration dependence of the inhibition in the presence of 'dystrophic' and 'normal' inhibitor one may conclude that the inhibitory effect is less pronounced in the case of inhibitor obtained from 'dystrophic' muscle.

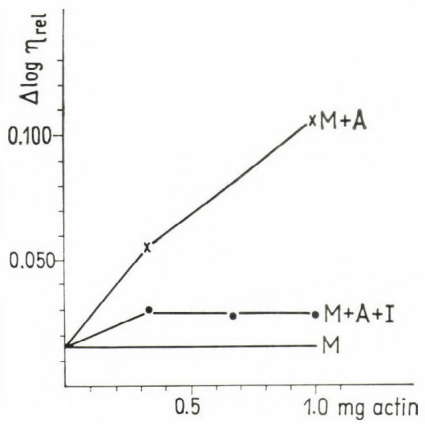

Fig. 3. The ability of actin to become complex with myosin both in the presence and in the absence of the inhibitor protein factor. Conditions: 0.5 M KCl, 0.5 mM $MgSO_4$, 50 mM Tris-HCl, pH 7.4, final volume 4 ml, temperature 24 °C. G actin was mixed prior to incubation with the inhibitor factor in a weight ratio of 1 : 1. Abscissa: mg actin added to 3.0 mg of myosin. Ordinate: decrease in the logarithm of relative viscosity due to the effect of ATP. Binding between myosin and actin in the absence (×) and in the presence (●) of the inhibitor factor

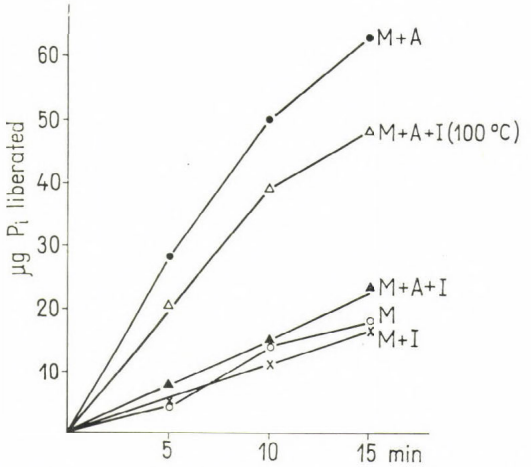

Fig. 4. The effect of actin on myosin ATPase activity, both in the presence and in the absence of inhibitor protein factor. Incubations were carried out at 24 °C in 5 ml, containing 0.05 M KCl, 50 mM Tris-HCl, pH 7.4, 3 mM $MgCl_2$, 2 mM ATP, 1.0 mg myosin, 0.50 mg actin and 0.45 mg inhibitor protein. G actin was mixed with the inhibitor prior to incubation. ATPase activity of myosin in the absence of actin (○), in the presence of actin (●), in the presence of actin and inhibitor factor (▲), in the presence of inhibitor factor alone (×), and in the presence of actin and heat treated inhibitor factor (△)

The actomyosin formation of actin which is mixed in G form with an appropriate amount of inhibitor factor is shown in the experiment represented in Fig. 3. Actomyosin formation was measured by determining the fall in viscosity obtained when ATP was added to a solution containing myosin and actin. It can be seen that there is no actomyosin formation in the presence of inhibitor of actin polymerization.

Figure 4 illustrates the effect of actin on the enzymic activity of myosin both in the absence and in the presence of inhibitor of actin polymerization. One can see the well-known activation of ATPase with F actin, but there is no change in the criginal low level of myosin ATPase activity in the presence of the inhibitor protein of actin polymerization. It is also demonstrated that heat treatment destroys the effect of the inhibitor.

Summarizing, this protein factor previously found in crude actin from dystrophic rabbit skeletal muscle, inhibits the formation of fibrous actin *in vitro* even under ionic condition when actin should be only in fibrous form. Furthermore, the actin loosing its ability to polymerize under the influence of the inhibitor protein is unable to form actomyosin and shows no effect on myosin ATPase.

This work is at a preliminary stage. The purification, the characterization and the comparison of the inhibitor factor with other proteins, especially β-actinin, needs further investigations.

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IMMUNOCHEMICAL STUDIES ON MYOSIN WITH THE AID OF QUANTITATIVE MICROCOMPLEMENT-FIXATION

by

ST. BRUGGMANN, K. BÜCHI and E. JENNY

INSTITUTE OF PHARMACOLOGY AND BIOCHEMISTRY, FACULTY OF VETERINARY MEDICINE, UNIVERSITY OF ZÜRICH, ZÜRICH, SWITZERLAND

INTRODUCTION

In the past, antibodies against a variety of myosins from different types of muscles and species have been induced mostly in the rabbit. The antisera obtained were used to localize myosin in the myofibril (Pepe, 1968), to establish its species and muscle specificity (Varga et al., 1960; Finck, 1965*a*, *b*; Gröschel-Stewart and Doniach, 1969) or to monitor its synthesis in cell cultures (Okazaki and Holtzer, 1965).

However, it soon became clear that according to immunological criteria of purity, both myosin preparations and antisera were heterogeneous (Starr and Offer, 1971; Pepe, 1972). One of the impurities has been identified and designated C protein (Offer et al., 1973), others are still unidentified.

While attempting to obtain a well-defined myosin–antimyosin system to study conformational changes of myosin heads, we purified white myosin (long. dorsi) of the rabbit to a high degree which induced antibodies in the guinea pig. Then we analyzed the antisera obtained by quantitative microcomplement-fixation. Some of the results are reported here.

MATERIALS AND METHODS

Myosins were prepared from A-filaments (Morimoto and Harrington, 1973) using the established methods of $(NH_4)_2O_4$ fractionation and column chromatography on DEAE-Sephadex (Offer et al., 1973). Polyacrylamide-gel-electrophoresis (Starr and Offer, 1971) revealed that all the impurities were eliminated except a protein with a molecular weight of about 100,000 daltons at a concentration of less than 0.3%. White myosin of the rabbit was complexed with methylated bovine serum albumin, suspended in Freund's adjuvant and subcutaneously injected into guinea pigs at intervals of one month for six months. Three mg of myosin was injected at a time. The antisera obtained after six months were analysed for purity and specificity by quantitative microcomplement-fixation (Levine, 1967). Conditions for the ATPase assay are indicated later in Figs 3 and 4. Other muscle proteins and protein systems were prepared according to established methods: Actin (Hartshorne et al., 1967), α-actinin (Arakawa et al., 1970), β-actinin (Maruyama, 1965), n-tropomyosin (Ebashi, 1964), the relaxing protein system (Schaub and Perry, 1969), creatin-

kinase (Dawson and Eppenberger, 1970), AMP-deaminase (Smiley et al., 1967), C-protein (Offer et al., 1973). Myokinase was a commercial product (Boehringer, A. G., Frankfurt/Main).

RESULTS AND DISCUSSION

The antisera did not cross-react with actin, α-actinin, β-actinin, n-tropomyosin, the relaxing protein system, creatin-kinase, AMP-deaminase and myokinase. A spurious cross-reaction was found with C-protein. This activity was eliminated by immunoabsorption with purified C protein.

The antisera show a high muscle specificity (Fig. 1). However, some antigenic determinants seem to be in common with red and cardiac myosin of the same species. A high muscle specificity has been reported by several authors (Gröschel-Stewart and Doniach, 1969; Finck, 1965*c*; Gröschel-Stewart, 1971; Samuels, 1961), and few are the claims to the contrary (Abandowitz and Basrur, 1970; Holtzer et al., 1959).

Myosins are assumed to be class-specific (Varga et al., 1960). In agreement we did not find a cross-reaction with white myosin of the chicken (Fig. 2). Myosins are reported to have a low species-specificity in mammals (Abandowitz and Basrur, 1970; Gröschel-Stewart, 1971; Engel and Horwath, 1960). Indeed, a strong cross-reaction with white myosins of corresponding muscles (long. dorsi) of the pig and the dog is evident (see Fig. 2). However, our results clearly show differences in antigenicity.

