SYMPOSIUM ON MUSCLE

Symposia Biologica Hungarica 8

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Akadémiai Kiadó, Budapest

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SYMPOSIA BIOLOGICA HUNGARICA 8

Edited by

E. ERNST and F. B. STRAUB

This book contains scientific material discussed during the Symposium, i.e. introductory lectures of about one hour each, and topics discussed during all of the remaining time.

The introductory lectures give a general survey of the present situation in muscle research within various special fields, current issues and recent experimental methods. The general discussions following the introductory lectures contain, besides the main topics of debate, several partial results claimed informally by the contributors. The partial results, in spite of their diverging character, have helped in the elucidation of fundamental questions (such as the submicroscopic structure of muscle, the functional role of muscle proteins and of the inorganic substance of muscle as well as the energetics of muscle functioning).

Owing to its wide scope, the book is of interest in the field of biochemistry, biophysics, biology, physiology, and of experimental medical science.

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BY

E. ERNST

Member of the Hungarian Academy of Sciences

F. B. STRAUB

Member of the Hungarian Academy of Sciences

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CONTENTS

LIST OF PARTICIPANTS

AUBERT, X. M.	Laboratoire de Physiologie générale, 6. Dekenstraat, Leuven, Belgium
BÁLINT, M.	Biochemical Institute of Eötvös L. University, Budapest VIII, Múzeum krt 4, Hungary
BANGHA, I.	Institute of Pathological Anatomy and Cancer Research, Budapest VIII, Üllői út 26, Hungary
BELÁGYI, J.	Biophysical Institute, University Medical School, Pécs, Rákóczi út 80, Hungary
BENDALL, J. R.	Low-Temperature Research Station, Cambridge, Downing St., England
BENSON, E. S.	University of Minnesota Minneapolis, Box 198 Mayo, Minneapolis, USA
BIRÓ, J.	Urological Clinic of University Medical School, Budapest VIII, Üllői út 78/b, Hungary
BIRÓ, N. A.	Biochemical Institute of Eötvös L. University, Budapest VIII, Múzeum krt 4, Hungary
BOWEN, W. J.	National Institutes of Health Bethesda, Md, USA
BRINLEY, F.	Johns Hopkins University, 725 N. Wolfe St., Baltimore, Md, USA
COUTEAUX, R.	Laboratoire de Biologie Animale, Faculté des Sciences, 12 Rue Cuvier, Paris 5, France
CSILLIK, B.	Institute of Anatomy, University Medical School, Szeged, Hungary
DAVIES, R. E.	University of Pennsylvania, School of Veterinary Medicine, Department of Animal Biology, Philadelphia 4, Pennsylvania, USA
DELLUVA, A. M.	University of Pennsylvania, Inst. of Biochemistry, USA
DRABIKOWSKI, W.	Department of Biochemistry, Nencki Institute of Experimental Biology, Pasteur St. 3, Warsaw 22, Poland
DYDYNSKA, M.	Nencki Institute of Experimental Biology, Pasteur St. 3, Warsaw 22, Poland
EBASHI, S.	Department of Pharmacology, University of Tokyo, Tokyo, Japan
EDMAN, K. A. P.	Department of Pharmacology, University of Lund, Lund, Sweden

Ernst, E.	Biophysical Institute, University Medical School, Pécs, Rákóczi út 80, Hungary
Fazekas, A.	Biochemical Institute, University Medical School, Budapest VIII, Puskin u. 9, Hungary
Garamvölgyi, N.	Biophysical Institute, University Medical School, Pécs, Rákóczi út 80, Hungary
Gárdos, G.	Central Research Laboratory of National Blood Transfusion Service, Budapest XI, Daróczi út 24, Hungary
Gerendás, M.	Central Research Laboratory of National Blood Transfusion Service, Budapest XI, Daróczi út 24, Hungary
Gergely, J.	Retina Foundation, Institute of Biological and Medical Sciences, 20 Staniford St., Boston, Mass., USA
Giles, B. G.	Unilever Research Laboratory, Colworth House, Sharnbrook, Bedford, England
Gladner, J. A.	National Institutes of Health, PHS, DHEW NIH Bldg 4, D-26, Bethesda, Md, USA
Guba, F.	Institute of Chemical Structure Research, Budapest VIII, Puskin u. 11, Hungary
Gutmann, E.	Institute of Physiology, Czechoslovak Academy of Sciences, Prague 4, Budejovicka 1032, Czechoslovakia
Hanson, J.	King's College, University of London, 26 Drury Lane, London W. C. 2, England
Hardwicke, P.	King's College, University of London, 26 Drury Lane, London W. C. 2, England
Harsányi, V.	Institute of Chemical Structure Research, Budapest VIII, Puskin u. 11, Hungary
Hasselbach, W.	Max Planck Institute, 69 Heidelberg, Jahnstrasse 29, GFR
Hoyle, G.	Biology Department, University of Oregon, Eugene, Oregon, USA
Huxley, H. E.	Medical Research Council, Laboratory of Molecular Biology, Hills Road, Cambridge, England
Ivanov, I. I.	Department of Biochemistry, Military Medical Academy, Leningrad D-187, Liteiny prosp. 9, Flat 34, USSR
Johnson, W. H.	Department of Biology, Rensellaer Polytechnic Institute, Troy, New York, USA
Jöbsis, F. F.	Department of Physiology and Pharmacology, Duke University, Medical Center, Durham, North Carolina, USA
Kakol, I.	Nencki Institute of Experimental Biology, Pasteur St. 3, Warsaw 22, Poland

KATONA, G.	Biochemical Institute, University Medical School, Budapest VIII, Puskin u. 9, Hungary
KIRSCH, H.	Humboldt University, Institute of General Biology, 104 Berlin, Hessische Str. 3–4, GDR
KOMINZ, D.	National Institutes of Health, NIH Pacific Office, United States Embassy 1, Enokisaka, Akasaka, Tokyo, Japan
KÖVÉR, A.	Physiological Institute, University Medical School, Debrecen, Hungary
LÁBAS, E.	Biological Research Institute of the Hungarian Academy of Sciences, Tihany, Hungary
LAKI, K.	National Institutes of Health, PHS, DHEW NIH Bldg 4, D-26 Bethesda, Md, USA
LÁNG, I.	Hungarian Academy of Sciences, Biological Class, Budapest V, Nádor u. 7, Hungary
MARUYAMA, K.	Biological Institute, University of Tokyo, Komaba, Meguro, Tokyo, Japan
MÜHLRAD, A.	Biochemical Institute of Eötvös L. University, Budapest VIII, Múzeum krt 4, Hungary
NIEDETZKY, A.	Biophysical Institute, University Medical School, Pécs, Rákóczi út 80, Hungary
NIEMIERKO, W.	Nencki Inst. of Experimental Biology, Pasteur St. 3, Warsaw 22, Poland
OPLATKA, A.	Weizmann Institute of Science, Rehowoth, Israel
PAGE, E.	University of Chicago, 950 East 59th Street, Chicago, Illinois 60637, USA
PEPE, F. P.	Department of Anatomy, University of Pennsylvania, Philadelphia, Pennsylvania 19104, USA
PINAEV, G. P.	Institute of Cytology, Acad. Sci. USSR, Leningrad
RICE, R.	Mellon Institute, 4400 Fifth Avenue, Pittsburgh, Pennsylvania 15213, USA
RINALDI, A.	Department of Anatomy, Vanderbilt University, Nashville, Tennessee, USA
RÜEGG, J. C.	Max Planck Institute for Medical Research, Heidelberg, Jahnstrasse 29, GFR
SALÁNKI, J.	Biological Research Institute of the Hungarian Academy of Sciences, Tihany, Hungary
SLEATOR, W. W.	Washington University School of Medicine, 660 South Euclid St. Louis, Missouri 63110, USA
SPRONCK, A.	University of Liège Laboratory of General Biology, 22 quai Van Beneden, Liège, Belgium
SRETER, F. A.	Retina Foundation, Institute of Biological and Medical Sciences, 20 Staniford St., Boston, Mass., USA
STRAUB, F. B.	Institute of Medical Chemistry, University Medical School, Budapest VIII, Puskin u. 9, Hungary

Strickholm, A.	Department of Anatomy and Physiology, Indiana University, Bloomington, Indiana, USA
Strzelecka, G.	Nencki Institute of Experimental Biology, Pasteur St. 3, Warsaw 22, Poland
Szabolcs, M.	Central Laboratory of the University Medical School, Debrecen, Hungary
Székessy, V.	Biochemical Institute, University Medical School, Budapest VIII, Puskin u. 9, Hungary
Szent-Györgyi, A. G.	Department of Biology, Brandeis University, Waltham, USA
Szöőr, A.	Physiological Institute, University Medical School, Debrecen, Hungary
Tankó, B.	Biochemical Institute, University Medical School, Debrecen, Hungary
Tigyi, J.	Biophysical Institute, University Medical School, Pécs, Rákóczi út 80, Hungary
Tregear, R. T.	Oxford University, Department of Zoology, Parks Road, Oxford, England
Varga, E.	Physiological Institute, University Medical School, Debrecen, Hungary
Virágh, Sz.	Central Research Institute of Medical Sciences, Budapest IX, Üllői út, Hungary
Vodnyánszky, L.	Biochemical Institute, University Medical School, Budapest VIII, Puskin u. 9, Hungary
Vorobyev, V. I.	Institute of Cytology, Acad. Sci. USSR, Leningrad
Wilkie, D. R.	University College London, Department of Physiology, Gower St., London W. C. 1., England
Worthington, C. R.	University of Michigan, Physics Dept., Physics-Astronomy Bldg., Ann Arbor. Michigan, USA
Zs. Nagy, I.	Biological Research Institute of the Hungarian Academy of Sciences, Tihany, Hungary

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WELCOMING ADDRESS BY THE PRESIDIUM OF THE HUNGARIAN ACADEMY OF SCIENCES

F. B. Straub

Ladies and Gentlemen, Dear Friends,

Before starting the proceedings of this Symposium I have to fulfil the pleasant duty of conveying to you the greetings of the Presidium of the Hungarian Academy of Sciences. Allow me to say these words first in Hungarian and I shall translate them into English later.

Tisztelt Vendégeink, tisztelt Hallgatóság!

A Magyar Tudományos Akadémia Elnöksége képviseletében szeretettel üdvözlöm az izomszimpózium résztvevőit és külön szeretettel üdvözlöm azokat a vendégeinket, akik más országokból jöttek el hozzánk, akik közül sokaknak ez az első alkalma, hogy a magyar tudományos élet egy szektorával megismerkedhessenek.

A Magyar Tudományos Akadémia az elmúlt évtizedekben egyre nagyobb és nagyobb erőfeszítéseket tett, hogy a tudományos kutatás számára elengedhetetlen nemzetközi kapcsolatokat előmozdítsa. Ennek számos formája között igen nagyra értékeli az Elnökség a szimpóziumokat, melyeken egy-egy jól körülhatárolt probléma megvitatására egybegyűlnek a világ minden részéről azok a kutatók, akik munkájukkal ezt a területet előbbre vitték és előbbre viszik. Egyetértünk azokkal, akik a szimpoziumokban látják a tudományos véleménycsere leghatékonyabb formáját.

Az izomszimpozium rendezését Budapesten azért is látjuk aktuálisnak, mert ennek az iránynak nálunk hagyományai vannak, és ma is jónéhány kutatóhely foglalkozik ilyen irányú témákkal. Reméljük, hogy a szimpozium ezeket a munkákat segíteni fogja.

Kívánjuk, hogy itt az Akadémia évszázados üléstermében, de ugyanúgy egész magyarországi tartózkodásuk alatt vendégeink megtalálják az igazi tudományos együttműködés feltételeit. Kívánjuk, hogy a már meglevő tudományos és baráti kapcsolatok a szimpozium alatt tovább erősödjenek és újak keletkezzenek. Úgy gondoljuk, hogy az ilyen találkozók előmozdításával Akadémiánk célkitűzését teljesíti: a tudomány és a népek közötti megértés szolgálatát.

A Magyar Tudományos Akadémia Elnöksége eredményes munkát kíván Önöknek.

Ladies and Gentlemen, Dear Friends,

On behalf of the Presidium of the Hungarian Academy of Sciences I have the honour to cordially welcome the participants of the International Muscle Symposium, and in particular those of our guests who have come from foreign countries, many of them having the first opportunity to get acquainted with a sector of Hungarian scientific life.

During the past decades the Hungarian Academy of Sciences steadily increased its efforts to encourage international co-operation, one of the essential conditions of scientific research. Among its various forms the Presidium greatly appreciates that of organizing a symposium where clearly defined problems are discussed by scientists gathered from all over the world who have contributed and are contributing to this special field.

We welcome the Muscle Symposium in Budapest, as this field of research has its traditions in our country, and a number of our laboratories are engaged in related studies also today. We hope that our Symposium will give great impetus to these studies.

We sincerely wish that our guests may find the appropriate conditions for real scientific co-operation here, in the century-old conference room of the Academy, as well as during all their stay in Hungary. We sincerely wish that the Symposium may strengthen old scientific and friendly relations and give rise to new connections. We believe that by encouraging such meetings, we are fulfilling the task of our Academy, i.e. to serve science and a better understanding between nations.

The Presidium of the Hungarian Academy of Sciences wishes you successful work!

INTRODUCTORY LECTURE

F. B. STRAUB

INSTITUTE OF MEDICAL CHEMISTRY, UNIVERSITY MEDICAL SCHOOL, BUDAPEST

Ladies and Gentlemen,

I am now coming to the official part of our program in which I am listed as the first speaker on a non-committal topic called introduction. Some of you may have wondered how I come to talk to this Symposium on Muscle, when I am no more working in this field. The main justification of this talk is that I happen to be at present the secretary of the Biology Section of our Academy. The secretary of the Biology Section is responsible for the general scientific policy in this field and as part of this job ex officio organizer and host to this Symposium. I would not like to misuse the privileges of a host and to bore our guests, nevertheless, I feel I am bound to say a few words about our intentions.

Those words of greetings which I have given you in the name of the Presidium reflect my conviction that a symposium is one of the best forms of international scientific exchange. But one has to admit that the content of this word: symposium, is rather ill-defined. Obviously, its greatest asset — and this is always so — is that specialists working on a particular problem come together from all over the world. Now, as the official part of a symposium usually does not take more than eight hours of a day, fortunately, there remain some free hours during which people come together and are able to talk about their problems. As I see it, even a dull and uninteresting symposium may fulfil its purpose outside the conference room.

Some organizers of symposia are, however, ambitious people, and they ask the question: would it not be possible to utilize for the purpose of the symposium those hours of daylight, too, which are devoted to the official program? There are a number of ways to do so. One suggestion could be to do away with the opening ceremony and start talking business right from the beginning. Yet it would be hard to find organizers who would miss a chance of making a speech if they had got such a fine chance. Besides, it is never too long and, moreover, it fills the time until those who have lingered over their breakfast, come to the lecture hall.

To abandon this lighter tone, I am coming now to a more serious problem. We have come together to talk about muscle. Should we invite everybody to deliver a talk of a limited time on anything connected with the problem of muscle contraction? Or should we select just a few problems and ask some people to start a certain line by a summary of their views and let the discussion be the main stream of the Symposium? When Professor Ernst first suggested the idea of this Symposium, he proposed to choose the second alternative. I agreed with him in this rather experimental

method, because I have attended several symposia which were just diminutive congresses, where lectures were delivered, one after the other, not much discussion followed, and the meeting could be called a symposium only because of the limited number of participants. Very much could be said in favour of this type of symposium, it is mostly very authoritative, it is a rich source of information. However, there is the psychological problem that speakers tend to emphasize the important aspects of their findings, such lectures rightly emphasize what we know and mostly neglect the problems which we still cannot answer.

We do not believe that a *prima facie* case can be made for the alternative method, which we suggest to be adopted. Only the results can prove it either way. What we want to achieve by this method is clear: much discussion and many-sided discussion of the problems. May I ask the collaboration of all of you in achieving this aim? We cannot boast of being experts in organizing this type of symposium, we can assure you only of our good intentions. Therefore, any comments and suggestions for improvement will be appreciated. Mind you, as I mentioned earlier, most symposia are valued for the discussions which usually go on in the free time and what we suggest is to use some of the official time of the proceedings to accomplish the same, informal and open discussions.

Still, I have to confess the second justification for standing here. Some of you may remember that I was one of the earlier generation, the members of which are mostly present in this room, which at Szeged, in the laboratory of Professor Albert Szent-Györgyi had worked on muscle protein during the much troubled years of the 1940s. I personally am partly responsible for the protein actin. During the ten years while I was working on actin we never had a chance to come together with others working in this field, apart from other members of our own laboratory. The outstanding quality of Szent-Györgyi's fascinating personality and his scientific leadership helped us to achieve some success, in spite of the isolation during the war and in the early post-war period. When we discovered the adenine nucleotide in actin, it was in a period when our laboratory at Szeged was not heated properly in winter and we had to work in winter-coats, while not having much apparatus apart from test tubes. Incidentally, there was a glass-blower in the building and we had some glass tubes, so viscosimeters could be constructed. This is the main reason why we worked out the microdetermination of ATP as a viscosimetric method. As far as I know the method has never been used by anybody else. Now I mention these petty hardships only to make it clear that during those times it was not the apparatus and not the heating that we missed most, it was the contact with fellow scientists which made us very uneasy. And with this thought I am coming back again to the main aim of this Meeting. The biochemistry of muscle, the electron microscopy of muscle, the mechanical activity, the energetics of muscle and the role of ions in muscle are all specialized fields of study, and there are perhaps only a few people who could master all of these fields. Yet if we honestly join our forces, the resulting vector of force may advance the future work of all of us in the proper direction.

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STRUCTURAL ASPECTS OF THE STRIATED MUSCLE*

R. COUTEAUX

LABORATOIRE DE BIOLOGIE ANIMALE, FACULTÉ DES SCIENCES, UNIVERSITÉ DE PARIS, PARIS

Now that great strides have been made in studying the contractile mechanism of the striated muscle, as well as the processes of excitation and activation which initiate the muscular contraction, it may appear more opportune than ever to compare the structural and chemical properties of different kinds of striated muscle fibres, in order to account for the functional differences observed between them. Two categories of striated muscle fibres, the twitch and slow fibres, which coexist in several classes of vertebrates, seem particularly suitable for this purpose.

The fibres of both types have been carefully studied in the frog by many authors, physiologically and morphologically, and the differences observed between the fibres have been up to now more clearly analysed in this species than in any other. Therefore, to compare the twitch fibres and those which produce only slow contractions, I shall mainly use the results obtained on frog muscles. Until recently no information was available on the fine structures of slow muscle fibres of the frog because, to be conclusive, such a study had required a previous identification of the fibres. It was only in 1962 that this was done by isolating the fibres and estimating visually the speed of contraction of each before fixation.

The peculiarities of the slow-muscle fibres observed under the light microscope and the electron microscope (which are considered the best means available to-day to distinguish the slow fibres from the twitch fibres in the frog muscles) are as follows.

MYOFIBRILS

The M-line is absent in the slow fibres (no thickening of A-filaments and no cross links between them).

Differences can be observed at the level of the Z-line, not only in the Z-line itself but also in the structure and arrangement of the I-filaments on either side of the Z-line.

SARCOPLASMIC RETICULUM, T-SYSTEM AND TRIADS

Two separate networks of tubules, representing respectively the sarcoplasmic reticulum and the T-system, differ by their form and distribution from those of the twitch fibres.

* For lack of the detailed lecture this abstract is published.

The T-system of the slow fibres includes not only transverse tubules but also longitudinal tubules connecting the former. Experiments with ferritin particles indicate that the tubules of the T-system in the twitch and slow fibres of the frog muscles are probably connected with the surface membrane of the fibres, as they are in fish muscles (myotomes). Calculations have been made to estimate the surface area of the membranes limiting the tubules of the T-system. For a fibre 50 μ in diameter, the area of the T-system was calculated to be about 3 times the area of the surface membrane in twitch, whereas both of these areas seem approximately equal in slow fibres. This difference between twitch and slow fibres, which may be principally explained by the difference in diameter of the myofibrils, which are generally much larger in the slow fibres, agree satisfactorily with estimates from membrane capacitance measurements.

The triads, linking the tubules of the sarcoplasmic reticulum with those of the T-system, are much less frequent in slow fibres than in twitch fibres, occurring at only every 5th or 6th line of each fibril.

JUNCTIONAL AREAS OF THE SURFACE MEMBRANE

(a) *Myoneural junctions.* The endings of the small-nerve system, which are considerably smaller than the end-bushes which terminate on the twitch fibres, are numerous on each slow fibre and very irregularly distributed over the whole fibre length. The subneural folds of the postsynaptic membrane are almost absent in the slow fibres, and the amount of cholinesterases per unit of junctional membrane surface is much smaller than that at end-bushes.

(b) *Myotendinous junctions.* Contrary to the distribution of cholinesterases at myoneural junctions, the amount of cholinesterases attached to the folds of the myotendinous junctions is much greater in slow fibres than in twitch fibres.

Two other characteristics of the slow-muscle fibres must be pointed out: they have relatively few mitochondria and an extremely low content of lipids.

To what extent is it possible now to correlate the properties of the slow-muscle fibres mentioned above with their functional peculiarities?

The differences in structure, distribution and cholinesterase concentration observed between the myoneural junctions of slow and twitch fibres agree with the physiological findings, indicating that nerve stimulation does not produce action potentials in slow-muscle fibres and that the graded contraction of these fibres results from the depolarization produced by summation and electrotonic spread of numerous junctional potentials.

The reduction of the T-system and the relative rarity of triads must also be taken into consideration in interpreting the coupling of depolarization to contraction in slow-muscle fibres.

Even though important differences exist between twitch and slow-muscle fibres in the mechanism of activation, experimental evidence suggests that the rate of tension development in slow-muscle fibres depends also on the rate of reactions taking place at the level of the myofilaments themselves. At the present time, cytochemical data are lacking on this point.

GENERAL DISCUSSION

HUXLEY (Medical Research Council, Laboratory of Molecular Biology, Cambridge): The general picture of the fine structure of striated muscle that has been built up in the last 10 or 15 years is now very familiar, and I will not repeat it here in detail. In summary, we believe that the myofibrils contain overlapping arrays of actin- and myosin-containing filaments, and that cross bridges between the thick and thin filaments generate a relative sliding movement of one set of filaments past the other; the filaments themselves remain virtually constant in length. The tension generated by the cross bridges along a given thick filament add up in parallel so that the tension generated by the system as a whole varies according to the extent of overlap of the filaments, and also according to the number of cross bridges which have time to attach to the active sites on the actin molecules alongside them.

The force generated by the cross bridges must act in the appropriate direction. That is, all the elements in force in one half of the A-band must act in one direction and those in the other half of the A-band must act in the reverse direction, so that the actin filaments are moved towards the centre of the A-band from either side. Studies on the assembly of the myosin and actin molecules into synthetic filaments have indicated that the myosin molecules are arranged so that they all point in the same direction in the one half of the A-band, and that the direction of all the molecules in the other half of the A-band is reversed. In this way the orientations of the active sites in the two halves of the A-band would be reversed relative to each other, thereby achieving the necessary directional relationships of the forces that they can generate. Similarly, it has been shown that actin filaments have a structural polarity and that this polarity is reversed on either side of the Z-line. These electron-microscopic studies, though valuable in showing up some aspects of the arrangement of the molecules of the contractile proteins in the muscle filaments, were not able to show the details in the very regular repeats of the molecules that we should expect to find. This is probably because some disorder of the structure takes place during the preparation of the specimen in electron microscopy, especially in the thick filaments.

We are now using improved low-angle x-ray diffraction techniques to study the detailed internal structure of the thin and thick filaments in muscle, the detailed arrangement of the actin and myosin molecules in these filaments, and the changes that occur in the arrangement when the two proteins interact with each other, when the muscles contract, and also when they pass into rigor. In the latter condition all the ATP in the muscle has been used up and it is believed that permanent cross connections are established between the two types of filaments. In the former case, in contraction, we believe that there is repetitive interaction between the myosin cross bridges and the active sites on the actin filaments, and that this leads to the relative sliding motion of the filaments past each other. Dr K. Holmes and Dr W. Brown have contributed to various aspects of the work.

The low-angle x-ray reflections that we are now studying in detail are the ones which were first described about 14 years ago (Huxley 1952,

1953) as being given by living muscles; recently, we have greatly improved the technology associated with the recording of these reflections and are now able to obtain much better pictures in much shorter times. This is not the place to describe the techniques in detail, but in summary, we are employing a high-powered fine focus rotating anode x-ray tube (designed by Holmes and Longley) and a new type of camera consisting of a combination of bent quartz focussing monochrometer, which gives us very high resolution in one direction, combined with a total reflecting bent glass plate, which gives focussing in another direction at right angles to the first and therefore essentially leads to point collimation. The fastest of our cameras can record the pattern from collagen fibres in less than 30 seconds, and the main features of the muscle diagram in about 10 minutes.

The x-ray diagram given by surviving frog sartorius muscle contains a wealth of interesting, informative, and intriguing detail. The main feature of this diagram is a system of equal-spaced layer lines, which were in fact the reflection first seen in 1952. These were later identified by Elliott (1964) and Worthington (1959) as arising from the myosin filaments, and indeed there is good reason to suppose that the cross bridges (which should show up, as it were, with higher contrast against the surrounding sarcoplasm) will contribute strongly to this part of the pattern.

The pattern we see is the characteristic one to be expected from a helical distribution of scattering matter. That is, we see a characteristic variation of intensity along the different layer lines which themselves are separated by a distance corresponding to a helical repeat of 429·6 Å. We observe that there is a strong meridional reflection lying on the third order of this repeat, which would correspond to the true axial repeat, i.e. the axial repeat of the subunits which are arranged in the helical pattern. In this case the subunits would correspond to the cross bridges and the pattern shows that these must occur at intervals of 143·2 Å. The distribution of intensity along the layer lines can be identified with that expected from a so-called 6/2 helix, i.e. one in which we have pairs of cross bridges, one on either side of the filament, repeating at the 143·2 Å spacing, with successive repeats rotated relative to each other by 60°, so that the whole structure repeats at $3 \times 143{\cdot}2$ Å, i.e. the helical repeat. The distribution of scattering intensity along the layer lines corresponds to that to be expected with a centre of mass of the scattering centres at a radius of about 110 Å, which corresponds to the position of the cross bridges as seen by electron microscopy.

If the arrangement of the cross bridges were perfectly helical then the only meridional reflections which could occur would be the ones on the third, sixth, ninth, etc. layer lines. However, although the reflections on these layer lines are strong ones, there are meridional reflections on all the other layer lines, except the first, showing us that there is some degree of distortion from an ideal helix.

At wider angles we can see the well-known system of reflections originally reported by Astbury (1949) as being given by the actin structure and interpreted in a number of possible ways by Selby and Bear in 1956. It was later shown by Hanson and Lowy (1963) that the actin filament did indeed consist of a double helix of apparently globular subunits, in agreement with one of the models put forward by Selby and Bear (1956). The actin reflections

from living frog sartorius muscle can be indexed on a helical net in which the pitch of the helix is 355 Å $\times 2$ and in which there are 13 subunits per turn in each of the chains. There are, however, some lower angle reflections, which have similar characteristics to the other actin lines, and which have a spacing of about 400 Å, and it is not clear at present what exactly is the origin of these latter reflections.

When living muscles are stretched the spacings and relative intensities of both these systems of reflections are unchanged as was originally shown in 1952. This provides a very clear demonstration that changes in filament length do not occur during passive changes in length of the muscle.

So far we have been concerned with the most well-known features of these diagrams. However, if we examine them in greater details with cameras of improved resolving power, we can see that they contain a number of other well-defined reflections of a rather more complicated and intriguing nature. In the first place, there are strong reflections on the meridian of these diagrams from living muscles at spacings of 388 Å and 444 Å units. These do not correspond to the periodicity of either the actin or the myosin helices and apparently arise from some other system in the muscle. As the system which gives rise to these reflections clearly has exactly the same orientation as that which gives rise to the reflections we have already described, and as the reflections are quite strong ones, it seems very likely that they arise from some other component directly associated with the myofibrils rather than, say, with the mitochondria or with the reticulum. In the electron microscope, especially in relatively thick sections, a well-defined periodicity is visible in both the I- and A-band, which has long been recognized as having a value in the region of 400 Å. More accurate measurements made recently have shown that the periodicity in the I-bands corresponds to the shorter of these two new spacings, i.e. to the value of 388 Å in living muscle and that in the A-band corresponds to the longer spacing, i.e. 444 Å. Furthermore, it was subsequently shown by Cohen and Longley (1966) that a periodicity of about 380 to 390 Å could be detected in crystals of tropomyosin. There has been a good deal of evidence recently, indicating that tropomyosin and actin are associated together in some way in thin filaments, and it therefore seems that it is this associated tropomyosin that we are picking up in the low-angle x-ray diagram. There have also, of course, been indications that the tropomyosin may play some role in the regulation of the activity of the actomyosin and its repeating periodicity may, therefore, turn out to have some significance in relation to that of actin and myosin.

The periodic structure that gives a repeat of 444 Å in the A-band is rather more mysterious, for as yet no additional component has been identified in the thick filaments, apart from myosin. There is, on the other hand, a certain amount of evidence (Huxley 1960, 1963) that all the protein of the thick filaments cannot readily be accounted for by those myosin molecules having cross bridges which are visible in the electron microscope, and there might well be, therefore, either additional myosin molecules buried deeper in the filaments or some extra protein component. This should be investigated by protein chemistry.

A problem we have been aware of for some time is the question of how the length of the filaments in muscle is determined. The thick filaments

appear to have a very constant length of about 1·55 to 1·6 μ in the muscles from a large variety of animals. The filaments are built up of many hundred myosin molecules, and it does not seem to me plausible that such a precise and constant length could be achieved by a kinetic mechanism. It appears more likely that there is some structural mechanism involved which ensures that the filaments always assemble themselves so as to have this precisely pre-determined length, and that this might give rise to some of the extra periodicities.

Let us now turn to the diagram of muscles in rigor, i.e. muscles in which combination between the cross bridges and actin filaments alongside has taken place, and let us first of all consider the diagrams of muscles near to rest length or shorter, in which there is a large region of overlap between the actin and myosin filaments. If we look at the very wide-angle actin pattern, i.e. that at spacings of less than 50 Å, we find that there is virtually no detectable change in either the spacing or the intensity of these reflections, except for the first true meridional reflection at 27·3 Å, which is somewhat more intense in the rigor specimens. This indicates that the local packing of the actin monomers is virtually unaffected when the cross bridges attach to them. The actin reflection near to 59 Å moves nearer to the meridian as though it were becoming associated with scattering matter at a larger radius than that of the actin filaments alone.

In contrast with the behaviour of the actin reflections, the myosin filaments diagram is greatly altered when the muscle passes into rigor. We still observe a strong meridional reflection at 143·2 Å, showing that the average axial repeat of the cross bridges is still the same. But the characteristic pattern of regular-space layer lines at intervals of 429·6 Å is no longer present, showing us that a considerable change has taken place in the helical arrangement of the cross bridges. In the part of the diagram where the cross-bridge reflections originally showed so prominently (i.e. between 60 Å and 430 Å), we now see a series of somewhat more diffuse reflections, which can be indexed on an actin-like lattice, indicating that the position of the cross bridges now conforms to the repeat of the active sites along the actin filaments. Such a change can be achieved only if the cross bridges move, so the result shows first of all that the cross bridges move when the muscle passes into rigor and that they do not represent a constant pattern for all states of the muscle. Furthermore, there is quite a strong reflection now on a first layer line at 380 Å, and another reflection at 229 Å; this, together with the subunit repeat of 143 Å, can be indexed on a non-integral helix with 2·66 residues per turn, i.e. 8 residues in three turns of the helix at a true repeat of 1140–1150 Å. (I should say that a similar helix to this one was first detected by electron microscopy in insect flight muscle by Reedy) (in preparation). There are thus indications that a change in the myosin filament helix may occur when the muscle passes into rigor, but from these observations we cannot see whether this is due directly to the absence of ATP or to the combination with actin, or to the presence of calcium.

Now let us turn to the study of the x-ray diagrams given by actively contracting muscles. It is not possible to keep an isolated muscle contracting continuously in an oxygenated Ringer bath. The muscle must be left for a time between successive stimuli to enable it to recover. A schedule

often employed is one in which the muscle is stimulated for about one second at one minute intervals, and carefully dissected muscles can continue to respond satisfactorily in this way for 10 or 20 hours or more. By arranging that a shutter on the x-ray camera is open only when the muscle is generating tension, we can build up the required total exposure time of 10 or 20 minutes by adding together 600 to 1200 individual one-second contractions, so that the total time of the experiment will be 10 or 20 hours; as mentioned previously, the fastest cameras that we now use will record the main features of the low-angle x-ray diagram within this time. When the diagrams from actively contracting muscles are examined (isometric, and also with shortening by 10 per cent R.L.) we find that the strongest characteristic reflections of the axial pattern, namely, the 143·2 Å meridional reflection (showing the average repeat of the cross bridges) and the 59 Å off-meridional actin reflection are unchanged in spacing. There may be a small decrease in the intensity of the 143·2 Å reflection, but that of the actin reflection appears unchanged, and we have not so far detected any movement of this reflection nearer to the meridian. These results (Huxley, Brown, and Holmes 1965) (which were also independently obtained by Elliott, Lowy and Millman 1965) show that the repeating periodicities of the two types of filaments and hence the over-all lengths of those filaments are unchanged during contraction. On the other hand, however, we find that a large change is visible in the intensity of the off-meridional part of myosin layer lines, which all become very much weaker.

This indicates that a substantial change occurs in the helical pattern of cross bridges when the muscle becomes active, and that the arrangement of cross bridges changes to not merely a new, but equally highly-ordered form. Surprisingly, a large component of this movement must be either radial or circumferential rather than axial, for a random axial displacement of cross bridges would be expected to greatly diminish the intensity of the 143 Å meridional reflection. One can say that an unsynchronized movement of the cross bridges takes place so that a consistent helical repeat is not present over a substantial length of the filament at any given time. The fact that the 59 Å actin reflection is relatively constant in position, and that there is little sign in the diagrams we have obtained so far (with at present available exposure time) of any additional reflections characteristic of muscles in rigor, indicates that not all the cross bridges are attached to the actin filaments simultaneously during isometric contraction. If all the cross bridges *were* attached simultaneously this would indicate that the range of movement of the cross bridges (supposedly oscillating to and fro in contraction) lay in the region of 50–100 Å. If only, say, one third of the cross bridges are attached at any given time this would indicate a movement of 15–30 Å approximately and, therefore, it is important to obtain data from much longer exposures than have been possible so far, to investigate this matter further by setting limits on the proportions of bridges attached at any given time.

To summarize, the actin and myosin molecules are arranged in their respective filaments in a very regular helical manner. The pitches of the two helices in resting muscle are not the same nor are their subunit repeats equal. The presence of additional components is indicated by the x-ray diagram. When the muscle passes into rigor the position of the cross

bridges on the myosin filaments changes so that a substantial number of them fall into a pattern compatible with the helical arrangement of active sites on the actin filaments. The actin filaments themselves do not undergo any substantial change in their local packing, but there are indications that a small change in their long helical period from 355–380 Å may take place. When the muscles become active, the regular arrangement of the cross bridges is disturbed so that they no longer give rise to a readily detectable x-ray pattern and we can, therefore, say that muscular contraction is associated with movement of the cross bridges, though much further work is needed to define the exact nature of this movement more precisely.

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SZENT-GYÖRGYI: Dr Huxley, would you mind to elaborate on your observations that in rigor all the cross bridges appear to be linked to actin, while in the contracted muscle only a portion of them, even though all of the cross bridges have moved, as indicated by the lack of regular pattern of layer lines in pictures obtained from contracted muscles?

HUXLEY: The results would indicate either:

(i) that the process of activation itself disturbes or changes the arrangement of cross bridges sufficiently so that even when a relatively small proportion of them are attached to actin, a large reduction in the strength of the x-ray reflections related to those from resting muscle is produced, or

(ii) that the attachment of a relatively small number of cross bridges to the actin disturbes the rest of the structure sufficiently to weaken the x-ray reflections in the observed manner.

EDMAN: I should like to ask, Dr Huxley, whether it has been possible to do x-ray studies of the glycerinated muscle during contraction. I would be particularly interested to know whether the side-to-side distance between the filaments is altered in a way similar to that observed in the living muscle.

HUXLEY: So far it has not been possible to do x-ray studies of glycerinated muscle during contraction because of the difficulties of diffusion of ATP into samples large enough to give an x-ray diagram within a practicable time.

EDMAN: A comparative study of that kind would be of great interest in view of the fact that the length–tension relationship in the living muscle cell (Edman 1966) appears to be basically different from that found in the glycerinated muscle fibre (Edman 1964).

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AUBERT: Dr Huxley suggested that in a muscle in rigor the number of cross links between the two sets of filaments was probably larger than in a normal contraction. This implies, I suppose, that the muscle in rigor should be the stronger and able to resist with a larger tension to an imposed stretch (10 to 30 per cent). Now, this is certainly true at very short lengths (say 2/3 l_0, standard length) in the case of frog sartorius during iodoacetate rigor, but not at lengths equal to, or larger than, l_0. Could you explain the fact described by G. Maréchal?

HUXLEY: I do not think that it necessarily follows that resistance to stretch of a muscle in rigor should be greater than that of an actively contracting muscle. There could be two factors involved: first, the point at which the bridges break off during stretch of a rigor muscle may be such that they are not all broken simultaneously during stretch; secondly, it may be that a bridge during the splitting of ATP can generate more tension than a passively extended one. However, this is an interesting question and should be given further thought.

HASSELBACH: I wonder if you can give us some information, from your x-ray diagrams of the resting muscle, concerning the thermal motion of the myosin side bridges.

HUXLEY: There is certainly a fade out, particularly of the non-meridional part of the myosin x-ray pattern as one goes to higher angles, which indicates a much larger temperature factor than is present, for instance, in the arrangement of the protein subunits of tobacco mosaic virus. However, I would not like at present to make an estimate of the average movement involved.

RINALDI: Do the cross bridges correspond to the helical nature of actin and myosin and do they change during activity?

HUXLEY: Yes, the cross bridges correspond to the helical arrangement of myosin molecules in the myosin filaments, and a change in their arrangement does appear to take place during activity.

RICE: What is the thickness of the heavy meromyosin thin filament complex?

SZENT-GYÖRGYI: I do not think one can give a quantitative value for the diameter of the actin heavy meromyosin complex from these patterns, except that the filaments of actin heavy meromyosin complex are thicker than filaments of actin alone. This agrees with the electron-microscopic observations.

BIRÓ: I should like to ask, Dr Huxley, whether it can be taken as granted that the packing of the myosin molecules in the A-rods in the native state of the myofibril is as tight as it is seen in the well-known cross-section pictures obtained with fixed material. I put this question, because some of our experiments on the tryptic digestion of myofibrils, carried out in common with Dr Bálint, showed a surprisingly easy accessibility of the myosin built in myofibrils to the action of trypsin. This is in marked

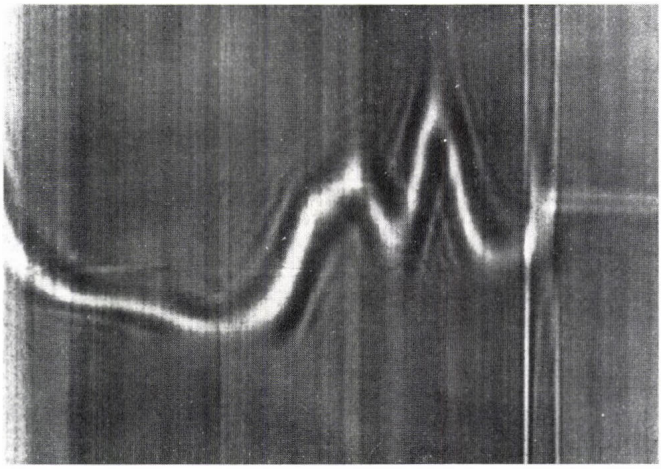

FIG. 1.—Ultracentrifugation of Hasselbach–Schneider extract of myofibrils digested in the presence of Ca ions; 5·5 mg/ml protein concentration, 198 000 g, 20° C, bar angle 55°; photographed 55 min after reaching full speed

contradiction with experiences showing that the tight aggregation of protein molecules interferes heavily with their proteolytic degradation. One such example is the case of actin which in unpolymerized state is rather easily digested by trypsin, while when polymerized it is resistant. A similar phenomenon is described by Kleczkowski and van Kammen (1961) in the case of aggregation of tobacco mosaic virus particles to virus rods. In view of these and similar findings, the observation of Szent-Györgyi and Holtzer (1963) that myofibrils treated with trypsin are completely dissolved in a few minutes is rather unexpected. We carried out similar experiments in milder conditions (with a trypsin to myofibril ratio of 1 : 190) and we obtained practically complete dissolution in a few minutes. This type of observation could possibly be explained by a mechanism whereby the myosin molecules on the surface of the A-rods would be fragmented and, as H-meromyosin is completely soluble and L-meromyosin is not quite insoluble in these conditions, the fragmented molecules move in solution thus exposing the deeper regions. By studying the digestion of myofibrils in the presence of Ca ions, however, we obtained results which could be more simply explained by assuming for the A-rods a loose gel-structure penetrable by trypsin molecules: when myofibrils are digested in the presence of 0·01 M $CaCl_2$ very little protein moves in solution (approx. 30 per cent) but this is not due to the lower rate of peptide bond splitting (Biró and Bálint 1966) in these circumstances. If we extract the undissolved protein by Hasselbach–Schneider solution (Hasselbach and Schneider 1951) and examine the extract in the ultracentrifuge the picture obtained shows that practically all myosin remaining in the myofibrillar structure is fragmented (Fig. 1). The faster peak is rather complex and its identity is hard to recognize. It could contain unfragmented myosin. The slower

peak, on the other hand, has an S_{20} value of 2·54; thus it is in all probability L-meromyosin. Its amount is roughly 30 per cent of the total protein extracted. Thus there can be very little, if any, unfragmented myosin. As this excessive digestion was obtained in undissolved state, we suggest that the A-rods in the native state form a loose gel-structure penetrable by trypsin molecules.

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HUXLEY: I think the observations on the tryptic digestion are very interesting ones. The myosin molecules are very probably fairly loosely packed within the thick filaments. On the other hand, the site on the myosin molecules which is susceptible to tryptic digestion, i.e. the region joining the light and heavy meromyosin parts of the molecule may perhaps lie close to the surface of the filaments, since a considerable part of the heavy meromyosin molecule is, we believe, projecting out sideways from the filaments in the form of cross bridges.

GERGELY: The inextractibility of the H-meromyosin of the digested myofibril could not be attributed to the fact that it remains bound to the actin structure, could it?

BIRÓ: The mechanism whereby H-meromyosin remains undissolved when the myosin is fragmented *in situ* is irrelevant from the point of view of my question. In fact we, too, believe that this insolubility is caused by the binding of the H-meromyosin parts to the actin structures. What is of interest is the fact that the myosin fragments remain in the myofibril but, in spite of this, virtually all random regions can be split by trypsin.

STRICKHOLM: I wish to ask, Dr Huxley, if anything is known about the nature of the bonds involved in the cross-linked filaments seen between actin and myosin. Are these filaments linked by covalent bonds, or might they perhaps be charged groups attracting between actin and myosin? Or, instead, could they be structural end-groups projecting outwards, which serve perhaps an enzyme function and are not involved in developing mechanical force?

HUXLEY: As far as I know there is very little definite chemical evidence concerning the nature of the bonds between actin and myosin, apart from the fact that SH-groups are important in them. As actin and myosin are generally agreed to be capable of forming a complex in solution, it seems reasonable to suppose that such a complex or combination would form in appropriate conditions within the muscle and that this combination is likely to be represented by the attachment of the heavy meromyosin projections on the thick filaments where they touch the actin monomers in the thin filaments. Of course, it is *possible* that the force in a muscle is developed in some entirely different structure which is invisible in most conditions (though the fact that synthetic actomyosin threads developed tension would indicate that these proteins are responsible for developing

mechanical force) but it seems more sensible to suppose that the force is developed at the points where our present structural information tells us that it could be developed and to see if we can build up a reasonable account of the properties of muscles on this basis. So far efforts in this direction seem to have been reasonably successful.

DYDYNSKA: The question which I should like to ask is if it is possible to tell something about the role of ADP bound to G-actin molecules in the process of forming cross bridges with myosin. In the course of glycerination of frog sartorii I tried to get the fibres completely free of ADP bound to actin and these fibres did not contract in ATP addition.

HUXLEY: I am afraid that our own results do not tell us anything at present about the possible role of bound ATP in actin in the formation of cross bridges with myosin. However, I wonder whether in the experiment mentioned by Dr Dydynska denaturation of the ATP-free actin may have taken place, for one might have supposed that subsequent addition of ATP would lead to the reattachment of nucleotide to the actin structure.

SZENT-GYÖRGYI: Both Oosawa and co-workers and Bárány and co-workers obtained preparations of F-actin which were capable of combining with myosin. The actomyosin complex formed could undergo superprecipitation in appropriate conditions.

GERGELY: Glycerination may cause other changes in the fibres, and the lack of contraction on the addition of ATP may be a reflection of these changes and not of the removal of ADP.

GUBA: I would like to raise some points in connection with Dr Huxley's report. As has already been reported (Guba and co-workers 1965a, 1965b), we have examined the ultrastructure of myofibrils of rabbit m. psoas by electron microscopy, using glutaraldehyde fixation. From the data obtained by measuring the diameter and spacing of filaments in cross-sections we have come to the conclusion that the primary filaments are formed from the fairly loose aggregation of protein molecules (predominately myosin). It seemed that the colloid state of these micellae changes during the muscle activity. Recently, we have got further evidence showing that the primary myosin micellae are far less rigid in comparison with the secondary ones. We have tried the freezing and thawing method (generally used to prepare polysomes from bacteria) to prepare filaments from isolated myofibrils. The thin filaments obtained proved to be myosin (Fig. 2). Another point I wanted to raise concerns the superprecipitation of actomyosin gel. It is common to talk about such a system as a model for muscle fibre. In an oriented actomyosin gel which exerts a considerable force during shortening, indeed, a filamentous build-up can be seen as revealed by electron microscopy. I should like to ask Dr Huxley if he has done diffraction experiments with oriented actomyosin gel and whether he thinks that in such a system a sliding mechanism could exist or not?

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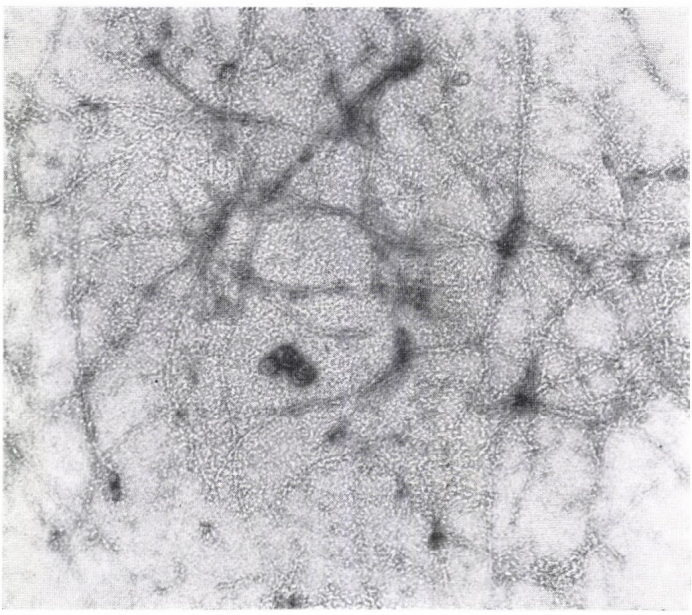

FIG. 2 — Filaments prepared from isolated myofibrils; × 74 000

HUXLEY: I am very interested to hear of Dr Guba's results about the character of the aggregation of the myosin molecules in the thick filaments. We, too, have found that the diameter of the thick filaments in resting muscle after glutaraldehyde fixation lies nearer to about 170 Å (in agreement with our earlier results by x-ray diffraction) rather than the 110 Å value apparent after osmium tetroxide fixation. However, a smaller diameter is still apparent in glutaraldehyde-fixed material from muscles in rigor.

Concerning Dr Guba's question about x-ray diffraction experiments on oriented actomyosin gels, we have not so far succeeded in orienting the artificial gels sufficiently well, to give usable low-angle diffraction diagrams. I think it is very likely that such gels contract in ATP by a sliding mechanism taking place in a somewhat less highly-organized system than a striated muscle. Our studies on the assembly of myosin molecules into thick filaments have shown that *in vivo* these can form with the required reversal of polarity in the arrangement of the myosin molecules at the centre of the filaments, so that they could draw in actin filaments from either end. No doubt, many of the filaments in actomyosin gels have this polarity reversal and so it is now becoming a little easier to see how such a system can have the property of always shortening in response to ATP addition, for the movement of actin filaments in two opposite directions towards each other must always lead to an over-all shortening of the system as a whole.

GARAMVÖLGYI: On the International Biophysics Congress in Vienna I presented some polarizing microscopic observations concerning the extensibility of the primary filaments (Garamvölgyi 1966a). This Symposium gives the possibility to speak about another serious structural problem

which is also in connection with the polarizing optical properties of the myofibril. It is a well-known fact that, although the Z-lines are birefringent, the contraction bands (C_z) appearing at the site of the Z-lines are of non-birefringent character.

It is also well-known that the insect flight muscle fibrils do not exhibit microscopically visible I-bands, except in the state of stretch, and so it is practically impossible to distinguish by light microscope their shortened or resting state. The Z-lines of short sarcomeres, lacking in I-bands, are non-birefringent, as are the C_z-bands of the vertebrate muscle. If, as a result of elongation, I-bands are present, the Z-lines become birefringent (e.g. Garamvölgyi 1966b).

Some years ago we produced artificial contraction bands, by removing the dense substance of the Z-lines and by adding ATP to the fibrils. The newly formed 'Z'-lines contain thick filaments running in a parallel direction and, notwithstanding, they exhibit a non-birefringent optical character (Garamvölgyi 1965b).

It is not easy to find a satisfying interpretation of this phenomenon. Let us see what may happen to the ends of the primary filaments, if they terminate at the A–I junction, when the A–I junction approaches the Z-lines in the course of shortening. According to an earlier assumption of Drs Hanson and H. E. Huxley, the ends of the primary filaments may bend, or crumple, resulting in irregularly-arranged zones on both sides of the Z-line (Hanson and Huxley 1956). The primary filaments which are bent could really extinct the double refraction of the zones on both sides of the Z-line, but they would not influence the character of the Z-line itself. On this basis we could expect double refracting Z-lines, edged by narrow non-birefringent zones, but not a non-birefringent contraction band.

The other possibility is the penetration of the primary filaments into the Z-line, thus bringing about a double overlap of the primary filaments, as suggested recently by Professor Hoyle. The interpretation is even more difficult in this case, because a double overlap of the primary filaments would result in an increase and not in an extinction of the birefringence of the contraction bands. This was clearly demonstrated by Dr Aronson, when he brought about the double overlap artificially (Aronson 1963).

The presence of the primary filaments inside the Z-lines of shortened sarcomeres, as demonstrated by the team of Professor Hoyle (Hoyle et al. 1965), can perhaps be interpreted in connection with my observations. I also found thick filamental portions inside the Z-line, corresponding to the primary filaments (Garamvölgyi 1963). It is very interesting that in the Z-lines of stretched myofibrils of the bee these Z-filaments are much thinner and one has the impression that their material has been drawn out from the Z-lines into both halves of the I-band (Garamvölgyi 1965a).

The Z-lines containing the very thick and regularly-arranged Z-filaments are non-birefringent, but those of stretched fibrils, in which there is a less regular array and less material, are double refracting. This is quite the opposite in relation to the change in birefringence of what we should expect on the basis of the theory of composed bodies. I shall try to find an explanation for this strange behaviour of the Z-lines on the basis of my sarcomere model recently proposed (Garamvölgyi 1965a). This model is based on the continuity of the primary myofilaments. I suggested that the thin portions

of the primary filaments may enter the Z-lines by rolling up, thus producing the thick Z-filaments of the short sarcomeres. In the case of relaxation, or passive stretch, the substance of the Z-filaments will be drawn out from the Z-lines. This means that, as a result of shortening, the amount of substance will increase inside the Z-lines (Garamvölgyi et al. 1964). Consequently, it seems probable that the substance entering the Z-lines would contribute to the extinction of the birefringence in the contraction bands.

The double refraction of regularly arranged systems consists of the intrinsic birefringence and of the form birefringence. If we suppose the primary thin filaments to shorten by rolling up, we may expect that at the beginning of shortening the spirals are extended, that means the twofold elevation angle of the spiral will be close to 180°. So the effect of the half turns will be increased by the opposite half turns and to this increased intrinsic double refraction the form birefringence is to be added. By further shortening, there will be a position at which the opposite half turns will be perpendicular and thus they will mutually extinct the effect of each other. As a consequence, the intrinsic birefringence will be zero, while the form birefringence may steadily grow by the increase in the diameter of the spiral. In the final state of shortening the turns of the spiral will stand perpendicularly to the filament axis and so there is a possibility of explaining the abrupt change in the polarizing-optical character, for the intrinsic birefringence of opposite sense may extinct the form double-refraction thus resulting in a total double refraction of practically zero.

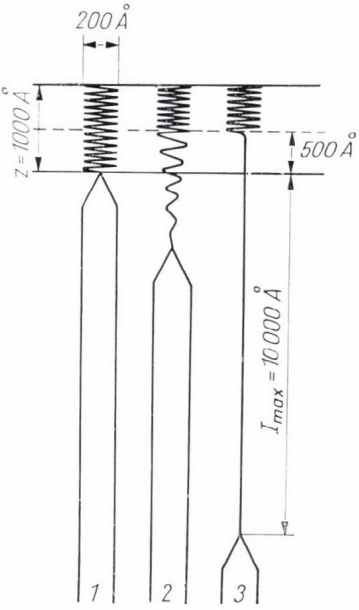

FIG. 3. — Schematic representation of the suggested mechanism of shortening and stretch of primary myofilaments in the region of the Z-line. *1* shortened, *2* moderately stretched, *3* stretched to the maximum I-band length. The secondary filaments have been omitted

My interpretation is, of course, nothing else than a mere speculation based on results obtained by light and electron microscopy, that is to say, an experiment to synthesize the different observations. I were very glad if I could direct the attention of the Symposium to this basic question. Finally, let me do an informative reckoning to check whether there is, or not, enough space inside the Z-lines for the suggested rolling up of the filaments. The diameter of the very thick Z-filaments of the short sarcomeres is about 200 Å. The width of the Z-line is 1000 Å, but we can take only its half, because two half I-bands belong to each Z-line. The maximum length of the I-band is about 1 μ, i.e. 10 000 Å (Fig. 3). From these values we can obtain how many turns are needed to take up the thin filaments connecting the primary filaments to the Z-lines: $2r\pi n = 10\,000n \sim 17$. Now let us see the possible maximum own diameter of the filaments rolling up: 500/17 = 30 Å; a quite realistic value for thin filaments.

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Hoyle, G., McAlear, J. H. and Selverston, A. (1965) *J. Cell Biol.* **26,** 621

TREGEAR: In a few x-ray experiments upon a glycerol-extracted insect flight muscle, performed with Dr K. C. Holmes and Dr M. K. Reedy (Tregear and co-workers 1966) we stretched the muscle by 5 per cent in rigor, in the hope of observing changes in the meridional periodicities. No changes were seen, yet electron-microscopic observations by Mr D. C. S. White (unpublished) show that the A-band does increase in length during such extension. These observations need repetition under carefully controlled conditions, but *prima facie* they indicate either that the A-filaments slip in a quantised manner, or that they are pulled out from some source in the thickened ends of the filaments or in the Z-disc itself.

REFERENCE

Tregear, R. T., Holmes, K. C. and Reedy, M. K. (1966) *Nature* **207,** 1276

GARAMVÖLGYI: I think that a slight elongation of the primary filaments in the region of the A–I junction would not change the x-ray diagram considerably. Until the 'no-overlap point', i.e. until 5 μ sarcomere length in the insect flight muscle, I am practically not in conflict with the assumption of the constancy of axial spacing of both the myosin and actin filaments. I also observed that the length of the A-band is practically constant in the sarcomere length range of 3–5 μ. On the other hand, my contraction scheme does not require the shortening of the secondary filaments. I did not perform any x-ray diffraction experiments, but on the basis of my model I should theoretically expect the same result as obtained by the teams of Drs Hanson and Hugh Huxley.

HUXLEY: In the case of the very stretched flight muscles, have you carried out filament counts in the I-bands to establish whether the number of filaments there correspond to the number of normal thin filaments expected for the particular number of thick filaments present in that particular fibril, plus the number of additional filaments to be expected in the A-filaments are continuous up to the Z-line?

GARAMVÖLGYI: Filament countings have been performed only in moderately stretched fibrils. In these fibrils there is a regular double-array of filaments in the I-band too, i.e. the sites of the primary filaments are not empty, but filled out by thin filaments. In highly-stretched fibrils the counting is very uncertain. Some thin filaments can imitate one single filament by apparent uniting and also accidental dots can be counted as filament profiles. I do not regard filament countings reliable in stretched myofibrils.

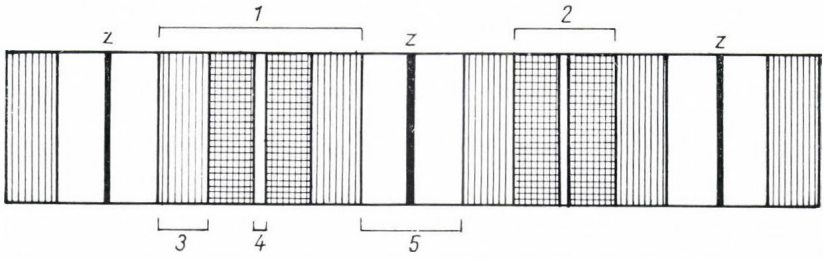

FIG. 4. —Two interpretations of some observations:

	Garamvölgyi	*Hanson*
1	E-band ≡ A-band of unstretched sarcomere	A-band + two half I-bands
2	dense part of A-band	A-band
3	stretched myosin filaments	half I-band
4	additional elongation zone	H-zone
5	I-band	irreversibly stretched zones

HANSON: I have made some observations similar to those of Dr Garamvölgyi, but I have interpreted them differently (Hanson 1956). Figure 4 is based on one of Dr Garamvölgyi's photographs (Garamvölgyi 1966). I used blowfly (*Calliphora*) indirect flight muscle. I want to mention now some of the evidence supporting my interpretation. After extracting myosin from the fibril, the optical density of the I-zone was considerably higher than that of the S-zone, suggesting that the actin filaments terminate at the border between the I- and S-zones. This was supported by two further findings. Potassium iodide extraction of the 'ghost' resulted in a uniformly low optical density from one Z-line to the next. When a solution of rabbit myosin was applied to a fibril from which myosin had been extracted, the rabbit myosin was taken up by the I-zone but not, apparently, by the S-zone.

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Hanson, J. (1956) *J. Biophys. Biochem. Cytol.* **2,** 691

GARAMVÖLGYI: I cannot agree with your interpretation. The first point of your figure says that I have confused the I-bands with the E-zones. I have checked this question very carefully (Garamvölgyi 1966a). In the polarizing microscope I found the I-bands to be practically non-birefringent on all sarcomere lengths, but the E-zones exhibited a still considerable double-refraction, i.e. about 50 per cent of the original birefringence of the A-band. Thus the E-zones can originate from the stretch of the A-band and they cannot be confused with the non-birefringent I-bands.

Concerning your fourth point I can say that I accept your statements on the constant length of both the A-band and the I-segment, at a certain sarcomere length range. If this is really so, the whole A-band has to get the character of an H-zone at about 5 μ sarcomere length. Now, the additional decrease in density in the middle of the A-band appears beyond this limit. According to your theory, the entire A-band, not only its middle, is equal

to the H-zone at these sarcomere lengths. Let me point out that a quite similar decrease in density, at the same site, in correspondingly highly-stretched frog myofibrils has been observed recently by Dr Sally Page (1965).She also stated that these zones cannot be H-zones. I fully agree with her statement!

Finally, your fifth point shows quite clearly the source of our present controversy. In 1956 you found irreversibly stretched zones in fly muscle fibrils (Hanson 1956). Quite different relations are ruling in the muscle of the bee. In this species even extreme degrees of stretch are reversible, at least in relation to the sarcomere length (Garamvölgyi 1966b). Thus my I-bands cannot be irreversibly stretched zones. I propose to check jointly the reversibility of stretch in our objects used, because this seems to be a decisive factor in our controversy.

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Page, S. (1965) *J. Cell Biol.* **26,** 477

HANSON: In one of your longitudinal sections the thick elements in the Z-line lie at an angle to the fibril axis and to the filaments. How do you explain this?

GARAMVÖLGYI: The fact that in the electron micrograph presented the axis of the primary Z-filaments slightly declines from the myofibrillar axis is merely accidental and not usual. It is due to distortion. I presented some other electron micrographs in which the primary filaments cross straight the Z-line (Garamvölgyi 1963, 1965).

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HOYLE: In recent studies on the structure of rabbit psoas muscle and of their Z-discs we have concluded that there is a basic framework of very thin filaments. According to the model we have developed, based on these findings, there is one very thin filament for each actin and two for each myosin. The latter may be located down the core or outside of the myosin. Such filaments could result in an appearance very similar to that which you observe, and also to the very interesting appearance of isolated primary filaments of flight muscle shown by Dr Guba.

In view of the theories of the structure of the Z-disc of vertebrate muscle presented by Knappeis and Carlsen, Porter and Franzini-Armstrong and Reedy, it would be of great interest to know the fine structure of that of flight muscle. Have you examined this, and in particular, have you considered the mode of attachment to the disc of the fine ends of your primary filaments?

GARAMVÖLGYI: In the Z-line of the insect flight muscle there is a hexagonal and not a tetragonal array. The paper of Dr Auber and Prof. Couteaux (1963) dealt with the structure of the Z-line of this kind of muscle. I also observed the same arrangement.

REFERENCE

Auber, J. and Couteaux, R. (1963) *J. de Microscopie* **2**, 309

HOYLE: Have you any information as to whether or not the fine filament which you find emerging from the myosin filament is present also in vertebrate muscle?

GARAMVÖLGYI: No, I have no information on that. My statements concern the insect flight muscle and I want to avoid a mechanical generalization. It is true, however, that the problem of the Z-line → C_z-band transformation represents a serious problem concerning vertebrate muscle, too. Also there is a change in the polarizing-optical property. I regard it extremely necessary to examine the structure of the vertebrate C_z-band. I just started to do a work of this kind.

GUBA: I should like to present one slide which I think can throw some light on the question of the continuity of thick filaments in the insect muscle (Fig. 5). The preparation of filaments followed Dr Huxley's method (Huxley 1963).

The starting material was locust thoracic muscle. As can be seen in the picture, the thick filaments taper and they seem to have a thin core which is surrounded by the aggregation of another material, likely myosin. One can imagine that there is a continuous filamentary system in the sarcomere of insect myofibril to which myosin molecules giving the appearance of primary filaments are attached. This explanation would be in agreement with Dr Garamvölgyi's observations presented here.

REFERENCE

Huxley, H. E. (1963) *J. molec. Biol.* **7**, 281

HANSON: What is the fine structure of the thin extension of the primary filament?

HUXLEY: With reference to your very interesting micrographs, in which thin filaments can be seen extending in the same straight line from the end of thick filaments of the insect flight muscle, and where other thin filaments, presumably actin, are also visible in the background, have you found any instances where on the same micrograph the characteristic actin double helical structure is clearly visible in the unstretched thin filaments, and where no such indications of actin structure are visible in the thin filament extending from the end of the thick one?

GUBA: I am afraid I cannot give you a definite answer to your very important question. The resolution of our micrographs is in most cases not satisfactory enough for us to see a definite difference between the two kinds of filament. Nevertheless, it seems to us that in some of our micrographs one can see the double helical structure of thin filaments but there is no such indication in the core of the thick filaments.

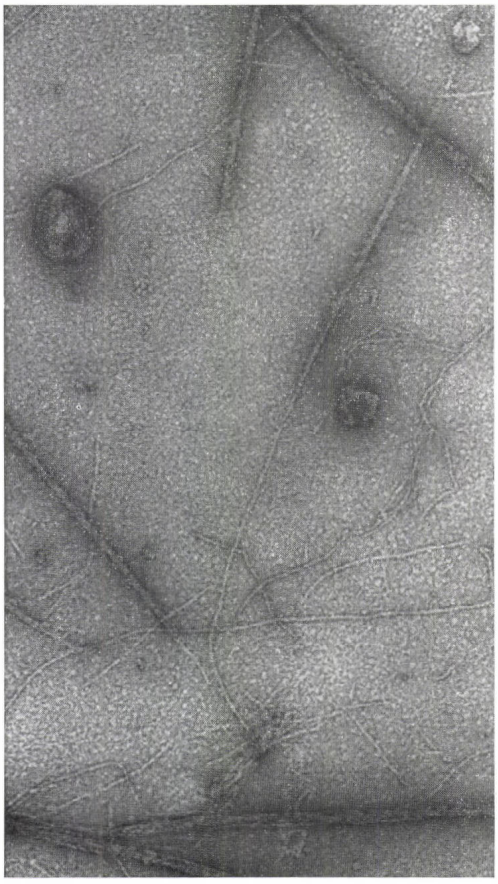

FIG. 5. — Locust thoracic muscle filaments; ×60 000

MARUYAMA: I should like to point out a minor problem, Dr Hanson. You mentioned that you could remove myosin from insect muscle fibres. But, according to Gilmour and Calaby, and also to my experience, myosin alone cannot be dissolved directly from the insect muscle. Always actomyosin is dissolved.

HANSON: I was speaking loosely when I said I had extracted myosin. I apply a solvent that extracts myosin from rabbit fibrils and I observe a decrease in the optical density of the A-band, but I have not determined yet if the material extracted is myosin or actomyosin.

HOYLE (Biology Department University of Oregon):* A simple picture of the ultrastructure of striated muscle has recently emerged, particularly from the electron-microscopic and x-ray diffraction studies of H. E. Huxley. He considers that contractile force is developed by interaction between the filaments and favors the view that the mechanism is related to movements of bridges located on the myosin at regular intervals, which extend between the myosin and actin filaments. A molecular hypothesis which invokes alternating cycles of shortening and extension of these cross bridges has been advanced by Davies (1963). A different mechanism, but also based on the cross bridges as providing motile force has been proposed by Reedy et al. (1965), specifically to apply to insect flight muscle. They suggest that the cross bridges turn inwards towards the center of the sarcomere after first attaching to the actin.

In my laboratory we have attempted to test the universality of Huxley's model with the hope of throwing some light on the mechanism itself. We have examined a total of 25 different muscles, five from vertebrates, the remainder from three different species of insects and six species of crustacean. The argument we use is that those structures which are truly fundamental in striated muscle should be common to all of them. Evolution

* With the collaboration of Patricia Anne McNeil, Benjamin Walcott and Allen Selverston.

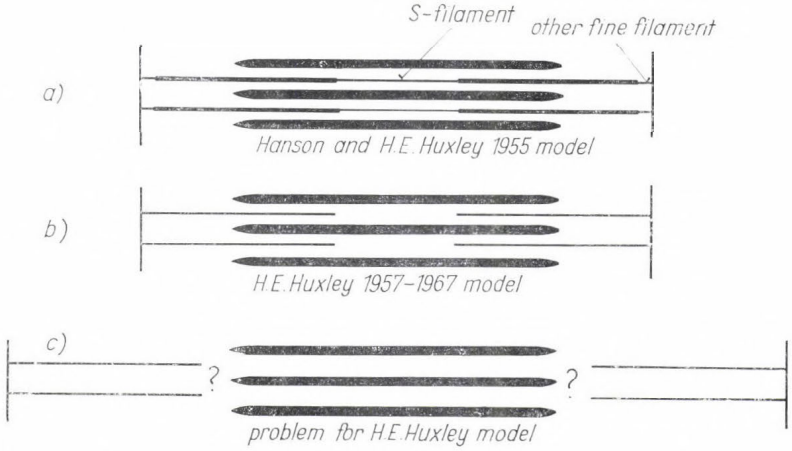

FIG. 6. — Models of muscle ultrastructure (see text)

has provided an important method for testing the validity of so-called universal concepts.

Two immediate difficulties are encountered in regard to Huxley's structural model. Firstly, when this model (Fig. 6) is stretched beyond about 40 per cent it falls apart. No actual striated muscle fibres we have tested tear at this degree of stretch. This might be because the sarcolemma and internal membranes hold the pieces together. For instance, the sarcoplasmic reticulum (SR) might have elastic properties. However, we should like to point out that it seems to us that relatively few striated muscles are composed of fibrils. The fibres are permeated, to a greater or lesser extent, by SR, but we find that this rarely forms the continuous 'sausage skin' envelope around clusters of filaments which is customarily implied for a length of more than a few sarcomeres. Also, SR is almost absent in some very slow striated fibres (Hess 1965). To test this possibility Allen Selverston, in my laboratory, isolated and stretched single glycerinated fibrils in the presence of ATP.

Selverston found that barnacle fibrils can be stretched in the presence of ATP to well beyond the overlap point. The lengths of the sarcomeres in such a stretch are not equal, some stretching much more than others and a few not at all (Fig. 7). The most heavily extended ones reach almost 3 times their rest length. The sarcomeres are apparently held together by very fine elastic filaments (Fig. 8).

In order to explain similar and related findings, especially elasticity of fibrils from which myosin had been extracted, Hanson and Huxley proposed the existence of elastic filaments. These were termed S-filaments and they were incorporated in a structural model. In Hanson and Huxley's illustration of the arrangement of the filaments during an isometric contraction (Fig. 5 in Hanson and Huxley 1955) an additional elastic filament, to which no letter has been given, is shown, connecting the thin (actin) filament to the Z-band. S-filaments were also included in the model

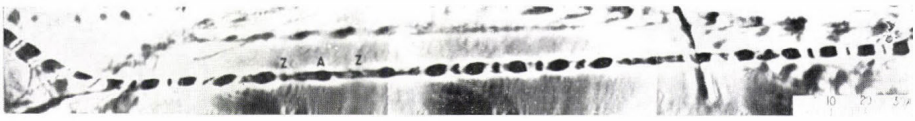

FIG. 7. — Single, glycerinated fibril from *B. nubilus*, partially split and stretched in the presence of ATP

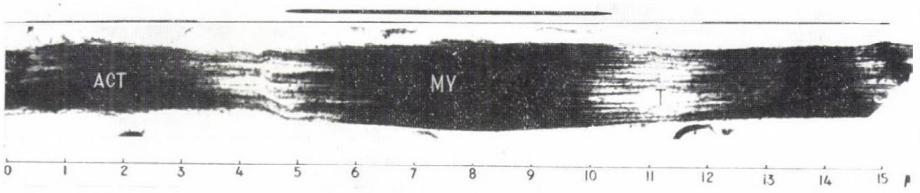

FIG. 8. — Electronmicrograph of sarcomere of *B. nubilus* fibril stretched beyond overlap point (see Fig. 7)

of A. F. Huxley. However, in his definitive account of the structure of rabbit psoas muscle, H. E. Huxley does not ever mention S-filaments (H. E. Huxley 1957), nor in several subsequent accounts (e.g. in *The Cell*, ed. by Brachet and Mirsky, 1960) was any discussion offered regarding the reasons for suddenly changing the model. Mysteriously, however, there are passing references to S-filaments in Page and Huxley (1963). Apparently, the reasons were: a failure to observe them in the electron microscope, coupled with the observation that in the heavily contracted muscle the actin filaments overlap in the center of the H-zone, which it would be hard for them to do if linked at their ends.

In our work, which we hope to publish in detail as soon as individual studies have been completed, we have noticed fine filaments not only in the gaps between the actin and myosin of highly-stretched sarcomeres (examples in Figs 9, 10 and 11) but also extending across the H-zones (Figs 10 and 11). Also, in very highly-stretched fibrils of barnacle a zone of low density appears between the Z-band and I-band filaments (Fig. 9). This suggests a partial detachment of actin filaments from the disc region and continuity by another, thinner, filament. We have seen no evidence of detachment in fibers fixed during isometric contraction as was suggested in Fig. 10 of the paper by Hanson and Huxley.

I-bands, especially of fibers fixed under stretch, also appear to contain very fine filaments lying in parallel with the ordinary thin filaments. We have considered many possible ways in which these fine lines could be developed as artifacts, but no single artifactual explanation fits all the cases.

Accordingly, I wish to propose that striated muscle contains, in addition to actin and myosin, a third filament located in parallel with them. I do not consider this to 'link the ends of the actin filaments', except in so far as it may do so indirectly because of adhesion to the actin, so that it cannot be identified with the hypothetical S-filament. Rather, it continues from Z-disc to Z-disc, perhaps even from insertion to insertion.

FIG. 9.—Fine filaments (T) in gap regions of sarcomeres stretched beyond the overlap point. Heavily stretched rabbit psoas fiber. The inserts at upper right show a single actin filament (A) and a single T-filament (T)

I propose to denote it by the capital letter T (for very thin). A diagram of the proposed location is shown in Fig. 12.

A close inspection of the micrographs published by H. E. Huxley (1957) suggests to me that fine filaments other than actin are visible in them, especially in the H-zones. Unfortunately, T-filaments are extremely thin, and neither very electron-dense nor readily-stainable with heavy metal

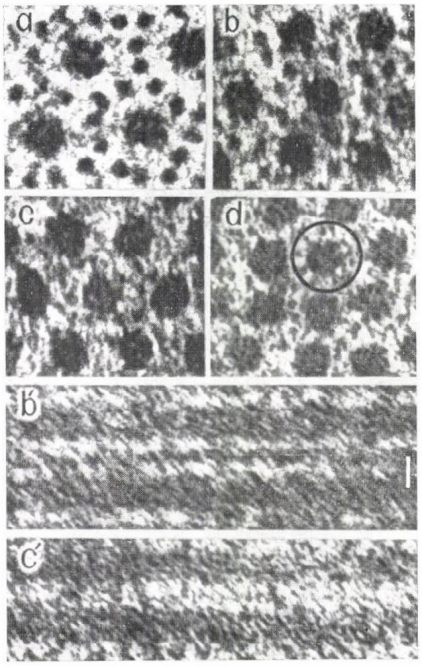

FIG. 10.—T-filaments in an insect fiber from the anterior coxal adductor of *Schistocerca gregaria*. *a* T. S. at rest length, *b*, *c* T. S. of same fiber, slightly stretched, in overlap region (*b*) and H-zone (*c*), *d* T. S. in presumed previous overlap region of heavily stretched fibers. Orbit of T-filaments is encircled, *b'* L. S. of region comparable to *b*, *c'* L. S. of region comparable to *c*

ions, so exact work on them is proving difficult. In our own work measurements of the thickness of T-filaments have been made by making recording densitometric measurements on electron-microscope plates in gap regions of sarcomeres stretched beyond the overlap point (Fig. 13). A very narrow slit was used. The measurements are bound to give higher than the true values, owing to the obliquity of alignment of the filaments, kinks, photographic grain, adhering particles, etc. The measurements fall in the range of 15–40 Å (Fig. 14); the higher values can be expected to be in error by up to 100 per cent. They are slightly larger in less heavily stretched material than in the most extended. The very thin filaments lying between the myosins in the H-zones of sarcomeres at rest length appear 20–40 Å thick. It would seem obvious that to study these filaments one should simply dissolve away the myosin and examine H-zones of the fibers electron-microscopically. When this is done, however, different results are obtained depending upon the speed, and extent of extraction, the degree of agitation, etc. (Ridgway and Walcott, unpublished.) Many observers have noted only isolated I-bands, with residual Z-discs in such material. Walcott and Ridgway, in my laboratory find that rabbit psoas muscle shortens during extraction of myosin by solutions containing ATP, if free to do so. The former material contains many

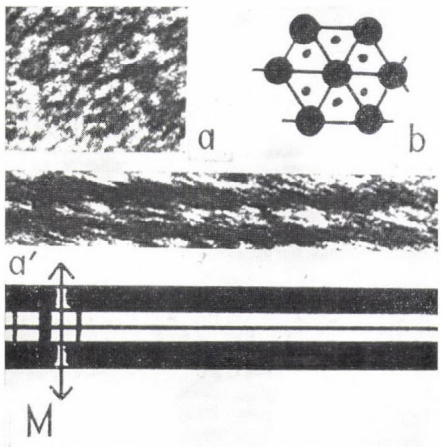

FIG. 11.—T-filaments in rabbit psoas. *a* T. S. M-band region, *b* diagram to explain appearance of *a*; fine filaments are considered to traverse the H-zone, passing between cross-bridges formed between myosin filaments; *a'* L. S. with interpretive diagram. Arrow shows region of section

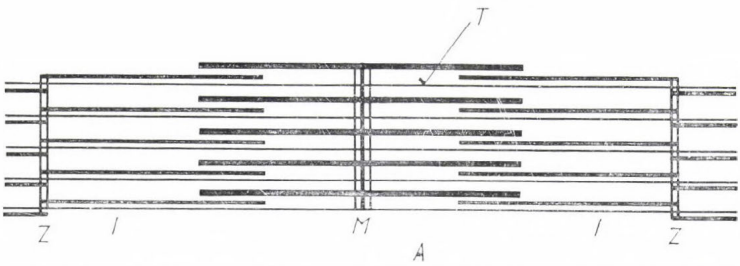

FIG. 12.—Structural model of a sarcomere including a third (T) filament

sarcomeres which are still relatively intact, and at various lengths, so that stages between actin/myosin overlap and extensions may be compared. In stretched sarcomeres the H-zones are seen to be bridged by fine filaments. It is difficult to see how the contraction which occurs during extraction can be explained in any other way than by a shortening occurring in an element such as the T-filament. Shortening which occurs in rigor mortis may require a similar explanation.