Antisera inhibit the K^+- and the Ca^{2+}-activated ATPase of the corresponding myosin (Figs 3 and 4). At the same concentrations of antisera and myosin the inhibition is equal for both types of ATPase. This result

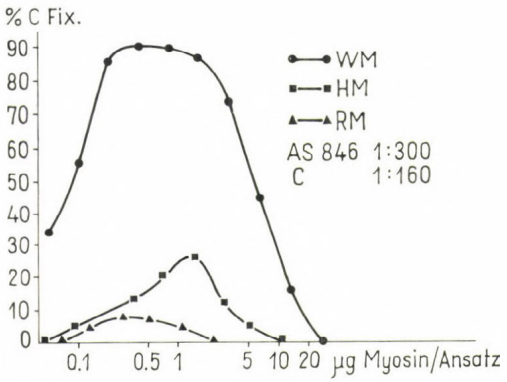

Fig. 1. Microcomplement-fixation with antisera against white (longissimus dorsi) myosin of the rabbit, using white, red and cardiac myosin of the rabbit as antigens

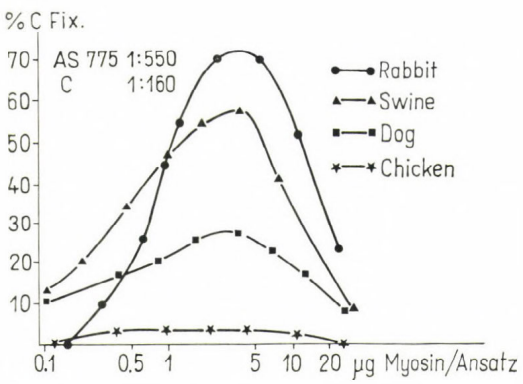

Fig. 2. Microcomplement-fixation with antisera against white (longissimus dorsi) myosin of the rabbit, using white myosins of the rabbit, the swine, the dog and the chicken as antigens

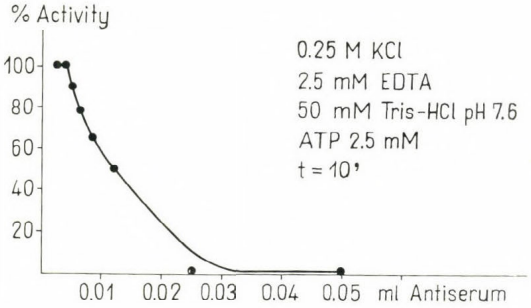

Fig. 3. Inhibition of the K^+ activated ATPase of white (longissimus dorsi) myosin of the rabbit with an antiserum induced in guinea pigs with the same type of myosin

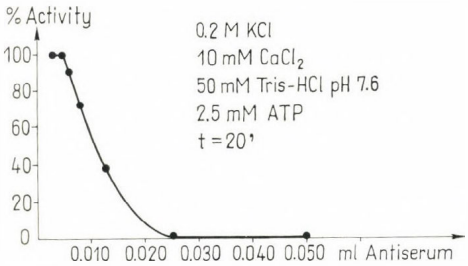

Fig. 4. Inhibition of the Ca^{2+} activated ATPase of white (longissimus dorsi) myosin of the rabbit with an antiserum induced in guinea pigs with the same type of myosin

proves that at least some antibodies in our antisera are directed to the active site of the ATPase (or at least a site very close to it) of myosin (Cinader, 1967) and that the same population of antibodies seems to inhibit both types of ATPases. An inhibition of myosin-ATPase by antisera has been reported by some authors (Gröschel-Stewart and Doniach, 1969; Gröschel-Stewart, 1971; Lowey and Steiner, 1972), none by others (Samuels, 1961; Kahlbrock Nass, 1962). These conflicting results can be reconciled by our observation that inhibiting antibodies in sufficient quantity appear rather late in the immunization process. So it might depend upon the time of collecting sera that an inhibition is found or not. Using HMM instead of integral myosin all results reported here could be repeated.

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THE DIFFERENTIATION OF RED AND WHITE SKELETAL MUSCLE IN THE RABBIT

by

G. Müller, M. Ermini and E. Jenny

INSTITUTE OF PHARMACOLOGY AND BIOCHEMISTRY, FACULTY OF VETERINARY MEDICINE, UNIVERSITY OF ZÜRICH, ZÜRICH, SWITZERLAND

Since the work of Herrmann and Nicholas (1948), Herrmann et al. (1949), De Villafranca (1954) and Trayer and Perry (1966), it is known that the enzymatic activity of the contractile proteins of skeletal muscle increases during development. It was also claimed that there exists a unique form of embryonic myosin with low ATPase activity from which subsequently the various kinds of myosins differentiate.

Being interested in the process of differentiation, we have studied the enzymatic maturation of myosin and myofibrils of both a predominantly white (m. gastrocnemius, 'fast twitch'), and red muscle (m. soleus, 'slow tonic') from the rabbit during development.

In addition we followed the appearance of red and white fibres in both muscles on the basis of succinodehydrogenase activity (SDH) in histochemical sections.

In Fig. 1 the specific activities of the K^+ and Ca^{2+}-stimulated ATPases of myosin are plotted in relation to age. No distinction is possible between the myosin activities of gastrocnemius and soleus around 5 to 2 days before birth, but within 6 to 9 days after birth adult levels are reached in both muscles.

Most conspicuous is the change in activity of the K^+-ATPase with a 10 to 15 fold increase for the gastrocnemius, and a 5 to 7 fold increase for the soleus. The Ca^{2+}-ATPase changes less dramatically though appreciably.

We conclude that the actual differentiation of myosin into its white and red types happens within this period of development. This is confirmed by the electrophoretic analysis of the myosin light chains on SDS polyacrylamide gels. At the age of 6 to 9 days myosin of the gastrocnemius already exhibits the three light chain bands typical for white muscle myosin, while that from the soleus shows only two. Before birth both myosins show two double bands in the molecular weight range of 20,000 and 27,000 daltons. Considerably more time is needed for myofibrils to reach adult levels of enzymatic activity (Fig. 2). Again no distinction is possible around birth between the two muscles, but within 17 to 20 days after birth the myofibrils of the gastrocnemius are 5 to 7 times and those of the soleus 2 to 3 times more active.

It seems that maturation of myosin and the other myofibrillar proteins does not take place at the same time or that development of protein interactions is retarded.

Experiments on the interaction between actin and myosin as well as between the regulatory proteins and actomyosin during differentiation

would have to be done. Simultaneously these proteins should also be examined quantitatively and structurally.

The histochemical distinction between red and white fibres on the basis of their metabolic situation, i.e. by the SDH activity of mitochondria, is not possible earlier than 17 days after birth (Fig. 3). At this stage the gastrocnemius can already be distinguished from the soleus which contains almost exclusively red fibres. As shown in Fig. 4, however, in the gastrocnemius the ratio of white to red fibres continues to increase from 0.4 at around 20 days up to 4 at about 4 months of age.

The results presented here allow 3 steps of differentiation to be distinguished in the rabbit skeletal muscle; first on the molecular level development of the enzymatic activity of the myosin molecule, second on the structural level of the myofibrils as indicated by the actomyosin ATPase activities, and third on the metabolic level as revealed by the distribution pattern of a mitochondrial enzyme, the SDH.