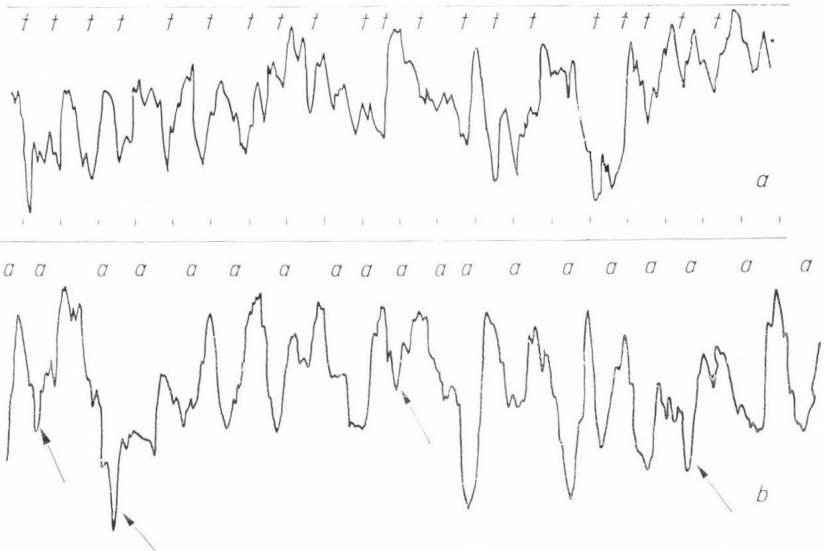

FIG. 13.—Parts of recording densitometric analysis of plate from which Fig. 9 has been printed. *a* Trace along the direction X- - - - - - - - -X: the portion shown is at left in the micrograph. Horizontal line corresponds to minimum light transmission, increasing downwards. Marks below tracing are at intervals of 100 Å. At points marked *t*, fine lines (T-filaments?) may be seen on the print; *b* Ditto, along the direction Y- - - - - - - - -Y, also the left-hand edge. Note the clean, broader peaks, each corresponding to the appearance of an actin filament in the print. Arrows point to sharp deflections which may represent T-filaments overlying A-filaments

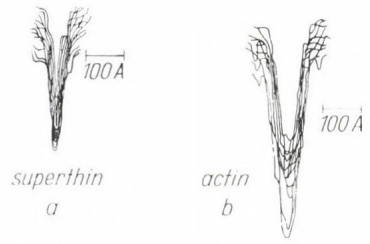

FIG. 14. — Superimposed traces of the clearest peaks obtained in the recording part of which is shown in Fig. 13. *a* Peaks occurring in the gap region and attributed to extended T-filaments. *b* Peaks occurring in the I-band associated with actin filaments

The real thickness of the T-filaments may be expected to vary with the degree of stretch, though the extent may be quite small, depending upon their chemical constitution and the manner in which they shorten. At their sharpest and thinnest they appear to be less than 20 Å in diameter. This thickness would be appropriate to a protein structure with a double or triple α-helix. They could, for example, be collagen-like. It may be worth while to point out that tropomyosin-B, which is present in significant amounts, perhaps as high as 15 per cent of total protein (Perry 1964), may have a double α-helix structure (Rowe 1964) and be about 15 Å in diameter. The location of tropomyosin-B within the fibril has not yet been determined, though recent evidence places it in parallel with actin, to which it binds readily (Pepe 1966).

I would like also to comment on a number of other aspects of structure. Firstly, regarding the Z-discs. It is already clear to us that there is not only one, but several different kinds of Z-disc fine structure. One kind of fast striated fibers of a copepod has recently been found by Dr Pat Dudley (and confirmed by ourselves) completely to lack a dense line in the Z-regions (Fig. 15). We are working together on the detailed arrangement of filaments in the center of the I-bands in these fibres. Z-discs of the rat diaphragm have been shown by Reedy (1966) to have a woven arrangement of fine filaments, but, although we have seen this pattern also, we find a simple window or cross-lattice arrangement to be commoner (Fig. 16). In this arrangement, fine links connect each actin filament of a square set of four by the shortest route, not diagonally across to an actin filament from the next sarcomere as in the model of Reedy. Sometimes, the two patterns occur mixed together in patches in the same disc.

Regarding ordinary thin (actin) filaments, they are present in rabbit psoas in a ratio of 2 thin to 1 thick (myosin). Arthropod striated muscles commonly have ratios of 3 : 1, 4 : 1 or 5 : 1 and we have found one crab muscle in which it is 7 : 1. Those with a 3 : 1 ratio show the familiar insect flight muscle pattern, with one actin in between each myosin. Where the ratio is higher than 3 : 1 there is less order, though in the 4 : 1 (10 actins per orbit) and the 5 : 1 (12 actins per orbit) there are occasional neat orbits. Rather, they occur in lines, showing the square pattern which is commonly found near Z-discs; myosin filaments are placed irregularly among them (Fig. 17). This pattern was found in the pink portion (there is also a white portion) of the tiny muscle which raises the very long (up to 10 cm) eye-stalk of the crab *Podophthalmus vigil*. I was attracted to study this muscle because although it is very short (2–3 mm) it has to produce extremely precise length changes in order to enable the eye to follow a moving object. To do so, it must operate

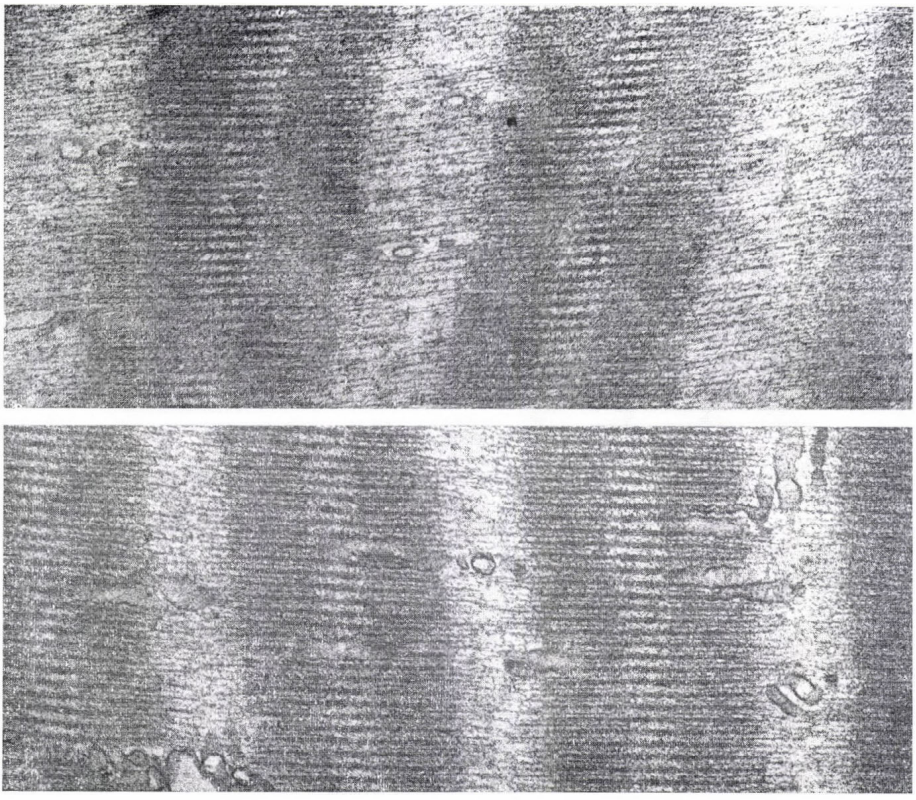

FIG. 15.—Electron micrographs of striated muscle sarcomeres lacking marked Z-discs. Antennal rotator of 47 1/2 h nauplius of *Dorcpygus seclusus*. Material supplied by Dr Patricia Dudley

against a large inertial load at a great mechanical disadvantage. The implications of the high actin–myosin ratio in this specialized muscle for theories of contraction are obviously of considerable interest.

The A-band lengths of the fibres from the various muscles studied range from about 1·3 μ to 12 μ. We find that in no fibres are sarcomere lengths as fixed as is usually stated. For example, in rabbit psoas we have found sarcomeres with A-bands varying from 1·3 μ to 2·0 μ.

It has been possible for us to prove conclusively (Hoyle et al. 1965) that the Z-discs of striated fibers of *Balanus nubilus* expand, probably by an active process, in association with contraction (Fig. 18), creating spaces within the disc through which thick filaments are able to pass easily. The filaments from adjacent sarcomeres interdigitate across the Z-region, thereby permitting contraction down to a small fraction of the rest length. We have recently observed comparable passing of thick filaments across the Z-region in sarcomeres of the anterior rotator of the swimming leg of *Portunus sanguinolentus* (Fig. 19). These occurred during an isometric contraction I shall refer later to the significance of uneven contractions of sarcomeres.

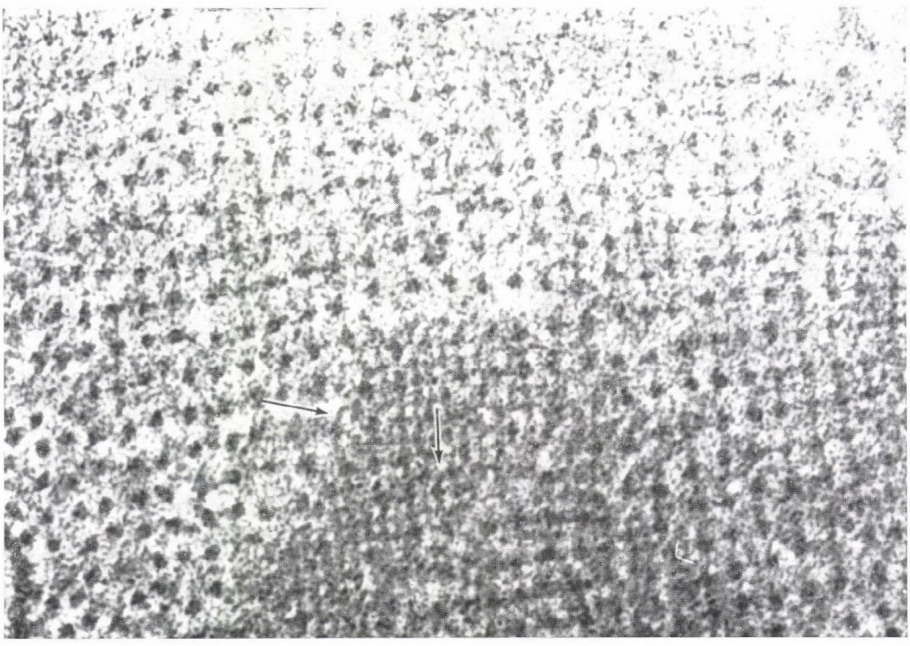

FIG. 16.—Square 'window' lattice of Z-discs seen in transverse section. Actin filaments of one half-sarcomere are situated exactly in the centers of those of the contiguous half-sarcomere. Very fine filaments continue across the disc from the terminating actin filament. Fast fiber from garter snake

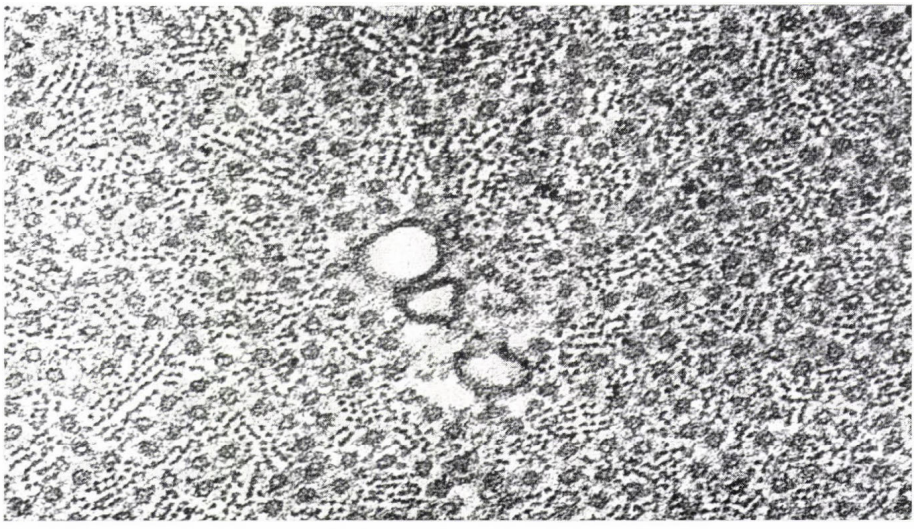

FIG. 17. — Transverse section of the overlap region of a fiber having a 7 : 1 actin – myosin ratio, from pink portion of elevator of the eye-stalk of *Podophthalmus vigil*

The point about giving you these preliminary details is to show that even striated muscle exists in a wide variety of forms at the level of fine structure. It may be unwise to assume that the features found on the first fibre to be investigated in detail should be expected to serve as a basis for a universal hypothesis, and it seems desirable to proceed by making comparative studies.

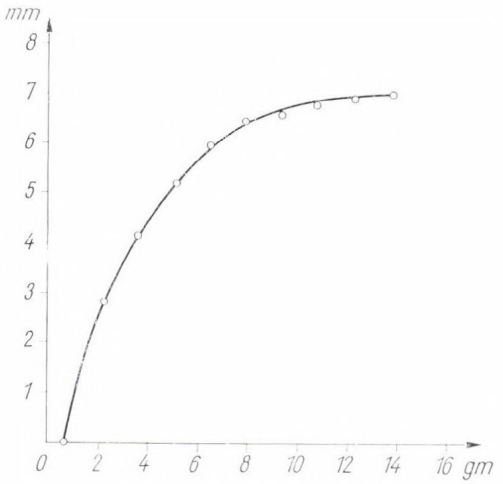

FIG. 18.—Stress–strain curve of series elastic component of a single fiber of *Balanus nubilus* stimulated to maximum contraction. Fiber length 41 mm. The maximum length of elastic component is 17 per cent of the rest length of whole fiber

The initial reason for embarking on the electron-microscopic studies referred to above was that physiological studies on single arthropod fibers had revealed features which were not compatible with the simple model. For example, the great extent of easily reversible contraction; this has been simply explained on the basis of opening up of Z-discs. Another major problem concerned the series elasticity, both of resting and active muscle (Hoyle 1965). Single fibers possess longitudinal elasticity at lengths at which the sarcolemma is markedly kinked. This was one reason for the postulation by Hanson and Huxley of the S-filament. Following excitation of the fibers, the contractile component shortens against a series elastic (SE) component. There are standard methods for measuring the length and stress–strain curve of this component; in fast frog muscles it is only a few per cent of the total length and is considered to be entirely accounted for by tendinous elements. In some arthropod fibers, by contrast, I find the apparent length of SE at full stretch under isometric tetanic force to be up to 20 per cent of the total length (Fig. 20). In the same fibers the tendons snap when stretched by only 0·5 per cent. The elastic component must therefore be located within the contractile portion of the muscle and it is much too long to be accounted for by Z-discs. The extent of distortion of internal passive elastic elements required to develop tensions equal to those developed during stimulation is so large, that it should easily be detectable. In an attempt to measure the expected shortening we have rapidly fixed a few kinds of muscle fiber with glutaraldehyde whilst stimulating them to develop graded or maximum tension. The fibers were held at rest length and force was monitored continually. Some interesting features could be observed in the fixed material, especially that obtained when the final tension developed at the moment of total death of the fiber was sub-maximal. These features were found in both vertebrate and invertebrate muscles. It is apparent that at the moment of fixation there are variable lengths of individual sarcomeres of single

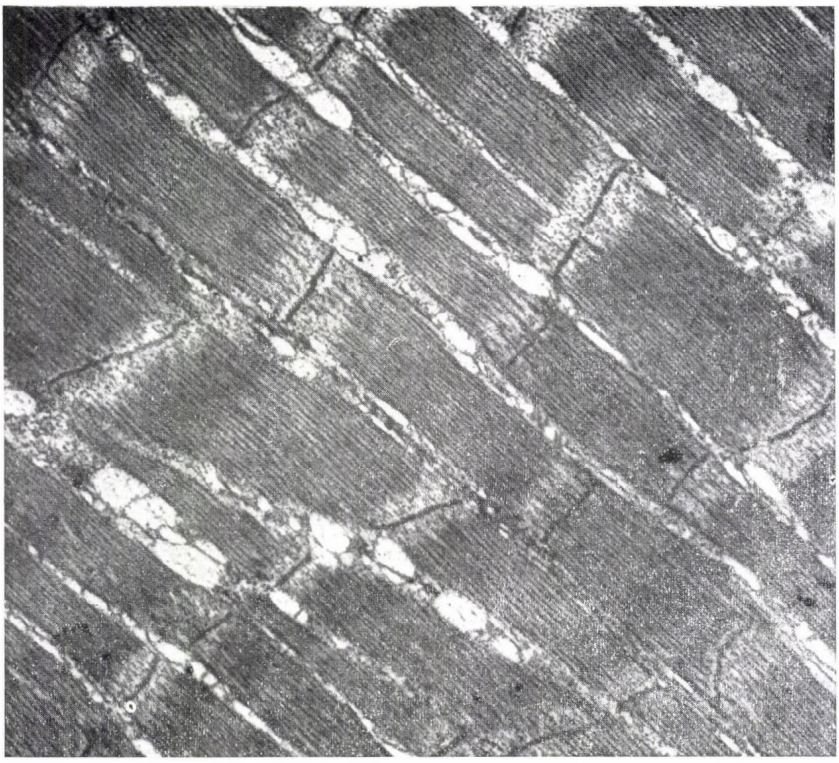

FIG. 19. — L. S. crab muscle fiber (light pink portion of anterior rotator of swimming leg of *Portunus sanguinolentus*) stimulated throughout fixation to give a large graded contraction

fibrils. This was also found by Page and Huxley (1963) for vertebrate fibers. Also, all fibers we have examined show distortions of the A-band which fall into a few catagories such as I have described in a recent paper (Hoyle 1967). The results amply fulfil the prediction that the elastic element is associated with the sarcomeres and is stretched by their unequal contraction. We may now consider the extents to which each of the possible sources may contribute. A structure of the S-filament type could have played the major role, though by the time I had made the elasticity measurements S-filaments were said not to exist. This led us to make the electron-microscopic investigations in which T-filaments were found.

I next considered the question whether the variation in lengths of the sarcomeres was sufficient to allow a simple passive role of the T-filaments in force transmission. In order to develop a force of 75 per cent P_{max} by passive stretching, even when aided by the sarcolemma, a whole fiber must be stretched to over 40 per cent l_0. The total equivalent stretch of sarcomeres in the fixed material was obtained by adding together the lengths of sarcomere greater than l_0, as seen in electron micrographs, and determining the fraction of the total length. This is only about 18 per cent,

FIG. 20. — L. S. garter snake muscle fiber (ribs-skin) stimulated by action of fixative. Note resemblance to Fig. 18

much less than the stretch required to develop tension equal to the force of the contractile elements.

Furthermore, there should be a simple relationship between the amount of graded tension and the extent of distortion, which should be greatest at highest tensions. This is not borne out, for at highest tensions there is rather less distortion. Two possible explanations will be considered, both invoking changes in the T-filaments associated with excitation. One is that stretches occur in parts of the T-filaments, which are not also reflected in over-all changes in length of actin and myosin filaments, i.e. the T-filament is an independent element, subject to local shortening and stretches which are temporarily stabilized during continued stimulation. Temporary changes in length of the sarcomere, or of one set of filaments would be needed to bring about this situation unless the T-filaments are themselves

contractile. The other possiblity is that the T-filaments undergo a change in their elasticity upon stimulation, tending to shorten. Thus, both alternatives may invoke active contractility on the part of the T-filaments. In that case they may play a role normally in the development of tension. All the major recent concepts of the molecular basis of contraction arise from the widespread acceptance of the newer H. E. Huxley model in which actin and myosin are stated to be the only longitudinal structural components, so that force must be postulated to be generated by interaction between them, unless by active lateral expansion of the fiber, which for several reasons is improbable.

Acceptance of the presence of T-filaments immediately raises the possibility of their active involvement in contraction; even without the above requirements, our present evidence for T-filaments may be summarized as follows.

(1) Elasticity is present at lengths which do not stretch the sarcolemma.
(2) Elasticity is present in fibers from which the sarcolemma has been dissected away. In some kinds of fiber the sarcolemma contributes only 15–20 per cent of the elasticity (Buchthal and Weis-Fogh 1956).
(3) The length of the series elastic component in maximally stimulated arthropod fibers is as great as 14 per cent of the fiber rest length. It is even greater during sub-maximal stimulation. The tendinous insertions of these fibers are inelastic, so the individual sarcomeres must be a major source of elastic material. Since myosin and actin filaments are inelastic, another component must provide the elasticity.
(4) Sarcomeres of all striated muscles tested, both vertebrate and invertebrate, can be stretched to beyond the overlap point of actin and myosin without disrupture.
(5) Fibrils from which myosin has been extracted are very extensible and elastic.
(6) Fine filaments may be detected in electron micrographs in the gap region between the actin and myosin of heavily stretched fibers, whatever the extent of stretch.
(7) In electron micrographs similar fine filaments may sometimes be seen in the H-zones, lying between thick filaments, in both longitudinal and transverse sections.

Although the evidence for T-filaments is by no means complete yet, I nevertheless feel that it is already compelling, and consider it to be worth while to discuss the possible role of T-filaments. A passive role as an elastic element is highly probable. It may be that in resting muscle T-filaments are already somewhat stretched as a result of stretched antagonistic radial components, also elastic, tending to reduce the diameter of the fibril and cause its elongation. For reasons given above, an active role in contraction also seems possible, and we should consider the possibility that T-filaments might even play a major role in contraction. A similar filament might be the basis of contraction in smooth muscle, and of contractile elements of cells other than muscle cells in which actin and myosin are not found. All the difficulties which have been encountered in explaining contractility in smooth muscle would disappear if a contractile very thin filament occurs in them.

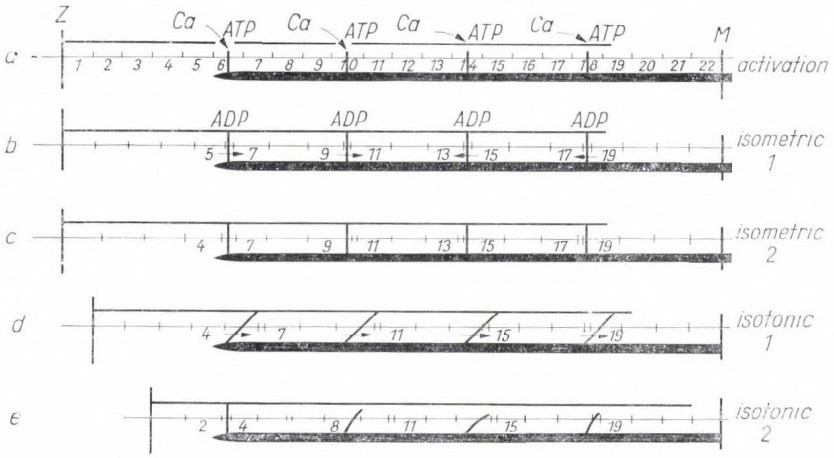

FIG. 21.—Hypothesis of contraction involving cross bridges between actin and myosin as enzymatic elements which may also contribute to force. Conformational changes in T-filaments cause shortening. *a* Under isometric conditions neighboring segments of the elastic T-filament are stretched. Net directions of stress are indicated by small arrows. Equilibration of the tensions will bring other regions of the T-filament within range of specific active sites.—Isotonic contractions result in shortening, which bends the bridges inwards (*d*) until they break (*e*), springing back to re-form

For striated muscle I would like to propose the involvement of the T-filaments in the form of a hypothesis. A full statement will be presented elsewhere.

At the start of a contraction cross bridges form between inelastic actin and myosin filaments when free calcium ions are present (Fig. 21). The Ca-actomyosin complex thus formed possesses its characteristic strong ATPase activity and causes the splitting of nearby ATP molecules. The energy thereby liberated causes in turn a configurational change in the nearby T-filaments, where a unit length then shortens. The force developed is transmitted via the remainder of the T-filament to the Z-discs, which are drawn inwards, producing the relative movement of contraction. The T-filaments provide, on this hypothesis, the source of length change. The force developed, or resistance to stretch, is shared by the T-filaments and by established cross bridges.

The ends of the bridges attached to the actin will become turned inwards, towards the center of the sarcomere. The bridges are presumably elastic, but when stretched beyond a critical point the attachments to actin will break. They will then reform attachments at different sites on the actin and the process will be repeated. The conformational change in the T-filament will persist as long as the calcium ion concentration is sufficiently high.

The only direct evidence that can be quoted at the present time, which may be considered to support the hypothesis, is the finding of Reedy and his co-workers (1965) that in insect flight muscle fixed in a state of contraction, the

cross bridges are turned inwards from the myosin filaments. This is the opposite direction to that required of bridges which themselves generate the shortening, unless they function by rotating, as these authors propose to explain their finding. In flight muscles T-filaments may be seen crossing the H-zone (D. S. Smith, B. J. Walcott, personal communications; see also Fig. 5 in Reedy and co-workers 1965).

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GUBA: In connection with Dr Hoyle's contribution I should like to call your attention to our findings concerning the ultrastructure of myofibrils in selective extraction of proteins (Guba 1964). We have found that after the removal of myosin and tropomyosin from the myofibrils a sheave-like structure remained with the Z-disk in the middle. These features show a great similarity to the isolated I-bands (Fig. 22). The continuity of the sarcomere is still visible but the diameter of thick filaments decreases considerably. The removal of actin abolishes the sheave-

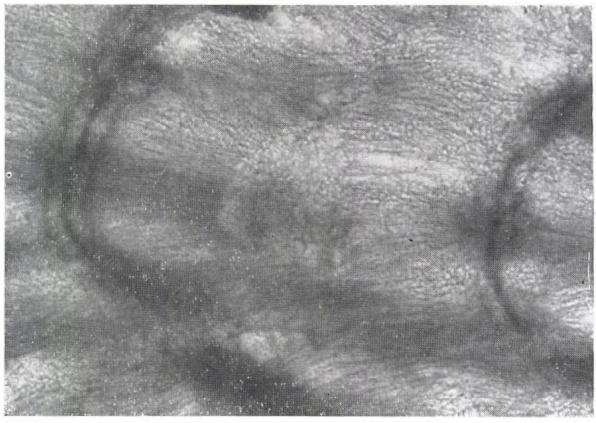

FIG. 22.— ×36 300

like structure, but a uniform filamentary system continues running through the whole sarcomere (Fig. 23). The electron density is very low. The filament diameter is less than 50 Å. On examining the material of these filaments, they seem to consist of a new protein. This backbone protein is named fibrillin. On the removal of fibrillin, the filamentous system left behind after extraction of myosin, tropomyosin and actin is completely destroyed. On the basis of our observations, we have drawn the conclusion that there is an ultra-thin and continuous filamentous system in the myofibril of rabbit m. psoas. This conclusion is in agreement with Dr Hoyle's observations, though we think that these filaments, like those found in the insect muscle, give a highly elastic core of primary filaments.

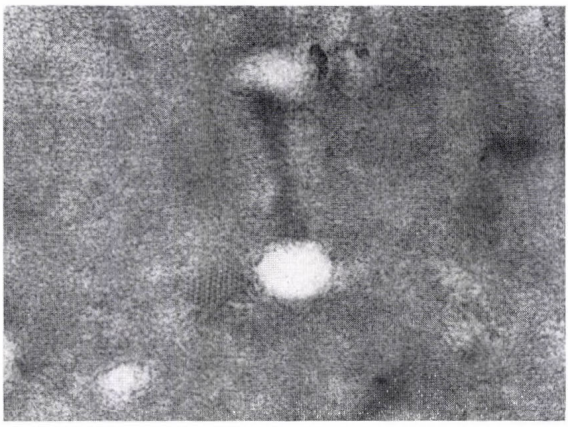

FIG. 23. — × 70 000

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EDMAN: We have recently made some measurements of the series elastic component in isolated semitendinosus fibres of the frog, which may be of interest in this context. The purpose of the experiments has been to find out to what degree the mechanical properties of the series elastic element are dependent on the state of activity of the contractile unit. The measurements which I am referring to were carried out at 2·15 μ sarcomere spacing at +4° C. Great care was taken to reduce the stray compliance of the recording device and its connections with the fibre. In the conditions given there was only 4–5 per cent reduction in the sarcomere spacing when the fibre was transferred from rest to full activity. For further technical details see Edman (1966) and Edman and co-workers (1966).

The rigidity (dP/dL) of the series elastic element was determined by recording the drop in tension that occurred in response to a rapid controlled release of the fibre at various tension levels (P). Figure 24 shows dP/dL plotted against P for one fibre. The open circles refer to measurements carried out during the rising phase of the tetanic contraction, i.e. during maximum activity. The open triangles are recordings performed during the rising phase of the twitch. The filled circles and the filled triangles are measurements made during the decay phase of the tetanus and the twitch, respectively, i.e. at instances when the intensity of the active state was very low.

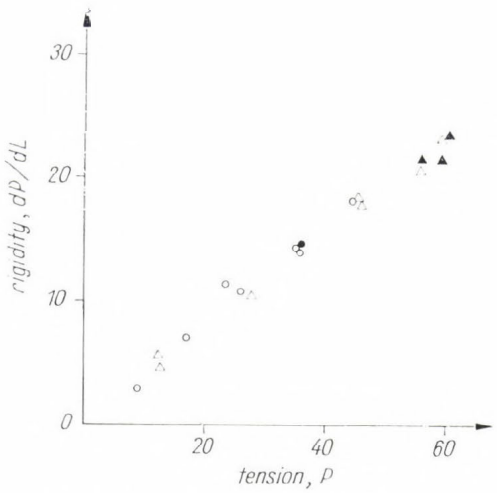

FIG. 24. — Rigidity of series elastic component of the isolated semitendinosus fibre of the frog. Ordinate: drop in tension (per cent of maximum tetanic output) in response to a controlled quick release. Abscissa: tension level (per cent of maximum tetanic tension) at which release is performed. Symbols: see text

As can be seen, there is no substantial difference between the data obtained during twitch and tetanus. Furthermore, the rigidity of the series elastic component, at a given P, is the same irrespective of whether the measurement is made during the rising phase of the contraction or during relaxation. The results thus seem to make evident that the mechanical properties of the series elastic element are virtually independent of the state of activity of the contractile unit. A very small portion of the series elastic compliance of the fibre is, therefore, likely to reside in the active, force-producing structures of the contractile system.

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HOYLE: The classical work of A. V. Hill has clearly established that in certain fast amphibian twitch muscles stimulated maximally by plate electrodes there is relatively little series elasticity. Most of this might be accounted for by tendinous attachments. In the invertebrate muscles and slow snake muscle to which I have referred, the excitation is not made as completely or as synchronously. As to why we should find an effectively long elastic element in the invertebrate fibers even when they are maximally stimulated, I can only speculate. It may be that the T-filaments are unevenly shortened and stretched, owing to a synchronous early excitation; this inequality would be temporarily stabilized by cross bridges between actin and myosin. Upon releasing the muscle from isometric contraction, the heavily stretched portions would be free to shorten, elastically. Perhaps activation introduces a component, such as cross bridges, which are inelastic, thereby masking the appearance of changed elasticity in the contractile component. In an incompletely activated fiber the latter would be more apparent.

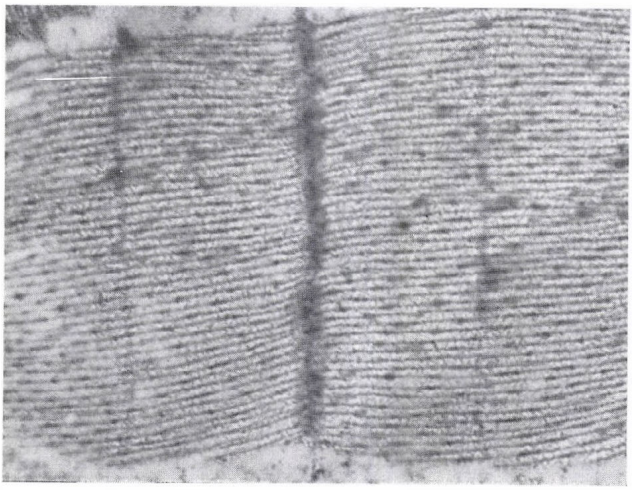

FIG. 25

RICE: Why do you use the term 'collagen-like' for your elastic fiber? Collagen is very inelastic.

HOYLE: I agree that it is not very appropriate and suppose that I was unduly impressed by the remarkable demonstration of large reversible length changes given by Dr Oplatka, in treated collagen strands. The basic molecular unit of collagen, interpreted as Collagen II, with its triple α-helix, is the kind of structure which one might expect to find in the T-filaments, although the basic unit would have to be much more extensible than collagen is.

ERNST: As to the conception concerning the two independent components of a sarcomere, i.e. thick and thin filaments, I want to show the following slide (Fig. 25). The thorax-muscle of the honey bee was prepared in Pringle's solution, put in a mixture of CO_2 + aether sulphuricus (approx. $-70°$ C). After thawing, it was fixed, dehydrated and embedded in araldit, the sections were stained with phosphotungstic acid. It is to be seen that thin filaments continuously go through the Z-lines, as was shown by us earlier (Ernst and Benedeczky 1962), on the one hand, and that the material of the so-called thick filaments is as if it had split into several parts localized without exception along the thin lines. By this I should like to remind you of my standpoint, according to which I had raised the question 'whether our electron-microscopic pictures really show the structure; namely, the picture is one thing, and the structure of three dimensions is another'.

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RINALDI: Judging from the model for contraction proposed by G. Hoyle, I cannot find any evidence which is in contradiction with the sliding filament theory advanced by H. E. Huxley and J. Hanson. There does not appear to be any disagreement in the newly proposed model as it is a sliding system, and a change in conformation with the proteins involved has been introduced by H. E. Huxley, earlier at this meeting, as an integral part of contraction.

The comments that types of muscle, differing from a variety of organisms, which appear not to fit the conventional characterization of the sliding filament model, do not agree with the interpretation of an even more unconventional system, that of the foraminiferan, *Allogromia laticollaris*, either.

This organism extends pseudopods from its body in a network as far as 17 mm into the environment. These pseudopods exhibit a two-way motion within them, which reaches speeds of 15 μ/sec, corresponding to what H. E. Huxley has indicated to be a theoretical maximum for filaments sliding past one another. Granules can be observed approaching each other from opposite directions; they bump into one another and then continue their motion towards the tip or body of the organism.

Electron-microscopic studies have revealed that these pseudopods are composed of filaments of varying sizes. The range of sizes is from 180 Å to 35 000 Å in diameter. More important, however, is the fact that the filaments form a bridge from one to another. Studying the kinetics of this system, coupled with its morphology, which is certainly unconventional in the sense of the specific array of the skeletal muscle, one is forced to conclude that the basis for this motion is that of one fibril past another, thus supporting the earlier proposal for fibrillar motion postulated by the sliding filament hypothesis in a very unconventional muscle system.

HOYLE: Contractile systems must have evolved from a common element present in animal cells. Doubtless, the muscle field would benefit if more attention were paid to such non-muscular contractile systems. I think it is already apparent that the common element is likely to be a set of very fine filaments. The orderly array seen in striated muscle would be concerned with improving the synchronization and therefore speed and force of contraction in the elemental unit.

LAKI: Glycerol-treated muscle fiber contracts when placed into neutral Nessler's reagent. In these conditions it is the I-band that shortens.

In this reagent the fibers can be stretched 3 to 4 times of their original length and may be set at that length by transferring the fibers into water. In this stretching procedure essentially the I-bands elongate, and the extent of stretch is so great that it is unlikely that there is an overlap between A- and I-band filaments. Nevertheless, such elongated, set fibers contract and lift weight when put back into the neutral Nessler's reagent. However, while under load the elongated fiber contracts only to its original length, it appears that when the filaments begin to interdigitate the energy is dissipated and thus only the unloaded fiber can contract. Apparently, in this reagent the sliding of the filaments instead of being responsible for generating force, dissipate it.

DAVIES: Please, Prof. Hoyle, do not use the term configurational change which in chemistry refers to changes in bonds as in D-L transformations.

The correct term is 'conformational change' which refers to rotation around bonds as in α-helix formation.

HOYLE: I shall be glad to adopt whatever terms are acceptable to the chemists. [My use of 'configurational' derives historically from the paper by Jean Hanson and H. E. Huxley (1956).] Even biochemists have used 'configurational' (e.g. Mandelkern and co-workers 1965).

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WILKIE: I am not quite clear about some of the details of your theory. Can you please tell me how it is possible to have two different types of cross link, one for developing tension and the other for shortening? I do not see how they can be arranged inside the muscle in such a way that shortening and force are kept separate. When the muscle contracts, and lifts a load, it seems to me the links that shorten must also overcome the full external force.

HOYLE: The theory does not call for two different types of cross link. On this theory both shortening and force must be developed by a conformational change in units of a continuous thin filament. However, the initial step is the establishment of cross links between actin and myosin. Such links will add to the stiffness of the muscle, and sum with the subsequent changes in longitudinal elements in resisting stretch, except when the links become bent inwards towards the center of the sarcomere as a result of the local shortening. On this basis, the tension-rise, which starts with the establishment of cross links, will occur in advance of length changes. We have found such a separation experimentally, in single crustacean fibers.

HUXLEY: It was not completely clear from Professor Hoyle's lecture, in which muscles and under what conditions he observes his 'super thin' filaments. Specifically, could he say whether he observes such filaments in rabbit and frog striated muscle when there is still overlap between the thick and thin filaments? Can he see super thin filaments in cross-section near the centre of the A-band in moderately stretched frog and rabbit muscle? In circumstances where he sees filaments in the gap formed by the extensive stretch of vertebrate muscle, what is the number of these filaments per fileal relative to the number of thin and thick filaments?

HOYLE: We believe that we can see T-filaments in all of the different kinds of muscle examined, at all lengths. This included rabbit psoas and sartorius and frog sartorius. Unfortunately, in no single case are the filaments outstandingly clear, although most observers who have examined all our electron micrographs of each muscle have been satisfied that they are there.

In frog and rabbit muscles which have been moderately stretched one can often see a faint orbit of very thin filaments in the H-zone. In the M-band region cross bridges between the myosin filaments lead to the appearance of six triangles around each myosin. In some places a small dot, which may

be the T-filament in transverse section, appears in the center of each triangle (see Fig. 11 on p. 38).

The number of superthin filaments in the gap regions of most of the material is almost exactly equal to the number of actins seen in the I-band. Also, the dots seen in the H-zone orbits are similar in number to the actins in normal overlap zones. However, there are fewer T-filaments than actins in the *Podophthalmus* eye raiser. Thus, it seems that the number of T-filaments may commonly, though not always, be similar to that of actin filaments. However, we do not wish to be too firm yet about these figures and keep hoping to find a method which will stain them more satisfactorily.

JOHNSON: I believe that there is a further difficulty in the operation of your model as you have shown it here. Dr Huxley has shown that, as shortening proceeds, the actin filaments from opposite sides of the sarcomere meet in the center and then pass each other. If a connection exists between the ends of the actin filaments and if this connection undergoes a conformational change which leads to shortening, one would expect that the ends of the actin filaments would be unable to pass each other unless a different mechanism is postulated for shorter sarcomere lengths. In fact, at length below that at which the actin filaments meet, your model would predict folding back of the ends of these filaments. This clearly does not occur; at least not in frog muscle.

HOYLE: On my model there is not considered to be more than a loose connexion between the T-filaments, which are independently attached to the Z-discs, or even pass right through them and the actin filaments. Thus, the latter are free to move independently, so there is no difficulty in explaining the overlap of actins during the extensive shortening.

DAVIES: Does Dr Huxley accept Prof. Hoyle's interpretation of Dr Huxley's electron micrograph? Also does he accept that Prof. Hoyle's super thin filaments actually exist?

HUXLEY and SLEATOR: What are the technical resources of your group?

HOYLE: We have been working intensively on muscle since January 1965, when we took delivery of a Siemens Elmiskop 1 Å. This instrument is giving better than 5 Å resolution. Our material is fixed in glutaraldehyde and post-fixed in osmium tetroxide, embedded in epon and sectioned with Dupont diamond knives using a Porter-Blum MT2 ultramicrotome. The sections examined are grey in colour and stained by the usual combinations of heavy-metal salts. All this should represent a significant technical advance on the situation achieved by Huxley in the work he did in 1956.

I should like to draw attention to the fact that the famous longitudinal sections of Huxley, on which our modern ideas about muscle are largely based, show a sarcomere length of barely 1 μ. The *in vivo* sarcomere length of rabbit psoas fibrils is 2·4 μ. Therefore, these sections have been compressed during sectioning by more than 100 per cent. Whilst this has resulted in a clear image of high contrast, it is nevertheless a highly distorted one.

HUXLEY: I believe Davies' question was whether I had seen any indications of very thin filaments in muscle similar to those reported by Dr Hoyle. Dr Hoyle showed a number of micrographs of various types of muscle in which he believed these filaments were visible. I was not quite sure on some occasions which type of muscle was being described, but I think I am right in saying that in muscles from frogs and rabbits the super thin

filaments were only visible in those which had been stretched to the point whereby a gap developed between the ends of the thin and thick filaments. My own observations on muscles have been restricted to those from frog and rabbit, and some insect fibrillar muscles, and therefore I cannot comment on Dr Hoyle's observations on other species. The presence of gap filaments was, however, of course reported long ago both by A. F. Huxley and by Sjöstrand, and presumably the filaments described by Dr Hoyle are the same as those reported by these other authors. I have not, myself, looked for filaments in this gap region, so I cannot speak from first-hand knowledge. I think it seems very likely that some type of filament is visible here, but whether this represents a genuine third type of filament in the muscle, or whether it represents thin tapered extensions of the thick filaments or a few thick filaments or thin filaments which have come out of register, or whether it represents some structures formed between the ends of the thick and thin filaments during fixation, still seems undecided.

I was interested in Prof. Hoyle's comments about section compression during the cutting of very thin sections. I have found that when section thickness lies below 200 Å and the sections are cut with a knife edge set perpendicular to the long axis of the muscle and longitudinal sections cut, then the flow of the plastic takes place rather, I imagine, like the flow when a lathe tool is applied to a metal block, and a very considerable foreshortening of dimensions occurs without, however, any substantial evidence of damage to the tissue. If Dr Hoyle can cut sections of, say, 150 Å thickness without such flow occurring, I would be most interested. The only reliable index of section thickness I find is the selection of single layers of the filament lattice of the type which contain pairs of thin filaments in between adjacent thick ones.

Ivanov: Dr Couteaux in his very interesting report has said that 'experimental evidence suggests that the rate of tension development in slow muscle fibres depends also on the rate of reactions taking place at the level of the myofilaments themselves'.

In this connection I should like to remind you about our work made in collaboration with Dr Strelina and Prof. Gukov, published in 1957. The single muscle fibers from so-called slow and twitch bundles of m. ileofibularis of the frog were electrically stimulated. The character of contraction was registered on moving film. After the evaluation of the character of the contraction (tetanic or slow contraction), the fibers were extracted with distilled water or 50 per cent glycerol for several days.

Then the extent and rate of contraction upon addition of ATP were determined. It was observed that in so-called tonic bundles of m. ileofibularis of the frog most fibers belong to the mixed or intermediate type. They contain in sufficient quantities probably two substrates: actomyosin and a substrate exhibiting a viscous after-effect. Depending on the character of stimulation, these fibers are capable of different types of slow or rapid contractile reaction.

However, a very low percentage of the fibers belongs to the purely tonic type. These fibers which exhibited slow contraction upon electrical stimulation contracted very slowly also upon addition of ATP. Thus it was shown that the type of contractile reaction of muscle fibers is determined first of all by the character of their protein substrates.

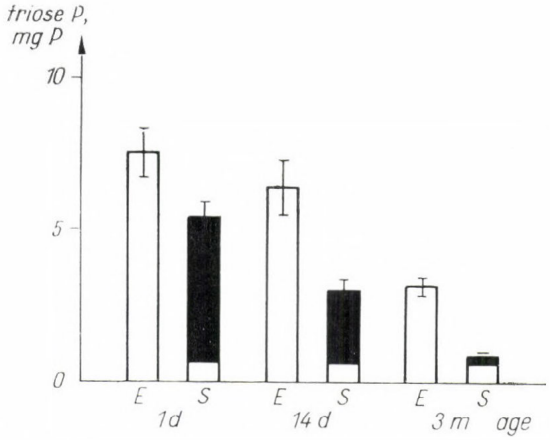


FIG. 26.—Aldolase activity of developing rat muscles

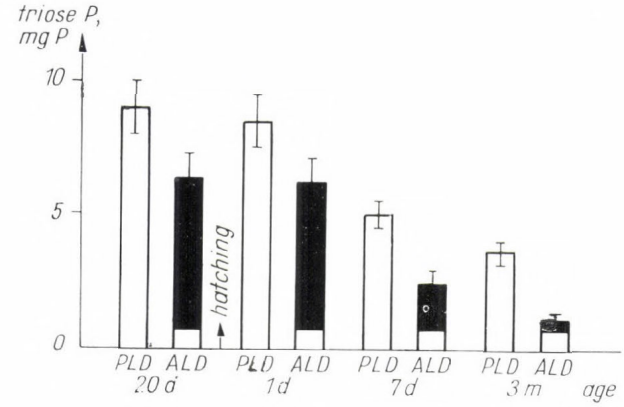
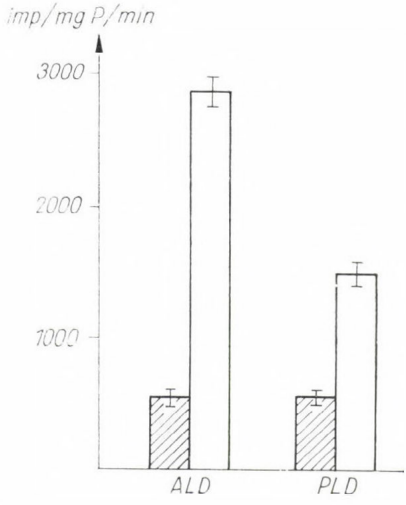


FIG. 27 Aldolase activity of developing chicken muscles



GUTMANN (Institute of Physiology, Czechoslovak Academy of Sciences): The question discussed by Professor Couteaux concerned the essential differences between slow-tonic and fast-twitch fibres of the frog and the question of different structures in these two fibres. From a physiological point of view the basic differentiation rests especially on (a) the existence

FIG. 28.—Incorporation of radioactive ^{35}S methionine into the proteins of the ALD and PLD of the chicken 1 h after intraperitoneal injection of ^{35}S methionine (200 μCi on 100 g of body weight). White columns = number of impulses per mg protein per min, in mg of precipitated protein. Black columns = activity of free amino acids in impulses per mg of fresh wet tissue

or the absence of propagated action potentials, (b) the contracture responses, e.g. to acetylcholine. — But the differentiation of slow tonic and fast twitch based only on electrophysiological findings will not help us very much to get a general outline of differentiation. However, if we compare fast and slow muscles of different animals from a metabolical point of view, it will be seen that a general metabolical differentiation of fast and slow muscles can be worked out, whatever species we are considering. The fast muscles show a predominance of the enzymes of glycolysis and this we shall find, notwithstanding whether they are twitch fibres in the classical sense or not. Figure 26 shows that aldolase activity is higher in the fast mammalian extensor digitorum longus (EDL) and in the fast posterial latissimus dorsi (PLD) in the chicken (Fig. 27). Glycogen levels are again higher in the fast EDL and in the fast PLD of the chicken. On the other hand, the slow soleus muscle of the rat has a higher turnover in proteins. Incorporation of ^{35}S methionine (Fig. 28) is increased in the slow soleus muscle of the rat (a twitch muscle) and in the slow anterior latissimus dorsi (ALD) of the chicken (a tonic muscle). This cannot be due only to the increased substrate supply or permeability, as is with the free amino-acids. Moreover, the slow tonic muscle (Fig. 29) has a higher concentration of ribonucleic acid and a higher level of proteolytic enzymes (Gutmann and Syrovy 1966). The physiological significance

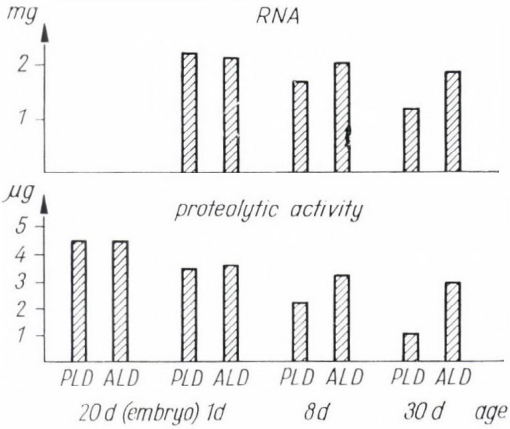


FIG. 29.—Content of RNA (mg/100 mg proteins) and proteolytic activity (µg tyrosine liberated/mg proteins) of developing chicken muscles. Each value is the mean from six animals

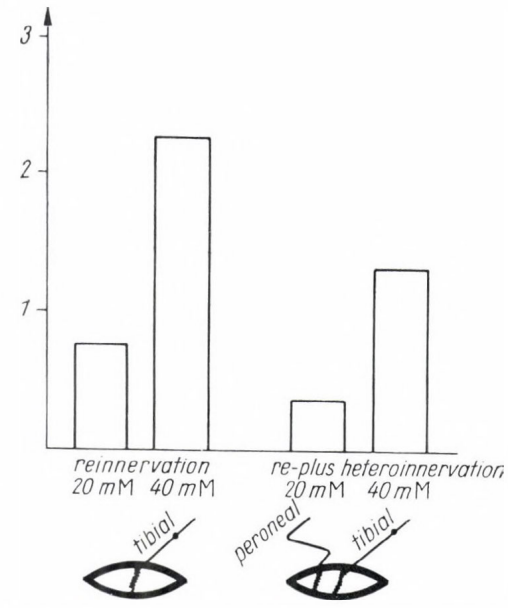


FIG. 30.—Caffeine contracture expressed in g of tension developed isometrically in vitro in the soleus muscle, 6 weeks after implantation of the peroneal nerve and simultaneous crushing of the tibial nerve on one side and after crushing the tibial nerve only on the other side. The values show tension developed by the contracture after adding a 20 and 40 mM solution of caffeine to the bathing solution

of the predominance of protein metabolism appears in the different adaptations to functional demands. The fast muscle is adapted to speed and has, therefore, a predominance in glycolytic processes, the slow muscle is adapted to maintenance of tension which is apparently related to a higher turnover in proteins. Finally, I should like to point out the importance of the innervation for the development of the metabolic differentiation of fast and slow muscles. The metabolic differentiation is lost with denervation and recovered with re-innervation. There is also a loss of differentiation in old age (Gutmann 1964). Miledi (1966a, b) has shown that the tonic frog muscle can accept fast innervation, when transplanted. We have used a different procedure in mammalian rat muscles. It is known that a muscle cannot accept accessory innervation, while this can be achieved in a denervated muscle. If the tibial nerve innervating the slow soleus muscle is crushed and if simultaneously an accessory 'fast' nerve (normally innervating the m. extensor digitorum longus) is implanted into the soleus muscle, a hyperneurotisation can be achieved, i.e. we have then a muscle with two end-plates, a 'slow' (reinnervated by the regenerated tibial nerve after nerve crush) and a fast one (innervated by the implanted peroneal nerve). The fast implanted nerve changes the contracture behaviour of the slow muscle. The slow soleus muscle, but not the fast extensor digitorum muscle, reacts with a contracture to caffeine. However, the contracture to caffeine in the slow soleus muscle is markedly decreased after implantation of the fast peroneal nerve, i.e. in the case of re-innervation and heteroinnervation of the soleus muscle (Fig. 30). Nerve influences are thus able to change the process of excitation-contraction coupling in the muscle (Gutmann and Hanzliková 1966a, b).

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SRETER (Retina Foundation Institute of Biol. Med. Sciences, Boston): Evidence obtained during the last few years suggests that myosins from white, or fast (WM) and red, or slow (RM) muscles (Seidel et al. 1964, Barany et al. 1964, Gergely et al. 1965, Sreter et al. 1966) differ in their molecular structure. Recently, we have compared the effect of various preparative procedures on the ATPase activity of myosin (Sreter et al. 1966). The activities are higher if EDTA is added to all solutions used in the course of the preparation regardless of whether one uses the classical Szent-Györgyi procedure based on repeated precipitation of myosin at low ionic strength, or ammonium sulphate fractionation, or ammonium sulphate fractionation in the presence of 2M LiCl (Luchi et al. 1965).

Since the increased ATPase activity in the presence of EDTA might have been due to the removal of impurities, it appeared of interest to compare white and red muscle myosins prepared in the presence of EDTA,

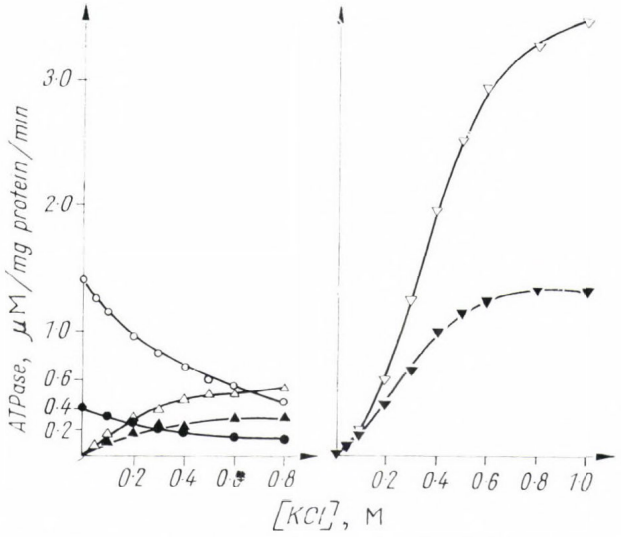

FIG. 31.—Effect of KCl concentration on myosin ATPase activities. Assay medium: 0·07 M Tris, 4 *m*M ATP, pH 7·5, 0·1 mg myosin per ml, 37° C. Key: △, ▲ no activator; ○, ●, 10 *m*M $CaCl_2$; ▽, ▼ 5 *m*M EDTA. Open symbols = white muscle myosin; filled symbols = red muscle myosin

in order to see whether impurities might have played a role in the observed differences between RM and WM. The data to be presented will show that the differences observed exist in these preparations, too, suggesting that the most likely explanation is indeed a difference in some aspects of the protein structure.

Figure 31 shows that, with highly active preparations, the ATPase activity of myosin from white muscles is two to three times greater than that of myosin from red muscles. This difference appears both in the calcium-activated and in the EDTA-activated system. It should be noted that the calcium-activated ATPase of both types of myosins decreases with increasing KCl concentration, an effect which has been attributed (Warren et al. 1966) to the structure-disrupting effect of ions. In contrast

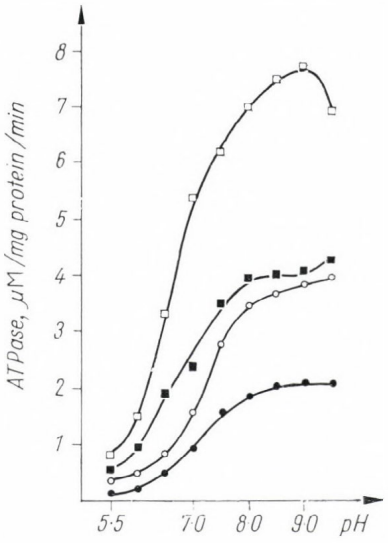

FIG. 32.—Effect of NH_4Cl and pH on EDTA-activated myosin ATPase activities. Assay medium: 0·05 M Tris, pH 7·5, 4 *m*M ATP, and 0·2 mg myosin per ml, 37° C. Key: □, ■ 0·3 M NH_4Cl + 5 *m*M EDTA; ○, ● 0·6 M KCl + 5 *m*M EDTA.

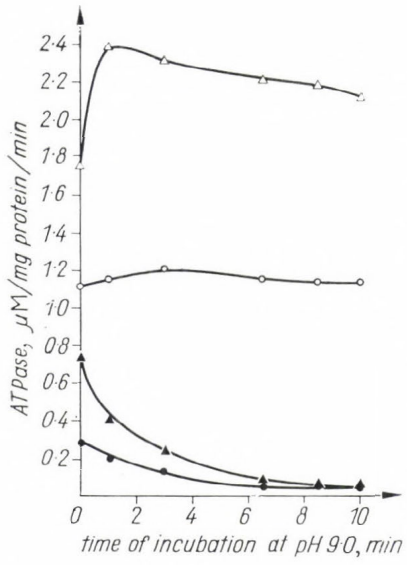

FIG. 33.—Effect of exposure to alkaline pH on myosin ATPase activity. Myosin (1·25 mg/ml) was incubated at 25° C for the times indicated on the abscissa in a medium containing 0·5 M KCl, and 0·1 M Tris, pH 9·0. ATPase assays were carried out with 0·2 mg of myosin per ml in a medium containing 0·5 mM Tris, 25 mM KCl, 10 mM $CaCl_2$ at pH 9·0 (△, ▲) and at pH 7·5 (○, ●). Open symbols = white muscle myosin; filled symbols = red muscle myosin

with this, in the absence of $CaCl_2$, KCl behaves as an activator of both myosins.

It should be added that results from several laboratories, obtained during the last few years, indicate that EDTA activation could be attributed to the removal of traces of Mg that inhibit ATPase (Muhlrad et al. 1964, Offer 1964, Martonosi and Meyer 1964). It would then seem that measurements in the presence of EDTA might produce a more reliable measure of ATPase activity than those carried out in the presence of calcium, since in the latter case differences in trace amounts of contaminating Mg^{++} could lead to different results. EDTA activation is particularly strong in the presence of ammonium ions, as was found earlier (Kielley et al. 1956) for what one would now describe as predominantly white myosin containing unknown amounts of contaminating RM (Kielley et al. 1956).

In agreement with our earlier results (Gergely et al. 1965) showing essentially identical dependence of pH on ATPase activity of WM and RM, the same is true for EDTA-activated ATPase (Fig. 32). In carrying out these studies it is essential to add ATP to the assay mixture before adding myosin. In the absence of ATP inactivation of RM (Fig. 33) ATPase takes place on incubation at pH greater than 7 (Sreter et al. 1966). Inour previous work on conventional myosin preparations, we concluded that the inactivation at high pH is an intrinsic property of RM. The persistence of this effect in presumably more highly purified preparations obtained in the presence of EDTA corroborates this view.

We are now engaged in studies aimed at the elucidation of the molecular basis for the differences between WM and RM. We have some evidence relating to differences in the reactivity of SH groups in the two types of myosin (Sreter et al. 1966), and we are exploring another phenomenon reflecting structural differences, namely, the slower decrease in the viscosity of RM on digestion with trypsin compared with the rapid decrease in the case of WM (Gergely et al. 1965, Gergely 1953). It appears that, although the over-all rate of liberation of non-protein nitrogen is essentially the same in WM and RM, those bonds whose splitting critically affects the formation of fragments of the myosin molecule possessing ATPase activity are hydrolyzed more slowly in RM.

In conclusion, I would stress that the existence of biochemical differences in muscles that appear to differ physiologically (Buller et al. 1960) opens up an interesting area of studies that might lead to a better understanding of the correlations between structure, chemistry and function.

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KÖVÉR (Institute of Physiology, University Medical School, Debrecen, Hungary): Dr Gutmann has mentioned in his comment, that the functional differences between tetanic and tonic muscles can also be reflected in the different characteristics of the structural organization of sarcoplasmic reticulum.

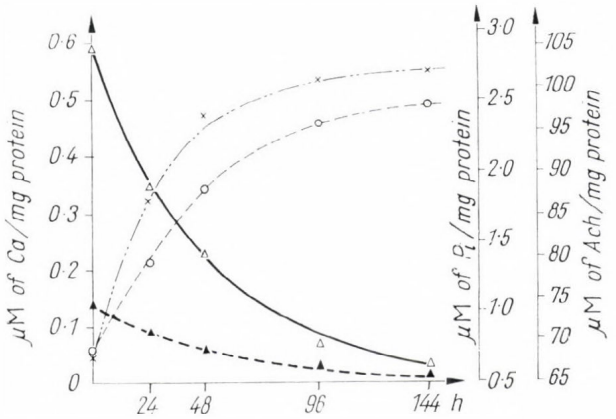

FIG. 34.—Changes in the characteristics of SRF during ageing; △ —— △ Ca^{++} uptake in the presence of 0·002 M potassium oxalate; ▲ — — — ▲ Ca^{++} uptake without oxalate; o — — — o ATPase activity; x— . . —x cholinesterase activity

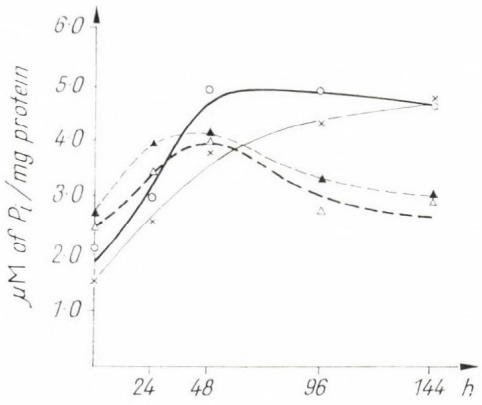

FIG. 35.—Effect of Ca^{++} on the ATPase activity of SRF during ageing; o——o ATPase activity without Ca^{++} and oxalate; △ — △ ATPase activity in the presence of 0·12 *m*M Ca^{++} without oxalate; x——x ATPase activity in the presence of 0·002 M oxalate without Ca^{++}; ▲ — — — ▲ ATPase activity in the presence of both 0·12 *m*M Ca^{++} and 0·002 M oxalate

In our earlier experiments it was found that the lower the cholinesterase activity of SRF, the higher is its Ca uptake. For example SRF prepared from the tetanic muscles of adult rabbit has a relatively low cholinesterase activity and a high Ca uptake, while the SRF obtained from fish (*Amiurus nebulosus*) muscle possesses a more marked cholinesterase activity and a definitely lower Ca uptake.

The correlation between Ca uptake and enzyme activity is verified by our observations obtained during the ageing of fish SRF. According to Martonosi, the decrease in Ca uptake demonstrated in the course of ageing can by no means be dependent on any change occurring in the phospholipid content of the SR-membrane. In Fig. 34 it may be seen that enzyme activity increases proportionately to the decrease in Ca uptake. This rise in the enzyme activity suggests a disorganization of the membrane structure. In the following figure (Fig. 35) it can be observed that during ageing the capability of Ca^{++} to activate the SRF-ATPase also changes. Namely, in the first few days, the ATPase activity markedly increased in the presence of Ca^{++}, whereas in the later period, Ca^{++} inhibited the SRF-ATPase.

In other experiments, the correlation between Ca uptake and enzyme activity was investigated during ontogenetic development. Practically no Ca uptake was found in the SRF prepared from the striated muscles of rabbits 1–2 days old (Fig. 36). At the same time, these preparations showed a relatively high cholinesterase activity. During postnatal life, the increase in Ca uptake was accompanied by a decrease in enzyme activity. This figure demonstrates further that the Ca uptake increases significantly already between the 4th and 6th days, whereas the enzyme activity shows no noticeable change at all. It may be assumed, therefore, that this increase depends on the production of some deionizing factors.

To approach the problem from another angle, we investigated the effect of tryptic digestion on the properties of fish SRF, which was digested by trypsin at pH 7; samples taken at different periods were inhibited by a soy bean inhibitor and Ca uptake, ATPase and cholinesterase activity as well as the Ca activation of ATPase were measured. Figure 37 shows that tryptic digestion lasting half a minute results in the cessation of Ca uptake, in a definite rise of enzyme activity, and in the inhibition of ATPase by Ca^{++}. Another half a minute later, however, enzyme activity again decreases, the Ca uptake again increases to 30–40 per

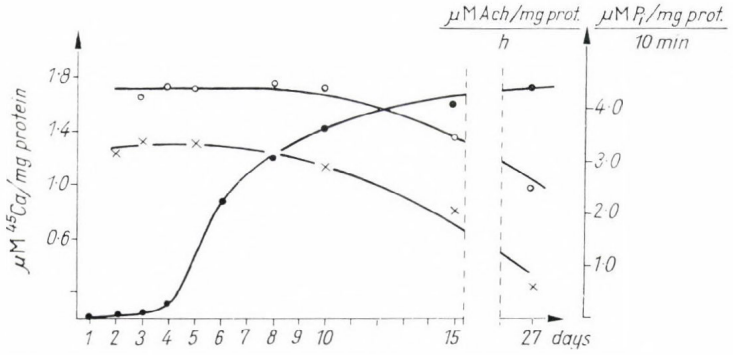

FIG. 36.—Change of Ca^{++} uptake, ATPase and cholinesterase activity of rabbit SRF during ontogenetic development; ● —— ● Ca^{++} uptake; o —— o ATPase activity; x —— x cholinesterase activity

cent of the original level, and even the activation of SRF-ATPase by Ca^{++} reoccurs. These results suggest a rearrangement of the SR-membrane. In the later phase of tryptic digestion the increase in the enzyme activity is accompanied by the complete disappearance of Ca uptake and by the inhibition of SRF-ATPase by Ca^{++}.

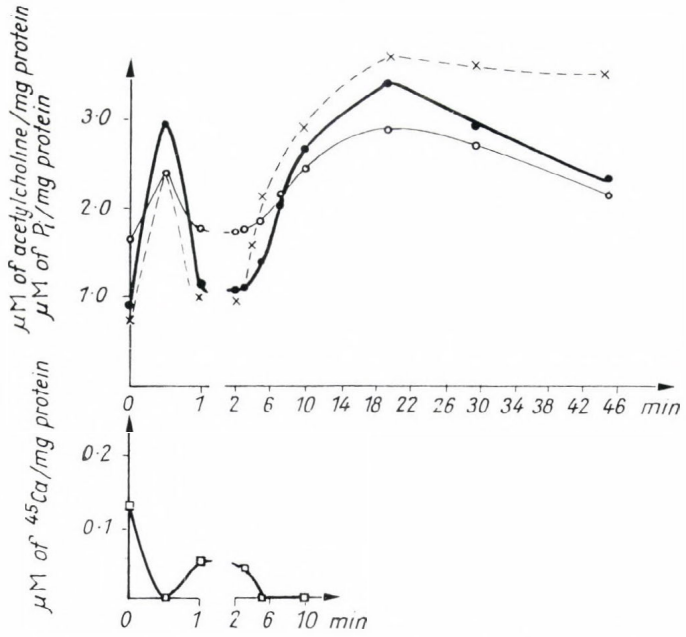

FIG. 37.—Effect of tryptic digestion on Ca^{++} uptake, ATPase and cholinesterase activity of fish SRF; o —— o ATPase activity; 0·06 mM Ca^{++}; ● —— ● ATPase activity without Ca^{++}; x --- x cholinesterase activity

FIG. 38.— Acetylcholinesterase activity of the A-alpha subneural apparatus in the rat gastrocnemius. Note the synaptic gutters (arrows) surrounded by two strongly enzyme-active lines, consisting of hundreds of semicircular units (organites). ×2 000. Thiolacetic acid technique

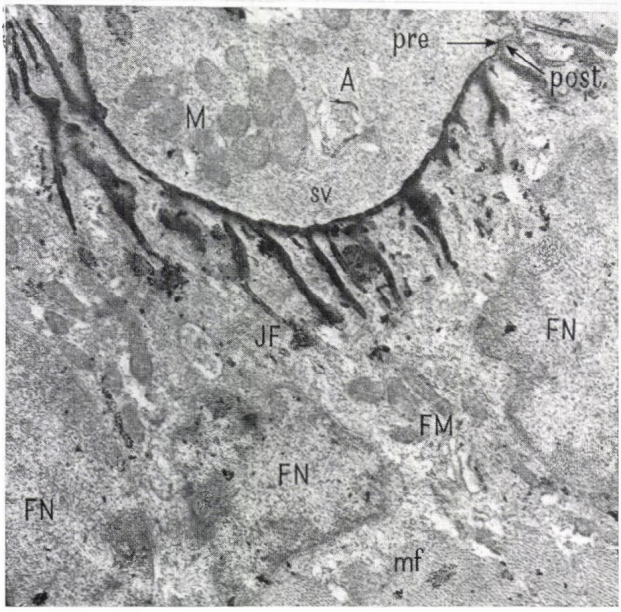

FIG. 39.—Acetylcholinesterase activity of the A-alpha subneural apparatus in the rat diaphragm. Electron-histochemical preparation, substrate: acetylthiocholine iodide. In this picture, the synaptic gutter appears in cross-section. A = axon, M = axonal mitochondria, *sv* = synaptic vesicles, JF = junctional folds, *pre* = presynaptic membrane, *post* = post-synaptic membrane, both membranes exhibiting the same kind of enzyme activity; FN = fundamental nuclei, FM = fundamental mitochondria *(télosomes)*, *mf* = myofilaments. ×22 000

All these results mentioned call our attention to the role of cholinesterase molecules in the building-up of the SR-membrane, and they indicate a close relation between ATPase and cholinesterase in the regulation of the Ca-binding capacity of SRF.

CSILLIK (Institute of Anatomy, Szeged, Hungary): I should like to comment on Prof. Couteaux's magnificent presentation on the innervation apparatus of the muscle, and at the same time, I should like to ask, Prof. Gutmann, a question. The subneural structures we have seen this morning by Couteaux represent those in amphibian muscles. In mammals, the synaptic gutters of the muscle surface membrane are slightly different: they are not straight, like in frog muscles, but have a more or less regular rounded form (Fig. 38). The gutters themselves are built up of semicircular units (organites), exhibiting a strong acetylcholinesterase activity. This or-

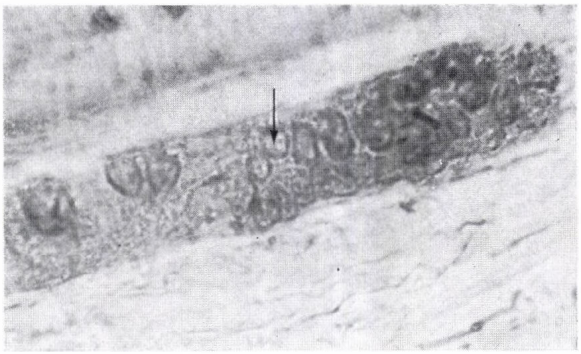

FIG. 40.—Acetylcholinesterase activity of the A-gamma subneural apparatus on an intrafusal muscle fibre of a muscle spindle in the cat's lumbrical muscle. Note the relatively low enzyme activity of the grape-like units (arrow). ×2000. Acetylthiocholine technique

ganization characterizes (with few exceptions) all of the mammalian skeletal muscles, both tonic and tetanic (red and white) ones. Electron microscopically, as shown in Fig. 39 obtained by my colleagues Dr Joó, Dr Kása and myself, the enzyme acetylcholinesterase is located in both the pre- and post-synaptic membranes of the junction. Since the post-synaptic membrane is thrown into multiple folds, the amount of the post-synaptic contingent of the enzyme is many times (10–15×) higher than that of the pre-synaptic compartment. We have good reason to assume acetylcholinesterase to be associated with the acetylcholine receptor. The high amounts of enzyme and receptor in the branching system of the post-junctional folds raises the question of a specific function; theoretical considerations lead us to suppose a post-junctional amplification mechanism to be active here as a device to strengthen the action of pre-synaptically released acetylcholine (Csillik 1965).

An entirely different kind of post-synaptic structures is present in the intrafusal muscle fibres of muscle spindle organs, innervated by thin A-gamma nerve fibres of the small nerve system. Light-microscopically, the acetylcholinesterase activity of these grape- or ring-shaped structures is much weaker than that of the aforementioned A-alpha junctions, and their structure is very similar to those seen in tonic amphibian muscles (Fig.

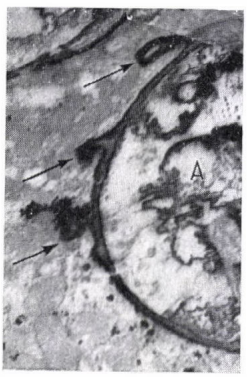

FIG. 41.—Acetylcholinesterase activity of the A-gamma subneural apparatus of a muscle spindle in the cat's lumbrical muscle. Electron-histochemical preparation, substrate: acetylthiocholine iodide. Cross section of one of the grape-like units appears in this electron micrograph. A = axon, arrows = short junctional folds. Note the enzyme reaction of both preand post-synaptic membranes and the fact that most part of the latter (except for the two short folds) is smooth. ×22 000

FIG. 42. — × 45 300

40). As seen in electron-histochemical sections, the branching system of junctional folds in these endings is virtually missing (Fig. 41). Thus, though the acetylcholinesterase activity of both pre- and post-synaptic membranes in this latter junction is practically identical with that in A-alpha junctions, the resulting total activity is many times less, whereas the pre-post ratio of the enzyme is 1:1 or 1:2, as contrasted to the 1:10 or 1:15 ratio in A-alpha endings. It appears to us that the absence of junctional folds (and, thus, the absence of a post-synaptic amplificatory mechanism) might be, at least partly, responsible for the small junctional potentials characterizing A-gamma nerve endings, differing in many aspects from the end-plate potential of A-alpha endings.

Coming back to Gutmann's presentation, I feel that the newly formed end-plates in muscle fibres with a 'double innervation' are more similar to grape-like A-gamma endings than to the (normally present) A-alpha endings. If this is really so, the unfolded A-gamma endings might exhibit electrical characteristics different from those of the original (folded) nerve endings. I would suggest, therefore, to check these newly formed motor end-plates not only by light microscopy but also by means of electron microscopy and electron histochemistry, in order to obtain sufficient basis for the evaluation of the electrical characteristics of muscle fibres with a double innervation.

FIG. 43. — ×56 600

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VIRÁGH (Central Research Institute of Medical Sciences, Budapest): Many muscle cells of the atrioventricular conducting tissue in *Rhesus macacus* heart contain leptofibrils. These leptofibrils are practically similar to those observed in the skeletal muscle of young birds, in the heart muscle cells of rat embryo, in the conducting system of the sheep heart, and also in the human muscle spindle. Leptofibrils were found not only in muscle cells but also in endothelial cells of arterioles of rat myometrium.

The leptofibrils of the Macacus heart conducting tissue cells are of wavy appearance (Fig. 42), consequently, their whole length may be seen in one single ultra-thin section only occasionally. In the monkey heart the longest leptofibril observed was measured to be 3 μ, although the leptofibrils are slender structures they may run tight parallel to each other forming 1·5 μ thick bundles at the periphery of the sarcoplasm. In longitudinal section of the leptofibrils 1000 to 1300 Å light periods alternate with 250 Å dark periods. The light periods consist of 40 to 50 Å thick filaments which entering the dark periods seem to ramify or to thicken.

The muscle cells which contain the leptofibrils are not fully differentiated. They have slender and ramifying myofibrils as well as numerous 50–80 Å

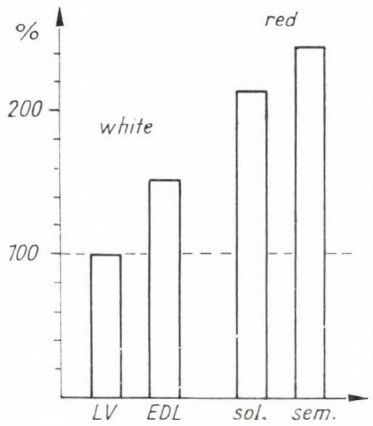


FIG. 44. — Proteolytic activity in different rabbit muscles (100% — lateral vostus). LV = m. lateral vostus; EDL = m. extensor digitorum longus; sol = m. soleus; sem = m. semitendinosus

thick filaments dispersed in the sarcoplasm. Dispersed filaments may be predominant in some of these cells. The leptofibrils lie usually near and parallel to the sarcolemma (Fig. 42). It has been observed in numerous electron micrographs that leptofibrils connect the myofibrils or the dispersed myofilaments with the sarcolemma.

In the conducting tissue cells single myofilaments often appear to leave the myofibril and enter a neighbouring myofibril or may remain as individual filaments dispersed in the sarcoplasm. Thin filaments leaving the myofibrils may be arranged into leptofibrils beginning mostly at the I-band level or more seldom at the A-band level. They are often oriented parallel to the typical myofibrils and their dense period may adhere to the Z-line of them when the leptofibril bypass the sarcomere. Some of the leptofibrils seem to collect the irregularly dispersed thin filaments. This is seen especially well in cells containing mainly dispersed filaments.

The leptofibrils are usually attached to the free surface of the cell where the sarcolemma is surrounded by and exposed to the connective tissue; they are not attached to the intercalated discs. They are very probably only the marginal portions of the dense periods which are in direct contact with the sarcolemma. Leptofibrils run sometimes perpendicularly to the cell border and under such circumstances are attached to them with the last dense period. Thicker leptofibrils may adhere step by step to the sarcolemma (Fig. 43).

The chemical composition and possible functional role of the leptofibrils are unknown. Our electron micrographs show clearly that the thin filaments of the myofibrils may directly continue in leptofibrils which strongly support the idea that the filaments of the leptofibrils are also actin filaments. Up till now there is no convincing evidence that the leptofibrils play any role in contraction. Our observations clearly demonstrate that they connect myofibrils to the sarcolemma. The same was found by Gruner in the muscle spindle.

Leptofibrils have been found in embryonic heart and young skeletal muscle cells so far. They may be also components of adult muscle fibres in which the contractile apparatus is not fully developed. This is the case in the conducting tissues of the heart and in the muscle spindle.

DRABIKOWSKI (Department of Biochemistry, Nencki Institute of Experimental Biology, Warsaw, Poland): I should like briefly to comment on Dr Gutmann's lecture, namely, to give an example of species differentation of proteolytic activity. Professor Gutmann showed us that in chicken muscles proteolytic activity was higher in the red muscles than in the white ones. However, in rats, as was later found in Professor Gut-

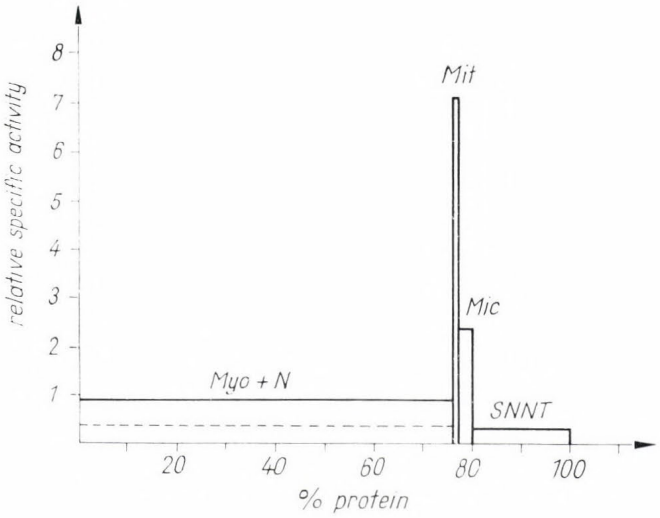


FIG. 45. — Distribution of proteolytic activity in subcellular fractions of the white rabbit muscles; *Myo* + N = fraction 0–600 g (dotted line denotes the activity in purified myofibrils); *Mit* = fraction 600–12 000 g, *Mic* = fraction 12 000–100 000 g, SNNT = final 100 000 g supernatant. Relative specific activity = the ratio of activity in the particular fraction to the activity in the initial homogenate

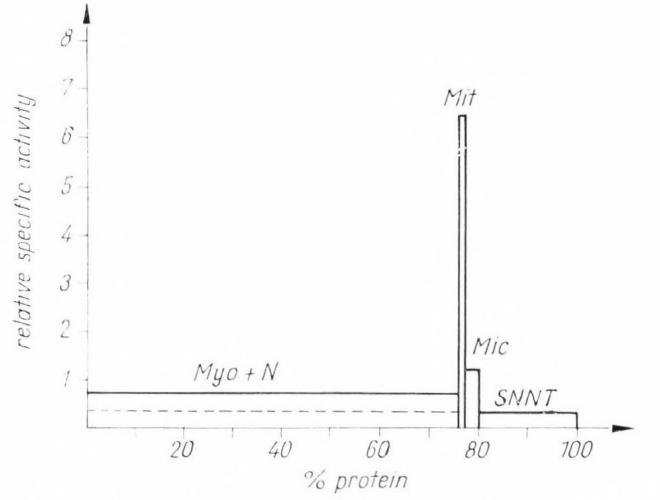


FIG. 46. — Distribution of proteolytic activity in subcellular fractions of the red rabbit muscle. Symbols as in Fig. 45

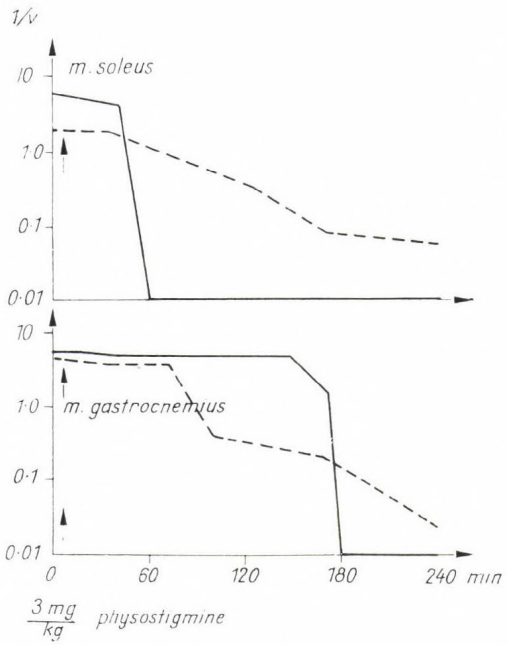
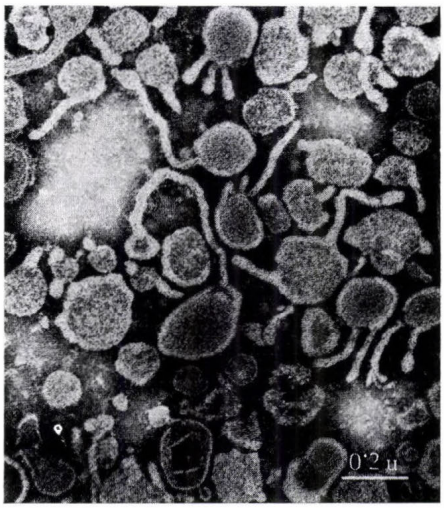


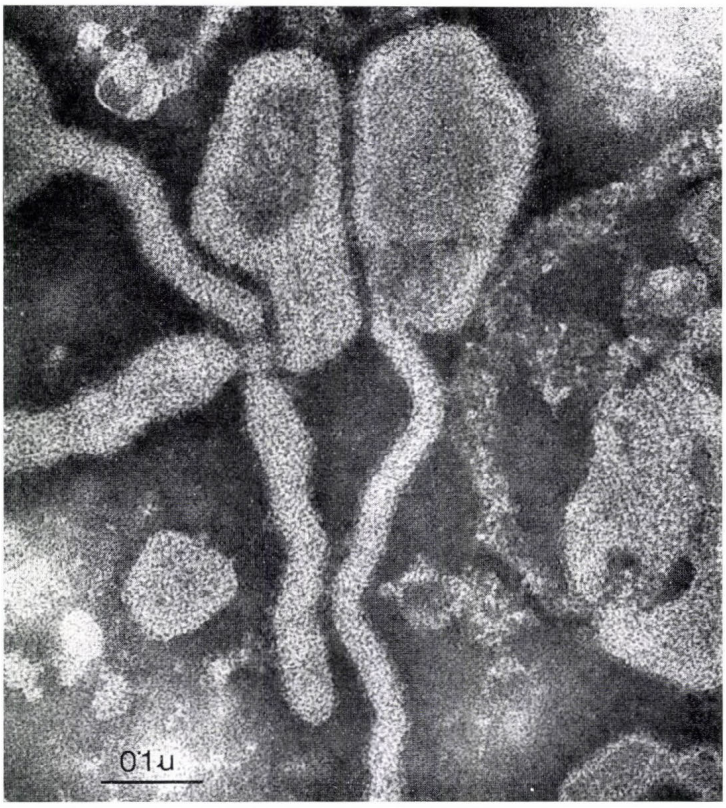
FIG. 47. — Changes in the direct (dotted line) and indirect (full line) excitability of the two muscles. (The abscissa shows the time, the ordinate the excitability expressed as the reciprocal value of the threshold in volts)

mann's laboratory, white muscles reveal higher proteolytic activity than red ones. On the other hand, recent investigations of Dr Hajek and myself showed that in this respect rabbit muscles are similar to chicken muscle, namely the proteolytic activity in red muscles (m. soleus and m. semitendinosus) is twice as much as the activity in the white muscles (Fig. 44). In spite of this, the distribution of proteolytic activity among subcellular fractions is the same in both kinds of rabbit muscle (Figs 45 and 46). One can see that the highest proteolytic activity (6–7 times higher than that in the initial homogenate) is located in the mitochondrial portions. Several pieces of biochemical information obtained by us seem to indicate that in the striated muscles endogenous proteolytic enzymes are localized in lysosomes, similarly to other tissues, as was shown by de Dure and his co-workers. On the other hand, as far as I know, no electron-microscopic pictures of muscle were published in which lysosome-like particles were seen.

VARGA (Physiological Institute, Medical University Debrecen, Hungary): Prof. Couteaux demonstrated a few very striking pictures about the differences in the nerve supply of tonic and tetanic muscles. This afternoon Prof. Gutmann presented data dealing with the differences in neuromuscular junctions of different kinds of muscle. In connection with this problem I should like to mention a few observations from our laboratory. We compared the effect of different anticholinesterases on cat soleus and gastrocnemius. As can be seen in Fig. 47 after 3 mg/kg body weight the indirect excitability decreased on the m. soleus and after one hour it became inexcitable. However, at the same time, there was no change observable as far as the indirect excitability of gastrocnemius was concerned. It changed only two hours later. We obtained similar results in the case of prostigmine, atropine and a few other drugs of different structures. In our opinion the obtained differences are explainable, assuming that the neuromuscular junction of the soleus is not so differentiated, and therefore, more permeable to any kind of drug. Besides this, of course, one may assume that there are also some differences in the sensitivity of the two different receptors.



a



b

FIG. 48. — Negatively stained (1 per cent phosphotungstic acid, PTA) preparations of mouse fragmented sarcoplasmic reticulum. *a* general view; note the elementary particle on mitochondrial fragments, *b* details of characteristic vesicular elements in negatively stained fragmented sarcoplasmic reticulum; note the small particles on the surface

GERGELY (Retina Foundation, Institute of Biological and Medical Sciences, Boston, Mass, USA): In connection with Dr Kövér's comments regarding the sarcoplasmic reticulum (SR), I should like to briefly report on some studies which were carried out in our laboratory in collaboration with Drs Ikemoto and Sreter (1966).

One of the problems in studying vesicles originating in the sarcoplasmic reticulum is that of purity, and this is particularly acute in the case of red skeletal and cardiac muscles which have a high mitochondrial content. Electron-microscopic examination of negatively stained (Huxley and Zubay 1960) specimens of preparations that have Ca-uptake, ATPase and relaxing activity — usually referred to as grana, microsomal fraction or fragmented sarcoplasmic reticulum (FSR) — makes it possible to check their purity and reveals some interesting features of the particles present.

We have used a method of homogenization that was originally devised for keeping mitochondria intact (Von Korff 1965) in order to minimize admixture of mitochondrial fragments in fractions obtained between 6 000 and 25 000 g. The predominant structures (Fig. 48*a*) have a globular head with a diameter of 0·1–0·2 μ, to which one or more tails are attached. In preparations of this type mitochondrial fragments show up clearly by virtue of the so-called elementary particles attached to them (Fernandez-Moran 1962). The more numerous elements originating presumably in the SR are readily distinguished from such mitochondrial fragments by the lack of these particles, although under higher magnification details of the surface structure are clearly visible (Fig. 48*b*). These consist of particles of much smaller size and appear similar to those described for other microsomal particles (Beneditti and Emmelot 1965, McLennan et al., in press).

Following incubation with ATP, calcium and oxalate, structures that are light in the electron micrographs appear presumably corresponding to Ca-oxalate deposits (Fig. 49). Owing to Ca-uptake by the vesicular FSR (Hasselbach 1964a), these deposits appear only in the globular portion, although quite frequently the tail portions appear to be outlined much more sharply under conditions of Ca-uptake.

Since ATPase activity is characteristic of these particles, and indeed ATPase activity has been considered as the basis of active calcium transport (Hasselbach 1964b), we tried to localize the ATPase sites by incubation in a medium containing lead salts. The earliest deposits attributable to lead phosphate appear at the junction of the tail and the globular part, suggesting that these are most the active sites of ATPase activity (Fig. 50).

On longer incubation, deposits appear more diffusely in the globular portion. In order to eliminate possible artifacts (cf. Gillis and Page, in press), we have carried out control experiments in media containing inorganic phosphate and under these conditions the deposits seen on incubation in the presence of ATP are absent. Thus, it may be safe to conclude that the deposits obtained in the presence of ATP appear at the sites where ATP is hydrolyzed rather than as a result of the absorption of lead phosphate formed by the reaction with phosphate present in the medium.

Trypsin has been known to destroy the relaxing activity (Lorand et al. 1957), what one now would describe as FSR. We have carried out trypsin

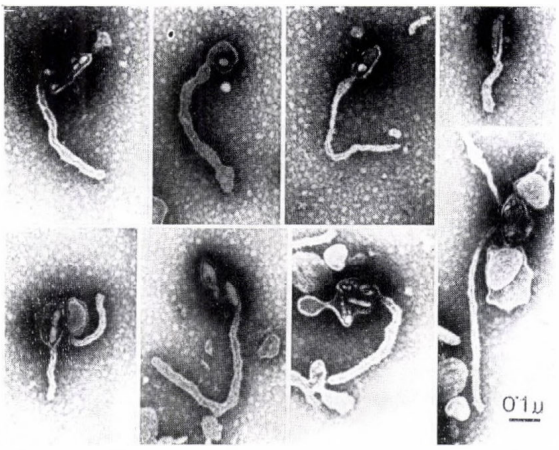


FIG. 49.—Vesicles of the fragmented sarcoplasmic reticulum loaded with Ca-oxalate. Note that accumulation of the material of low electron density is seen only in the globular portion of the vesicle (see text). Negative staining with 1 per cent PTA

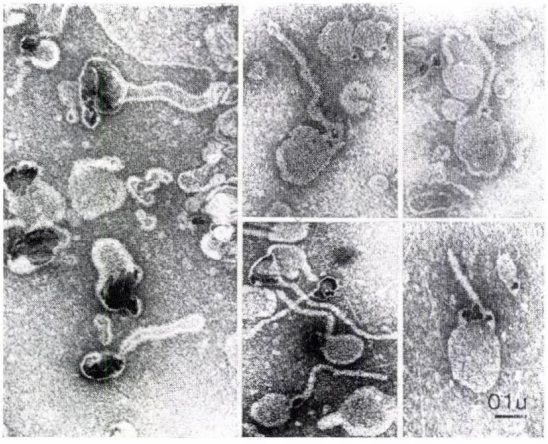


FIG. 50. — Localization of ATPase activity in vesicles of the fragmented sarcoplasmic reticulum

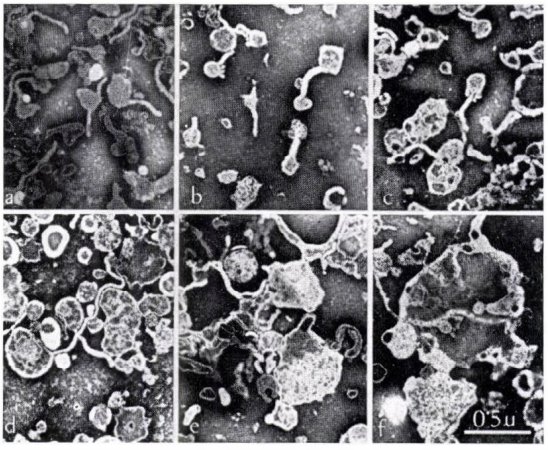
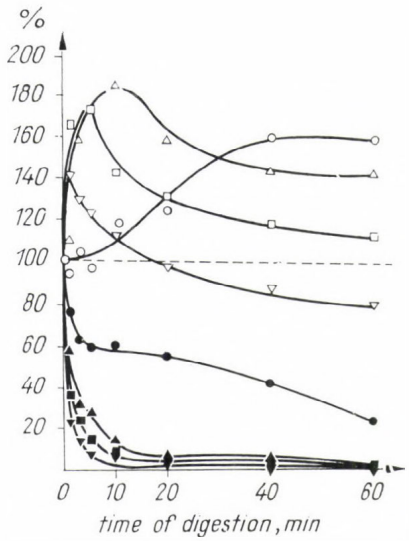


FIG. 51. —Structural changes in FSR vesicles on digestion with trypsin. Vesicular protein–trypsin ratio 1/100. Time of incubation: *a* 0 min, *b* 5 min, *c* 10 min, *d* 20 min, *e* 40 min, *f* 60 min



trypsin/prot.	ATPase	Ca uptake
1/300	○	●
1/150	△	▲
1/50	□	■
1/25	▽	▼

FIG. 52. — The effect of trypsin on ATPase and Ca-uptake activity of fragmented sarcoplasmic reticulum

digestion using various levels of trypsin and our results agree in some ways with those of Dr Kövér, although, as you will see, at some points they do not. Structurally, trypsin digestion first leads to alterations in the tail portion, resulting eventually in the separation of the tail from the globular portion. On longer digestion the destruction of the globular portion also takes place (Fig. 51).

Concomitantly with these structural changes, calcium uptake decreases but the ATPase shows an increase, the temporal sequence of the changes being somewhat dependent on the ratio of trypsin to the vesicular protein (Fig. 52). At lowest trypsin concentrations there is an almost instantaneous 40 per cent decrease in calcium uptake with very little change in ATPase activity. During the next twenty minutes calcium uptake decreases gradually, while ATPase increases considerably. At higher trypsin–protein ratios calcium uptake drops very rapidly and at the same time ATPase activity increases; on longer digestion ATPase activity eventually decreases, but only at the highest concentration does it drop below the initial level. We have never observed the biphasic effect reported by Dr Kövér.

These changes could be interpreted as being either due to some uncoupling between ATPase and calcium uptake at the active sites, or to selective destruction of the calcium uptake sites with the alteration of the structure resulting in either the appearance of new ATPase sites, or an increased activity at the existing ones.

It would be interesting to speculate on the meaning of the appearance of lead phosphate deposits in the presence of ATP at the junction of the tail with the globular portion, in terms of the latter being the principal site of ATPase activity. In view of the fact that trypsin alters the tail portions first, and that these changes are accompanied by changes in calcium uptake, it would seem that — although calcium deposits are never seen in the tail portions — there may be some functional connection between the tail-like portions and the globular portion where the calcium deposits appear.

Finally, I should like to say a few words about the possible connection between some features of the SR *in vivo* and the particles seen with the electron microscope. The particles containing a globular head and a tail-like appendix might suggest that the former correspond to the terminal cistern of the SR *in vivo*, whereas the tail-like portions represent the tubular, longitudinal, more central parts of the SR. The parallelism between the

localization of the Ca deposits in terminal sacs (Costantin et al. 1965, Hasselbach 1964b) and in the globular portions in negatively stained preparations of FSR makes this tentative correspondence even more attractive. However, at this stage this has to be considered as being purely conjectural and more work will be needed to establish correlations between various aspects of the particles found in preparations of FSR and the structurally and functionally distinct parts of the SR *in situ*.

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STRUCTURAL PROTEINS AND THEIR INTERACTION

S. EBASHI

DEPARTMENT OF PHARMACOLOGY, FACULTY OF MEDICINE, UNIVERSITY OF TOKYO, TOKYO

The revolutionary discoveries made in Szeged a quarter of a century ago were not only the first step in the molecular biology of muscular contraction, but also the first success in pursuing the physiological function on a molecular basis (Szent-Györgyi 1941, Banga and Szent-Györgyi 1941, Straub 1942). Today, it is a matter of common knowledge that the interaction between actin and myosin in the presence of ATP is the fundamental process underlying muscular contraction. Further, a polymerized form of actin (Straub 1942) or myosin (Noda and Ebashi 1960) appears to be almost identical or very similar to the actual myofilaments in myofibrils (Hanson and Lowy 1963, Huxley 1963, Zobel and Carlson 1963). Thus, it would appear that a precise study of the actin–myosin system might provide a total picture of muscular contraction in living muscle.

Recently, however, investigations intending to bridge the gap between the molecular mechanism and physiological contraction have led to the conclusion that the important aspect of muscular contraction cannot be represented solely by the myosin–actin interaction, but requires the participation of other structural proteins (Ebashi 1966). Since my lecture will be followed by other papers which will effectively cover the general aspect of muscle biochemistry and biophysics, I should like to confine this presentation to recent studies on new structural proteins which have been found and studied in our laboratory.

Native tropomyosin (a factor sensitizing actomyosin to Ca ions).—It is well established that a minute amount of Ca ions regulates the contraction–relaxation cycle (Weber et al. 1963, Ebashi 1965). Responsiveness to Ca ions or Ca-removing agents can be demonstrated not only with myofibrils and glycerinated fibres, but also with such a simple system as myosin B, which has been called natural actomyosin. However, since synthetic actomyosin does not possess this important property (Perry and Grey 1956, Weber and Winicur 1961), it is apparent that the actin–myosin interaction is devoid of some fundamental feature of muscle contraction. We investigated this problem and found that another structural protein is needed, in addition to actin and myosin, to show the responsiveness to Ca ions (Ebashi 1963, Ebashi, S. and Ebashi, F. 1964). The factor is very similar to the tropomyosin of Bailey (1948), in its physico-chemical properties, but differs in that the ordinary tropomyosin does not show this physiological activity.

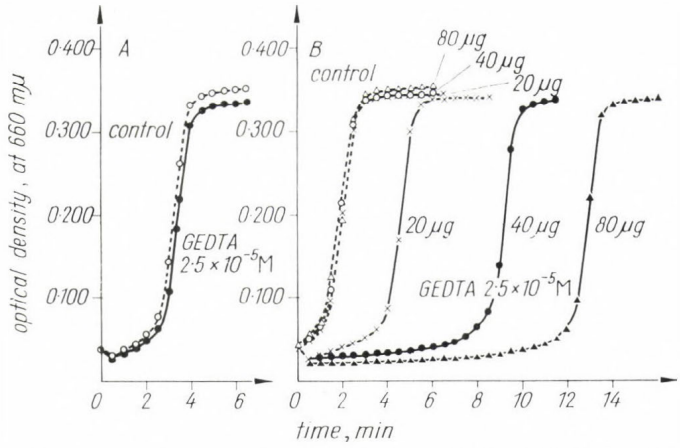

FIG. 1.—Response of trypsin-treated myosin B to GEDTA in the absence (*A*) or the presence (*B*) of native tropomyosin. — GEDTA: glycoletherdiaminetetraacetic acid (EGTA). Figures on curves indicate amounts of native tropomyosin in μg/ml. Trypsin-treated myosin B, 0·78 mg/ml. For other details see the original paper (Ebashi S. and Ebashi F. 1964)

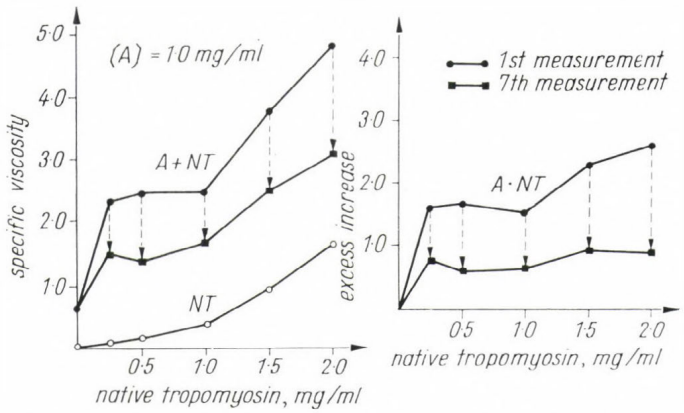

FIG. 2.—Effect of native tropomyosin on viscosity of F-actin solution. Curves on the left = actual values of specific viscosity of solutions. Curves on the right = calculated from the curves on the left. A · NT = $\eta_{sp(A+NT)} - \eta_{sp(NT)}$

The new protein, tentatively named native tropomyosin,* is sensitive to trypsin digestion as is the ordinary tropomyosin (Laki 1960). By using this trypsin-sensitive property, the presence of native tropomyosin in myosin B and myofibrils has been demonstrated (Fig. 1).

* According to D. R. Kominz, *tropomyosin extraite à μ 1* of Hamoir (1955) and Δ-protein of Amberson et al. (1957) might be related to native tropomyosin (personal communication). Azuma and Watanabe (1965) have shown that 'metin' prepared by Szent-Györgyi and Kaminer (1963) contains this protein as a minor component.

Native tropomyosin has a remarkable effect on F-actin. For example, it elevates the viscosity of F-actin distinctly (Ebashi and Kodama 1966) (Fig. 2). Flow-birefringent studies have revealed that the interaction is very anomalous and cannot be compared with any other interactions between structural proteins of muscle. According to electron-microscopic photographs, F-actin filaments from which native tropomyosin has been exhaustively removed are more pliable than usual and show a tendency of clinging to each other. The addition of native tropomyosin to such filaments restores their former rigid state.

TABLE I

Amino-acid compositions of tropomyosin-related proteins

	Native tropomyosin	Tropomyosin (Kominz et al. 1957)	Troponin
Asp	83	89	83
Thr	20	26	22
Ser	34	40	31
Glu	201	212	159
Pro	10	2	26
Gly	21	11	43
Ala	94	108	74
Val	29	27	37
Met	22	16	27
Ileu	37	29	33
Leu	85	95	65
Tyr	15	15	12
Phe	10	4	23
His	9	5	17
Lys	106	113	100
Arg	49	41	66
(NH_3)	(66)	(64)	(60)
	825	833	818

Troponin (a factor promoting the aggregation of tropomyosin).—From a quantitive standpoint, the physico-chemical properties of native tropomyosin are to a certain extent different from those of ordinary tropomyosin. The former has a higher viscosity and an increased sedimentation constant ($S_{20,w}$ at $\mu = 0{\cdot}27$ is approx. 4·7) than the latter. There is also some difference in their amino-acid compositions (Table I). Investigation inquiring into this problem has revealed that native tropomyosin could be separated into two components (Ebashi and Kodama 1966), one is the ordinary tropomyosin and the other a protein of globular nature, which we have named troponin (Ebashi and Kodama 1965, 1966). The ratio between troponin and tropomyosin is around 1 to 2–3. Troponin, like tropomyosin, is very sensitive to trypsin digestion (Figs 3 and 4).

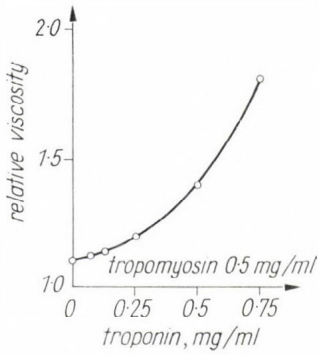

FIG. 3.—Effect of troponin on viscosity of tropomyosin. For the details see the original paper (Ebashi and Kodama 1965)

If troponin is added to tropomyosin, there occurs a remarkable increase in viscosity (Fig. 3) and sedimentation constant (Fig. 4). On the basis of these results together with those from flow-birefringent studies (Fig. 5), the conclusion can be drawn that troponin facilitates the aggregation of tropomyosin. The amino-acid composition of troponin, which in comparison with that of tropomyosin is distinct, accounts for the difference in amino-acid compositions of native and of ordinary tropomyosin preparations (Table I). Troponin itself does not interact with F-actin alone, but only in the presence of tropomyosin does it exert the same effect as does native tropomyosin on F-actin (Ebashi and Kodama 1966).

Troponin combined with tropomyosin shows essentially the same effect as that of native tropomyosin on the actin–myosin interaction (Fig. 6). Thus, the troponin–tropomyosin complex demonstrates almost all of the properties of native tropomyosin. This means that Bailey's tropomyosin (Bailey 1948), the function of which has long been unknown, is now definitely connected with the physiology of muscle in combination with troponin.*

As stated above, trypsin is a good agent to eliminate native tropomyosin from myosin B or myofibrils. Since it is shown that native tropomyosin consists of two components, viz. troponin and tropomyosin, the question arises as to which component would be attacked by trypsin preferentially. The investigation of this problem has revealed that mild trypsin treatment abolishes troponin completely, but leaves much of tropomyosin unaffected. This explains the embarrassing fact that troponin itself can exert a definite native tropomyosin-like effect on trypsin-treated myosin B (Figs 7 and 8).

TABLE II

Contents of structural proteins

Proteins	g/100 g of muscle*
Myosin	5·5—6·0
Actin	~2·0
α-actinin	0·8—1·0
β-actinin	~0·2
Tropomyosin	0·5—0·7
Troponin	0·2—0·3

* Estimated from the yield

α-Actinin (a factor promoting the interaction between actin and myosin). —The crude extract used to prepare native tropomyosin contains a large amount of another protein,

* Mueller (1966) is of the opinion that native tropomyosin may be a type of tropomyosin preparation which contains a larger amounts of sulfhydryl groups than is usual. We cannot accept this opinion because of various reasons, for example: (1) the activity of native tropomyosin is not parallel with the sulfhydryl contents; (2) tropomyosin preparation of high sulfhydryl contents is not effective (S. Watanabe, personal communication); (3) tropomyosin preparations made by the original procedure of Bailey, which Mueller followed, very often contains considerable amounts of native tropomyosin.

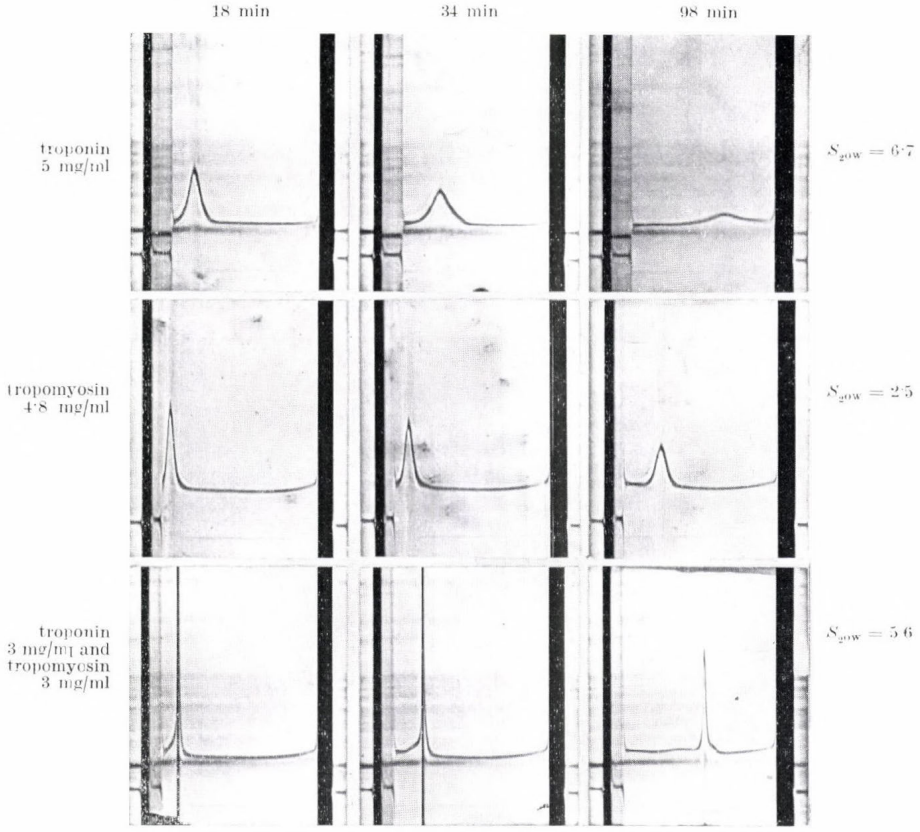

FIG. 4.—Sedimentation diagrams of troponin, tropomyosin and troponin–tropomyosin complex. Ionic strength 0·27, pH 6·8 (0·02 M Tris-maleate)

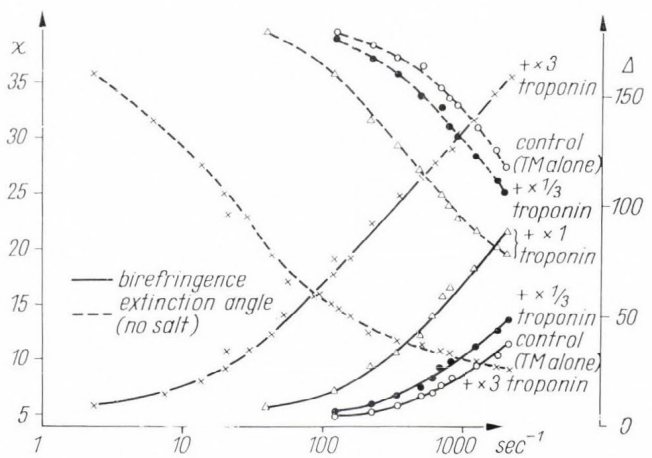

FIG. 5.—Effect of troponin on flow birefringence of tropomyosin. Tropomyosin 0·6 mg/ml

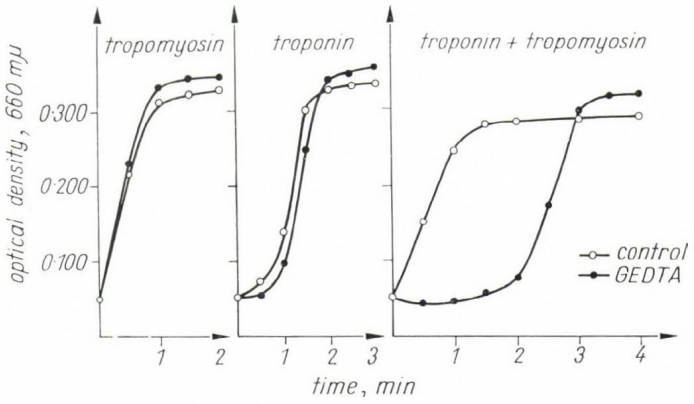

FIG. 6.—Effect of troponin–tropomyosin complex on superprecipitation of synthetic actomyosin. Tropomyosin 0·06 mg/ml, troponin 0·03 mg/ml, actomyosin 0·7 mg/ml, GEDTA 1×10^{-4} M

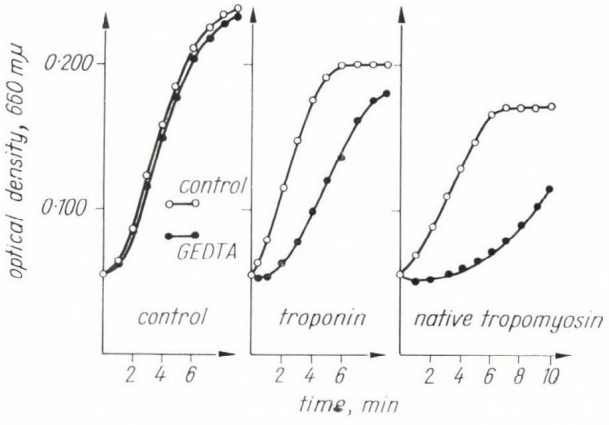

FIG. 7.—Effect of troponin on superprecipitation of trypsin-treated myosin B. Troponin 0·03 mg/ml, native tropomyosin 0·06 mg/ml, trypsin-treated myosin B 0·65 mg/ml, GEDTA $2{\cdot}5 \times 10^{-5}$ M

named α-actinin (Ebashi et al. 1964, Ebashi, S. and Ebashi, F. 1965). The yield of this protein from muscle is more than one third of actin (Table II). Nonomura (1967) has shown that preparations obtained by ammonium sulphate fractionation consist of three components, of which the sedimentation constants, $S_{20,w}$, are 6 S, 10 S and 25 S. All components have almost equal physiological activity. Molecular weights of the 6 S and 25 S components are about $1{\cdot}6 \times 10^5$ and $3{\cdot}2 \times 10^6$ respectively. All evidence indicates that these components are of a globular nature.

If an appropriate amount of α-actinin is added to F-actin, the latter gelatinizes immediately at cold temperature. In case of excess addition of α-actinin F-actin is precipitated(Ebashi, S. and Ebashi, F. 1965). According to electron-

microscopic studies, α-actinin seems to facilitate lateral association of F-actin filaments.

In addition to its effect on F-actin, α-actinin promotes the rate and the extent of superprecipitation of synthetic actomyosin (Kominz et al. 1957, Mueller 1966). The action of α-actinin is more pronounced at high concentrations of Mg ions (Fig. 9). Native tropomyosin, and tropomyosin to a lesser extent, both inhibit the action of α-actinin, but in the presence of high concentrations of Mg ions, the inhibition is much less (Fig. 10). Like β-actinin, the amino acid composition of α-actinin resembles that of actin. The properties of β-actinin, another new structural protein found by Maruyama (1965), will be described later.

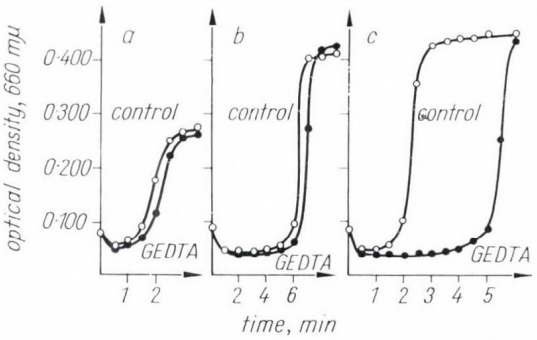

FIG. 8.—Effect of tropomyosin extracted from trypsin-treated myosin B on superprecipitation of synthetic actomyosin. *a* control, *b* tropomyosin extracted from trypsin-treated myosin B 0·03 mg/ml, *c* *b* + troponin 0·03 mg/ml. Actomyosin 0·75 mg/ml, GEDTA $2{\cdot}5 \times 10^{-5}$ M

It is interesting that all three proteins, native tropomyosin, α-actinin and β-actinin, show a profound effect on the physico-chemical properties of F-actin, but not on those of myosin. There is good evidence to say that all these proteins, at least the first two, are located in the thin filament including the Z-band. Thus, the thin filament may not be considered as merely a filament of F-actin, but a complex of these structural proteins. Furthermore, Hama et al. (1960) have shown that the properties of natural F-actin are considerably different from those of ordinary F-actin; e.g., the former is far more labile being more readily denatured. All these facts and considerations strongly indicate that the properties of F-actin filaments in solution do not provide complete information concerning the properties of the thin filament *in vivo*.

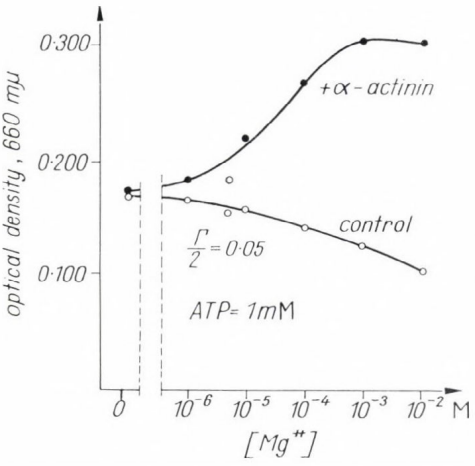

FIG. 9.—Effect of α-actinin on superprecipitation at varied concentrations of Mg ions. Curves indicate the maximum turbidity. α-actinin 0·125 mg/ml, actomyosin 0·75 mg/ml

Localization of new structural proteins in myofilaments.—Two methods were used for the determination of the localization of native tropomyosin and its components in the myofilament (Endo et al. 1967). One included the ordinary antibody technique using FITC (fluorescein isothiocyanate) or ferritin as the marker. The

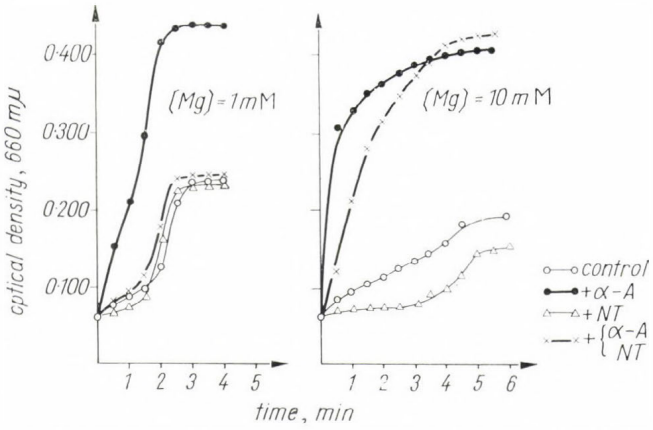

FIG. 10.—Competition between α-actinin and native tropomyosin in superprecipitation at different concentrations of Mg ions. α-A = α-actinin 0·25 mg/ml, *NT* = native tropomyosin 0·06 mg/ml, actomyosin 0·75 mg/ml

other method involved combining the protein itself with FITC and then allowing further combining of this complex with myofibrils, from which the protein under investigation had been removed previously. As described above, trypsin first eliminates troponin preferentially, then tropomyosin. This procedure is quite fitted for our purpose.

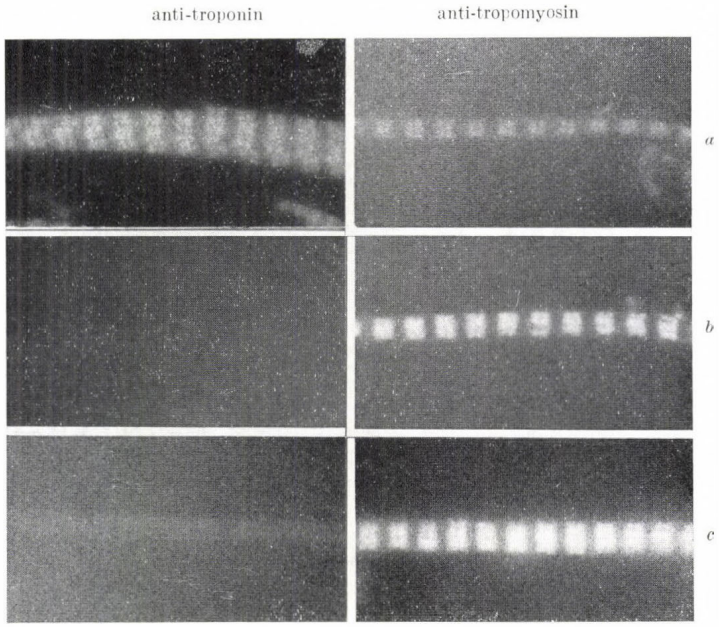

FIG. 11.—Fluorescent microphotographs of FITC-labelled antibodies of troponin and tropomyosin. *a* FITC-antibodies were absorbed by actin, *b* absorbed by troponin, *c* myofibrils were pretreated by unlabelled anti-troponin

FITC-labelled native tropomyosin binds to the entire thin filament region and the Z-band of trypsin-treated myofibrils. The unstained region corresponds to the H-zone exactly and its width increases with the degree of stretching of fibres. After mild trypsin treatment, FITC-troponin binds uniformly to the thin filament region, but not to the Z-band. If treated with trypsin further, the region unstained by FITC-troponin gradually increases around the Z-band. FITC-tropomyosin binds to only the Z-band region after mild trypsin treatment. Further treatment with trypsin increases the area in which FITC-tropomyosin binding occurs.

All these facts suggest that troponin is located along the whole thin filament but not at the Z-band. Tropomyosin seems to exist along the entire thin filament including the Z-band.

The results obtained with FITC-antibody of troponin are consistent with the above supposition (Fig. 11). However, the distribution of antibody of tropomyosin is, to a certain extent, puzzling. It always binds to the entire thin filament region as expected (Fig. 11), but less pronounced to the overlap region and only occasionally to the Z-band. At present we do not have a definite explanation for this.

If we observe the binding of anti-troponin with the electron microscope, a definite periodicity of approximately 400 Å can be observed along the whole thin filament (Figs 12 and 13). This indicates that the well-known periodicity of 400 Å along the thin filament is at least partly due to the presence of troponin.

In the case of α-actinin (Masaki 1965), only one method, i.e. the labelled antibody technique, is used at present. According to this,* the localization of α-actinin is confined to the Z-band region. This is certainly a reasonable location of α-actinin in view of other biochemical evidence. However, the

TABLE III

Localization of structural proteins of regulatory nature

Proteins	Localization
Troponin	thin filament with 400 Å periodicity
Tropomyosin	thin filament and Z-band
Native tropomyosin (troponin–tropomyosin complex)	thin filament
α-actinin	Z-band
β-actinin	?

* The antibody of α-actinin was shown to bind also to the M-line (Ebashi 1966). However, it was revealed later that this was due to the antibody of a different protein contaminating the preparation of α-actinin used for immunization. The investigation as to whether the contaminated protein is identical with the M-line substance is now in progress.

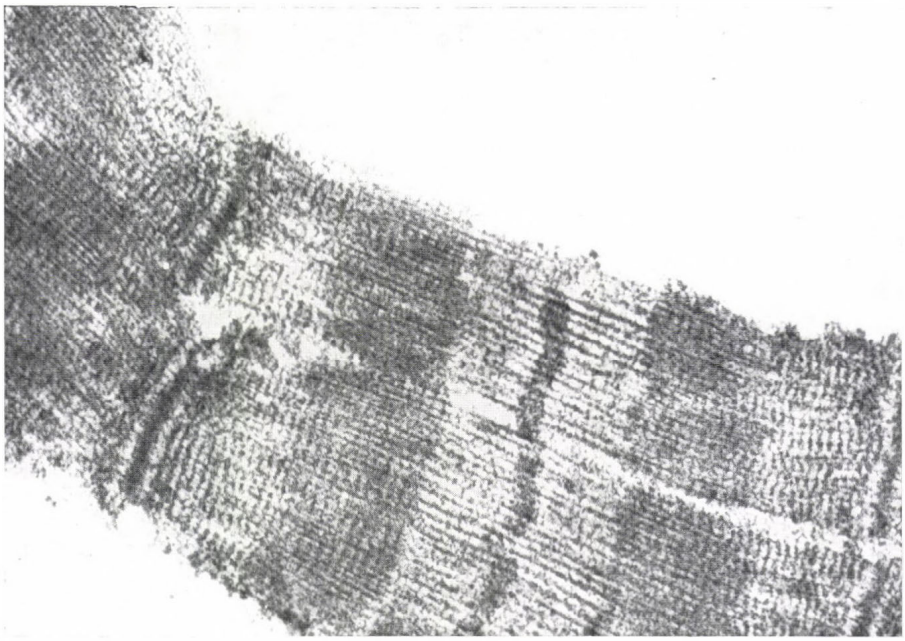

FIG. 12.—Electron micrographs of moyfibrils treated with ferritin-labelled antibody of troponin. Osmium fixation and double staining with uranyl acetate and lead (Milloning). Approx. ×42 500

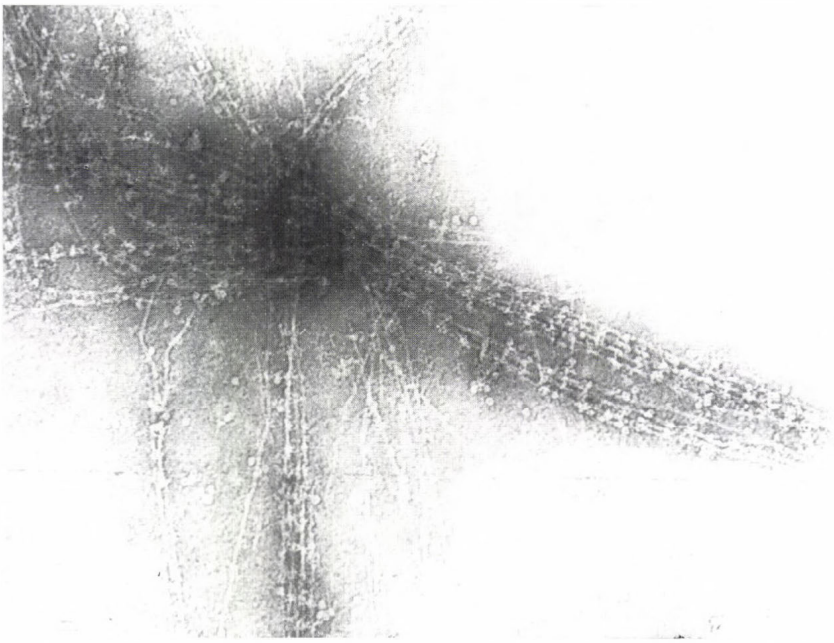

FIG. 13.—Electron micrographs of separated filaments treated with ferritin-labelled antibody of troponin. Negative staining with uranyl acetate. Approx. ×70 000

amount of α-actinin in muscle is too large to be limited only to the Z-band region (Table II). In this respect further investigation is still needed (Table III).

Concluding remarks.—The significance of the structural proteins described here as well as those to be reported later might be considered subsidary compared with the essential nature of actin or myosin in muscle contraction. However, it may also be true that the interaction between actin and myosin would never develop to a real contraction in living muscle without the aid of these proteins. In other words, the more emphasis is laid upon physiological aspects, the more the roles of these proteins can be appreciated.

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ORGANIC SUBSTANCES OF THE STRIATED MUSCLE

I. I. Ivanov

DEPARTMENT OF BIOCHEMISTRY, MILITARY MEDICAL ACADEMY, LENINGRAD

If I venture to take the floor after the brilliant report made by Prof. Ebashi, it is only for the reason that the results of the investigation I want you to take notice of, may possibly be considered an extension of the cardinal researches in the field of muscular biochemistry which have been carried out in Szeged. Our investigation concerns muscular proteins studied in a comparative biochemical aspect or, to be more exact, in phylo- and ontogenesis. There is no doubt that even a simple quantitative determination of actomyosin-complex proteins contained in various types of muscle at different stages of ontogenesis may suggest a number of questions which would scarcely arise if no other but skeletal muscles of adult animals, say rabbits, were examined.

As early as 1948–49, in our study on the chemical compound of skeletal, cardiac and smooth muscles, we noticed that the protein fractional composition of those muscles was markedly peculiar. Slowly contracting smooth muscles capable of producing viscous after-effect, which not unfrequently turns into obturatory function not related to any considerable increase in energy metabolism, proved to differ from rapidly contracting striated somatic muscles in having a low content of actomyosin. This, as well as many other facts discussed previously, allowed us, as early as 1949, to suggest that there exists in the smooth tonic muscles a special substrate of viscous after-effect, or a substrate of indefatigable counteraction to distension, which is not identical with the ordinary actomyosin-substrate of contractile activity. It will be recalled that Parnas, Bailey, Tsao, Rüegg and many other investigators came to similar conclusions when they were studying the smooth obturatory muscles of invertebrates (molluscs).

Particularly from our data, Tsao concluded that tropomyosin B can possibly participate in the obturatory function performance in smooth muscles of vertebrates. Do not forget: what we said about the existence of a special viscous after-effect substrate concerned only smooth tonic muscles. As to the mechanism of producing various types of tonus in striated skeletal muscles, I have no chance of discussing it here. I would like only to mention that in so-called tonic bundles of m. iliofibularis of the frog most fibres belong to the mixed or intermediate type. They contain, probably, two substrates: actomyosin and a substrate exhibiting a viscous after-effect. Depending on the character of stimulation, these fibres are capable of different types of slow or fast contractile reaction. Only a very low percentage of the fibres belongs to the purely tonic type which I mentioned yesterday in my comments to Dr Couteaux's report. All the same, one

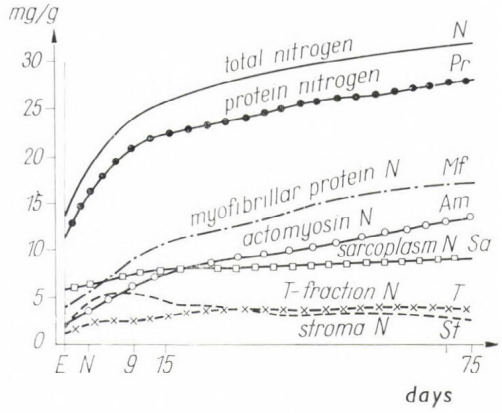

FIG. 1.—Ontogenetic changes in protein fraction nitrogen content of striated muscle in rabbit. *E* = embryos, *N* = newborn animals

should think that data characterizing the behaviour of protein fractional composition of smooth tonic muscles might be of interest to the specialists who are carrying on research into the mechanism of producing and maintaining skeletal muscle tonus of various types.

Studies pursued in our laboratory, and published between 1959 and 1962, have substantiated a previously established fact, i.e. the low level of actomyosin-complex proteins in smooth visceral muscles as well as in striated muscles at early ontogenetic stages. The cardiac muscle occupies an intermediate place, keepingcloser to skeletal muscles of adult animals as far as actomyosin content is concerned.

To examine the protein fractional composition of various types of muscles in ontogenesis we have made use of our modification of muscle protein salt fractionation, as this method enabled us to estimate in a tissue sample, apart from total and protein nitrogen and proteins of sarcoplasm, stroma, and myofibrils (actomyosin complex), the content of myofibrillar proteins soluble in salt media of low ionic strength.

The protein fraction last referred to was previously termed extraprotein. We have termed it T-fraction, since it is not with individual protein but with highly heterogeneous protein fraction that one has to do with here. Chemical compound of T-fraction as well as some of its properties will be discussed a little later.

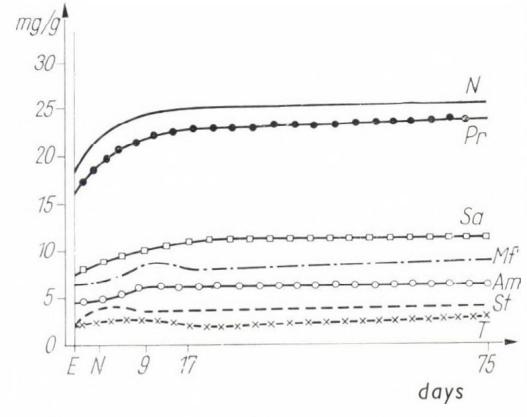

FIG. 2.—Ontogenetic changes in protein fraction nitrogen content of cardiac muscle in rabbit

And now, let me show you a few diagrams to demonstrate the dynamics of ontogenetic changes occurring in protein fractional composition of skeletal and cardiac muscles in rabbit (Figs 1, 2 and 3).

You can see that the protein fractional composition of the cardiac and particularly of the skeletal striated muscle undergoes considerable ontogenetic changes; the increase in total and protein nitrogen being especially characteristic. This age increase in tissue protein content is mainly due to

the reduction in percentual water content which occurs in the organism of a developing animal.

Thus, the curves of ontogenetic changes in protein content in individual fractions must differ in form and incidence depending on the terms in which the protein content is given: in milligrams of nitrogen per gram of wet tissue, or in percentages of total (or protein) nitrogen. Most characteristic of the skeletal muscles in rabbit is an increase in both the absolute and the relative (percentual) content of myofibril proteins, which is observed at definite stages of development. This increase in the main occurs at the expenditure of a marked increase in the amount of actomyosin at the time when fibres with a rapid tetanic contractile response are being formed in the muscle tissue.

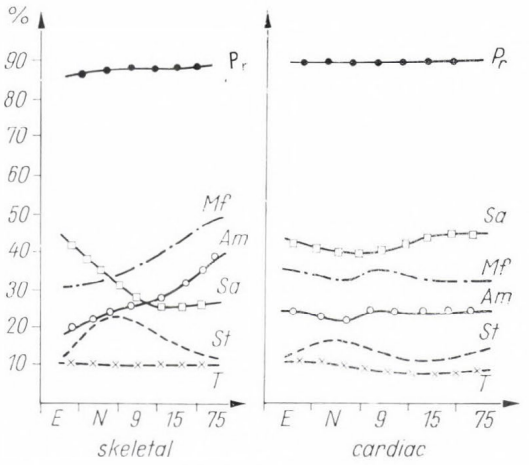

FIG. 3.—Ontogenetic changes in protein fraction nitrogen content of skeletal and cardiac muscles from total nitrogen of tissue in rabbit

These results corroborate the earlier observations of Ivanov and Kashavina (1948), and of Hermann and Nicholas (1948).

One should, however, remember that there exist striated somatic muscles which reach functional maturity as early as at the moment of birth. To this sort of muscles belong e.g. the muscles of mastication and of suckling in mammals as well as the skeletal muscles of animals, such as guinea pigs, which, in the moment of their birth, are already capable of independent life. The muscles of these animals are characterized, at the moment of birth, by contractions of the tetanic type; their actomyosin content does not differ much from that of the muscles of adult individuals.

The cardiac and smooth muscles, which are characterized by the developing of their contractile function as early as in the embryonal period, do not show any marked ontogenetic changes in the content of the examined fractions, the actomyosin-complex proteins included. All this gives evidence of a certain correlation existing between the fractional composition of muscle proteins and the nature of functional activity of various types of muscle at corresponding stages of ontogenesis.

The T-fraction proteins in these types of muscle do not undergo any considerable ontogenetic changes. Therefore, the ratio actomyosin/fraction T markedly increases in skeletal muscles and does not change in cardiac muscle in ontogenesis.

The figures characterizing the fractional composition of skeletal and cardiac muscles in adult rabbit are given in Table I. For lack of time, I shall not discuss them here. The only remark I will make in passing is that the protein fractional composition of the cardiac ventricular muscle, both by our and literature data, essentially differs from that of the auricular muscle,

TABLE I

Fractional composition of protein in the skeletal and cardiac (ventricular) muscles of a mature rabbit (determined after Ivanov and co-workers 1959)

Fraction		Skeletal muscle	Cardiac muscle (ventricles)
Total N of the tissue:			
a) mg N/g of tissue		32 — 34·5	25·0 — 27·6
b) % N of fraction to total N of tissue:		—	—
Protein N	a)	28·3 — 30·2	23 — 24·99
	b)	88 — 89	89·8 — 90·5
Non-protein N	a)	4	2·21 — 2·82
	b)	10 — 12	9·0 — 10·2
N of sarcoplasmic proteins	a)	8·95 — 9·7	8·5 — 11·24
	b)	27·6 — 28·6	34 — 40·7
N of myofibrillar proteins	a)	17·0 — 17·3	8·0 — 8·5
	b)	50 — 53	30 — 33
Actomyosin N	a)	13·5	6·1
	b)	39 — 41·8	22·1 — 23·9
N of fraction T	a)	3·6 — 3·8	2·3 — 2·5
	b)	11·1 — 11·3	8·3 — 9·4
Stroma N	a)	2·8 — 3·48	5·36 — 5·5
	b)	8·6 — 10·1	19·5 — 23·0
Ratio AM/T		3·5/1 — 3·8/1	2·6/1

where e.g. the content of myofibrillar proteins is considerably lower and the content of stroma proteins is considerably higher than in the left ventricle.

Now, some considerations about the methods used for the quantitative determination of muscle proteins. It should be stressed that, for many reasons, none of the available modifications of the muscle protein fractionation proves perfect.

Special difficulties arise in those cases where the methods of salt fractionation are employed for the quantitative determination of proteins in the embryonal skeletal muscle (as well as in the smooth muscles of the viscera). The term actomyosin may be associated then with a fraction that corresponds, by the way of its forming, to the actomyosin of a functionally mature skeletal muscle, only in a conventional manner; so, for instance the embryonal actomyosin fraction comprises a considerable amount (after Robinson 1952), about 60 per cent, of nucleoproteins of the cellular nuclei. These proteins are extracted from the muscle brei, in addition to actomyosin, by salt solutions of high ionic strength, and they fall out with actomyosin in the precipitate upon dialysis or dilution of the extract. Hence, the methods devised for the quantitative determination of the actomyosin-complex proteins in a functionally mature striated muscle tissue will naturally poverstate the actomyosin content in the embryonal muscle tissue as well as in the smooth visceral muscles of adult individuals.

The term myofibrillar proteins cannot be accepted without reserve either, as far as smooth muscles of vertebrates are concerned. As is well known, all longitudinal myofilaments of these muscles are actin. According

to Panner and Honig (1966), in smooth muscle cells 'myosin exists as dimers which connect and interact with two relatively distant filaments'. However, by treating minced smooth muscular tissue with salt solution of high ionic strength, one may extract some amount of actomyosin associated in the same way as in striated filaments with globular proteins. By analogy with skeletal muscles we termed the above proteins T-fraction proteins as well.

From the methodological point of view, of great importance is also the question whether a water-soluble modification of actomyosin may be found in smooth muscles (Needham and Schoenberg 1964, Schirmer 1965).

This problem was recently discussed in Mrs Dorothy Needham's report published in the *Proceedings of Royal Society*.

Allow me to remind you of the fact that, according to the authors referred to, mincing smooth muscles by grinding with quartz sand in salt solution of low ionic strength (0·05 M NaCl) may result in getting a special water-soluble variety of actomyosin, Laszt and Hamoir's tonoactomyosin, which little by little or rapidly (if crystalline NaCl is added) turns into ordinary water-insoluble actomyosin. At the same time, after Jean Hanson and co-workers (1957), it can be believed that tonoactomyosin solutions are suspensions of fragmented actin and myosin filaments, which are released when the muscle is homogenized.

We, in our turn, are inclined to assume that tonoactomyosin does not exist in the capacity of individual protein.

In our laboratory, Dr G. F. Degtyareva has demonstrated that the water-soluble variety of actomyosin may be extracted from smooth stomach muscle of rabbit not only by grinding a tissue sample in salt solutions of low ionic strength but also by treating thin slices of stomach tissue by the same solution. The extracts obtained in this way, in accordance with literature sources, gradually gelatinate, when stored.

However, it is interesting to note that if the extract of water-soluble actomyosin obtained is immediate, while its viscosity is still low, ultracentrifugated with an acceleration of 106 000, a thick viscous layer will be found in the bottom of the test tube; electron-microscopic examination will readily reveal there some structures that bear a strong resemblance to the fragmented F-actin filaments. No protein of tonoactomyosin properties remains then in the supernatant fluid.

All this allows of assuming that, in fact, by treating a homogenized smooth muscle tissue with salt solutions of low ionic strength, we obtain a suspension of fragmented contractile protein filaments or a mixture of actin and myosin as dimers; they pass into solution together with water-soluble sarcoplasm proteins. When stored such solutions gradually convert into ordinary actomyosin viscous colloidal gel.

Whatever the ultimate decision about the existence of a water-soluble variety might be, the facts established by Laszt and Hamoir, and by D. Needham and co-workers, the facts that prove that a part of actomyosin may pass into solution together with sarcoplasmic proteins extracted from smooth muscular tissue samples, appear to be of great importance.

It is true that the amount of actomyosin passing in this way into solution depends on the ionic strength, pH, and salt composition of the solution and therefore we are able to minimize it by varying these conditions.

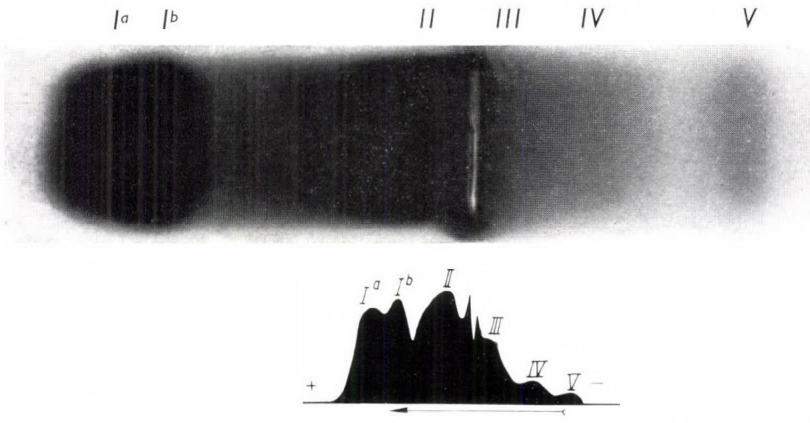

FIG. 4.—Electrophoregram and densitogram of myocardium T-fraction proteins in dog

But all the same, for an exact quantitative determination of protein fractional composition in smooth muscle one should foresee a chance of some actomyosin having penetrated into the sarcoplasmic protein fraction, and accordingly, introduce necessary corrections into the ultimate calculations. This fact, which had never been considered before, may be responsible, as well as other causes, for a rather considerable discrepancy in figures referred to by different authors to characterize the protein fractional composition of smooth muscles (the content of sarcoplasmic proteins and of actomyosin).

These last years the attention of many investigators has been drawn to myofibrillar proteins soluble in salt media of low ionic strength. These proteins were given different names by different authors. Since the point in question is a highly heterogeneous system and not the individual protein, we have termed these proteins as fraction T, as has already been said above.

It is very likely that the free space between separate myosin and actin protofibrils of skeletal muscles easily observed on electron micrographs, is filled with a motile sol formed just by these proteins. Thus, the T-fraction proteins seem to secure in a sense the constancy of the interior milieu of myofibrils. According to our data published between 1959 and 1962, that fraction contains about 40 and 50 per cent extremely labile proteins, which are very near to the sarcoplasmic X-globulins of Weber; it also contains tropomyosin and other not yet identified proteins. We have investigated the proteins of T-fraction by fractional salting out method, electrophoresis in agar gel, enzyme electrophoresis, ultracentrifugation, flow birefringence and by tracer isotope method. A part of these data have been lately published in Russian.

First of all it should be noticed that the T-fraction proteins, at least the bulk of them, do not display flow birefringence and, thus, they appear to be globular proteins. Using the method of ammonium sulphate salting out, after Derien, ultracentrifugation and electrophoresis in agar gel, a strongly pronounced heterogenicity of T-fraction proteins was demonstrated. A great part of them had a small molecular weight and did not precipitate during the 160 minutes of preparative ultracentrifugation with 56 100 revolutions per minute.

Now, I should like to show you an electrophoregram in agar gel and a densitogram of T-fraction proteins extracted from the myocardium of dog (Fig. 4).

It is evident from this electrophoregram that the T-fraction proteins prove to be indeed a heterogeneous system which usually consists of IV–VI components. I^a and I^b subfractions are so alike with regard to their electrophoretic motility that in some cases it is difficult to distinguish one from the other. One of them seems to be tropomyosin (Fig. 5).

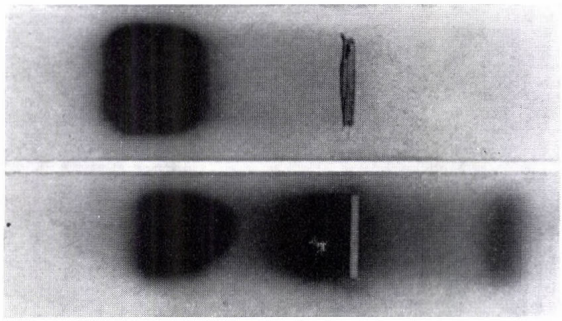

FIG. 5.—Electrophoregrams of tropomyosin sample(I) and of myocardium T-fraction proteins (II) in dog

TABLE II

Percentual distribution of protein in individual subfractions of myocardium T-fraction in dog

$I/I^a + I^b$	II	III	IV	V
37·5	42·2	15·4	3·9	1·0

Peak II (Fig. 4) contains a great amount of protein. This protein (or proteins) readily undergoes spontaneous denaturation when concentrated. In this respect it resembles Weber's sarcoplasmic X-globulins. Much less protein falls to the share of the cathode components (III, IV and V). The nature of these proteins has not yet been identified.

In one of our experiments, carried out together with Dr Markelov, the percentual content of proteins in various components of fraction T extracted from the myocardium of dog was on the electrophoregram as follows (Table II).

The composition of fraction T extracted from skeletal and smooth muscles of rabbit is rather similar to that of fraction T extracted from myocardium. However, the percentual distribution of protein in individual subfractions varies a little or even rather significantly.

In a parallel electrophoretic fractionation of sarcoplasmic proteins and T-fraction proteins (Fig. 6) one can see well that the proteinogram of fraction T markedly differs from that of sarcoplasm.

Thus, we must reject the suggestion that fraction T consists of sarcoplasmic proteins which have resisted isolation in treating the muscular homogenate with a salt solution of low ionic strength.

The conclusion that the composition and properties of T-fraction proteins are not identical with those of sarcoplasm is corroborated by the data obtained in studying enzymic activity and isoenzymic spectres of sarcoplasmic and myofibrillar proteins soluble in salt media of low ionic strength.

In our laboratory, Dr N. F. Korovkin, I. M. Markelov and N. P. Mikhaleva have revealed cholinesterase, lactate dehydrogenase, aldolase, aminotransferases (alanine and aspartic-amino-transferases), glucose-phosphat-

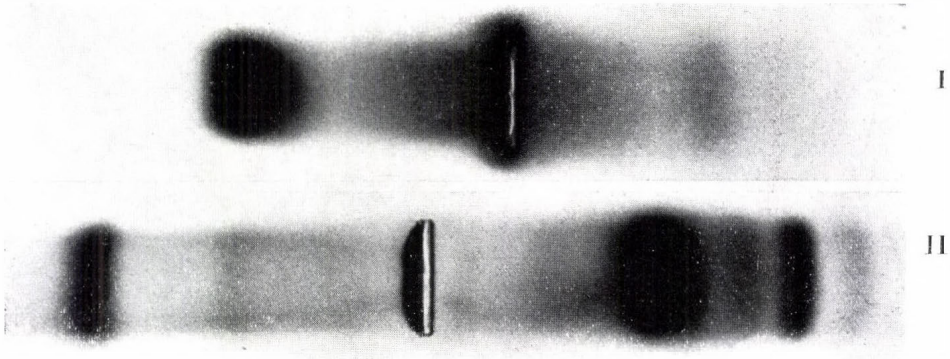

FIG. 6.—Electrophoregrams of T-fraction (I) and sarcoplasmic proteins (II) of myocardium in dog

isomerase and some other enyzmes to be present both in sarcoplasmic and T-fraction proteins. However, their activity estimated in milligrams of protein proved to be quite different.

Specific enzymic activity of sarcoplasmic proteins was considerably higher. Thus, it is evident that most of the enzymes which appear in the blood plasm, e.g. in myocardiac infraction, are to be considered, in accordance with known data, enzymes of sarcoplasmic and not of myofibrillar origin.

Moreover, it was proved that the isoenzymic spectres of fraction T proteins and of sarcoplasm are different, too. As an example, allow me to demonstrate the distribution of lactate dehydrogenase in the enzym-electrophoregram of sarcoplasmic and T-fraction proteins in skeletal muscles (Fig. 7).

The materials presented leave no doubt that myofibrillar proteins soluble in salt media of low ionic strength considerably differ in their properties and composition from proteins of muscle fibre sarcoplasm.

Since the proteins of fraction T cannot be extracted from muscular brei by water or salt solutions of low ionic strength, though they are readily soluble proteins, a suggestion arises of their being closely associated with myosin and actin filaments. These proteins are very likely to form complexes which may be termed native contractile proteins.

As has already been said, the peculiar features of smooth muscle contractile activity, in particular the capacity for indefatigable counteraction to distension, may only depend on the fact that contractile proteins form complexes with suitable components of T-fraction which may be tropomyosin.

A further detailed study of individual components of T-fraction proteins seems to be especially interesting, as this fraction contains globular proteins, liable to a rapid turnover.

These data were obtained in studying skeletal muscles of 6-day and 18-day-old rabbits as well as the smooth muscle of pregnant uterus. It should be recalled that the incorporation intensity of radioactive amino acids, both in total muscle protein and in individual protein fractions, in embryos and newborn animals is several times higher than in adult individuals. This fact is certainly due to an intensive synthesis of muscular proteins at early stages of ontogenesis. In animals that have reached full growth, the amino acid

tracer incorporation in muscle proteins, on the other hand, proves to be relatively low. Lack of time prevents me from giving detailed description, so I would like to sum up in short the most important conclusions we arrived at.

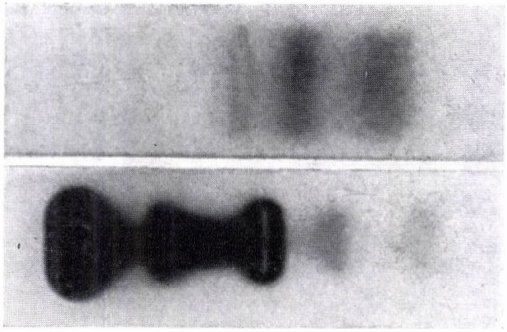

FIG. 7.—Enzymelectrophoregrams of lactate dehydrogenase of T-fraction (I) and sarcoplasmic proteins (II) of skeletal muscle in rat

In experiments on 18-dayold rabbits, the most intensive radioactive glycine incorporation, 6 hours upon injecting labelled amino acid (20 μCi on 100 g weight), was observed in proteins of fraction T, proteins of sarcoplasm and proteins of actomyosin complex. The radioactivity of stroma was not high. In one of the experiments the ratio of various fraction activities expressed in impulse per minute was as follows: 685(T) : 559(Sr) : 553(AM) : 104(Str).

Although a single determination of the various protein fractions' activity performed always 6 hours upon radioactive glycine injection, can hardly give a good idea of the dynamics of synthesis and turnover in some or other protein structures in a living organism, the above data are still of interest;

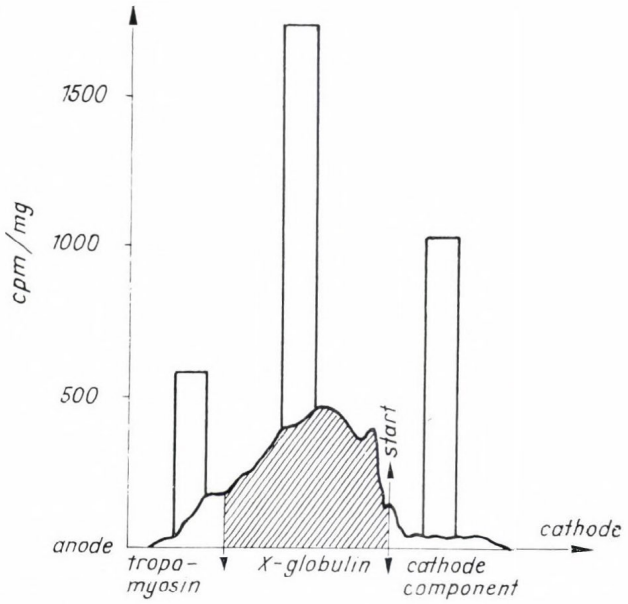

FIG. 8.—Densitogram of T-fraction proteins in skeletal muscles and distribution of ^{14}C-glycine activity. The latter shown by columns and calculated per 1 mg of subfraction protein

they show that fraction T, or at least some of its protein components, belong to metabolically active muscle proteins.

In our subsequent work carried out together with Dr J. J. Keerig we have concentrated our attention upon studying the incorporation of labelled amino-acid in individual protein components of T-fraction into skeletal muscle. In that series of experiments, the fraction T was isolated from 18-day-old rabbit skeletal muscle, concentrated and then exposed to electrophoresis on agar gel. The radioactivity of individual protein subfractions was assessed directly on a dried agar plate. The activity was expressed by the number of decays in minute per 1 mg of dry protein. Radioactive glycine was found to appear especially fast in the subfraction of labile globulins of fraction T (Fig. 8).

A marked incorporation in that subfraction was already noticed 30 minutes after the beginning of the experiment. Other components of fraction T, particularly tropomyosin, did not show any noticeable radioactivity at that period of time (30 min). In the tropomyosin area of the electrophoregram radioactivity manifested itself afterwards and during our experiments its maximum value did not reach that of the T-fraction globulin component.

The same picture is seen in the distribution of activity between myometrium protein fractions of rabbit in the first half of pregnancy.

Thus, the labile globulins of fraction T as well as Weber's X-globulins do belong to the fast synthesizing ones and undergo fast turnover. Hence it seems quite possible that they play the role of some sort of depot to store amino acids for a pressing synthesis of the actomyosin-complex proteins at certain stages of ontogenesis or, possibly, when an intensive training of muscles provokes an increase in their mass.

I do not certainly consider this statement proved but it seems to be quite possible. In any case we do not agree with Meierson (1963) that this function can be attributed to tropomyosin.

The data obtained induce us to turn our attention to a problem which is still under discussion. As is well known, the investigations of Hogness, Cohn and Monod (1955), Rotman and Spigelman (1954), carried out on rapidly propagating bacterial cells, have shown that, in spite of the well-known conception of Schoenheimer, the bacterial proteins do not appear to be, practically, in the state of dynamic balance with the amino acids of the environment. This has made for the belief that the turnover of protein structures in the multicellular organism, which was observed in experiments using the isotope method, is only a reflection of the permanent dying off and new formation of various cellular elements whose destruction is followed by a release of amino acids and their subsequent contribution to the amino acid pool of the organism. By this conception, the assumption that the cytoplasmic proteins can be made use of, without destruction of the cells, as a source to supply amino acids to other tissues and organs is disproved.

There exist, however, a few facts showing that the idea of utilizing, in the organism of animals, the proteins of living cells as suppliers of amino acids for the purpose of endogenic protein nutrition is not impossible. It should not be forgetten that the marked development of the mass of skeletal muscles when trained, and the decrease in their volume and weight because of forced inactivity, is not associated, as the morphologists know, with changes occurring in the number of muscular fibres. It is a real increase or decrease in

the proportions and mass of every striated muscle fibre that we have to do with here.

Thus, as far as the striated muscle tissue of animals is concerned, there are no reasons to reject the idea of a possible utilizing by an organism one type of proteins to synthesize others, particular to a given tissue.

Investigations in that direction are going on in our laboratory.

My time is up.

Some data and considerations on the ontogenetic changes occurring in the form and proportions in the contractile protein particles have been given in the theses of my report. Additional material will be presented by my pupil and friend—Dr G. P. Pinaev.

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GENERAL DISCUSSION

Hanson (King's College, University of London): Vertebrate skeletal muscle, sectioned and examined in the electron microscope, shows an axial periodicity of about 400 Å that has been known for many years. Recently, two periodicities have been distinguished in such sections, one associated with the actin filaments (Carlsen et al. 1961), the other with the myosin filaments (Huxley 1964). It is the one associated with the actin filaments that I want to discuss now. For various reasons it seems unlikely that it could be due to the helical form of the actin filaments (Hanson and Lowy 1964). I do not intend to repeat the arguments now but to present some new evidence that is emerging from current studies of F-actin prepared from rabbit skeletal muscle.

When one takes a solution of F-actin and raises the concentration of Mg^{++} or of Ca^{++} to about 0·1 M, the protein precipitates (Bárány et al. 1954). I have examined the structure of these precipitates; they are visible in the light microscope. Before showing you the electron micrographs I should remind you of the structure of a single F-actin filament (Fig. 9); this can be represented by a model consisting of two strands of globular molecules twisted round one another, with the cross-over positions of the two strands spaced at regular intervals (Hanson and Lowy 1963).

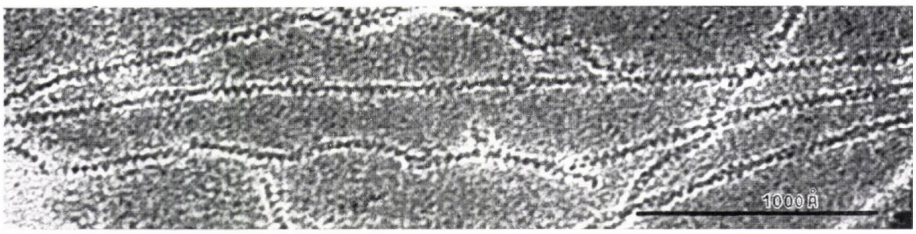

FIG. 9. — Electron micrograph of F-actin negatively stained with uranyl acetate. Single filaments

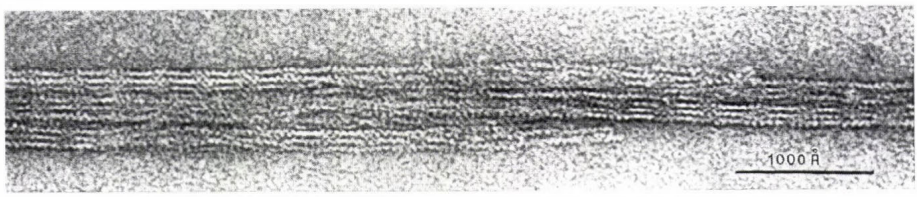

FIG. 10. — Actin prepared by Straub's method and purified (cf. Fig. 11). Aggregates formed by 0·07 M $MgCl_2$

The aggregates formed at high Mg^{++} concentration are flat structures in which the actin filaments lie side by side, often with the cross-over positions in transverse alinement (Fig. 10). The actin used for Fig. 10 was made by the Straub method, and kept at 0° C during extraction from the acetone-dried residue; it was purified by two cycles of depolymerisation and repolymerisation. The same actin preparation was used for the section shown in Fig. 11. The precipitated protein was centrifuged, fixed in 1 per cent OsO_4 (buffered with veronal acetate at pH 7·0), dehydrated in ethanol, stained with phosphotungstic acid and imbedded in Araldite. No details of the structure of the actin filaments are visible in sections of this material. In particular, there is no sign of the helical form of the filaments (cf. Fig. 10).

Figure 12 shows a section from a different block prepared in exactly the same way as the last one (Fig. 11), except that the actin was prepared differently. Now all the aggregates show a conspicuous cross-striation. Measurements of this periodicity are highly variable, presumably because the randomly oriented aggregates undergo different amounts of compression during sectioning. But fortunately microtome knives can have flaws: measuring aggregates oriented with the long axis at right angles to knife marks in the section, one obtains much less variable values for the spacing of the striation. In glutaraldehyde-fixed material the spacing measures about 410 Å, whilst in material fixed in OsO_4 the value is about 370 Å. These results are similar to those of Page and Huxley (1963) for the periodicity associated with the actin filaments in sections of intact muscle fixed in glutaraldehyde or in OsO_4.

The actin which formed the striated aggregates shown in Fig. 12 was made by the potassium iodide method of A. G. Szent-Györgyi (1951) and

FIG. 11.—Section through aggregates of purified Straub actin

was not purified. Figure 13 shows the same unpurified actin prepared by a negative contrast method. It is not easy to see the helical structure of the individual actin filaments or to see how the helices are alined; perhaps this is due to the presence of other material that obscures the fine structure. The lateral alinement of the actin helices does not appear to be worse in the purified Straub actin (Fig. 10) than in the impure KI actin (Fig. 13), yet the purified material does not show striations in sections, whereas the impure material does.