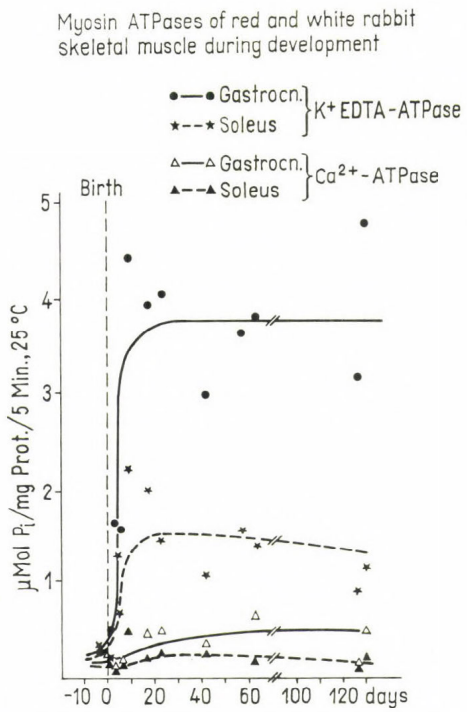

Fig. 1. Myosin ATPases. Myosin was prepared according to Perry (1955). For the K^+-stimulated ATPase the incubation mixture contained in a total of 2.0 ml: myosin 50–100 μg; Na_2-ATP, 4 mM; KCl, 0.6 M; EDTA, 10 mM; Tris-HCl, pH 7.6, 50 mM; and for the Ca^{2+}-stimulated ATPase: as before, but instead of KCl and EDTA, $CaCl_2$, 10 mM

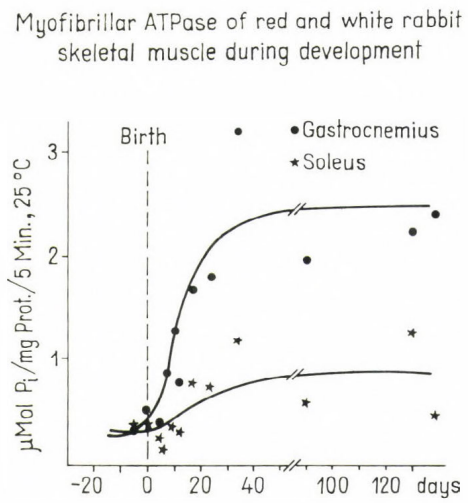

Fig. 2. Myofibrillar ATPases. Myofibrils were prepared according to Perry and Zydowo (1959). The incubation mixture contained in a total of 2.0 ml: myofibrillar suspension, 0.3–0.7 mg of protein; Tris-HCl, pH 7.6, 25 mM; Na_2-ATP, 2.5 mM; $MgCl_2$, 2.5 mM

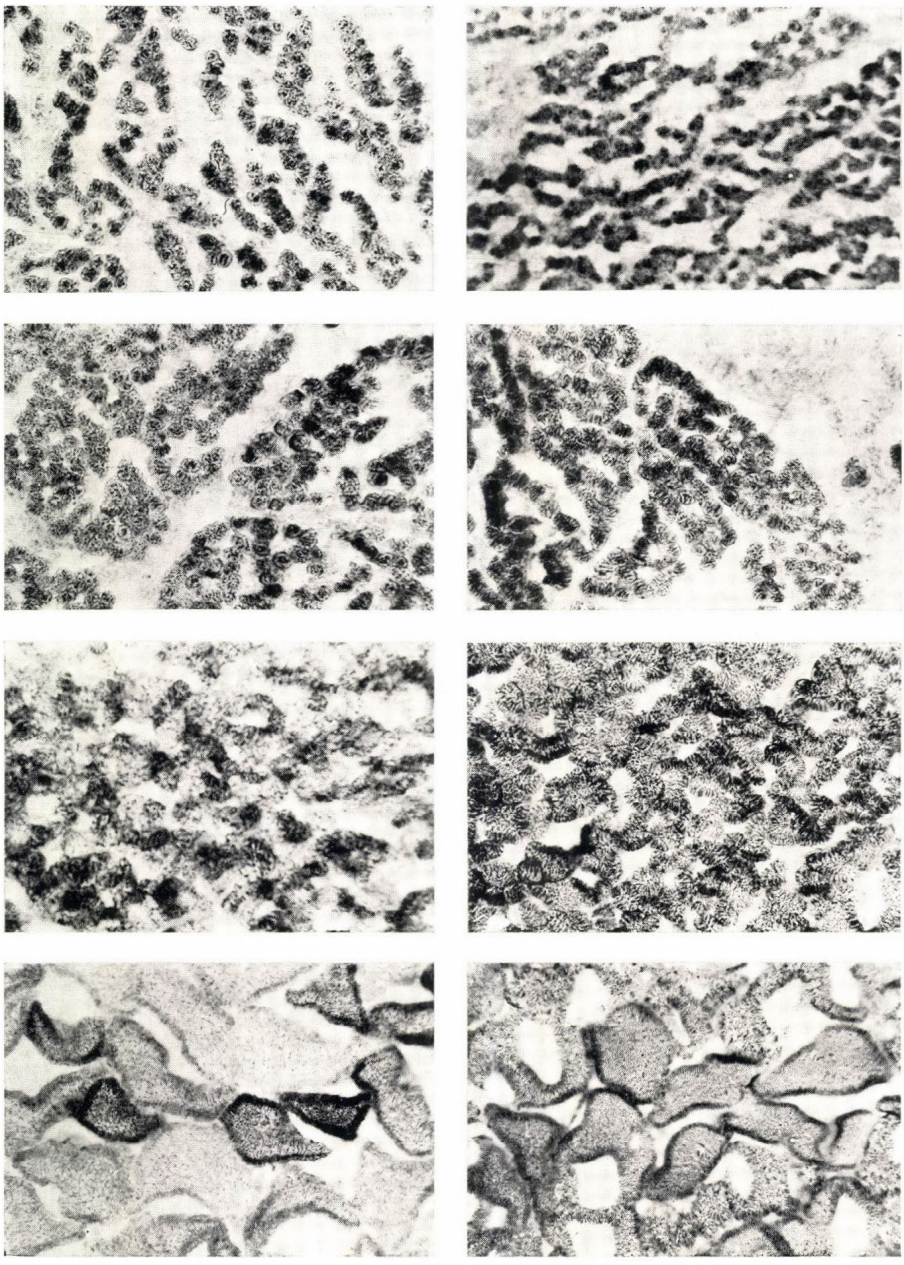

Fig. 3.Rabbit red and white fibres of gastrocnemius and soleus muscles during development. 10 μ cryostate sections were stained for succinodehydrogenase (SDH) reaction according to Nachlas et al. (1957). Red fibres: dark; white fibres: weakly stained. Distinction is possible only on the 17th day after birth. $A = 0$ day (birth); $B = 6$ days; $C = 17$ days; $D = 130$ days

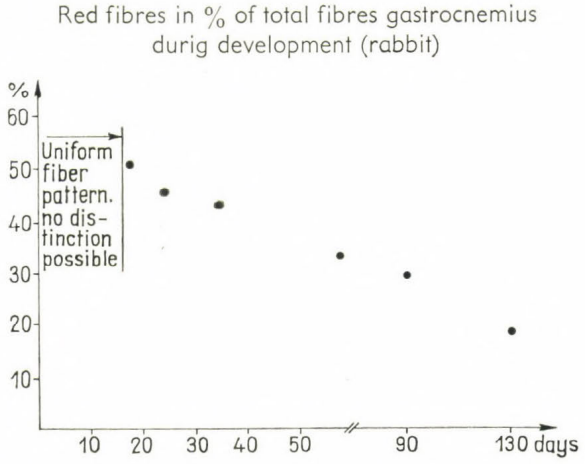

Fig. 4. Red fibres in per cent of total fibres in rabbit gastrocnemius during development. Distinction was made on the basis of SDH-reaction

It seems that the metabolic differentiation is dependent on and must be preceeded by structural and functional differentiation of the contractile apparatus.

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Symp. Biol. Hung. 17, p. 143 (1974)

HEART MYOSIN ATPase AND LIGHT SUBUNITS (A COMPARATIVE STUDY)

(ABSTRACT)

by

C. Delcayre and B. Swynghedauw

INSERM-U 127 GROUPE DE RECHERCHES SUR LE MÉTABOLISME ET DES VAISSEAUX, PARIS, FRANCE

Heart myosin ATPase (10 mM $CaCl_2$–0.60 M KCl pH 7.6) was found to be twice to three times higher in rats (423 nmoles/min/mg) than in guinea pigs (268) or in dogs (139). It correlates with the maximum velocity of shortening at zero load of the myocardial muscle, as determined by other authors.

These cardiac muscle myosins have the same two light subunits (M.W.: 18,000 and 27,000) in SDS polyacrylamide gel electrophoresis; one of them (M.W.: 18,000) exists in guinea pig, dog and rabbit, as two different molecules having a different charge, as shown in urea electrophoresis. Therefore, the rat heart myosin apparently does not possess the phosphorylated light subunit described by Perry in rabbit heart or skeletal myosin.

This work suggests that the phosphorylation of myosin light subunit correlates with the physiological properties of the muscle.