In summary, aggregates of synthetic actin filaments formed by raising the Mg^{++} ion concentration can show, in sections, a conspicuous axial periodicity that resembles the periodicity associated with natural actin filaments in sections of intact vertebrate skeletal muscle. This striation is found in preparations of unpurified KI actin, but it is not found in preparations of purified Straub actin. Examination of negatively-stained aggregates indicates that the striation is not due to the helical structure of the actin filaments themselves. These results suggest that the periodicity seen in the intact

FIG. 12.—Section through aggregates of unpurified KI actin

muscle is most likely due to other material associated with the actin filaments.

Possibly this other material is tropomyosin B (see Hanson and Lowy 1963, 1964). Cohen and Longley (1966) have found that tropomyosin precipitates as cross-striated tactoids in 0·1 M Mg^{++} or Ca^{++}. In negatively stained preparations they found that the periodicity measured about 400 Å.

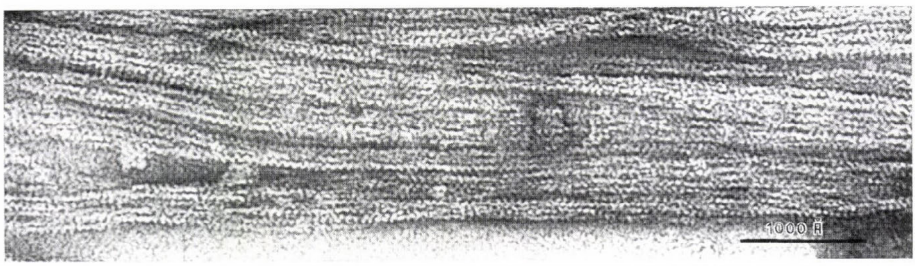

FIG. 13.—Actin prepared by KI method and not purified (cf. Fig. 12). Aggregates formed by 0·07 M $MgCl_2$

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GERGELY: Do you have information concerning the effect of the extraction temperature on the appearance of striations in the sectioned actin aggregates?

HANSON: The Straub actin was extracted at 0° C, the KI actin at about 4° C.

DRABIKOWSKI: Dr Hanson, have you checked the microscopic picture of crude actin extracted both at room temperature and 0° C? In our earlier work with Dr Gergely it was observed that purified actin obtained from water extract from acetone-dried muscle powder extracted at 0° C did not contain tropomyosin; on the other hand, if extraction was performed at room temperature tropomyosin could be demonstrated. Our recent determinations have showed, however, that the tropomyosin content in crude actin preparations, i.e. in the water extracts from acetone-dried muscle powder, does not differ substantially depending on the temperature of extraction. Crude actin preparations extracted at room temperature contain on the average 25 per cent of tropomyosin and, when extracted at 0° C, about 20 per cent of the latter protein. Room-temperature extracted actin after one polymerization and depolymerization cycle still contains about 15 per cent of tropomyosin, whereas in the case of 0° C-extracted actin most of the tropomyosin remains in the supernatant during centrifugation of F-actin in Spinco, so that the obtained purified actin contains, in agreement with our previous results, only 2–4 per cent of tropomyosin. The reason for this difference is not clear at present. One may assume that temperature does not influence the extraction of tropomyosin and only some unknown factor, being extracted at room temperature, is necessary for the actin-tropomyosin interaction.

Perhaps the comparative examination under the electron microscope of F-actins obtained from crude and purified actin, extracted at either temperatures, will help to elucidate both the results mentioned above, and the observation of Dr Hanson concerning the differences between Straub-actin and KI-actin.

HANSON: I have looked at sectioned aggregates of Straub actin, extracted either at 0° C or at room temperature, but in neither case purified. I have not yet studied enough preparations to be certain about the results.

MARUYAMA: I should like to make a brief supplement to Dr Ebashi's presentation on the new structural protein of striated muscle.

β-actinin, actin-dispersing factor, acts on F-actin so as to inhibit the network formation and to regulate the particle length of polymerizing F-actin (Maruyama 1965a, 1965b, 1966).

Recently, an electron-microscopic study on the action of β-actinin has been performed in collaboration with Drs Nonomura and Otsuki in Dr Ebashi's laboratory. Usual F-actin of Straub type showed very long particle length, more than 3 μ. When polymerized in the presence of a suitable amount of β-actinin, F-actin particles of 0·5–1·2 μ in particle length were observed under electron microscope. Much shorter F-actin particles of 0·2–0·3 μ were obtained, either when G-actin was polymerized or when preformed F-actin was sonicated in the presence of a large amount of β-actinin (one half of actin in weight). A detailed study along this line is in progress.

As reported before (cf. Dr Ebashi's presentation) actin and α- and β-actinin have very similar amino acid composition. These three proteins were denatured by heat and treated by trypsin. The resultant peptide products were compared by paper chromatography. Peptide maps of these proteins were shown to be very similar to each other. This work has been carried out by Dr H. Hama in our laboratory. Dr Masaki in Dr Ebashi's laboratory has recently shown that these three proteins are distinguishable by immunological tests. It is very probable that actin and both actinins are very similar proteins whose functional sites are different.

We have reported that F-actin can be isolated from myofibrils without depolymerization process (Hama et al. 1965). It was shown that this 'natural F-actin' contains both α- and β-actinin. α-actinin could be removed by repeating sedimentation and washing of natural F-actin, whereas β-actinin was not washed out. The action of trypsin to digest actinin bound to natural F-actin was not so remarkable as in the case of KI-extracted F-actin. β-actinin bound to Straub F-actin was easily freed off by washing or by trypsin treatment.

We strongly feel that the understanding of structure and function of the thin or I-filaments of myofibrils requires the elucidation of mechanism of the action of α- and β-actinins on Straub type of actin.

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GUBA: As I mentioned yesterday, after removal of the known structural proteins (i.e. myosin, tropomyosin, actin) one can see a continuous filamentary system in myofibril left behind. We have tried to isolate the material of these filaments. Our starting material, to avoid contamination from other cell elements as far as possible, was isolated and thoroughly washed myofibrils. Care was taken to avoid denaturation of proteins during their extraction. The residue after removal of the proteins mentioned previously was washed with 5 vol of a solution of 0·1 M KCl (pH 4·5). After centrifugation from the sediment a homogeneous protein was extracted and characterized as has already been described (Guba 1965).

The amino acid composition of the protein calculated for a g mol/10^5 g protein was as follows: Cys/2 1, Asp 81, Thr 61, Ser 53, Glu 86, Pro 51, Gly 82,

Ala 67, Val 50, Met 38, Ileu 54, Leu 60, Tyr 34, Phe 40, His 19, Lys 51, Arg 54, Try 10, Sarcosine 4. Total: 900. Polar groups: 440.

The exact proof of the presence of sarcosine (i.e. it is not a consequence of a decomposition) requires further investigations. The apolar character of fibrillin seems to be expressed. The protein has a sedimentation constant

$$S^0_{20} = 4{\cdot}60(\pm 0{\cdot}08)\mathrm{S}$$

The diffusion constant is

$$D^0_{20} = 4{\cdot}36(\pm 0{\cdot}08)10^{-7}\ \mathrm{cm^2/sec}$$

Intrinsic viscosity is

$$\lim_{c \to 0} \frac{\eta_{\mathrm{spec}}}{c} = 0{\cdot}25$$

During slow precipitation the protein forms well-ordered aggregates as revealed by electron microscopy.

The extracted protein does not show any enzymatic function and there is no experimental evidence for any interaction between the fibrillin and the other proteins of myofibril. Thus its role in the function of striated muscle is probably a structural one.

Nevertheless, it seems most probably that the isolated protein, because of the isolation method, differs from the native one. To clear up this problem experimental work is under way.

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Hanson: Dr Guba, how do you extract fibrillin? Could the extracts be contaminated with material from the Z-lines?

Guba: We think that, owing to the excessive purification of our starting material, there cannot be much of the Z-substance in fibrillin.

Kominz (National Institutes of Health Pacific Office, United States Embassy, Tokyo): I should like to talk about phase-changes related to ATPase activity, the interaction of Ca^{++} and native tropomyosin with myosin, and some relationships between swelling and phase-changes in muscle proteins.

Figure 14 illustrates the effect of mercurial titration on the Ca^{++}-activated ATPase of myosin (Kominz 1965). If ATP or pyrophosphate is added prior to mercurial, the loss of ATPase activity is prevented. Ultracentrifugation experiments showed that the protective action of ATP is through the prevention of transformation of the slow sharp myosin peak into faster-sedimenting forms (Kominz 1965).

At low ionic strength, prior to the addition of mercurial, ATP has the opposite effect on the mercurial inhibition of the Mg^{++}-activated ATPase of actomyosin (Kominz 1966). Here we see that the addition of mercurial in the presence of ATP causes a greater loss of ATPase activity than the addition of mercurial in its absence (Fig. 15). It is proposed that ATP bound at a site other than the active center affects the mercurial-induced configurational change—interacting repulsively with a negative group at

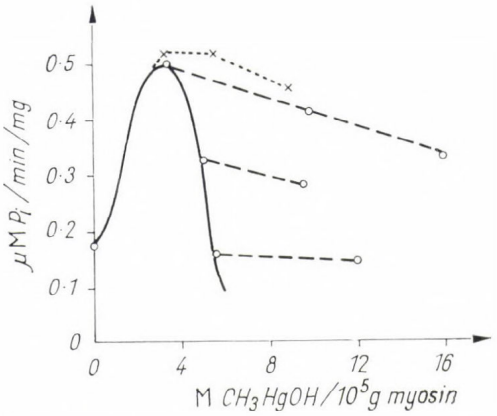

FIG. 14.—Effect of methylmercuric hydroxide on Ca^{++}-activated ATPase activity in the presence or absence of ATP or pyrophosphate. Assays performed by pH-stat at pH 7·0 and 20° C, on system containing 0·25 M KCl, 5 *m*M Ca^{++} and 2 *m*M ATP. Symbols: o myosin, × myosin treated first with pyrophosphate; solid line = methylmercuric hydroxide added in the absence of ATP; dashed line=methylmercuric hydroxide added in the presence of ATP

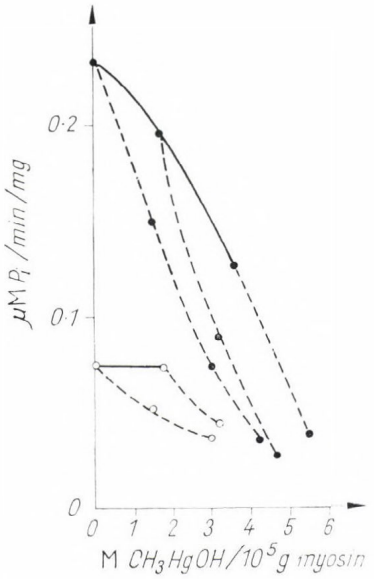

FIG. 15. — ATP-modified effect of methylmercuric hydroxide on Mg^{++}-activated ATPase at 0·017 ionic strength. Symbols: solid line= methylmercuric hydroxide added in the absence of ATP; dashed line = methylmercuric hydroxide added in the presence of ATP. Synthetic actomyosin

low ionic strength and attractively after that cation-binding removes the negative charge.

If one examines the Mg^{++}-activated ATPase of mercurial-titrated actomyosin in the presence of native tropomyosin (Fig. 16), one finds that the inhibitory effect of native tropomyosin is more sensitive to mercurial than is the ATPase activity.

Interaction with native tropomyosin has been studied by Maruyama, Ishikawa and Ebashi (Maruyama et al. 1964). They have found a broad zone of ionic strength in dependence on the inhibitory action of native tropomyosin, which is narrowed markedly on trypsin digestion of the myosin. I have found (Kominz 1966) that in the region of sensitivity to native tropomyosin, there is a dependence of this sensitivity upon the concentration of native tropomyosin which strongly suggests a binding process (Fig. 17). To demonstrate whether this binding is to myosin or to actin, experiments utilizing radioiodine-labelled native tropomyosin were performed (Kominz and Maruyama, in press). It was clearly seen that binding took place only to actomyosin (Fig. 18) or to actin (Fig. 19). Therefore, the interaction of the myosin center reflected by the Ca-sensitivity must occur either with native tropomyosin bound to actin or with a part of the actin molecule made reactive owing to this binding of native tropomyosin.

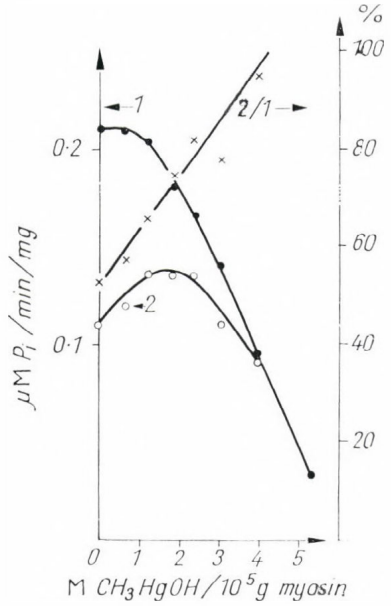

FIG. 16.—Effect of methylmercuric hydroxide titration on Mg^{++}-activated ATPase of myosin at 0·013 ionic strength in the presence of native TM. Symbols: ● actin + myosin + native TM, ○ same + EGTA, × ATPase (+ EGTA)/ATPase (untreated); left ordinate for ● and ○, right ordinate for ×

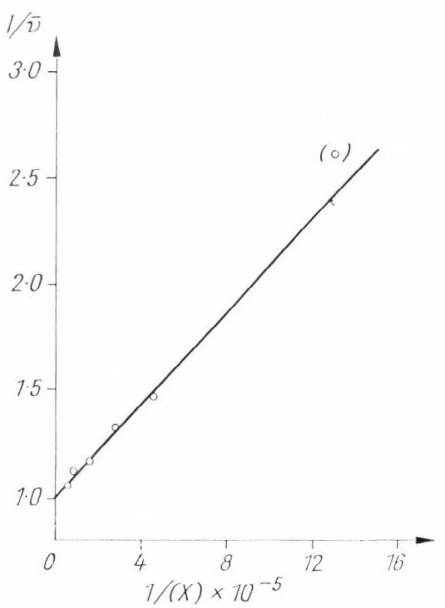

FIG. 17. — The reciprocal of the inhibition of the Mg^{++}-activated ATPase plotted against the reciprocal of the native TM concentration

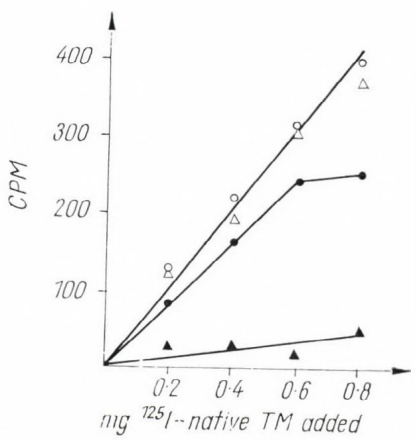

FIG. 18.—Binding of ^{125}I-labelled native TM to actomyosin and myosin. Symbols: ○ original myosin solution, △ myosin precipitate, ● original actomyosin solution, ▲ actomyosin precipitate

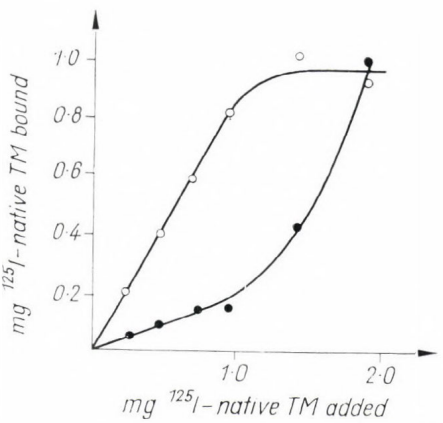

FIG. 19.—Binding of ^{125}I-labelled native TM to actin. Symbols: ○ mg native TM bound to 3 mg F-actin pellet, ● mg native TM left in supernatant solution

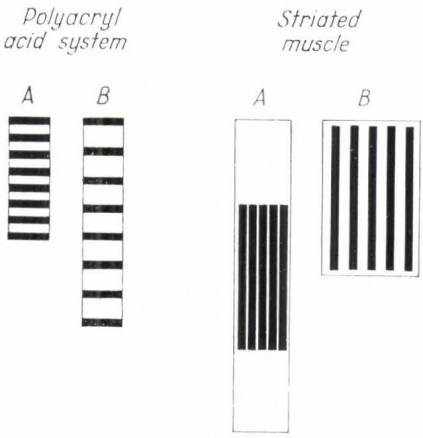

FIG. 20. — Comparison of the swelling properties of a block made from alternate layers of cross-linked polyvinyl alcohol and polyacrylic acid with the postulated swelling properties of a sarcomere of striated muscle. Left: black foils are polyvinyl alcohol; white foils are polyacrylic acid. Right: black rods are rigid α-helical LMM portions of myosin; white space between the rods contains amorphous HMM portions of myosin. *A* state of reduced swelling, *B* state of increased swelling

Although I have proposed that the swelling of myosin could be related to the phase-transition involved in muscle contraction (Kominz 1965), I have been able to produce evidence for such a Ca^{++}-triggered reversible swelling only in one case. This is concluded on the ground of changes in ultracentrifuge patterns of heavy meromyosin resulting from certain papain digestion procedures (Kominz et al. 1965). We have found that in the presence of low levels of pyrophosphate, the addition of 10^{-5} to 10^{-4} M Ca^{++} can cause a slowing of a part of the peak, interpretable as a swelling of 10 to 30 per cent. Removal of Ca^{++} by EGTA causes a reversal of the process, and Mg^{++} cannot replace Ca^{++} in producing the swelling.

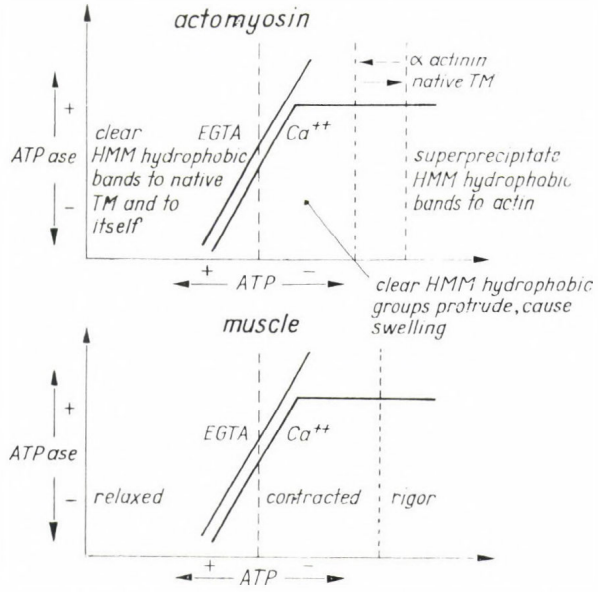

FIG. 21.—Schematic comparison of zones of ATP concentration where the Ca^{++}-EGTA antagonism is effective for muscle and for actomyosin

A simplified model illustrating how such swelling might cause muscle contraction is offered in Fig. 20. On the left is the experimental model of Kuhn et al. (1960); black zones are inert polyvinyl alcohol; white regions are cross-linked polyacrylic acid foils which swell on charging owing to water uptake resulting from osmotic effects. On the right is a representation of striated muscle, the black zones are light meromyosin rods and the white regions between them are swollen heavy meromyosin cross-bridges. If the heavy meromyosin did not occupy essentially all of the region between the myosin filaments, water transfer would take place within this region rather than between I-zone and A-zone, resulting in a marked lag of contraction behind swelling. Calculations based on an estimate of each half-sarcomere containing 150 trimers of heavy meromyosin with a swelling of 4·4 (Kominz et al. 1965), give only 30–40 per cent occupancy of this space; more refined calculations wait on further data.

Although electrostatic effects such as those employed by Kuhn and co-workers can be invoked to produce osmotic swelling, similar osmotic effects can be produced by breaking hydrophobic bonds in a protein and introducing the hydrophobic groups into water. Immobilization of water structure when such hydrophobic groups are introduced can produce positive second virial coefficients (Smith P. K. and Smith, E. R. B. 1937) analogous to those produced by charge effects.

A summary of some of the concepts presented here is presented in Fig. 21. Native tropomyosin allows removal of Ca^{++} to inhibit the ATPase. It also lowers the level of ATP where superprecipitation commences. It is proposed that clearing of actomyosin gels or swelling of glycerinated myofibrils is analogous to contraction of living muscle, and that superprecipitation of actomyosin gels or contraction of glycerinated myofibrils is analogous to rigor of muscle. Some evidence in support of such concepts is at hand (Hotta and Terai 1966), and experimental procedures could be brought to bear on most of them.

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SZENT-GYÖRGYI: How much native tropomyosin could actin bind in your experiments?

KOMINZ: A binding of approximately 1 g of native tropomyosin to 3 g of actin was indicated in these experiments.

GERGELY: Is it possible that some of the changes in the sedimentation pattern that you attribute to conformation changes are in fact due to aggregation?

KOMINZ: These small ultracentrifugal changes could be due to aggregation if they involved end-to-end reaction between highly asymmetric molecules. Since they presumably involve swollen, fairly symmetric molecules, a conformational change is indicated.

EDMAN: A shift of water from one part of the sarcomere to the other should be expected to upset the osmotic equilibrium between inside and outside at the I-band region according to your model. This would tend to produce an intake of water at that region of the sarcomere and, of course, counteract shortening of the I-region. In fact, the sarcomere would increase its volume, or would it not?

KOMINZ: According to the x-ray studies of Gerald Elliott on living muscle, shortening is accompanied by an increase in diameter so that the volume of the sarcomere remains constant; that is, on contraction there is no net flow of water into or out of the sarcomere. The current hypothesis is that this isovolumicity results from a water flow between the I- and A-regions, and that the swelling of the A-region is due to a triggered increase in osmotic pressure there. It proposes that the sites of action are the H-meromyosin amorphous regions, and that the mechanism is by an alteration of the internal non-covalent bond structure, such as a reversible rupture of several hydrophobic bonds.

The preceding description has referred to intact muscle. In the case of myofibril preparations, it is quite likely that water could enter the A-region laterally and an increase in volume could occur. Such a process would counteract shortening, as you suggest. Shortening then would occur by some irreversible process analogous to rigor, when the ATP concentration drops below a threshold level.

GERGELY: Dr Paine, in our laboratory, has obtained evidence that the interaction between actomyosin, and the Ebashi factor present in it, is counteracted by 0·6 M KCl. If actomyosin is dissociated by ATP or inorganic pyrophosphate in 0·6 M KCl, and the actin centrifuged down, most of the EGTA-sensitizing factor appears in supernatant.

DRABIKOWSKI (Department of Biochemistry, Nencki Institute of Experimental Biology, Warsaw): In connection with Dr Gergely's remark I should like to add one comment. The fact observed in Dr Gergely's laboratory that,

TABLE I

Percentage of tropomyosin in actin

Kind of actin	Initial	In pellets after centrifugation					
		in KCl, [M]			in $(CH_3)_4NCl$, [M]		
		0·1	0·3	0·5	0·1	0·3	0·5
LT + BTM	26·1	24·8	15·7	6·2	21·8	21·2	18·7
	26·3	25·5	16·0	8·0	29·2	21·4	19·5
RT	10·7	10·8	6·9	0	9·6	—	—
	11·0	13·4	10·9	4·9	13·3	—	12·3
	14·1	17·5	—	11·9	17·3	—	16·9
	13·9	15·2	—	2·9	14·3	—	12·0

during ultracentrifugation of myosin B in 0·5 M KCl in the presence of ATP, EDTA sensitizing factor (native tropomyosin) remains in supernatant can be easily understood in view of our recent results showing that at 0·5 M KCl the dissociation of the F-actin–tropomyosin complex takes place. One can see in Table I that when either room-temperature actin (denoted in the table as RT), or the mixture of low-temperature purified actin (LT) and Bailey's tropomyosin (BTM) were centrifuged in Spinco at 0·1 M KCl, the amount of tropomyosin in the pellets was the same as in the initial solution. When, however, the concentration of KCl was increased, the amount of tropomyosin sedimented together with F-actin markedly decreased. However, this was not the case when, instead of KCl, actin was polymerized and centrifuged in the presence of $(CH_3)_4NCl$.

In connection with Dr Ebashi's lecture I should like to present some recent data obtained in our laboratory, concerning the influence of tropomyosin on the α-actinin–F-actin interaction.

Two types of actin were used in these studies depending on the temperature of extraction of crude actin from acetone-dried muscle powder. The

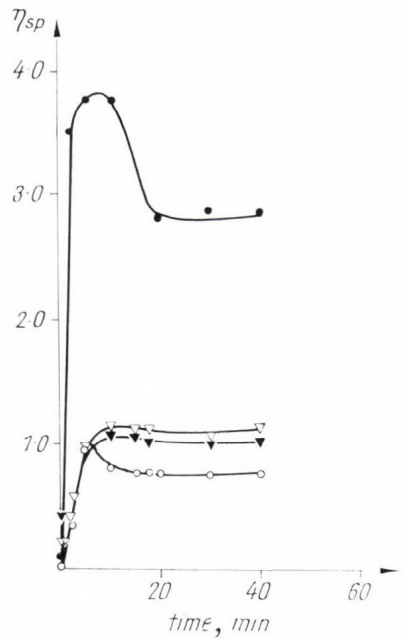

FIG. 22.— The effect of α-actinin and tropomyosin on the polymerization of low-temperature (LT) G-actin. 3 ml samples were polymerized at zero time with 0·1 M KCl + 1 mM $MgCl_2$ at 21° C and the viscosity was measured. Symbols: ○ LT-actin, ● LT-actin + α-actinin, ▽ LT-actin + TM, ▼ LT-actin + TM + α-actinin

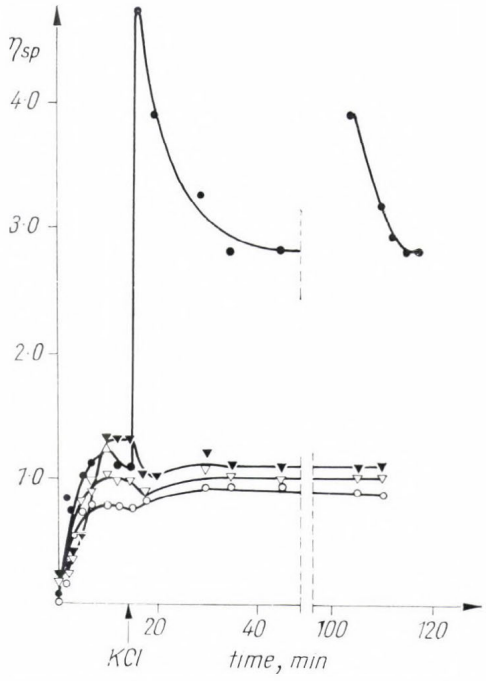

FIG. 23.—The effect of α-actinin and tropomyosin on the polymerization of low-temperature (LT) G-actin. 3 ml samples were polymerized at zero time with 1 mM $MgCl_2$ at 21° C and the viscosity was measured. Arrow indicates the time of the addition of 0·1 M KCl. Symbols: as in Fig. 22

first, denoted as low-temperature actin and obtained from crude actin extracted at 0° C, contained only traces of tropomyosin; the second, denoted as room-temperature actin and extracted at room temperature, contained about 15 per cent of tropomyosin.

In the first series of experiments the effect of tropomyosin purified according to Bailey, of α-actinin, and the joint effect of these two proteins on the polymerization of low-temperature G-actin was examined (Fig. 22). One can see that the viscosity of the obtained F-actin reaches somewhat higher final value in the presence of tropomyosin than in its absence, owing to the interaction between these two proteins. When polymerization of actin was brought about by 0·1 M KCl, with or without 1 *m*M $MgCl_2$, α-actinin caused a considerable increase in viscosity. The gel obtained showed a distinct thixotrophy; namely, viscosity partially dropped during repeated measurements and appeared to increase again during standing. When, however, besides α-actinin, tropomyosin was added to actin the effect of α-actinin was completely abolished.

In 1 *m*M $MgCl_2$, in spite of the polymerization of actin, almost no effect of α-actinin on the viscosity of actin was found (Fig. 23). After subsequent addition of 0·1 M KCl the obtained pattern was similar to that obtained during polymerization with 0·1 M KCl + 1 *m*M $MgCl_2$; namely, the immediate anomalous increase in viscosity in the presence of α-actinin and the lack of the effect of this protein in the presence of tropomyosin were seen.

In the next series of experiments the effect of α-actinin and tropomyosin on the viscosity of low-temperature F-actin was examined. Figure 24

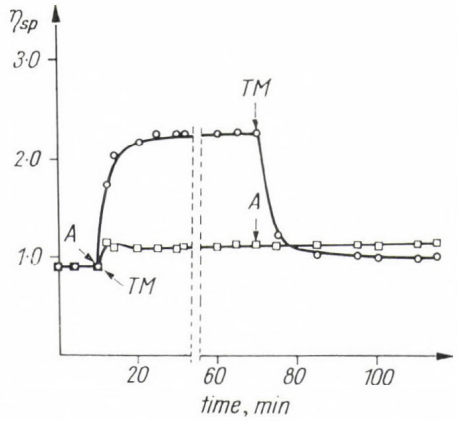

FIG. 24.—The effect of α-actinin and tropomyosin on the viscosity of low-temperature F-actin. In one sample of F-actin (○) the changes in viscosity were measured after the addition of α-actinin (indicated as A), and the subsequent addition of tropomyosin (indicated as TM). In the second sample of F-actin (□) the addition of α-actinin followed the addition of tropomyosin

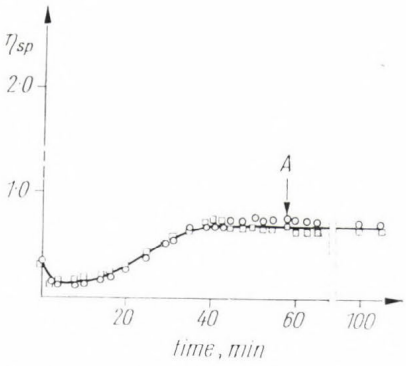

FIG. 25.—The effect of α-actinin and tropomyosin on room-temperature actin. One sample of room-temperature G-actin (○) was polymerized at zero time with 0·1 M KCl and the viscosity was measured. After polymerization at time indicated by arrow α-actinin was added. To the second sample of room-temperature G-actin (□) α-actinin was added and polymerization was initiated at zero time by the addition of 0·1 M KCl

shows that the viscosity, which increased considerably after addition of α-actinin, dropped to the initial level after subsequent addition of tropomyosin. On the other hand, α-actinin added to the complex of F-actin and tropomyosin did not cause any increase in viscosity.

In agreement with the above results no effect of α-actinin was found either when room-temperature G-actin was polymerized in the presence of α-actinin or when the latter protein was added to already polymerized room-temperature F-actin (Fig. 25). The experiments presented above show that the effect of α-actinin on F-actin, first observed by Maruyama and Ebashi, depends on the kind of salt which had been used for the polymerization of actin. Contrary to 0·1 M KCl, in the presence of 1 *m*M $MgCl_2$ or 0·1 M potassium iodide, α-actinin does not increase the viscosity of F-actin.

Besides, these experiments suggest that a competition between α-actinin and tropomyosin for the same, or closely located, binding sites in the actin molecule may exist. They seem also to support the previous suggestion of Hanson and Lowy that the strands of tropomyosin lie outside the actin filaments in the grooves between the strands of the latter protein.

EBASHI: Since Mg ions greatly favour the action of α-actinin, it is desirable that you would repeat your experiment also using high concentration of Mg ions, say 10 *m*M.

HUXLEY: In the table showing the quantities of different proteins present in 100 g of wet muscle (cf. p. 80) Professor Ebashi gives actin as 2 g and α-actinin as 0·8—1·0 g. One can estimate the actin content of muscle in a different way, from observations of the number of subunits visible along the actin filament (assuming them to be G-actin monomers of molecular weight 60 000, which seems compatible with their size and shape) and the number of filaments in a given volume of muscle. This gives a value of approximately 3·5 g per 100 g of whole muscle. I wonder if the lower value quoted by Professor Ebashi might reflect the presence of a considerable quantity of partially denatured actin with the α-actinin fraction, whose activity really resided in a smaller component, as Professor Ebashi believes is the case in native tropomyosin.

EBASHI: The figures in that table are the values estimated from the actual yields. I am also interested in the fact that the yield of actin has never reached the value calculated from other evidence.

HUXLEY: Concerning the general question of the modification of the interaction of actin and myosin and of myosin ATPase, it might be useful to remember that the actin and myosin molecules are each arranged in helical fashion in separate filaments so that the number of molecules which can interact will depend considerably on the three-dimensional structure of the filaments and on their flexibility. In this connection it is interesting to remember that the helical pitch of the myosin filaments in a frog sartorius appears to change when the muscle goes into rigor and the proteins, actin and myosin combine so that it has a value of 380–390 Å and that this figure is approximately equal to the x-ray and electron-microscope periodicity shown by the extra component in the I-filaments. This is the periodicity found by Professor Ebashi and Dr Maruyama in actin filaments when treated with troponin, which control the calcium sensitivity of the actomyosin ATPase.

SZENT-GYÖRGYI: The other estimate of actin content Dr Huxley referred to is based on the bound ADP content of muscle. This is a value in which several laboratories, including Perry, Biró, Mommaerts and ourselves, agreed. We obtained a value of about 4·5 μM/g protein of a washed myofibril, which would correspond to an actin content of about 27 per cent. It would appear, if one adds the considerable amounts of actinins and other factors to the known muscle proteins in the proportion of your estimate, one gets more protein out of muscle than one started with. This is rather worrysome and may suggest that perhaps the activities ascribed to these factors are due to smaller amounts of contamination in the fractions.

EBASHI: Although no one has succeeded in extracting such a large amount of actin, I think that the ADP content provides so far the best estimate of actin content. In this respect I essentially agree with you. However, even if we take this into consideration, the total amount of proteins listed in the table may be around 11 g from 100 g of muscle and certainly less than the amount of structural proteins contained in 100 g of muscles. All the experiments including immunoelectrophoretic studies definitely indicate that in the α-actinin preparation the major component itself is of the active principle.

PEPE: Dr Ebashi, do you know whether your anti-actin antibody reacts with troponin?

EBASHI: We have no direct experience concerning this.

PEPE: The reason I ask is that a few years back Dr Hugh E. Huxley and I got the same results with anti-actin staining as you have shown with anti-troponin (Pepe and Huxley 1964). In addition, in connection with Dr Maruyama's presentation — recently I have found that on addition of anti-actin antibody to a suspension of purified F-actin filaments, the F-actin filaments aggregate. The aggregates show the same periodicity along the length of the filaments as is seen when I-segments of muscle are stained. As the amount of antibody is increased relative to the F-actin, the aggregates become shorter but still retain the periodicity of staining. At high ratios between antibody and F-actin the filaments depolymerize until short segments of the order of 3 000 Å or so are obtained. I am not sure whether this may have some relationship to Dr Maruyama's work.

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EBASHI: The fact that the anti-troponin is located at the thin filament with 400 Å periodicity could be compatible with your results with anti-actin.

HANSON: Dr Pepe, what do you precisely mean by the 400 Å periodicity?

PEPE: It is the 400 Å period associated with the actin filaments in the I-band. The anti-actin antibody accentuates this periodicity.

GARAMVÖLGYI: (Biophysical Institute, University Medical School, Pécs): The great majority of the work performed on the structural proteins of the myofibril deals with the proteins localized in the A-band and in the I-segment,

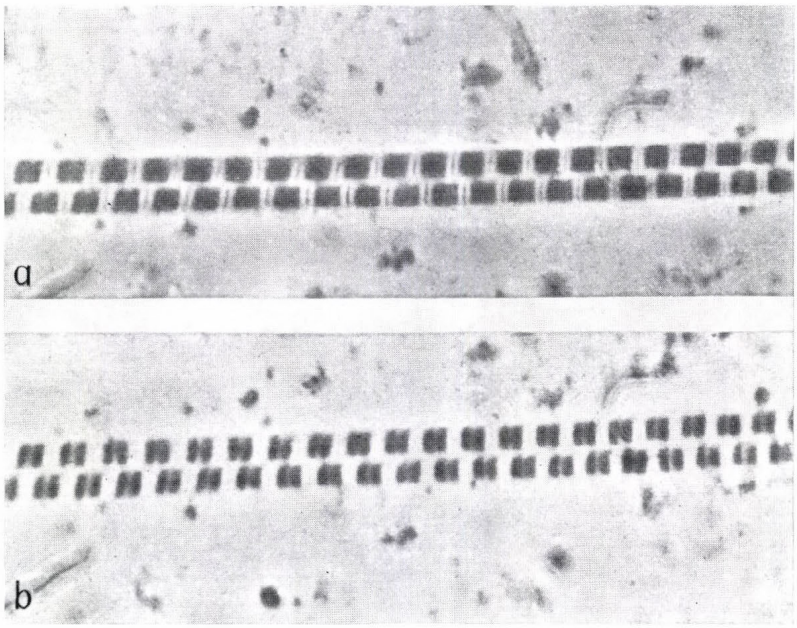

FIG. 26.— *a* Two myofibrils of the flight muscle of the bee. Phase contrast. *b* The same fibrils after the action of 0·1 per cent lipase enzyme, pH 7·2. The Z- and M-lines are missing

but very little is known about the proteins of the Z- and M-lines. It is really very difficult to bring these structural elements in a state in which their material could be determined by the usual methods of protein chemistry.

I would like to present some preliminary observations obtained in a current work which we are conducting together with Dr Ch. Trombitás, from the Laboratory Centre of our University. Although we are only at the start of this work, I regard it necessary to submit this way of protein isolation to the criticism of the Symposium, even at this early stage.

This work is based on the following preliminary observations. In order to remove the Z- and M-lines we treated insect flight muscle myofibrils with lipase or trypsin, both at pH 7–7·2 (Garamvölgyi 1961). By both kinds of enzymatic treatment we observed the same structural changes, i.e. after the treatment both the Z- and M-lines were missing (Fig. 26).

It is to be noted, however, that the effect could be achieved with lipase more easily, that is in a lower concentration and at a considerably higher speed, than with tryptic digestion. I do not regard it probable that the effect of lipase could be attributed to eventual proteolytic contaminations.

In the electron microscope we could also observe the lack of the Z- and M-lines (Fig. 27). Transverse sections of lipase-treated myofibrils have shown that, together with the Z- and M-lines, the set of the

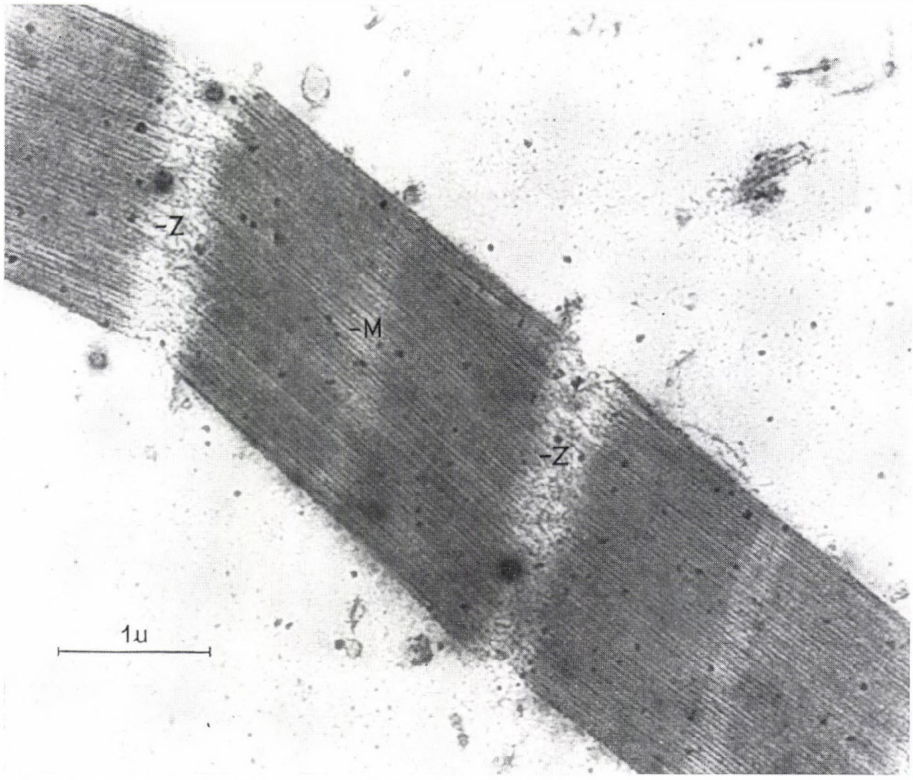

FIG. 27.—Electron micrograph of a myofibril after lipase digestion. Note the empty sites of the Z- and M-lines

secondary filaments is also missing (Garamvölgyi 1965). It seems that practically only the primary filaments survived the digestion. This suggestion seems to be supported by the fact that lipase-treated myofibrils can be fragmentated very easily. Some seconds of mechanical disintegration are sufficient for the complete destruction of the myofibrillar organization. In the medium we found isolated primary filaments (Fig. 28). This may be attributed to the lack of the secondary filaments and thus to the interruption of the actin–myosin linkages. It seems that the presence of the secondary filaments is absolutely necessary for the regular array of the A-band.

On this basis we supposed the Z- and M-lines to be built up from protein and lipid constituents alternating. By the destruction of the latter (i.e. through treatment with lipase) we hoped to liberate the protein component. We prepared a myofibrillar suspension from the thoracic muscle of about 100 bees and washed it in 50 ml of Pringle's solution 20 times by repeated centrifugation, in order to remove the sarcoplasmic proteins. To the last supernatant we added lipase enzyme in a 0·05 per cent final concentration. This solution served as control. To the sediment we added lipase in the same concentration. After the action of the enzyme it was centrifuged once more

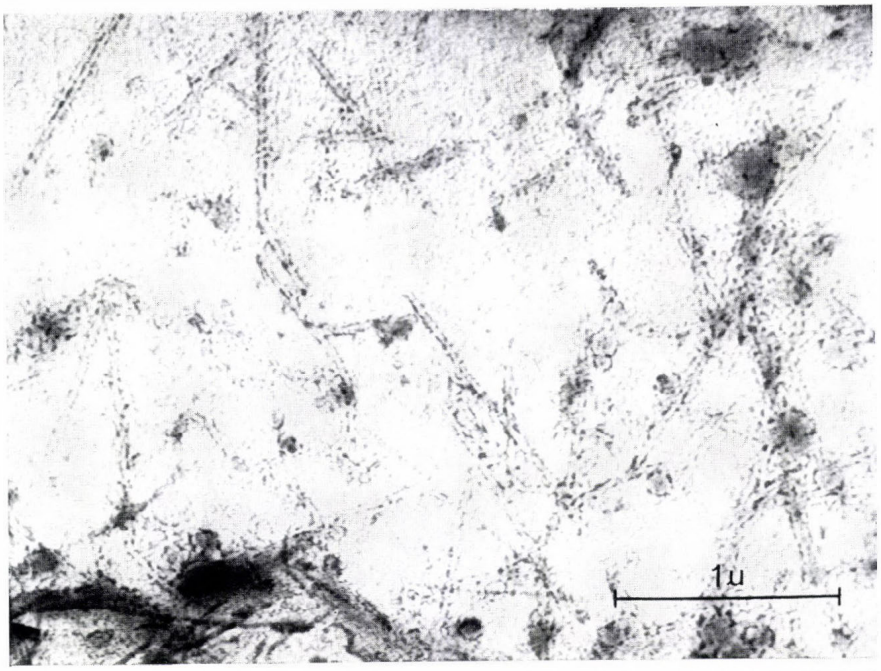

FIG. 28.—Primary myofilaments isolated by extremely short homogenization of lipase-digested myofibrils. Uranyl acetate staining

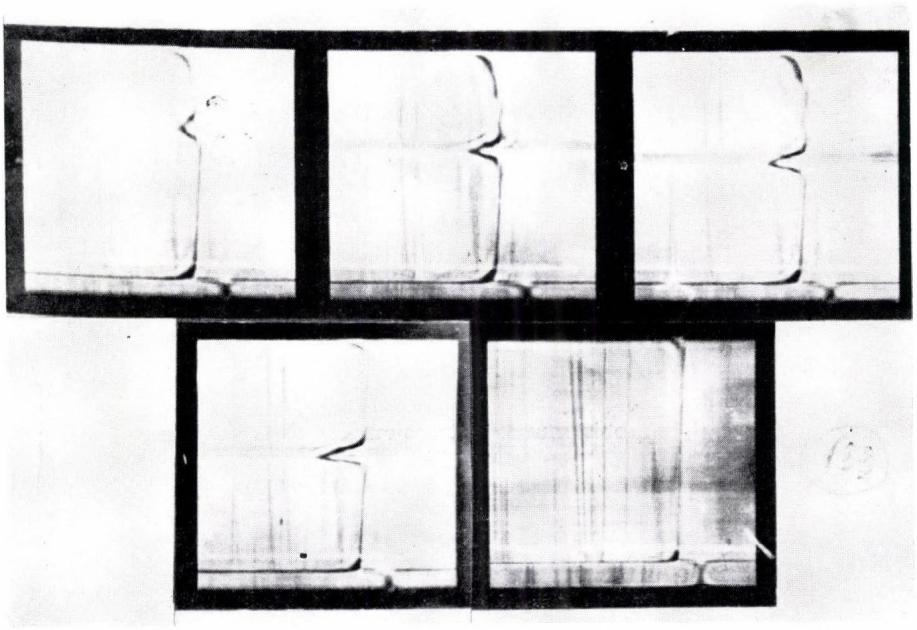

FIG. 29.—Ultracentrifuge record of the lipase extract of bee myofibrils. The control (bottom) does not contain any peak

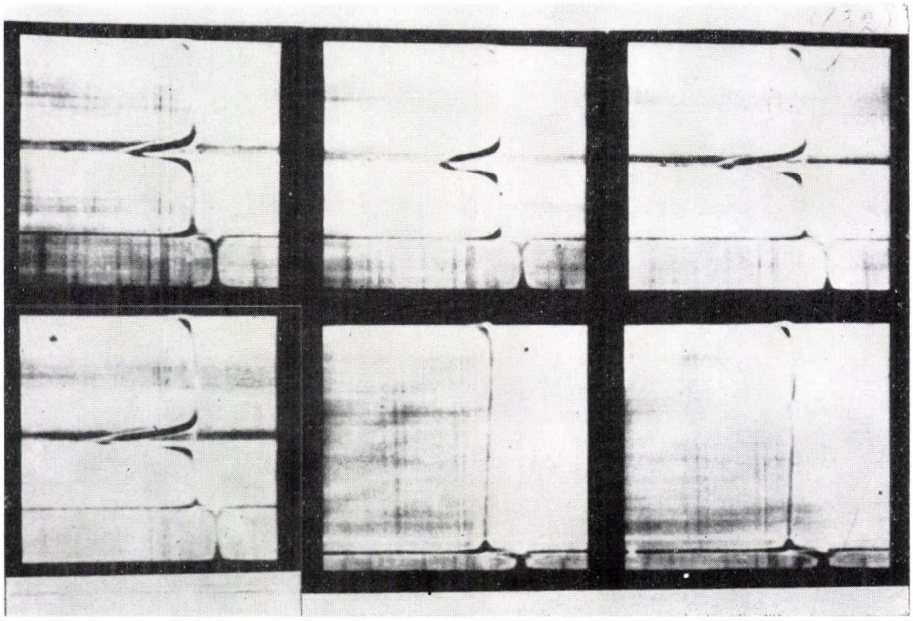

FIG. 30.—The same as Fig. 29, but showing extract of rabbit muscle

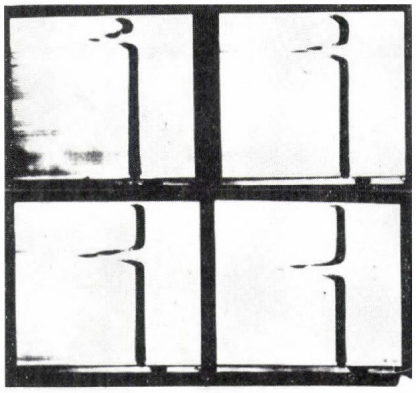

FIG. 31.—Ultracentrifuge record of rabbit muscle extract exhibiting a double peak

and this solution contained the myofibrillar extract. Both solutions were ultracentrifuged. In the control we did not obtain any recognizable component, but in the test solution there is a considerable peak, sedimenting at measurable speed. That means, we could find the substance liberated by the destruction of the lipids (Fig. 29).

We performed similar experiments in a quite similar way also on rabbit psoas muscle and we obtained the same result (Fig. 30).

The experiments probably did not result in one single uniform component. In some cases we could observe a double peak in the ultracentrifuge record (Fig. 31). The solution may contain the substance of the secondary filaments and also probably that of the Z- and M-lines. We hope to be able in the future to separate the individual components, to determine their physico-chemical constants and, if possible, to check their sites inside the myofibril.

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EBASHI: As was mentioned in my lecture, a crude preparation of anti-α-actinin contains another antibody which combines with the M-line. We are now carrying on studies to inquire whether the antigenic protein of the latter is actually the M-line substance.

DRABIKOWSKI: Did you use lipase or phospholipase to treat the muscles (or myofibrils — I do not remember now). As far as I know most of the lipids in muscles are phospholipids which will not be split by lipase.

GARAMVÖLGYI: It was lipase enzyme. Our purpose was the destruction and not an investigation into the lipid component. I am not an expert in lipid biochemistry, thus, I cannot characterize the properties of the latter.

PINAEV (Institute of Cytology Acad. Sci. USSR, Leningrad): Artificial actomyosin is usually obtained by mixing a small volume of an actin solution with a larger volume of a myosin solution in proportion of 1:2·5. Its properties are generally accepted as being identical with those in a solution of natural actomyosin.

Natural actomyosin is obtained by twenty-four-hour extraction of muscle mince with a Weber solution.

However, the results obtained from the measurements of both of these actomyosins with the usual birefringence technique show that they are considerably dissimilar in their physico-chemical properties (Fig. 32).

The extinction angle α and the value of birefringence Δn of the actomyosins were measured at various velocity gradient of flow (g). The gradient of velocity was changed from ten to one hundred and fifty reciprocal seconds. Flow birefringence values of actomyosins are given in the figures presented as a function of the velocity gradients.

Our experiments showed that values of the extinction angle in the case of the artificial actomyosin solutions range from eight to six angular degrees, and that they are not noticeably affected by the variation of gradient.

In the case of the natural actomyosin the same characteristics lie between nineteen to ten. That is, the value of α is higher.

The same divergence was observed in measuring the birefringence effect (Δn). The values of Δn for the artificial actomyosin are very high: by g of twelve inverse seconds they reach the value of 105×10^8. But Δn of the natural actomyosin is twice lower.

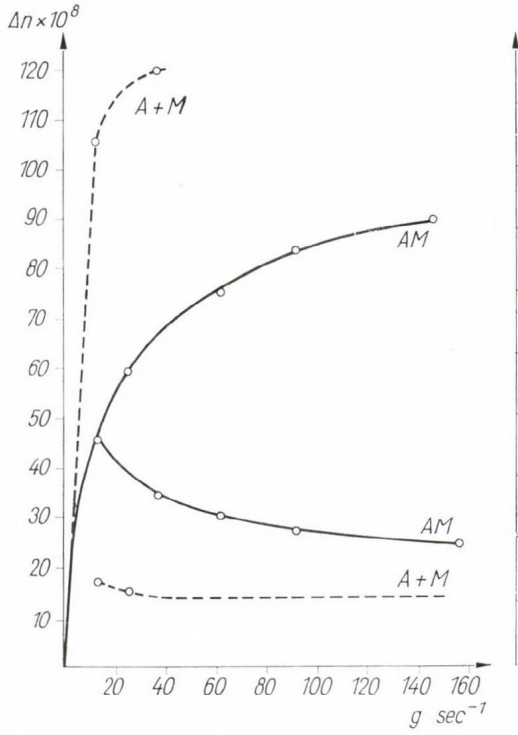

FIG. 32. — Flow birefringence of the artificial and natural actomyosin. A + M = actin + myosin (artificial actomyosin), AM = natural actomyosin

It seems that protein particles in the artificial actomyosin solution are larger and more asymmetric than those of the natural actomyosin.

Our results are, therefore, essentially at variance with the widely spread opinion that there is no difference between these two actomyosins.

It should be noted, however, that Ivanov and others have already reported about the stability of the artificial actomyosin to high pressure. They have found it being higher than that of the natural actomyosin.

One may ask, what is the cause of these differences? Why do the particles of these proteins differ in their shape and size?

The birefringence effect of actomyosin solutions seems to be caused by the actin component. In 1960 Noda and Ebashi showed that myosin does not exhibit birefringence at low velocity gradient in solutions of 0·6 M KCl and pH 7·4.

But the F-actin obtained with the method of Straub exerts a very strong birefringence under the same conditions. As shown in Fig. 33, the velocity gradient has no influence on the curve for the actin, which has shifted to the zone of small values of α. This fact indicates the fast orientation of the actin particles and their large and asymmetric macromolecular character.

The curve of the extinction angle for actin lies somewhat lower than the similar curves for the two actomyosins.

Moreover, it is not affected by the relation of actin to myosin in the artificial actomyosin. Four correlative curves correspond to the solutions of various proportions, i.e. 1 : 2·5, 1 : 5, 1 : 8, 1 : 10.

The uppermost curve represents the natural actomyosin solution. As criterium for identification of the two actomyosins their contractile abilities were usually taken. But the properties said above may not possibly influence the contractile abilities of the actomyosin complex.

Observations of Atmarin and Vorobyev support our explanation of the fact. They showed that the combination with actin was not the only condition for getting a contractile complex.

The contractile ability of actomyosin can be indirectly estimated by the decreasing viscosity or birefringence of protein solutions in the presence of ATP.

Therefore, the appropriate measurements were made. As shown by Figs 34 and 35, the value of the extinction angle really increased in both cases due to the ATP added. Consequently, as we have thought, their reaction with ATP is independent of the qualitative characteristics.

The kinetics of the reaction was investigated in order to check whether the observed variations in the run of curves for the orientation angle could be explained as a consequence of the action of the ATP added. With the increase in g we have not got, however, the reduction in the α values, on the contrary, a distinct increase. This effect may be attributed to the greater polydispersity of the solutions studied. Moreover, it points again to the dissimilarity of the two proteins.

It is necessary here to note one more observation made during our studies as being of some importance and therefore analyse the curves again as follows. It is clear from Figs 32 and 33 that the curve of α-value as a function of g runs for the actin lower than the respective curves for the artificial actomyosin.

The explanation of the phenomenon may lie possibly in the interaction of actin with the myosin particles, with the formation of entirely new set of particles of a somewhat less asymmetry.

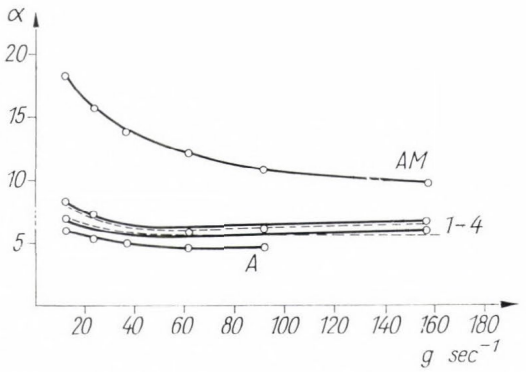

FIG. 33.—Curves *1–4* actin/myosin in proportions 1:2·5, 1:5, 1:8, 1:10

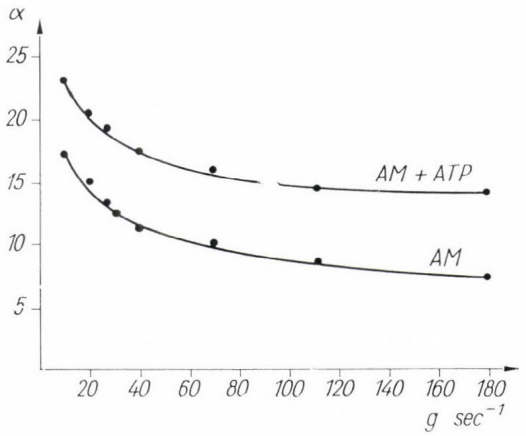

FIG. 34

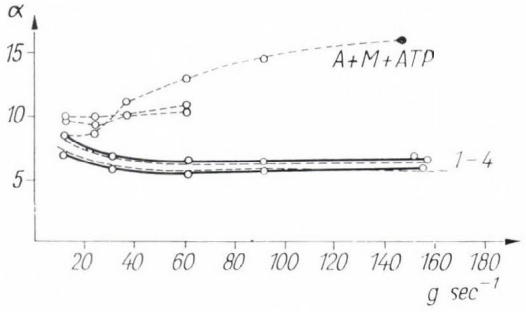

FIG. 35

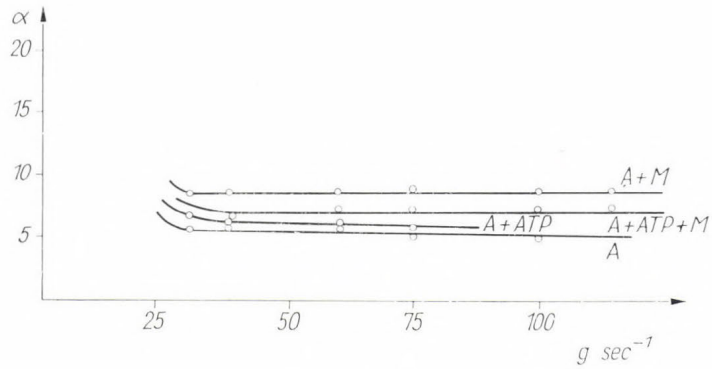

FIG. 36

At present the hypothesis that ATP added to actomyosin solutions dissociates actomyosin into two components: actin and myosin, is considered as already proved.

But, if it were so, the curves of the extinction angle should shift into the region of the lower α values, which corresponds to actin or remain at least unchanged. We are, however, confronted actually with quite a contrary effect: that is, the said curves for the actomyosin, after ATP has been added to the solutions shift still deeper into the zone of the larger α-values.

It follows from this that the shift of the curves may be attributed with a reasonable degree of certitude to the less asymmetric character of the macromolecules. Thus, we were obliged to check whether the myosin, which is supposed to be present in the solution in the dissolved state, has no appreciable effect on the birefringence of the actin. In order to clarify the problem, the extinction angles of the solutions containing actin and myosin in the dissociated conditions were measured with the results as follows (Fig. 36).

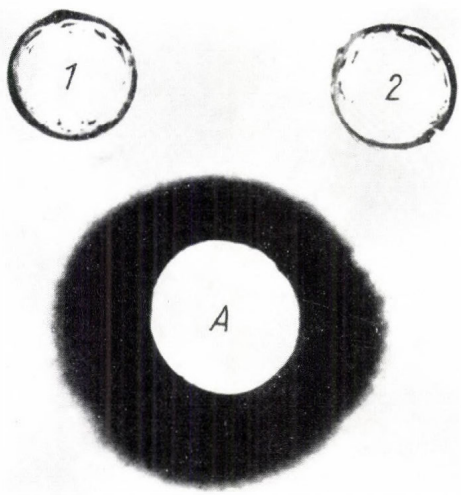

FIG. 37. — Precipitation reaction in an Ouchterlony plate. *A* antimyosin, *1* myosin, *2* sarcoplasmic cholinesterase

The lowest curve represents actin, the next one the same actin but with the ATP added; further, there is the curve representing actin, plus ATP, plus myosin. It is the curve where the myosin should not react with the actin. The last one is the curve for the artificial actomyosin solutions.

The curves of that entire set run close to one another and the values

represented by them range from 7 to 8 degrees, that is, in the region of values far lower than the values of twelve or fifteen degrees for artificial actomyosin in the presence of ATP.

It follows that free myosin cannot be, in fact, the cause of the marked drop of the birefringence values resulting on interaction between actomyosin and ATP.

These data permit us to suggest the following:

(1) the combination of actin with myosin is accompanied by the change of their former structure,

(2) the action of ATP on the actomyosin cannot be thought of as just its disintegration into its two components: actin and myosin.

MARUYAMA: Did you check the dependence of extinction angle on protein concentration? The reason why I ask this is that we have obtained much higher α values of myosin B at low velocity gradients (Noda and Maruyama 1958, Haga et al. 1966). We have observed that the extinction angle of synthetic actomyosin was much lower than that of myosin B, because F-actin of Straub type is very long in particle length, while F-actin from myosin B is 1–2 μ in length (Maruyama 1965).

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SZÖŐR (Physiological Institute, University Medical School, Debrecen): In connection with the paper of Prof. Ivanov I should like to show our observations referring to the distribution of cholinesterase activity among different fractions obtained from the striated muscle of rabbit (Table I).

TABLE I

	Homogenate	Extract of sarcoplasmic proteins	Extract of myofibrillar proteins	Fraction T	Residue
	mg acetylcholine/g muscle/hour				
1.	2·85	1·24	1·32	0·06	0·20
2.	3·65	1·40	1·80	0·09	0·22
3.	2·62	1·00	1·20	0·10	0·30
4.	4·65	1·90	2·30	0·15	0·30

This table shows the cholinesterase activity of muscle homogenate of the sarcoplasmic protein fraction extracted at low ionic strength, the myofibrillar fraction extracted with Weber solution, the fraction T separated from the myofibrillar fraction and residue. The cholinesterase activity is expressed in mg ACh hydrolysed fractions referred to one gram muscles.

It can be seen that the cholinesterase activity is chiefly found in the sarcoplasmic and myofibrillar fractions. The cholinesterase activity in fraction T is only 2–4 per cent that of the muscle homogenate.

Studying the immunological behaviour of fractions we found that there are significant differences in the antigenic properties of fractions with cholinesterase activity.

Chicken immunsera obtained against purified rabbit myosin produced a precipitation reaction with the homologue antigen but did not react with the purified sarcoplasmic cholinesterase (Fig. 37).

Therefore, it can be supposed that the two kinds of cholinesterase differ from each other in their antigenic properties.

Symp. Biol. Hung. 8, pp. 125–150 (1967)

UNIVALENT IONS AND WATER IN MAMMALIAN HEART MUSCLE: AN EVALUATION OF THE MEMBRANE THEORY AND THE FIXED CHARGE THEORY

E. PAGE

UNIVERSITY OF CHICAGO, CHICAGO

I might begin by commenting on the origin of the title of this lecture. Two years ago, I had the pleasure of sharing a laboratory at the Harvard Medical School with Prof. Joseph Tigyi of the University of Pécs, who, as secretary of the organizing committee, has done so much to make our present meeting successful. Prof. Tigyi is a disciple of Prof. Ernst. Our contact with him led us to reexamine many of the assumptions underlying the way we had approached the behavior of ions in heart muscle. Our dominant interest has been the description of mammalian heart muscle, and we have had no emotional or other commitment to any theory. There is now available a significant amount of information about ion transport in mammalian heart muscle. It therefore seemed useful to see how this information is affected by the difference in theoretical approach which Prof. Tigyi and I brought to muscle physiology.

Some ten years ago, we began to develop an isolated preparation of mammalian heart muscle, a preparation suitable for investigating the behavior of inorganic ions in this tissue. At that time there were two theories which could serve as a framework for the analysis of our data: the so-called membrane theory and the fixed charge theory (Ling 1962, Troshin 1958). We chose to analyze our data by the membrane theory. In so doing, we soon found that many of our assumptions were, in fact, unsound, or that we were dealing with approximations which were subject to very large uncertainties. It might be anticipated that this experience would tend to enlist us among the supporters of the fixed charge theory and among the opponents of the membrane theory. Surprisingly enough, that is not the case, and the reason is one which we could not have foreseen when we began our work. What has happened is that, concurrently with our own studies and in intimate relation to other branches of muscle physiology, there has sprung up a new field, one that has, in effect, revolutionized our perspective of muscle. I refer to the organization of muscle at the level of electron microscopic ultrastructure, and the way this organization affects the concepts of membrane transport in heart muscle. By looking at some of the observations on ion transport in the light of what is now known of cardiac ultrastructure, we arrived quickly at the conclusion that the questions of greatest interest in heart muscle exist whether one interprets the data by the membrane theory or by the fixed charge theory; and that although the membrane vs. fixed charge controversy cannot be considered a settled question for heart muscle, it is now irrelevant for the most interesting problems in the field.

Anyone who wants to study ion transport in a different muscle must decide almost from the start how he will treat the organization of the tissue. The need for such a decision arises from the fact that it is desirable to express the driving forces for ion movement in terms which have their counterparts in the physical chemistry of aqueous electrolyte solutions and of ion exchanger membranes, terms with units of concentrations, activity, electrical potential. The requirement to define the system with which one is working is not, of course, unique for muscle or for a biological material. For a muscle, the problem is illustrated by the definition of the intracellular concentration of an ion, for example that of potassium ion

$$[K]_i = (K_T - K_o)/(W_T - W_o)$$

where $[K]_i$ = the cellular concentration of K ions (in *m*M/kg cell water)
K_T = the total content of K in the extracellular compartment (in *m*M)
W_T = the total weight of water in the muscle (in kg)
W_o = the weight of water in the extracellular compartment (in kg).

The definition contains some of the important assumptions of membrane theory. Among these are that it is meaningful to divide the potassium in heart muscle into cellular and extracellular fractions; that the method used for such a partition does in fact accomplish its purpose; that the intracellular potassium content, the numerator in the defining equation, and the intracellular water content, the denominator in the equation, are each sufficiently homogeneous so that their quotient, a quantity with units of concentration, can reasonably be used to express one of the driving forces which act on the potassium ion and cause it to move across the cell membrane.

These are substantial assumptions.

When we began our studies, we were confronted with two practical questions: how exact are the assumptions for heart muscle, and how useful are they in predicting the behavior of ions in heart muscle? The investigation of how exact these assumptions are for cat heart muscle has led us down a long road, as it has a number of other groups working with other forms of heart muscle. During our own travels down this road, we have applied to cat heart muscle many of the now classical methods of membrane physiology; we have developed certain methods of our own; we have made electron microscopic observations on cat heart muscle; and we have begun to reinterpret our observations on ion transport, taking into consideration what we now know of cardiac ultrastructure.

The subject may be introduced by reviewing the assumption that the water and electrolyte content of heart muscle can be conveniently divided into two fractions: a cellular fraction, and an extracellular fraction. When a light microscopist looks at sections of fixed and stained heart muscle, he sees cells and the spaces between cells. When a physiologist speaks of the extracellular space, he thinks in terms of the volume of muscle determined with an extracellular tracer. That is, he measures the volume of muscle which equilibrates by diffusion with a solute that is thought to be excluded from the cells. The classical physiologists assumed that the extracellular volume so measured is roughly identical with that of the spaces

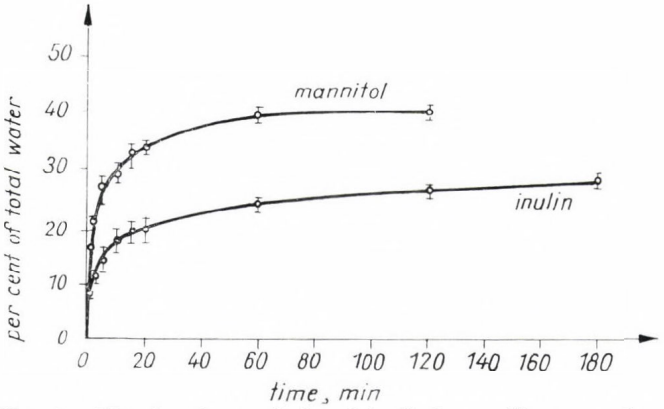

FIG. 1.—Uptake of mannitol and inulin by papillary muscles from the right ventricle of the cat heart (Page 1962)

among cells as observed by light microscopy. The classical molecular species used as extracellular tracers include inulin, sulfate and various carbohydrate molecules like sucrose, mannitol, sorbitol, or raffinose.

When the extracellular volume of cat heart muscle is measured as a function of time (Page 1962), the results are distinctly non-classical, as shown in Fig. 1. The extracellular compartment was measured with an independent method with two molecules which were known to remain outside cardiac cells (Page 1962). The upper curve is the extracellular volume measured with mannitol, a small molecule, the lower curve that measured with inulin, a large molecule. It is evident that a greater fraction of muscle water is accessible to mannitol than to the much larger inulin molecule. The observations suggest that the extracellular compartment is inhomogeneous with respect to the diffusion of molecular probes of different sizes; that is, portions of the compartment are readily accessible to the diffusion of small molecules like mannitol, but are only slowly, if at all, permeable to large molecules like inulin.

These conclusions hold, whether one analyzes the results on the basis of membrane theory or on the basis of fixed charge theory. But if one chooses, on the basis of membrane theory, to calculate intracellular concentrations, it becomes important to decide which measure of the extracellular compartment is the correct one. The point is illustrated in Table I. The table (Page

TABLE I

Dependence of cellular concentrations on extracellular volume

Extracellular tracer	Intracellular concentration (mM/kg cell water)		
	$[K]_i$	$[Na]_i$	$[Cl]_i$
Inulin	162±3	43±5	46±2
Mannitol	208±6	5±2	17±2

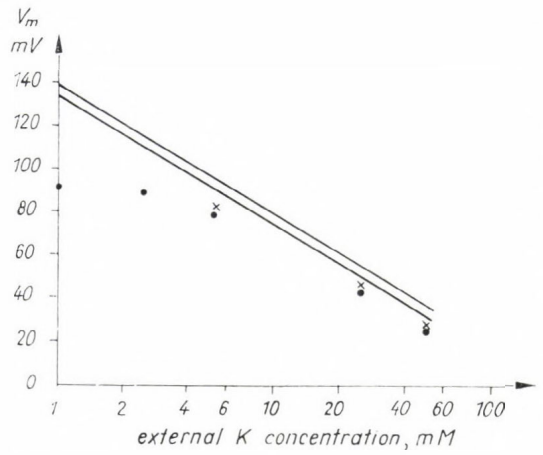

FIG. 2.—Comparison of V_m, the resting potential of cardiac cells from cat papillary muscle, with V_K, the potassium equilibrium potential, as a function of the external potassium concentration (Page 1962a). Measurements were made in the steady state with respect to cellular concentrations of potassium, sodium and chloride, the cellular potassium concentration being approximately constant throughout. Points are resting potentials obtained with microelectrodes in solutions whose chief anion was chloride; crosses are the results of corresponding measurements in isosmolal, chloride-free sulfate solutions. The upper and lower lines give V_K calculated on the basis of extracellular spaces estimated with mannitol and inulin, respectively. Temperature 27·5° C

1964) shows that the concentration of potassium is much larger and that the concentrations of sodium and chloride are much smaller when mannitol is used to measure the extracellular space than when inulin is so used. Such differences introduce an unacceptable indeterminacy into the calculated driving forces on ion movement, as illustrated in Fig. 2. The figure compares the resting potential of cat ventricular cells (V_m) as measured with microelectrodes (Page 1962a), with the potassium equilibrium potential, V_K, which is the potential difference calculated from the limiting case of the Nernst equation for a potassium-permeable membrane. It is defined by the logarithm of the ratio of intracellular to extracellular potassium concentrations according to the relation $V_K = (RT/F) \ln ([K]_i/[K]_o)$, in which R, T, and F are, respectively, the gas constant, the absolute temperature, and the Faraday. The experiments (plotted in Fig. 2) were made at progressively increasing external potassium concentrations, $[K]_o$, under conditions in which the intracellular potassium concentration, $[K]_i$, was held constant. As in many other excitable and non-excitable tissues the absolute value of the resting potential measured with microelectrodes under *in vitro* conditions is smaller than the potassium equilibrium potential. The difference has been the subject of much discussion among muscle physiologists and is important in interpreting the origin of the resting potential. It is evident from Fig. 2 that the extent of this difference depends on whether the intracellular potassium concentrations are calculated on the basis of mannitol as the measure of extracellular volume (the upper curve), or on the basis of inulin (the lower curve). In other respects the data for mammalian heart muscle resemble those for other striated muscles and for nerve (Hodgkin 1958). In particular, at physiological and lower external potassium concentrations the slope of the semilogarithmic plot of resting potential vs. external potassium concentration departs markedly from the line predicted on the basis of the Nernst equation.

Since the discrepancy between measured potentials calculated from the intracellular potassium concentrations is of such importance in defining the driving forces on the potassium ion, it seems useful to consider one source of this discrepancy which may be particularly significant in mammalian heart muscle. If the true value of $[K]_i$ were lower than the calculated value, the potassium equilibrium potential would, in fact, approach the resting potential measured with microelectrodes, that is, the distribution of potassium would approach an equilibrium distribution. The critical assumption is that all of the potassium in the cell exists in a single state which is roughly comparable to the state of water in a salt solution (or at least cannot be differentiated from such a state by the usual methods of membrane physiology). This assumption is probably inexact for mammalian heart muscle. It has been our experience with papillary muscles from the cat's heart that from 12 to 25 per cent of cellular potassium behaves differently from the rest. The difference can be shown in many ways. For example, in very hypertonic solutions, cardiac cells will lose most of their potassium, but not a critical fraction of 12–25 per cent (Page and Storm 1966). Similarly, after prolonged inhibition of sodium transport, either by incubation at 0° C, or by exposure to large concentrations of the cardiac glycoside ouabain, cells will exchange all but 12–25 per cent of their potassium for sodium (Page, unpublished observations). Our working hypothesis is that this inhomogeneity of cellular potassium in mammalian heart muscle is due predominantly to potassium associated with the mitochondria. This hypothesis rests on the now rather extensive literature on ion transport in mitochondria isolated from mammalian heart muscle, including some early work by Ulrich (1959, 1960), by Weatherall (1962), and by Schreiber and co-workers (1960), and in particular the recent observations from the laboratories of Brierly and co-workers (1966) and of Pressman (1965) and Cockrell and co-workers (1966). These studies indicate that mitochondrial potassium ions in heart muscle participate in an elaborate, energy-dependent ion exchange. It can also be calculated from estimates of the potassium content per mitochondrion and of the number of mitochondria per cell that 12–25 per cent of total tissue potassium is a reasonable figure for the fraction of cellular potassium associated with mitochondria.

Several laboratories, including our own, have shown that in the steady state when cellular ionic composition is constant, all or nearly all of the potassium in the cardiac cell exchanges at the same rate with radioactive potassium outside the cells (Carmeliet 1961, Weidmann 1965, Goerke and Page 1965, Taylor 1962). The observation that under one set of conditions all of cellular potassium exchanges at a single rate appears at first sight to be inconsistent with the presence of a significant potassium fraction associated with mitochondria. However, the observation means only that mitochondrial K-exchange is, under these conditions, much faster than the step which is rate-limiting for the exchange of cellular potassium as a whole. In other words, mitochondrial potassium exchange is concealed by the much slower exchange across the cell membrane, or by some other rate-limiting exchange. On the assumption that most of mitochondrial potassium is present in some relatively poorly hydrated state, it is possible to recalculate potassium concentrations for the cytoplasmic potassium (that fraction of cellular potassium which is not associated with mitochondria). The result of this calculation shows that the potassium equilibrium potential so computed for cat heart muscle cells comes very close to that measured with microelectrodes. This somewhat speculative digression illustrates that it is not necessary to invoke a fixed charge theory for *all* of cytoplasmic potassium in order to explain a discrepancy which is to date inadequately accounted for by the membrane theory.

Even after estimating the inhomogeneities in the ionic composition of the cytoplasm, it is still necessary to devise a meaningful partition of heart muscle into tissue compartments. There are a number of ingenious independent approaches to this problem in the literature. For example, Fisher and Young (1961) have applied hydrostatic pressure to the rat heart, both in the form of constant pressure and in graded increments of pressure. They found that at a pressure of 500 millimeters of mercury the extracellular solution, but not the intracellular solution, is pressed out of the heart. The volume of extracellular fluid so pressed out agrees with that obtained by conventional extracellular space measurements using sorbitol or raffinose. Faced

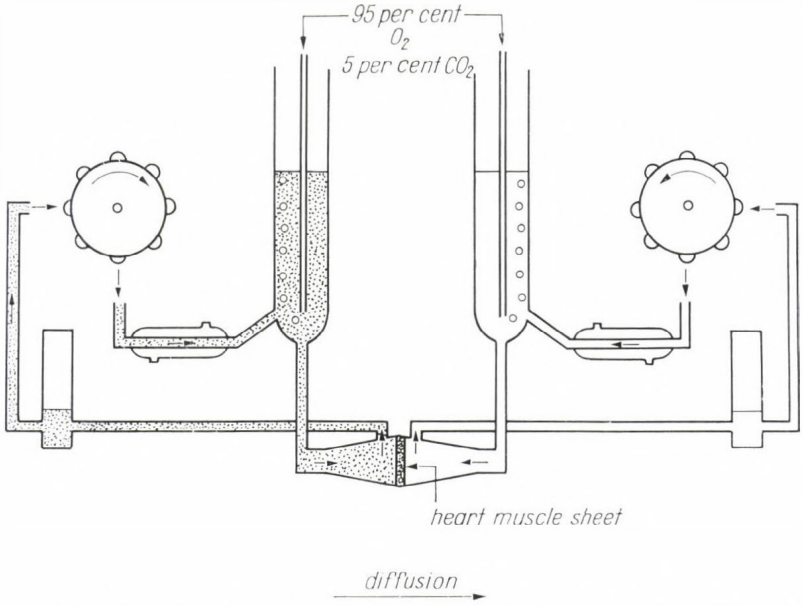

FIG. 3.—Experimental arrangement for measuring diffusion through a sheet of right ventricle from the cat heart (Page and Bernstein 1964). The sheet (arrow) separates the chamber into left and right conical halves. The glass reservoirs above the chamber can be sampled at appropriate intervals. Baffles are inserted between the pumps and the muscle chamber to minimize mechanical oscillations. See text for further discussion

with the necessity of defining the size and determining the nature of the extracellular compartment in cat heart muscle, we have pursued two lines of inquiry: first, we have tried to characterize more closely the diffusion of ions and unchanged molecules in this compartment; secondly, we have traced the continuities of the compartment in electron micrographs with the serial section technique of Sjöstrand (1958).