Symp. Biol. Hung. 17, p. 145 (1974)

STUDIES ON HUMAN HEART MYOSIN

(ABSTRACT)

by

C. KLOTZ, J. J. LEGER and B. SWYNGHEDAUW

INSERM-U 127 GROUPE DE RECHERCHES SUR LE MÉTABOLISME DU COEUR ET DES VAISSEAUX, PARIS, FRANCE

Human heart myosin was isolated less than 36 h after death. The cardiac myosin was slightly modified by post-mortem proteolysis since myofibrillar ATPase was unchanged 24–48 h after death and it was identical in human biopsies taken during open-heart surgery and in samples from autopsy. Km and Vm of myosin ATPase (10 mM $CaCl_2$–0.60 M KCl pH 7.1) were identical in human, pig and rabbit heart myosins. The Km of skeletal myosin was the same as that of heart myosin but Vm was higher. The percentage of light and heavy subunits, studied in SDS electrophoresis, was about the same in the different hearts. Light subunits of human heart myosin were isolated by electrophoresis in the presence of urea. There were usually two, sometimes three light subunits from human heart myosin with M.W., amino acid composition, UV spectra and per cent of helicity very different from those of pig heart. Myofibrillar ATPase was studied in 70 hearts and correlates with the degree of heart hypertrophy ($r = 0.33 - P < 0.02$).

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AFFINITY CHROMATOGRAPHY OF ACTIN, MYOSIN AND MYOSIN FRAGMENTS BY IMMOBILIZED ACTIN, MYOSIN AND NUCLEOTIDES

by

A. Oplatka, A. Muhlrad and R. Lamed

DEPARTMENTS OF POLYMER RESEARCH AND BIOPHYSICS,
THE WEIZMANN INSTITUTE OF SCIENCE, REHOVOT, ISRAEL

The common methods for the separation and isolation of the protein components of biological motile organelles such as muscle are generally based on differences in size, shape and electrical charge (e.g. ultracentrifugation, electrophoresis) or solubility (in a liquid medium or in a gel matrix) of the protein molecules. These differences obviously involve the whole molecule. There are, however, cases in which two (or more) protein species have practically the same size, shape and charge but differ with respect to a relatively small site which plays a significant physiological role e.g. myosins of red and white skeletal muscles of the rabbit. Needless to say that quantitative physico-chemical studies of myosin should be carried out with preparations which are uniform with respect to ATPase activity. This evidently means also that inactive molecules should not be present. In order to separate myosin molecules from different ATPase sites we must have at our disposal a technique which is sensitive to such differences i.e., which involves *specific* chemical interaction of the sites with a proper reagent. If the latter is immobilized to a column then differences in the affinity of myosin binding to the reagent and/or to its decomposition products may be detected upon eluting the myosin preparation.

In order to achieve this and other similar goals, we have immobilized F-actin [and also heavy meromyosin (HMM) and HMM subfragment-1 (S-1)] and ATP (several types of columns) as well as other nucleotides such as ADP and AMP-PNP to Sepharose (agarose) and other carriers. The 'acetone dried powder' obtained from muscle after the extraction of myosin and used for the preparation of actin, proved to be most suitable as a kind of 'immobilized F-actin'. Myosin and its fragments have had affinity chromatographed on actin and on nucleotide columns, while the bound myosin species were interacted with G- and F-actin. The procedures for the preparation of these columns have been described in several articles (Lamed et al., 1973; Lamed and Oplatka, 1974; Gadasi et al., 1974; Muhlrad et al., 1975; Lamed et al., 1975 and in papers submitted for publications).

The adsorbability to Sepharose-ATP of S-1 kept at 25 °C in a batchwise experiment in the presence of EDTA decreased with time at the same rate as the ATPase activity. In the case of heat-denatured HMM, the per cent decrease in adsorbability was smaller than the corresponding fall in ATPase activity and the eluent concentration required for desorption higher than for S-1. The conclusions derived were that (*i*) immobilized ATP can distinguish between active and heat-inactivated myosin heads and would not adsorb the latter; (*ii*) both active heads can bind ATP. Since it has been

claimed that HMM binds up to two ATP molecules per molecule it appears that each of the heads can bind one ATP molecule. We can thus eliminate the possibility that only one of the myosin heads can form a complex with ATP; (*iii*) the affinity of binding of HMM is stronger than that of S-1, since it can bind at two points (provided the density of available ATP groups is sufficiently high) thus enabling the immobilized ATP to distinguish also on the basis of the number of binding sites.

In column experiments, 'dead' and 'living' S-1 come out as two separate peaks (the first appearing in the void volume) using KCl, ATP or pyrophosphate gradients or by a stepwise application of these eluents, thus enabling the removal of inactive material (including other proteins) and the preparation of fully active S-1 (or HMM). Moreover, a partial resolution of a mixture of HMM and S-1 into two peaks could be achieved, the HMM appearing later. By collecting the protein from the ascending part of the double peak, obtained by a passing myosin preparation in which part of the heads have been chopped off by papain, we could obtain a pure preparation of one-headed myosin. The affinity chromatography of rabbit skeletal myosin and of myosin derivatives in which the light meromyosin rod is intact, requires a high ionic strength medium in which myosin is molecularly dispersed and, in addition, the presence of Ca^{2+} or Mg^{2+}. The use of bound ATP columns for the preparation of purified myosin species became a routine procedure in our laboratory since 1972.

Our next step was to examine whether the ATP and the other nucleotide columns can be sensitive to changes induced by chemical reagents. As is well known, chemical modifications have been extensively used in the study of the nature of the active site of myosin. Separation of molecules in which the active site was affected from those in which the reagent attacked other regions in the molecule as well as from intact molecules could be helpful in kinetic studies of the reaction, in particular for the elucidation of the number of functional groups affected in the active site and of the various kinetic constants associated with separate functional groups. Indeed, after partially trinitrophenylating (TNP-ating) myosin and HMM, we could isolate that part of the preparation in which the active sites have been modified. Analysis of the elution pattern (with respect to protein concentration and K^+- and Mg^{2+}-activated ATPase activities) at various times, in combination with a spectroscopic determination of the bound TNP groups, led to the conclusion (in accordance with a parallel study in solution) that the introduction of two TNP groups per myosin (or HMM) and one per S-1 is responsible for the marked changes in the ATPase activities. Using an ATP column we could follow the formation of mono- and di-TNP-ated myosin which adsorb differently on the column and to isolate the former which can still exhibit superprecipitation despite the fact that the Mg^{2+}-ATPase of its modified head is not activated any more by actin. Acetone powder columns could also sense a difference between native and TNP-ated S-1: the shapes of the elution peaks were quite different, as were the concentrations of Mg-pyrophosphate required for elution. A mixture of the two protein species could thus be partially separated. Using an ATP column we could also separate a mixture of skeletal and heart S-1. The best eluent was K-acetate. Myosin from rabbit uterus (smooth muscle)

was eluted from Sepharose-ATP at a much lower KCl concentration than skeletal muscle myosin. Our columns thus appear to be quite promising as a tool for the separation and purification of myosins from mixed muscles (red and white, smooth and skeletal) as well as of different isoenzymes from the same muscle. This would be especially important in developmental studies of both muscle and non-muscle cells.

It was intriguing to examine whether nucleotide and/or actin columns could sense any difference between the twin heads of a given myosin. The experiments carried out so far, using different eluents gave a single, quite symmetrical and homogeneous (with respect to ATPase activity) peak for S-1. This was observed also under conditions in which the bound ATP was split (i.e. in the presence of Ca^{2+} or Mg^{2+}). The latter experiments indicate that both myosin heads are capable of splitting ATP, apparently with the same activity.

HMM S-1 and its TNP-ated derivative have been chromatographed on immobilized ATP, ADP and AMP-PNP columns, in the presence and in the absence of Ca^{2+} or Mg^{2+}. Splitting of bound ATP was followed by using γ^{32}-P ATP columns. While the divalent cations had little effect on the chromatographic patterns in the case of the nonhydrolyzable ADP and AMP-PNP, they catalyzed splitting in the case of ATP and, at the same time, strongly increased the affinity of adsorption of the proteins. The protein-elution and the phosphate-release patterns were strongly correlated. It appears that, under conditions in which the bound substrate is split, the enzyme can bind also to the decomposition products of ATP. In other words, in what we call 'dynamic affinity chromatography' columns, the affinity of binding of an enzyme to an immobilized substrate under conditions favourable for the enzymic reaction is probably determined by some kind of a Michaelis–Menten constant rather than by the value of the equilibrium association constant to the intact substrate. Parallel studies of myosin-elution and phosphate-release patterns might prove useful for the elucidation of the detailed mechanism of ATP breakdown by myosin and its fragments.

The Ca^{2+}-activated ATPase activity characteristics of immobilized myosin, HMM and S-1 were not usually much different from those of the corresponding unbound species. However, changes could be observed in the pH profile and in the ionic strength dependence of the Ca^{2+}-ATPase. Immobilized myosin was capable of adsorbing G-actin; however, only about half of the actin could be desorbed. Very little F-actin was absorbed, probably due to the inability of the long-chain protein to penetrate the gel matrix. We failed in our attempts to restore the ATPase activity of the immobilized heavy core of myosin (obtained by LiBr or LiCl extraction of the light chains from Sepharose-bound myosin) by the readdition of the light chains. The fact that the added protein was nevertheless strongly adsorbed suggests the possibility of utilizing myosin columns from which the light chains have been removed for the isolation of the latter in studies of their biosynthesis and assembly.

Nucleotide and actin columns on the one hand, and myosin columns on the other hand, are now being tested for their capability to isolate and to concentrate myosin and actin, respectively, from various non-muscle cells where these proteins are much less abundant than in muscle. In order

to obtain the highest possible yields we prefer to chromatograph cell homogenates in the proper medium.

Columns of the type described may have other applications such as in the study of the stoichiometry and energetics of binding to each other of mechanochemical proteins (Oplatka, 1972; Kondo et al., 1972), the ATPase activity, actin-binding capacity etc. of myosin at a low ionic strength the aggregation of which may be prevented by immobilization (Oplatka, 1972); the interaction of myosin with bound G-actin which cannot polymerize (Oplatka, 1972).

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Symp. Biol. Hung. 17, p. 151 (1974)

LIGHT CHAINS OF MYOSIN IN RELATION TO MUSCLE FUNCTION

(ABSTRACT)

by

I. Syrovy

INSTITUTE OF PHYSIOLOGY, CZECHOSLOVAK ACADEMY OF SCIENCES
PRAGUE, CZECHOSLOVAKIA

The relationship between myosin ATPase activity and the electrophoretic pattern of the light chains of myosin was studied during development and after denervation. It has been shown that changes in myosin ATPase activity sometimes reflect the electrophoretic pattern of the light chains of myosin, in addition two myosins with different ATPase activities and the same electrophoretic pattern may exist.

For example, in denervated rabbit soleus muscle, the myosin ATPase activity increases and the electrophoretic pattern of the light chains of myosin becomes similar to that of myosin of fast muscle. On the other hand, in heart muscle the ATPase activity of myosin decreases during development without detectable changes in the pattern of the light chains of myosin.

The physiological significance of these results and the limitations of the study of light chains of myosin are still being discussed.

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THE INFLUENCE OF MOTOR NERVE ON THE COMPOSITION OF MUSCLE PROTEINS

by

Ö. TAKÁCS, MAGDA G. MÉSZÁROS and F. GUBA

DEPARTMENT OF BIOCHEMISTRY, MEDICAL UNIVERSITY, SZEGED, HUNGARY

The present investigation was udertaken to study the changes of the proteins of muscle after denervation, in special relation to its relative quantity, moreover, to study the submolecular organisation of structura proteins.

METHODS

Adult rabbits were used in our experiments. The animals were killed 2–6–8 weeks after denervation. The preparative procedure was different being adapted to the purpose of experiment. Specimens were processed or electron microscopy by conventional methods.

1. The preparation of cellular fractions was made according to Pollack and Bird (1968). After differential centrifugation a supernatant with the soluble proteins (U) and the 'nuclear', mitochondrial and lysosomal fractions were obtained.
2. To prepare myofibrils we used the method of Fukazawa et al. (1970), or alternatively our method elaborated later to observe the *in vivo* situations.
3. The sarcoplasmic proteins were extracted with isoosmotic glycerol solution according to Scopes (1968). The soluble proteins obtained were dialysed and liophylized. Aliquots of the dry material were dissolved in Tris buffer pH 7.2 or solubilized in 1% SDS-Tris buffer, to determine the molecular weight and the ratio of the components.

The electrophoresis of solubilized proteins was carried out according to Weber and Osborn (1969), or alternatively with the method of Talbot (1971), using 7% acrylamide gels.

RESULTS

The total protein content of normal muscle was 195 mg/g wet weight of muscle tissue on the average.

Following denervation the protein content decreased. After 8 weeks about 120 mg/g wet weight was measured.

The changes in the cellular fractions are demonstrated in Fig. 1. In muscles, six weeks after denervation the relative content of the nuclear fraction increased from 61% to 68%, while the mitochondrial and the

lysosomal fractions increased of about 125% and 100%, respectively (Fig. 1*a*). The soluble protein fraction decreased remarkably relative to the normal.

The proteolytic enzyme activity measured in the same fractions showed an absolute increase. The change was especially characteristic in the activity of the soluble protein fraction (Fig. 1*b*).

The protein patterns of the solubilized cell fractions are seen in Fig.

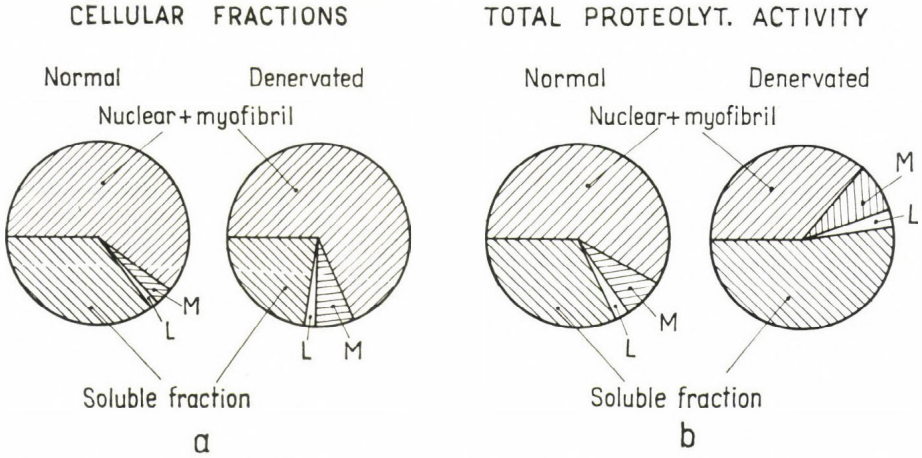


Fig. 1. Relative distribution of the cellular fractions *a* and that of the total proteolytic activity *b* of the normal and denervated muscle. Legends: see in text