The kinetics of diffusion into and out of the extracellular space of skeletal muscle have been described extensively by Harris and Burn (1949) and by Keynes (1954). We have taken a somewhat different approach and have chosen to consider stationary state diffusion through a sheet of heart muscle (Page and Bernstein 1964). The particular form of the diffusion equation used by us had previously been applied by others to diffusion through ion exchange membranes or through other porous media. The experimental arrangement is shown in Fig. 3. An otherwise intact sheet of right ventricular muscle from a cat heart is clamped between two sides of a fluid-filled chamber. A known volume of fluid is continuously and rapidly circulated through each side. The chemical composition of the solutions on each side is identical, but a radioactively labelled extracellular tracer like sucrose or sulfate is initially added to the left side only. Since the only communication between the two sides of the chamber is through the extracellular space, the labelled substance diffuses through the extracellular space from left to

TABLE II

Time course of diffusion through a sheet of heart muscle

$$Q^* = Dc_1 A_d t/\lambda^2 l - lc_1 A_d/6 - (2lc_1 A_d/\pi^2) \sum_{n=1}^{\infty}{}' \left[(-1)^n/n^2\right] e^{-Dn^2\pi^2 t/\lambda^2 l^2}$$

$$\text{Intercept} = \lambda^2 l^2/6D$$

$$\text{Slope} = Dc_1 A_d/\lambda^2 l$$

Q^* = cumulative amount of radioactivity which has diffused across the sheet in time t
l = thickness of the sheet
A = total area of the sheet
c_1 = counts per minute per unit volume in the initially radioactive reservoir
D = diffusion coefficient of the labeled species in free aqueous solution
A_d = an area associated with the diffusion channel so that the quantity lA_d is the volume of the diffusion channel
λ = a tortuosity factor for the extracellular diffusion channel

right; its rate of appearance in the right chamber can be followed by sampling this chamber at suitable intervals. Table II gives the appropriate solution of the diffusion equation for the time course of appearance on one side of the sheet of a substance diffusing through the sheet from the other side. Q^*, the cumulative amount of radioactivity which has diffused across the sheet in time t, is given by a non-linear build-up corresponding to the exponential series, which is the third term on the right, and by a linear asymptote corresponding to steady state diffusion and given by the first two right-hand terms. D is the free solution diffusion coefficient, c_1 the specific activity in the compartment of origin, l, the thickness of the sheet, A_d the area available for extracellular diffusion, and λ, an extracellular tortuosity factor, for taking into account the increase in the mean diffusion path arising from the fact that extracellular molecules must diffuse around rather than through cells. The equation has an intercept on the time axis, given by $\lambda^2 l^2/6D$, from which λ can be calculated, using a tabulated value of D. If λ is known, A_d may be calculated from the slope of the linear steady state asymptote by the third equation. Figure 4 shows the experimental time course for the diffusion of sucrose, superimposed on the plot of the diffusion equation. For the impermeant sucrose molecule the time course of appearance of radioactivity on the initially non-radioactive side of the sheet gives a virtually perfect fit for both the non-linear and steady state portions of the

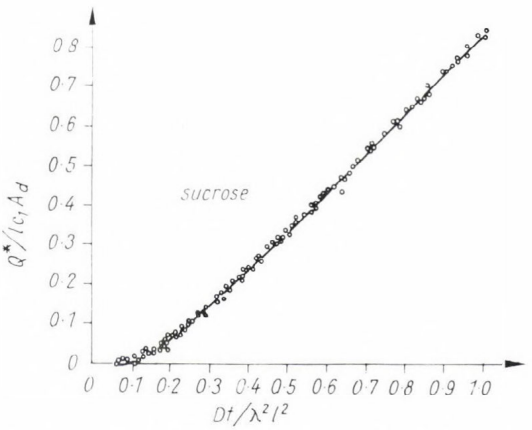

FIG. 4.—Experimental time course for the diffusion of sucrose through a sheet of right ventricle (open circles) superimposed on the plot of the diffusion equation (solid line) (Page and Bernstein 1964). Temperature 22·5° C

theoretical curve. For sodium and sulfate ions, to both of which resting heart muscle is relatively impermeable, similarly good fits can be obtained. Page and Bernstein have found that the tortuosity factor, λ, is the same for sucrose, sulfate and sodium. λ thus appears to be independent of the molecular species for molecules of this size. The value of λ so obtained can be used to calculate A_d/A, the fraction of the geometrical area of the sheet, which is available for extracellular diffusion; that is, the fraction of the face of the sheet not made up of cells. Table II tabulates λ and A_d/A for sucrose, sulfate, and sodium, three molecular species which do not readily enter resting heart muscle cells; A_d/A has also been obtained for molecules like water, urea, and glycerol, which do enter cells and equilibrate with cell water. Like the tortuosity factor, the fractional area available for extracellular diffusion is constant and independent of the molecular species used to determine it.

The fact that the tortuosity factor and the area available for extracellular diffusion are both independent of the molecular species suggests that the dimensions of the channels through which diffusion is occurring are large relative to the dimensions of the diffusing molecules. To test this point, it would be of interest to see if very large molecules can actually diffuse through the muscle sheet. The largest labelled molecule available to us, a dextran with a molecular weight of from 60 000 to 90 000, does indeed diffuse through the sheet. The size of this molecule can be roughly approximated by an equivalent sphere with a molecular diameter of 150 to 180 Å. This value suggests that the channels must have average dimensions very much larger than 150—180 Å in order that the dextran may diffuse through the channels in a finite time. In addition to this qualitative statement, it is possible to calculate the volume and water content of what may be termed the extracellular diffusion channel for small molecules. This is the area available for the extracellular diffusion of small molecules, multiplied by the thickness of the sheet. The water content of the diffusion channel for sucrose is 26 per cent of the total content of water in the muscle. This is about the same as the water content of the inulin space, but is only 2/3 of the sucrose space after 3 hours of equilibration with sucrose. On the basis of this method it can be concluded that 1/3 of the extracellular space has kinetic characteristics different from that of the other 2/3. This extracellular subcompartment would account for about 13 per cent of the water in the muscle, with a possible range of 7–19 per cent. The extracellular compartment of the cat's right ventricle is thus inhomogeneous with respect to the diffusion of ions and molecules within it.

What is the structural counterpart of such an inhomogeneity? To obtain an answer to this question, we have tried to develop more satisfactory methods of preserving cardiac fine structure for electron microscopy, and have applied the method of serial thin sections introduced by Sjöstrand (1958) to the cat's papillary muscle (Page 1966, 1966a). Before discussing the results of these experiments, we may summarize the conventional concepts of the ultrastructure of mammalian heart muscle. Figure 5 is a three-dimensional picture of a mammalian heart muscle cell from an article by Nelson and Benson (1963). The figure shows the longitudinal cell membrane or sarcolemma, the transverse cell boundary or intercalated disk, the myofibrils with their characteristic striations, the nucleus, and the mitochondria.

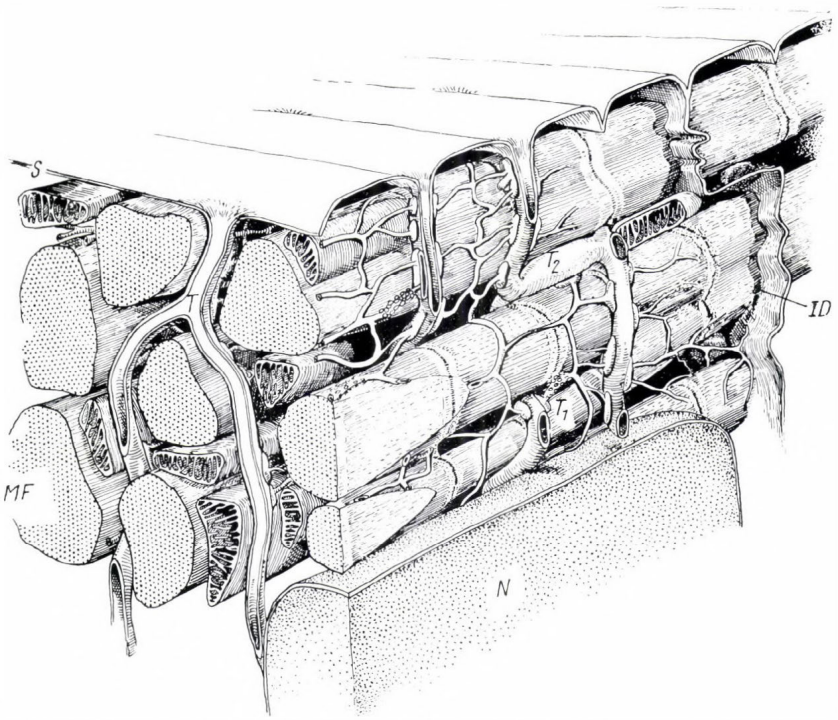

FIG. 5.—Drawing of the suggested three-dimensional structure of a mammalian myocardial cell proposed by Nelson and Benson (1963). T, T_1, and T_2 represent different parts of the transverse tubular system. Other labelled structures include the sarcolemma (S), a myofibril (MF), an intercalated disk (ID), and a nucleus (N). The smaller tubules around the myofibrils are sarcotubules whose interior is probably not continuous with that of the transverse tubules. The distribution of sarcotubules may be more complex than indicated here (Page 1966a)

The longitudinal cell membrane or sarcolemma has very large infoldings, the transverse tubular system first described in mammalian heart muscle by Lindner (1957), and subsequently shown in more detail by Porter and Palade (1957), by Simpson and Oertelis (1962), and by Nelson and Benson (1963). Karnovsky (1965) has demonstrated that under *in vivo* conditions the lumina of these transverse tubules are continuous with the spaces between muscle cells, that is, with the classical extracellular space. The situation in mammalian heart muscle is therefore similar to that found by H. E. Huxley (1964), by S. Page (1964), and by Endo (1964, 1966) in frog skeletal muscle—an extension of the extracellular solution into the interior of the muscle cell.

Figure 6 is one of a sequence of thin serial sections of a Purkinje cell from the papillary muscle of the cat's right ventricle (Page 1966a). We chose to do most of our studies with Purkinje cells for two reasons. First, the most extensive studies on the electrophysiology of heart muscle cells, notably those from the laboratory of Weidmann (1956), have been done in Purkinje cells. Secondly, Purkinje cells, which are somewhat smaller than cells of the

FIG. 6.—Electron micrograph (longitudinal section) of a Purkinje cell bundle consisting of two cells separated by an intercellular cleft (Page 1966a). Upper margin: classical extracellular space containing an erythrocyte within a capillary. Diads appear as dark patches along the longitudinal surfaces of the plasma membranes which face the classical extracellular space and the intercellular cleft. A transverse tubule in close association with a sarcotubule is seen in cross- section near the left lower margin of the lower cell. The tissue was fixed with buffered glutaraldehyde, post-fixed with osmium tetroxide and uranylacetate and stained with lead tetraacetate. Magnification $\times 16\,000$

working myocardium, can be more easily analyzed with the technique of serial sections. We confined our studies to Purkinje cells in which the myofibrils and the organization of the contractile apparatus are fully developed. The ultrastructure of these cells is not in all respects comparable to that of other types of mammalian heart muscle, but the features discussed here, which are relevant to the subject of ion transport, are also encountered in other types of mammalian heart muscle. Figure 6 shows two Purkinje cells, each about two microns in diameter, separated by an intercellular cleft. Each cell is bathed on one side by the classical extracellular space, that is, the space between cells, and on the other side, by an intercellular cleft. The figure also shows a cross-section through a transverse tubule—a structure lined by a membrane which is, by ultrastructural criteria, a plasma membrane (Sjöstrand 1963). The lumen of this transverse tubule can be shown by serial cross-sections to be continuous with the classical extracellular space on the one hand, and with the intercellular cleft on the other hand. The Purkinje cells are thus honey-combed with extensions of the extracellular solution from the interspaces between cells which appear at the top and bottom

of the figure. The extracellular extensions run not only transversely, as in Nelson and Benson's reconstruction, but also parallel to the long axis of the cell. Both transverse and longitudinal channels are of restricted diameter with a range of from 2000 to 3000 Å. By morphological criteria the contents of these channels should be able to exchange with the classical extracellular space; under steady-state conditions their contents should have the same ionic composition as that of the classical extracellular space. In evaluating the ion exchange of tubular systems in mammalian heart muscle, it should be emphasized that both the longitudinally and transversely oriented extensions of the classical extracellular compartment are very much wider in this tissue than the analogous transverse tubules of amphibian skeletal muscle as described, for example, by Peachey (1965).

Suppose it is desired to measure the exchange of cellular potassium with radioactive potassium. The isotope is introduced into the classical extracellular space (Fig. 6), and the rate at which the muscle as a whole becomes radioactive is followed; in other words, the potassium influx at constant cellular potassium content is measured. Alternatively, the muscle is exposed for a long time to radioactive potassium until all the potassium in the muscle has exchanged with extracellular potassium. The rate at which the muscle loses radioactivity to an unlabelled solution (the outflux of labelled potassium at constant cellular potassium content) is then measured. In cat heart muscle, influx and outflux are found to be the same. But what does such a potassium exchange mean? Is the cell membrane of the longitudinal surface facing the classical space between cells equally permeable to potassium and other ions as the membrane lining the transverse tubules or the intercellular clefts? Stated differently, is the pathway for radioactive potassium ions most nearly approximated by an in series arrangement (classical extracellular space, tubule, cell) or by an in parallel arrangement in which both the classical extracellular space and the tubules are in parallel with the cell? If the membrane permeability for potassium and other ions is uniform throughout, how are the kinetics of exchange of a cellular ion affected by the long diffusion path through the tubular extensions of the extracellular space? Does the diffusion channel for small extracellular molecules correspond to the spaces between muscle cells? And does the rest of the extracellular space, the 13 per cent of muscle water measured by the difference between the equilibrium sucrose space and the diffusion channel for sucrose represent the volume of the extracellular extensions, both transverse and longitudinal?

These questions remain unanswered. Figure 7 presents one example of an experimental problem indirectly related to such questions. The figure shows the rate of exchange of cellular potassium in cat papillary muscle as a function of the diameter of the muscle (Goerke and Page 1965). The total cellular potassium content is constant under the experimental conditions. The exchange of potassium is observed to increase strikingly as the diameter of these roughly cylindrical muscles becomes smaller. The results are the same whether the direction of radioactive potassium movements is into or out of the cells. After making these measurements, we counted the number of cell nuclei per cross section of a formaldehyde-fixed muscle as a rough index of cell size. We observed that muscles with smaller diameters have a larger number of nuclei per microscopic cross section as seen in the light

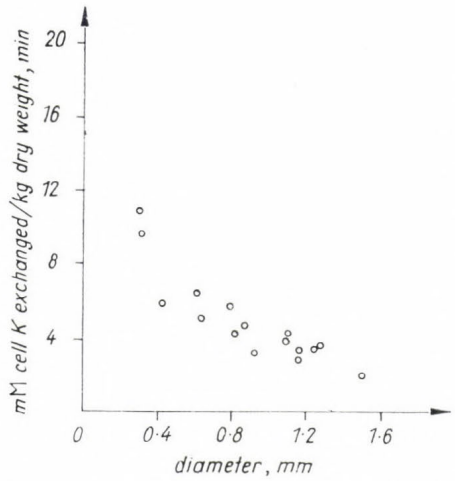

FIG. 7.—Dependence of the cellular potassium exchange in the steady state on the diameter of cat papillary muscles. Temperature 27·5° C, external potassium concentration 5·3 mM (Goerke and Page 1965)

microscope. On the assumption that the number of nuclei per cell is approximately constant, we concluded that muscles with smaller diameters have more cells and cells of smaller diameter, each cell having a greater ratio of surface to volume. Such cells would therefore have more cell surface available for exchanging cellular with extracellular potassium. The electron micrographs of papillary muscles suggest that this interpretation may be oversimplified. The cell surface area available for the exchange of ions has conventionally been approximated from the apparent surface area seen with the light microscope. The electron-microscopic finding that there are both transverse and longitudinal extensions of the spaces between cells raises two further questions: (a) What fraction of the surface available for the exchange of potassium ion is located in the tubular extensions of the extracellular space, and what fraction borders on the interspaces between cells? And (b) is the ratio of tubular surface to total cell surface greater for cells with small diameters than for cells with large diameters?

Any attempt to relate electron-microscopic data to a physiological measurement must face up to another problem, that of estimating to what extent fixation and the other procedures by which heart muscle is prepared for electron microscopy alter the shapes and the relative volumes of the various component tubular systems? We are now systematically measuring these effects. We find that conventional methods of fixation seriously alter the permeability and composition of heart muscle (B. Krames and E. Page, unpublished observations). We would therefore be very hesitant to estimate the volumes and areas in living heart muscle from volumes and areas measured in electron micrographs of fixed muscle. This is unfortunate, because it would be most interesting to know how the various tubular extensions behave under conditions like that shown in Fig. 8 (Page and Storm 1966). In the figure, the water content of cat heart cells (calculated on the basis of the mannitol space as a measure of extracellular water) is plotted against the reciprocal of the external osmolality. It is apparent that the cellular water content decreases in hypertonic solutions and increases in hypotonic solutions. The extrapolated intercept on the ordinate is not significantly different from zero. This observation is consistent with the interpretation that all of the water in the cell is free to respond to an increase in external osmolality by a net movement out of the cell. However, the uncertainty of the measurement is such that as much as 18 per cent of cell water could be in an osmotically inactive state ($P < 0{\cdot}1$). In very hypotonic solutions the heart muscle cell is no longer an osmometer; that is,

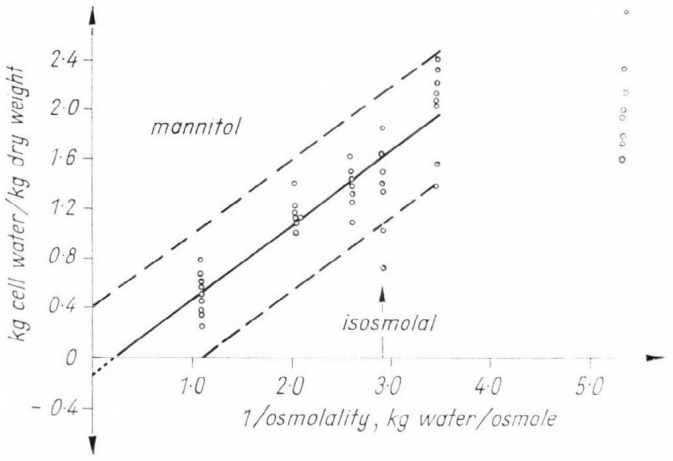

FIG. 8.—Cellular water content of cat papillary muscle as a function of extracellular osmolality (Page and Storm 1966). Cell volumes were calculated using the mannitol space to measure extracellular volume. External osmolality was varied by adding sucrose to a solution whose concentration of NaCl was half that of physiological cat Ringer's solution. Broken lines are two standard errors of the estimate from the least squares (solid line)

it no longer responds to a decrease in the activity of water outside the cell by a cellular uptake of water.

Girardier and co-workers (1964) have examined electron micrographs of rat heart muscle exposed to hypertonic or hypotonic solutions and then fixed with osmic acid. They conclude that raising the tonicity results in an increase in the ratio of mitochondrial volume to sarcoplasmic volume. This conclusion needs to be reviewed in the light of studies on isolated cardiac mitochondria by Packer (1960) and by O'Brien and Brierley (1965). These studies indicate that such mitochondria behave as perfect osmometers over a rather large range of extramitochondrial tonicities. Girardier has reported further that the transverse tubular system dilates markedly in hypertonic solutions. Such dilatations have also been described in the skeletal muscle of amphibians (Huxley et al. 1963, Freygang et al. 1964) and crustaceans (Girardier et al. 1963). The interpretations of this finding in mammalian heart muscle must await more precise information about the volumes of cardiac mitochondria, tubules, and cytoplasm *in vivo*, compared with their volumes after fixation and after the other procedures preliminary to electron microscopy.

Up to this point the discussion has been concerned with experimental observations whose existence as a phenomenon is independent of whether one chooses to analyze them according to the membrane theory, the fixed charge theory, or any other theory. The phenomena to be considered next have usually been formulated in terms of membrane theory, but they, too, can be observed without imposing a detailed physical model on the data. These phenomena include first, ion movements associated with the action poten-

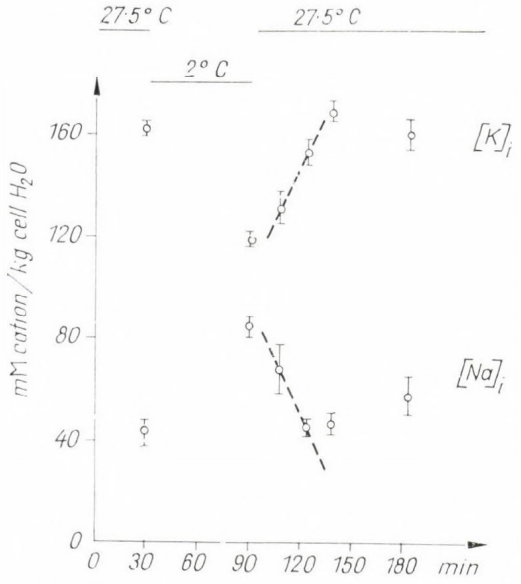

FIG. 9.—Effects of cooling and rewarming on the intracellular concentrations of potassium (upper plot) and sodium (lower plot) (Page and Storm 1965). The temperature variations are indicated at the top

tial, secondly, ion movements during active ion transport, and thirdly, measurements of the membrane capacitance in heart muscle.

The Hodgkin–Huxley theory of the ionic basis for excitation and impulse conduction in nerve and muscle (Hodgkin 1958) postulates that certain characteristic movements of Na and potassium occur in association with an action potential. Whatever position one may take on the theoretical model underlying the hypothesis, the observations of Hodgkin and Huxley and of many others in various types of nerve and muscle suggest that net movements of sodium and potassium should occur during excitation in heart muscle. Nevertheless, an increased influx of sodium and an increased efflux of potassium have not to date been demonstrated unequivocally in mammalian heart muscle (Goerke and Page 1965, Taylor 1962). Fluxes of potassium, which are for technical reasons easier to measure than those of sodium, can be estimated with fair precision. Some explanations for the fact that many observers have failed to observe an increased flux of potassium or sodium during excitation have been advanced in a monograph by U. Sjöstrand (1964). The particular possibility to be stressed here is that a major part of the transient ion movements which accompany the action potential may be occurring across the membranes lining the transverse and longitudinal extensions of the extracellular space. That is, the net fluxes may consist of localized movements into and out of spatially restricted regions. In these regions diffusion may be slow and is perhaps confined to unstirred layers at the surface of the membrane. The suggestion is neither new nor radical. Restricted diffusion in similar extracellular channels already is the basis of an extensive body of theoretical and experimental studies on frog skeletal muscle (Girardier et al. 1963, Sjöstrand 1964). However, the geometrical relationships and distribution of the extracellular channels in mammalian heart muscle differ from those in skeletal muscle (Page 1966a); their diameter is probably greater in heart muscle. Moreover, the cardiac action potential has a uniquely long repolarization phase and other characteristics differing from the action potential of skeletal muscle. In both types of muscle, we urgently need to know the composition of the solution in the channels, and yet a reliable experimental method of directly sampling this solution is not yet available.

The ability to sample the fluid in the tubular systems of heart muscle would also facilitate the study of ion movements usually considered under the heading of active transport. In heart muscle, as in skeletal muscle, there are several convenient ways of turning active ion transport off and on (Page et al. 1964, Page and Storm 1965). One way is to cool the muscle down to near zero degrees Centigrade. At such low temperatures the active transport of ions becomes slowed sufficiently to disturb the steady state. The cells take up sodium and lose potassium, and the resting potential falls. These processes are reversed by rewarming the muscle, which reactivates active transport. The fall in cellular K concentration and the rise in cellular Na concentration on cooling are illustrated in Fig. 9, which also shows the complete reversal of these changes about 30 minutes after rewarming. In Fig. 10 the recovery of the membrane potential is plotted against the time after rewarming of the muscle (Page and Storm 1965). The points represent the resting potential of cells at the surface of the muscle; the vertical line denotes the time at which muscles, previously kept in the cold for two hours, were suddenly rewarmed. The absolute value of the resting potential difference in the cold is low, but when the muscle is abruptly rewarmed the potential rises to control or higher values almost as fast as we can measure the response. The resting potential of surface cells returns to high (negative) values within the first minute. By contrast, the ionic composition of the muscle as a whole remains unchanged from its value at 2–3° C and does not fully recover until 20–30 minutes later. That is, the cellular sodium content is still high and the cellular potassium content is still low long after the absolute value of the resting potential has reverted to a normal or greater than normal value.

It is not strictly correct to compare the results of microelectrode measure-

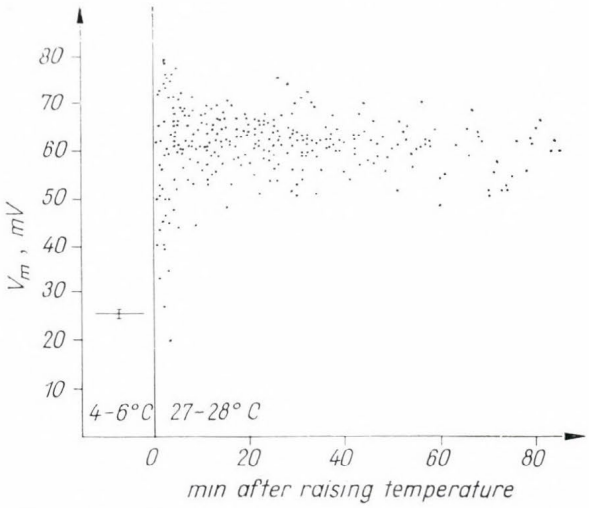

FIG. 10.—Effect of sudden rewarming on V_m (Page and Storm 1965). The period of rewarming is indicated by a vertical bar. V_m before rewarming is 25·6 ± 0·7 mV. The external K concentration used for this experiment was 10 *m*M

ments limited to cells at the surface of the papillary muscle with the membrane potentials and ionic composition of the muscle as a whole, which includes cells lying deep within the tissue. Nevertheless, it is probable that even in surface cells the recovery of the ionic composition lags behind the almost instantaneous recovery of the resting potential. The argument, which is discussed in detail elsewhere (Page and Storm 1965), rests on a calculation of the net-fluxes, that is, of the rates of net transport of potassium and sodium which would be required for recovery of the cellular ionic concentrations in the few seconds needed to restore the resting potential after rewarming. Such fluxes would have to be at least two orders of magnitude greater than any values ever obtained for heart muscle. The experimental observations may thus be summarized as follows: the absolute value of the resting potential is less than that of the potassium equilibrium potential, both under control conditions and in the cold, when active transport is presumably inhibited. But when the temperature is suddenly raised, that is, when active transport is 'switched on', the normal relationship between the resting potential and potassium equilibrium potential is reversed: the potential difference measured with microelectrodes temporarily exceeds the potassium equilibrium potential.

Such a reversal of the normal relationship between the resting potential and the potassium equilibrium potential has also been reported for frog skeletal muscle by various laboratories (Kernan 1962, Mullins and Award 1965, Cross et al. 1965, Adrian and Slayman 1966). We chose an interpretation of our observations on cat heart muscle, similar to the interpretations which have been made for frog skeletal muscle. That is, we suggested that immediately after rewarming the resting potential is determined at least in part by an 'electrogenic pump'. The term electrogenic pump usually refers to a hypothetical carrier mechanism which is capable of transporting ions into or out of the cell. It is characteristic of such a mechanism that it transports ions in such a way as to bring about a net transfer of charge, and thereby generates an electrical potential difference across the cell membrane. The cardiac glycoside ouabain is considered to be a rather specific inhibitor of carrier-mediated transport. Our suggestion of an electrogenic pump therefore receives some support from the observation that ouabain completely prevents the rapid restoration of the resting potential which normally follows the rewarming of cat papillary muscle. The suggestion is also consistent with earlier measurements by Délèze on the response of the membrane potentials of ungulate hearts to rewarming (Délèze 1960). But to postulate an electrogenic extrusion of sodium is only to open up a large number of questions about the kinetics and stoichiometry of such a mechanism. It is desirable to define the relationship of sodium extrusion rates to the intracellular and extracellular concentrations of sodium and potassium, as well as to quantitate the coupling between movements of sodium, potassium, calcium, and hydrogen ions. In spite of a number of careful studies (Langer 1966), even the steady state exchange of cellular sodium in mammalian heart muscle has not, in my opinion, been satisfactorily measured to date. The reason is the complexity of sodium exchange in this tissue, which has been confirmed in our laboratory (unpublished observations). The analysis of the time course of this exchange by conventional kinetic methods (Haas 1962) requires a number of arbitrary

assumptions. The basis for the difficulty is not a particular tissue model based on membrane theory or fixed charge theory, but rather our inability to perform a meaningful compartmental analysis on heart muscle, and our inability to relate ion movements and electrical potential differences to the appropriate anatomical portion of the membrane or to the appropriate organelle.

One ought not to leave a discussion of ion transport in heart muscle without mentioning one of the classical applications of membrane theory, the analysis of a cell as a cable. Fozzard (1966) has recently repeated Weidmann's (1952) original measurement of the membrane capacitance in cardiac Purkinje fibers and extended these measurements to higher frequencies. On the basis of an analysis similar to that published by Falk and Fatt (1964) for skeletal muscle, Fozzard has shown that the membrane capacitance behaves as if it had two components—one in parallel and one in series with the membrane resistance. He has presented evidence suggesting that the resistance of the salt solution in the transverse tubules may vary with the conductivity of the extracellular solution. Thus all of the classical interpretations of membrane theory as applied to mammalian heart muscle are undergoing modification to take into account the peculiarities of cardiac ultrastructure.

Where does that leave us with respect to a theoretical approach to ion transport in mammalian heart muscle? It seems prudent to restrict the answer specifically to mammalian heart muscle. For this particular tissue the application of membrane theory has proved useful in that the areas where classical membrane theory turned out to be inadequate are exactly the areas on which interest is focussed today. The most interesting observations are usually independent of the theoretical approach used—starting from another series of assumptions, an experimenter would demonstrate essentially the same phenomena. Moreover, there are important aspects of ion transport in heart muscle which do not at present lend themselves to theories about the nature of membranes or of cytoplasm. Two examples of such aspects are the Ca ion movements associated with excitation–contraction coupling, or the movements of calcium, potassium and hydrogen ions in mitochondria.

We might take the position that both the membrane theory and the fixed charge theory are sweeping generalizations, and as such they are of historical significance. But in cardiac muscle good measurements are, at this particular time, more important than sweeping generalizations, and many good measurements still remain to be made.

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TIGYI (Biophysical Institute, Medical University, Pécs): One of the most important suppositions of classical membrane theory is that all ions are uniformly distributed within the muscle fibre. I should like to present two experimental results which cannot be understood in this way.

1. Radioautographic localization of the potassium in the thin sections of frog toe muscles shows a selective accumulation of the potassium in the anisotropic sites of the cross striation (Fig. 11). These radioautographic results are not new, they confirm only the earlier results of Ernst and Tigyi-Sebes, which were performed with the method of hystochemistry and microincineration. If the concentration of potassium in the fibre differs according to the cross striation, how can we calculate the concentration values for the Nernst equation?

2. Prof. Page has shown the discrepancy between the potassium concentration of fibre and the resting potential in the case of cat heart papillary muscle. We have achieved similar results on frog muscles (*Rana pipiens* and *Bufo asiaticus*). Figure 12 shows the changes in the resting potential as a function of time if we change the osmotic concentration in the external fluid (by adding 24 g of sucrose to the 100 ml Ringer solution). In these experiments no significant change in potassium was measured, but the resting potential has dropped nearly to the half value of the original one. The resting potential increases again, after changing the hypertonic solution for normal Ringer, i.e. the phenomenon looks to be nearly reversible.

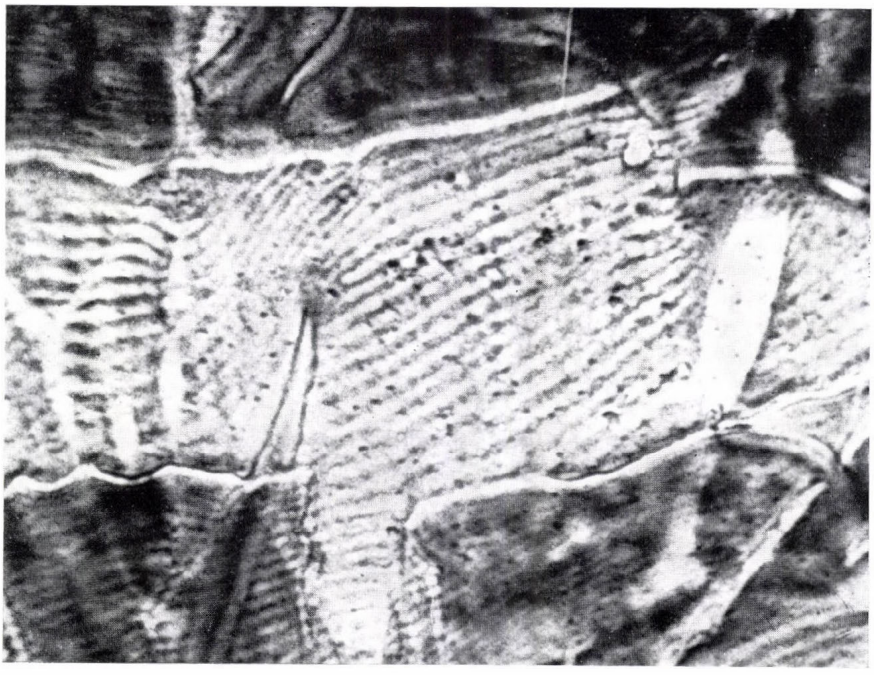

FIG. 11

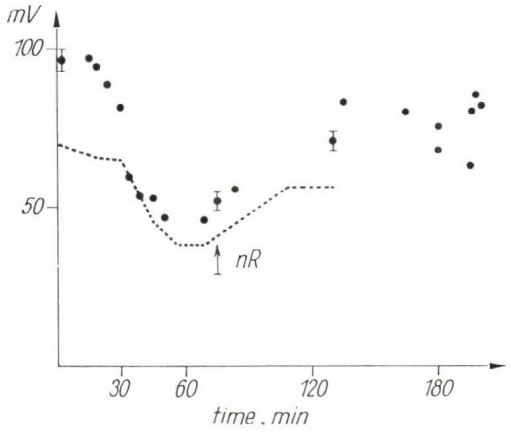

FIG. 12

The extracellular space (milieu, sucrose) and the chloride content did not show significant shifts, therefore, Kernan's argument (1964) against Koketsu and Kimura's experiments cannot be valid for our experiments.

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PAGE: One of the questions raised by Dr Tigyi's observations is what fraction of the potassium in a muscle cell exists as potassium bound to the proteins of the myofibril. I once made a calculation of this fraction for the potassium ion in the cells of mammalian heart muscle. Based on the Scatchard plots of potassium binding to myosin B, published by Lewis and Saroff, the potassium so bound would represent a minute fraction of the cellular content of this ion. Based on the much larger estimates for potassium binding, suggested by Banga, the total potassium associated with myofibrils should not exceed about 2 per cent of cellular potassium.

TIGYI: In our radioautographic experiment, the toe muscles were incubated 3–4 hours in a Ringer solution containing the potassium in a high specific activity (600 mC/g).

I estimate the percentage of the potassium exchange—according to our sartorius experiments—is about 30 per cent. I suppose, therefore, we get the information mainly about the localization of fibre-potassium in the cross striations.

VARGA: Dr Page showed a figure according to which he found significant differences as far as the measured and calculated membrane potential values are concerned. On the other hand, it is well known that Hodgkin and Horowicz found almost the same values as calculated on the basis of the Nernst equation on striated muscle. My question is whether in your opinion this difference is explainable, considering that you obtained these data on heart muscle?

STRICKHOLM: The observations on muscle membranes which show that the membrane potential has a slope of 58 mV for a tenfold change in external potassium do not necessarily indicate a membrane which is predominately potassium permeable. Such a conclusion is based on other additional assumptions, one of which is that the intracellular concentrations remain unchanged during an external ion shift. In addition, what is often recorded is the steady state membrane potential against external potassium. This is not the same as the instantaneous potential dependence of the membrane on an ion. Recently, Dr Wallin of Uppsala University and I have re-examined in crayfish giant axons the interpretation of the observations which show

a 58 mV potential change for a ten-fold change in external potassium. We found at elevated external potassium levels, where there is a 58 mV change for a ten-fold change in potassium, that the instantaneous change in membrane potential to a ten-fold change in potassium was only 20–30 mV and only with time (5–20 minutes) did the membrane potential approach a stable steady state giving a 58 mV change. During this approach to a steady state membrane potential, the intracellular ion concentrations were observed to shift. Therefore, each of the points on a plot of steady state membrane potential against external potassium represent entirely different cells and any conclusions drawn must take this into account. In the crayfish giant axon, the membrane appeared to be equally permeable to potassium and chloride in that region where the steady state membrane potential appeared to vary in a Nernst manner with respect to potassium. In muscle, therefore, similar caution must be exercised in interpreting the significance of the steady state membrane potential dependence on an ion.

AUBERT: I would remind Dr Page that Carmeliet (1961) has shown by direct experiments performed both in Weidmann's laboratory and in his own laboratory in Louvain, that at low K-concentration in the external medium the conductance and the permeability to potassium ions are decreased in the case of Purkinje fibres of the sheep heart. This fact helps to explain the departure from linearity of the resting potential vs log K concentration plot, according to the ionic theory of membrane potential.

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STRICKHOLM: The measurements which Weidmann made with intracellularly applied step currents to determine membrane impedances of cardiac muscle are generally quite difficult to interpret. Prof. T. Teorell of Uppsala has shown with artificial membranes that when a step current is applied across a membrane, the potential changes observed may not necessarily provide a measurement of membrane conductance or impedance. If the interactions between voltage, current, and water transport across membranes are considered, many alternative interpretations are possible as to the nature of the relationship between membrane potential and applied current, only one of which is that this relationship gives a measure of membrane impedance. That such difficulties of interpretation occur is seen in the voltage clamped frog sartorius muscle, where transient inward currents can flow without any corresponding impedance changes.

I am inclined to agree with the view of Dr Page which holds that the time relationship of the active ion currents in cardiac muscle have not yet been determined.

DYDYNSKA: I should only like to give a short comment in connection with the magnitude of the extracellular space in muscle. Using the sartorius of *Rana esculenta* (weighed, drained and not blotted) as skeletal muscle, in 100 mg tissue the dry materials amount to 18·5 mg, including 12·8 mg proteins. Extracellular water is 47 μl and fibre water 34·5 μl. Extracellular space was measured with sucrose that, according to Boyler, enters into the

fibres, but not into the myofibril space. The volume of myofibrils calculated from the data given above amounts to 51 per cent, which is far less than 80 per cent assumed by Dr Hanson in her calculation for rabbit muscles (1963). The relation between fibre water and protein content, however, is 2·70, and that is very similar to 2·75, calculated for rabbit muscle.

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BENSON: My colleagues and I have looked at the ultrastructure of heart muscle, using the electron microscope. The type of structure Dr Page describes (abundant intracellular clefts and very small cell diameters), in our experience is not characteristic of mammalian heart muscle with the exception of Purkinje cells. Mammalian heart muscle cells characteristically have diameters around 10 μ and do not contain the intercellular clefts seen in Purkinje cells. On the other hand, frog myocardial cells have numerous intercellular clefts, average cell diameters around 2 μ, sparse sarcoplasmic reticular and no transverse tubular system (Staley and Benson 1966). Also I would like to ask Dr Page to comment on possible relationships between the characteristic fine structure he finds in mammalian heart muscle and differences in the process of activation of contractile units in heart and skeletal muscle.

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PAGE: We have not ourselves looked at the ultrastructure of frog muscle with the electron microscope. With respect to Dr Benson's question about the relative magnitude of longitudinally and transversely oriented extensions of the extracellular solution in various forms of heart muscle, I think the answer must await a three-dimensional reconstruction of these tissues. F. S. Sjöstrand has developed methods which now make it possible to reconstruct a cardiac cell in three dimensions from the electron micrograph of (consecutive) serial thin sections (600 Å). These methods are tedious and rather difficult, but the amount of additional information more than compensates for the additional work. To answer Dr Benson's question about our ultrastructural observations on the diads involved in excitation–contraction coupling in mammalian heart muscle: we have found that diads, the organelles presumed to be the sites of localized Ca-release and Ca-uptake during excitation–contraction coupling and relaxation, are distributed very differently in cardiac muscle, as compared with frog skeletal muscle. In the cat heart, diads are present, not only along the transverse tubules, but also at the plasma membrane lining the cell surface and the longitudinally oriented clefts between cells. If the activation of the contractile elements is related to the release of calcium ions by the diads, the different geometrical distribution of diads suggest a different pattern of activation for mammalian cardiac muscle and frog twitch muscle, respectively.

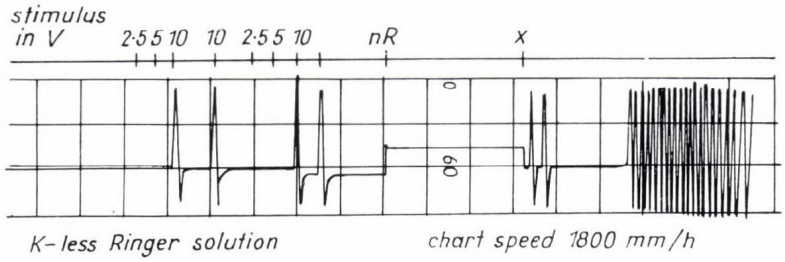

FIG. 13

ERNST: Concerning the question mentioned by Page that some part of the potassium content of the heart behaves itself differently from that of the other part, I should like to show you Fig. 13.

Frog heart was perfused with K-less Ringer solution. After a certain period, it contained but 1/2–1/3 of its original K-content, i.e. about 0·1–0·2 mg K, and stopped beating. In this condition electric stimuli caused single contractions without restoring the automaticity. But automaticity reappeared again when the K-less solution had been exchanged to a normal Ringer solution containing 0·1 mg K per ml. Thus the question arises, how it is to be explained that the heart stopped beating when it still contained a certain amount of K but it restarted beating due to the normal Ringer solution containing less K than the heart (Ernst 1966).

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Ernst, E. (1966) *Acta biochim. biophys. Acad. Sci. hung.* **1,** 55

WILKIE: I should like to raise the question, whether the permeability changes due to electric stimulation and/or to the potassium content newly given to the heart could explain Ernst's results.

ERNST: I am not on friendly terms with conceptions like permeability changes in membranes, which has never been precisely localized and concretely identified. But in this case the chief point is that similar experiments were performed also on pieces of the sinus venosus of the frog heart. And these pieces behaved in the same manner as the whole heart inasmuch as they stopped beating in a K-less solution and restarted beating in a normal Ringer's solution containing potassium.

BENSON: The possibility of explaining the different effects of the potassium contained in the heart itself and in the Ringer's solution could perhaps be found in the circumstance that they are localized in different compartments of the heart?

ERNST: And the explanation for the similar results gained on pieces of the sinus venosus?

PAGE: Prof. Ernst's description of the contraction of the frog heart which has been depleted of potassium reminds me of somewhat similar observations in cat heart muscle. By pre-incubating cat papillary muscles in the cold, the cellular potassium content can be reduced to low values and the intra-

cellular sodium concentration can be raised to a value which approximately equals the normal external sodium concentration. Nevertheless, such muscles begin to contract spontaneously as soon as the temperature is raised to 27·5° C.

BOWEN: In 1952, work in my laboratory showed that 10^{-6} M digoxin accelerated the ATP-induced shortening (synaeresis) of myosin B threads. In the following year, Robb and Mallow confirmed this finding.

In 1960, Skou and Post showed that ouabain affects the 'membrane, ATPase of crab nerve and human erythrocytes. Since ATPase are thought to be necessary for active ion transport, it is natural to speculate that it is through ion transport across membranes that cardiac glycosides exert their inotropic effect. Consequently, these results and speculation dispute the findings by myself and Robb that the glycosides exert their inotropic effect directly on the contractile protein. The problem gravitates to whether the inotropic effect is direct on the contractile proteins or on an energy supplying reaction. The effect has recently been reinvestigated in the laboratory of Dr Manuel Morales, San Francisco, by several investigators including myself. Myosin B was made by a process modified to give maximum purity of product. Rabbit skeletal muscle and beef heart were used. The contractile process was followed by Ebashi's method for assaying superprecipitation. This method was thoroughly perfected in San Francisco by Yasui and Watanabe.

Optical density changes in the superprecipitating actomyosin were followed by means of a Zeiss spectrophotometer used as densitometer. The changes in density were recorded in units of optical density on a Minneapolis-Honeywell Recorder. Rates of ATPase were studied by means of a pH stat in parallel but separate experiments. Protein concentration in the reaction mixture was 0·01 per cent and ATP concentration was 1 *m*M (to investigate rates of superprecipitation) and 0·05 *m*M (to investigate extents of superprecipitation).

The results show that the rate of superprecipitation was increased about 50 per cent and that the extent of superprecipitation was increased about 25 per cent by 10^{-9} M ouabain. Both the rate and the extent of superprecipitation were also increased by 10^{-4} M ouabain. The reason for this double optimum is unknown, but 10^{-9} M is as low or lower than the concentration of cardiac glycoside which will just show an inotropic effect on living heart muscle.

Determinations of ATPase activity in parallel experiments show that the same concentration of ouabain affects the ATPase activity of myosin B. The rate of utilization of ATP was increased 50 per cent when 10^{-9} M ouabain was included in the reaction mixture. The high concentration of 10^{-4} M ouabain also increased the ATPase activity. The ATPase activity of myosin A was not increased by any concentration of ouabain tested.

Digoxin at 10^{-10} M was associated with a large increase in the rate of superprecipitation. The effects of digoxin on ATPase activity of myosin were not tested.

A possible explanation of the effect of concentrations of ouabain and digoxin of such low magnitudes as presented above is offered in the unabridged paper in the September issue of *Circulation Research* (Vol. 19, p. 496) under the authorship of Stowring, Bowen, Mattingly and Morales.

Journal references to the authors cited above are also included in the complete report.

GERGELY: I wonder if you would comment on the fact that in the lower concentration range ouabain seems effective in a molar concentration that is perhaps 100 times lower than the molar concentration of myosin.

BOWEN: One can only say in reply to Dr Gergely's request that there may be reactive sites of importance to superprecipitation which are less concentrated than the superprecipitating protein. This point is emphasized by Stowring and co-workers. Also, the possibility exists that cardiac glycosides act as catalysts and are effective at concentrations lower than the substrate involved. The 0·01 per cent concentration of myosin B is possible 10^{-8} M if 10^7 is accepted as molecular weight, but if myosin A only is involved, the molarity is about 2×10^{-6}.

EDMAN: A rough estimate of the concentration of the cardiac glycoside in the extracellular fluid during digitalization would give us a value of 10^{-7}–10^{-8} M/litre. However, we know very little, so far, about the glycoside concentration inside the myocardial cell. There may well be an accumulation intracellularly, so your concentration of 10^{-6} M may not be unreasonably high. The very marked frequency-dependence of the inotropic effect of digitalis (Edman 1965) suggests that the most important component in the digitalis effect is an action on the excitation–contraction coupling rather than on the actomyosin system itself.

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Edman, K. A. P. (1965) *Ann. Rev. Pharmacol.* **5,** 99

BOWEN: Besides excitation–contraction coupling being involved in positive inotropism, cardiac glycosides are thought by some workers to act through an effect on membrane permeability. We are saying that perhaps the effect is directly on the contractile protein. Whichever system the effect is through it has been shown by Okita and his colleagues that digitalis does not accumulate in any tissue except those through which it is eliminated (Okita et al. 1955, Spratt and Okita 1958). Also, they found that digitoxin did not bind to tissue proteins such as cardiac. Goodman and Gilman withdrew their statement that cardiac glycosides may accumulate in heart tissue, in the second edition of their pharmacological text.

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Spratt, J. L. and Okita, G. T. (1959) *J. Pharmacol. exp. Ther.* **124,** 109, 115

SLEATOR: We have studied the effects of glycosides on living heart muscle and find that one way to summarize them is to say that glycosides appear to make the tissue more sensitive to calcium in the external medium. In your preparation, what is the relation between the ouabain effect and the concentration of calcium in the medium?

BOWEN: We may not have done the experiment you have in mind, but we did demonstrate that in the presence of 10^{-5} M $CaCl_2$ there was no specific effect of the cation on the ouabain effect.

TIGYI: I would like to show another difficulty of the membrane theory concerning the active muscle. As Hodgkin and Horovicz have published (1959) (sorry that neither of them is present at our Symposium), the amount of potassium and sodium exchange in a single fibre is in the order of magnitude 10^{-11} M/cm^2. Calculations based on these data should give an exchange of potassium in the active muscle, after ten thousand twitches, near to 100 per cent.

We could have never shown a higher exchange in potassium under the circumstances mentioned above when we stimulated indirectly. When using direct stimuli the exchange rises up to 40 per cent. Therefore, we suppose (Tigyi 1959) that the way of stimulation plays a very important role in the evaluation of the ion exchange measurements.

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Tigyi, J. (1959) *Acta physiol. Acad. Sci. hung.* **16,** 93

STRICKHOLM: Did you use summer or winter frogs in your experiments? Frog muscles are reputed to change their physiological and pharmacological properties from summer to winter.

TIGYI: We have used frogs of all seasons, and no significant differences were observed.

SRETER: I would like to ask Dr Tigyi's comment on Grob's experiment, in which he measured arteriovenosus potassium concentration difference in the stimulated forearm of human subjects. They reported a rather large potassium efflux indicating potassium depletion of the indirectly stimulated muscles.

TIGYI: In Grob's experiments, the circulation was a closed system, so the metabolic by-products of the contraction flowed repeatedly through the active muscle. As earlier studies of Ernst have shown, under such circumstances, a significant decrease in ions appears regularly. However, this effect is considered by us as a secondary phenomenon.

Symp. Biol. Hung. 8, *pp. 151–205 (1967)*

MECHANICAL ACTIVITY OF STRIATED MUSCLE

F. F. JÖBSIS

DEPARTMENT OF PHYSIOLOGY, DUKE UNIVERSITY MEDICAL CENTER, DURHAM

In this lecture I would like to advance some thoughts and experiments on muscle contraction viewed as a sequence of chemical reactions. Such an approach is certainly not new, but it might be considered that its application has been somewhat neglected so that it has played but a minor role in the physiological investigation of muscular contraction. This is not caused by a lack of interest on the part of muscle physiologists in the data yielded by biochemical studies, but may well be a long-term holdover of some historical trends.

In 1871, Bowditch enunciated his justly famous and much maligned all-or-none law. Justly famous, because it summed up in very simple form some cardinal features of cardiac physiology. Much maligned, because later it was also attempted to apply this simple law to skeletal muscle. But, over the years it has been shown that it is not a law applicable to the latter—not to entire muscles and not even to single muscle fibers. Only electrical membrane phenomena with an absolute refractory period appear to be governed by it. However, in this trend of thinking a theory was proposed in which the action potential supposedly produced a new state in the muscle. This was to occur completely and almost instantaneously in response to a single effective stimulus. By the electrical membrane events the primary mechanical components of muscle were supposedly changed from a flaccid to a tautly stretched condition, i.e. a new elastic body was created. The inherent potential energy of the new system was then used for tension development and shortening. Although a similar theory had been proposed before Bowditch's law, only afterward did the new version become dominant. This occurred especially when the concept appeared to be in agreement with certain heat measurements.

In 1923, the classic experiments of Fenn (1923) showed the fallacy of this theory. He reasoned that the degradation of the newly created elastic state to the resting state could result only in the utilization of the energy difference between the two states. Various forms of mechanical activity such as tension production, shortening and work could be performed at the expense of this fixed quantity of energy. But the sum of the three parameters should be the same under different mechanical arrangements. His experiments proved, however, that the total energy expended by the muscle depended critically on the weight of the load lifted during the contraction (Fig. 1). From a large amount of experimentation of this type it has become clear that Fenn's result is correct. However, the shape of the energy curve as a function of the load varies considerably depending

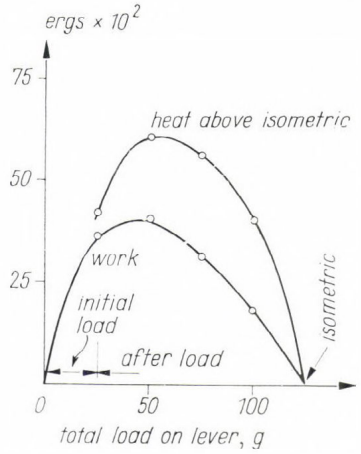

FIG. 1.—The Fenn effect. The extra heat liberated and the work performed are shown as a function of the load lifted by the frog's sartorius in short tetany (Fenn 1923)

on the type of muscle used, and the temperature at which the experiment is run. Fischer showed for instance, by measurement of heat production and of oxidative recovery metabolism that the energy utilized by minimally loaded isotonic contractions can be far lower than that used by isometric ones and that this difference increases with raised temperature (Fig. 2) (Fischer 1928, 1930, 1931). Nevertheless, with respect to the new-elastic-body theory, the important fact remains that energy utilization varies as a function of the load.

Fenn clearly pointed out that his results were incompatible with the new-elastic-body theory and that they led to a point of view that energy utilization depends on processes which vary with the shortening, tension and work parameters. This conclusion suggested that a sequence of reactions must take place with a time course extending throughout the period of mechanical activity and that the energy utilized in such reactions was influenced by the mechanical state of the muscle throughout the contraction.

In 1934–36, D. E. S. Brown (1934, 1936) showed that the well-known effect of hydrostatic pressure on muscle contractility was mediated primarily by a process occurring mainly before the mechanical reaction of the muscle (Fig. 3). The effect of high hydrostatic pressure rests on the occurrence of volume differences between the reactants and products of a chemical reaction. Since muscle contraction is accompanied by a decrease in volume, as shown by Ernst (1963), hydrostatic pressure tends to increase the extent of the reaction and thus the resulting tension is increased. By rapidly applying hydrostatic pressures of several hundred atmospheres at some variable time after the stimulus, Brown

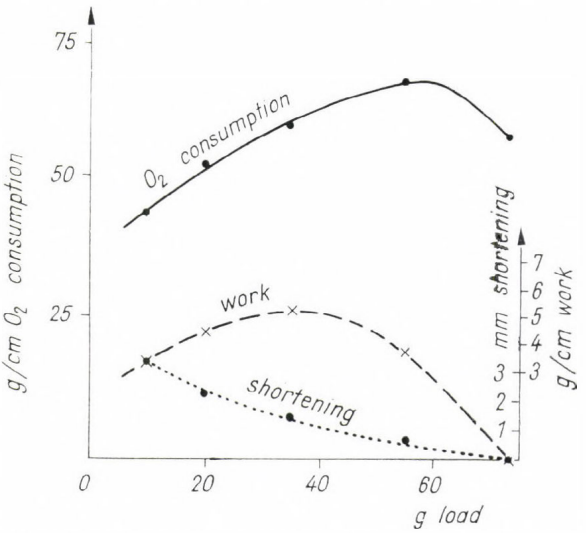

FIG. 2.—Shortening, work and O_2-consumption during oxidative recovery shown as a function of the load. Frog sartorius, 11·7°C. Each point is the result for a series of single twitches (Fischer 1931)

showed that application early in the contraction cycle produced the greatest tension increment. The effect was most easily summarized by plotting the extra tension developed in response to the pressure as a function of the time at which the pressure was applied. Thus he arrived at the time curve of a process that started immediately upon stimulation and reached a maximum very early in the rise of isometric tension. These results he interpreted as showing that the mechanical events are preceded by a reaction which is accompanied by a large decrease in the volume of the product as compared with that of the reactants. Since it occurs very early in the twitch Brown designated this the 'α-process'. His general interpretation was that under the influence of the hydrostatic pressure an increased amount of the products was formed by the α-process. This caused in turn a higher amount of tension production in the subsequent reactions. These experiments proved, therefore, that a sequence of reactions takes place, one of which results in the externally manifest mechanical response of the muscle.

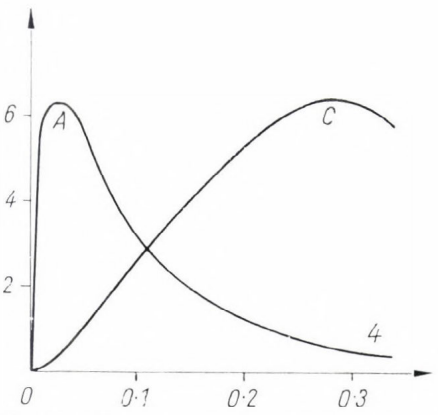

FIG. 3.—Time course of the α-process (*A*) preceding the development of mechanical tension (*C*) in the twitch. Ordinate: tension in arbitrary units; abscissa: time in seconds. Frog sartorius 0·5° C (Brown 1936)

Shortly afterwards, Dubuisson (1937, 1939) published the results of his novel experimentation on pH changes in contracting muscles. With the aid of a specially designed glass electrode that was in contact with the muscle, he demonstrated that a series of pH fluctuations occurred during and after the contraction (Fig. 4). The various phases of these changes were interpreted as evidence for the occurrence of a number of different reactions yielding or absorbing H^+-ions.

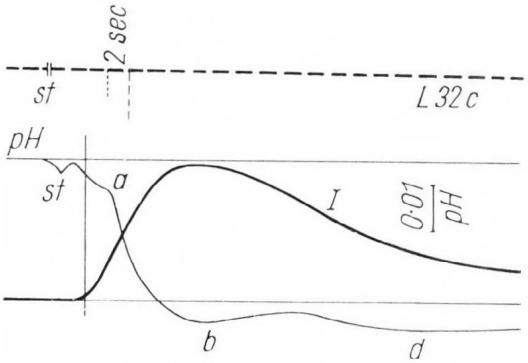

FIG. 4.—Changes in pH during and following the contraction of muscle from the frog's stomach (Dubuisson 1939)

These three experiments, demonstrating the Fenn effect, the α-process and the pH changes, might well be considered the starting point of a kinetic approach to muscular contraction. From this point of view the mechanical performance of striated muscle is the result of a number of sequential reactions, which are triggered by the action potential. Such a kinetic concept carries with it certain predictable consequences concerning the energe-

tics of muscular contraction. A few of these will be examined a bit later.

Although forty and thirty years have gone by, it can only be noted that the promise of the kinetic approach has not been fulfilled. Attempts have been made at the incorporation of new biochemical data into the kinetic schemes, but in a way this line of thought has remained on the fringe of the main stream of muscle physiology. Since the twenties and thirties, the breakthroughs of biochemistry—the discoveries of myosin, actin and their ATPase activity, for example, and more recently the role of calcium—have provided a much more extensive background for the interpretation of the sequence of events. Electron microscopy has provided a new anatomical foundation. With these new data in mind, but not necessarily straining for a rigorous interpretation, it might be illustrative to set up a reaction scheme. No claim of accuracy, originality or priority is made. In fact, the present attempt owes much to the analyses of Polissar (Johnson and co-workers 1954), of Morales and co-workers (1955) and of Goodall (1957).

INTRACELLULAR KINETICS OF CALCIUM

After the pioneering work of Heilbrunn and Wiercinski (1947), of Bozler (1952), and of Bianchi and Shanes (1959) and more recently of Annemarie Weber (1959, 1961), Ebashi (1961, 1962), Hasselbach and Makinose (1961), Podolsky and Hubert (1961) and Brown and co-workers (1963), it is generally agreed that calcium serves as the activating ion in muscular contraction. It seemed, therefore, of some value to attempt to measure the fluctuations of ionized calcium in the cytoplasm of the contracting muscle.

In 1963 Ohnishi and Ebashi (1963) showed that calcium accumulation by isolated vesicles of the sarcoplasmic reticulum was measurable by using murexide as a calcium indicator. At physiological pH murexide combines with calcium to give a colored complex, which has an absorption peak at a lower wavelength than Ca^{++}-free murexide (Fig. 5). Thus the addition of calcium produces a decrease in the optical density at 540 mμ and an

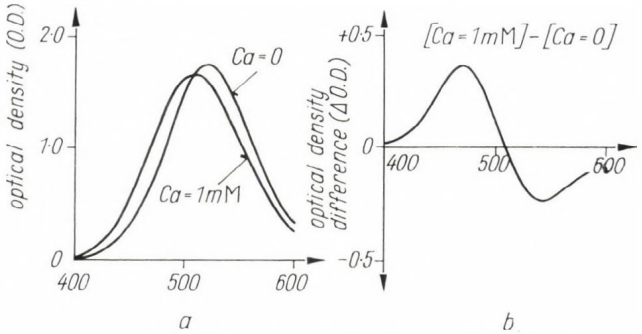

FIG. 5.—Spectral characteristics of murexide and Ca-murexide. Left: absorption spectra; right: difference spectrum pH 6·8, 20° C (Ohnishi and Ebashi 1963)

increase at 470 mμ (or transmission changes in the reverse direction). We have introduced the indicator into muscles of tropical toads by intraperitoneal injections of murexide solution for several days before sacrifice. The experiments were carried out by transmitting a monochromatic beam of light through the muscle, at either a peak or a trough wavelength of the murexide difference spectrum and comparing the optical changes with those at two adjacent wavelengths. The transmitted light was measured with a photomultiplier and recorded on a computer for transients averaging. The signals at the adjacent wavelengths were subtracted from the one recorded at the peak wavelength in alternate series of 5 to 10 contractions. In this way the scattering artifact was eliminated and a wavelength specific effect remained. (For further details of the method, see Jöbsis and O'Connor 1966).

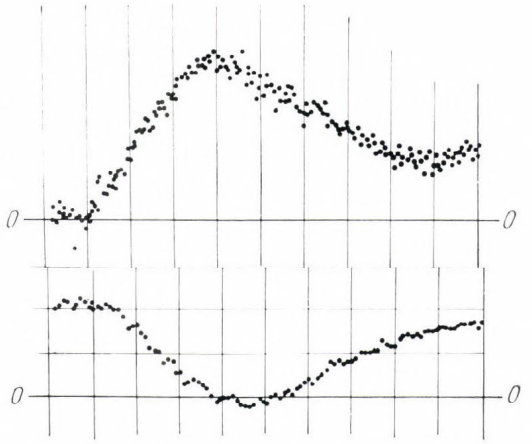

FIG. 6.—Kinetics of the Ca-murexide in the sarcoplasm of the toad sartorius muscle at 9—10°C. Top: 540 mμ (minus 505 and 580 mμ); bottom: 470 mμ (minus 440 and 505 mμ). Increase in light transmission is recorded in the upward direction. The vertical lines provide a time scale at every 25 msec. The stimulus was delayed 25 msec after the start of the sweep. Results on two preparations from different toads (Jöbsis and O'Connor 1966)

The bottom trace of Fig. 6 shows the result of one such experiment in which 470 mμ was alternated with 505 and 440 mμ as adjacent reference wavelengths. The downward deflection indicates a decrease in light reaching the photomultiplier, which agrees with an increase in Ca^{++} following the stimulus. The top curve of Fig. 6 shows the same type of experiment performed at the trough of the Ca-murexide difference spectrum (540 mμ minus 505 and 580 mμ). It is clear that the two traces show approximately the same event, but recorded as mirror images. At 540 increased Ca^{++} produces an increased light transmission (decreased OD), whereas at 470 mμ a decreased transmission (increased OD) would result from the formation of the Ca^{++}-murexide complex. Satisfactory results were obtained in this manner in about 50 per cent of the cases, whereas in the others no effect could be noticed. The number of successful experiments increased, however, by dissolving the injected murexide in dimethyl sulfoxide, an agent well-known to improve the permeation of large numbers of substances across biological membranes.

In Fig. 7 another experiment is shown with recording at 470 mμ as the measuring wavelength. The upper traces represent the time courses of the isometric twitch tension and of the light scattering. From this figure it is seen that the free Ca^{++} concentration increases immediately after the stimulus and comes to a maximum in 50 msec, i.e. at the very beginning of the contraction cycle. Subsequently, the Ca^{++} concentration falls to

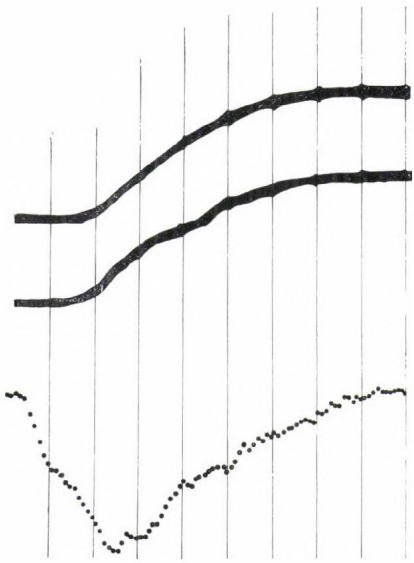

Fig. 7.—Time relations between tension development (upper trace), light scattering (middle trace) and the kinetics of the Ca-murexide complex, 470 mμ — (440 and 505 mμ). Time scale: 25 msec per division. Stimulus delayed 12 msec after the start of the sweeps. Toad sartorius, 12° C (Jöbsis and O'Connor 1966)

approximately the original level by the time the peak tension of the twitch has been reached (far right side of Fig. 7).

The localization and the relative distribution of the murexide in the cell are unknown at the moment. However, since the calcium concentration within the tubules of the reticular system is very high, any murexide located within that structure will be in the Ca-complex form. The same is the case for murexide that might be absorbed to external surfaces in contact with Ringer's solution since the latter contains 2 mM Ca^{++}. Only Ca^{++} changes in the sarcoplasm proper can be expected to be registered by color changes in the indicator such as illustrated in Figs 6 and 7.

The time course of the rise and fall in the sarcoplasmic concentration of free Ca^{++} strikingly resembles that of Brown's α-process (Fig. 3). The possibility exists, therefore, that the α-process is the reaction of free Ca^{++} with the contractile proteins. It might be of some interest to investigate the pressure dependence of this reaction.

Since calcium appears to serve as the activator of both muscular contraction and of the ATPase activity of actomyosin, the release of Ca^{++} can be presented as the first step in the sequence of reactions resulting in the mechanically evident aspects of the twitch. Although any scheme presently conceived will in all probability turn out to be incorrect or at the least grossly oversimplified, a first attempt can perhaps be made by showing the initial reaction between actin, myosin and Ca^{++} in the following way:

$$\begin{array}{l} A + M + Ca^{++} \rightleftharpoons AMCa + ATP \rightleftharpoons \ldots \\ \quad\;\; \uparrow\downarrow \\ \quad\;\; (Ca) \end{array}$$

This merely states that one of the earliest occurring events is the release of calcium from a storage compartment and its combination with actin and myosin to form a complex. The subsequent reaction with ATP is discussed further below. According to certain points of view, it is considered that in the relaxed muscle ATP is absorbed to the myosin molecule, specifically to the cross-bridges. This may well be the case, however, it is of no great consequence in our scheme, as it would merely mean that the second reaction has taken place before the release of calcium and that, for example, instead of myosin as the reactant we should write myosin-ATP. From a conceptual point of view little would have been changed.

TIME COURSE OF P_i LIBERATION

It is generally agreed that the hydrolysis of ATP to ADP and inorganic phosphate (P_i) plays a key role in the contraction. The work of Cain and Davies (1962) has shown that ATP breakdown is perhaps the first chemically measurable reaction in a contraction. Although these results do not preclude that an earlier energy-yielding reaction takes place for which ATP hydrolysis serves as a rephosphorylating reactant, it will be assumed here that ATP

FIG. 8.—Time course of pH change (top) and the myogram of the ileofibrillaris of the turtle at 22° C. Time marks every second (Distèche 1962)

breakdown is the primary, energy-yielding reaction in the contraction cycle.

ATP hydrolysis is known to be accompanied by a decrease in the pH. The studies by Dubuisson (1937, 1939) and the more recent ones by Distèche (1962) show the time course of the pH changes. These measurements were made by applying a pH electrode with a very thin, glass membrane to the contracting muscle. The records exhibit a decrease in pH starting soon after the occurrence of the stimulus. A trace obtained by Distèche from a turtle's ileofibularis is depicted in Fig. 8.

Since the early time course of the first acidification is important for the determination of the sequence of reactions, I have attempted to increase the resolution of the early kinetics by optical measurements utilizing the pH indicator bromcresol purple. The method is completely analogous to that for the Ca^{++} except that the indicator is allowed to equilibrate with the muscle after excision. Figure 9 shows the result for a toad muscle at about 12° C. In agreement with the earlier results a phase of rapid acidification is noticed, starting at about the time when the mechanical tension first rises. After

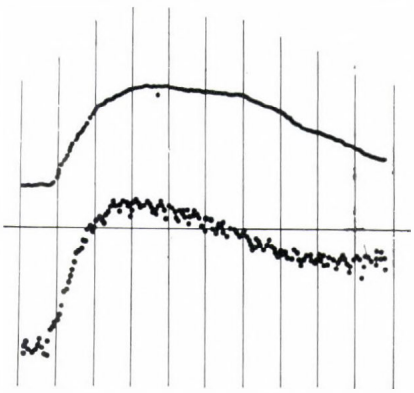

FIG. 9.—Early pH change in the toad sartorius muscle during the single twitch at 12° C. Measurement with bromcresol purple at 588 mμ (560 and 620 mμ reference wavelengths)

reaching a maximum the trace shows a gradual change toward alkalinization. This is interpreted as caused by the rephosphorylation of ADP from creatine phosphate. *In vitro* studies have shown that this last reaction is accompanied by a considerable pH change in the basic direction. In addition, an acidification related to glycolysis can be detected at a much later time (Dubuisson 1937, 1939, Jöbsis 1963).

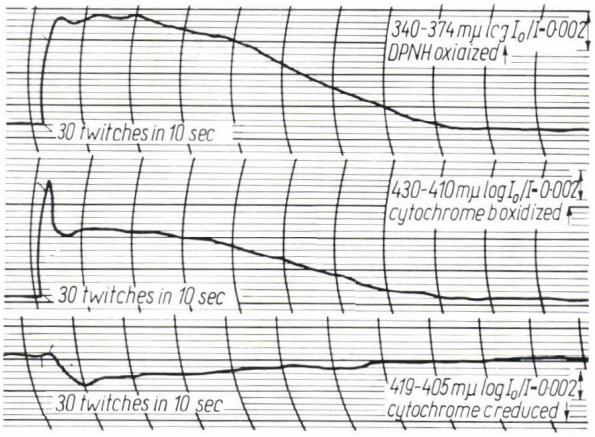

FIG. 10.—Spectrophotometric measurements of the kinetics of cytochromes *b*, *c* and of NADH (DPNH) in response to a series of twitches. Toad sartorius at 23° C, after aging treatment to inhibit glycolysis (Jöbsis 1963)

It appears, therefore, that inorganic phosphate is released early in the time course of the twitch but later than the onset of the transient Ca^{++} changes. The significance of this will be discussed at the end of the next section.

TIME COURSE OF ADP LIBERATION

As a further exploration of the time sequence of the intracellular events, the data on the increase in ADP concentration can be quoted. For this purpose I used the reaction of the respiratory chain contained in the mitochondria within the intact muscle. Chance and Williams (1955) showed that the steady-state percentage of reduction in several members of the respiratory chain of isolated mitochondria changes when ADP is added and oxidative phosphorylation is initiated. These changes can be measured spectrophotometrically since the oxidized and reduced forms of the chain components differ in light absorption characteristics. Thus these optical changes can be used as a signal for the arrival of ADP at the mitochondria during a contraction since ADP triggers oxidative phosphorylation, i.e. oxidative recovery metabolism. To this end

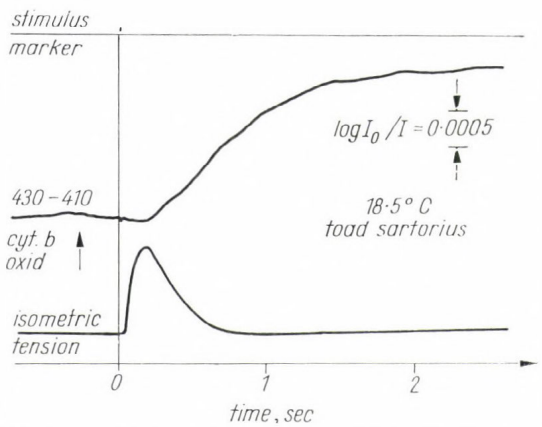

FIG. 11.—Onset of the spectrophotometrically measured cytochrome *b* in response to a single, isometric twitch. Toad sartorius, 18·5° C (Jöbsis 1959)

beams of a measuring and reference wavelengths are transmitted alternately through the muscle by a double-beam spectrophotometer. The difference between light absorptions at an absorption maximum of one of the respiratory chain components and at the reference wavelength is measured and recorded as a function of time. In Fig. 10 the cyclic responses of three respiratory chain components are shown as they result from a series of twitches (Jöbsis 1963). The cycles show the intensity and the time course of oxidative recovery metabolism which ends when all ADP has been converted back to ATP. (For a review of this technique see Jöbsis 1964.)

Analysis of the onset of the optical density changes is shown in Fig. 11, in which the response of cytochrome *b* was measured in correlation with the development of twitch-tension (Jöbsis 1959). Not in all cases was the correlation exactly as illustrated in Fig. 11, but generally the first appearance of ADP was signalled near the peak of the contraction.

From the response delay of the cytochrome *b* signal it is concluded that ADP is released at a later step in the reaction sequence than the inorganic phosphate. This suggests the following type of reaction scheme:

$$\begin{aligned} &A + M + \underset{\underset{Ca^{++}}{\uparrow\downarrow}}{Ca^{++}} \rightleftharpoons AMCa + ATP \rightleftharpoons AMCa\,ADP + P_i \rightleftharpoons A + M + \\ &+ Ca^{++} + ADP \end{aligned}$$

The cleavage of the final complex would be the end of the reaction cycle of the contractile system.

A KINETIC MODEL OF MUSCULAR CONTRACTION

The reaction scheme constructed above does not constitute a system in which the relevant biochemical data are strictly accounted for. Instead it is the simplest scheme that can be derived from these physiological measurements against a back-ground of known biochemistry. From the work of many investigators, specifically for instance the beautiful results reported here by Prof. Ebashi, it is clear that the model must be an oversimplification. Nevertheless, it would be illustrative to represent this system by the analogue computer simulation. The scheme developed above was represented as follows:

$$\begin{aligned} &A + \underset{\underset{(C)}{\uparrow\downarrow}}{C} \rightleftharpoons AC + ATP \rightleftharpoons AC\,ADP + P_i \rightleftharpoons A + C + ADP \qquad (A) \end{aligned}$$

in which actin and myosin have been used as a single entity, since from morphological data it appears that the two are not randomly distributed but are in restrained and close apposition. A representation according to mass law kinetics appears therefore not necessary and the two proteins have been lumped together as one entity: A. In the scheme C stands for an activator, i.e. Ca^{++}. The remainder of the analogue computer program is equivalent to the chemical scheme developed above. Again it is considered that ATP reacts as a reagent with the actomyosin-Ca^{++} (AC). ATP is regarded as being present in excess, methodologically this is equal to

incorporating it in the rate constant or as a moiety absorbed to A in the resting condition.

In Fig. 12 computer solutions are shown for the changes in concentration as a function of time after the stimulus. The curves are labelled according to scheme A. The additional curve, labelled P, represents the change in the permeability of the membranes of the endoplasmic reticulum. At the risk of gross oversimplification, it may be conjectured that during the action potential a number of 'holes' is created in the membranes of the reticulum and Ca^{++} diffuses into the sarcoplasm proper. Subsequently, these holes are repaired with first order reaction kinetics. The number of holes existing at any one moment would be indicated by the height of the P curve above the baseline.

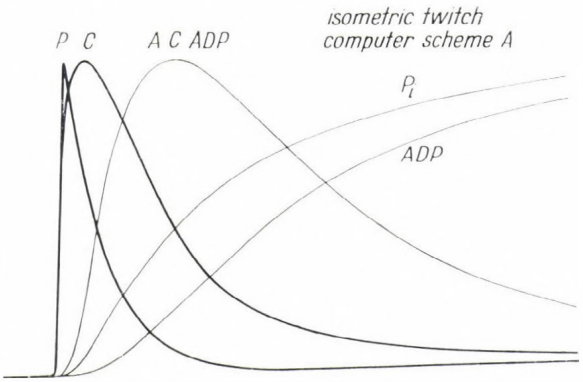

FIG. 12.—Analogue stimulation of simplified muscle contraction scheme (A). The first response to the single 'stimulus' is an initial increase in permeability. Symbols: P = permeability, C = Ca^{++}, A = actin + myosin, P_i = inorganic phosphate, ADP = adenosine di-phosphate. Abscissa = time, ordinate = concentration; both in arbitrary units. The intermediate AC ADP is considered analogous to the isometric twitch tension

It is the contention of the present approach that the isometric force of the myogram is directly proportional in time course and intensity to the transient concentration response of one of the intermediates in this scheme, specifically the AC ADP intermediate. The usual features of an isometric twitch are well represented by the AC ADP curve including a lag period, reminiscent of the latent period, an initially increasing rate of tension development and finally a more protracted fall of tension in the relaxation period. Although the latent period has generally been thought to represent the time required for the inward travel of excitation and for the diffusion of the activator to its site of action, no special provisions for such events have been made in this first simple model. Inclusion of these could easily allow for an increased duration of the latent period.

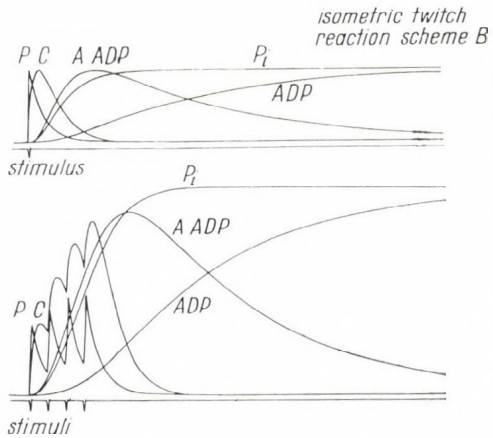

FIG. 13.—Analogue stimulations of a single twitch and a 4-stimulus tetanus with simplified reaction scheme (B). The intermediate A ADP is analogous to the isometric tension; otherwise as Fig. 12

A feature that does not agree too well with the present data is the relative time course of the intermediate AC ADP and of the disappearance of Ca^{++}. The re-absorption of Ca^{++} appears to be completed at approximately the time of the tension peak (Fig. 7). When the relative time courses of the 'contraction phase' and the 'relaxation phase' of the AC ADP complex are made to approach those of the actual twitch, it is practically impossible to achieve these time relations with this first model. Qualitatively this is understandable since in scheme A, Ca is an integral part of the tension producing intermediate AC ADP and upon the cleavage of this complex Ca^{++} is again liberated in the sarcoplasm. It is, therefore, of some interest to investigate the sort of change in the model that would generate time relations more easily matched to those of the Ca^{++} kinetics. As a first attempt I have assigned tension production to an intermediate which does not contain Ca^{++}. Such a scheme could be presented as follows:

$$\begin{array}{l} A + C \rightleftharpoons AC + ATP \rightleftharpoons A\,ADP + C + P_i \rightleftharpoons A + ADP \\ \quad \Updownarrow \\ \quad (C) \end{array} \qquad (B)$$

A re-interpretation of these data is shown for a single stimulus in the top of Fig. 13 and for a 4-stimulus tetanus in the bottom. In the twitch the tension intermediate (A ADP) reaches its maximum at the moment when the free Ca^{++} concentration (C) has virtually dropped to zero. The solution of the C transient is now almost identical with the time courses of the free Ca^{++} measurements (Figs 6 and 7) and with Brown's α-process (Fig. 13). Some difference is also evident in the release of P_i and ADP. The time courses now resemble the measurements of the change in pH and in the redox state of cytochrome *b*. Thus scheme B appears to be more in agreement with the experimental data than scheme A. It is, nevertheless, a bit early to make a final choice. More data on the Ca^{++} transients and more exhaustive studies of the analogue model are required. However, the above attempts at an interpretation of the data do not fail to illustrate the striking advantages of the kinetic model and should spur us on toward more serious consideration of this approach.

ENERGY UTILIZATION IN TWITCHES AND SHORT TETANI

From the viewpoint of muscle contraction as a system of sequential reactions, the question of the total turnover in the system leads to a new track both experimentally and in the model. Casting it in the form of a question on energetics one can ask: what is the relation of the total amount of ADP split to the parameters of the isometric contraction? As a first approach it was decided to study this question in the transition region between twitch and tetanus. To this end varying numbers of stimuli were delivered to the muscle at a tetanic rate and the total amount of the oxidative recovery metabolism was measured for each contraction. Fluorometric observations on mitochondrial NADH (DPNH) within the muscle tissue were utilized. In the model the same variable was manipulated and the final height of the ADP curve was used as the index to energy consumption.

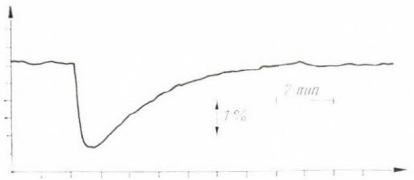

FIG. 14.—Fluorometrically monitored response of mitochondrial NADH (DPNH) to a single isometric twitch. The downward deflection denotes a decrease in fluorescence, i.e. a decrease in NADH (Jöbsis 1966)

It is possible to measure fluorometrically the fraction of mitochondrial NAD, that is in the reduced state, since this form (NADH) fluoresces under illumination with light around 340 mμ, whereas the oxidized form (NAD^+) does not. Thus the intensity of this fluorescence can serve as an index to the degree of reduction of mitochondrial NAD. Since the degree of reduction decreases when O_2 uptake increases, a loss of fluorescence is registered when oxidative recovery metabolism is taking place. It so happens that the NADH in the mitochondria suffers far less from fluorescence quenching than the glycolytic fraction, which is freely dissolved in the cytoplasm. For this reason respiratory chain NADH can be measured fluorometrically without interference from glycolytic NADH. This is not possible with the spectrophotometric technique. It is necessary to resort to special treatment of the muscle to produce results such as shown in Fig. 10 (Jöbsis 1963), whereas with the fluorometric method oxidative recovery metabolism can be measured routinely in untreated specimens (Jöbsis and Duffield 1967).

In Fig. 14 a fluorescence trace is presented as was obtained from a toad sartorius muscle with a fluorometer constructed for the purpose (Jöbsis et al. 1966). At the beginning of the trace the muscle was in a resting metabolic condition. A single twitch produced the decrease in fluorescence intensity shown by the cycle which indicates the time course and intensity of oxidative phosphorylation. (Note that the time course of the recovery metabolism is measured in minutes, whereas the duration of the twitch is barely one second).

A good amount of effort has been expended in studying the parameters of the optical signals of oxidative recovery metabolism. In the beginnings the peak height of the responses was used for correlation with the amount of activity. A severe drawback of this parameter is the fact that increasing numbers of twitches yield a saturation type standard curve. Although still usable, this is rather inconvenient and becomes increasingly less sensitive with larger amounts of activity. At a Gordon Conference, Professor Fenn suggested to me that the area under the response cycle might be a more convenient parameter and indeed it was found to be so, for the response cycles of cytochrome *c*. With the present instrumentation the time integral of the fluorescence cycle ($\int \Delta Fl$) is also directly related to the amount of energy spent by the preparation. An illustrative example is shown in the top left of Fig. 15 for the effect of one to three single twitches delivered at a rapid rate. Thus Prof. Fenn's parameter has proved very useful indeed. It can also be noted in Fig. 15 that the fluorometric response is stable in the presence of iodoacetic acid. This last fact is of great importance for the quantitative correlation of $\int \Delta Fl$ with the contractile effort, since it provides a check on possible interference stemming from the glycolytic rephosphorylation of ADP. Furthermore, it is seen that a large difference exists in the energy utilization of isometric and minimally loaded isotonic

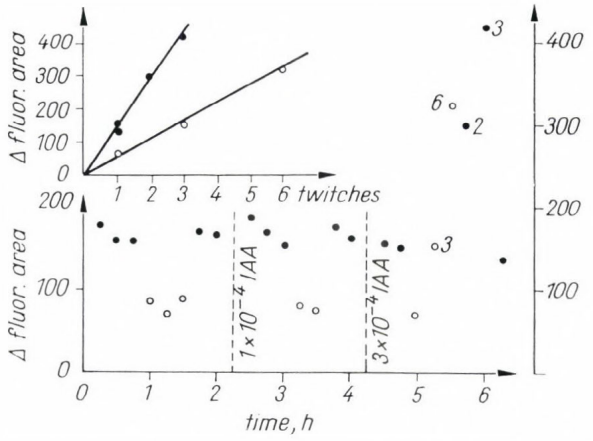

FIG. 15.—An experiment demonstrating the variability in the area under the fluorometric response curve as a function of time. Symbols: ○ minimally loaded isotonic twitches; ● isometric twitches. About 5 hours after the start of the experiment several series of multiple twitches were elicited. The results of the latter are shown graphically in the inset

contractions. I would like to discuss this point in tomorrow's session.

In a sequential system of reactions the initiation of the reaction by the addition of a limited amount of primary reactant results in the transient rise in concentration and subsequent relaxation of each of the intermediates. Intuitively, it does not sound unlikely that the area under such a curve is directly related to the total amount of reaction taking place. In fact it is possible to show mathematically that this is the case for a sequence of first order reactions, i.e. the area under the curve varies linearly with the added amount of primary reactant. In the case of the respiratory chain, the fall in NADH and its subsequent return to the resting base-line is quite clearly analogous to such a transient: ADP is the added reactant, NAD^+ the transient intermediate which is signalled as a loss of fluorescence. It is another matter, however, to prove the linearity of the area under the cycle (i.e. its time integral) with the total turnover in the system on a formal, mathematical basis or even by an analogue or digital computation. The respiratory chain, although a system of remarkable beauty, is not noted for its simplicity. Experimentally, however, the same type of relation can be shown to exist for the response of isolated mitochondria to the addition of different quantities of ADP. Thus the $\int \Delta Fl$ can be used as a direct parameter for the analysis of the total energy expenditure, which gave rise to the recovery metabolism. Competing reactions such as glycolysis have to be excluded. For the range of 1–4 isometric twitches glycolysis does not encroach noticeably upon oxidative metabolic recovery, but for higher amounts of activity glycolysis must be inhibited, for instance by iodoacetic acid (Jöbsis and Duffield 1967).

As a first attempt, the total amount of contractile effort was studied in the transition region between the twitch and the tetanus. To this end,

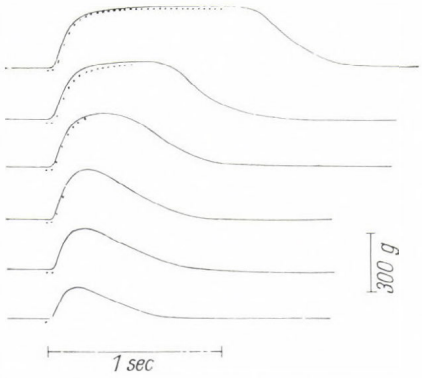

FIG. 16.—Mechanical aspects of the transition region between isometric twitch and tetanus. The dots beneath each myogram mark the moment of stimulation. Toad sartorius, 15° C

2, 3 or more stimuli were delivered at a tetanic rate to the muscle which responded with increasingly large twitch-like responses until a typically flat-topped, tetanic contraction was reached (Fig. 16). This type of experimentation was performed while monitoring the fluorometric response that followed each contraction. The results of one such an experiment are given in Fig. 17. The top graph shows the peak isometric force (P_0) and the time integral of the tension ($\int P_0$) as a function of the number of stimuli delivered at 32 cps. The latter amounts to the area under such curves as shown in Fig. 16. In the bottom graph the relation between the $\int P_0$ and the time integral of the fluorescence cycles ($\int \Delta Fl$) are shown. The correlation is highly significant and shows that in this transition region the energy utilization is directly related to the time integral of the isometric force (Jöbsis 1966).

Another interesting feature of these results is the fact that little room is left for a contribution from the activation process when energy utilization is correlated with the P_0. This amount of energy is generally thought to be a fixed quantum needed to produce a transition from the resting condition to the active state. The experimental spread in the data may conceal a small energy expenditure for this process but it cannot be more than about 10 per cent of that spent in the single isometric twitch. This is very low compared with the present day estimates of 33 to 50 or even 80 per cent from myothermic data and from a direct analysis of the various $\sim P$ stores of exercised and resting muscles. It appears, however, in line with certain ones of the estimates of Infante and co-workers

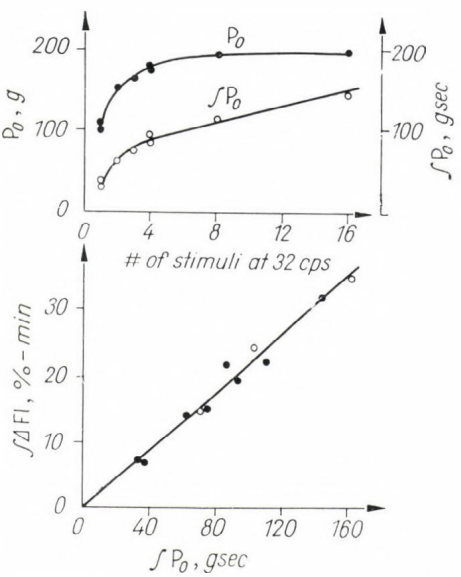

FIG. 17.—Effect of varying numbers of stimuli at a tetanic rate on mechanical and energetic parameters of isometric contractions. Top: the isometric force (P_0) and its time integral ($\int P_0$) as a function of the number of stimuli. Bottom: the time integral of the fluorescence cycle ($\int \Delta Fl$) as a function of the $\int P_0$. Symbols: ● the same contractions as shown in the top graph, o cumulative effect of 2, 3 and 4 single twitches in rapid succession, 1 cps (Jöbsis 1966)

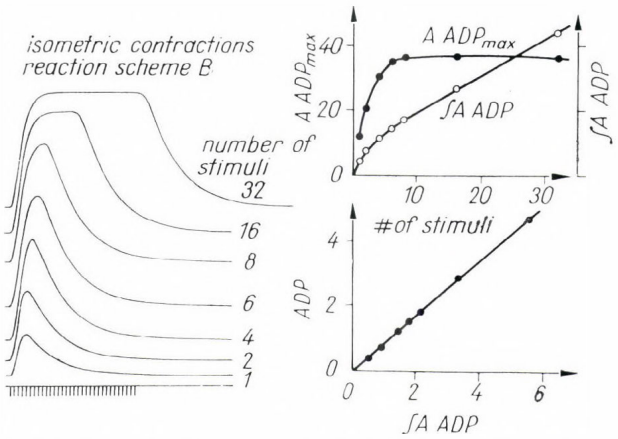

FIG. 18.—Computer simulation of the experiments of Figs 16 and 17 on the transitions between twitch and tetanus. The curves on the left show the effect of varying numbers of stimuli at a tetanic rate on the tension intermediate A ADP. The graphs on the top right show the peak of the A ADP curves and the areas under the curves as a function of the number of stimuli. The graph on the lower right shows the correlation between the area under the curves and the total energy turnover as measured by the height of the ADP curves (the latter are not shown in this figure)

(1964). The question of the activation energy is discussed more fully elsewhere (Jöbsis 1966).

The linearity of the energy expenditure with the $\int P_0$ is not unexpected if a system of sequential reactions is used as a model for muscle contraction. If it be considered that the tension is directly related in its time course and intensity to the time course and concentration of an intermediate in a sequence of reactions, then it is only to be expected that the total area under the mechanical response is directly related to the total amount of energy turnover that takes place. In other words, the same reasoning that applied to the NADH fluorescence as an index to the total response of the respiratory chain should also apply to the sequence of events giving rise to the mechanical performance of the striated muscle. This notion has been tested with reaction scheme B in a simulation of the above experiment. In the left hand side of Fig. 18 the A ADP intermediate is shown as is affected by the number of stimuli. In the right hand top graph the peak concentration of A ADP and the area under the curves are depicted as a function of the number of stimuli. Below is shown the correlation between the $\int$ A ADP and the total ADP produced in the reaction. It is interesting to note that all the curves strongly resemble the ones from the muscle experiment shown in Figs 16 and 17. Perhaps some small measure of credibility is thereby given to my very simple reaction scheme. What is more definitely established, however, is the fact that the time integral of an intermediate is linearly related to the total turnover of reactants in the transition region between twitch and tetanus.

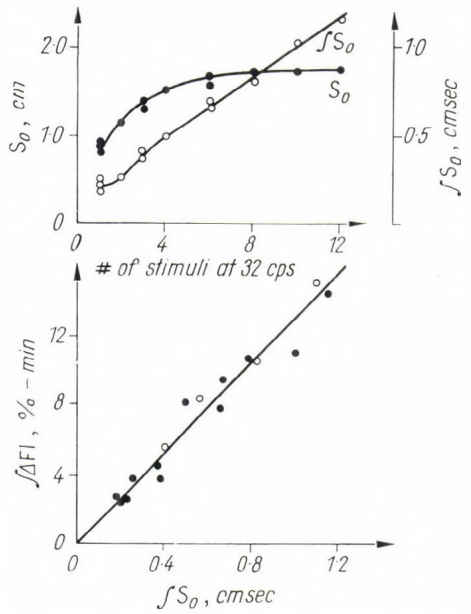

FIG. 19.—Effect of varying numbers of stimuli at a tetanic rate on the mechanical and energetic parameters of minimally loaded isotonic contractions. Top: the shortening distance (S_0, filled circles) and its time integral ($\int S_0$, open circles) as a function of the number of stimuli. Bottom: the $\int \Delta Fl$ as a function of the $\int S_0$. Filled circles: the same contractions as depicted in the top graph. Open circles: cumulative effect of 2, 3, 4 and 6 single twitches in rapid succession (Jöbsis 1966)

The same type of experimentation was also performed under minimally loaded isotonic conditions. The results of such an experiment are shown in Fig. 19. In the upper graph the mechanical parameters of the shortening distance (S) and the integral of the shortening curve ($\int S$) are presented as a function of the number of stimuli for the transition region between twitch and tetanus. The results of the concomitantly monitored fluorescence cycles provide a measure of the energy expended which, as shown in the bottom part of the figure, is again linear with the time integral — this time $\int S$ (Jöbsis 1966). In these experiments there is also a lack of evidence for a significant contribution of the activation process to the total energy expenditure.

In order to accommodate shortening as a separate function in muscle contraction it is merely necessary to specify that the tension intermediate A ADP can be used either for tension production or for shortening. This rather uncomplicated addition to the reaction scheme has not yet been tested on the analogue computer; it will be done in the near future.

A LIMIT TO THE APPLICABILITY OF THE KINETIC MODEL

The above experiments supply evidence that an intermediate of the reaction sequence directly underlies the mechanical phenomena observed in contracting striated muscle. It is necessary, however, to indicate a strict limit to this approach. When a muscle is tetanically stimulated for longer times than studied above, the noted linearity between the $\int P_0$ and the energy utilization as measured by the $\int \Delta Fl$ shows a sudden change in slope. In Fig. 20 the results of such an experiment are shown. In this case the muscle was stimulated for periods up to 3 seconds at a rate of 48 times per second. The early correlation, in the transition region between twitch and tetanus, is again a straight line through the origin, but the later part of the experiment does not follow this relation. In the computer model this behavior between $\int$ A ADP and total amount of ADP produced does not show the diphasic behavior. It is, therefore, postulated

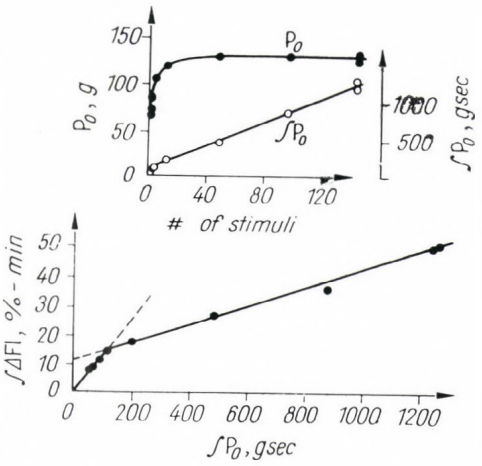

FIG. 20. Mechanics and energetics of longer lasting isometric tetani. Toad sartorii at 12° C. Tetanic stimulation at 48 cps; otherwise as Fig. 17

that a change in the reaction mechanism occurs. No specific data are available to indicate the type of molecular mechanism that is involved. The observations can, however, be described as the onset of a mechanically 'locked' condition or, in the terms of the concepts of Prof. Ernst (1963), it might be an indication that a 'crystallization' of the contractile proteins takes place. In both expressions it is implied that a new condition has occurred in which the muscle does no longer maintain static tension by continued splitting of ATP at the original rate. A mechanical arrangement has apparently been reached which depends on a much smaller continued input of power. Supposedly this lesser rate is related to the maintenance of the static condition. This phase of the work is under investigation at the moment. It appears from preliminary experiments that such a locking or crystallization does not take place when the muscle is shortening. A permanent or at least semi-permanent attachment of the actin and myosin molecules either at the cross bridges or in a differently arranged crystalline structure might underlie such a static tetanic condition.

In conclusion I would like to stress several points concerning the experiments and the kinetic model.

The calcium data have been obtained only recently and, necessarily, they suffer from a certain incompleteness and lack of extensive number of repetitive observations. This will be remedied by more work in the near future. However, the main challenge in this work is to perform some quantitative measurements on the amount of Ca^{++} released. This will require considerable modification of the instrumentation which will take some time. But such improvements should also benefit the pH and cytochrome *b* measurements.

One of the dangers of building the kinetic model so closely to a specific interpretation of the experimental data is the near certainty that this interpretation will be modified in the near future. This brings with it the great danger that either the model or the experimental results (and probably both) will be dismissed peremptorily when the biochemical interpretation is found to be in error. However, the experimental data should stand on their own and in fact they can do so quite well. The kinetic model is also independent of biochemical interpretations although perhaps not quite so obviously so. Its main importance is the illustration of the general concept that such a scheme can account satisfactorily for both the shape of the mechanical response and the total amount of the reaction as measured by energetic parameters. An awareness of this in the minds of the members

of this distinguished audience is all that is requested as the outcome of this presentation.

Now it is my most pleasant duty to acknowledge the valuable collaboration of James Duffield and Michael O'Connor in many aspects of the work reported here. I also owe a great debt of gratitude to Mrs Fieke Bryson and Mrs Margaret Grafton for dedicated technical assistance far beyond the call of duty. I am grateful to Dr E. A. Johnson for introducing me to the analogue computer and to Dr J. W. Moore for taking my education further in hand.

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EBASHI: First, I would like to congratulate you for the success in demonstrating the release of Ca-ions during the contraction of living muscle. If I compare the level of Ca-ions with that of contraction, which may be represented by the turbidity, and take the rate of Ca accumulation of the sarcoplasmic reticulum into consideration, I do not think that the sarcoplasmic reticulum is solely responsible for the disappearence of Ca-ions at that stage. In this respect I agree with you. The other systems including the contractile elements might also be responsible for the uptake of Ca-ions at an earlier stage.

JÖBSIS: Thank you very much indeed for your very kind congratulations, Dr Ebashi. Coming from you, any praise of an experiment concerning Ca measurements is very high praise indeed. About the rapid reabsorption of the Ca^{++}, I can only agree with you that it is surprising, so surprising in fact that I felt it necessary to change my way of thinking and, therefore, the kinetic model of muscle contraction. This new model provides a good explanation and a perfect fit to other data, whereas the old one did not.

(*Note added after the conference.* — Further experimentation and computation has made it clear that the above statements may be a bit too categorical. With some difficulty it is possible to pick a set of reaction constants for scheme A that will generate a Ca^{++} curve showing a rapid decrease followed by a long 'tail'. However, this is accompanied by a definite loss of fit between the shape of the isometric twitch and of the tension intermediate. This set of constants is, therefore, not correct, but the experience does suggest some caution in drawing conclusions from the model. In addition, new Ca^{++} experiments have made it clear that the latter part of the contraction is most sensitive to any inadequacies of the subtraction method. The exact shape of the trace is, therefore, the least reproducible in the relaxation phase of the twitch. A realization of these points makes it clear that further experimentation is needed to be performed, preferably with an improved instrumentation. This is being constructed at the moment.)

EBASHI: I should like to point out that a large amount of Ca would be sequestered in the Ca—actomyosin complex and that, consequently, the level in the sarcoplasm could show a rapid fall without much reabsorption by the reticular system. I should emphasize the last point, since I have some reason to think that pumping of Ca was delayed for some time after the stimulus.

JÖBSIS: I completely agree with your first point. In fact this state of affairs is incorporated both in scheme A and scheme B, in which free Ca^{++} is decreased by two reactions, one resulting in the Ca–actomyosin complex and the other being the reabsorption by the reticulum. The relative rate at which free Ca^{++} is first decreased depends on the relative reaction rates of these two processes. I have no evidence for or against the idea that the pumping rate is inhibited for some time after the stimulus. It is, however, possible to match the Ca-kinetics without recourse to such a delay, but then again it might also be possible to do so with a delay. I have never tried it.

HUXLEY: I would like some explanation of the lag period between tension development and ADP release.

JÖBSIS: Lags are part of any kinetic scheme. The further down the sequence of reactions we are, the longer the lag. In the experimental data we see ADP signalled by cytochrome *b* around the time of the tension peak. In the model the rapid increase in ADP is seen a bit earlier than that. I am not sure what underlies this difference. It is not terribly big and *some* time is needed for diffusion to and into the mitochondria, but that can account for only a small delay. Nevertheless, the point is that from any model in which ADP is part of the tension intermediate we would expect a lag period before we see a rise in the ADP concentration.

STRICKHOLM: I wish to ask Dr Ebashi if his experiments were on endoplasmic reticulum from the same muscle and animal as used by Dr Jöbsis. There are known variations in muscle types and functions and perhaps it might be that the endoplasmic reticulum has different properties in different muscles.

EBASHI: I have no experience with the sarcoplasmic reticulum of the toad. However, no essential difference has been found between those of the rabbit and the frog.

TIGYI: What, do you think, is happening in hypotonic solutions as regards the Ca change and the shortening?

JÖBSIS: We have studied neither hypertonic nor hypotonic solutions for their effects on Ca release. I realize that this is a very important question, especially the hypertonic situation, since it has been conjectured that Ca^{++} release is normal, whereas the actin–myosin interaction may be inhibited. Naturally, I do want to have a try at this type of experiment in the near future.

TIGYI: How, do you think, the measurements on pH changes reported by Dubuisson and those presented by you in the opening lecture coincide?

JÖBSIS: The present pH measurements and those of Dr Dubuisson and Dr Distèche are very closely comparable indeed. The only difference is some extra time resolution and the application to the toad sartorius muscle. The later phases of the pH changes were not recorded by me in these experiments. They are, however, reported in my 1963 paper and very closely comparable to the measurements made with pH electrodes.

KÖVÉR: Could you say anything about the events occurring during the effect of caffeine?

JÖBSIS: No, I have not had a chance to take a look at the effect of caffeine, but I fully agree that this would be a very important experiment to perform, since increased intracellular Ca^{++} levels are as often implied as the mode of action of caffeine.

HASSELBACH: What are your ideas concerning the compartmentation of calcium in relation to your observations?

Can you exclude that the colour change in Ca murexide is caused by the pH change due to contraction?

JÖBSIS: There is little evidence to be extracted from my data concerning Ca compartmentation in muscle. I think that I can eliminate the extracellular space and the intrasarcotubular space, since in both places the Ca concentration should be high enough to saturate the murexide. This leaves the sarcoplasm proper as the only candidate of a compartment from which the signal could arise.

I think that the difference in kinetics of Ca^{++} and H^{+}-ion release is

good evidence that pH changes are not responsible for the optical changes at 540 and 470 mμ. Although the light absorption by Ca-murexide is susceptible to pH it is not a terribly large effect for the very small pH changes with which we deal here.

BENDALL: Could the apparent reabsorption of calcium during the rising phase of a muscle twitch be explained by its temporary chelation by ATP and the contractile proteins?

Whether this is possible would depend on the relative binding constants of ATP and, actomyosin, on the one hand, and murexide, on the other. Do you know the calcium-binding constant of murexide?

JÖBSIS: Yes, I definitely think that the major factor in the rapid decrease in free Ca^{++} during the contraction phase is due to the utilization of Ca^{++} in the reactions of the contractile proteins. Even some chelation by ATP might be possible. I hesitate to quote the binding constant of calcium with murexide since I believe that some errors may have been made in its determination. We are now re-measuring this constant. It is, however, quite clear that the affinity is not terribly great at physiological pH (pH 6·8). This is an advantage since the murexide will not chelate the Ca^{++} away from the contractile proteins and the sarco-tubular system. It is also a disadvantage since only a small proportion of the murexide will be able to bind any Ca. This results in a relatively small signal. In a few months I hope to be able to give a more exact description of the system.

HANSON: Can you compare the time course of the active state with that of Ca^{++} release?

JÖBSIS: I would be very hard pressed to answer that question. I have never quite understood the concept, and human nature being what it is, I just do not believe in it. However, Dr Edman has a contribution to make concerning the active state and I would, therefore, like to defer to him.

EDMAN (Department of Pharmacology, University of Lund, Sweden): Little is known up to the present time, as to whether or not the *time course* of the mechanical activity in response to excitation of the cell is dependent on the degree of extension of the sarcomeres. The degree of activity of the contractile unit, the 'active state' according to Hill's terminology, has been experimentally defined in this study* as *the capacity to produce tension*. With the approach used it has been possible to determine almost the entire time course of the active state, even a substantial part of the rising phase, in a single cell at the sarcomere level.

The fibre was mounted horizontally between a tension transducer and a light isotonic lever in a thermostated (2–3° C) perspex trough (Edman 1966). All mechanical recordings were carried out at selected sarcomere spacings determined for the middle segment of the fibre. The sarcomere length was measured at rest for various degrees of extension of the fibre.

The fibre was paced to contract at 1 1/2 minute intervals. A series of four stimulating pulses (frequency: 3–6 cps) produced a stepwise increase in the tension almost to the tetanic level. In the fourth cycle a quick release was produced, which allowed the sarcomeres to shorten by 0·1 μ. Figure 21 illustrates a number of releases carried out at different times after the

* In collaboration with Dr A. Kiessling whose present address is: Department of Physiology, Karl-Marx-University, Leipzig

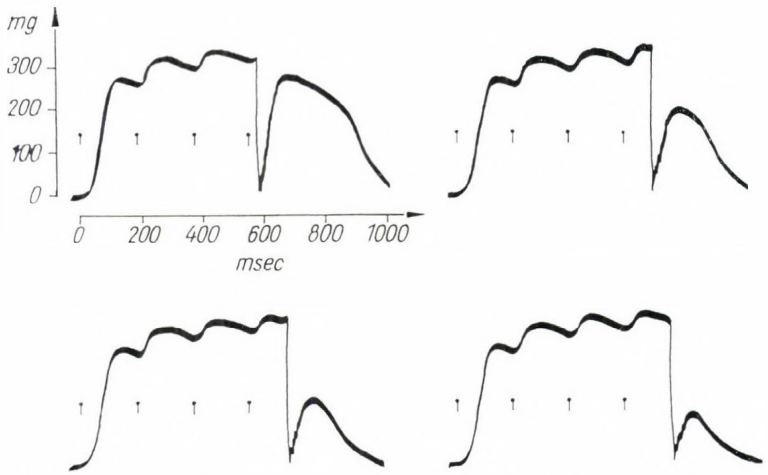

FIG. 21.—Vertical bars: stimulation signals. Fibre cross-section: 90×200 μ^2

fourth stimulus at 2·15 μ sarcomere spacing. There was an abrupt fall in tension, followed by a recovery of tension at the 0·1 μ shorter sarcomere length (2·05 μ). The isometric output at the peak of the redeveloped contraction, i.e. at a time when the contractile system was stationary, represents the capacity of the contractile unit to produce tension at that moment after the stimulus (cf. Ritchie 1954). The tension values obtained in this way provide points on the decay phase of the active state curve.

The approach used for determining the rising phase of the active state curve is shown in Fig. 22, illustrated in a series of four partially sum-

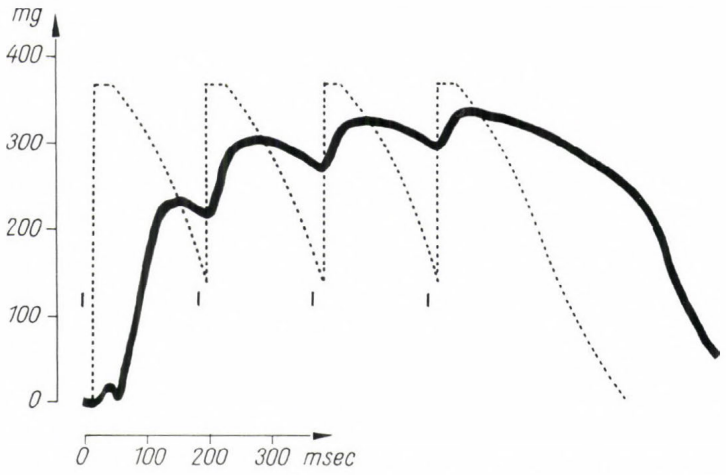

FIG. 22.—Same fibre as in Fig. 21. Vertical bars: stimulation signals

mated twitches produced by the same fibre as in Fig. 21 at 2·05 μ sarcomere spacing. By the onset of each new activity period in the series the tension was reversed from decay to rise with the formation of a distinct trough in the myogram. The trough occurred when the contractile unit was neither lengthening nor shortening; it, therefore, represents the capacity to produce tension at that moment after the latest stimulus. The dotted lines in Fig. 22 denote the individual active state curves in the series and have been included to illustrate that the troughs in the myogram are related to the rising phase of the active state curve. By plotting the trough tensions with the preceding stimulus as a common origin, the rising phase of the active state can be defined.

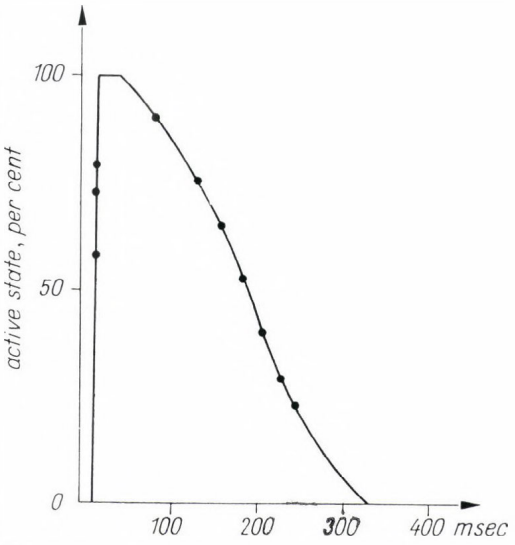

FIG. 23.—Ordinate: intensity of the active state, percentage of maximum (tetanic tension). Abscissa: time after stimulus, msec

Figure 23 shows the active state curve in an isolated fibre at 2° C and a sarcomere spacing of 2·05 μ. The experimental values on the rising phase of the curve refer to the troughs in Fig. 22. The decay phase is based on the quick-release measurements described in Fig. 21. The plateau represents the tetanic output at the sarcomere length considered. The curve illustrates the general features of the active state curve as observed in these preparations. It is of particular interest to note the very rapid rise in the mechanical activity. Full intensity is achieved within a few (4–5) msec after the onset of the activity. A much longer time, on the other hand, is taken up by the decay phase. In the example illustrated in Fig. 23 about 200 msec

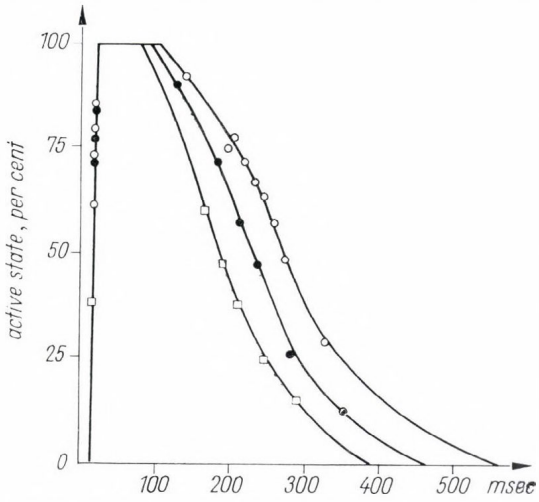

FIG. 24 .—Ordinate: see Fig. 23; abscissa: time after stimulus, msec

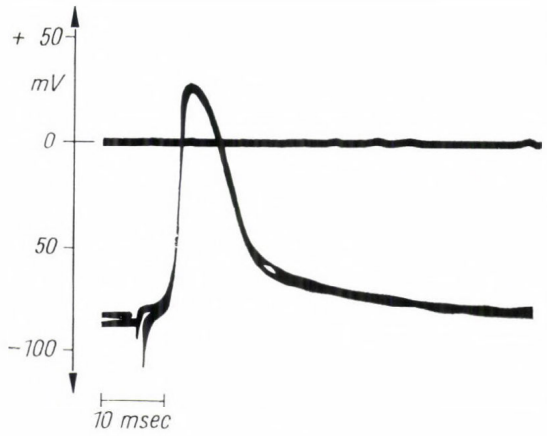

FIG. 25

were needed for the active state to decline from 90 to 10 per cent of maximum activity. As has been demonstrated previously (Edman et al. 1966), the time of onset of the decay as also the steepness of the decay phase, at a given temperature and sarcomer length, vary considerably from fibre to fibre.

Figure 24 illustrates the active state curve defined at three different sarcomere lengths (2·42, 2·04 and 1·70 μ) in the same fibre at 2° C. In determining the decay phase (see above) the same amount of release (0·1 μ/sarcomere) was used in all cases. The maximum activity, i.e. the tetanic output, has been expressed as 100 per cent at all lengths investigated. There was no detectable change in the rising phase of the active state curve by altering the degree of extension of the fibre. The duration of the activity, however, was progressively decreased by a reduction in the sarcomere spacing due to an earlier onset of the decay phase of the active state at the shorter lengths. In six complete experiments performed the duration of the active state at 1·9 μ sarcomere length was 48–84 (mean: 71) per cent of the active state duration existing at 2·6 μ, as measured at 50 per cent of maximum activity.

Previous experiments on the frog's semitendinosus fibre (Edman and Grieve 1966, Edman et al. 1966) would seem to indicate that the duration of the active state, *at a given sarcomere spacing*, is quantitatively related to the duration of the action potential. It was, therefore, of interest to find out whether or not the action potential was affected by altering the degree of extension of the fibre. In order to test this point, intracellular recordings of the action potential were performed at various sarcomere lengths in the same fibre using a standard glass electrode, or in some experiments for measurements below slack length (2·3–1·8 μ), by means of a movable, Brady–Woodbury type of glass electrode. Shifting from one length to another within the range 2·9–1·8 μ did not have any significant effect on the action potential. Figure 25 shows two superimposed action potentials recorded at 2·9 μ and 2·35 μ sarcomere spacings. As can be seen, the two potentials are almost identical.

The results have shown that the degree of extension of the cell *is* a determinant of the time course of the active state in the skeletal muscle fibre. Little is known at the present time, however, as to the mechanism by which the length dependence of the mechanical activity is achieved. It is clear that the effect on the active state course produced by a length change in the fibre is not to be attributed to a change in the duration of the action potential. The degree of extension of the cell, thus, probably controls a more intimate step in the excitation–contraction process. There is strong

support from previous experiments (Edman et al. 1966) for the idea that the time course of the active state is governed by an activator agent released into the intracellular compartment at the depolarization of the plasma membrane. On this basis, the present findings suggest that the rate of metabolism of the activator, e.g. the speed by which it is inactivated, or eliminated from the myofibrillar space, is dependent on the length state of the sarcomeres.

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JÖBSIS (Department of Physiology, Duke University Medical Center, Durham): Thank you, Dr Edman, for the very clear presentation of these interesting data. I must confess that I was perhaps a bit arch in my first response to Dr Hanson's question and would like to make amends quickly before we discuss Dr Edman's contribution.

If we do want to consider the active state the following remarks may be relevant. (i) The active state may correspond in activity and in time course to the tension intermediate, but becomes manifest through a long lag period created by a large series elastic component. However, I see little evidence of this. (ii) The activity and time course of some other intermediate such as AMCa (or AC in the model) may correspond to that of the active state. This appears much more likely. The cross bridges have formed, but the deformation reaction that generates tension still needs to take place. That reaction has its own time course and this may account for the relative sluggishness of the tension development. The series elastic component may also contribute but does not need to be the main reason for this sluggishness. (iii) The active state in the toad sartorius is probably not maximum after a single stimulus. There are several reasons for thinking so, but this is not the time to go into them. The result would be that the active state curve would not be flat-topped but rounded, i.e. it would look very much like any of the reaction intermediates.

In conclusion then I would like to say that it may be possible to illuminate the thinking about the active state by information gained from the kinetic interpretation. However, since I do not feel that the active state concept has been very fruitful, with the exception of Dr Edman's elegant work, I do not believe that I will spend much effort on interpreting its meaning.

BRINLEY: I am impressed by the really elegant mechanical experiments which Dr Edman has performed on single fibers, and my question should not be considered as implying any sort of criticism. It has been shown by Huxley and Peachy (1961) and by Gordon, Huxley and Julian (1966) as well as by others, that the degree of overlap of sarcomeres (and hence presumably the tension development) is non-uniform along the length of a single fiber. I ask, therefore, if the results of single fiber experiments in

which the measured active state tension is a sort of average tension measured over all of the sarcomeres can be used to discuss the behavior of sliding filaments in a single sarcomere.

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EDMAN (Department of Pharmacology, University of Lund, Lund): The sarcomere spacing varies by about ± 2 per cent of the mean value in the middle segment of the fibre, i.e. the portion (approximately 90 per cent of the fibre length) considered in the present study. It is also well known that the sarcomeres at the ends of the fibre (approximately 10 per cent of the fibre length) are shorter than in the rest of the fibre. As was described previously (Edman 1966) there are very uniform changes in the band pattern during contraction. The degree of homogeneity of the sarcomere spacing within the middle segment existing at rest is thus maintained during activity, and there is no yielding of the sarcomeres in the end regions. These findings hold true whether the fibre develops tension at a stretched length ($<3{\cdot}0\ \mu$) or at a shortened state. The almost complete reproducibility of the sarcomere pattern from one contraction to another is evident from *E* and *F* in Plate 1 in Edman (1966). It is a requirement, of course, that the fibre is in a perfect functional condition during these measurements. Any damage to the fibre's function produced, for example, by too frequent or too strong stimulation, is manifested by local yielding.

On the basis of this information we conclude that it is adequate to correlate the tension output of the fibre with the mean sarcomere length of the middle segment of the fibre. The length–tension relation obtained in this way is no doubt valid for interpretation of the mechanical behaviour of the cell at the level of the individual sarcomere. One certainly has to recognize the variability of the sarcomere length that exists, but the error introduced due to this variability would not be greater than a few per cent in the way that we have arranged the measurement.

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Edman, K. A. P. (1966) *J. Physiol.* **183,** 407

It was followed by a discussion about the details of Edman's contribution. Participants in sequence: Wilkie, Ebashi, Edman.

DAVIES (School of Veterinary Medicine, Department of Animal Biology, University of Pennsylvania): This work was done with Dr M. J. Kushmerick and, in part, Mr R. E. Larson, and was on single contractions at 0° C of isolated frog (*Rana pipiens*) sartorius muscles which had been pretreated with 0·38 *m*M 2,4-fluorodinitrobenzene (FDNB) for 40 min at 0° C. This treatment completely inhibits ATP-creatine-phosphotransferase and allows the direct measurement of ATP breakdown in single contractions of muscles. All measurements of ATP breakdown were based on the increase in inorganic phosphate. Experiments have now been carried out in several ways. (i) Muscles were given one supramaximal electrical pulse, allowed to

contract unloaded, then re-extended when the twitch was over and re-stimulated at 1 sec intervals. This was done for 5, 10, 20 or 30 contractions on groups of muscles which were compared with their unstimulated paired controls. The results were all similar and the change was $-0{\cdot}08 \pm 0{\cdot}009$ μM ATP/g/activation. (ii) When the muscles were unloaded and allowed to stay fully shortened after the first pulse, the result was $-0{\cdot}054 \pm$ $\pm 0{\cdot}0075$ μM ATP/g/activation. (iii) In experiments similar to those in (ii), except that the muscles were stimulated at 10 pulses/sec, the result was $-0{\cdot}021 \pm 0{\cdot}010$ μM ATP/g/pulse. (iv) In similar experiments but at 50 pulses/sec the change was $-0{\cdot}0065 \pm 0{\cdot}002$ μM ATP/g/pulse and 25 per cent of this occurred after the last pulse had been given.

After allowance for the differences amongst the various series of experiments, it seems that the best figure for a single complete activation is $-0{\cdot}05 \pm 0{\cdot}01$ μM ATP/g/activation. This is highly significantly different from zero, whereas in our earlier experiments both with frog rectus abdominis and with sartorius we could not distinguish between 0·0 and $-0{\cdot}1$ μM ATP/g/activation (Cain et al. 1962, Infante et al. 1964). If the pulses are given rapidly then the amount per pulse becomes smaller. This all fits with the view that this ATP is required to pump calcium back from the cytoplasm to the sarcoplasmic reticulum and that if the second pulse is given in quick succession then less calcium is liberated as a result of that pulse. However, the rate of ATP usage per unit time remains high so it appears that the calcium pump is continuously active in a muscle that is being continuously stimulated electrically.

Hill (1949) gives the value for the activation heat as 1·2 mcal/g/activation and at a $-\Delta H$ of 10 000 cal/M ATP this would require 0·12 μM ATP, and it is of interest that Dydynska and Wilkie (1966) and Wilkie found that the total initial heat production is similar in normal and FDNB-treated sartorius muscles. Thus activation heat does not come directly from the breakdown of ATP. This is confirmed by experiments in salines made three times hypertonic with sucrose where the muscles still transmitted a normal action potential but failed to contract. The change was $-0{\cdot}006 \pm 0{\cdot}008$ μM ATP/g/pulse at 5 or 20 pulses/sec which is not significantly different from our earlier finding (Cain et al. 1962) for the frog rectus abdominis of $0{\cdot}0 \pm 0{\cdot}01$ μM phosphorylcreatine/g/pulse at 12 pulses/sec.

In the first series of experiments the muscles did a lot of shortening but very little work and, on the basis of Hill's new formula for shortening heat (Hill 1964), the calculated shortening heat would have been equivalent to $-0{\cdot}154 \pm 0{\cdot}009$ μM ATP/g/contraction based on 10 000 cal/mole for $-\Delta H$ for ATP. This figure includes the heats of ionization of the products and fits the recent findings of Dydynska and Wilkie (1966) and Wilkie. Even if the whole change in ATP were due to shortening heat and all the ATP were used in such a way as to give maximum heat output, i.e. 0 per cent thermodynamic efficiency (work/$-\Delta F$); the difference is $0{\cdot}074 \pm 0{\cdot}0126$, which is very highly significant. The important thing is that this is not any *extra* ATP utilization above that in an isometric twitch but the *whole* of the ATP used for all the processes. Thus, under these conditions it appears that it is impossible that shortening heat could have come directly from the breakdown of ATP. Only by assuming a sufficient breakdown of ATP during contraction followed by a *resynthesis* during

relaxation could these results fit. However, this possibility has already been tested and found not to be so (Cain et al. 1962, Infante 1962, Infante et al. 1964).

Thus it seems that shortening heat is not degraded free energy from ATP and this conclusion fits with the calculations for shortening heat based on hydrogen-bond formation given in *Nature* (Davies 1963), where shortening heat is believed to be transformed entropy.

Muscle is remarkable in that the faster it moves the less ATP it uses per unit distance it shortens. We were interested in the efficiency with which the energy liberated from this ATP is utilized for mechanical work at different speeds.

We chose the most disadvantageous assumption to get the efficiency by first comparing the external work done with the total ATP used concomitantly. At constant velocities from 0·6 to 3·0 cm/sec during tetanically-stimulated maximally working single contractions on a Levin-Wyman ergometer at 0° C the apparent efficiency was constant at about 55 per cent based on an average value of $-\Delta F = 10\,000$ cal/M ATP at the estimated concentrations and pH in the muscle cytoplasm (Kushmerick and Davies 1966). We now find that at lower and higher speeds the efficiency falls dramatically.

At low speeds the muscle approaches the isometric situation and must be continuously doing internal work because of the to and fro micro-dithering in apparently fused isometric tetani that was discovered by Nicolai (1936).

At high speeds the reduction in efficiency can be accounted for by the theory published earlier (Davies 1963). The shape of the force–velocity curve is accounted for by the occurrence of a velocity dependent reaction. The theory is based on the assumption that in activated muscle a calcium link is formed between actin and a postulated flexible rapidly coiling polypeptide of H-meromyosin. For electrostatic reasons this peptide then contracts to *form* an alpha helix and causes the relative movement between actin and myosin which is believed to be the basic reaction of muscle contraction. This linkage is less likely to occur at high relative-velocities so fewer and fewer links will form at high speed, which accounts for the reduced force developed and the reduction in the amount of ATP split per g per unit distance moved. At the highest speeds the actin site will tend to have moved through much of the possible distance along which the flexible peptide can operate (100 Å), so by the time the linkage has formed, the distance through which the peptide can pull and do work will be much reduced. Thus the external work done per *molecule* of ATP (which is believed to *break* the alpha helix and partially extend the peptide towards the β configuration) will be reduced and the efficiency (work/$-\Delta F$) will fall as has now been observed.

If an allowance is made for the ATP usage associated with the electrical stimulation (25 pulses/sec) during these isovelocity contractions (calculated at $-0{\cdot}01$ μM ATP/g/pulse) the efficiency for external work becomes even more velocity dependent and reaches values of as much as 90 per cent at velocities from 1–2 cm/sec at 0° C and near zero at the lowest and highest velocities of shortening. This is very remarkable but nevertheless seems to be true.

Thus we have shown (i) that at 0° C in DNFB-treated frog sartorius muscles the ATP change associated with activation is $-0{\cdot}05 \pm 0{\cdot}01$ μM ATP/g/activation; (ii) that shortening heat does not come directly from the breakdown of ATP; and (iii) that the efficiency (external work/$-\Delta F$) of a working muscle is extremely velocity dependent. After allowance for activation it is near zero at the lowest and highest speeds and reaches 90 per cent at the middle ranges.

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WILKIE: Were the experiments on the chemical equivalent of the shortening heat performed on series of contractions or by freezing the muscle at the peak of a single unloaded shortening?

DAVIES: Our experiments were performed on a series of contractions. However, since the direct ATP equivalent of the shortening heat was greater than the total observed ATP breakdown in the whole series of contractions, it would only be possible to account for shortening heat as degraded free energy from ATP if there were a resynthesis of ATP during relaxation, and we know this does not occur.

We have begun experiments in which the muscle is frozen at the peak of single unloaded shortenings. The results agree with the previous series in that the ATP breakdown is too small to account for shortening heat, if it were degraded free energy rather than transformed entropy. However, it will be necessary to make many more experiments before the results have a statistical validity equivalent to the first series. It is true, as sug-tested by Prof. A. V. Hill, that experiments of this type obtained from measurements on muscles that have shortened but not relaxed, will be most compelling.

GERGELY: I should like to ask, if it is indeed the case that the shortening heat does not have a chemical counterpart in ATP or creatine phosphate breakdown, if it is possible to decide whether the counterpart is an entropy or an enthalpy change in the contractile system itself. In either case, one would expect that to reverse these changes there should be a corresponding chemical counterpart during relaxation. Is there any experimental evidence bearing on this point?

DAVIES (School of Veterinary Medicine, Department of Animal Biology, University of Pennsylvania): Our experiments show that shortening heat cannot be degraded free energy from the breakdown of ATP itself. This means that some extra reaction must occur in which there is a large difference between the enthalpy and the free energy change. This, of course, means that there is a big entropy change in the reaction, so the answer

to your question really should be 'both'. The heat output is due to the decrease in entropy.

A specific mechanism has been put forward in *A Molecular Theory of Muscle Contraction* (Davies 1963). In this theory the assumption is that the energy from each ATP is used to break 46 hydrogen bonds in an α-helix to form a flexible polypeptide. The peptide is postulated to be part of the cross bridge of H-meromyosin and to exist in resting muscle in an almost randomly coiling form, although it would spend part of its time in the β-configuration. When calcium becomes available in the sarcoplasm, it is believed to form a link between the bound ATP of this peptide and the bound ADP on the actin filaments. The net result of this is to shorten the polypeptide into an α-helix. This should cause the formation of 46 hydrogen bonds, and I believe that this is the source of the shortening heat. The details are given in the *Nature* paper. The theory even predicted that shortening heat should be less during very lightly-loaded and hence very rapid contractions, when many cross bridges will not interact even once. This was later observed by Hill (1964).

The important thing is that the heat output for the formation of hydrogen bonds under these circumstances was based on a figure of around 1500 cal/M, whereas the free energy change is only about one-seventeenth of the associated heat change. This situation has been discussed in great detail by Dr Wilkie (1960) in his review *Thermodynamics and the Interpretation of Biological Heat Measurements*. In this case the small changes in free energy, as the α-helices are formed serially between myosin and actin, are associated with a large heat output because of the entropy transformation. In the molecular theory of muscle contraction this is worked out and there is remarkable agreement between the observed and the calculated shortening heat. In fact, allowing for the more recent values for the enthalpy of hydrogen bond formation during the formation of α-helices in aqueous solution, ΔH is probably about -1200 rather than -1500 cal/M (Hermans 1966). This new figure gives an even better agreement than before, i.e. observed 4·4 mcal/g, calculated 4·1 mcal/g, whereas the previous calculated figure was 5·3 mcal/g for the particular situation discussed in that paper.

Another most important conclusion is that the ratio of the enthalpy to the free energy is such that there should be a heat uptake almost equivalent to the heat output when the helix is broken. This is necessary to reconcile the findings of Carlson, Hardy and Wilkie (1963) and of Hill (1964).

It is interesting that this heat uptake, which thus abolished the shortening heat in the over-all contraction–relaxation cycle, occurs during the time the tension relaxes to zero. It does not matter whether the muscle returns to its original length. It is the final extension of the peptides to the resting situation which, according to the theory, caused the heat uptake, and this would be so whether the muscle remained short or long.

The experimental evidence bearing on this point is precisely the apparently contradictory findings of Hill, and of Carlson, Hardy and Wilkie. Since both Hill and Wilkie, and others, agree that shortening heat really is real during the time the muscle is shortening, it seems best to assume that both sides are correct, and it is this assumption that is quantitatively accounted for by the theory. This led to our experiments which were designed to test whether, in fact, shortening heat is degraded free energy from ATP.

Now that we know that it is not, this finding seems to be strong support for the molecular theory or any other possible mechanism which would lead to similar predictions.

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BENDALL: Could you give any detail on the nature of activation heat in relation to the over-all efficiency of the contractile process?

DAVIES: The source of the activation heat is not certainly known, although I have suggested that it is associated with the release of calcium from the sarcoplasmic reticulum. Our belief is that the ATP associated with activation is needed to pump the calcium back again after it has been released, and this occurs during the decay of the active state. Our best figure for a single complete activation is $-0{\cdot}05 \pm 0{\cdot}01$ μM ATP/g/activation (at 0° C in frog sartorius muscle treated with 2,4-fluorodinitrobenzene). This usage of ATP necessitates a significant correction factor in the observed over-all mechano-chemical efficiency of single isovelocity concentrations. Thus, the over-all efficiency of 55 per cent becomes about 90 per cent for the mechanical generator at a velocity of 2 cm/sec based on the assumptions I have presented previously.

SPRONCK (Laboratory of General Biology, University of Liège, Liège): The time course of phosphorylcreatine (PC) splitting at 2° C in frog sartorii (*Rana temporaria*), poisoned with 0·4 mM monoiodoacetic acid (IAA) has been followed by chemical analyses: chromatography (Hanes and Isherwood 1949), and colorimetric determinations of creatine (Ennor and Rosenberg 1952) and inorganic phosphorus (Berenblum and Chain 1938a and b). These results have been previously published (Spronck 1965) and will be briefly recalled to mind here.

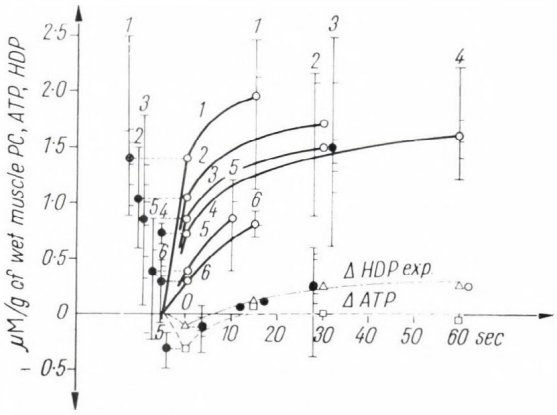

FIG. 26.—Time course of PC splitting (curves *1* to *6*, ○), of ATP and HDP breakdown and synthesis (ΔATP, □ and ΔHDP_{exp}, △) during and after 5 isometric twitches (frog sartorii, 2° C); zero time corresponds to the end of the last relaxation; an increase in the amount of C, ATP and HDP is indicated as positive. The results concerning each pair of muscles (at rest and stimulated) are grouped along the vertical lines bearing the same numbers as the corresponding curves. Each small horizontal dash represents an experimental value. It has been necessary to the clarity of the figure to displace some of the vertical lines along the time axis

The muscles are frozen at liquid air temperature, 0, 10, 15, 30 and 60 seconds after five isometric twitches (1 per sec), zero time being taken at the end of the fifth relaxation period.

Post-contractile PC hydrolysis could be detected in each of the six groups of frog investigated (curves *1* to *6* in Fig. 26).

Although large differences are observed in the amount of PC split at zero time, the time course of PC breakdown after mechanical activity seems to follow a rather constant pattern, as shown in Fig. 27 where the differences between the average PC split at zero time (0·875 μM/g) and the amount hydrolyzed at 10, 15, 30 and 60 seconds are plotted against time.

Since we were working on IAA poisoned muscles, it appeared interesting to follow the time course of fructose-1-6-PP (HDP) and triose-P metabolism, the accumulation of which involves an ATP breakdown. The HDP metabolism has been investigated by chromatographic analysis but the corresponding amounts of triose-P have been estimated from the equilibrium constant

$$K = \frac{C^2 \text{triose-P}}{C\,HDP}$$

determined by Meyerhof and Lohmann (1934).

During mechanical activity, the sartorii use 0·875 μM of PC/g, 0·130 μM/g of HDP disappear, and a few determinations of ATP show a hydrolysis of 0·33 μM/g. If we assume that most of the ADP produced by stimulation is rephosphorylated according to the reactions PC + ADP → C + ATP and HDP + ADP → HMP + ATP one concludes that at the end of the last

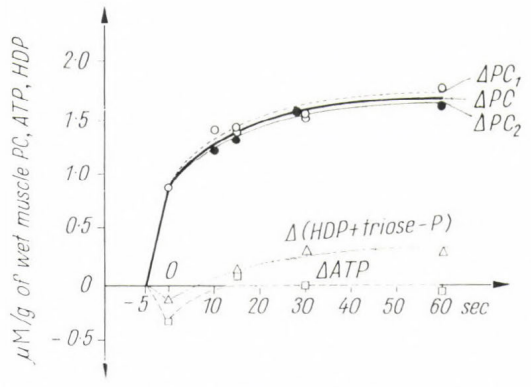

FIG. 27.—Is derived from Fig. 26. ΔPC_1 (o) corresponds to the individual post-contractile hydrolysis increments observed at each time interval; the value shown for zero time corresponds to the average of the zero time determinations; ΔPC_2 (●) corresponds to the average of the ordinates read on curves *1* to 6 in Fig. *26*, and ΔPC is the mean of ΔPC_1 and ΔPC_2

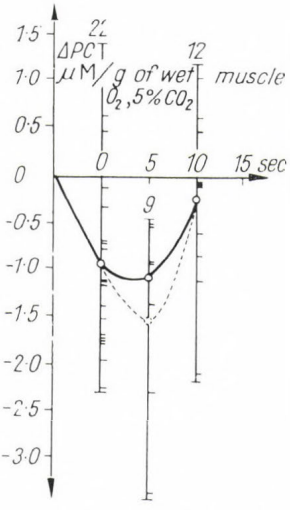

FIG. 28.—Time course of PC metabolism with 5 per cent CO_2 in O_2, during and after 5 isometric twitches at 2° C

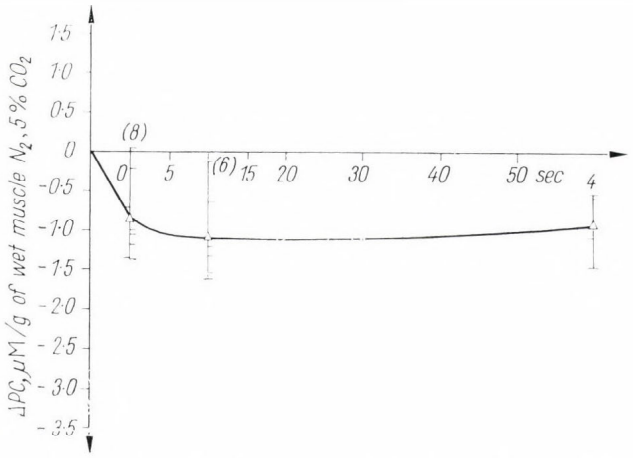

FIG. 29. — Time course of PC metabolism with 5 per cent CO_2 in nitrogen, during and after 5 isometric twitches at 2° C

twitch, the muscles have actually split 0·875 + 0·130 + 0·330 = 1·335 μM/g of ATP, of which 0·875 only have been paid with PC, so that the 'debt' of PC amounts to 0·130 μM/g + 0·330 μM/g = 0·490 μM/g.

During the minute which follows, the muscles go on splitting PC at a decreasing rate: 0·825 μM/g being consumed after 60 seconds. They resynthesize the HDP lost during mechanical activity in less than 10 seconds and accumulate afterwards 0·335 μM/g of the same. Assuming that HDP synthesis corresponds to a loss of an equivalent amount of ATP, rapidly paid with PC, there is left 0·825 μM/g — 0·335 μM/g = 0·490 μM/g of PC to compensate for the ATP not resynthesized during activity, which can be shown to be recovered in 15 or 20 seconds after the end of the last contraction.

The post-contractile PC splitting detected by chemical analysis might be correlated with the negative delayed heat observed by Hartree (1932), D. K. Hill (1940) and A. V. Hill (1961). It is also an argument in favour of the interpretation of the alkalization observed by Dubuisson (1939) and Distèche (1960) and attributed to Lohmann's reaction.

It is generally admitted, but without a complete experimental proof, that PC recovery proceeds from the ATP through Lohmann's reaction. If this is actually the case, and if the alkalization observed by Dubuisson (1939) and Distèche (1960), represents actually the PC splitting by Lohmann's reaction, then the fact that this alkalization appears in IAA-poisoned as well as in unpoisoned muscles implies that PC recovery must be coupled with other chemical events so that no pH change occurs.

Recent experiments by Dydynska and Wilkie (1966) have shown that, in normal muscle, in O_2 at 0° C, the PC split during a 30 sec tetanus (5·9 μM/g) is restored with a roughly exponential time course whose half-time is about 10 minutes and which lasts 40 to 50 minutes. This fact may be supported by Infante, Klaupiks and Davies (1965) who have shown that

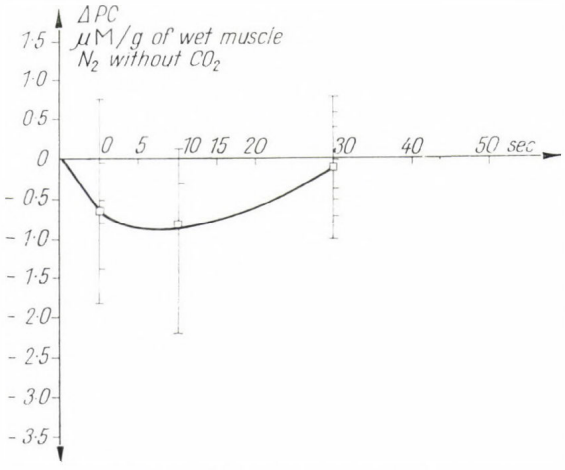

FIG. 30.— Time course of PC metabolism in pure nitrogen, during and after 5 isometric twitches at 2° C

all the PC split during 20 or 40 minute incubation in 0·25 *m*M 2,4-dinitrophenol (80 per cent of the total PC), was resynthesized within 25 minutes after removal from the drug, at 20° C.

With the same techniques as those we used for PC hydrolysis and after the same and small mechanical activity, it is possible to follow the time course of PC recovery in muscles which are not poisoned with IAA.

Figure 28 shows the quick recovery of PC in aerobic conditions (95 per cent O_2, 5 per cent CO_2), 80 per cent of the PC split during 5 isometric twitches being resynthesized 10 seconds later already.

In anaerobic conditions (obtained by placing the muscle into a closed chamber in a previously nitrogen-gased Ringer bicarbonate solution, where it is submitted to further gassing for 45 minutes) PC recovery after 5 twitches occurs in 100 per cent nitrogen only (Figs 29 and 30) and more slowly than in aerobic conditions.

Considering our results in nitrogen, one is inclined to think that they fit with the fact that the apparent equilibrium constant

$$k' = \frac{[\mathrm{PC}] \cdot [\mathrm{ADP}]}{[\mathrm{ATP}] \cdot [\mathrm{Cr}]}$$

decays *in vitro* and in the presence of 0·02 M $MgSO_4$, from 1·4 at pH 9·7 to 0·1 at pH 8, to 0·01 at pH 7·4 and to 0·00525 at pH 7 (Noda et al. 1954) so that PC recovery seems to be easier at alkaline pH. In fact, we do not observe any PC recovery with 5 per cent CO_2 in N_2 (Fig. 29), in which conditions the frog sartorius muscle accumulates lactic acid, and has an approximate internal pH of 6·9 (Hill 1955). On the other hand, Fig. 30 shows that in pure nitrogen, that is to say at a more alkaline pH, PC recovery is possible.

However, in aerobic conditions (5 per cent CO_2 in O_2), the recovery proceeds quickly though the pH is probably only slightly higher than 7. At such pH, the *in vitro* equilibrium constant k' is lower than 0·01, but it is impossible to reach such a small value from the actual concentrations of ATP, ADP, PC and Cr in muscle, unless one admits the existence of compartments in which ADP concentration would fall to a very low value (at least the tenth of usual amount). If this were so, calculations show that PC recovery is then possible through Lohmann's reaction even at pH 7·0 (Fig. 31).

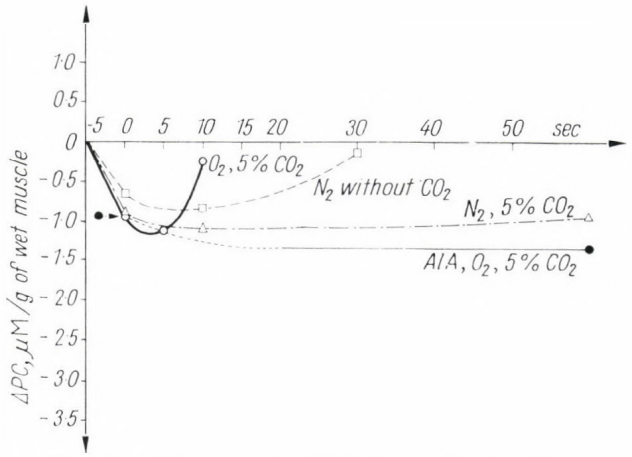

FIG. 31.—Time course of PC metabolism in all our experimental conditions

Evidences for this compartmentation exist. For instance, Hohorst, Reim and Bartels (1962) have observed, even in IAA, in which conditions muscle sets more alkaline when contracting, a considerable diminution of k' all along a 60 sec tetanus. They conclude to a functional compartmentation of ATP and ADP, so that at least 85 per cent of these do not react either with creatine kinase or with other kinases at a rate comparable to the breakdown of PC.

It is well known from the work of Jacobs, Heldt and Klingenberg (1964) that a high level of creatine kinase exists in isolated mitochondria free from any cytoplasmic contamination. The enzyme is separated by means of agar gel electrophoresis as an isoenzyme of extramitochondrial kinases. If one accepts the mitochondria to be a cellular compartment, it is possible that this creatine kinase involves a mitochondrial PC recovery, either thanks to a very low ADP concentration due to ATP production by the oxidative metabolism, or through another unknown and oxidative process of the mitochondrial phosphate metabolism. We, therefore, propose ourselves in our forthcoming experiments to determine the pH influence upon PC recovery after 5 isometric twitches, at 2° C, when O_2 assists this anabolic process and the whole metabolism proceeds freely.

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WILKIE: What do you think is the reason for the difference between your recent results and the earlier work of Carlson and Siger and Mommaerts? (References given at the end of my lecture.) Both of these groups of investigators reported that hexose phosphates did not form in IAA-poisoned muscles at 0° C except after prolonged stimulation.

SPRONCK: It is known that, in 1960, Carlson and Siger reported that no HDP was formed at 0° C, but this assumption was not based on analytical data. They indicate that Lundsgaard, in his early work, found an increase in Pi and little or no esterification of Pi as HDP and they come to the same conclusion using their determinations of Cr, PCr and Pi only. On the other hand, Mommaerts, Seraydarian and Maréchal; Maréchal and Mommaerts come to the same conclusion after identical determinations. These authors only mention two determinations of hexose monophosphate and hexose diphosphate from which it seems to me impossible to conclude that the HDP level remains constant after one tetanus of 6 seconds or 6 tetani of one second.

JOHNSON: What do you think is the relation between your experimental results and the pH change mentioned in Prof. Jöbsis' lecture?

SPRONCK: Dr Jöbsis mentioned in his lecture that it was possible to show that one twitch produces an increase in the intracellular ADP level. This fact means that the breakdown of PC begins later than that of the ATP and is in complete agreement with the post-contractile PC breakdown that we have detected by chemical analysis.

Dr Jöbsis recalled also to mind the pH changes observed by Dubuisson and Distèche during muscle activity, that is to say, a rapid acidification starting as soon as the action potential and a much slower alkalization starting generally after a few twitches and lasting longer than mechanical activity. The rapid acidification is attributed to ATP breakdown and agrees fully with the increase in ADP observed by Jöbsis. The alkalization is assumed to correspond to PC breakdown and our results are an argument in favour of this interpretation.

DYDYNSKA: What I am interested in your results is the question if the muscle which recovered in nitrogen was in Ringer solution, because I found no recovery after tetanic contraction in N_2, when the muscle was in pure gas and I thought its cause lay in the accumulation of lactic acid which was not allowed to flow out of the muscle and in Ringer solution it could go out of the fibres.

SPRONCK: The anaerobic conditions are obtained by placing the muscle in about 250 ml of Ringer bicarbonate solution and by gassing them for

40 minutes at 2° C, so that lactic acid which could be formed at the end of the treatment is allowed to diffuse in the Ringer solution. This solution is removed 5 minutes before the electric stimulation. In these conditions, chemical determinations have shown that the amount of lactic acid present in muscle is still low at the beginning of the first twitch and the relatively small activity performed by the muscles in our experimental conditions does not give rise to a sufficient amount of lactic acid as to stop PC recovery. I believe however you are right to think that the accumulation of lactic acid may stop PC recovery if the muscles are kept in pure nitrogen and if they perform a more important mechanical activity. In these conditions, the lactic acid may reduce the pH to such low values as to stop the PC recovery through the Lohmann's reaction.

BENDALL: In connection with Dr Spronck's remarks, I should like to draw attention to the fact that during the onset of rigor mortis in intact skeletal muscle, at any temperature between 5° C and 37° C, the creatine phosphate (PC) level begins to drop from the moment the blood supply is cut off and anaerobiosis prevails. However, the pH at this intial stage may be as high as 7·20, and in such a case the PC level will have dropped almost to zero before the pH has fallen below 6·50. Glycolysis in rabbit, pig, beef, pigeon and whale muscles, for example, continues beyond this stage until the pH has fallen from 5·8 to 5·5. In spite of this substantial glycolysis, the ATP level begins to fall more or less rapidly as soon as the PC level has fallen below about 3 μM/g of muscle. The interesting point is, though, that anaerobic glycolysis cannot maintain the PC level, let alone increase it, at the high *initial* pH values of 7·2 or above.

AUBERT (Laboratoire de Physiologie générale, Leuven): One of Dr Jöbsis' propositions, in the course of his excellent presentation, struck me as slightly biased. He showed us that the time integral of the fluorescence changes was strictly proportional to the tension–time integral. From this accepted fact, he concludes that there is no place for an activation energy, for, said Dr Jöbsis, he had 'proven' that the time-integral of fluorescence was proportional to the total energy used up, and so no energy was left for activation. As a matter of fact, what he has shown us was that this time integral measured according to certain specifications, was proportional to the number of twitches delivered by the muscle in a short period of time. It is my contention that two postulates are implied in this procedure:

(1) We are told that the fluorescence traces returns to the base line after 8 minutes at 12° C, when the integral is assumed to be complete. But in my limited experience on the fluorescence of frog muscle, the oxidation is very often followed by some reduction lasting for a rather long time. And most people working on the respiration of muscle or on its recovery heat would certainly not accept that the processes of restoration are already complete after 8 minutes at 12° C. This may introduce a first error.

(2) On the other hand, is it quite certain that the total energy increases in strict proportion with the number of stimuli or twitches? If not, this might introduce an element of non-linearity in the calibration of the fluorescence curve. Now, my experience in heat production is that in twitches as well as in tetanic contractions, previous activity alters the energy output of successive contractions. To prove that point, I present two figures, which might have a more general interest.

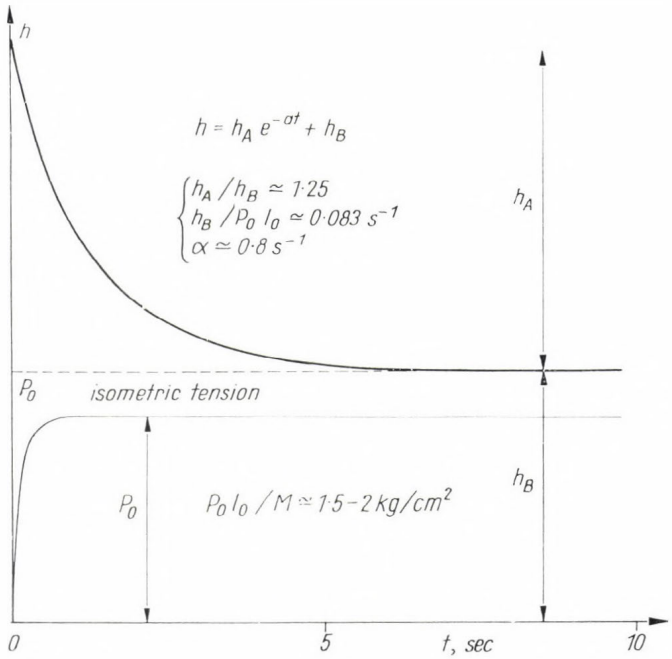

FIG. 32

Figure 32 summarizes the evolution of the rate of heat production in an isometric contraction with the labile heat (h_A) and the steady heat (h_B) reached after a few second contraction (frog sartorius at 0° C). This of course could agree with Dr Jöbsis' statement that the energy output increases more rapidly during a short period of stimulation than during a long one integrated and averaged.

Figure 33 shows the effect of repeating the same stimulation after a given interval following the first tetanic contraction. The tension curve (not shown in the picture) is practically the same after intervals of 15 seconds upwards. Nevertheless, it is obvious that the labile heat production has been seriously reduced by the first burst of activity, although the steady state of heat production is exactly the same in both cases. Thus there is no fatigue in the usual sense. By comparing the effect of interval (15, 30, 60, and 120 sec), it can be seen that even after two minutes the labile heat has not yet completely recovered. This takes more than 15 minutes in our experiments.

This result shows (i) that the same tension time can be obtained with different energy production (and the measure of recovery heat has shown that the heat deficit is a real economy of energy, not an entropy effect), and (ii) that if the labile heat had something to do with activation heat, its chemical effect could only be appreciated in the very first contraction of a completely restored muscle.

JÖBSIS (Department of Physiology and Pharmacology, Duke University, Medical Center, Durham): All of the points that Prof. Aubert brought up

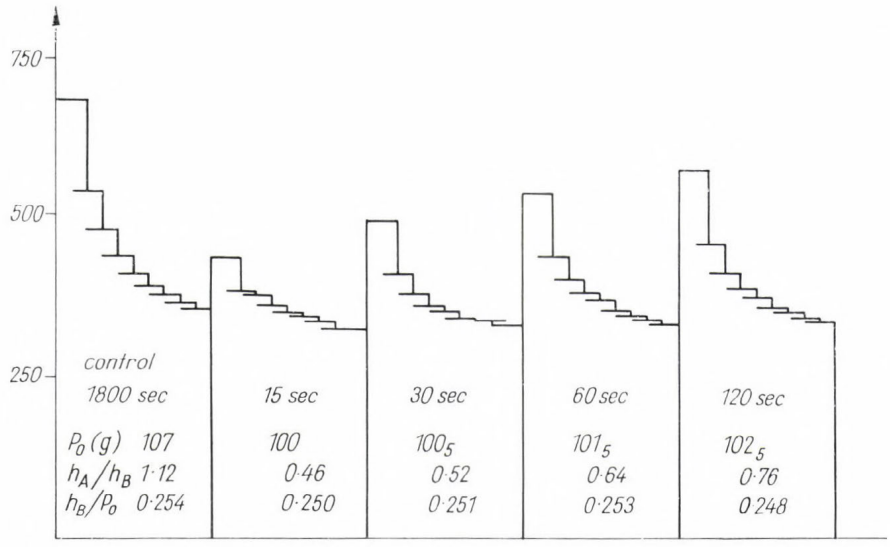

FIG. 33

are important ones, indeed. Each requires a clear and definite answer: (i) The first point that I would like to take up may be paraphased: "Is the energy utilization linear with the number of twitches?" Heat measurements shown by Prof. Aubert prove that this is not the case for long tetani. In addition, it is noted that the same holds for twitches. I certainly was not aware of this fact. I can only say that for twitches the $\int \Delta Fl$ is linear with the number; I have never investigated tetani in this manner. This point must have been measured for twitches with myothermal techniques. Dr Wilkie, do you know of such an experiment and what was the outcome?

WILKIE: Answers that he does not know of a published experiment but that from his own experience he would say that the heat development is linear with the number.

JÖBSIS: Thank you, Dr Wilkie, that settles the case for small numbers of twitches. It may be different, of course, for a twitch following a long tetanus but that case was not included in my presentation. However, Prof. Aubert's data on the tetanus are very important in their own right. I cannot help but speculate that the increased efficiency of the second tetanus may have some connection with the persistence of some condition favoring the earlier occurrence of the 'locked' or crystalline state. (ii) Prof. Aubert pointed out the problems encountered by the occurrence of oscillations in the fluorometric response. These fluctuations are caused by oscillations in the glycolytic activity which are reflected in oscillating ADP levels which in turn produce oscillations in respiratory chain NADH. When they occur the $\int \Delta Fl$ is sharply diminished and looses its usefulness as a linear index to the recovery. In Fig. 34 the lower curve shows this effect. After 6–8 twitches in rapid succession oscillations occur and the $\int \Delta Fl$ is curtailed sharply. At a more leisurely rate (middle curve) oscillations do not occur but the $\int \Delta Fl$ is diminished, owing to glycolytic recov-

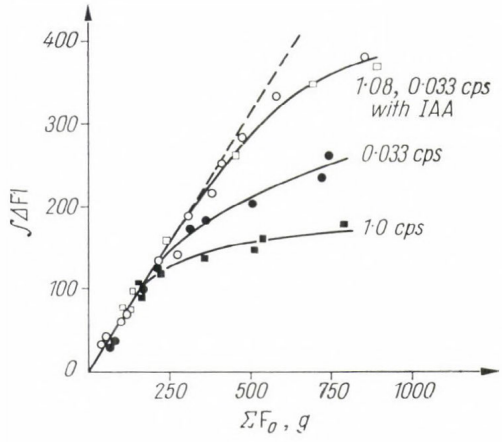

FIG. 34

ery. The top curve shows that the administration of iodoacetate allows a much larger range of activity. The final curtailment is probably related to the catabolism of AMP arising after myokinase activity. In all the experiments reported so far I have stayed within the linear range as tested by an occasional group of 3, 4 or more twitches. (iii) The fact that the oxidative recovery metabolism of one twitch is completed in 8 to 10 minutes may only be a reflection of the fact that the temperature is 12° C rather than 0° C and that the toad sartorius contains a 3–5 times higher concentration of respiratory chain components than the frog sartorius. Using a temperature coefficient (Q_{10}) of 2·5 we arrive at about 20 minutes at 0° C and using a 3 times lower respiratory chain concentration one would estimate a full hour for complete oxidative recovery in the frog sartorius at 0° C. It is clear then that, if erroneous, the 8 minute figure is too long rather than too short.

DAVIES: How long did your tetani last in the experiments that you have just described?

AUBERT: Four to five seconds, at 0° C. Please, note that the labile heat is already declining during the first second of tetanization, and secondly, that the energy needed to maintain a tetanic contraction during one second at 0° C (measured as heat or as creatine phosphate hydrolyzed) is about the same as the energy used up in a single twitch. The condition of the muscle in my experiments is thus not so widely different from those of Dr Jöbsis.

DAVIES: In that case there would be a considerable formation of inosines which occurs after the first 1·5 or 2 seconds in a maximally stimulated frog sartorius muscle which has been treated with 2,4-fluorodinitrobenzene.

HUXLEY: According to your scheme, at the peak of tension development the calcium level in the muscle has already returned again practically to zero. Nevertheless, if a muscle is given a quick release at this point a large recovery of tension can take place. This would imply that within a given sarcomere, the links in your scheme must all operate in series as a muscle shortens, and the scheme would appear to exclude systems in which links can undergo several cycles of activity during a single shortening. This is, I believe, at variance with the observed amount of ATP split and the number of enzyme sites in the muscle.

JÖBSIS: There is nothing in the model that would limit the activity to a single turnover. In fact I would guess that there would be several. However, scheme A would more easily generate the several cycles of activity required by the $\sim P$ utilization per twitch. I believe, that you

analyze it correctly that in scheme B an operation in series would more easily explain the experiment to which you refer.