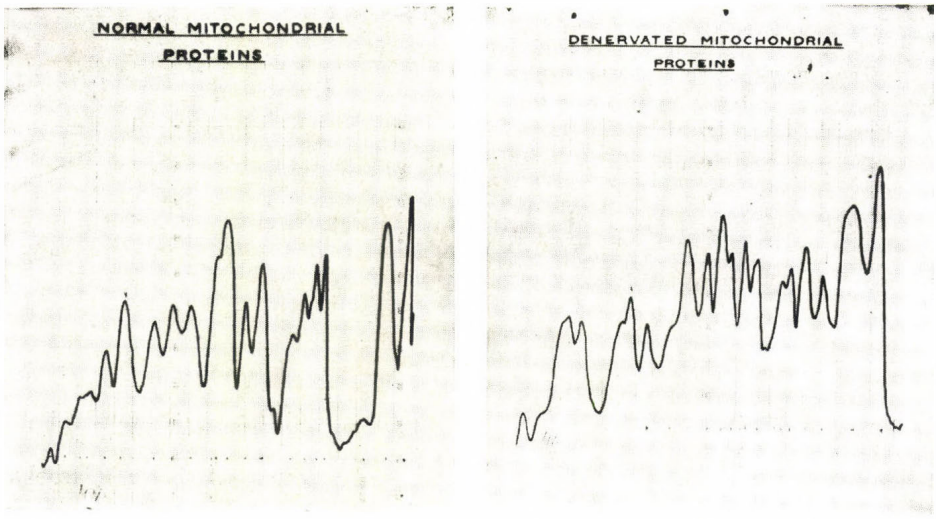
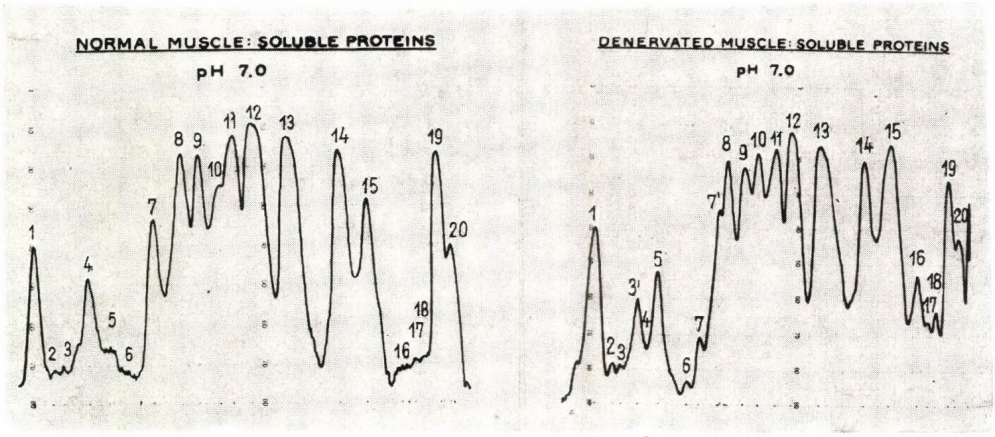


Fig. 2. Densitometric scan of the normal *a* and denervated *b* mitochondrial proteins. Methods: according to Talbot and Yphantis (1971)



a *b*

Fig. 3. Densitometric scan of the normal *a* and denervated *b* sarcoplasmic proteins. Methods: according to Talbot and Yphantis (1971)

2*a* and *b*. The normal mitochondrial protein profile seems to be composed of 20 proteins with molecular weights ranging from 10,000 to 150,000 daltons. The identification of components on electrophoretograms is difficult because under the conditions of solubilization the native proteins are decomposed into single polypeptides. One can note the abundance of some components corresponding to about 130,000, 50,000 and 29,000 daltons, respectively. The 60% of the components originated from the organelle. The matrix is characterized by the 127,000 dalton components. A broad bend of low molecular weight LMW component represents the 40% of the intermembrane space material. The outer membrane consists mainly of 49,000 dalton components. The inner membrane is composed chiefly of the 29,000 dalton protein.

Denervation leads to a very striking alteration in the relative quantity of the fastest component corresponding to the intermembrane space material, and that of the 29,000 dalton component (inner membrane).

Changes are also found in the relative quantity of the 49,000 dalton component. The electrophoretic pattern of lysosomal proteins consists of 15 components. After denervation the relative amount of the main components was altered. Changes are demonstrated in the fastest and middle components.

The changes in the relative distribution of the sarcoplasmic proteins are demonstrated in Fig. 3. It is evident from the results that denervation leads to a change in the relative quantity of some sarcoplasmic proteins. Table I lists the identified proteins numbered in order of decreasing mobility on SDS gels. Figure 4 shows the band pattern of normal myofibrillar proteins and Table II summarizes the results of the proteinograms obtained from the solubilized structural proteins. The main components, myosin heavy chains and actin are present in constant relative quantities. Significant alterations are observed in the relative quantities of the myosin light chains and that of the troponin subunits.

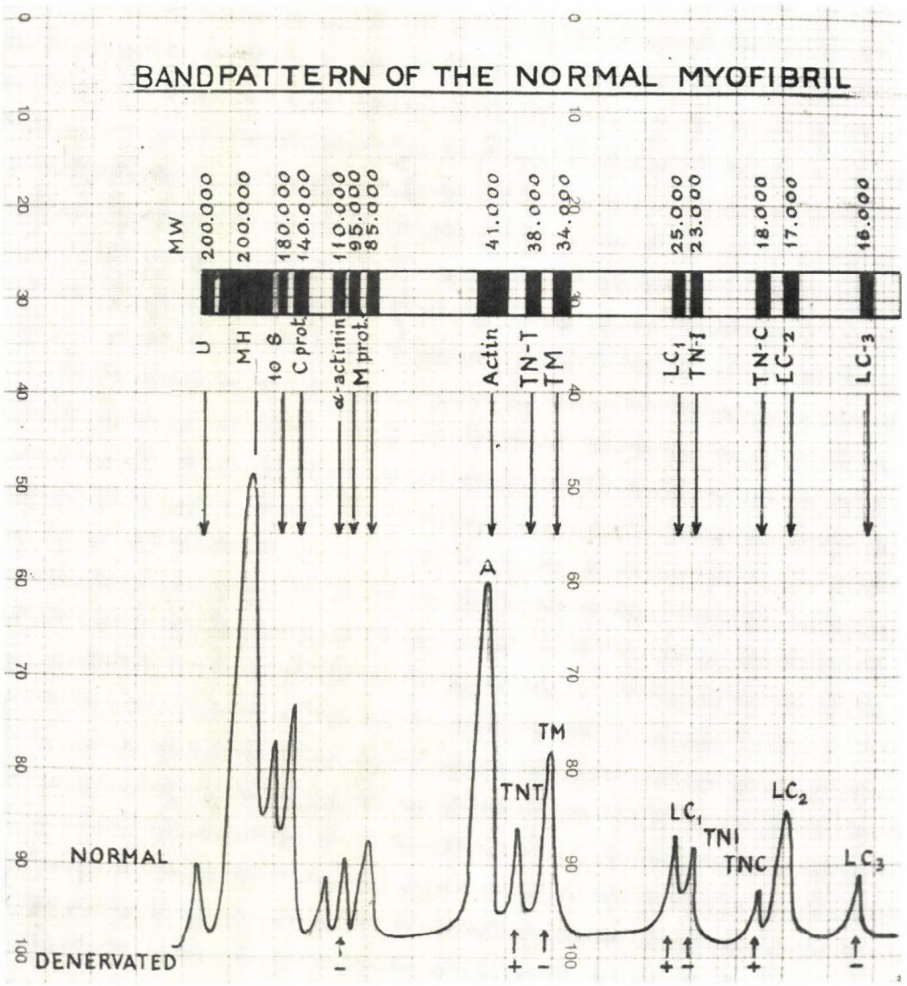
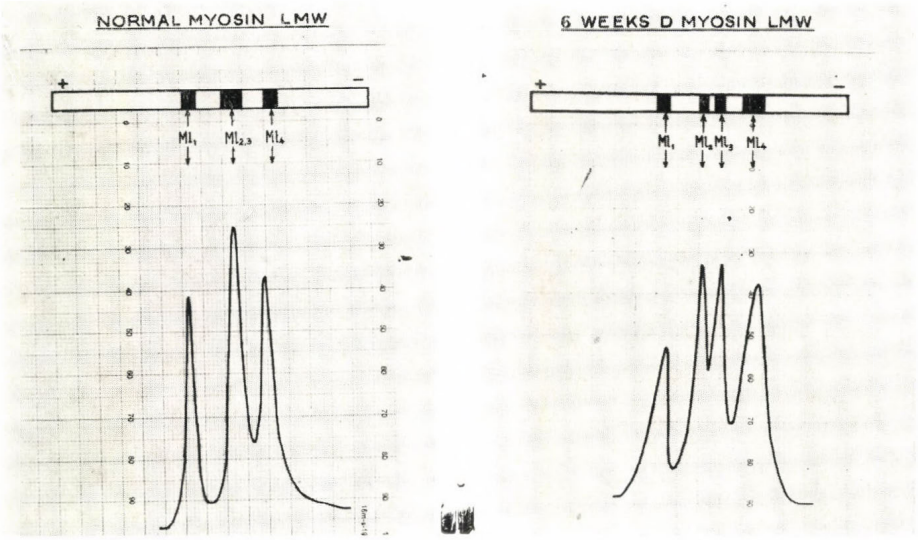


Fig. 4. Band pattern and scan of the normal myofibrillar proteins. Changes in the relative proportions of the denervated protein fractions are indicated by arrows. (+ or −)

To analyse this problem, we isolated the myosin light chains according to Perrie et al. (1973). The electrophoretic separation was performed in the presence of 8 M urea in Tris-HCl buffer (pH 8.3). Figure 5 demonstrates the results, while Table III shows the relative quantities of components calculated as the mean of several measurements.

Substantial changes are observed in the light chains of myosin obtained from denervated muscle. It is remarkable, that the quantity of LC_3 decreases and at the same time the LC_2 component appears in double band pattern. We have measured the phosphate content of the DTNB components in the myosin of normal and denervated muscle. This value accounts for about one mole phosphate per mole of normal DTNB light chain and



a *b*

Fig. 5. Band pattern and scan of the normal *a* and denervated *b* myosin LMW preparates. Methods: according to Weber and Osborn (1969)

Table I

No.	Protein	Mw×10³	No. of Su	Mw×10³ Su	Trend of change
1	Myoglobin	18.0	1	18.0	+
2	Myokinase	21.5	1	21.5	+
3	?				—
4	Triosephosphate isomerase	54.0	2	27.0	0
5	'F' protein	30.5	1	30.5	+
6	Phosphoglycerate mutase	66.0	2	33.0	0
7	α-Glycerol phosphate dehydrogenase	67.0	2	33.5	—
8	Lactate dehydrogenase	140.0	4	34.5	—
9	Glyceraldehyde-3 phosphate dehydrogenase	144.0	4	36.0	—
10	Aldolase	160.0	4	40.0	+
11	Creatine kinase	82.0	2	41.0	0
12	Enolase	83.0	2	41.5	—
13	Phosphoglycerate kinase	48.5	1	48.5	0
14	Phosphoglucose isomerase	108.0	2	54.0	—
15	Pyruvate kinase	228.0	4	57.0	+
16	AMD-deaminase	270.0	4	67.0	+
17	Phosphofructose kinase	295.0	4	74.0	0
18	Phosphorylase B kinase	1000.0	12	80.0	+
19	Phosphorylase B	185.0	2	94.0	—
20	?				—

Table II

Distribution of the structural proteins

No.	Protein	MW	Normal rel.%	Denervated rel.%	Trend of change
1.	Myosin LC_3	15,500	2.00	0.9	—
2.	Myosin LC_2	17,000	8.12	8.60	0
3.	Troponin-C	18,000	3.22	6.00	+
4.	Troponin-I	23,000	5.14	3.80	—
5.	Myosin LC_1	25,000	4.37	5.00	+
6.	Tropomyosin	34,000	10.55	5.00	—
7.	Troponin-T	38,000	5.69	7.80	+
8.	Actin	41,000	13.27	12.30	0
9.	β-Actinin	85,000	5.10	6.00	0
10.	M Protein	95,000	3.76	6.00	+
11.	α-Actinin	110,000	3.23	2.40	0
12.	C Protein	140,000	6.50	4.20	—
13.	10 S Actinin	180,000	6.30	8.00	+
14.	Myosin-H	200,000	20.40	20.00	0
15.	Unknown	200,000	2.85	3.90	+

Table III

The relative amount of LMW components of purified myosin

Light chains	LC_1	LC_2	LC_3
	Ml_4	Ml_3 Ml_2	Ml_1
	MW: 25,000	MW: 18,600	MW: 15,000
Normal	38–39%	43–44%	20–21%
Denervated	40–44%	23% 21%	12–16%

about 0.5 mole phosphate per mole of denervated DTNB light chain. According to Perry the DTNB light chain appears as a single or double band pattern in 8 mol urea gels. The double band pattern (Ml_2–Ml_3 form) corresponds to the dephosphorilated DTNB light chain while the former (Ml_2 form) represents the phosphorylated chain.

Our findings are in accord with Perry's experiments. After denervation the dephosphorylated DTNB light chain exhibits the Ml_2–Ml_3 band pattern in urea gels and contains less phosphate.

Summing up the results it seems reasonable to assume that these changes are presumably due to the suspension of the trophic and motoric functions of the nerve, suggesting that denervation leads to the dedifferentiation of skeletal muscle.

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ATPase ACTIVITY AND SUBUNITS OF MYOSIN IN THE MYOCARDIUM OF RATS CONDITIONED BY SWIMMING

by

I. MEDUGORAC

INSTITUTE OF PHYSIOLOGY II., TÜBINGEN, FRG

Chronic haemodynamic overload of the heart by physical training results in the enlargement of this organ, which according to investigations of our work-team (Hepp et al., 1974) is accompanied by a moderate increase of the cross-sectionally related, myocardial contractile capability.

In opinion of many authors, this process of heart hypertrophy, caused by physical training, involves significant changes of the ATPase activity of contractile proteins (Wilkerson and Evonuk, 1971; Hearn and Gollnick, 1961, Bhan and Scheuer, 1969).

There appeared to be a correlation between the contractile capability of the muscle and the specific ATPase activity of the contractile proteins (Seagren et al., 1971; Chandler et al., 1972). In response to work overload of the organism-specific myosin ATPase activity increased (as detected in the above-mentioned investigations) whereas during muscular dystrophy (Lobley and Perry, 1972) and heart failure (Luchi et al., 1969; Chandler et al., 1972) this activity is decreased.

The mechanism, changing the enzymatic activity of myosin, and the reciprocal changes in the myosin molecule, enabling this dynamic of the ATPase activity and force-velocity relation, are of considerable interest but still unknown.

The aim of the present investigations was to determine to what degree the activity of the ATPase of rats ventricular myocardium is influenced by a training period of several weeks. Furthermore, eventual structural alterations in the myosin molecule as a possible basis of the changed enzymatic activity are of particular interest.

Several groups of young Sprague-Dawley rats underwent daily swimming training. After 8–12 weeks, i.e. after a total training period of 100–120 h, the trained rats (SH) showed an 12% average increase in heart weight as compared to controls (CH) of the same age.

Fresh ventricular tissue was obtained from rats anesthetized with pentobarbital.

Actomyosin was isolated using three vol. of Weber–Edsall solution by extraction of 24 h at 4 °C and purified by repeated precipitation.

Myosin was obtained from actomyosin according to Weber (Weber, 1956). The degree of myosin purity was tested in SDS-acrylamid-gel electrophoressis (Fig. 1). Actomyosin and myosin ATPase activity was studied at room temperature in the presence of various Ca^{++} and K^{+} concentrations, 2mM ATP, 20 mM TRIS/HCl buffer pH 7.5 and 0.2–0.4 ml (0.4–0.8 mg) protein solutions.

Differences in collagen concentration between SH and CH were not detected on the basis of hydroxyproline determination.

In all enzymatic preparations the ATPase activity significantly increased after swimming training, especially in case of different Ca^{++} and K^{+}-concentrations (Table I).

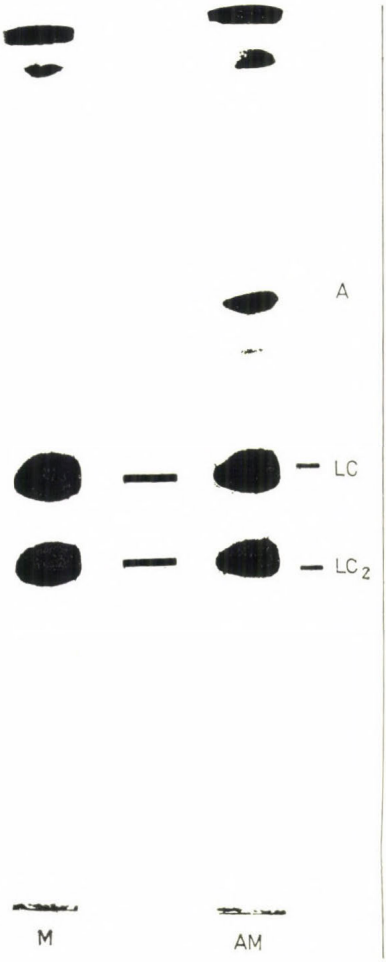

Fig. 1. Comparison of sodium dedecylsulfate polyacrylamid gel (10%) of cardiac actomyosin (AM) and myosin (M) from rats in 8 M urea (according to Weber and Osborn, 1969). AM=actomyosin; M=myosin; A=actin; LC_1 and LC_2=light chains. The preparation of myosin contains only traces of actin