(*Note added after the conference.* — New measurements and calculations have shown that the distinction between scheme A and scheme B is not as easily made as previously thought (cf. my answer to Prof. Ebashi's comment). It is, therefore, better to state that the choice between schemes A and B is not completely settled, although B appears favoured. The experiment that Prof. Huxley brought up requires thoughtful attention when a final choice must be made.)

GERGELY: I think that Dr Jöbsis has presented some extremely interesting and elegant experimental data, but, impressed as we may be with these, we should bear in mind that the kinetic scheme proposed by him may be only one of many possibilities and that, for instance, in the case of the presence of ADP in the active intermediate I do not see any direct evidence on the basis of the kinetic analysis. I may be wrong, but I think this is a point one ought to bear in mind.

JÖBSIS: Yes, it is very important indeed to emphasize that the kinetic schemes presented above are only two possible ones. There undoubtly are others. But I do not believe that simpler ones can be constructed and until further evidence forces a more complicated scheme, I would like to stick to the simplest one. The notion that ADP is part of the complex that I like to call the 'tension intermediate', comes from my measurements of the delay of the cytochrome *b* response. I believe that those data are correct and I do not know of any measurements that directly contradict mine. The *in vitro* actomyosin ATPase data yield some information, some of which is in favour and some against my conclusion. Besides, it is hard to bridge the gap between extracted components and the *in vivo* situation. In conclusion then, I agree with you that it would be a pity if my data were overshadowed by a faulty theory. I will have no scruples if modification of the theory is required as more data become available. Until that time, however, I will have to do with the presently available measurements.

HANSON: I do not understand why any ADP reaches the mitochondria; why is it not captured en route by creatine phosphokinase, for example?

JÖBSIS: Creatine phosphokinase, myokinase and some other $\sim$ P buffering systems can only partially cut down the ADP rise resulting from the contraction. They are equilibrium systems and a new equilibrium will result featuring a slightly higher ADP concentration. In fact, it has been shown that only a small per cent of the maximum ADP concentration is signalled by the mitochondria and that is just about the level one expects from calculations that take the creatine phosphokinase system into account. Now, there is a distinction to be made here. The maximum concentration signalled in this way is only a small per cent, yet the total aliquot of ADP will in the end be re-phosphorylated by the respiratory chain system. What happens is that the $\sim$ P buffering systems smear out the concentration; make it lower at the moment of the maximum concentration but keep feeding in ADP from the creatine phosphokinase system during the later parts of the oxidative recovery period.

TREGEAR: Chaplain, Abbott and White showed that in glycerol-extracted insect flight muscle, activated by ATP and calcium, the addition of ADP raised the tension in the muscle, reversibly. This is unlikely to be a myokinase effect, as addition of further ATP did not have the same effect. It is consistent with Dr Jöbsis postulate of an ADP-intermediate.

JÖBSIS: That is a very interesting and relevant observation, Dr Tregear, and if I may be so free as to inject my own point of view, it might well be worth considering that high ADP levels evoke the locking process in a long tetanus.

BENDALL: Points out that in mammalian muscle the ADP effect is not present.

JÖBSIS: Since the mammalian muscle does not show this behavior it may be necessary to postulate that the insect preparation contains an exceptionally high rate constant for the back reaction of the final cleavage of the ADP tension intermediate.

VOROBYEV (Institute of Cytology Acad. Sci. USSR, Leningrad): A study of conformational changes in protein fibers and synthetic polymers made it possible to regard muscle contractility as a process connected with changes in configuration of myofibrillar proteins, accomplished according to the phase transition type.

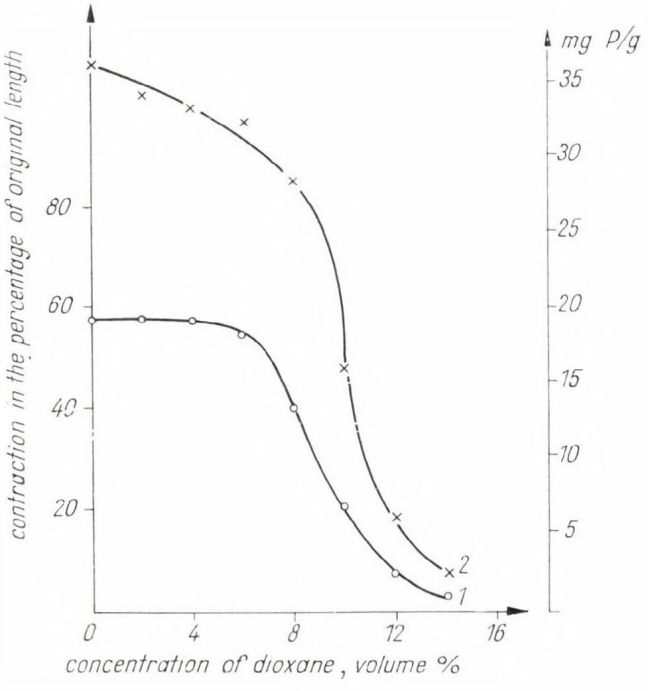

FIG. 35

To reveal the character of conformational changes upon muscle contraction we studied the influence of various concentrations of some organic solvents on the extent of contraction of glycerinated muscle fibers at the action of ATP.

Flory and Hoeve tried to use this method for the analysis of the conformational changes during muscle contraction. The results of their experiments with ethylene-glycol and glycerol were interpreted as an evidence in favour of the phase transition during muscle contraction.

In our work we checked the effect of the organic substances which are known to disturb the secondary and tertiary structures of protein macromolecules: dioxane-, formamide, dimethyl-formamide, ethylene-chlorhydrine and urea.

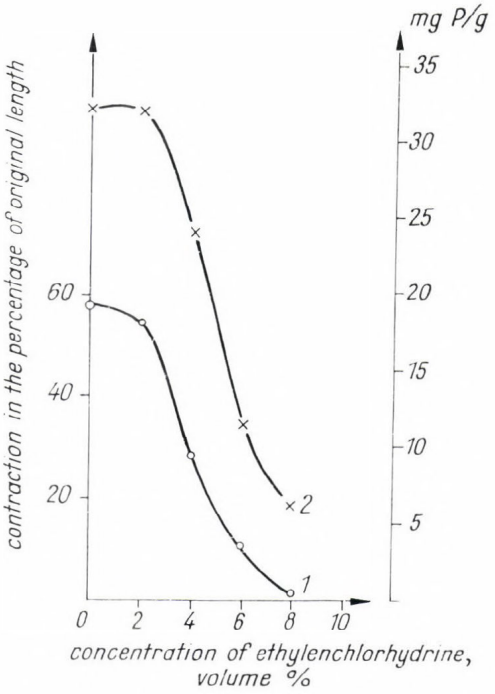

FIG. 36

Figure 35 represents the dependence of the extent of contraction of glycerinated muscle fibres upon the addition of ATP and the dependence of the rate of dephosphorylation of ATP on the concentration of dioxane in the medium.

It may be clearly seen that dioxane, affecting the tertiary structure of globular proteins without influencing the α-helix, induces sharp changes in the extent of contraction. The ATPase activity changes in a similar way although it does not strictly correlate with the extent of contraction: it begins to decrease already at such dioxane concentration as does not effect the extent of contraction.

At the action of ethylenechlorhydrine (Fig. 36), which is known to be an agent causing spiralization in globular proteins (it effects the tertiary structure via the secondary one), the extent of contraction and the rate of dephosphorylation of ATP proved to be of similar character, i.e. they depend on the range of concentration.

In the case of dimethyl-formamide, which affects the tertiary structure and causes protein spiralization, a sharp change in contraction was observed at a narrow range of concentrations from 12 per cent to 7 per cent (Fig. 37).

From these experiments it is difficult, however, to evaluate the nature of forces participating in co-operative transitions which accompany contraction. But, the action of such low polar solvents as dioxane, ethylene-chlorhydrine and dimethyl-formamide apparently alters hydrophobic bonds and therefore affects first of all the tertiary structure of myofibrillar proteins.

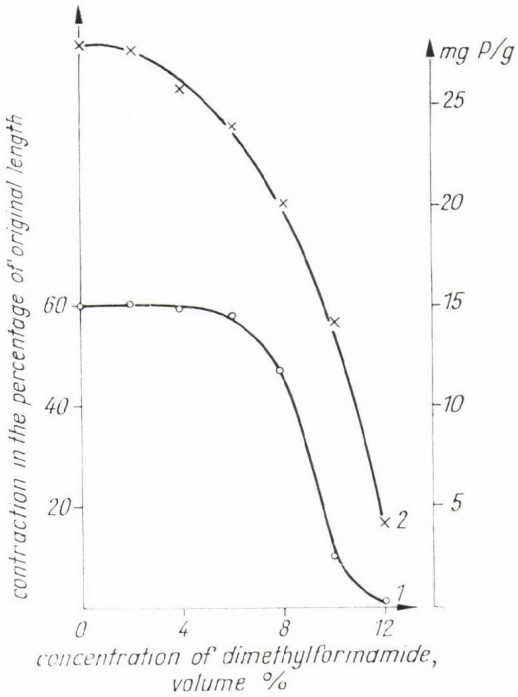

FIG. 37

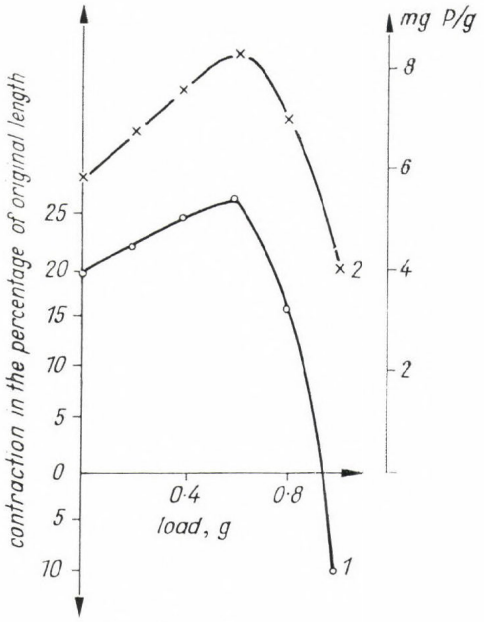

FIG. 38

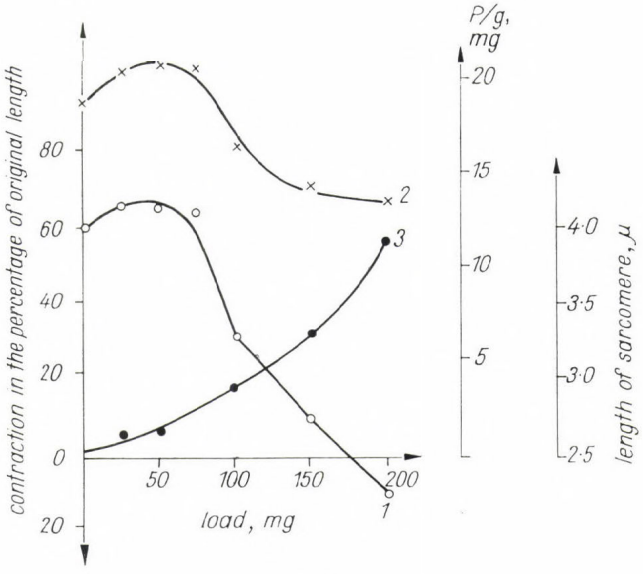

FIG. 39

Another experimental approach to the investigation of the phase nature of muscle contractility is related to the effect of external forces on conformational transitions of order–disorder type.

A theoretical survey of phase transitions in biopolymers with the ordered inner structure permits the conclusion that the loading should influence the process of conformational transition.

It means that if co-operative changes which have the character of phase transition really have taken place upon muscle contraction, they should be sensitive to the action of external forces.

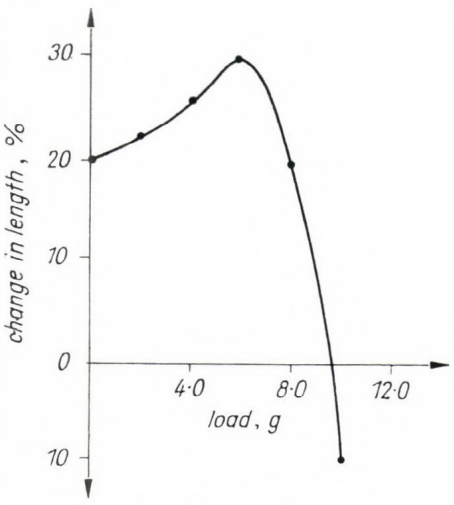

Fig. 40

Figure 38 represents the data obtained from experiments with glycerinated bundles of m. ileo-fibularis of the frog. It is evident that an increase in loading first leads to the increase in the extents of contraction and ATP splitting and then results in their fall.

Figure 39 represents results obtained from the experiment with the fiber bundles of m. psoas of the rabbit. On the right of the ordinate is given the length of sarcomere in the originally stretched fibers. The sarcomere length was measured with a diffraction method with the help of a goniometer. As can be seen, at stretching the length of sarcomer increases, the maximum of contraction and ATPase activity corresponding to the sarcomer length of 2·7 μ. It seems that at this length of the sarcomere protofibrills have an opportunity to overlap in such a way that it provides most completely a mechano-chemical function of the muscle.

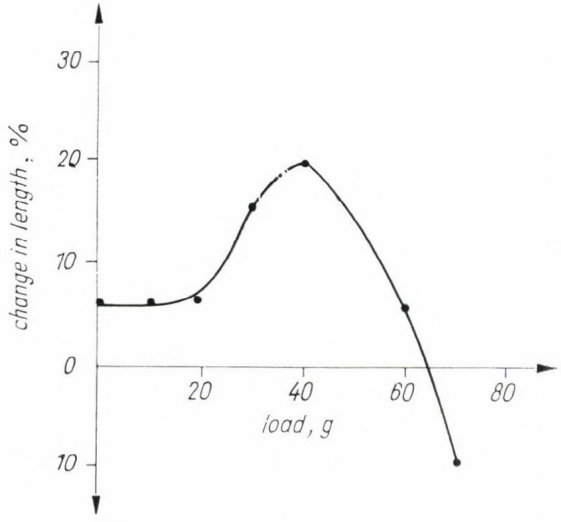

Fig. 41

Thus, these experiments reveal connections among mechanical, structural and enzymatic factors determining muscle activity.

We have studied the effect of loading on thermal contraction of muscles (m. sartorius of the frog *Rana temporaria*). The muscles were killed either by usual glycerinization procedure, when

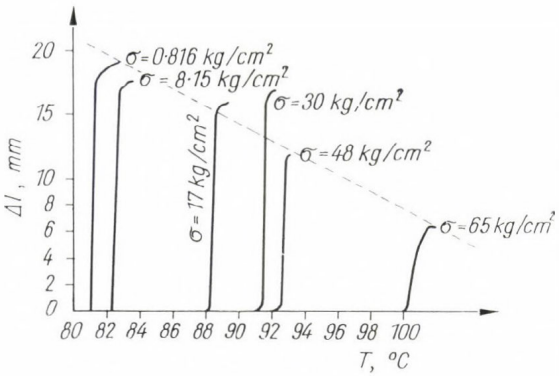

FIG. 42

native contractility is retained, or through gradual heating up to 90° C, when muscles lost contractility.

Figure 40 shows the dependence on the load of length changes in muscle killed at heating. It is seen that under load of 3–4 gramms the muscle contracts stronger than under small load. Further increase in loading results in the reduction in contraction.

Figure 41 gives the results of experiments with glycerinated muscles. Thus at some range of loading killed muscles contract if heated in agreement with Starling's law.

Nothing of the kind was observed while experimenting with other synthetic or natural polymer fibers. In Fig. 42 the changes in length of collagen fibers upon thermal contraction are plotted versus temperature of heating. Figures above the curves designate tension. It is seen that with the increase in loading shifts occur only in the temperature region of transition toward high temperatures, whereas the degree of thermal contraction falls from the very beginning of loading.

Thus, the ability of a muscle to follow Starling's law is probably defined not by the properties of its native proteins but by the unique structural organization of its fibrillar proteins in myofibrills.

EDMAN: I am puzzled by the length–tension curve as you presented it for the glycerinated frog's skeletal muscle fibre. It would mean that up to a certain level the fibre is capable of lifting a heavier load further than a smaller load. That certainly does not agree with the mechanical behaviour of the living vertebrate skeletal muscle cell. Nor does it agree with the length–tension diagram obtained from glycerinated psoas fibres contracting upon ATP (Edman 1964). Is your diagram possibly composed of data from different muscle preparations, which have happened to add up in this way, or is this length–tension curve really a constant finding with your preparation?

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Edman, K. A. P. (1964) in: *Biochemistry of Muscle Contraction*. Ed. by J. Gergely, Little, Brown and Co., Boston

VOROBYEV: I must confirm that not only on glycerinated frog's muscle fibers, but also on glycerinated fibers of rabbit's m. psoas, we constantly observed that at a certain range of load the extent of contraction upon addition of ATP increases with the augmentation of loading. With an

increase in loading within the same range ATP splitting by glycerinated fibers was found to increase (Vorobyev and Ganelina 1963, Vorobyev 1966).

I should like to stress that experimental results may be reproduced easily in any series of glycerinated fibers used. Each point on the curves I demonstrated is the mean value of the results of several experiments. An increase in the extent of contraction and ATPase activity proved to be statistically reliable.

I wish to draw Dr Edman's attention to the fact that an increase in loading results in the stretching of muscle fibers. Hence, at the action of various loads, fibers are found to be in various initial states. This is represented in the slide I demonstrated. While the mean initial length of a sarcomere in unloaded fibers was 2·5 μ, that of the fibers in which the greatest extent of contraction was observed, was 2·7 μ. This, I suppose, corresponds to the length of a sarcomere in the resting living muscle fiber. Pay attention to the fact that this is the initial length of a sarcomere where the maximal ATP splitting by glycerinated fibers was noted.

The data obtained may be interpreted as follows. Upon stretching of fibers thin myofilaments seem to slide along thick ones. It appears that at a certain degree of stretching (certain loading) a most favourable geometrical correlation is established between active centres of myosin and actin myofilaments. Moreover, I suppose that for the most effective work the myofibrils should be given a certain stress which would provide an appropriate conformation of macromolecules of mechanochemically active proteins. It is shown that the load itself affects conformational transitions in biopolymer fibers stabilizing or, on the contrary, unstabilizing certain conformations (Birshtein et al. 1961, Vorobyev 1964).

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(Vorobyev and Ganelina) Воробьев, В., Ганелина, Н. (1963) *Цитология* **5**, 672

JÖBSIS: Do I have it correct that you do not utilize an energy source such as a ~P compound in these experiments? If so, where does the energy derive from?

VOROBYEV: You have misunderstood the data I spoke about. It was pointed out that the contraction observed was caused by the addition of ATP. Moreover, having observed ATP splitting in the course of contraction we established that, as indicated in my reply to Dr Edman, the rate of ATP splitting by glycerinated fibers depends on the load applied. This may be seen on the slides I demonstrated.

DYDYNSKA: All that I want to tell about is simple biochemical evidence which perhaps may be useful when one wants to board the question of localization of nucleotides in the muscle fibre. Glycerinated muscle sartorii of the frog, *Rana esculenta*, and the glycerin in which they were embedded were analysed day after day and it was found that after the efflux from muscle of about 50 per cent of adenine nucleotides during first 24 hours further efflux slowed down, showing apparent inhibition which lasted to about the 20th day. From the 20th to 200th the quantity of adenine

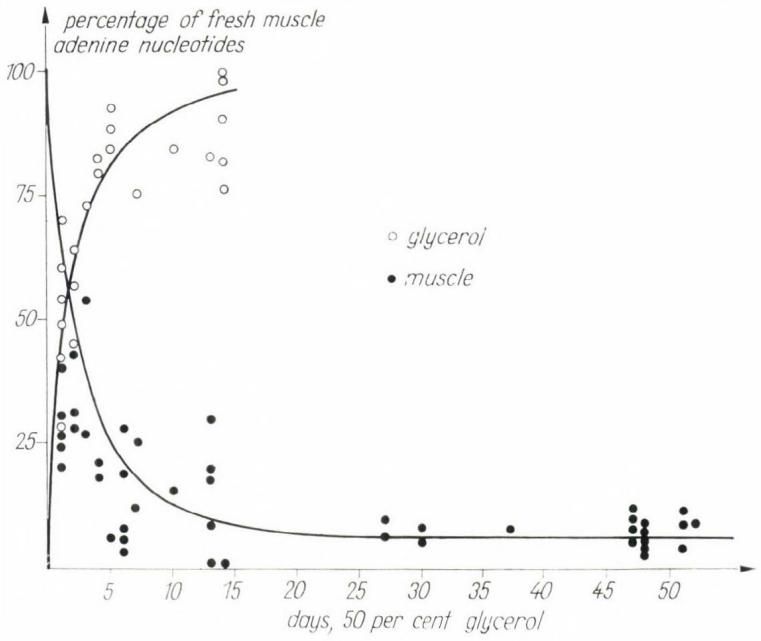

FIG. 43

nucleotides found was very stable and amounted to 0·43 ± 0·11 μM/g, which on the basis of chromatographic analysis could be stated as ADP, bound to actin present in the fibres. The lag of efflux of adenine nucleotides out of the glycerinated muscles may be explained in the way that the part of nucleotides compartmentilized between myofibrils stuck to them during rapid dehydration following the first osmotic shock, after embedding in the 50 per cent glycerin, and could flow out only some time later when glycerin entered the whole muscle (Fig. 43).

BOWEN: Mr Chairman, I wish to discuss some characteristics of glycerol-treated rabbit psoas muscle fibers which have been methylated by dimethyl sulfate. Two years ago experiments were reported (Bowen, Martin 1964, Bowen 1964) which showed that methylated fibers lost their ATPase activity but not their ability to undergo ATP-induced shortening; however, this shortening was not restricted to induction by ATP. The large complex anions of ADP, AMP and pyrophosphate also induced shortening. These latter compounds are entirely incapable of causing shortening of untreated glycerinated fibers.

These results raised the question of whether methylation changed the striated structure of the muscle fibers to an unorganized amorphous mass which shorten by tightening of random coils. This was very much in need of answering, because when the methylated fibers were transferred to water for washing, they became greatly swollen. The length did not change.

Methylation was done in solutions of dimethyl sulfate buffered to pH 4·2 by 0·028 M Na acetate (Saroff et al. 1953). At this pH, it was considered

TABLE I

Lengths of A- and I-bands of glycerol-extracted muscle fibers before and after ATP- or PP-induced shortening with and without methylation by dimethyl sulfate

Condition and treatment of the glycerinated fibers	Lengths of A- and I-band and H-zone			Length of sarcomere
	A	I	H	
	μ	μ	μ	μ
Non-methylated; before ATP, mounted in 0·044 M KCl	1·25	1·55	0·6	2·8
Non-methylated; after 4 mM ATP, 0·044 M KCl	1·0	0·6	0·3	1·6
After treatment with DMS; in water	0·9	2·1	—	3·0
DMS-treated, 10 mM ATP	0·65 to 0·9	0·35 to 0·7	—	1·25
DMS-treated, 4 mM ATP	1·25	0·65	0·3	1·9
DMS-treated, 10 mM NaPP	1·0	0·6	—	1·6

that methyl radicals reacted with the carboxyl sites of the protein molecules. First, muscle fibers were examined by phase microscopy at 1600× magnification before and after methylation and after shortening with ATP and Na pyrophosphate. Photographs of the fibers (to be published) revealed that methylation did not transform them into homogeneous structures. After the fibers had become swollen, the photographs revealed that the uptake of water was mostly, if not entirely, restricted to the I-bands. After transfer to solutions of ATP or pyrophosphate, the I-bands of the methylated fibers shortened and to about the same extent as non-methylated glycerinated fibers (Table I). When the swollen fibers were put into ATP or pyrophosphate solution, the striated structure returned to normal except that Z-lines were no longer visible. H-zones did reappear if the concentration of ATP was low (4 mM), but did not appear if the concentration was high (10 mM). When this happened, the A-bands also shortened but only by that length which the H-zones had occupied, before their disappearance (Table I). This indicates that the H-zone has an organized structure of protein which is

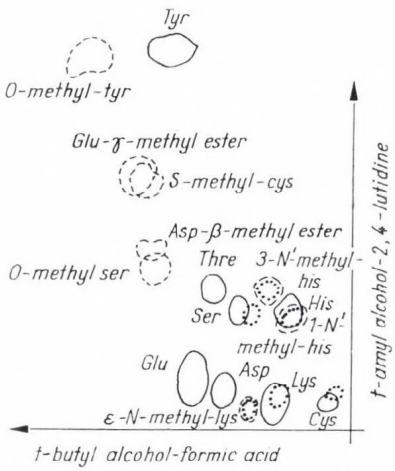

FIG. 44.— Chromatogram and autoradiogram of hydrolysed methylated glycerol-treated muscle fibers. *Solid lines* enclose spots of naturally occurring amino acids from hydrolysates. *Broken lines* enclose spots of methyl derivatives of amino acids added as standards. *Dotted lines* enclose radioactive spots from hydrolysates

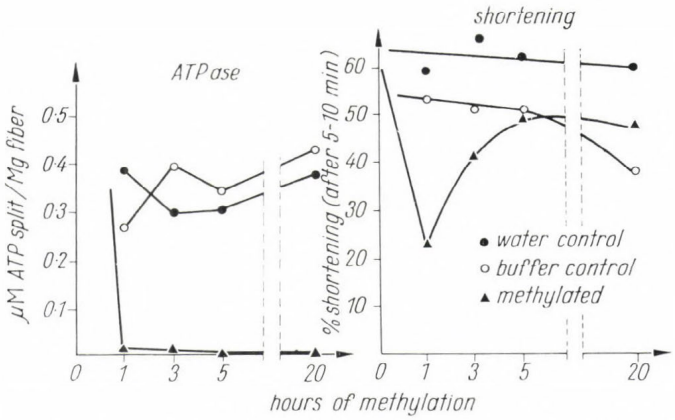

FIG. 45.—Effects of duration of methylation by dimethyl sulfate on the ATPase activity of glycerol-treated muscle fibers and on their shortening ability. Controls include fibers treated with water and with buffer only

less reactive to dimethyl sulfate than actin is. It would, therefore, be less affected by the shortening-inducing compound.

It is, therefore, to be concluded that methylated fibers shorten when complex anions are applied by undergoing sliding or interdigitation of the fine filaments into the thick filaments of the A-band.

The problem of where on the actomyosin molecule methylation occurs was studied by methylating with C^{14}-labelled dimethyl sulfate. The kind(s) of sites to which the labelled CH_3-radical had attached was investigated by autoradiography and paper chromatography (Irreverre and Martin 1954, Piez et al. 1956) of fiber hydrolysate (Fig. 44). The conclusion can be made that aspartic and glutamic acids, histidine and lysine are methylated with these methods and may therefore be involved in ATPase activity of actomyosin. Upon methylation, these provide a positively charged site, the neutralization of which provides an electrostatic attraction which shortens the fibers.

In Fig. 45 the results show that during the methylation process, the shortening associated with the splitting of ATP is inhibited during the first hour to about 1/3 of what occurs in untreated glycerinated fibers and then increases to about 4/5 of normal ATP-induced shortening. Figure 45 also shows that the ATPase activity of the fibers is reduced drastically during the first hour and continues to decline to zero activity at a slower rate. While this reduction in activity is occurring, the fibers regain shortening ability when ATP or pyrophosphate is applied.

These results, together with those of 1964, have led us to consider the shortening of methylated fibers by ATP, ADP, AMP, and pyrophosphate an anionic shortening. That is, it is caused by the binding of anions to excessively positively charged fibers and fibrils. These results also support the idea that shortening of non-methylated glycerinated fibers is caused, at least in part, by binding of an anion such as that of ATP to the fibers as was proposed long ago by Morales and Botts (1953).

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STRICKHOLM (Department of Anatomy and Physiology, Indiana University, Bloomington; and Department of Physiology, University of California, School of Medicine, San Francisco*): In striated muscle, it has been observed that local depolarization of small areas of the muscle cell surface produces a non-propagated localized contraction (Pratt 1930, Gelfan 1933, Steinman 1937, Huxley 1957, Huxley, Straub 1958, Huxley, Taylor 1958). This coupling of surface membrane excitation to intracellular contraction occurs only when the membrane is depolarized at those regions where the intracellular triad or transverse tubule structures approach the surface membrane. Thus in the frog sartorius muscle (*Rana temporaria*), it was found that depolarizing the membrane at the A band did not produce intracellular contraction while membrane depolarization at the center of the I-band or Z-disc, where the transverse tubules approach the surface, did produce contraction. These contractions were graded with depolarization intensity and had a maximum inward penetration of 10 μ which lasted as long as the membrane depolarization was maintained (Huxley and Taylor 1958).

The above experiments were later repeated by Strickholm (1961, 1962 and 1963) with a local quasi-voltage clamp technique which permitted small regions of the muscle cell surface to be voltage-clamped within a few millivolts with the simultaneous measurement of membrane current (Fig. 46). These later experiments on the North American frog sartorious muscle (*Rana pipiens*) gave different results than those observed on the European frog (*Rana temporaria*). As observed before, local contraction had occurred only beneath the excited membrane. In contrast to the other observations, this contraction appeared to involve the entire diameter of the I-band or Z-disc and had a depolarization threshold at which an entire I-band showed motion or not at all. The surface membrane above adjacent Z-lines also had different depolarization thresholds for contraction initiation. In contrast also to previous observations, this contraction was twitch-like, since it terminated and relaxed during a maintained depolarization. Although membrane depolarization appeared to be the initial trigger, it was observed that with a failing surface membrane, where the initial inward membrane currents were reduced, a stronger membrane depolarization was necessary to initiate contraction. This stronger depolarization increased the transient inward current toward its previous level. This suggested that a critical inward membrane current density was perhaps involved in coupling membrane excitation to contraction (Strickholm 1962).

* Address at the time of the Symposium

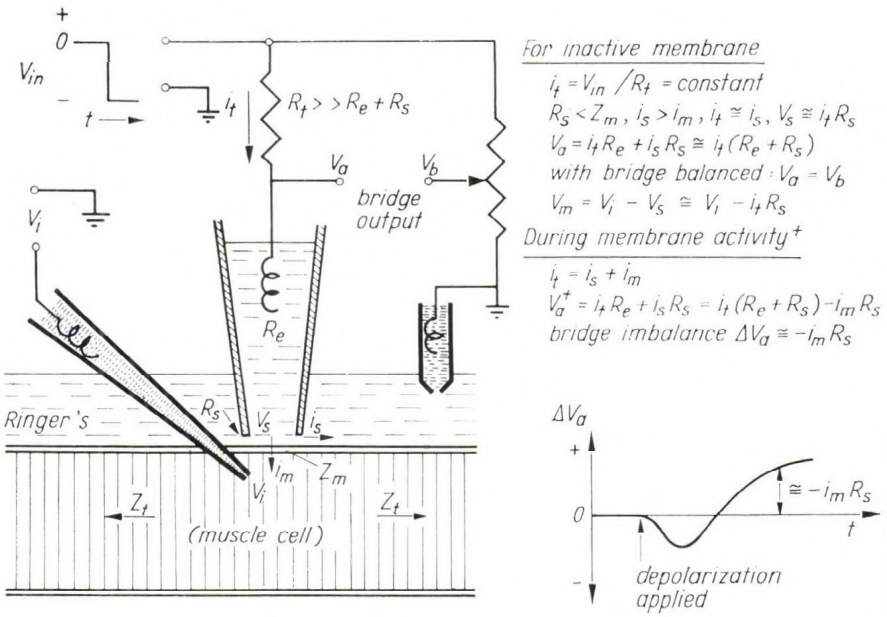

FIG. 46.—The local quasi-voltage clamp method

Subsequent research has indicated that contraction–coupling under a local regional voltage clamp is quite variable, not only across species and with different muscle types such as fast and slow, but also with similar fibers such as the frog sartorius which presumably consists of all fast fibers. These variations in the frog sartorius appear related to the seasons and can also occur with time as the muscle fibers deteriorate. Thus muscles examined after overnight cold storage in Ringer's rarely show similar contraction-coupling behavior as do freshly isolated muscles.

In summer frogs (*Rana pipiens*), local membrane depolarization of fast muscle fibers produces a local contraction, which involves only the sarcomeres lying beneath the excited membrane with no lateral spread of contraction (Fig. 47). This contraction typically involves motion in the entire diameter of each I-band. At a critical membrane depolarization which is different for each I-band, the entire diameter of each excited I-band either contracts or not at all. The contraction is twitch-like with relaxation occurring during a maintained surface membrane depolarization. Although the contractions appear all or none at initiation, they are probably graded since a strong depolarization produces more observable light scattering in the contracting regions than a weaker depolarization. There appears to be normally no gradation of contraction along the diameter of each I-band. However, in failing fibers, a gradation of contraction is observed.

Depolarization of the surface membrane of frog slow fibers produces a local contraction which ordinarily involves only those I-bands which are in the myofibril unit lying beneath the excited surface membrane (Fig. 47). The contractions are graded in that a weak membrane depolarization

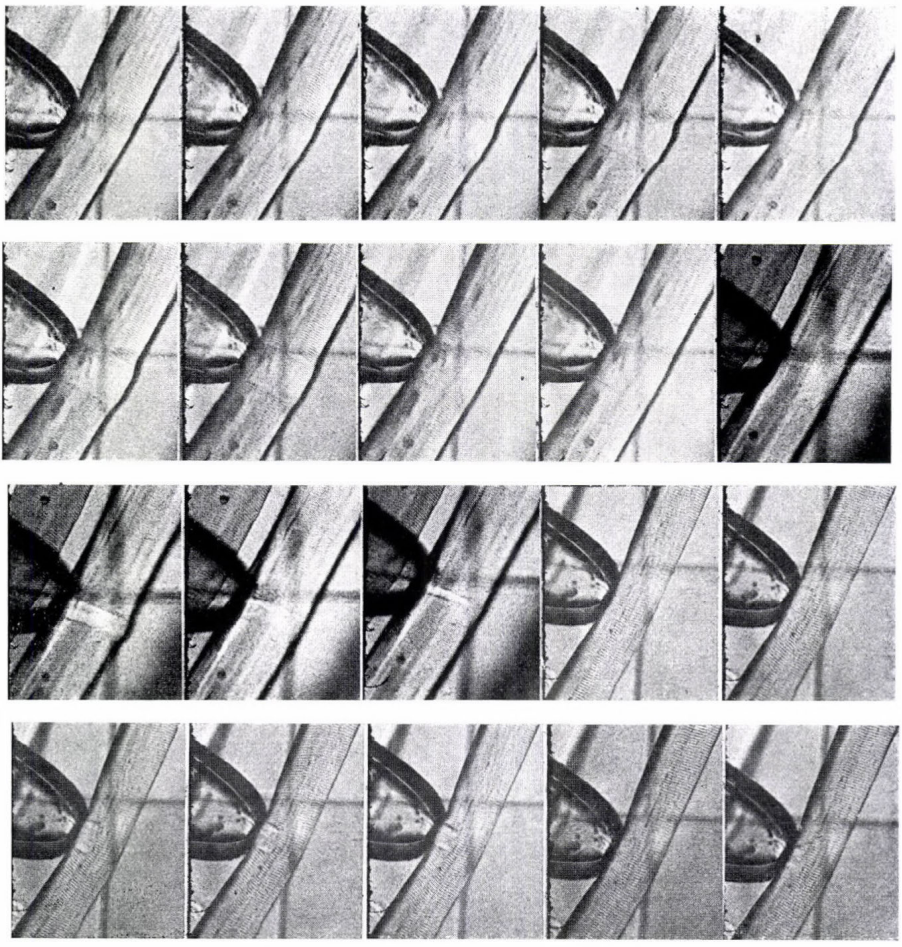

FIG. 47.—Cinephotography of locally excited frog muscle (*Rana pipiens*). Photography through polarizing microscope, polarizers parallel, $T = 20°$ C. *a* Fast fibers. A strong 10 millisec depolarization applied at zero time. Note bulging of muscle; *b* same fiber as in *a* but at a slightly different location. One second depolarization applied at zero time showing relaxation during depolarization; *c* same site as *b* but with a weaker depolarization showing fewer I-band contractions; *d* slow fiber. One second depolarization showing maintained sarcomere contraction; *e* same site as *d* but with a weaker one sec depolarization

produces contractions penetrating only a few microns inward, while with a sufficiently strong depolarization the entire diameter of a myofibril unit may contract. Occasionally, at a critical membrane depolarization, the entire subsurface myofibril unit will either contract or not at all in a manner similar to the fast fibers. However, the contracting myofibril units ordinarily do not relax under a maintained depolarization. With a strong depolarization, contraction often spreads to deeper adjacent myofibril units.

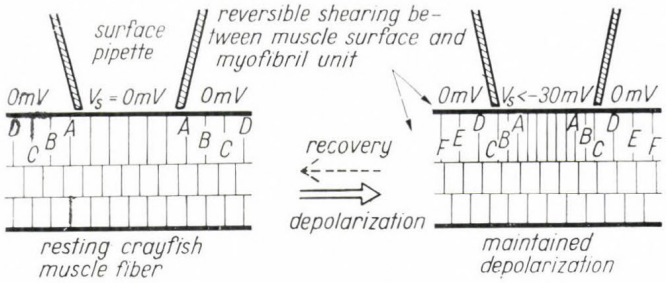

FIG. 48.—Reversible shearing between myofibril unit and surface membrane during local depolarization

The contraction in these deeper myofibril units usually relaxes under a maintained depolarization, while contraction of the immediate submembrane myofibril unit is maintained.

In the leg muscles of crayfish (*Procambarus clarkii*), local depolarization of the surface membrane produces a local graded contraction similar to that in slow fibers of frog. The depth of contraction penetration increases as the depolarization is increased. The local contraction also spreads slightly longitudinally to involve regions which are not under the excited surface membrane. This is in contrast to that observed in frog fibers where contraction appears normally confined to only the I-bands beneath the excited membrane region.

If a large surface region of crayfish muscle is excited which covers perhaps 10—20 I-bands over a myofibril unit, contraction of the I-bands under the excited membrane will pull relaxed sarcomeres, which are not under the excited surface membrane to beneath the excited membrane. The moment these relaxed sarcomeres enter the region beneath the excited surface membrane, contraction occurs which pulls additional relaxed sarcomeres under the excited membrane region. With a sufficiently strong depolarization, this process continues until the sarcomeres are maximally contracted to a length approximately equal to the A-band. In this process there is no movement of the cell surface. This contraction is reversible and suggests that with contraction, the transverse tubules are either reversibly sheared from the cell surface or that each myofibril unit can shear or slide reversibly away from the transverse tubule system and endoplasmic reticulum (Fig. 48). The surface pipette can be moved along the surface membrane to lie above where the myofibril units have sheared from the cell surface with no change in the contraction–coupling properties, which also indicates reversibility of the process.

According to our studies about the relation of membrane current to contraction–coupling we suppose: this intracellular generated potential may be related to the time course of intracellular Ca^{++} release and disappearance in toad sartorius muscle described by Jöbsis at this Symposium. Contraction–coupling is therefore tentatively postulated to involve a coupling of the action potential to the transverse tubule system, either by involving the leakage current or simply by depolarization. A signal which might

also involve the leakage currentagated prop down th ise tubule system, transferred to the endoplasmic reticulum which when excited releases Ca^{++} ions resulting in muscle contraction. When the endoplasmic reticulum recovers from excitation it recovers its Ca^{++} binding or accumulating capacity and relaxation begins when calcium is removed from the intracellular space.

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ENERGETIC ASPECTS OF MUSCULAR CONTRACTION

D. R. WILKIE

UNIVERSITY COLLEGE, LONDON

The distinctive function of muscle is to convert chemical energy into mechanical force and work. I propose to discuss the energetic aspects of this conversion under the following headings, and to ask a number of questions which I hope will then provide a framework for the subsequent general discussion.

(1) *Energy balance.* Over the whole cycle of contraction and relaxation it is now possible to account quantitatively for the energy output observed (heat + work) in terms of the measured breakdown of phosphocreatine (PC). It is therefore possible to calculate the *in vivo* heat of hydrolysis of PC with high accuracy, and to infer an approximate value for the heat of hydrolysis of ATP. The relevant thermodynamics are quite straightforward since only the First Law is involved, and this says simply that the energy output in the form of heat, h, and work, w, over any specified interval of time must be given by:

$$h + w = n_1(-\Delta H_1) + n_2(-\Delta H_2) + \text{ etc.}$$

where n measures the number of moles of each reaction for which ΔH gives the enthalpy change in kcal/mole (see Wilkie 1960).

It is simplest to work under conditions where the chemical changes in the muscle have been simplified so that only one chemical reaction is contributing substantially to the output of energy. This is thought to be the case in muscles at 0° C poisoned with iodoacetate and nitrogen so that both glycolysis and oxidative phosphorylation have been prevented. The only doubt is over the question whether hexose phosphates are formed in substantial amounts. Earlier reports by Carlson and Siger (1960) and by Mommaerts, Seraydarian and Maréchal (1962) claimed that this occurred only after considerable activity, while Spronck (1965, and at this Symposium) appears to have demonstrated this reaction after only five twitches. The matter remains somewhat doubtful, so it has been assumed in what follows that the hydrolysis of PC is effectively the only reaction that occurs. The first attempts to equate energy output with PC breakdown were mainly concerned with isometric contractions in which no external work was performed (Carlson and co-workers 1962, 1966).

In the series of experiments reported here, various other types of contraction have also been investigated. The technique for measuring heat in experiments of this kind has been improved by the introduction of thermopiles of the self-calibrating 'wafer' type (Wilkie 1963).

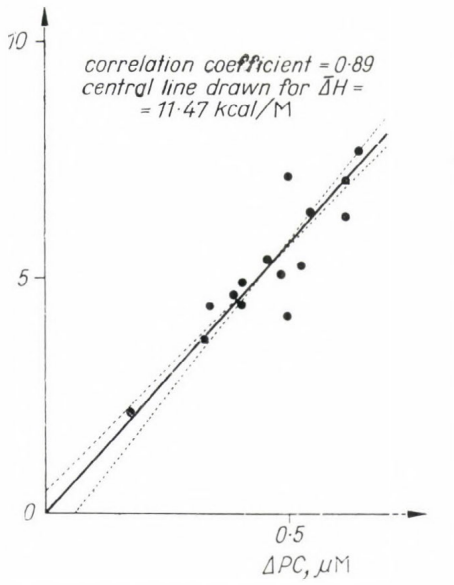

FIG. 1.—Energy output, mcal; ordinates: plotted against phosphocreatine breakdown, Δ PC, μM; abscissae: following 30 isometric twitches. N_2 and IAA-poisoned frog sartorius, 0° C

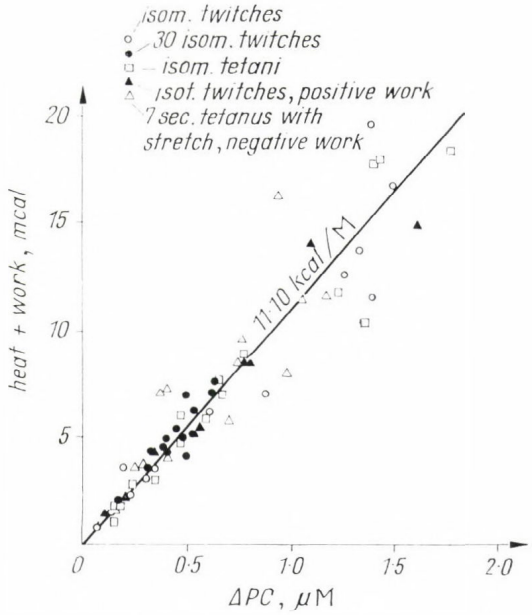

FIG. 2.—Ordinates, abscissae and experimental conditions as in Fig. 1, but various different types of contraction, as indicated in the figure

Figure 1 shows the relation between total heat output and PC splitting in muscles that have undergone a series of 30 twitches at 3 sec intervals. In order to spread out the points and thus to reveal the functional relation between the variables, it is essential to choose muscles of widely varying weight. The correlation between heat and PC split is high ($r = 0{\cdot}89$, $P \ll 0{\cdot}001$) and it is clear that the points are well fitted by a line passing through the origin with a slope of 11·5 kcal/mole.

In Fig. 2 the same type of method has been applied to various other types of contraction, as explained in the legend, and including the performance of positive work—which by the Fenn effect augments the energy output to 130 per cent of its isometric value—and of negative work which suppresses the energy output to about 70 per cent of the isometric value. In all cases the relation between energy output and ΔPC is the same and corresponds to an *in vivo* enthalpy change of 11·1 kcal/mole—the best estimate available at present.

It had been hoped (see Dydyńska and Wilkie 1966) that a value for the *in vivo* enthalpy change during ATP splitting could be found from experiments similar to those just described, save that the muscles had been poisoned with fluorodinitrobenzene (FDNB), where there was reason to think that ATP splitting provided the sole source of energy. However, it is only for the first second or two of contraction that the chemical changes are so simple, and during the subsequent 40 sec that were required to remove the muscles from

the thermopile many other reactions occurred. It was observed in these experiments that the heat produced per gram of tension developed in a twitch was the same in FDNB-poisoned as in normal or IAA-poisoned muscles. In the first case the energy came from the ATP split; in the latter two cases it came from the splitting of an equal number of moles of PC. Thus it can be inferred that the *in vivo* enthalpy change for ATP splitting must be about the same as that for PC splitting, i.e. about 11 kcal/mole.

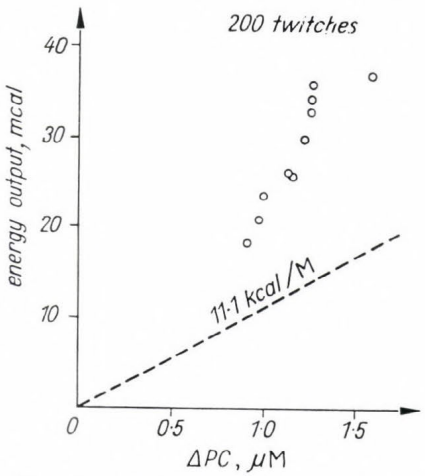

FIG. 3.—Frog's sartorius in N_2 but otherwise unpoisoned, showing the changes that follow a series of 200 twitches. More energy is given out than can be accounted for by the PC split

The difficulty in drawing up an energetic balance when several different reactions are going on is simply that the errors in all the chemical determinations soon accumulate to such a degree that the functional relationship is obscured. That this need not always be the case is shown in Figs 3 and 4, which show the results of experiments on unpoisoned muscles in nitrogen, where the energy for contraction is derived from two sources—the breakdown of PC and the formation of lactate. As shown by comparing the points with the line in Fig. 3, appreciably more energy is given out than can be accounted for by the PC split. The discrepancy, as shown in Fig. 4, is roughly proportional to the lactate produced, and indicates a ΔH value of about 23 kcal/mole for this reaction. However, this value should be considered tentative at present, until more consideration has been given to the effect of the changes in hydrogen ion concentration.

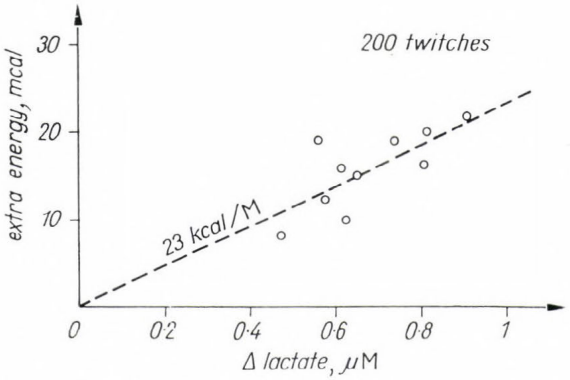

FIG. 4.—The same experiments as in Fig. 3. The extra energy produced, over and above that expected from PC breakdown, has been plotted against the lactate produced

One major problem that remains is whether the equivalence of PC splitting and physical energy that is found for the whole cycle, is also found from moment to moment within the cycle.

If it is, this suggests that we are concerned with a single chemical flux, whose speed and extent are modulated by the mechanical conditions of contraction. If not, then the disparity indicates the existence of hitherto unknown chemical or physical processes whose store of energy makes up the difference. The experiments described in an earlier session by R. E. Davies suggest that there may indeed be such a discrepancy and that only a small part of the heat observed in a muscle that is shortening rapidly can be accounted for by the ATP or PC split up to that time. If these results are confirmed it will establish the existence of a process associated with large heat changes that must be reversed during the later stages of contraction or during relaxation. Linked to this is the question whether it is meaningful to divide the total energy flux into elements corresponding to activation heat, shortening heat, etc. Does the energy flux vary with muscle length in the manner that would be expected of a system of sliding filaments? What fraction of the energy flux is linked to the contractile machinery, and what to ancillary services such as the pumping of calcium that is believed to switch the contractile process on and off? Does this pumping require a continuous expenditure of energy all the time a muscle is active, or is it required only once, during relaxation? The answer to some of these questions may well be provided by experiments such as those described by Dr Jöbsis.

(2) *Efficiency.* The efficiency of their muscles is clearly a matter of the greatest importance to animals and men; nevertheless both in mammals and in frogs only 20 to 25 per cent of the free energy available from the oxidation of carbohydrate can be converted into mechanical work. The first concern of an engineer confronted by an inefficient machine is to find out in which part of the machine the major wastage is occurring. When a physiologist asks the same question about muscle the answer is most unsatisfactory. We simply do not know what fraction of the total wastage arises during the rapid conversion of chemical to mechanical energy during contraction, and how much during the far slower—purely chemical—recovery process, in which the depleted store of PC is rebuilt. Even the existence of this unsatisfactory state of affairs is not widely recognized. Fundamentally the difficulty arises because the concept of efficiency is inextricably bound up with the Second Law of thermodynamics. In a system such as a muscle, which converts chemical energy to mechanical work and operates at uniform temperature, the proper measure of efficiency (e) (see Wilkie 1960) is

$$e = (\text{work obtained})/(\text{free energy used up})$$

In terms of the measured heat, h, and work, w, it can easily be shown that

$$e = \frac{w}{w + h} \times \frac{\Delta H}{\Delta F}$$

where ΔF is the free energy change in kcal/mole. During the actual process of contraction, everything in this equation is known except ΔF, and until ΔF is unknown, the efficiency cannot be calculated. Satisfactory measure-

ments are available of the free-energy change *in vitro* under standard conditions (see George and Rutman 1960; Huennekens and Whitely 1960). However, it is not at all plain what value one should adopt for the ΔF of ATP splitting or PC splitting *in vivo*, where the standard free energy change must be corrected for the concentrations (or strictly, the activities) of the participating reactants, and also for the secondary reactions associated with the movement of hydrogen ions. In consequence (see Wilkie and Woledge 1966) the value of ΔF *in vivo* is uncertain over the range $-10{\cdot}3$ to $-12{\cdot}8$ kcal/mole, and perhaps beyond it.

For the whole process of contraction followed by oxidative recovery it is known that $\Delta F \simeq \Delta H$ so it can be deduced from measurements of work and heat that the genuine thermodynamic efficiency is about 0·2 in the case of frogs' muscle. Unfortunately, we cannot know the efficiencies of the initial and the recovery processes separately until the appropriate value of ΔF for the initial process is established. Considering ΔF as unknown—perhaps to an exaggerated degree—the consequences are shown in the following table:

ΔF, kcal/mole	-5	-10	-15
initial efficiency	0·88	0·44	0·29
recovery efficiency	0·23	0·46	0·69

Thus according to the value adopted for ΔF, the inefficiency in the muscular machine as a whole may reside almost entirely in the initial chemical $\rightarrow$ $\rightarrow$ mechanical conversion, or it may arise in the purely chemical reaction sequences that occur during recovery. To some chemists the very idea of efficiency is unacceptable when it is applied to reactions of fixed stoichiometry, such as are presumed to occur during the metabolic processes of recovery. Nevertheless, inefficiency does arise in such reactions: it has a clear definition—the entropy created or free energy dissipated; and to measure it should not be beyond our powers! After all, it is only at equilibrium that $\Delta F = 0$. In order that a reaction should run spontaneously, it is essential that the concentrations of reactants and products should be displaced from their equilibrium values in such a way that the net value of ΔF becomes negative. It is this free energy change that drives the reaction; and in driving the reaction, part or all of the free energy becomes degraded into heat. This degradation is the source, and also the measure, of the inefficiency of the process.

This idea is illustrated in Fig. 5a, in connection with the Lohmann reaction by which, during contraction, ATP is rapidly regenerated from ADP and PC.

$$\mathrm{ADP} + \mathrm{PC} \rightleftarrows \mathrm{ATP} + \mathrm{C}$$

The equilibrium constant of this reaction has been assumed to be about 19 (Carlson and Siger 1959) and the calculated curves show how the equilibrium concentrations of PC and of ATP would fall as the muscle was progressively exhausted, assuming that no regeneration of ATP occurred, e.g. in a muscle poisoned with iodoacetate and nitrogen. Consider the muscle as it passes from its resting state (*1*) to a partially exhausted state (*2*). The final concentrations of ATP and PC must lie on the equilibrium curves, but in order to move from (*1*) to (*2*) the concentration of ATP must have

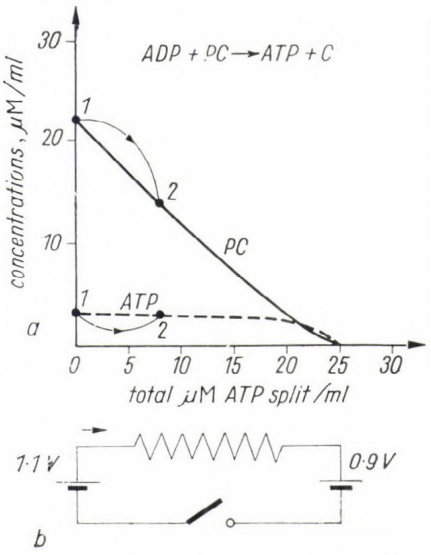

FIG. 5.—*a* Theoretical curves to show how the concentrations of ATP and PC fall as a muscle is progressively exhausted; *b* a theoretical model to illustrate the energetics of two coupled reactions

been lower, and that of PC higher, than the equilibrium values, as indicated qualitatively by the lines with arrows. It is this displacement from equilibrium that provides the free energy whose degradation to heat drives the reaction forwards. This situation has not yet been fully studied from the quantitative point of view, but it may provide a method of calculating the efficiency of a purely chemical process. The free-energy change in passing from (*1*) to (*2*) depends only on the concentrations at those two points and is independent of the path followed in between. This leads to the rather surprising conclusion that the efficiency of the process is not dependant on the speed of the reaction.

The situation is formally similar to that depicted in Fig. 5b, in which two ideal galvanic cells of different voltage can be connected electrically. For every 96 500 coulombs that flow, 1 gram equivalent of reaction will occur in cell (*1*) in the spontaneous 'downhill' direction, and an equal quantity of reaction will be produced in cell (*2*) in the 'uphill' direction. The speed of these coupled reactions can be varied by altering the value of the resistance, but in every case the free energy degraded per gram equivalent of reaction is the same, 96 500 × 0·2 joules. The efficiency is therefore always the same, 0·9/1·1 = = 0·82. Varying the electrical resistance is analogous to varying the amount and activity of enzyme in the chemical case.

(3) *The mechanism of energy conversion.* How exactly do the cross bridges convert chemical into mechanical energy? About this—the most fundamental question in muscle physiology—we have very little to guide us. The only man-made systems for direct conversion of energy depend on ionic changes in polyelectrolyte gels or collagen fibres, as will be demonstrated by Dr Oplatka; and although it is true that ionic changes accompany the splitting of ATP, these seem to be incidental and not related to the extraordinary, and so far completely unexplained, specificity of ATP in energy-using biochemical reactions. Theoretical models such as those of A. F. Huxley and R. E. Davies indicate that with our present level of knowledge (or ignorance) some of the observed behaviour of muscle may be accounted for on the basis of widely different premises. Specific tests for either theory do not seem possible at present. Such theories are called on to explain an awkward range of phenomena—not merely the conversion of energy from one form to another but also the dependence of the chemical flux on mechanical conditions (shortening heat and Fenn effect) and also the suppression of chemical flux that results from performing work *on* the

active muscle. Classical thermodynamics provides a somewhat arid—though altogether necessary—set of boundaries within which all theories must operate. Perhaps the modern development of irreversible thermodynamics, with its emphasis on fluxes and the relation between them imposed by coupling, will be more successful in indicating not merely boundaries but also possible mechanisms of operation.

Irreversible thermodynamics deals among other things with the situation that exists in a contracting muscle, where a flux of chemical reaction is linked in some way to an output of mechanical energy.

The rate at which free energy is being degraded into heat is called the dissipation function, i.e.

$$D = A \times h + P \times v$$

where A = affinity, $(-\Delta F)$, g.cm/mole
h = rate of reaction, mol/sec
P = force, g wt
v = speed of shortening, cm/sec.

Note that all the terms in the equation have the dimensions of power. In a contracting muscle, P is a negative number; thus D is less than $A \times h$ and only part of the flux of chemical free energy is being dissipated into heat, while the remainder is transformed into mechanical power, $P \times v$.

Dr S. R. Caplan (1966) has recently proposed a theory to account in some detail for the way in which chemical and mechanical flux are linked to each other. This theory is attractive in that it successfully predicts the shape of the force–velocity curve and also predicts a relationship (whose existence has been long suspected, and recently demonstrated experimentally by R. C. Woledge, 1966) between the curvature of the force–velocity curve and the thermodynamic efficiency. If the theory could be substantiated, therefore, it would resolve the uncertainty discussed earlier about the separate efficiencies of the initial and of the recovery processes.

Dr R. C. Woledge and I (Wilkie and Woledge 1966) have therefore tried to compare the theory with some experimental facts about muscle. Caplan assumes, as is usual, that the muscle obeys linear phenomenological equations:

$$v = L_{11} P + L_{12} A \text{ (cm/sec)}$$

and

$$h = L_{21} P + L_{22} A \text{ (mol/sec)}$$

where the L are constant coefficients. All the quantities are positive except P. In words, both the speed of shortening and the rate of chemical reaction are helped by a large affinity and hindered by a large force. Without going into details, the model system evolved from these equations has two degrees of freedom, while the muscle has only one—if the force is specified, for example, speed and chemical flux are completely determined. Caplan has accordingly introduced another restriction into his model in the form of a *regulator* which imposes an additional relation between input and output. The characteristics of this regulator were deduced on very general grounds, and it was then found that this led to a relation between force and speed that was identical with A. V. Hill's well-established equation for the force–

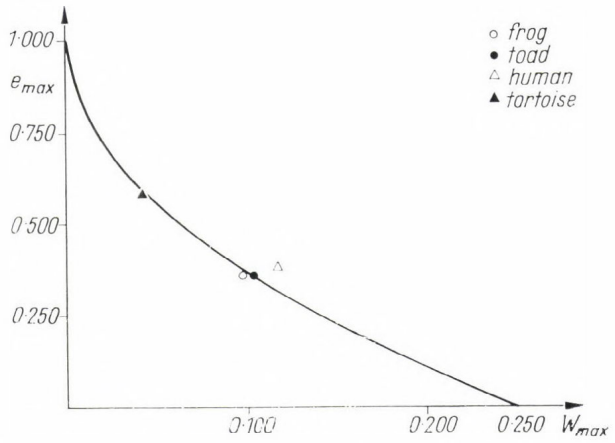

FIG. 6.—Relation between maximal efficiency (ordinates) and curvature of the force–velocity curve (abscissae). This curvature is measured as W_{max}, which is defined as

(maximum value of $P \cdot V$)/($P_0 \cdot V_0$)

where P = force, V = speed, P_0 = isometric force, V_0 = unloaded speed. The smaller the value of W_{max}, the more curved is the force–velocity curve

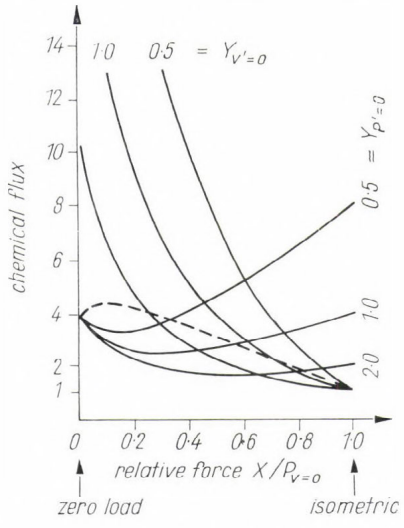

FIG. 7.—The variation of chemical flux as a function of the force exerted by the muscle. The interrupted line shows what is observed experimentally. The full lines show the predictions from Caplan's theory, making various assumptions about the initial conditions

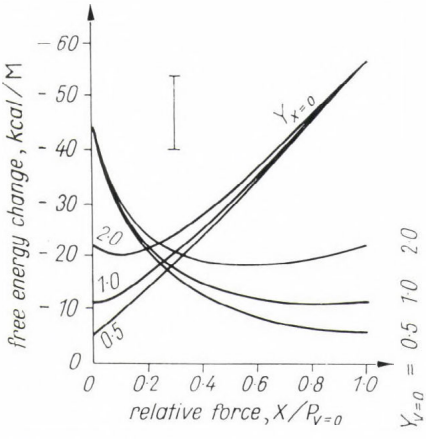

FIG. 8.—The variation of free-energy change as a function of the force exerted by the muscle. Curves drawn from Caplan's theory, making various assumptions about the initial conditions. The vertical bar shows the change in free energy that could be derived from a 100fold change in concentration ratio

velocity curve. Most attractive of all, from our point of view, was the fact that the thermodynamic efficiency could be directly predicted from the shape of the force–velocity curve. This theoretical relation is shown by the curve in Fig. 6, where the ordinate shows the maximum value of the efficiency and the abscissa is a measure of the degree of curvature of the force–velocity curve, more curved to the left, less curved to the right. Experimental values of (work)/(work + heat) are available for the frog, toad and tortoise, and can be inferred for man. In order to convert them into measurements of the efficiency it is necessary to know or assume the value of $\Delta F/\Delta H$. If this is taken as 1·3 (a perfectly reasonable value on other grounds) then Fig. 6 shows quite a good fit between the experimental points and Caplan's theoretical curve.

The 'regulator' in Caplan's model works by varying both the chemical flux, h, and the affinity, A, as a function of the force on the muscle; and this provides further means for comparing theory and experiment. In Fig. 7 the interrupted line shows roughly how the observed chemical flux varies as the load on the muscle is changed. The full lines are predictions from the theory making various assumptions about the initial value of $\Delta F/\Delta H$. Clearly the theoretical and experimental curves cannot easily be reconciled. Finally, Fig. 8 shows how the affinity of the chemical reaction must vary with load. There is no experimental curve to serve as a basis of comparison, but even without one it seems fair to comment that such enormous variations in the free energy of the driving reaction could hardly occur in reality. The vertical bar shows how small a change is produced by even a hundredfold change in the concentration ratio. However, it is certainly to be hoped that some modification of this general scheme may be found that will preserve some of the virtues of Caplan's theory while removing some of its discrepancies. In the meantime, while we are searching around for clues in physical chemistry, there are many other purely biological problems to consider. Do all the different types of muscle contract by essentially the same means? If so, discovering what they have in common may point out what is essential for a contractile machine. How about the restrictions within which animals, under the stress of evolution, have arrived at the compromises best suited to their conditions of life? Did the tortoise pay for his highly efficient muscles by accepting slow speed, or a very curved force–velocity relation, or both? And why was he obliged to pay?

I wish to acknowledge the generosity of the welcome trust in providing the travel grant, which enabled me to attend the Symposium in Budapest. Finally, I am deeply indebted to the Hungarian Academy of Sciences for their generous hospitality.

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GENERAL DISCUSSION

DAVIES: Commented on the problem of the shortening heat. In the whole contraction cycle the appearance of the shortening heat is very disputable. (Manuscript of comment has not arrived.)

TIGYI: According to Prof. Wilkie's lecture, the heat + work/mcal values do not differ in the various types of contraction. It means, implicitly, no experimental evidence of the phenomenon 'heat of shortening' published by A. V. Hill.

I would like to mention our microcalorimetric experiments which never showed any sign of the heat of shortening (Tigyi 1954, 1959). On the contrary, the heat production of strictly isometric contraction (at resting length) compared with the loadless isotonic one was measured to be always about 30 per cent higher. The quantitative values are shown in Table I.

TABLE I

Comparison of heat production of 'isotonic' and 'isometric' tetani of the m. semimembranosus of frog

No.*	Type of contraction	Heat produced mcal/g
1	Isotonic	26·3 ± 2·3
2	Isometric	29·8 ± 2·2
3	Isotonic	17·5 ± 1·8

* Numbers mean the sequence of experiments.

Similar results are gained also when comparing the isotonic and isometric twitches.

To explain the extra heat production of isometric contraction, we suppose the existence of the 'heat of crystallization' of myosin as a possible reason (Ernst 1963). Many other experiments, e.g. volume measurements, birefringence studies, heat production of stretched myosin threads, etc. support this hypothesis.

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The comment was followed by a discussion about the details of the shortening heat, mainly the methodological peculiarities were discussed. Participants in order: Wilkie, Tigyi, Aubert, Tigyi, Ernst, Wilkie, Hoyle, Wilkie.

ERNST: The measurement of the heat accompanying the so-called isometric contraction, if described in this simple way, does not permit to establish any exact connection between the mechanical and energetic side of contraction. First of all, the relative length of the muscle (compared with the resting length) needs to be given, because the muscle stretched over its resting length increases its heat production and metabolism and (e.g. lactic acid) even without being stimulated. Furthermore, the myosin or actomyosin of the active muscle being hindered in shortening undergoes crystallization, which is accompanied by heat production as has been shown by us on living fibrils and also on threads made from extracted actomyosin (Ernst 1963).

Thus, the heat accompanying a so-called isometric contraction cannot serve as a quantitative datum concerning the bioenergetics of the muscular activity.

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GERGELY: Commented on the efficiency of ATP + $C_v \rightarrow$ ADP + CP reaction and the postulate of an intermediate. (No manuscript.)

WILKIE: Argued with stoichiometric evidence.

PAGE: Dr Wilkie, as you know, one of the assumptions underlying the application of the thermodynamics of irreversible processes to a system is that the state of the system be not too far removed from a state of equilibrium. Since you have applied non-equilibrium thermodynamics to muscle, can you estimate for us how realistic this assumption is for a contracting muscle?

WILKIE: According to my own supposition these phenomena are in a state not far from equilibrium, therefore, the formulas of the thermodynamics of irreversible processes are applicable.

AUBERT: Is it not so that the non-equilibrium thermodynamics and the phenomenological equations (including Onsager's symmetry relation) can be usefully applied only when the sum of products of conjugate forces by fluxes is proportional to the rate of entropy creation?

Now, I do not see clearly how you can assimilate the product of force by velocity to a rate of free energy dissipation.

OPLATKA: Theoretical considerations of contraction of collagen fibres were produced with the demonstration of a model 'diffusion controlled contraction'. (Manuscript did not arrive.)

JÖBSIS: I would like to say a few words about some experiments on the Fenn effect. Yesterday I introduced two ideas concerning the energetics of muscular contraction. The first was the adequacy of the time integrals of the force and of the shortening as indices to the total energy turnover in twitches and short tetani. The other was the apparent lack of a distinct contribution from activation to the overall energy utilization. Today I would like to present data on the application of these concepts to the interpretation of the Fenn effect.

The total energy utilizations of contraction under varying afterloads were compared by measuring the area under the fluorometric response cycle

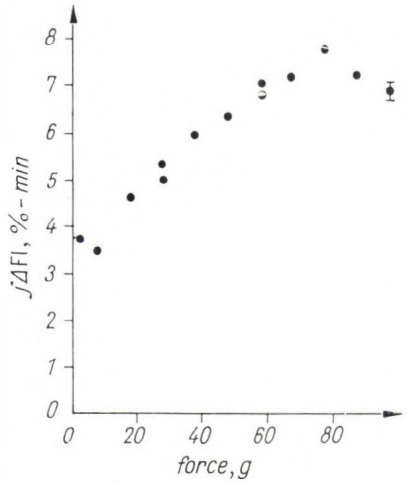

FIG. 9.—Relative energy utilization in the single twitch as a function of the force developed during the contraction. Toad sartorius, 11° C. Initial load 2·0 g; all other loads are after-loaded. Four cycles were recorded for the isometric twitch; the bar shows the spread of the data. Two cycles were recorded for the minimally loaded isotonic condition; the $\int \Delta Fl$ was virtually identical for these two cycles (Jöbsis and Duffield, in press)

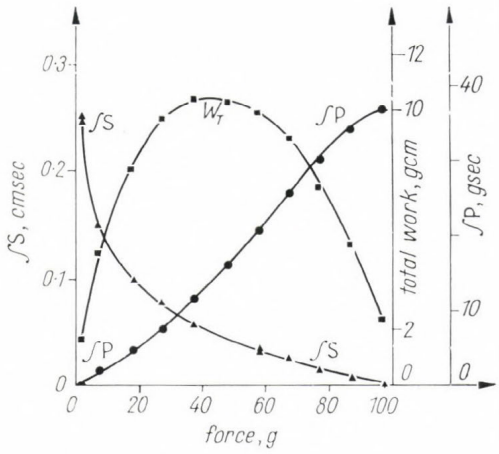

FIG. 10. — Total work (W_T) and the time integrals of shortening ($\int S$) and of force ($\int P$) as a function of the force developed during the contraction. Data from the same experiment as Fig. 9. The measurements of most duplicate points were virtually identical and could not be differentiated in the drawing (Jöbsis and Duffield, in press)

$\int \Delta Fl$). In Fig. 9 the data of such an experiment for the single twitch of the toad sartorius is shown on the usual ordinates of energy as a function of the load. This set of data shows that the minimally loaded isotonic contraction is much less expensive energetically than the isometric one. The original work of Fenn shows the opposite trend, i.e. the minimally loaded isotonic contractions use more energy. However, the present data are in agreement with other measurements. I do not wish to review the status of the field on this question but curves similar to Fig. 9 occur more frequently at higher temperatures than at 0° C. The use of toad rather than frog sartorii is also a contributing factor. The fluorescence data show a minimum energy utilization near the completely isotonic and a maximum at approximately 80 per cent of the isometric tension. A maximum at some intermediate load rather than at one of the extremes of the scale appears to be common to all published curves.

Simultaneously, we have measured the shortening and tension parameters of the contraction. Shortening measurements speak for themselves and the time integral of this is shown in the left hand curve of Fig. 10. The tension was measured with strain gages on the isotonic lever. These showed the tension rising until that of the load was matched. Subsequently, this tension level was maintained during the period that the load was lifted and returned again to the after-load screw. The end of the entire twitch was marked by the final relaxation of tension. The areas

under the tension ($\int P$) and shortening ($\int S$) aspects of the twitch were measured with a polar planimeter. The amount of work (W_T) was calculated from the external work done by lifting the load + the work involved in deforming the lever and finally the internal work estimated according to the method of Jewell and Wilkie (1958). (Further details are reported in Jöbsis and Duffield 1967.)

I would like to propose now that these three parameters, $\int S, \int P$ and W_T are sufficient to describe the Fenn effect curves. The specific hypothesis is, therefore, that the three curves of Fig. 10 can be scaled and summed so as to produce a curve closely matching the experimental points of Fig. 9. In order to do this, the fluorometric response cycle ($\int \Delta Fl$) is set at

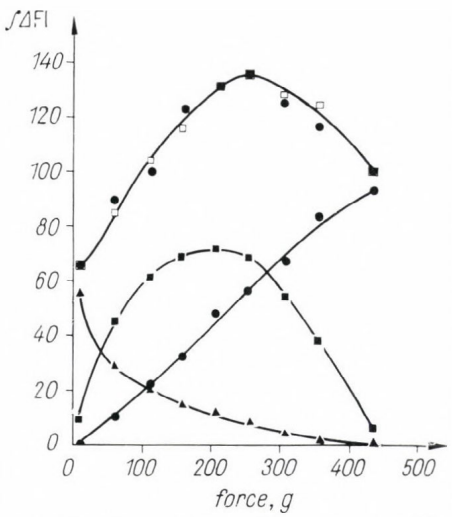

FIG. 12—Comparison of the energy utilization (●) in short tetani with the scaled and summed contraction parameters (□). Bull frog sartorius, 15° C. Initial load 10·0 g; all other loads are afterloaded. Stimulation by 4 supramaximum shocks delivered at 24 cps. W_T (●); $\int S$ (▲); $\int P$ (●) in lower curves. Quadruplicate and triplicate points have been averaged at the 10 g isotonic and at the isometric points (Jöbsis and Duffield, in press)

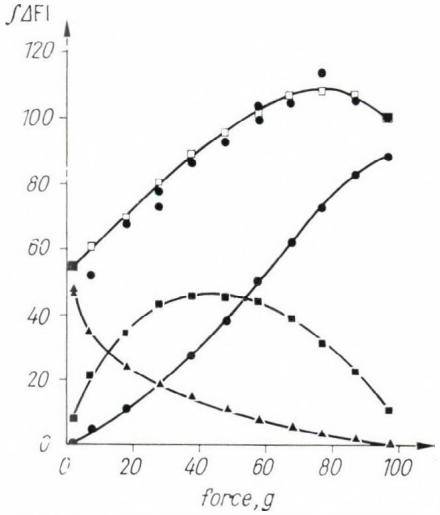

FIG. 11—Comparison of the energy utilization (●) with the summed W_T, $\int S$ and $\int P$ parameters after scaling (□). Same experiment as in Figs 9 and 10 (Jöbsis and Duffield, in press)

100 per cent at the isometric end of the scale and the other values are related to it. The value of the sum of $\int S$, $\int P$ and W_T are set equal to the experimentally found $\int \Delta Fl$ value at 3 loads (the minimally loaded isotonic, the isometric case and a load halfway between them). The three simultaneous equations thus produced are solved and the three scaling factors are found. Applying these factors to the remaining experimental points of the three parameters at various loads yields, therefore, three new curves of $\int S$, $\int P$ and W_T. If the hypothesis is correct, the sum of these three parameters at any load should now be close to the value of the $\int \Delta Fl$ at that load. In other words, the degree of matching of the $\int \Delta Fl$ data and the summed, scaled $\int S$, $\int P$ and W_T data indicates the degree of confidence (or lack of it) in our hypothesis.

In Fig. 11 the results of this procedure are illustrated. The lower three curves are the parameters depict-

ed earlier in Fig. 10, but this time after the scaling procedure. The open squares in Fig. 11 represent the value of the summed, scaled parameters and the filled circles the experimental $\int \Delta Fl$ points. The fit is quite satisfactory for the hypothesis that the main energy utilization in the twitch is directly related to the sum of $\int S$, $\int P$ and the total work. The deviations from absolute coincidence appear to be a matter of experimental error rather than a trend suggesting an overlooked contribution or a completely erroneous working hypothesis.

An attempt was made to find conditions yielding more closely the sort of curve described by Fenn, in which all isotonic contractions utilize more energy than the isometric ones. We found that the bullfrog sartorius at approximately 15° C is, with some difficulties, amenable to fluorescence analysis. At this temperature short (four-stimulus) tetani yield curves that begin to be reminiscent of those of Fenn (see the top of Fig. 12). The presentation in the figure is completely analogous to that of the experiment with the toad sartorius depicted in the first three pictures. Again it is clear that the summation of the scaled parameters yields a curve in satisfactory agreement with the experimentally determined variation of energy utilization with the load.

In none of these experiments was the activation energy a necessary or useful parameter in the energetic picture. In fact, a sizable contribution, i.e. more than approximately ten per cent of the total energy involved in the isometric or isotonic twitch, could not be fitted in, while a good fit was maintained between the experimental data and the calculated values. Since I showed it yesterday that the $\int S_0$ and $\int P_0$ are parameters intimately connected with the kinetic model, the present analysis of the Fenn effect should tend to strengthen the interest in the kinetic approach as a fruitful perspective on muscular contraction.

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GERGELY: I wonder if Dr Jöbsis sees an amount of energy directly related to a change in length.

JÖBSIS: No, not really. What I see is an amount of energy related to the time taken in shortening and to the amount of shortening that has taken place. It is inherent in my statement that energy utilization is directly related to $\int S\mathrm{d}t$ (or to $\int \Delta l\mathrm{d}t$). The way I visualize it is that during rapid shortening each cross bridge is broken only after a small amount of over-all shortening has taken place. In the case of the lifting of a heavier load, shortening is rather slow and some bridges are made and broken, owing to the elastic stretching of other parts of the fibre, i.e. without yielding external shortening. I consider that during the maintenance of the shortened state there is a continued internal tension development, but this is against the internal restraints of membranes and tendons and even against the folded myofilaments. In other words the situation is much like the isometric case, except that the shortened muscle is producing internal tension rather than external tension. In addition, of course, the internal tension generated is much less

than in the isometric contraction at rest length, because the overlapping of actin filaments, etc. prevents the formation of the maximum number of cross bridges. These types of processes are the ones that result in the linearity between energy and $\int S$.

AUBERT: Prof. Wilkie has put the question of equivalence in the chemical and the physical energy from moment to moment within the cycle of contraction and relaxation. As shown by Prof. Davies and his colleagues the use of the drug 2,4-fluorodinitrobenzene (FDNB) opens the way to the solution of this problem, as it allows us to block the Lohmann reaction, leaving ATP as the main source of energy for the contraction. The complexity of the chemical reactions that continue in the poisoned muscle has, up to now, made impossible to draw up an accurate energetic balance sheet, but an interesting point has emerged from the study of the time course of heat production in such poisoned muscle.

As I recalled yesterday, after Dr Jöbsis's presentation, the development and the early stages of the maintenance of tetanic tension is associated with a heat production rate of the type

$$h = h_A \exp(-\alpha t) + h_B$$

where h_A is the so-called labile fraction and h_B the steady level obtained after a few seconds of tetanisation. Now, when frog sartorius muscles have been poisoned for one hour at 0° C with 0·4 *m*M FDNB—a concentration sufficient to block completely the Lohmann reaction, according to direct controls by Maréchal—the steady rate of heat production h_B falls progressively with every new tetanus, whereas, the labile fraction h_A remains practically unaltered, even after four contractions.

It is thus possible that the labile heat production is correlated with the use of ATP at the level of the contractile proteins, while the steady rate should be correlated with all the reactions reforming ATP or disposing of its breakdown products. Nevertheless, we cannot reject an alternative explanation, i.e. that the labile heat has something to do with the movements of calcium ions (either release of Ca^{++} or triggering of the calcium pump).

DAVIES: Asked about the duration of the tetanus.

AUBERT: Four to five seconds.

DAVIES: In that case there would be a considerable formation of inosines which occurs after the first 1·5 or 2 seconds in a maximally stimulated frog sartorius muscle which has been treated with 2,4-fluorodinitrobenzene.

AUBERT: There was still plenty of ATP at the end of each tetanus according to Maréchal's analysis of our muscles. But even if the muscles, badly altered as a result of activity under poisoning, had their secondary reactions the striking fact remains that the initial part of the next contraction is not changed as seen from the heat production.