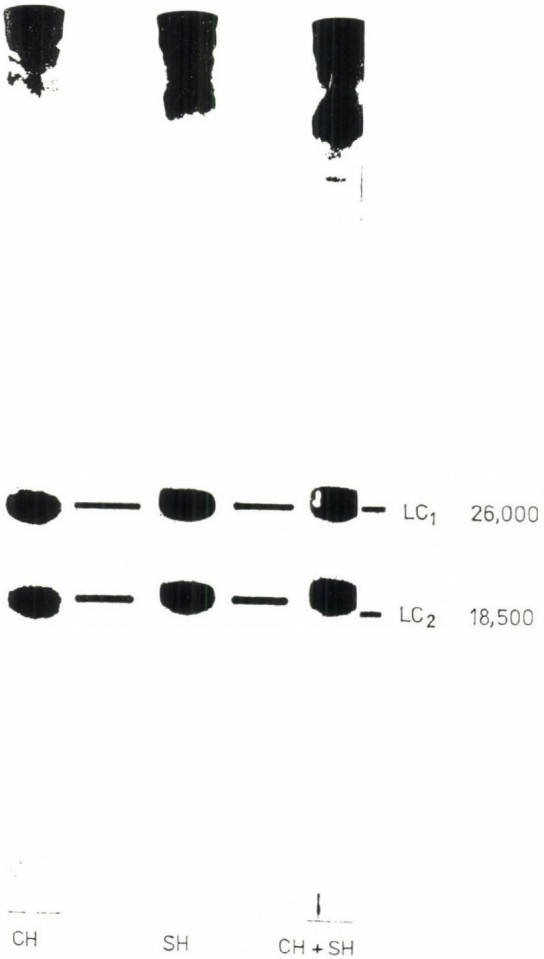

Fig. 2. SDS-gel electrophoresis of cardiac myosin (in 8 M urea) from each group: trained (SH) and control (CH) rats as well as from a myosin mixture in the ratio 1 : 1 (SH + CH). The pattern of both LC from SH and CH coincided completely in the myosin-mixture. No differences occur in molecular weights of light chains of SH and CH

Table I

Comparison of the myosin and actomyosin Ca^{++}-ATPase activity of SH and CH ($\bar{x} \pm s_{\bar{x}}$)

KCl (mM)	Myosin (6)					
	SH (24) CH (20) SH/CH			SH (24) CH (20) SH/CH		
	$CaCl_2$ concentration (mM)					
	10			20		
	Myosin and actomyosin ATPase activity Pi (mμ mol/mg protein/min)					
30	360 ±39	228 ±21	1.57/1	312 ±18	232 ±22	1.34/1
90	404 ±09	315 ±35	1.28/1	320 ±13	264 ±29	1.21/1
360	296 ±20	232 ±21	1.28/1	272 ±23	240 ±22	1.13/1

KCl (mM)	Actomyosin (7)					
	SH (28) CH (24) SH/CH			SH (28) CH (24) SH/CH		
	$CaCl_2$ concentration (mM)					
	10			20		
	Myosin and actomyosin ATPase activity Pi (mμ mol/mg protein/min)					
30	406 ±25	234 ±29	1.73/1	416 ±17	256 ±26	1.62/1
90	504 ±20	328 ±39	1.54/1	552 ±14	336 ±34	1.64/1
360	400 ±29	272 ±30	1.47/1	400 ±30	277 ±28	1.47/1

ATPase activity measured at 25 °C, 5 min.
Standard conditions: Tris-HCl pH 7.5, 20 mM; ATP 2 mM; myosin and actomyosin 0.2–0.8 mg in final volume of 2 ml.
Number of separate actomyosin and myosin preparations examined and number of SH and CH are in parentheses.
SH = Hearts of rats conditioned by swimming
CH = Hearts of control rats

The work of Barany (Barany, 1967) suggests a significant correlation between maximum shortening velocity of the muscle (V_{max}) and Ca-ATPase activity.

In our investigations on swimming rats the length tension diagram of the left ventricular muscle strips showed an increase in the isovolumetrically developed tension as compared to the controls of the same size.

Although it seems that the activity of the myosin Ca-ATPase *in vitro* corresponds with the myosin ATPase activity causing contraction *in vivo*, we merely suppose a relation between our biochemical and mechanical findings.

These findings showed, however, that the specific ATPase activity of the ventricular myocardium is influenced by physical training. Because the purified myosin of SH exhibits changes in specific activity as compared to CH, one is compelled to look for the cause in the structure of the myosin molecule itself.

Because of the ATPase activity of myosin, the interaction between light chains (LC) and heavy chains (HC) is indispensable (Dreizen and Gershman, 1970; Gershman and Dreizen, 1970; Frederiksen and Holtzer, 1968; Gaetjens et al., 1968; Stracher, 1969). The decisive significance of

the specific activity is due to the light chains (Kim and Mommaerts, 1971). Hence, a change in enzyme activity might be due to possible alterations in these light components.

Using SDS-acrylamid-gel electrophoresis we found that both trained and untrained animals possessed two LC with a mol. wt from 26,000 (LC_1) and 18,000 (LC_2). On the basis of the evaluation of many gels differences in relation to the molecular weight of the LC of myosin of CH and of SH could be excluded.

This agreement can be demonstrated again very easily by separating light chains electrophoretically from both groups (SH and CH) and then as a mixture in the ratio 1 : 1 in acrylamid gels. The fact that the bands of LC_1 and LC_2 of CH + SH myosin are completely congruent in gel with mixed myosin, excludes any difference in molecular weights between LC in CH and SH (Fig. 2).

However, on the basis of the densitometry of 30 identical gels of each group, we could observe, that the relation of LC_1 and LC_2 has changed in the hypertrophied heart of trained animals. We found that the relative amount of the electrophoretically slower component (LC_1) with a mol. wt of 26,000 was significantly higher in myosin of the trained animals than in the myosin of control rats. Whereas the ratio of LC_1 : LC_2 in control animals is 124 : 100, a ratio of 146 : 100 is found in SH (Fig. 3).

These changes in the relation of the light chains to each other are possibly related to the increase in the ATPase activity of SH.

The myosin molecule is a dynamic protein. As shown in several studies it is neither synthetised nor degraded as a functional unit. Single subunits (both HC and LC) are built up and degraded separately and at different rates (Sarkar and Cooke, 1970; Morkin et al., 1973; Brivio and Florini, 1971; Wikman-Coffelt et al., 1973).

This dynamics of synthesis, the degradation of the molecule and the different turnover rate of the single subunits may be an important mecha-

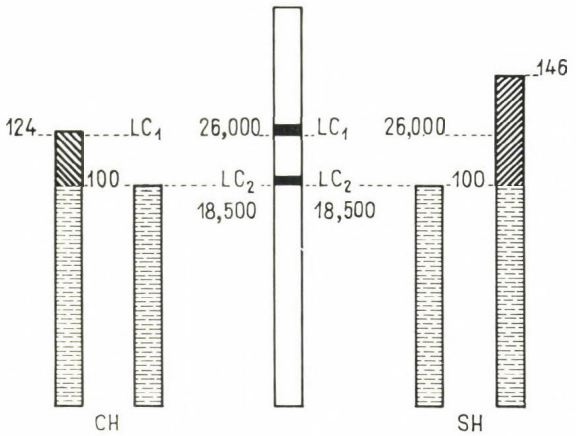

Fig. 3. Average shift in the relations LC_1 and LC_2 in SH (146 : 100) in comparison with CH (124 : 100). 30 gels of both control and trained rats were evaluated

nism which regulates the enzyme activity. As the synthesis of myosin increases considerably as a reaction to the enhanced muscular activity (Morkin et al., 1972), it becomes clear that during increased haemodynamic load, there might be a displacement in the relative portion of single subunits. Whether these shifts in the relations between the single chains are responsible for the dynamics of the ATPase activity, remains open to discussion.

In any case, our findings suggest that the increased Ca-ATPase activity, due to physical training, is accompanied by structural changes in the myosin molecule.

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