Debate came about the role of Ca ions, the multiplex factors of the isometric twitch, and about the lack of shortening heat. Participants were in sequence: Davies, Aubert, Gergely, Jöbsis, Wilkie, Jöbsis.

TREGEAR: A synchronous insect flight muscle works in life by oscillation, and the similar behaviour of glycerol-extracted fibres shows that this is a property of the contractile material. The glycerol-extracted preparation will work for a few seconds at an amplitude, delivering an amount of

work per cycle near to that in life. However, the glycerol-extracted muscle will only work at a much lower oscillation frequency than the live muscle. The system will only work while the tension is kept below a critical level, which may often be reached by stretching the muscle by 4–6 per cent in activating solution. In relaxing solution, on the other hand, the muscle may be extended by at least 15 per cent without impairing its function.

Two facets of this system are of particular interest. The first is that the enzymic sites appear to be activated by stretching of the sarcomere both to split ATP and to generate tension after a delay. This sensory function requires relative movement of the filaments of only a few hundred Å, and is not dependent on close contiguity between the A-filaments and the Z-disc.

The second is that on being activated in this way by an oscillation, the muscle can drive approximately 1–3 kcal mechanical energy out of the filament array for every mole ATP split. If the muscle is oscillated too fast this efficiency falls off.

RÜEGG: The rate of ATP consumption in relation to power output of the muscle machine may be determined in glycerol-extracted insect fibrillar muscles (Rüegg, Tregear 1966) which perform oscillatory work when they are sinusoidally stretched in ATP salt solution (Jewell, Rüegg 1966). Fibre bundles of about 7–10 fibres from the dorso longitudinal muscle of the tropical waterbug *Lethocerus maximus* were sinusoidally stretched in a solution containing 5 *m*M ATP, 5 *m*M $MgCl_2$, 70 *m*M KCl, 20 *m*M histidine pH 6·5, 4 *m*M EGTA-calcium buffer. The oscillation amplitude was about 3 per cent of the resting length and the frequency 2/sec. Increasing the calcium ion concentration up to about 10^{-6} M increases both power output and oscillation-induced extra ATPase in proportion when the fibres are driven to oscillate (Fig. 13). At higher Ca^{++} concentration (about 10^{-5} M) the power output decreases to about 38 $\pm$ 20 per cent though the extra ATPase induced by oscillation is further activated to 139 $\pm$ 10 per cent of the value at the optimal calcium ion concentration. Thus, at high calcium ion concentration the efficiency is decreased but the interpretation is complicated by the uncertainty about the intrafibrillar ATP

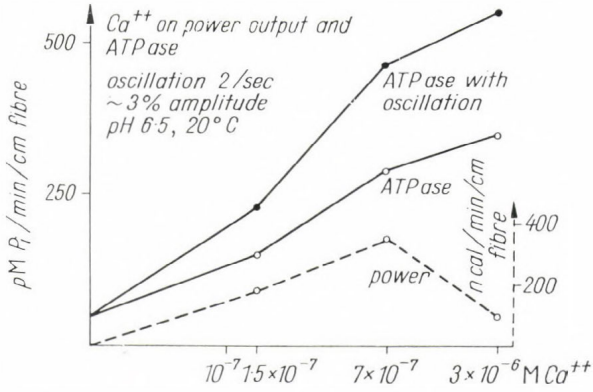

FIG. 13.—Effect of Ca^{++} concentration on power output and ATPase activity of oscillating and non-oscillating glycerinated insect fibrillar muscle

and ADP level and by the possibility that the work of part of the active fibres may be absorbed by non-oscillating fibres of the fibre bundle core. Investigations with single fibres and with ATP restituting systems are in progress. It is interesting, that the oscillatory extra ATPase (and also the stretch ATPase; see Rüegg and Tregear 1966) show the same dependence on the concentration of ionized calcium as the actomyosin ATPase. These findings are consistent with the view that the oscillatory extra ATPase is essentially an actomyosin ATPase. It depends on actin–myosin interaction and it may well arise from an oscillation-induced increase in the interaction rate between actin and myosin.

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HASSELBACH: Have I understood right that the ATPase of your fibres is activated owing to the stretch of the A-filaments?

TREGEAR: Yes.

In the discussion of Tregear's presentation Bowen and Huxley have participated in addition. (Manuscript did not arrive.)

VOROBYEV: I should like to note that Dr Tregear's results correlate with our experiments about which I spoke yesterday.

In 1962 we managed to observe an increase in ATPase activity of glycerinated muscles (m. sartorius of the frog) upon stretching. Later on, we showed that loading influences the enzymatic properties of glycerinated muscle fibres (m. ileo-fibularis of the frog and m. psoas of the rabbit). Small loads accelerate the splitting of ATP by fibres, while the action of large loads reduces the ATPase activity.

In Figs 38–39 (cf. pp 192-4) you can see the results of experiments with glycerinated muscle fibres of the rabbit psoas. The bundles of glycerinated fibres were stretched by the load. The length of the bundle under definite load and the length of sarcomere (curve *3*) were measured. Then ATP was added. After a certain period of time we measured the extent of contraction of the bundle (curve *1*) and the amount of inorganic phosphate split from ATP (curve *2*). Simultaneously 10–12 experiments were performed. Each point of the curves is the mean of several experiments. You can see that the maximum of ATPase activity was attained when fibres were stretched under the load of 50 mg to the sarcomere length of about 2·7 μ.

Now, about the interpretation of this phenomenon. I shall demonstrate the results of our experiments in which deformation of actomyosin or myosin molecules in solution was induced by the application of hydrodynamic field of flow. Experiments were carried out in the device for the study of double birefringence in flow. In this experiment (Fig. 14) we have compared a time course of ATPase activity of myosin at the action of hydrodynamic field (gradient of flow $g = 3100$) and without its application (at zero rate gradient).

As can be seen ATPase activity of myosin is increased upon the action of hydrodynamic field. With time the increase in enzymatic activity is com-

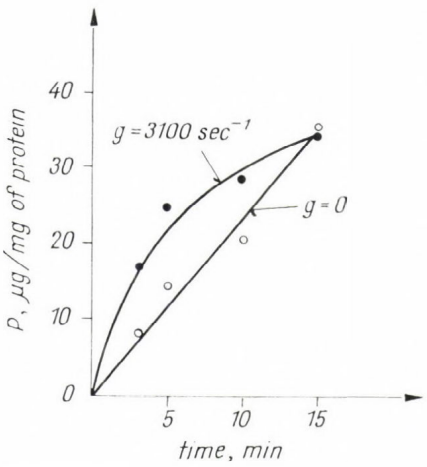

FIG. 14

pensated by its decrease due to the denaturation of myosin in the hydrodynamic field.

I think that we cannot explain this effect only by such geometrical factors as the extent of the overlapping of myofilaments. Mechanical deformation is known to influence a conformational transition in macromolecules. I suppose that the increase in ATPase activity upon stretching in our experiments and in Dr Tregear's experiments gives rise to conformational changes in myosin or actomyosin. These changes probably are located in the bridges between myosin and actin myofilaments.

After the presentation questions were asked by Wilkie and Gergely.

VOROBYEV: Glycerinated muscle fibres of m. psoas of the rabbit and m. ileo-fibularis of the frog were prepared with the ordinary method of A. Szent-Györgyi. The bundles of fibres were worked repeatedly in solutions of 0·13 M NaCl, $1{\cdot}5 \times 10^{-3}$ M $MgCl_2$, 0·05 M $CaCl_2$, 0·02 M tris buffer, pH 7·2. The bundles were placed in a special vessel with the same solution with $3{\cdot}6 \times 10^{-3}$ M ATP. The extent of contraction of the control bundles upon the addition of ATP was about 60 per cent.

Experiments with myosin were performed both in solution of high ionic strength (0·6 M KCl) and in 0·15 M KCl (condition in which Dr H. E. Huxley performed a linearly ordered aggregate of myosin). No noticeable differences in the ATPase activity upon application of hydrodynamic field in 0·6 M KCl and 0·15 M KCl were observed. We have observed a slight increase in the effect with actomyosin in the solution of 0·15 M KCl in comparison with the actomyosin in the solution of 0·15 M KCl.

Comments were made, in addition, by Bowen and Jöbsis.

RINALDI: Demonstration of a film about the movements of granulae in *Allogromia laticollaris*.

The motion picture film illustrates two-way motion existing in the pseudopods of *Allogromia laticollaris*, a living foraminiferan. As previously suggested, I consider this organism to possess a 'primitive muscle'. The two-way motion existing in the pseudopod is similar to the two-way motion envisioned, as I interpret it, in the muscle cell by the sliding filament hypothesis. The filaments in the muscle cell move only a few microns, but the subfibrils of the foraminiferan pseudopods move millimeters. Furthermore, the pseudopods can transport materials on their surface and each subfibril is surrounded by a unit membrane. The subfibrils are able to anastomose to one another. The subfibrils are of different shapes and dimensions; their range is from 120 Å to 25 000 Å. I have extracted these fibres with 50 per cent glycerol and water, but the addition of ATP is so disturbing to such a fine network that I cannot determine whether they are affected. I have not succeeded in identifying actomyosin-like components in these pseudopods

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FACTS, IMPLICATIONS AND PERSPECTIVES

E. Ernst

BIOPHYSICAL INSTITUTE, UNIVERSITY, PÉCS

Dear Colleagues

Now, after discussing structure, chemistry, mechanics and energetics of the striated muscle, we have arrived at the point when it seems timely to try to summarize or *synthesize*. For these the Organizing Committee reserved this afternoon, i.e. this plenary meeting, on the one hand, and the whole day tomorrow when during the excursion each participant will have the opportunity to discuss any questions with other colleagues.

1. My task is now to begin the discussion dealing with synthesizing, and so I cannot help asking: Am I now really able to synthesize? As to this question, permit me, please, to remember my speculations at the start of my scientific career, more than half a century ago. It seemed to me that the most profound problem of science was the activity of our nervous system, the leading laboratory of making science; accordingly, I decided to occupy myself first of all with the function of nerves. Because of the outstanding importance of method the question of how to record the activity of the nerve seemed to be the first one. I beg to remind you, that at that time the fact had long been known that *a tetanic* contraction of the muscle could be brought about by stimulating the nerve *with* d. c. That means that the *continuous* process of stimulation is followed by the *discontinuous* process of the tetanic contraction. This interesting phenomenon surely played a role in Frey's hypothesis (Frey 1883), according to which 'the ability should be ascribed to nerves to transform the constant course of d. c. into separate impulses of excitation'. That meant to me that the discontinuity in the tetanic contraction of the muscle originated from the discontinuity of the nerve-function, i.e. that *muscle represents a reliable recorder of nervous activity*. Therefore, I thought I should enter upon investigations into nerves only after studying and 'getting to know' muscle. Shortly, muscle seemed at that time to me the anteroom to my future work on nerves, and now after half a century, I wonder whether I am further than the anteroom to muscle.

But, at any rate, I see a *great development* in many fields of myology and also our Symposium has given evidence of this development. Therefore, permit me, please, gratefully to thank in the name of the Organizing Committee all the participants of this Symposium and first of all the worthy colleagues who accepted the task of delivering the introductory lectures. I think all participants agree also that the Organizing Committee gratefully thanks the International Union of Pure and Applied Biophysics for having consented to organize this Symposium under its auspices. I should like to propose to send a letter to the Union thanking for its consent.

1.1. And now, a few words for the development; first of all, in the field of structure. Let me, please, remind you of the fact that even the basic microscopic structure of the muscle fibre was being debated in the twenties, and either the fibrils or the cross-formations of the fibre were looked upon as artifacts due, among others, to the method of preparation (Ernst 1963). In contrast to that, now we are discussing units 100—1000 times smaller than the fibril itself, we are approaching step by step the investigation into units of macromolecular size (Huxley 1966b). And, what is still more, even single atoms of K, Na and Ca could recently be localized inside the microstructure, moreover, I hope, inside the submicrostructure in the near future (Ernst 1966, Tigyi 1966, Winegrad 1965).

These last results belong also to the chemical structure of the muscle. In this field first of all myosin and actin are to be mentioned; myosin was known long ago, but the newer development concerning the proteins of the muscle is very conspicuous. I will not speak more about this topic, the participants are much more able, than I, to evaluate the advances in this field (Ebashi 1966, Ivanov 1966).

Mechanical activity and energetics were the subjects of the second part of our Symposium. Apart from what has been achieved in detail, development can be seen also in the circumstance that today everybody looks upon the muscle problem as a complex of structure, chemism, mechanism and energetics. That is a very important fact of development contrasting with previous efforts, made by some authors to solve the muscle problem on the slender basis of one or another experimental result and disregarding all other experimental facts.

1.2. But our old Master, A. V. Hill (1916) said in 1915: muscle physiology is no longer a child who is satisfied with *half-statements*, it has grown up and has to reckon with exact quantities and absolute values. Accepting Hill's admonition and continuing it in a less diplomatic version, *we ourselves*, as grown-up research workers, after getting beyond the teen-age of biological science, should properly perform our work in myology. Let me, please, mention in short, e.g., the late lactic acid theory of muscle contraction to show what I mean. Of course, nobody wants to discredit the valuable experimental facts concerning lactic acid production accompanying muscular activity, but this theory neglected all other experimental facts, that is why this theory from the beginning seemed to me to be based on half-statements. The more so since Embden showed that lactic acid was produced even *after relaxation*. Meyerhof (1930), this great scientist of biochemistry, endeavoured to weaken the refuting force of this important contradictory *fact* by producing the *expression* of 'Extra-Milchsäure' ('extra lactic acid'). And now, forty years later, Davies' comment (Davies 1966), describing the ATP breakdown during muscular activity, demonstrates also a delayed breakdown of ATP occurring after the mechanical work is completed (Infante and Davies 1962). Let me raise the question: will the prospects for 'extra ATP breakdown' differ from those for 'extra lactic acid'?*

* Some data mentioned during our Symposium show the role of ATP played in contraction quite differently from what it has originally been supposed to be. (Cf. Bowen's comment, p. 198; Bowen 1966). These and other *facts* corroborate to the experimental results and considerations of Ernst's team rejecting the original ATP-theory of contraction (Ernst 1963, Szabolcs 1954, Ernst and Metzger-Török 1962).

1.3. I should not like to pause at this special point but shall discuss in short the more general question: what is the *ratio between facts and inferences* in the myology of our days. Trying to answer I feel, first of all, my obligation to explain why I used the expression implications and not inferences in the program of our Symposium. The simplest way to answer would be, of course, to refer to my imperfect knowledge of the English language, which is nearly similar to that of German. Concerning the latter, I was instructed 30 years ago, after giving a lecture about volume diminution, at the invitation of the Biological Society in Vienna. After my lecture I was, of course, praised etc., but a German colleague, with whom I was on friendly terms, addressed me as follows: 'I congratulate you, Mr. Ernst, you have spoken excellent Hungarian.' I can imagine what the words of congratulation of a sincere English colleague will be after my present lecture.

Coming back to the word implications, I confess, in this case I deliberately used this expression instead of inferences, because I wished to emphasize that an important question is sometimes not clarified by a certain assumption but, on the contrary, becomes much more complicated. Both expressions—implication and complication—contain the Latin verb *plicare*, the meaning of which manifests itself in the Latin sentence *anguis se plicat* (translated: snake folds itself). Please, do not think I wanted to allude to somebody or to something, all the less, since I am known in friendly circles to be a weak shooter. But, notwithstanding, I can sometimes succeed in hitting the target.

1.4. Half-statements often are produced by emphasizing a certain experimental result *one-sidedly;* striking examples are to be found, e.g., in cases when experimental results that have been published earlier are described again as new scientific progresses. Furthermore, we often are witnesses to the situation in which an assumption trying to explain an experimental result—so to speak—covers up other experimental achievements; or something similar manifests itself on the part of the critics. An example presents itself in the case of the hypothesis according to which the process of shortening is brought about by movements of certain submicrostructural units *exclusively in longitudinal direction* of the fibril.* But many earlier experiments about contraction of muscle *in vivo* demonstrated simultaneously with shortening also a blow-out of the contracting fibre in transversal direction (Fig. 1). Therefore, the explanation of shortening should explain also the widening.— Another example of one-sidedness is the way in which the role of Ca in muscular activity is sometimes explained, the role of K or Na being simultaneously disregarded.**

* Passing over the strong contradictory comments of Ernst (1966), Garamvölgyi (1966), Guba (1966) and others, I want to emphasize especially Huxley's opinion: the sliding hypothesis is not for explaining everything, . . . the supposed activity of the cross bridges does not belong to the hypothesis. — This moderate statement of Huxley pronounced at the end of our Symposium may be confronted with his statement made one week earlier at the International Biophysical Congress in Vienna (Huxley 1966a): "The active sliding motion of actin filaments past myosin filaments during contraction is brought about by processes occurring on . . . cross bridges . . ." The conspicuous difference is to be ascribed—I should like to believe it—to the discussions held at our Symposium.

** When discussing the role of Ca, I want to mention my dear colleague, Anne Lánczos, who was one of the first to describe the role of Ca in activity. (She was killed in 1944 by the fascist murderers.)

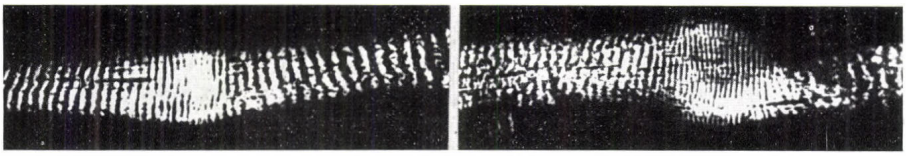

FIG. 1.—Contraction of a cross-striated muscle-fiber (Hydrophilus)

Perhaps one of the most characteristic instances of one-sidedness in myology of today consists in neglecting the importance of water in muscle physiology. This time I do not want to discuss what the connection between the water-content of muscle and its irritability is; (which connection appears as the effects of drying, or of glycerin, or of perfusion with hypertonic solutions, etc.; Ernst 1963). Neither shall I dwell upon the very interesting phenomenon demonstrating a long tetanus-like contraction of the muscle perfused with hypotonic solution (Ernst 1963). Well, what I am going to mention is how water can be looked upon as a link transmitting energy over long distances, e.g. 100 Å; further the conception about the Grotthus-type mechanism explaining the role of water in the transmission of electrons over long distances (Horne 1964). In this process the protons of the water molecules are assumed to play the role, especially in the inner shell of the hydrate water, e.g., of monovalent cations or of radicals (Reynolds and Lumury 1955, Horne and Axelrod 1963). But, I myself should not like to commit the fault of one-sidedness and therefore I only remark that the problem concerning the bound or free water in muscle deserves keen interest(Pócsik 1966).

By emphasizing the fault of one-sidedness I do not, of course, disapprove of it in experimental work concerning a certain single question, but in solving the general problem of the striated muscle one-sidedness has never played a promoting role. For, please, do tell me what progress was brought about by and what result remained from the Engelmann–Pauli–Fürth's swelling theory, or from the Berzelius–Fletcher–Hopkins–Meyerhof–Hill lactic acid theory ?! Many thousands of papers, seemingly proving these theories or written in favour of them, are buried in oblivion.

2. Of late decades research work in myology was directed towards *microscopic* or molecular dimensions. This tendency was, as we know, very successfull, but one-sidedness in this case can easily bring on a situation in which one cannot see the macroscopic wood for the microscopic trees. Therefore, permit me, please, to continue with *macroscopic aspects*.The more so, because the fact sometimes seems to have become forgotten that *muscle is a macroscopic device of nature to perform work* and not a topic of human science by which some workers in it can acquire fame.

2.1. Muscle *performs movements and transforms them*, like machines (e.g. a sewing machine). Thus exact investigation and mathematical formulation of these movements form a substantial part of myology. Accordingly, I should like to bring into prominence the contraction curve of the muscle (Fig. 2) as it is produced by experiment: this time course of shortening can be expressed by the formula

$$\frac{r}{R} = \sin \frac{\pi}{2} \frac{t}{T} \tag{1}$$

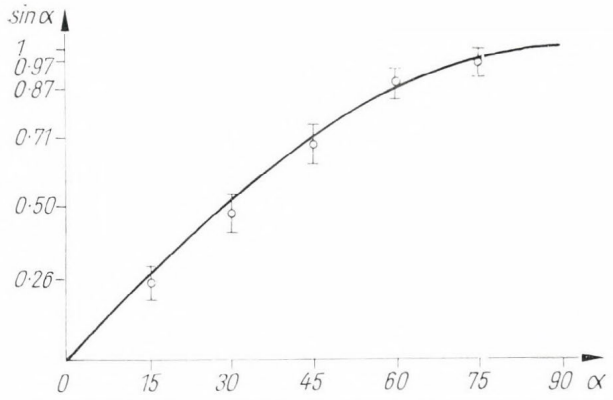

FIG. 2.—Sine-curve and the curve of shortening (twitch)

where R and T denote the maximal shortening of a twitch and the time belonging to it, hence the fraction r/R signifies the relative shortening in the relative period t/T; Fig. 2 shows that the standard deviations of the experimental values of shortening are relatively small, so this formula of the macroscopic process of shortening can be looked upon as approximately expressing the experimental facts.* Therefore, the value of any hypothesis, explaining shortening by submicroscopic or molecular movements depends, in my opinion, on how far they agree with this macroscopic law.**

2.2. Muscle *produces and transmits* force like machines. The force exerted by active muscle is an unequivocal function of the extent of shortening; the explicit forms of this function are different according to the different experimental results described by different scientists, from Schwann to Hill, in the last 150 years. The simplest formula can be derived on the basis of Schwann's experiments shown in Fig. 3; the corresponding mathematical expression reads

$$\frac{p}{P} = 1 - \frac{r}{R} \tag{2}$$

where P denotes the maximum muscular tension, hence p/P the relative tension depending on r/R (see above).

These data refer to different contractions of a muscle, and the same function between force and shortening is valid for different stages of the same contraction. This *general law based on experimental facts* says, as known, that the more the muscle has shortened during contraction the less is the force exerted by it. *Any hypothesis trying to explain the molecular or submolecular origin of force produced in contracting muscle must not contradict this fact.* Accordingly, e.g. the hypothesis explaining contrac-

* On the basis and by means of this macroscopic law and mathematical formulation also the time course of the tetanic contraction can be exactly described (Ernst 1963).

** Thus the molecular aspects are inseparably connected with macrohappenings, as is generally right and practical *to study real biological phenomena and not single aspects of them.*

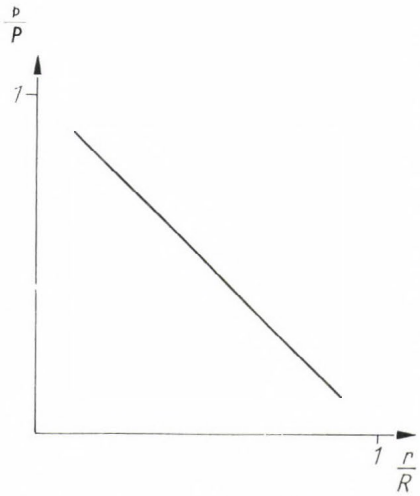

FIG. 3.—p/P as a function of r/R

tile forces as due to attraction between opposite (electric) charges is, in this simple form, inconsistent with experimental facts, or else the muscle would exert the greater force the more it has shortened.

2.3. The muscle-machine simultaneously exerting force and producing displacement, *performs work*; hence *theoretical maximum work and mechanical efficiency* should be determined data, by means of which the quality of machines can be properly judged. As to the first, by means of equation (2), the proper differential equation and the integral of it

$$W = \int_0^R p \, dr = P \int_0^R \left(1 - \frac{r}{R}\right) dr = \frac{1}{2} PR$$

can be obtained. That is, the maximum work which a muscle can perform in a (tetanic) contraction is given by half product of the maximum muscle force (when there is no shortening) and maximum shortening (when no force is exerted).*

But, experiments on *excised muscles* give smaller values for the effective work w amounting, in general, to half of W. Thus, mechanical efficiency in these cases is

$$\eta_m = \frac{w}{W} \sim 0{\cdot}50$$

But mechanical efficiency, computed for work performed *under proper physiological circumstances*, can come close to the unit (Ernst 1963). This, on the other hand, contradicts values which can be read in the literature (Davies 1966), and are computed on the basis of heat development or some metabolic processes. But that is erroneous; to clarify the situation we have to consider that all known metabolic processes and heat production can be brought about *by mere passive mechanical stress* of a muscle. Moreover, stretching even a bundle of threads of actomyosin is accompanied by heat development, as shown by Fig. 4. Consequently, all speculations or quantitative data, wanting to demonstrate a functional connection between contraction and heat development, neglect these experimental facts. The data given at this Symposium by Jöbsis (1966), Davies (1966) and especially by Wilkie (1966) support the author's views (Ernst 1963).

* E.g. for the biceps of a man

$$P \sim 10 \text{ kp cm}^{-2} \times 12 \text{ cm}^2$$
$$R \sim 10 \text{ cm}$$

and therefore

$$W \sim 6 \text{ mkp}.$$

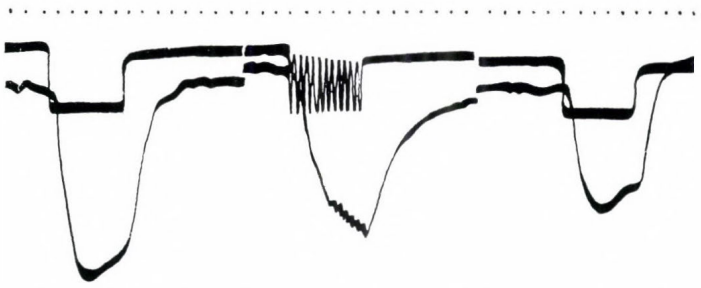

FIG. 4.—Lengthening and heat production of a bundle of actomyosin threads. Top: time = sec each, middle: lengthening, below: heat development. The second curve shows frequent stretching

2.4. By the high value of the mechanical efficiency of its work, muscle corresponds to a very good machine. But muscle is much more than that, in so far as it has turned out to be a *unique, excellent device surpassing the data of physics by its biological characteristics*. Namely, when it is repeatedly engaged mechanically, it becomes enabled to perform greater work than before, and it brings this about by increasing its own substance. This biological process, called *hypertrophy*, accompanies mechanical activity. Hypertrophy having long been known, for many generations, did not succeed in being considered in muscle science according to its importance. Notwithstanding, this question leads us to interesting considerations.

a) Hypertrophy of the muscle can be assumed to be brought about by means of *mechano-chemical* coupling. Although the process of mechano-chemical coupling does not seem to have been elaborated properly in science, notwithstanding, in the case of muscle, it can be treated even semi-quantitatively. To my sorrow, I have no time enough to discuss this topic in detail.

b) Figure 5 demonstrates an electron-microscopic picture showing *polysomata* lying between submicroscopic units of muscle (Cedergren and Harary 1964). Thus, the question can be raised whether the ribosomes, being in the immediate vicinity of active muscle subunits, are directly stimulated by them to greater activity.

Besides that, neither can the possibility be excluded that the polysomata are stimulated through submicroscopic *nervous elements* which are supposedly present inside the muscle and stimulated by its activity. Furthermore, activity is regulated by the nervous elements contained in the fibres, spindles and tendons, and therefore muscle can be looked upon as a *cybernetic device*.

3. According to what has been said up to now, muscle represents an excellent macroscopic machine. Hence, I expect the greatest development of myology when muscle is investigated according to its basic nature, i.e. as a working machine, exceeding all quantitative data of technique and physics by virtue of its biological means. Along this line should be understood important questions of the praxis in sport and labour, such as fatigue, stiffness, contracture or spasm, rupture of muscle, the isometric

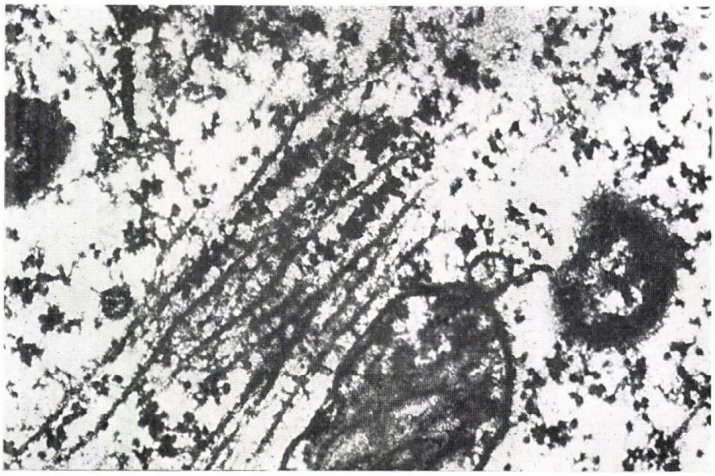

FIG. 5.—Polysomata between filaments of a fibril

training — and in clinical work such as myotony, degeneration, etc. (Ernst 1966).

Well, I am now already speaking about perspectives. Prof. C. F. Powell of Bristol, delivering a lecture on *The Role of Science in European Civilization* at a CERN-meeting (Counsil European Recherches Nucleaires), spoke about the future of science to be organized for whole continents or even on a global scale. Our present claim is, of course, much less, but we are compelled to see that our muscle science—despite doubtless developments—is fallen behind the times. Let us just consider the immense gap between the development of electricity and myology, both being about three hundred years old: electricity has grown to be the industrial giant of our age and our delegate in the Universe.

3.1. And myology? It is true that many hundreds of thousands of research-workers are today occupying themselves with muscle, and that there is a continuously increasing flood of publication about muscle. But permit me, please, to raise the question whether the real development of myology is growing proportionally to the literature about myology? Further, it is also true that many symposia have been organized to promote the development of myology; these, in general, contained a lot of lectures more or less independent of each other; and some reports of these symposia are, indeed, very good and interesting but, the question arises whether the symposia could in future become more efficient.

That is why our Organizing Committee decided that only introductory lectures should be held at this Symposium, and discussions should try to fix and *formulate the present state* of some topics to be debated. Thus I beg to propose the following as examples: (i) the real state of the *thick and thin filaments* inside the whole fibril; (ii) what *the medium* is in which the filamentous material is embedded *in the sarcomer;* (iii) what the mathematical law of *macroscopic* shortening is and how far the hypothesized *submicroscopic movements* correspond to it; (iv) what the mechanism and the mechan.

ical consequences are of the *crystallization of the muscle* proteins; (v) how is it to be explained that a *muscle stretched* to a certain degree is unable to exert active force on stimulation; (vi) *mechanism* and consequences *of hypertrophy*.

3.2. It would mean, in my opinion, a great progress if our Symposium — better to say its review appearing, I hope, in the near future—would inform colleagues that our discussions had succeeded in producing clear cut formulations either of the agreements concerning some topics or of the opposed standpoints. On the basis of such a result future symposia could perhaps fix for discussion one or another problem and foreshadow that the aim of the next symposium would be to clarify the problem previously assigned. Working along these lines we shall perhaps arrive at the stage where international symposia are able not only to determine what to discuss, but even to suggest what to investigate.

Thanking you for your kind attention I should like to ask you to try to formulate the results of our discussions concerning the debated questions and among them some of those proposed by me.

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GENERAL DISCUSSION

GUBA: In spite of brilliant experimentations which give support to the sliding filament theory there are several points in which more understanding would be very welcome.

I think many of us got evidence that the two opposite-standing sets of thin filaments of a sarcomere could pass each other at shortened sarcomere length, getting in the double overlap zone.

One can find this overlap of thin filaments in myosin extracted myofibrils too (Figs 6 and 7).

These and some other findings are strong supports for the sliding mechanism. It is inevitable to consider some kind of sliding during the contraction. The question to be answered, as it appears to me, is whether the sliding of filaments is the driving phenomenon of the contraction or it is a consequence of some other, more fundamental events. To come closer to this problem one has to consider as many observations as possible and see whether they are in harmony with the sliding theory or not. It seems to me that there are facts which are incompatible with the concept of a simple sliding mechanism as driving phenomenon of the contraction and which throw some light on the possible colloidal changes in myosin during the contraction.

(1) The continuity of the filaments in the myofibril. — (a) As has already been published we have done some work on the continuity and colloid state of filaments in the myofibrils as seen under the electron microscope.

The structures observed in the electron microscope have been discussed from the point of view of the sectioning technique, too. We concluded that cross sections give more reliable data than the longitudinal ones. The ultrastructure of rabbit m. psoas myofibrils and especially the transitions of bands in the sarcomere have been investigated in the relaxed and contracted states in adequate cross-sections. Glutaraldehyde fixation, Epon embedding and adequate cross-sectioning were used. Measurements were performed on the sizes of filaments and on the distance of the primary filaments in the relaxed and contracted states.

The results of our measurements are summarized in Tables I, II and III. From these data at least the continuity of the primary filaments throughout

TABLE I

Data on the sizes of the filaments in the relaxed muscle

	H		A		A-I junction		I	
	Å	SD	Å	SD	Å	SD	Å	SD
Lattice constant (distance of the primary filaments)	360	20	360	18				
Diameter of the primary filaments	160	21	120	24	70	24		
Diameter of the secondary filaments			65	4	65	10	80	10

The number of filaments was calculated for 1 μ^2 surface

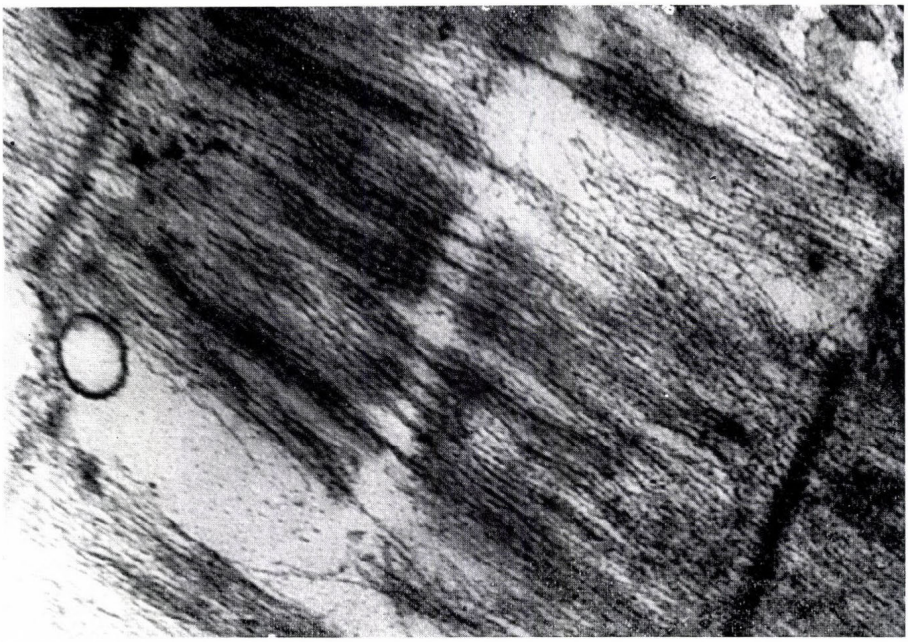

FIG. 6.—Relaxed muscle fibre (m. psoas of rabbit) after extraction of myosin. The opposite-standing sleeve-like sets of thin filaments separated by a gap are clearly seen

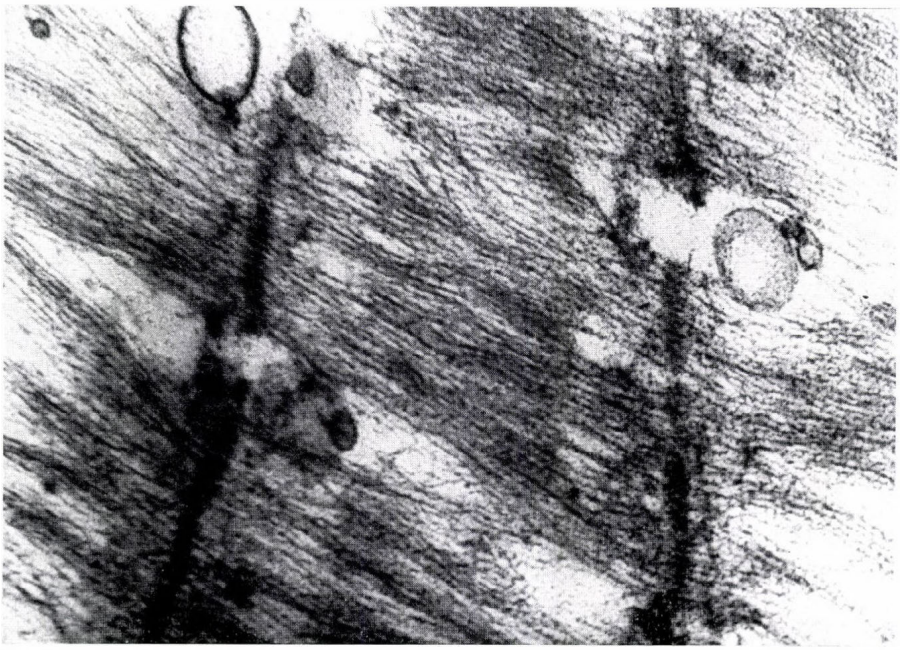

FIG. 7.—Contracted muscle fiber after extraction of myosin; the opposite-standing sets of thin filaments are overlapping in the centre of sarcomere

TABLE II

Number of filaments in 1 μ^2 area in the relaxed muscle at different parts of the sarcomere

	A		A–I junction		I		Z	
	n/μ^2	SD	n/μ^2	SD	n/μ^2	SD	n/μ^2	SD
PF	1045	32	—	—	—	—	—	—
SF	2100	46	—	—	—	—	—	—
F	—	—	2800	253	2300	215	2400	420

The area occupied by the myofibrils determined with a planimeter was found to be 92 ± 2 per cent of the fibre

TABLE III

Data on contracted myofibrils

	H		A at H		A at C	
	Å	SD	Å	SD	Å	SD
Lattice constant (distance of the primary filaments)	415	30	—	—	360	21
PF ⌀	200	12	—	—	115	8
SF ⌀	—	—	50	10	90	7
SF (number) : PF (number)	?		Rate	SD	Rate	SD
			2·8	0·5	2·0	0·1

The area occupied by the myofibrils was found to be 72 ± 15 per cent of the fibre

the sarcomere can be concluded. The conclusion drawn is consistent with the observations of Auber and Couteaux (1963) and that of Garamvölgyi's presented here at this Symposium.

(b) It seems that the isolated primary filaments of insect muscle have a thin core as revealed with negative staining technique (Fig. 8). This core exists very likely in the primary filaments of vertebrates too.

(c) As was demonstrated at the Monday afternoon session and has been published (Guba 1964, Guba and co-workers 1966), after the extraction of myosin, actin and tropomyosin a continuous filamentous structure is left in the myofibrils connected to the adjacent Z-membranes (Ernst, Benedetzky 1962).

These observations suggest that we have to assume the continuity of the filaments in the myofibril.

(2) The rigidity of filaments. — (a) In Tables I and III our measurements of the size of filaments in relaxed and contracted myofibrils are summarized. From this data we can estimate the quantity of protein represented by the filaments. There is a fairly great difference in the protein content of relaxed and contracted myofibrils. A relative increase in the protein content of myofibrils can be found during contraction by this estimation. An obvious explanation for the discrepancies may be that the contraction is associated with a change in the gel structure of myofibrils.

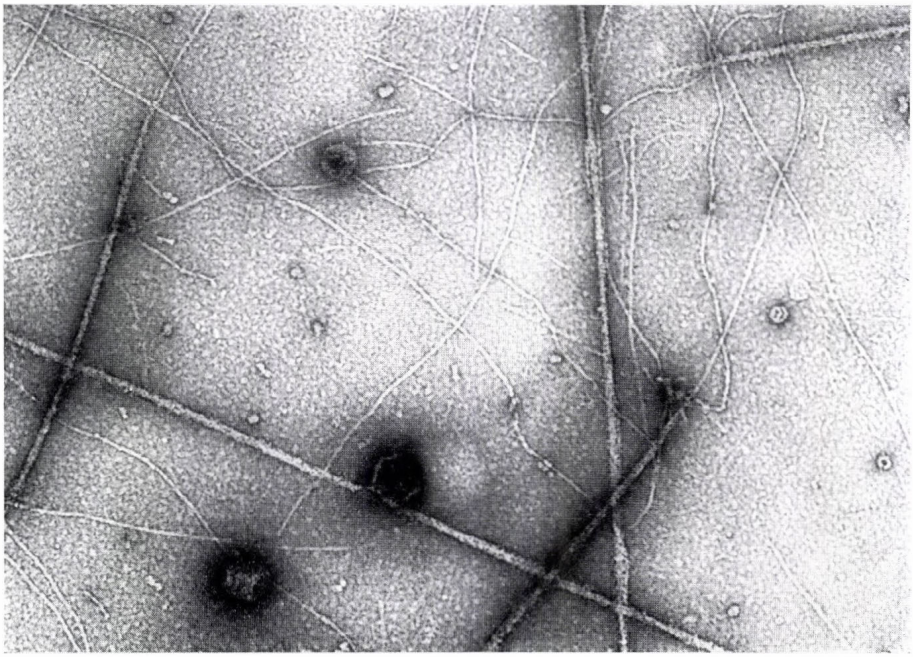

FIG. 8. — Primary and secondary filaments from insect muscle fibre: filaments are disintegrated and suspended in a relaxing solution of Huxley (Huxley 1963). The thin core of primary filaments is clearly seen (arrows)

(b) Garamvölgyi has shown that in the stretched insect flight muscle a part of the A-band material is extruded giving extra bands at both sides of the E-band. These E-bands show up birefringence and very likely they consist of the myosin from the thick filaments.

(c) Using the electron micrographs (the negative plates) for mass determination (Bahr and Zeitler 1965, Vajda and co-workers 1966) we could measure the distribution of the dry mass of A-band and that of I + Z-bands at different sarcomere lengths. These results are summarized in the *Observations on the Relative Protein Content of Myofibrils of Different Sarcomere Length* (Vajda and co-workers 1965; Fig. 9).

The interpretation of these curves led us to the conclusion that during the contraction of myofibrils (at least in vertebrates) adjoining the sliding filaments there is a definite movement of the proteins toward the Z-membranes. The amount of the moving protein is about 20 per cent of the A-band material.

(d) Several authors have demonstrated that during the contraction an overlap of the thin filaments occurs but no one has shown as far as I remember a bending of the thick filaments by the Z-membranes in shortened myofibrils. It seems to me from the observations discussed above that the rigidity of the two kinds of filaments differs greatly. The thin filaments are more rigid and possibly there is a change in the gel structure of myosin filaments during contraction.

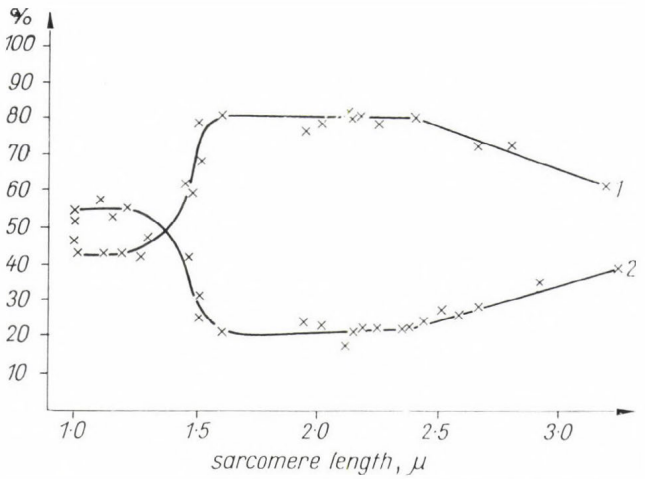

FIG. 9. — Protein content (percentage) of the different bands of rabbit myofibrils as the function of sarcomere length. *1* A-band, *2* I-band + Z-disc (or contraction band)

(3) The possibility of a phase transition in contraction. — There have been several observations even recently (Hoeve and co-workers 1963), which point out a phase change during contraction.

(4) The special characteristics of myosin. — All problems concerned above point to the special role of myosin in contraction.

It has been well known for long that the myosin, a water soluble protein, has special physical-chemical properties. These properties are governed by other proteins (especially by actin) and by uni-, di-, and polyvalent ions.

One of the special characteristics of myosin is that it can be converted into a hydrophilic gel by diluted salt. In this gel the myosin micellae contain over 95 per cent water and most of the water is within the micellae. The micellar structure of myosin depends on the proteins and ions being present. For instance, Fig. 10 shows the micellar structure of pure myosin in the standard solution of Dr Huxley (1963), Fig. 11 demonstrates the effect of ATP (standard salt solution + 5×10^{-3} M ATP). Figure 12 shows the well known arrowhead structure of actomyosin and Fig. 13 the structure of myosin in the presence of tropomyosin.

The most striking change which one can produce in actomyosin gel is its dehydration, the superprecipitation. This transition is very difficult to follow by the usual hydrodynamic methods. Nevertheless, there are observations (Maruyama and Gergely 1962) on the clear phase of this transition which show that a linear aggregation of myosin proceeds the superprecipitation.

Some time ago we started to investigate the colloidal characteristics of superprecipitation using hydrodynamic and electron microscopic methods as well (Guba and co-workers 1966). The main points we have found are as follows:

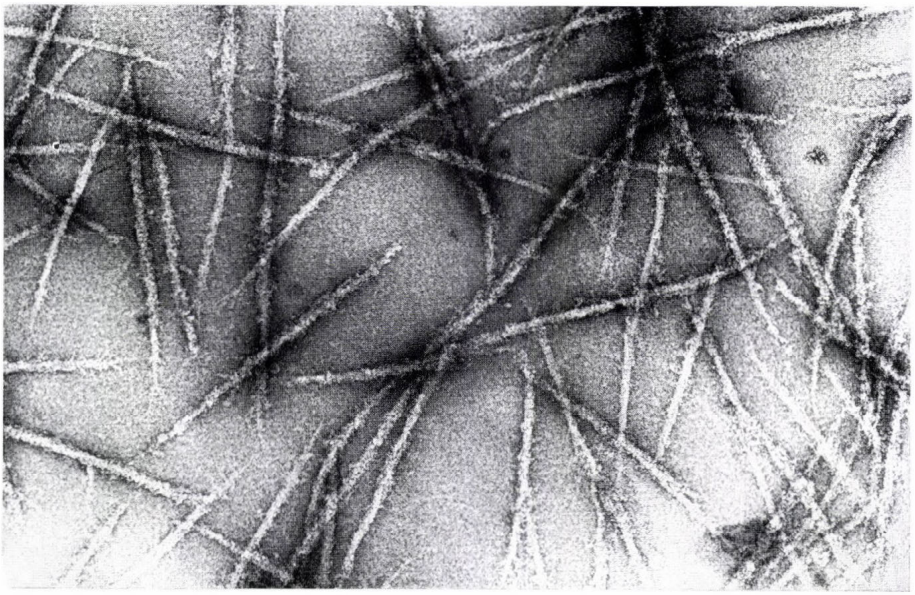

FIG. 10. —Micellar structure of isolated myosin in the standard solution of Huxley (Huxley 1963)

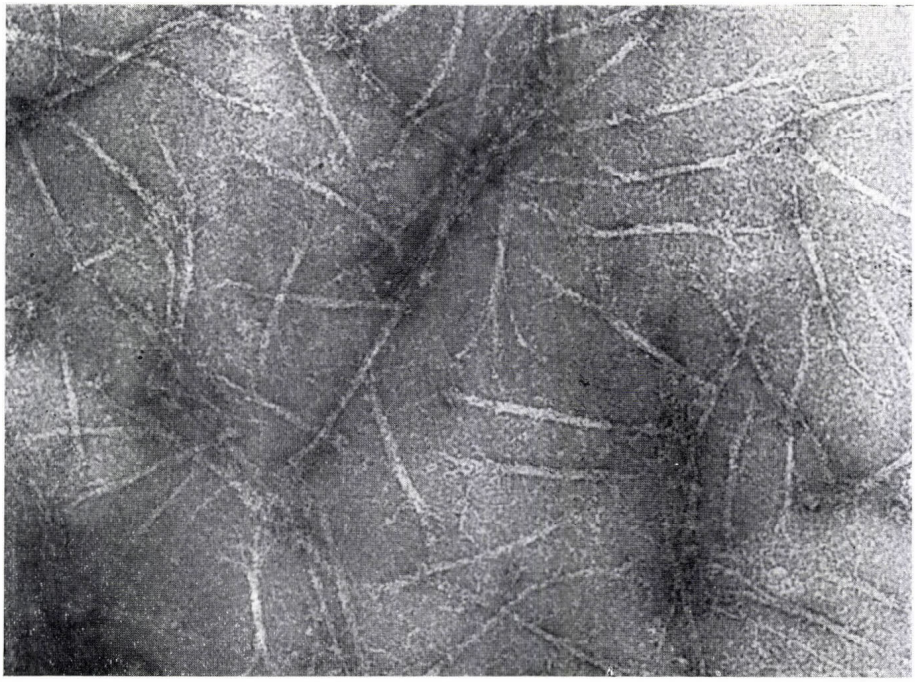

FIG. 11. —Micellar structure of soluted myosin in standard solution containing ATP (5×10^{-3} M)

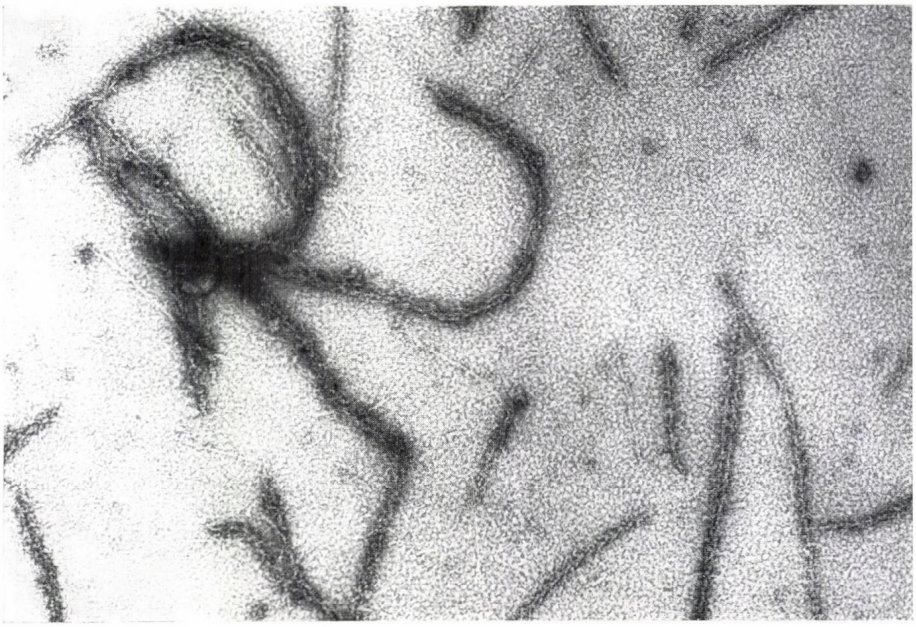

FIG. 12. —Micellar structure of myosin in the presence of F-actin (the arrowhead structure)

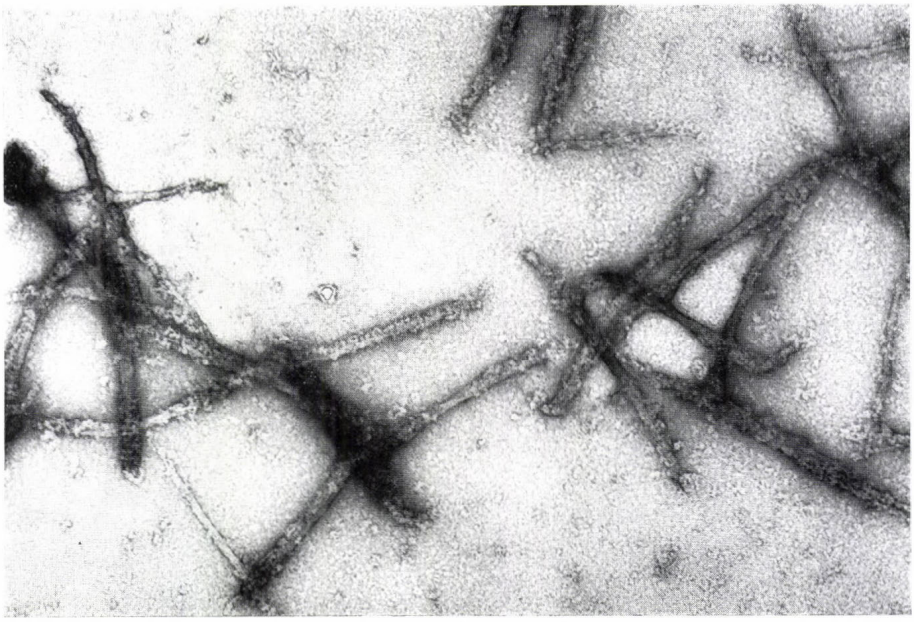

FIG. 13. —Micellar structure of myosin in the presence of tropomyosin. Note the periodicity (approx. 150 Å) of the micellae

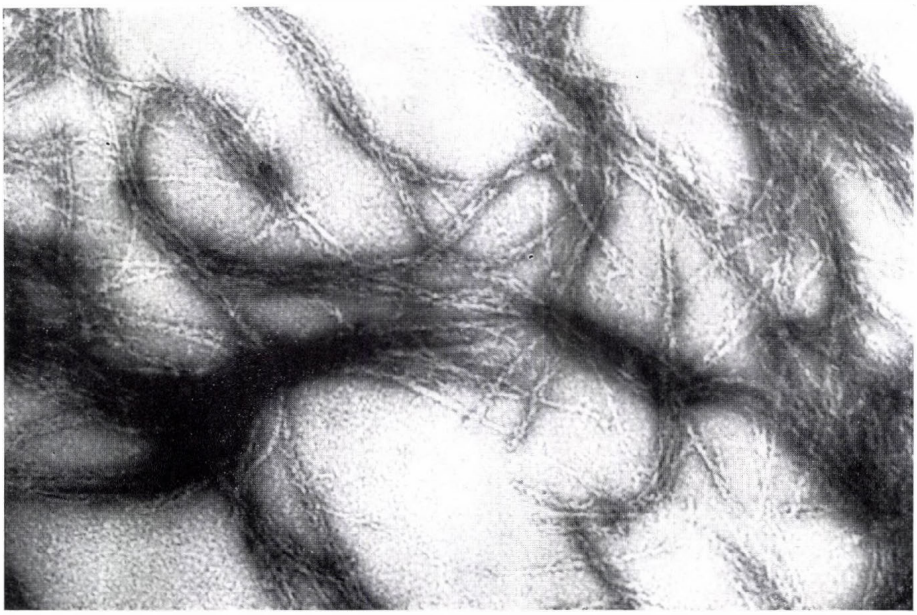

FIG. 14. —Micellar structure of actomyosin by low ionic strength (KCl 10^{-1} M, $MgCl_2$ 10^{-3} M)

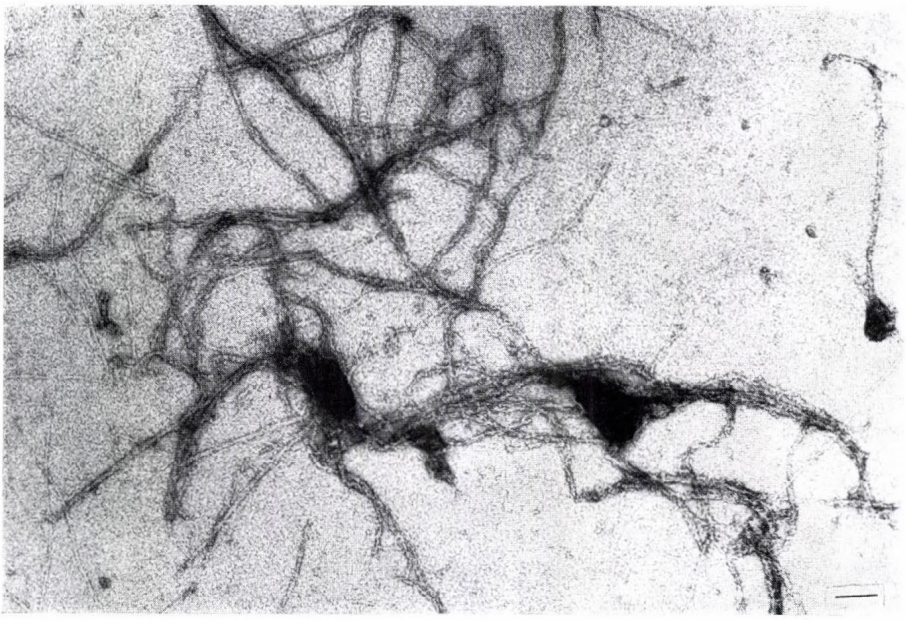

FIG. 15. —Micellar structure of myosin–actin mixture in the clear phase (HCl 5×10^{-2} M, TRIS 5×10^{-2} M, $MgCl_2$ 4×10^{-3} M, KCl 8×10^{-2} M, Cystein 1×10^{-4} M, ATP 1×10^{-2} M)

FIG. 16. —Superprecipitated actomyosin gel. Note the released thin filaments (presumably F-actin) and the amorphous clumps (dehydrated myosin?)

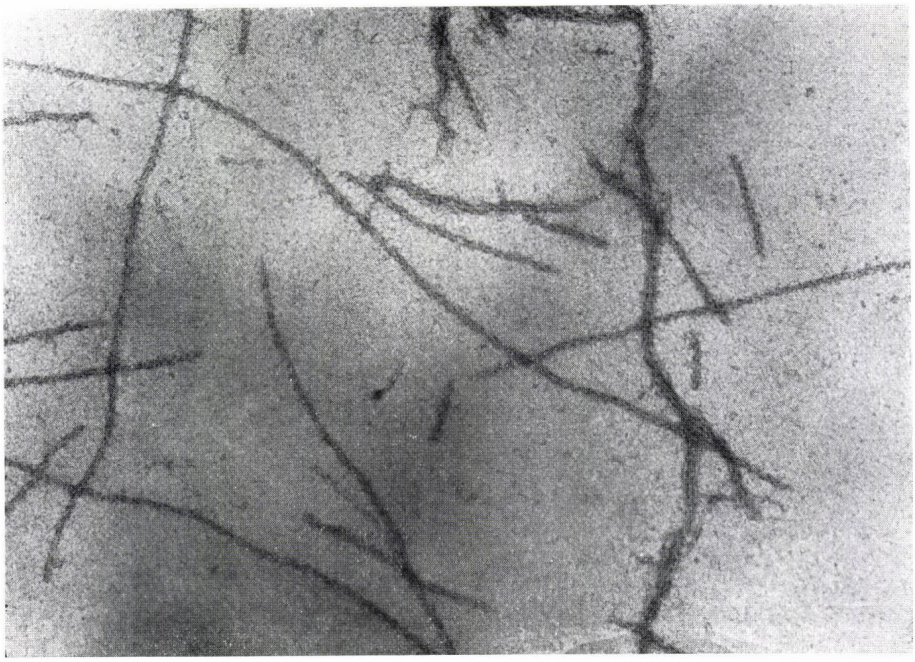

FIG. 17. —Arrowhead structure of redissolved superprecipitated actomyosin gel by high ionic strength (0·5 M KCl)

(a) A relatively small amount of F-actin could induce a relatively large amount of myosin to go in superprecipitation.

(b) In the starting gel it is hard to recognize the arrowhead structure of the micellae (Fig. 14).

(c) It is difficult to distinguish actin and myosin filaments among the uniformly thin filaments of the clear phase (Fig. 15).

(d) In the superprecipitated gel an amorphous part can be seen, besides the thin filaments, presumably released F-actin (Fig. 16).

(e) The ratio of the protein to water in the starting gel (before superprecipitation) is 1 to 100, which changes to 1 to 17 in the superprecipitated actomyosin. So superprecipitation is associated with the dehydration of the system.

(f) The redissolved superprecipitate at an ionic strength of 0·5, pH 7, shows the arrowhead structure of actomyosin (Fig. 17).

If we try to put together all the observations mentioned above (and others not being discussed here) and if we think that the superprecipitation of actomyosin is the basic driving phenomenon of muscle contraction we may draw a picture of the contraction as follows.

Under the conditions of the relaxed muscle, actin exists in F-actin form situated in the thin filaments. These filaments have a fairly rigid and orderly structure. The highly hydrated myosin molecules are associated in a loose gel form on a core which runs through the whole sarcomere. This core consists of fibrillin and gives the rubber elasticity of the relaxed muscle. Myosin and actin are dissociated in the relaxed muscle for the presence of ATP and absence of Ca-ions. By excitation a part of the myosin gets in association with F-actin, which induces a special oriented aggregation of these mobilised myosin molecules. This oriented myosin structure is still hydrated and behaves like a stretched helical spring. The spring-like myosin micellae in the presence of salts and ATP dehydrate and superprecipitation takes place. The superprecipitation causes the spring to perform contraction work and the unaltered (thick and thin) filaments to slide. Relaxation requires Ca ions to be pumped out and ATP to hydrate the myosin molecules.

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Szent-Györgyi: I would be very cautious in postulating movement of myosin during contraction. Several years ago at the Dedham Conference Dr Johnson and I had proposed a migration of myosin towards the lateral portions of the A-band in order to explain the staining pattern obtained in sarcomers at various muscle length using fluorescent antibody prepared against myosin. We suggested that myosin was discontinuous in the center of the A-filaments, and the two halves of the myosin containing structures were attached to the contralateral actin filaments. We assumed that contraction is due to a shift of myosin towards the lateral portions of the A-band and the movement would carry along the actin filaments of the opposite half of the sarcomere. Dr Stephens who at that time was a graduate student with Dr Inoue and myself worked out a method to destroy selectively various portions of the sarcomere using UV irradiation. His results which were published in the *J. of Cell Biology* showed that the contraction patterns of the damaged sarcomeres were in agreement with the sliding filament theory but did not fit in with our theory of contralateral attachment. Even more damaging to our theory was the work of Mr R. H. Colby, a graduate student from the University of California who spent several months with Dr Inoue, Dr Sato and myself. He has carried out a very careful study of the intrinsic birefringence of the A-band and I-band at various sarcomere lengths. Mr Colby has found that the intrinsic birefringence of the A-band is about ten times as great as that of the I-band. Contraction did not lead to a decrease in the birefringence in the center of the A-band. On the contrary, there was a slight increase at the regions where the double overlap of actin-filaments would be excepted to take place. In stretched sarcomeres the lateral portions of the A-band showed a slightly stronger birefringence than the center. A result one would expect from the overlap region between actin and myosin filaments. Other studies which we performed with Dr Johnson also supported the sliding filament theory and were contradictory to our expectations. For instance, we found that the staining pattern remained the same in free contractions and in loaded contractions of pre-stretched muscle. These results to our mind exclude a shift in the location of myosin unless something else is responsible for the birefringence in the A-band — a very unlikely possibility. Therefore, we do not believe in the correctness of our theory any more. The changes in fluorescent band pattern must have some other type of explanation. It is very gratifying, indeed, that the recent experiments of Dr Pepe led him to an interpretation which is logical and self-consistent and explains the unusual features of our antibody studies.

In summary, I doubt very much that any large-scale movement or migration of myosin accompanies contraction and to my mind the quantitative predictions of the sliding filament model are consistent with and explain a great many of the relevant experimental observations.

Guba: I agree with you, one should be very cautious with experimental results. Nevertheless, I think the quantitative electron microscopy allows us to measure a relatively small rearrangement of myofibrillar dry mass, an amount which is undetectable with other methods.

Huxley: What is the periodicity seen in myosin aggregates in the presence of tropomyosin?

Guba: It is about 150 Å.

DAVIES: Many theories of muscle contraction have been put forward recently which can account for a few facts about muscle but cannot explain others, and are certainly wrong.

In order to clarify the situation I request that a list be prepared containing all the major facts. This list can then be used to test the value of new hypotheses. I have already started such a list and request that everyone send me (with references please) facts that need explaining. The final list would be submitted to IEG No. 4. for publication. I think that even in this respect the Information Exchange Group would be a very useful instrument to help in clarifying the situation. — I am also collecting a list of all theories of muscle contraction, and again request that everyone who knows of one should write to me about it.

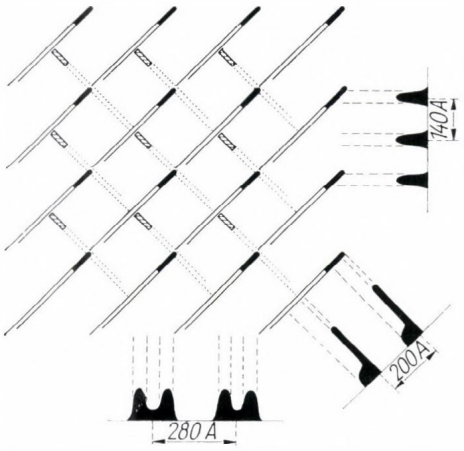

FIG. 18.—The basic structure of tropomyosin B crystals

GERGELY: I should like to second Dr Davies' comments concerning the Information Exchange Group on muscle. In a field such as ours, in which people from several disciplines come together, it is particularly useful to have a ready means of sharing information that would otherwise be scattered in journals outside one's own fields of immediate interest.

KOMINZ: I should like to call your attention to the fact that information of basic importance to an understanding of muscle mechanisms may be present in studies on pathological conditions published in clinical journals.

A familiar myopathy has recently been described which is characterized by the presence of metachromatic rod-like structures in otherwise normal muscle cells (Shy and co-workers 1963). Because of these abnormal inclusion bodies, the myopathy has been labelled 'nemaline' (Greek: nema=thread).

Electron-microscopic examination in one laboratory revealed a transverse spacing of 145 Å (Shy and co-workers 1963, Engel and co-workers 1964); however, in a second laboratory no transverse spacing could be seen (Afifi and co-workers 1965), while a third laboratory reported a transverse spacing of 175 Å (Gonatas and co-workers 1966). In the last case, cross-sections revealed a lattice structure with 200 Å spacing; because of this and the demonstration of very early rod-like structures as fusiform enlargements of the Z-band, it has been suggested that the rod-like structures are composed of tropomyosin B (Gonatas and co-workers 1966). This gives us a basic insight into the structure of tropomyosin B crystals (Fig. 18): how can a periodicity of 145 Å and one of 175 Å both be possible in such crystals? If the 400 Å long tropomyosin B molecules aggregate end-to-end in staggered longitudinal rows, with alternate planes oriented at approximately 90° as illustrated, the electron-microscopic spacings of Hodge (1959) and Huxley (1963) can be explained. The distance between the planes could vary from 145 to 175 Å

depending on the fixation process, just as Elliott (1964) found a distance of 150 to 200 Å between the planes of paramyosin ribbon.

This myopathy offers further challenges to our understanding of muscle structure: why do not all muscles suffer from extension of their Z-bands laterally? Does tropomyosin B usually exist only in the Z-band and native tropomyosin elsewhere, and does the presence of troponin inhibit the crystallization? Or is there some genetic difference in the primary structure of the tropomyosin of nemaline myopathy patients?

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BENSON: I would also like to call attention to the reported effects of the antimalarial agent plasmacid on skeletal muscle. As reported by Price and co-workers, the administration of this agent to rats was followed by a selective loss of actin filaments and Z-bands.

HANSON: Dr Szent-Györgyi has withdrawn a hypothesis; I want to withdraw a fact. Dr Lowy and I, studying actin filaments in the electron microscope, concluded that the pitch of the helix was about 2×350 Å (Hanson and Lowy 1963). I confirmed this for preparations made with the methods that we had used, but I have found that other methods can give different results. Using both synthetic and natural filaments, I found values much higher than 350 Å in preparations negatively-stained with uranyl acetate applied. Lower values around 350 Å are found if the stain is potassium phosphotungstate (pH 7·0) or if the preparation is fixed in osmium tetroxide before staining with uranyl acetate.

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RINALDI: I would like to ask a specific question, Prof. Huxley. Do you have any concept according to which you think one filament will drive over another? I am not concerned about the organism and prefer an answer which you picture as being operational in the highly structured muscle cell as you know it.

I wish an answer pointig out the system you consider could move one filament over another, further what you feel the cross bridges contribute to the system and the physical-chemical properties of actin and myosin, which you consider will move one filament relative to another. You must have some visualization of the mechanics of such motion.

HUXLEY: In order that a relative sliding movement of actin past myosin filaments may take place, the myosin cross bridges must go through a cycle of attachment to and detachment from sites on the actin filaments.

As the actin filaments move past the myosin filaments the cross bridges will attach sequentially to a series of actin monomers along the actin filaments. The simplest way to suppose that a relative sliding force is developed is to postulate that a change in the configuration of the cross bridge takes place either when it becomes attached to an actin monomer or when, having already become attached to an actin monomer, splitting of ATP takes place at the enzyme site on the myosin. One would postulate that the structure of the cross bridges was such that when this configurational change took place, a component of the resultant movement was directed longitudinally closely towards the M-line. Next, one would suppose that the cross bridge then detached from the actin filament which it had pulled along, returned to its original configuration and re-attached to a new site on an actin filament ready to begin a new cycle of movement and ATP splitting again. This is a hypothetical mechanism which can serve as a basis for experiments designed to test whether or not it is true.

HOYLE: Dr Huxley, you use the expression 'sliding-filament theory' as a package carrying a double deal, part of which is a theory of the mode of development of motile force by cross bridges. Originally, in striated muscle there are two parameters which remain constant during moderate stretch and shortening. These are the A-band length and the distance between the margins of two adjacent H-zones as measured across the Z-disc. The theory that these changes result from the sliding of two sets of interdigitating filaments each having fixed lengths is virtually a proven fact now, but remains the 'sliding filament theory'. As to how force development and shortening are brought about, these are additional matters. There are several theories extant which invoke sliding of filaments in contraction, but which do not utilise cross bridges as mechanically active units. Would it not be more appropriate, then, to keep 'sliding filament theory' non-committal as to mechanism, and to use a different expression such as 'cross bridge shortening hypothesis' for your ideas about the molecular mechanism of shortening?

HUXLEY: The sliding filament theory of the mechanism of contraction in striated muscle was given this name because the theory postulates that contraction is brought about as a *consequence* of a relative sliding movement of the thick and thin filaments past each other. That is, it supposes that the force for contraction is a relative sliding force between the two types of filament. It supposes that the structures present in the thick and thin filaments represent the working parts of the contractile machinery and that contraction is a result of events in those structures and not in another structure, real or imaginary, elsewhere in the muscle. I think it is clear then that when we use the term sliding filament theory we are not being guilty of any ambiguity. We are calling attention to the fact that the observable band pattern changes can be explained on the basis of the sliding filament mechanism and we are hypothesising that the force for contraction is developed by the sliding process itself rather than for instance, that the sliding motion is a consequence of a shortening force being developed somewhere else. If we are asked to describe the theory in greater detail, we will discuss the protein composition of the filaments and point to the cross bridges as being the most likely seat of the relative sliding force between the filaments which we require.

STRICKHOLM: I would like to inquire of Drs Hoyle and Huxley, both who have spent great effort examining muscle with the electron microscope, why Dr Hoyle has reported seeing thin filaments, while Dr Huxley has not? Is this due to a difference in electron microscope resolution, or is there some other explanation?

HUXLEY: I believe Dr Strickholm's question was whether I had seen any indications of very thin filaments in muscle similar to those reported by Dr Hoyle. Dr Hoyle showed a number of micrographs of various types of muscle in which he believed these filaments were visible. I was not quite sure on some occasions which type of muscle was being described, but I think I am right in saying that in muscle from frogs and rabbits the super thin filaments were only visible in muscles which had been stretched to the point whereby a gap developed between the ends of the thin and thick filaments. My own observations on muscles have been restricted to those from frog and rabbit, and some insect fibrillar muscles, and therefore I cannot comment on Dr Hoyle's observations on other species. The presence of gap filaments, however, was of course reported long ago both by A. F. Huxley and by Sjöstrand, and presumably the filaments described by Dr Hoyle are the same as those reported by these other authors. I have not, myself, looked for filaments in this gap region, so I cannot speak from first-hand knowledge. I think it seems very likely that some type of filament is visible here, but whether this represents a genuine third type of filament in the muscle, or whether it represents thin tapered extensions of the thick filaments or a few thick filaments or thin filaments which have come out of register, or whether it represents some structures formed between the ends of the thick and thin filaments, during fixation, still seems undecided.

I was interested in Prof. Hoyle's comments on section compression during the cutting of very thin sections. I have found that when section thickness lies below 200 Å and the sections are cut with a knife edge set perpendicular to the long axis of the muscle and longitudinal sections cut, then flow of the plastic takes place rather, I imagine, like the flow when a lathe tool is applied to a metal block, and a very considerable foreshortening of dimensions occurs without, however, any substantial evidence of damage to the tissue. If Dr Hoyle can cut sections of, say, 150 Å thickness without such flow occurring, I would be most interested. The only reliable index of section thickness I find is the selection of single layers of the filament lattice of the type which contains pairs of thin filaments in between adjacent thick ones.

HANSON: The results of my attempts to find a 'backbone' are open to other possible interpretations. After extracting the material of the thick filaments (in vertebrate skeletal myofibrils) and then removing actin with potassium iodide, we found the fibrils to remain continuous. I examined them negatively-stained and found neither myosin filaments nor actin filaments, but a sparse network of very thin structureless filaments connecting successive Z-lines. However, I do not know if this material was present in the intact fibril or if it represents an insoluble and modified residue of the primary and secondary filaments, or was derived from interfibrillar material.

GUBA: We were very anxious to handle the proteins during the extractions so as not to denature them.

GARAMVÖLGYI: Let me continue the comments of Drs Hanson and Huxley.

They were speaking of the S-filaments designated as hypothetical. In the last few years I presented some papers on this subject (Garamvölgyi 1965, Garamvölgyi and Kerner 1966). I am in the position to say that in the bee muscle fibrils deprived of their myosin filaments there are well recognizable filaments just at the site where they have been supposed by Drs Hanson and Huxley. So the S-filaments are not merely hypothetical. I do not know exactly, how I was able to observe them, but in the insect flight muscle there are very clear conditions and due to the fact that I did not remove the extracting solution, perhaps a certain kind of impregnation rendered them visible. I still use the term S-filaments for them, although I am not convinced that they would necessarily belong to the set of the secondary filaments. Prof. Huxley's more recent statement on the existence of a double overlap of the secondary filaments does not make this arrangement probable. I am inclined to accept that the S-filaments form the basic framework of the myosin filaments. This is in agreement with my proposed sarcomere model and is similarly in aggreement with Dr Guba's statement concerning the backbone protein of the myofibril. In this connection may also be pointed out the work of Auber and Couteaux (1963), who found an axial subfilament inside the light core of the primary myofilaments in transverse sections of fly myofibrils.

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HUXLEY: Since Dr Hanson and I made our original suggestions about the existence of S-filaments, I have made various attempts to see them in the electron microscope, and it has been the lack of success of those attempts, together with the finding of the double overlap of the secondary filaments in strongly shortening muscles, which has led me to have considerable doubts about the real existence of S-filaments in rabbit and frog muscles. In many cases residual filaments could be seen crossing the H-zone in muscles from which myosin had been extracted. However, these H-zone filaments were somewhat erratically positioned, i.e. there was no indication that one such filament was present per thin filament, rather that in some places one would see a dense clump of filaments, and in others a complete absence of them. Now the procedure in carrying out this type of experiment necessarily has to be the following: that a muscle fibre or a small bundle of fibres is treated with a suitable solution for extracting myosin, and that the extracting solution is then replaced by a fixative either with or without the use of an intermediate washing solution. It is rather difficult to be absolutely sure that the last traces of myosin have been removed from the muscle; there is always a possibility that a small amount of myosin remains inside the muscle fibres still in solution and that when the ionic strength is lowered again, either by the fixation itself or by previous washing with lower ionic strength solution, that this myosin will aggregate on to the actin filaments on either side of the H-zone gap and that in other places these aggregates will extend across the gap and

produce the appearance of gap filaments. It is also possible that the original extraction of the myosin may not be absolutely complete and for these reasons I never felt sufficiently convinced of the reality of the filaments one can see in extracted muscles from frog and rabbit. I am not completely familiar with the precise extraction conditions employed by Dr Garamvölgyi, and also by Dr Guba in his work on fibrillin, but it does occur to me that some residual myosin might conceivably be present.

GUBA: In our experiments on fibrillin we tried to follow the extraction of myosin besides other tests by determining the total ATPase activity, i.e. that of the extracted proteins plus that left behind after extraction. We used only those materials in which there was no significant change in the total ATPase activity. According to these experiments no appreciable amount of myosin could remain in myofibrils which show the continuous very thin filaments between the adjacent Z-discs.

HOYLE: In our work with barnacle giant fibers we have come to believe that there is a substantial amount of internal shortening and stretching during an isometric contraction. There is thus an appreciable heat of shortening under these conditions. One would expect less internal shortening in fast vertebrate twitch fibers, but, nevertheless, it might be appreciable. It is not considered in treatments of isometric contraction. What, in your opinion, would be a realistic estimate of the effective shortening in the muscles with which you are familiar?

WILKIE: It depends very much on how inextensible the connections to the muscle are. If they are very inextensible, about 3 per cent. If no special precautions are taken, it can easily rise to 10 per cent or even more.

EDMAN: Our results from measurements of the force–velocity curve in isolated fibres of the frog's semitendinosus muscle disagree with those presented by Dr Hoyle. We have recorded the speed of shortening during after-loaded isotonic tetani by recording the movements of markers placed on the cell surface in the middle segment of the fibre. The movements of the markers were recorded by means of cine-camera (65 fps, 2·5 msec exposure time), all measurements being performed at 2·1 μ sarcomere spacing at 4–5 per cent. The velocity data plotted against the load can be fitted by Hill's equation with $V_{max} = 5{\cdot}86$ μ/sec and $a/P_0 + 0{\cdot}41$ (Edman and Grieve 1966). There was no sign of distortion of the curve at low loads as Dr Hoyle's data showed. I wonder if the relatively low velocities at small loads that you obtained were due to high inertia in the recording system?

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HOYLE: The force–velocity curves obtained from single barnacle fibers showed a flat portion at low loads suggesting a maximum speed capability over a range of loads. Since this effect might have been influenced by inertia of the recording system, the latter was improved, and lever inertia further reduced. The curves then obtained actually showed a small dip, i.e. lower speeds at the lowest loads, suggesting a positive influence of load in enhancing response.

BENDALL: During this general discussion on the work of the Symposium, I think it might be as well to recall the following aphorism and indeed to inscribe it, together with the dictum of Empedocles, as a preface to the report of the proceedings. It was coined by the famous English schoolman, William of Occam in the 14th century, when he, as some of us here, had become exasperated by the multiplicity of heavenly beings, invented daily and almost hourly by his colleagues.

It states, "Entities should not be multiplied beyond necessity". It is known as Occam's Razor, and will be seen on careful inspection to have a very sharp cutting edge, well-suited to shaving off the beard-like excrescences which, unheeded tend to sprout from the fact of even the most elegant of the hypotheses.

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